Intra-specific map development and molecular analysis for agronomically important traits in chickpea (*Cicer arietinum* L.)

THESIS SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

BY

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RESEARCH GUIDE

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SEPTEMBER 2009

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INTER-INSTITUTIONAL COLLABORATIVE RESEARCH EFFORT

Research work embodied in this thesis was carried out at

National Chemical Laboratory, Pune

In collaboration with

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And

University of Agricultural Sciences (UAS), Dharwad

Scientists involved from each institute

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MPKV: Dr. Jamadagani & Dr. L. B. Mhase

UAS: Dr.R. L. Ravikumar

SEPTEMBER 2009

CERTIFICATE

Certified that the work incorporated in the thesis entitled **"Intra-specific map development and molecular analysis for agronomically important traits in chickpea (*Cicer arietinum* L.)**" submitted by **Mrs. Radhika Prabhakaran** was carried out under my supervision. The materials obtained from other sources have been duly acknowledged in the thesis.

Dr. Vidya S. Gupta
(Research Supervisor)
September 2009
Pune

DECLARATION

I hereby declare that the thesis entitled "**Intra-specific map development and molecular analysis for agronomically important traits in chickpea (*Cicer arietinum* L.)**" submitted for the degree of Ph.D. to the **University of Pune**, has not been submitted by me for a degree at any other university.

Radhika Prabhakaran

September 2009

Pune

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ACKNOWLEDGEMENT

First and foremost I would like to express my gratitude to Dr. Vidya S. Gupta who was not only my guide but also my mentor, for her academic guidance and support till date.

I would also like to thank Dr. P. K. Ranjekar, who initiated my way into NCL. His interest and involvement in my work and concern for my welfare has greatly motivated me.

Dr. Naren, who was my advisor as well as a good friend, I value his concern and support at all times. I would like to give my special thanks to Dr, Meena D. Lagu for her constant support and timely help to make things available. I would also like to mention my gratitude to Dr. Mohini Sainani and Dr. Ashok for the advice, motivations and encouragement.

I am also happy to express my thanks to Dr. Sivaram, Director of NCL for providing me the research facilities during the tenure of my work. I would also like to acknowledge the Council of Scientific and Industrial Research for providing me with financial assistance. I would also like to convey my gratitude to the McKnight Foundation, USA, for supporting my research.

I thank Dr. Bhushan Dholakia for clarifying the doubts about QTLs and also proof reading my thesis draft. Also a very special thanks to Manjie without whom I would have lost my way during the analysis part of the thesis.

I also enjoyed the company of my friends Abhijit, Aditi, Ajay, Arun, Atul, Ashwini, Charu, Elan, Gauri, Gayatri, Hemlatha, Hemangi, Krishna, Manasi M, Manasi T, Medha, Mona, Neeta, Neha, Nikil, Nishi, Pankaj, Pavan, Prabhash, Prajakta, Pradeep Soni, Prashant H, Prashant P, Priya, Ram, Ramya, Rashmi, Rasika, Rebecca, Richa, Rupali, Sagar, Savita, Sheetal, Sirsha, Sofia, Supriya, Suhas, Vaiju, Varsha and Yashwant. Special thanks to Ajit for his constant wishes at every occasion and Jagtapji for the help he rendered in the external work. Neighbors in the other departments also need a special mention for having friendly conversations on the lonely corridors.

Dr. Jamadagni, Dr. Mhase from MPKV Rahuri & Dr. Ravikumar, UAS, Dharwad, are also gratefully acknowledged for developing the plant material required for this study and phenotyping the agronomic traits.

I also wish my father was here, whom I missed to share this special ending of a long journey. My sincere gratitude to my mother, who gave the platform for my higher education. My brother and sister also need to be remembered who really waited for this day. My entire family, who constantly asked about the progress through phone calls, really needs to be thanked.

The completion would not have been possible without the constant encouragement and moral support given by my husband Prabhakaran and my daughter Sanjana. It is their sacrifice and persistence which has made me successfully complete this work. Fulfilling the dream they cherished and struggled for all these years will be a true repayment.

I acknowledge several friends and well wishers, whom I have not mentioned above and whose best wishes have always encouraged me. Lastly the Almighty, without whom, this moment would not have come true.

During the course of this work, I have experienced moments of excitement, joy and also depression, which have definitely improved my outlook in life and scientific research. I am sure someone will continue the research I started and my best-wishes would always be with the person.

Radhika

LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
APS	Ammonium per sulphate
ASAP	Allele specific associated primer
BAC	Bacterial artificial chromosome
Bp	Base pair
˚C	Degree Celsius
CIM	Composite interval mapping
cM	Centimorgan
DAI	Days after infection
DNA	Deoxyribonucleic acid
dNTP	Deoxy ribonucleotide tri phosphate
EDTA	Ethylene diamine tetra acetate
Foc	*Fusarium oxysporum* f.sp. *ciceri*
Foc1	*Fusarium oxysporum* f.sp. *ciceri* race1
Foc4	*Fusarium oxysporum* f.sp. *ciceri* race4
GEI	Genotype x Environment interaction

Ha	Hectare
HCL	Hydrochloric acid
IAA	Iso-amyl alcohol
IPCA	Interaction principal component axes
IM	Interval mapping
KCL	Potassium chloride
Kg/ha	Kilogram per hectare
LG	Linkage group
LOD	Log of the odd (Base 10 logarithm of the likelihood ratio)
$MgCl_2$	Magnesium chloride
MIM	Multiple Interval Mapping
M-QTl	Main effective QTL
NACl	Sodium chloride
NaOH	Sodium hydroxide
NIL	Near isogenic line
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
QTL	Quantitative trait loci
RIL	Recombinant inbred line

rpm	Revolutions per minute
SSR	Simple sequence repeat
TAE	Tris-acetate EDTA
TEMED	Tetramethylethylenediamine
TRIS	Tris-hydroxymethyl aminomethane
U	Unit
λ 260, λ 280	Absorption at 260nm, 280nm

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Chapter I

Introduction and review of literature

Chapter 1 Introduction and review of literature

1.1. Introduction

Family Fabaceae, comprising about 650 genera and 18,000 species (Pohill and Raven, 1981) is the most important family of Dicotyledonae with an annual world market value of two billion US $ and is second to cereals in world food production (Winter et al., 2003). In India, legumes are one of the source of high quality protein for the rural poor and are also used as fodder, forages and green manure. They are as well used for soil recovery and improvement through symbiosis with soil bacteria.

1.1.1. Chickpea, *Cicer arietinum L.*, an important food legume

Chickpea is a self pollinating, diploid (2n = 2x = 16), annual legume (Arumuganathan and Earle, 1991) with a small genome size of 740 Mbp. It is a deep rooted crop belonging to the family *Fabaceae*. Chickpea is one of the first domesticated grain legume crops of the old world (Van der Maesen, 1987). The *Cicer* species occur right from sea level (e.g *C. arietinum, C. montbretii*) to over 5000m (*C. microphyllum*) near glaciers in the Himalayas. The cultivated species, *C. arietinum* is found only in cultivation and cannot colonize successfully without human intervention. Two types of chickpea (Fig. 1.1) are recognized; desi (colored, small seeded, angular and fibrous) and kabuli (beige, large seeded, rams-head shaped with lower fiber content). Kabuli are usually utilized as whole grains and desi as whole seeds, de-hulled splits (dal) or flour. Seeds are ground to flour and used in confectionery. Young shoots or green pods are shelled for the peas and eaten as a snack or vegetable. Chickpea is also known for its use in herbal medicine and cosmetics. An acrid liquid from the glandular hairs of the plant contains about 94% malic acid and 6% oxalic acid and is used medicinally. Chickpea germplasam is maintained at two International centers (ICRISAT, India and ICARDA, Syria) and at National centers including the Vavilov institute, Russia and USDA-ARS Regional Plant Introduction Station, Pullman, U.S.A. Tremendous variation for economically important traits has been documented and improved cultivars have been developed and released by using the core germplasm. (www.grepicrisat.org/cgiar/chickpea)

Desi **Kabuli**

Figure 1.1: Desi and kabuli seeds of

. chickpea

1.1.2. Center of origin and distribution

The regions of Turkey and the ancient city of Jericho domesticated this crop around 7,500 B.C, which is considered as its centre of origin (Ladizinsky, 1975). Botanical and archeological evidence shows that chickpea was first domesticated in the Middle East and then was widely cultivated in India, Mediterranean area, the Middle East, and Ethiopia since antiquity. Chickpea has majority of its cultivation in dry areas of the Indian subcontinent (Saxena, 1990) and India is the principal chickpea producing country with a share of 90% in this region. Presently, the most important chickpea producing countries are India (63%), Pakistan (9%), Turkey (6%), Iran (4%), Mexico (3%), Myanmar (3%), Ethiopia (2%), Australia (2%), and Canada (1%). Chickpea, India's most important food legume is currently grown in about 6.7 m ha in India and 11.67 m ha worldwide (FAOSTAT 2008). In India, during the past 30 years, the chickpea area has remained stagnant, however the production has increased from 4.2 m t (2003) to 6.3 m t (2007)

and yield (717 to 845 kg/ha) (FAOSTAT 2008). There is a reduction in the chickpea area in northern India but it is largely compensated by increase in the chickpea area in central and southern India. (Fig.1.2)

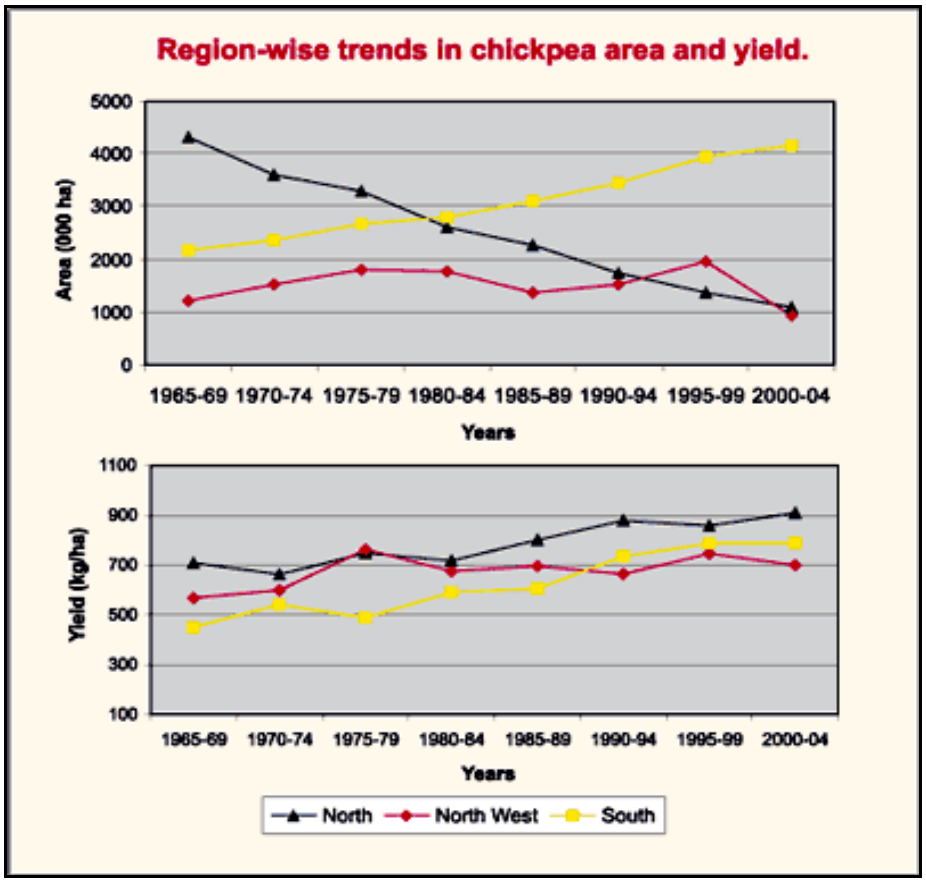


Figure 1.2: Region-wise trends in chickpea area and yield in India (FAOSTAT, 2006)

1.1.3. Morphology

Chickpea (*Cicer arietinum L.*) has a deep tap root system, which enhances its capacity to withstand drought conditions; it is well adapted to areas having relatively cooler climatic conditions and a low level of rainfall. The aerial portion is profusely branched, erect or spreading, reaching a height of 0.2-1 m, appearing glandular pubescent, olive, dark green

or bluish green in color. Leaves are imparipinnate, glandular-pubescent with 3-8 pairs of leaflets with rachis ending in a terminal leaflet. The inflorescence consists of solitary flowers, sometimes two per inflorescence and borne on 0.6-3 cm long peduncles, 7-10 mm long calyx; while the bracts are triangular or tripartite; the corolla is 0.8-1.2 cm long and varies from white, pink, purplish (fading to blue), or blue. The staminal column is diadelphous (9-1) with a sessile, inflated and pubescent ovary (Duke, 1981; Cubero, 1987; van der Maesen, 1987). The seeds (1-2 or maximum 3) are contained in a pod which is rhomboid ellipsoid, inflated and glandular-pubescent. The seed color varies from cream, yellow, brown, black, or green. Seeds may be rounded to angular with a smooth or wrinkled, or tuberculate seed coat, which is laterally compressed with a median groove around two-thirds of the seed forming a beak at the anterior end. During the cryptocotylar germination, cotyledon tips remain in the seed coat in intimate contact with the endosperm (Duke, 1981; Cubero, 1987; van der Maesen, 1987).

1.1.4. Season

The yield from chickpea is high when grown on sandy and loam soils having an appropriate drainage system. The production of chickpea is affected in excessive cold conditions. Chickpea is sown in the months of September to November in India and is considered as a rabi crop. The desi type chickpea reaches physiological maturity by 95-105 days and Kabuli type by 100-110 days. The plant is harvested when its leaves start drying and shedding and can be done manually or with the help of a harvester. In India, it is harvested in February, March and April. The required optimum conditions include 18-26°C day and 21-29°C night temperatures and annual rainfall of 600-1000 mm (Duke, 1981; Smithson *et al*., 1985).

1.1.5. Nutrition

Raw chickpea seeds contain per 100g: 357 calories, 4.5-15.69% moisture, 14.9-24.6g protein, 0.8-6.4% fat, 2.1-11.7g fiber, 2-4.8g ash, 140-440mg calcium, 190-382mg phosphorous, 5- 23.9mg iron, 0-225mg beta-carotene equivalent, 0.21- 1.1mg thiamin,

0.12-0.33mg riboflavin and 1.3-2.9mg niacin (Fig. 1.3). Sprouting is said to increase the proportionate amounts of ascorbic acid, niacin, available iron, choline, tocopherol, pantothenic acid, biotin, pyridoxine, inositol and vitamin K. It contains 17% to 24% protein with essential amino acids such as tryptophan, methionine and cysteine. It is a good source of carbohydrates and proteins, together constituting 80% of the total dry seed weight. For the chickpea-growing world as a whole, availability of nutrients from chickpea is calculated to be 12 calories and 0.6g proteins per seed. Chickpea is the most hypocholesteremic agent where germinated chickpea is reported to be effective in controlling cholesterol level in rats (Geervani, 1991). Glandular secretions of the leaves, stems and pods contain of malic and oxalic acids, which are supposed to lower the blood cholesterol levels. Additional medicinal applications of this legume crop include use for aphrodisiac, bronchitis, cutamenia, cholera, constipation, diarrhea, dyspepsia, flatulence, snakebite, sunstroke and warts.

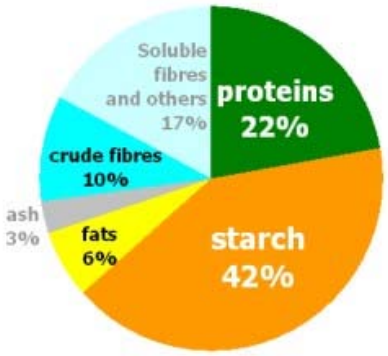


Figure 1.3: Nutrient composition of chickpea seeds

Chickpea is better at fixing atmospheric nitrogen than lentil. In India, chickpeas are also grown as a second crop after rice (Van der Maesen, 1972). This legume crop has several agronomic properties that are beneficial to cropping systems with respect to its capacity to increase soil fertility due to its ability to fix nitrogen only in association with *Cicer* specific *Rhizobium* species *Bradyrhizobium,* while the strain which infects lentil and field pea does not work. It also stimulates *Mycorrhiza* populations in the soil to the benefit of other crops in rotation, thus avoiding excessive use of chemical fertilizers and

soil acidification. Because of its capability to dissolve calcium phosphates by the root exudates rich in citric acid, this crop is unique in mobilizing phosphorous from sources that are not available to other crops.

1.1.6. Production constraints of chickpea

The major problem in chickpea is that its production falls short of demand leading to a decline in its per capita availability from 27 to 17g. This gap is further increasing due to population growth in India and in Asia which are the main consumers of chickpea. Biotic and abiotic stresses represent the most important factors causing low production of chickpea (Singh *et al*., 1994a). For example, the number of pathogens affecting chickpea has shown a three fold increase during last 15 years (Nene *et al*., 1996). The rise in import of pulses and their value (Fig 1.4) in India shows the increasing demand and the need for improvement and research in legumes.

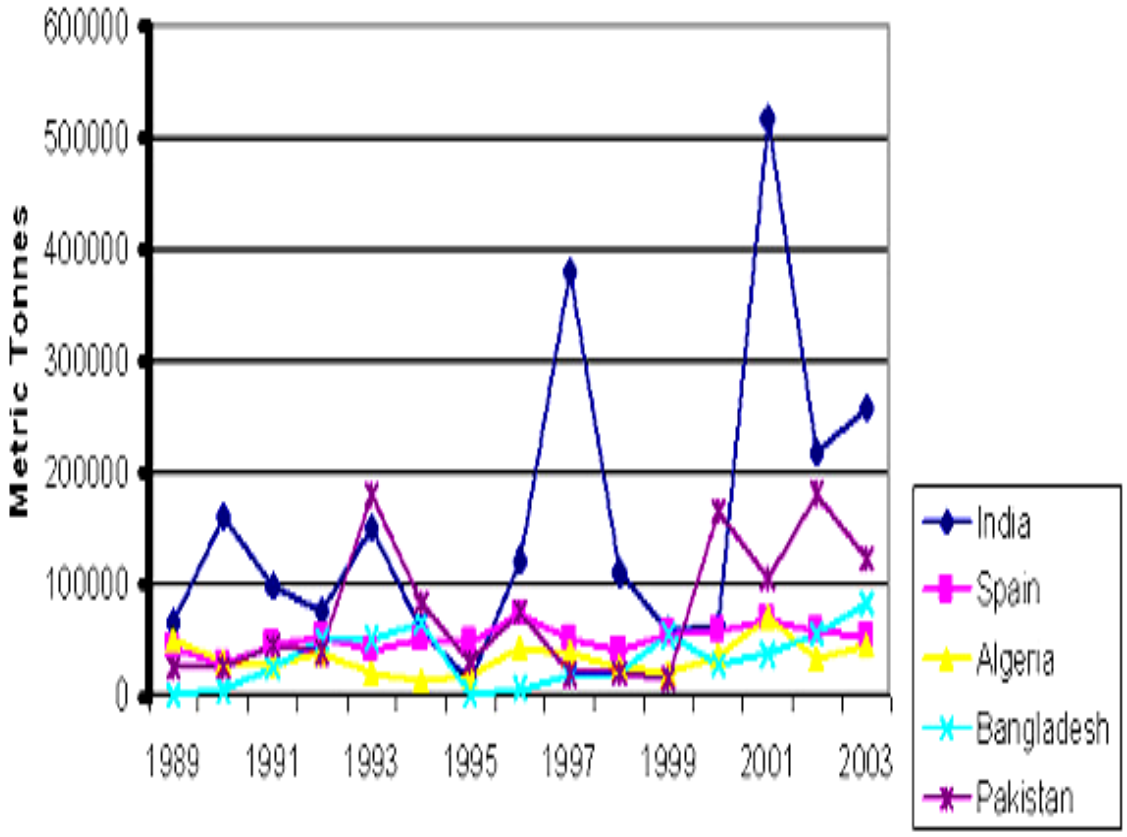


Figure 1.4: Leading chickpea importing countries (FAO, 2005)

The main fungi that affect chickpea are *Fusarium oxysporum* Schlechtend.:Fr. f.sp. *ciceri* (Padwick) Matuo & K. Sato, causing the plant to wilt and ascochyta blight caused by *Ascochyta rabiei* (Pass.) Lab. Ascochyta blight is the most serious disease in North India, Pakistan, the U.S. and the Middle East (sometimes causing l00% losses) (Smithson *et al*., 1985). Other fungi known to attack chickpea include leaf spot (*Alternaria* sp.), *Ascochyta pisi*, rust (*Uromyces ciceris-arientini*), gray mould (*Botrytis cinera*), powdery mildew (*Leviellula taurica*), *Pythium debar-yanum*, *P. ultimum*, dry root rot (*Rhizoctonia bataticola*), foot rot (*Sclerotium rolfsii*) and wilt (*Verticillium albo-atrum*). Viruses isolated from chickpea include alfalfa mosaic, pea enation mosaic, pea leaf roll, pea streak, bean yellow mosaic, and cucumber mosaic (Duke, 1981; Kaiser, 1988; Smithson *et al*., 1985; Van Emden *et al*., 1988). Among pests, pod borer (*Helicoverpa armigera*) is the most important pest of chickpea, which feeds on leaves and developing seeds (Smithson *et al*., 1985). Additional insect pests attacking chickpea include cutworms (*Agrotis* sp.), lesser armyworms (*Spodoptera exigua*) and leaf minor, groundnut aphid (*Aphis craccivora*), pea aphid (*Acyrthsosiphon pisum*), cowpea bean seed beetle (*Callosobruchus maculatus*), and Adzuki bean seed beetle (*C. chinensis*). In general, estimates of yield losses by individual pests, diseases or weeds range from 5-10% in temperate regions and 50-100% in tropical regions (Van Emden, 1988).

Drought stands to be the number one problem among the abiotic factors in major chickpea growing regions because the crop is grown on residual moisture and is eventually exposed to terminal drought (Johansen *et al*., 1997). In West Asia and North African countries, low temperature causes freezing injury or death or delayed onset of podding and reduces yield tremendously (Singh, 1987). Heat and salinity problems are also important following drought and cold stresses (Singh *et al*., 1994). Additional insurmountable constraints in all grain legumes including chickpea are three processes, namely; photorespiration, nitrogen fixation and photosynthetic energy relationships which operate against high grain yields. Photorespiration occurs in the light and consumes about 30% of the products of photosynthesis in all grain legumes. Secondly,

the symbiotic relationship between the legume plant and the *Rhizobium* to fix nitrogen diverts the carbohydrates from the plant to the bacteria and reduces potential grain production by about 10%. Lastly, the plant needs more energy to produce a given amount of oil and protein than starch. This energy intensive process ultimately manifests itself in lower yields in grain legumes (Hymowitz, 1990). Therefore, taking into account various limitations on chickpea production, better genotypes of this legume crop are being developed through conventional and modern agricultural practices all over the world.

Application of biotechnological approaches can contribute efficiently to solve or reduce the above mentioned problems. Successful application of biotechnology to biotic or abiotic constraints of the legume crops will require both a good biological knowledge of the target species and the mechanisms underlying resistance / tolerance to these stresses (Dita *et al*., 2006). Various biotechnological approaches such as genetic engineering and marker assisted breeding are being exploited for chickpea improvement. In this review, more emphasis has been given to molecular markers and marker assisted breeding in chickpea.

1.2. Biotechnology tools for molecular marker-assisted breeding

1.2.1. Marker types and qualities

Mapping and sequencing of plant genomes would help to elucidate gene function, gene regulation and their expression (Mohan *et al*., 1997). Development of high density integrated genetic linkage maps based on molecular markers is a prerequisite for use in marker assisted selection (MAS) and positional cloning of agronomically important genes in crop species (Azhaguvel *et al.*, 2006). For a molecular marker to be useful to plant breeders, it must be assayed readily and reproducibly in a range of laboratories (King, 1994). Linkage analysis is one of the basic and indispensable methods in genetics (Mohan *et al*., 1997). Linkage can define the genetic distances between consecutive traits which may be recognized based on parental differences in appearance, enzyme activities, restriction fragment lengths or nucleotide sequences at an allelic locus and their

segregation in the population (Mohan *et al*., 1997). Linkage maps based on morphological and isozyme markers were constructed for rice, maize, wheat and many other cultivated plants. Although the information on the conventional map is important to know the location of gene/s corresponding to pheonotypical traits; their usefulness is limited by the low number of morphological markers which are available to the plant breeder for crop improvement programs. Polymorphism in the nucleotide sequence is usually sufficient for it to function as molecular marker in mapping. These polymorphisms are revealed by molecular techniques such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeat or microsatellite (SSR), random amplified polymorphic DNA (RAPD) and cleavable amplified polymorphic sequences (CAPS). Genetic maps have been constructed in many crop plants using these markers on a single segregating population. An ideal molecular marker technique should have the following criteria: (1) be polymorphic and evenly distributed throughout the genome, (2) provide adequate resolution of genetic differences, (3) generate multiple, independent and reliable markers, (4) simple, quick, inexpensive with possibility to automation, (5) need small amounts of tissue and DNA samples, (6) have linkage to distinct phenotypes and (7) require no prior information about the genome of an organism (Agarwal *et al*., 2008). Unfortunately no molecular marker technique is ideal for every situation. Techniques differ from each other with respect to important features such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements, cost etc.

1.2.2. MAS for single gene trait

The effectiveness of MAS depends on the strength of linkage between the marker and the gene locus controlling the trait of interest, and genetic control of the trait (Hayward *et al*., 1994). Isozymes and DNA markers have been widely used to tag gene(s) conferring resistance to various fungal, bacterial and viral diseases in different crop species. The molecular markers and their applications in plant breeding programmes have been reviewed extensively (Gupta *et al.*, 1999; Melchinger, 1990; Mohan *et al*., 1997). Various

factors, including fungal inoculums levels and environmental conditions such as soil, temperature and/or moisture level often influence disease development in field experiments. Molecular markers permit genotyping of individuals at seedling stage itself for the trait of interest in a disease free environment. In chickpea, several gene(s) conferring resistance to biotic stresses as well as agronomic traits have been tagged using molecular markers.

Although the use of MAS may be helpful for crop improvement, its practical application in legumes for the genetic improvement of resistance or tolerance to stress has been limited, being mainly hampered by lack of investment and the genetic complexity of most stress-related traits (Dita *et al*., 2006). There are some exceptions where MAS has already facilitated breeding efforts for some legume crops against important biotic stress. For instance MAS was successfully used for the breeding of resistant soybean to cyst nematode (Diers, 2004), resistant pinto bean to common bacterial blight (Mutlu *et al*., 2005) and of resistant narrow-leafed lupin (*Lupinus angustifolius* L.) to phomopsis stem blight (Yang et al., 2002) and anthracnose (Yang *et al*., 2004). The usage of MAS in chickpea has resulted in few important outcomes such as, gene of flower colour by the SSR marker GAA47 by Cobos *et al*. (2005); single gene for double pod through another SSR marker TA80 by Rajesh *et al*. (2002b) and Cobos *et al*. (2005). Besides the phenotypic traits, the technique was extensively used in the resistance gene localization of various races of Fusarium wilt pathogen (races 1, 2 and 3 by Gowda *et al*., 2009; Sharma *et al*., 2004b; races 4 and 5 by Winter *et al*., 2000).

Breeding durable resistance to biotic and/or abiotic stresses is a major task for plant breeders and pyramiding different resistance or tolerance genes into a genotype is one way of achieving this (Dita *et al*., 2006).

1.2.3. Gene pyramiding assisted by MAS

There are numerous examples of introgression and pyramiding of favorable alleles and QTLs in legumes. However, only in a few cases MAS has been used to assist gene pyramiding to overcome stress. Most relevant work has been carried out in common bean

breeding for rust and anthracnose resistance (Faleiro *et al*., 2004). Similarly, molecular markers linked to the majority of genes conferring anthracnose resistance (*Co-1* to *Co-10*) have been described, thereby providing an opportunity to pyramid them in a resistant cultivar through MAS (Kelly and Vallejo, 2004). There are RAPD markers linked to the 11 genes (*Ur-1* to *Ur-11*) conferring rust resistance and these markers are being used to incorporate and pyramid rust resistance into common bean cultivars, and /or to combine rust resistance with resistance to other diseases, such as BCMV, BGMV, common bacterial blight, and /or anthracnose (Singh, 2001; Stavely, 2000).

In the quest for resistant cultivars to multiple stresses, combining several biotechnological approaches such as transgenesis or mutagenesis and MAS, to pyramid multiple resistance genes appears as a powerful strategy (Dita *et al*., 2006). Such an approach was recently achieved in soybean to manage insect resistance, resulting in the enhancement of resistance levels to corn earworm (*Helicoverpa zea*) and soybean looper (*Pseudoplusia includens*) in eight soybean lines in which two major insect-resistance QTLs and a synthetic Bt gene (*cry1Ac*) were combined (Walker *et al*., 2004). The general knowledge of abiotic stress QTL in legumes is still at an early stage so that gene pyramiding has not been applied yet. Nevertheless, advances achieved in non-legume crops such as tomato, in which many salt stress tolerance QTL have been identified and validated, open the possibility to transfer all of them to obtain a single improved cultivar (Foolad, 2004). In addition, combining molecular markers with other technologies had improved the efficiency of MAS. Recently, the combination of MAS with biolistic transformation was used in rice to achieve multiple resistance against blast and bacterial blight disease (Narayanan *et al*., 2004).

Abiotic stresses generally involve perturbation of various cellular functions and activation of complex metabolic pathways, and are conferred by polygenic traits (Kassem *et al*., 2004; Lee *et al*., 2004). This complexity together with the lack of good sources of natural tolerance makes this an area that is not readily amenable for conventional breeding strategies. Recent progress achieved in non-legume plants supports the potential use of transgenic approaches to produce tolerant lines (Jiang *et al*., 2004; Shou *et al*.,

2004; Umezawa *et al*., 2004). For instance, the use of transgenic, mutagenic and genetic approaches strongly improved the understanding of the genetic and molecular mechanisms of salinity tolerance in plants, and this will help develop crops, including legumes, with improved tolerance (Foolad, 2004). As a result, it was found that over-expression of a single-gene controlling vacuolar or plasma membrane Na+/H+ antiport protein, in transgenic Arabidopsis, tomato and rape seed provided them with a high level of salt tolerance under greenhouse conditions (Shi *et al*., 2003; Zhang and Blumwald, 2001).

1.2.4. Potential of genomic research

In recent years, an impressive number of advances in genetics and genomics have greatly enhanced the understanding of structural and functional aspects of plant genomes and have integrated basic knowledge in ways that can enhance our ability to improve crop plants to our benefit. Genomics is a new science that studies genome at a whole genome level by integrating the traditional disciplines of genetics with new technologies from informatics and automated systems (Fig. 1.5) (Varshney *et al*., 2005). Various sequencing projects to enhance the knowledge of major crops are present in the public domain and combining the new knowledge from genomic research with traditional breeding method is essential for enhancing crop improvement. Complete genome sequences of Arabidopsis (The Arabidopsis Genome Initiative, 2000) and two rice cultivars representing both the indica and japonica subspecies (Goff *et al*., 2002; Yu *et al*., 2002; International Rice Genome Sequencing Project, 2005) have become available. Whole genome or gene space sequencing is being carried out for several plant species such as maize (http://www.maizegenome.org/), sorghum (Bedell *et al*., 2005), wheat (http://www.wheatgenome.org/), tomato (http://sgn.cornell.edu/help/about/tomato_sequencing.html), tobacco (http://www.intl-pag.org/13/abstracts/PAG13_P027.html), poplar (http://genome.jgi-psf.org/Poptr1/), Medicago (http://www.medicago.org/genome/) and lotus (http://www.kazusa.or.jp/lotus/). Superior varieties can result from the discovery of novel genetic variation, improved selection techniques or the identification of genotypes with new or improved attributes caused by

superior combinations of alleles at multiple loci (Varsheny *et al*., 2005). The improvement of important food legumes such as chickpea, by modern biotechnology, has been lagging behind than cereals and other model plants. Important pulses such as chickpea are of monophyletic origin (Zohary, 1999) therefore, they possessed highly invariable genomes which further hampered their analysis for a long time. Nevertheless, the advent of DNA marker technology has allowed progress in the genomics of these crops. Transfer of knowledge from model plants and advanced crops together with high-throughput technologies will catalyze the analysis of entire transcriptomes and proteomes which will foster the development also in the neglected crops and substantially add to their agricultural value.

1.3. Chickpea improvement through MAS

1.3.1. Molecular marker aided genetic mapping of chickpea

Development of high density integrated genetic linkage maps based on DNA markers, is a prerequisite for use in mapping, MAS, positional cloning and mapping of QTLs in agronomically important crop species. Although an impressive amount of progress has been made in linkage mapping in chickpea (Table 1.1), a saturated linkage map with all the important traits is still awaited. Intra-species polymorphism among the cultivars is very low therefore inter-species crosses have been extensively used to develop linkage maps as revealed in the Table 1.1 and references quoted therein. The mapping era for chickpea initiated with isozymes and morphological loci (Gaur and Slinkard, 1990a, 1990b) however, due to unavailability and environmental impact, the urge for better marker system identification became essential. The first molecular map of chickpea using STMS markers was reported by Winter *et al.* (1999) with the objective of obtaining a framework map of chickpea, based on co-dominant markers, to which more dominant markers can be added. In this study, initially locus specific primer pairs were designed for 22 microsatellite containing regions from *C. arietinum* which also generated amplification products in *C. reticulatum*, the closest relative of *C. arietinum*.

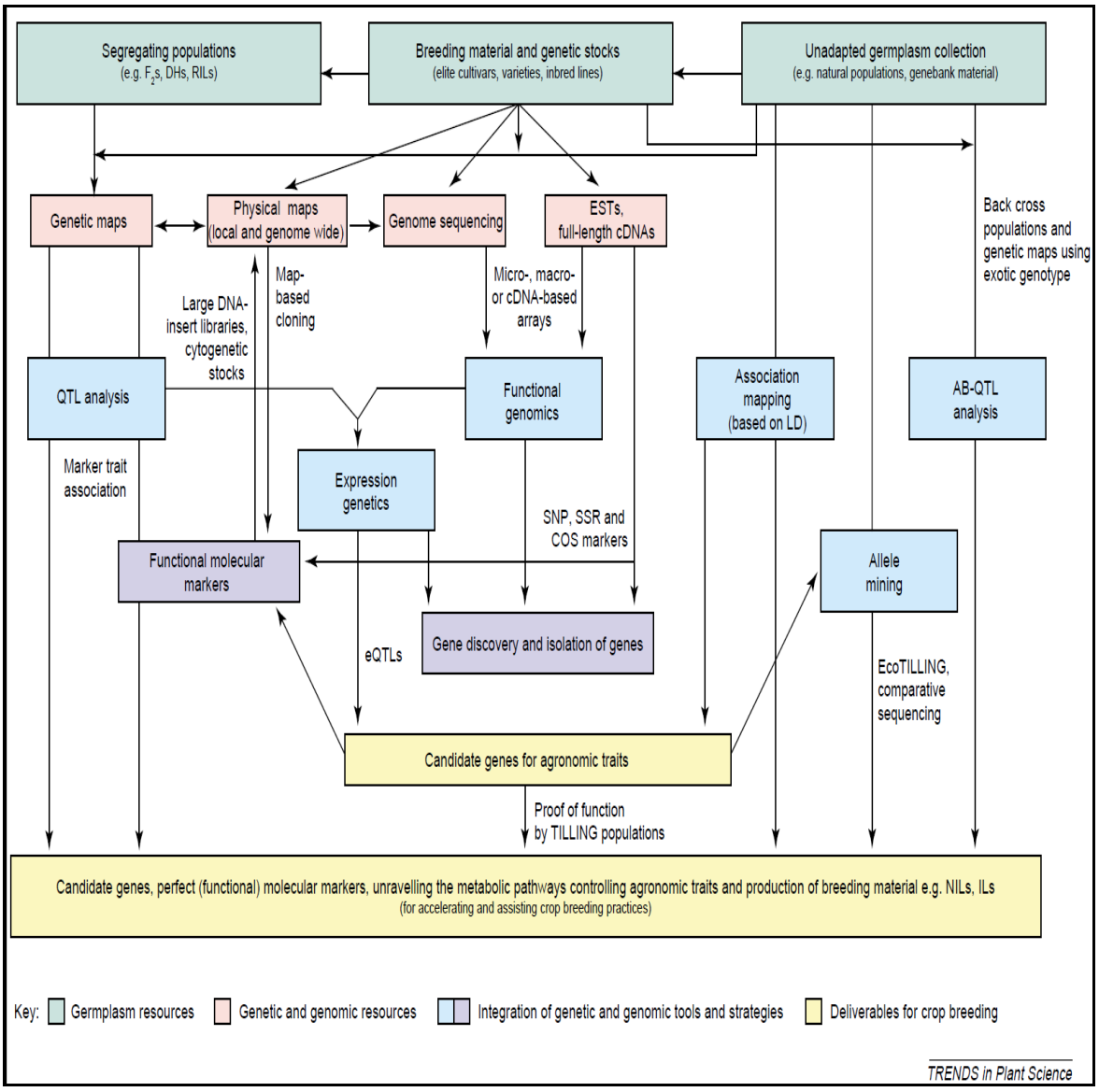


Figure 1.5: An integrated view of exploitation of genomic resources for crop improvement via various genetic and genomic strategies. Abbreviations: AB-QTL, advanced backcross QTL; COS, conserved orthologous set; DHs, doubled haploids; eQTLs, expression QTLs; ESTs, expressed sequence tags; ILs, introgression lines; LD, linkage disequilibrium; NILs, near isogenic lines; QTL, quantitative trait locus; RILs, recombinant inbred lines; SNP, single nucleotide polymorphism; SSR, simple sequence repeat or microsatellite; TILLING, targeted induced local lesions in genome. (Varshney *et al*., 2005)

Characterization of the simple sequence repeats from *C. arietinum* genome and amplification of the alleles in the wild progenitor made them a favorite choice for linkage map construction of the *Cicer* genome. The map generated using these STMS markers covered 613 cM, whereas the previous chickpea map covered 550cM (Simon and Muehlbauer, 1997). Later, Winter *et al*. (2000) constructed an extensive inter-specific linkage map with different marker systems and two tightly linked Fusarium wilt resistance genes. A total of 354 markers covering 2077.9 cM in eight large and eight small linkage groups at an average distance of 6.8 cM between markers, remains as the reference map of all the succeeded inter and intra-specific linkage maps. The concept of using more than two populations and map integration became the most reliable method of linkage map construction (Cobos *et al.,* 2005). Currently International Chickpea Genomics Consortium (www.icgc.wsu.edu) has undertaken the chickpea map integration task with the existing linkage maps. The most appropriate SSR markers are being used in the map construction. Till date around 510 chickpea SSRs are commercially available (28 primers reported by Huttel *et al*., 1999; 174 primers by Winter *et al*., 1999; 95 primers by Sethy *et al*., 2003, 2006a, b; 200 primers by Lichtenzveig *et al*., 2005 and 13 primers by Choudhary *et al*., 2006). Recently sixty EST-SSRs have been developed by Choudhary *et al*., 2009. Beyond the intra and inter species comparison, synteny studies between pea, lentil and chickpea revealed the existence of common linkage groups with homologous loci (Kazan *et al,*. 1993; Simon and Muehlbauer, 1997). Only five conserved groups were detected between chickpea, lentil and pea. Most pronounced synteny occurred at LG-7 of chickpea map with 2 clusters of loci syntenic to pea LG-7 (Weeden *et al*., 1993) and lentil LG-5. Reduced synteny between chickpea, pea and lentil reflects their taxonomic relationships: pea and lentil (2n=14) belong to the tribe *Vicieae;* chickpea (2n=16) to the monogeneric tribe *Cicereae.*

1.3.2. Map integration

The knowledge of the localization of molecular markers is extremely important for tagging genes, marker assisted selection, synteny mapping etc. It is also useful to estimate precisely the genetic relationships between cultivars in the context of plant registration

Table 1.1: Important genetic maps of chickpea (Modified from Varshney *et al*., 2007)

Mapping population	Features of genetic map	Genome coverage	Reference
F_2 inter-specific (*C.arietinum* x *reticulatum*) (*C.arietinum* x *C.echinospermum*)	7 linkage groups with 3 morphological and 26 isozymes	200 cM	Gaur and Slinkard, 1990a, 1990b
F_2 inter-specific (*C.arietinum* x *C.reticulatum*)	8 linkage groups with 5 morphological and 23 isozymes	257 cM	Kazan *et al*., 1993
F_2 inter- specific (*C.arietinum* x *C.reticulatum*) and F_2 (*C.arietinum* x *C.echinospermum*)	10 linkage groups with 9 morphological, 27 isozymes, 10 RFLP and 45 RAPD loci	527 cM	Simon and Muehlbauer, 1997
RIL, inter-specific (*C.arietinum* ICC-4958 x *C.reticulatum* PI489777)	11 linkage groups and 120 STMS	613 cM	Winter *et al* .,1999
RIL, inter-specific (*C.arietinum* ICC-4958 x *C.reticulatum* PI489777)	16 linkage groups with 118 SSR, 96 DAF, 70 AFLP, 37 ISSR, 17 RAPD, 8 isozyme, 3 cDNA, 2 SCAR and 3 morphological markers	2078 cM	Winter *et al*.,2000
RIL, inter-specific (*C.arietinum* FLIP 84-92C x *C.reticulatum* PI489777	9 linkage groups with 89 RAPD, 17 ISSR, 9 isozyme and one morphological marker	982 cM	Santra *et al.,* 2000
RIL, inter-specific (*C.arietinum* ICC-4958 x *C.reticulatum* PI489777)	8 linkage groups, integration of 55 SSR and one RGA	1175 cM	Tekeoglu *et al*., 2002

Mapping population	Features of genetic map	Genome coverage	Reference
RIL, intra-specific (*C.arietinum* ICCV2 x *Cicer arietinum* JG-62	14 linkage groups with 68 SSR, 34 RAPD, 4 ISSR and 5 morphological markers	297 cM	Cho *et al* ., 2002
RIL, intra-specific (*C.arietinum* ILC 1272 x *C.arietinum* ILC3279)	8 linkage groups with 52 SSR, 3 Ascochyta blight resistance loci	–	Udupa and Baum, 2003
RIL, inter-specific (*C.arietinum* ICC-4958 x *C.reticulatum* PI489777)	Incorporated 47 disease resistance gene specific markers to Winter et al. (2000) 2500 cM, total 296 markers, 12 linkage groups	2500 cM	Pfaff and Kahl, 2003
F_2, intra-specific (*C.arietinum* ICC12004 x *C. arietinum* Lasseter	8 Linkage groups with 54 SSR, 3 ISSR, 12 RGA loci	535 cM	Flandez- Galvez *et al*., 2003a
F_2, inter-specific (*C. arietinum* Lasseter x *C.echinospermum* PI527930)	8 linkage groups with 14 SSR, 54 RAPD, 9 ISSR, 6 RGA loci	570 cM	Collard *et al*., 2003
RIL, intra-specific *(C.reticulatum* PI 359075 x *C. arietinu*m FLIP 84-92C	11 linkage groups with 53 SSRs	–	Cho *et al* ., 2004
RIL, intra-specific (*C. arietinum*- two populations, CA2139 x JG-62, CA2156 x JG-62)	10 linkage groups with 118 RAPD, 13 SSR, 3 ISSR and 4 morphological markers	330.03 cM	Cobos *et al* ., 2005

Mapping population	Features of genetic map	Genome coverage	Reference
RIL, inter-specific (*C. arietinum* Hadas x *C.reticulatum* Cr-205	9 linkage groups with 91 SSR, 2 CytP450 markers	345 cM	Abbo *et al*., 2005
F_2, intra-specific (*C.arietinum* ICCV-029 x CDC Frontier)	8 linkage groups with 144 SSRs	1285 cM	Taran *et al*., 2007a

and protection (Dillmann *et al*., 1997). For all these applications, there is a need to obtain a saturated map which could be of nearly universal use for different genetic backgrounds (Lombard and Delourme, 2001). To achieve this objective, the construction of consensus maps synthesizing the information provided by multiple segregating populations has many advantages over mapping based on a single population (Lombard and Delourme, 2001). In this integration process, large number of loci are mapped, thus increasing the number of potential useful markers in various genetic backgrounds and provides larger genome coverage. Loci order and map distances can be assessed more precisely, and possible chromosomal rearrangements between the different parents used can be identified. The validity of the construction of a consensus map based on individual populations where a difference occurs in recombination frequency was questioned by Beavis and Grant (1991). However, if the marker order between individual maps and the consensus map is conserved, the composite map remains valuable (Lespinasse *et al*., 2000). As no single population would segregate for all the economic traits of interest, genes for those traits need to be mapped on linkage maps developed from different segregating populations. As the map becomes saturated with more and more markers, complex traits could be dissected and utilized efficiently in breeding programs. Further, establishment of gene-specific markers on the map could be useful for marker-assisted selection and positional cloning of agronomically important genes. Chickpea mapping literature completely lacks map integration attempts. Nevertheless, as a pioneer attempt, two recombinant inbred line (RIL) populations derived from intra-specific crosses with a common parental line, (JG-62) were employed to develop a chickpea integrated genetic

map (Cobos *et al.,* 2005). Joint segregation analysis including a total of 160 markers and 159 individuals resulted in 10 LGs comprising 138 markers and a genome coverage of 427.9 cM.

Individual as well as integrated maps become economically important if it constitute any desirable trait of the end use. Wilt being one of the major devastating diseases of chickpea, following part is focused on the genetics of wilt resistance, the genes controlling the resistance and the markers that are used in tagging the resistance genes.

1.4. An important biotic stress of chickpea: Wilt due to *Fusarium oxysporum*

1.4.1. Races of Fusarium oxysporum

One of the major hurdles in the production of high quality and yield of food crops is the difficulty in control of plant diseases; an aspect that concerns the producer and consumer as well. This is because many fungal pathogens have developed resistance against the active ingredients of a wide spectrum of fungicides, and there is a common perception that pesticides are undesirable. It is in this context that the application of biotechnology would be a better choice to minimize the incidence of disease in agricultural crops (Lyon and Newton, 1999). Fusarium wilt, caused by *Fusarium oxysporum* Schlechtend.:Fr. f.sp. c*iceri* (Padwick) Matuo & K. Sato, is one of the most important biotic stress of chickpea and has the potential to cause upto 100% yield loss. The disease is prevalent in the Indian subcontinent, Ethiopia, Mexico, Spain, Tunisia, Turkey, and the United States (Halila and Strange, 1996; Nene *et al*., 1989; Westerlund, 1974). *F. oxysporum* f. sp. c*iceri* is a vascular pathogen that perpetuates in seed and soil, and hence is difficult to manage by the use of chemicals. Pathogenic and genetic variability in the pathogen was characterized using differential lines and DNA markers (Haware and Nene, 1982; Rubio *et al*., 2003; Sivaramakrishnan, 2002 Barve *et al*., 2001; Gurjar *et al*., 2009). Efforts were also made to develop race-specific DNA-based markers for certain races (Barve *et al*., 2001; Gurjar *et al*., 2009). The pathogen has eight races (Sharma *et al.,* 2007). Races 1A,

2, 3, and 4 have been reported from India, and races 0, 1A, 1B/C, 5, and 6, from the United States and Spain. Cultivar specialization (race) of *F. oxysporum* f. sp. *ciceri* was first reported in India (Haware and Nene, 1982).

Resistance to wilt in chickpea has been shown to be race specific and governed by major resistance genes (Upadhyaya *et al*., 1983). Introgression of race-specific wilt resistance in modern cultivars is a basic objective in chickpea breeding programmes. Three independent genes conferring resistance to Fusarium wilt race1 have been reported (H1/h1, H2/ h2, H3/h3). Complete resistance is conferred when one of the H1/h1 or H2/h2 genes is present as recessive homozygous and H3/h3 as a dominant one (Upadhyaya *et al*., 1983; Smithson *et al*., 1983). The dominant alleles at both *h*1 (*H*1-) and *h*2 (*H*2-) loci result in early wilting, while recessive at either one (*h*1*h*1 *H*2- or *H*1-*h*2*h*2) produce late wilting (Upadhyaya *et al*.,1983a, b; Brinda and Ravikumar, 2005). Lately, three independent genes for race 2 (Gumber *et al*., 1995; Kumar, 1998), two for race 4 (Tullu *et al*., 1999) and one for race 0 and 5 (Tekeoglu *et al*., 2000) have been reported. Marker-assisted selection for wilt resistance should greatly accelerate pyramiding of race specific wilt resistance genes for developing durable resistance in new chickpea cultivars.

1.4.2. Mapping of Fusarium wilt resistance genes of chickpea

The first attempt of tagging Fusarium resistant loci (*Foc*1) with DNA markers was performed by the identification of two RAPD markers ($UBC170_{550}$ and $CS27_{700}$) at 7% recombination from the resistant loci by Mayer *et al.* (1997). Since this was a RAPD marker, more precise ASAP primer was designed out of it. This marker was mapped in the LG-6 of the map of Simon and Muehlbauer (1997). Consequently, this LG corresponded with the LG-2 of the map made by Winter *et al.* (2000). Applying ISSR markers to an F_6 derived population originated from Foc0, Foc4 and Foc5 resistant ICC-4958 and susceptible PI-489777 cultivars, Ratnaparkhe *et al.* (1998a) showed a linkage between a marker $UBC855_{500}$ and Foc4 resistant loci at a distance of 5.2 cM. This marker was coupled at 0.6 cM to CS27 of Mayer *et al*. (1997) indicating a clustering of resistant genes for races 1 and 4. In this study the resistant locus for race 5 was mapped to the

same LG as race 1and 4 resistant genes, 41 cM away from the race 4 resistant locus. Such co-localization was expected as Tullu (1996) already demonstrated close linkage between resistant genes for races 1, 2, 4 and 5 in a narrow cross between chickpea accessions WR315 and C104. Later, Sharma *et al*. (2004b) showed a tight linkage between *Foc1*, *Foc3*, and *Foc4* genes on LG-2 with the SSR markers, TA96, TA27, CS27 and CS27A. Recently, Gowda *et al*. (2009) identified *Foc*1, *Foc2* and *Foc3* on LG-2 with SSRs TA110, H3A12, TA96, TA194, and H1B06y, respectively. Thus, to date, random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSRs) and simple sequence repeats (SSR) based on the polymerase chain reaction (PCR) technique, have been reported to produce markers closely linked with genes for resistant to races 1, 2, 3, 4 and 5 (Mayer *et al*., 1997; Ratnaparkhe *et al.,* 1998a, b; Tullu *et al*., 1998, 1999; Tekeoglu *et al*., 2000; Winter *et al*., 2000, Gowda *et al* ., 2009) However, due to the different types of mapping populations and the software used to analyze the linkage data, the identified markers slightly changed its order on the respective linkage maps (Fig. 1.6). Presently, however; the marker density in and around the Fusarium resistant gene cluster is not as high to allow map based cloning.

The final journey of this review could not be concluded without the discussions of the most important aspect of plant biotechnology, the creation of new and better plant varieties by means of natural variation. The overall value of a plant variety is determined by many small and subtle characteristics that are quantitative and polygenic in addition to a few major characteristics that may be qualitative and monogenic.

1.5. Genetic architecture of Quantitative traits in plant

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 (Mackay, 2004, Laurie *et al*., 2004). Genetic architecture is defined by the knowledge of the genes controlling a trait, and QTL of moderate and even small effects are now being resolved to the gene level (Salvi and Tuberosa, 2005; Fridman *et*

al., 2004). QTL mapping studies have yielded useful biological information in terms of the importance of pleiotropy versus linkage for specific traits (Monforte and Tanksley, 2000; Chung *et al*., 2003) and co-linearity in the organization of crop genomes (Gale and Devos, 1998). Furthermore, QTL mapping has served as a launch pad for the discovery of the underlying genes through map-based cloning of QTL (Frary *et al*., 2000), candidate-gene analysis (Pflieger *et al*., 2001), or comparative mapping (Paterson *et al*., 1995). Knowledge of the approximate locations of QTL has been used as a starting point for fine mapping by non-QTL mapping approaches or for studying candidate genes that are close to the identified QTL and that may be the actual genes that affect the quantitative trait.

Mapping populations may be constructed based on parents that segregate for multiple traits. This is advantageous because QTLs controlling different traits can be located on a single map. RILs or DH populations are ideal for these purposes (Beattie *et al*., 2003; Khairallah *et al*., 1998; Marquez-Cedillo *et al*., 2001; Serquen *et al*., 1997; Taran *et al*., 2002). The most important experimental design factor is the size of the population used in the mapping study. Larger the population, more accurate is the mapping study and more likely it is to allow detection of QTLs with smaller effects (Haley and Andersson, 1997; Tanksley, 1993). An increase in population size provides gains in statistical power, estimates of gene effects and confidence intervals of the locations of QTLs (Beavis, 1998; Darvasi *et al*., 1993). There are many factors that influence the detection of QTLs segregating in a population (Asins, 2002; Tanksley, 1993). The main one is the genetic property of QTLs that control traits. Only QTLs with sufficiently large phenotypic effects will be detected; QTLs with small effects may fall below the significance threshold of detection. Another factor is the distance between linked QTLs. QTLs that are closely-linked (approximately 20 cM or less) will usually be detected as a single QTL in typical population sizes (<500) (Tanksley, 1993). Increasing the efficiency of MAS for quantitative traits calls for improved field experimentations / designs, robust mathematical models and sound statistical approaches.

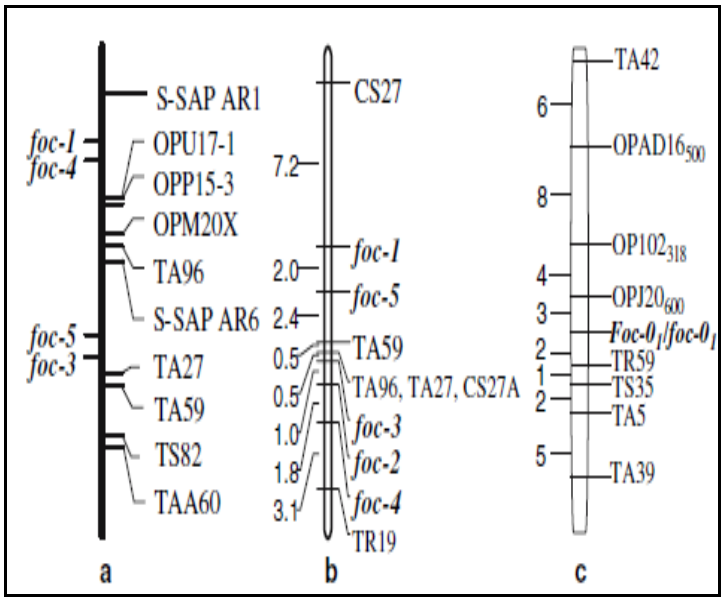


Figure 1.6: Linkage map of six wilt resistance genes in chickpea genome. Fig 1.6a depicts position of four wilt resistant genes (*foc-1, foc-3, foc-4* and *foc-5*) on an inter-specific linkage map (Millan *et al*., 2006), Fig. 1.6b shows position of five wilt resistant genes (*foc-1, foc-2, foc-3, foc-4* and *foc-5*) on an intra-specific linkage map (Sharma and Muehlbauer, 2005) and Fig. 1.6c shows location of race 0 resistant gene (*Foc-01/foc-01*) on linkage group LG-3 (Cobos *et al*., 2005). Fig. 1.6a, b corresponds to LG-2 of Winter *et al.* (2000) whereas (Fig. 1.6c) LG-3 of Cobos *et al*. (2005) corresponds to LG-5 of Winter *et al*. (2000). (Sharma and Muehlbauer, 2007)

1.5.1. MAS for improvement of quantitative traits

The preliminary step of QTL mapping is to produce an extensive framework map and the position of the QTL needs to be mapped precisely relative to closely spaced flanking marker. There are several more steps required, because even the closest markers flanking a QTL may not be tightly linked to a gene of interest (Michelmore, 1995). Theoretically, all markers that are tightly linked to QTLs could be used for MAS however, due to the cost of utilizing several QTLs, only markers that are tightly linked to no more than three QTLs are typically used (Ribaut and Betran, 1999). Even selecting for a single QTL via MAS can be beneficial in plant breeding; such a QTL should account for the largest proportion of phenotypic variance for the trait (Ribaut and Betran, 1999; Tanksley, 1993). Furthermore, all QTLs selected for MAS should be stable across environments (Hittalmani *et al*., 2002; Ribaut and Betran, 1999). As a final step, identified markers

should be validated by testing their effectiveness in determining the target phenotype in independent populations and different genetic backgrounds (Cakir *et al*., 2003; Collins *et al*., 2003; Jung *et al*., 1999; Langridge *et a*l., 2001; Li *et al*., 2001; Sharp *et al.*, 2001).

The latest trends are to combine QTL mapping with methods in functional genomics, developed for the study of gene expression. These techniques include expressed sequence tag (EST) and microarray analysis, which can be utilized to develop markers from genes themselves (Gupta *et al*., 2001; Morgante and Salamini, 2003). The use of gene sequences derived from ESTs or gene analogues, described as the 'candidate gene approach,' holds much promise in identifying the actual genes that control the desired traits (Cato *et al*., 2001; Pflieger *et al*., 2001; Yamamoto and Sasaki, 1997). EST-derived and SNP (single nucleotide polymorphic) markers are usually integrated into existing maps that have already determined the locations of QTLs (Hayashi *et al*., 2004; Ishimaru *et al*., 2001; Skiba *et al*., 2004; Wang *et al*., 2001; Zhang *et al*., 2004). For a maximal efficiency of MAS, direct QTL-allele-specific markers (such as STS markers derived from cloned QTL alleles) are needed (Sorrells and Wilson, 1997). Although success in terms of cloning of QTL alleles is highly limited, map-based cloning and candidate gene approaches would increasingly facilitate isolation and characterization of agronomically important QTLs (Lynch and Welsh, 1998). There have been few reports of successful map-based positional cloning of QTLs like for glucose and fructose content in tomato (*Brix 9-2-5*) (Fridman *et al*., 2000), fruit weight in tomato (*fw 2.2*) (Frar *et al.,* 2000), heading date in rice (*Hd1*) and (*Hd6*) (Takahashi *et al*., 2001), etc. Cloning the genetic determinants of QTLs is expected to bridge the missing link in understanding the relationship between genotype and phenotype.

1.5.2. G x E interaction on QTL mapping

The evaluation of QTL by environment interaction (Q × E) continues to be a major constraint on the efficiency of MAS (Beavis *et al*., 1996). Significant GE interactions have been reported by comparing QTLs detected in multiple environments (Stuber *et al*., 1992; Zhuang *et al*., 1997). In these studies, the appearance of QTLs being detected in one environment but not in another was considered to be an indication of GE interaction

(Zhuang *et al*., 19971). However, it has been shown that QTLs readily detected in different environments may still have significant GE effects (Yan *et al*., 1998). Genotype by environment (GE) interaction plays an important role in determining the adaptation and fitness of genotypes to the physical environment. GE interaction has received considerable attention in crop breeding programs as they are closely related to the stability of varieties. A large number of studies were carried out in the past to estimate the amounts of GE, and to evaluate varietal stability in a number of crop species (Finlay and Wilkinson, 1963; Eberhart and Russell, 1966; Zhang and Geng, 1986). Experiments that are replicated across sites and over time (e.g. different seasons and years) may enable the researcher to investigate environmental influences on QTLs affecting trait(s) of interest (George *et al*., 2003; Hittalmani *et al*., 2002; Jampatong *et al*., 2002; Lindhout, 2002; Paterson *et al*., 1991b; Price and Courtois, 1999).

Methods for QTL mapping based on phenotypic data from a single environment or on mean values across multiple environments are well developed (Lander and Botstein, 1989; Haley and Knott, 1992; Zeng, 1993, 1994). The ANOVA is an additive model that describes main effects effectively and determines if GE interaction is a significant source of variation, but it does not provide insight into the patterns of genotypes or environments that give rise to the interaction. The AMMI model analysis combines the ANOVA (with additive parameters) and PCA (principal component analysis) (with multiplicative parameters) into a single analysis. The AMMI model analysis is useful in making cultivar recommendations, specifically by mega environment analysis, in which the best performing cultivar for each sub region of the crop's growing region is identified (Zobel *et al*., 1988; Gauch and Zobel, 1997). Biplot graphs, which show markers of both genotypes and environments, are used to present AMMI analysis results (Gauch and Zobel, 1997; Ebdon and Gauch, 2002b). Crop breeding programs should take GE interaction into consideration and have an estimate of its magnitude, relative to the magnitude of G and E effects. Furthermore, the identification of the cultivar that yields best at a specific growing environment would be useful to breeders and producers.

1.5.3. Analytical methods to resolve QTLs

A variety of methods are available for QTL analysis in crop plants (Tanksley, 1993, Michelmore *et al*., 1991). The earliest and simplest methods for QTL analysis included single-marker regression or tests for independence in segregation of molecular marker and the trait of interest. A serious limitation of this approach is, confounding of the effect of one QTL by many others that influence the trait. Another serious limitation is that a QTL with major effect and loose linkage cannot be distinguished from a QTL with minor effect and tight linkage. A milestone in QTL analysis, therefore, was the QTL interval mapping (Lander and Botstein, 1989), which had several advantages over the above mentioned traditional methods involving regression analysis or tests of independence. This approach needs a molecular genetic map and utilizes, in a single analysis, information from all the markers of a linkage group, thus permitting detection of QTL in each interval lying between any two flanking markers that individually may show no association with the trait. Modifications of simple interval mapping (SIM) have also been suggested in the form of composite interval mapping (CIM) (Zeng, 1994) and multiple interval mapping (MIM) (Kao *et al.,* 1999) that take care of some of the limitations of SIM. Statistical approaches for locating multiple QTL are more powerful than single QTL approaches because they can potentially differentiate between linked and/ or interacting QTL. When the alleles of two or more QTL interact (epistasis), this has great potential to alter the quantitative trait in a manner that is difficult to predict. One of the most extreme cases is the complete loss of trait expression in the presence of a particular combination of alleles at multiple QTL. The ultimate challenge in the search for multiple QTL is to consider every position in the genome simultaneously, locate a potential QTL that might act independently, be linked to another QTL, or interact epistatically with other QTL.

QGene and MapManager QTX are commonly used computer programs to perform single-marker analysis (Nelson, 1998; Manly *et al*., 2001). The use of linked markers for analysis compensates for recombination between the markers and the QTL, and is considered statistically more powerful compared to single-point analysis (Lander

and Botstein, 1989; Liu, 1998). Many researchers have used MapMaker (Lincoln *et al*., 1993b) and QGene (Nelson, 1998), to conduct SIM and QTL Cartographer (Basten *et al*., 1994, 2001), MapManager QTX (Manly *et al*., 2001) and PLABQTL (Utz and Melchinger, 1996) to perform CIM. The results of the test statistic for SIM and CIM are typically presented using a logarithmic of odds (LOD) score or likelihood ratio statistic (LRS). The determination of significance thresholds is most commonly performed using permutation tests (Churchill and Doerge, 1994). Briefly, the phenotypic values of the population are 'shuffled' whilst the marker genotypic values are held constant (i.e. all marker-trait associations are broken) and QTL analysis is performed to assess the level of false positive marker-trait associations (Churchill and Doerge, 1994; Hackett, 2002; Haley and Andersson, 1997). This process is then repeated (e.g. 500 or 1000 times) and significance levels can then be determined based on the level of false positive marker-trait associations. Before permutation tests were widely accepted as an appropriate method to determine significant thresholds, a LOD score of between 2.0 to 3.0 (most commonly 3.0) was usually chosen as the significant threshold.

1.5.4. Chickpea genome and QTL dissection

Molecular markers are especially advantageous for traits where conventional phenotypic selection is difficult, expensive, or lacks accuracy or precision (Varshaney *et al*., 2007). Molecular mapping and identification of molecular markers associated with genes and QTLs for traits are prerequisites for the MAS. Though not excellent, some progress has been made in the area of development of molecular markers or construction of genetic maps in chickpea. As a result, molecular markers linked to a few abiotic or biotic stress tolerance / resistance as well as agronomic traits had been identified in the past.

Genetic mapping of chickpea has focused on tagging agronomically relevant genes such as ascochyta blight resistance (Tekeoglu *et al.,* 2002; Udupa and Baum, 2003; Collard *et al*., 2003; Flandez-Galvez *et al*., 2003b; Millan *et al.,* 2003; Cho *et al*., 2004; Iruela *et al*., 2006), *Fusarium* wilt resistance (Benko-Iseppon *et al*., 2003; Sharma *et al*., 2004) and yield-influencing characters such as double podding and other morphological

characters (Cho *et al.,* 2002; Rajesh *et al.,* 2002b; Abbo *et al*., 2005; Cobos *et al.,* 2005). Root characters and drought tolerance has been studied by molecular markers extensively using an intra-specific population (Chandra *et al.,* 2004). For improving cold tolerance, AFLP markers have been identified in an F_2 progeny of a cross between the chilling sensitive cultivar Amethyst and the chilling tolerant ICCV-88516 (Clarke and Siddique, 2003). In the case of flowering, a major gene (*efl-1*) for time of flowering was reported by Kumar and van Rheenen (2000), and another report (*ppd*) by Or *et al*. (1999). The latter gene controls time to flowering through photoperiod response (Hovav *et al*., 2003). Cho *et al.* (2002) mapped a QTL for days to 50% flowering on LG-3. Another QTL was also located on this linkage group in an inter-specific RIL population and explained 28% of the total phenotypic variation (Cobos *et al*., 2007). In addition to the above mentioned traits, molecular mapping for other traits is in progress in many laboratories.

1.6. Concluding remarks

Although during the past few years, significant progress has been made in the area of genomics of legume crops, there is still a need to develop more SSR, SNP or DArT markers and dense genetic maps. Further, the generation of some BAC (Rajesh *et al*., 2004) and BIBAC libraries (Lichtenzveig *et al*., 2005) offers the possibility to develop genome wide or local physical maps to isolate genes for resistance/tolerance to biotic/abiotic stresses as well as agronomic traits (Yuksel and Paterson, 2005). Thus molecular breeding through existing tools in combination with continuous incremental changes such as improvements in genetics and biometrics, plus revolutionary changes including automation of breeding trials and computerization of phenotyping will be very useful for legume improvement (Dwivedi *et al*., 2006). In addition, recent studies show strong correlation between the degree of synteny and phylogenetic distance in legumes (Young *et al*., 2003; Wang M.L. *et al*., 2004b; Choi *et al*., 2004). Therefore, advances in the area of genomics of medicago and lotus may be used to transfer information on genes involved in nitrogen fixation and other physiological processes of agronomic importance to legume crops by utilizing the comparative genomics approach combined with bioinformatics (Varshney and Tuberosa, 2007). However, the extent to which genetic

knowledge from model systems will readily translate into economic impact in related crops remains to be empirically demonstrated (Thro *et al*., 2004; Koebner and Varshney, 2006). Although genomics holds great potential for improving breeding efficiency, the high costs associated with genomics research are a critical factor hindering further applications of genomics to crop improvement particularly for inbreeding and/or minor crop species. Nevertheless, there are several success stories on the development of improved superior cultivars. In the coming years, it is anticipated that the decreasing cost of genotyping and sequencing coupled with further advances in molecular platforms and bioinformatics, will allow genomics to become an integral part of crop breeding and to improve selection efficiency (Varshney *et al*., 2005). The global demand for pulses in 2020 is estimated as 23.8 Mt with a target yield of 1095kg/ha (Paroda and Praduman Kumar, 2000). A combination of productivity enhancement through crop improvement enhanced with biotechnological tools, integrated crop management and expansion of area to new niches and production systems are needed to achieve this target (Varshney *et al*., 2007).

1.7. Objectives of the thesis work

Although India is the major producer of chickpea in the world, it fails to meet the domestic demand. To meet the growing demand it is essential to increase the chickpea productivity which can be achieved by reducing the loss caused by biotic and abiotic stresses and develop the agronomically superior and high yielding varieties. In view of improving the chickpea productivity, research work on map development, mapping of Fusarium wilt and yield related traits was initiated at Plant Molecular Biology group of National Chemical Laboratory, in collaboration with various other chickpea breeding research organizations. The importance of the chickpea crop in Indian perspective and the need for improvement of chickpea yield by direct or indirect methods necessitated the work, which was carried out keeping in mind the following objectives:

1) Construction of chickpea integrated linkage map using JG-62 x Vijay (population I) and Vijay x ICC-4958 (population II) RIL populations.

2) QTL analysis of yield and yield related traits *viz.* plant height, plant spread, branches per plant, pods per plant, seed weight, yield per plant and days to maturity in Vijay x ICC-4958 population.

3) Tagging of Fusarium wilt resistance genes in chickpea (*Cicer arietinum* L.) using Vijay x ICC-4958 population.

1.8. Organization of thesis

Considering the above objectives, I have organized my thesis in the following order.

Chapter 1: Introduction and review of literature

Chapter 2: Development of an integrated intra-specific map of chickpea *(Cicer arietinum* L.) using two recombinant inbred line populations.

(a) Development of individual linkage maps for populations I and II using PCR based molecular markers like RAPD, ISSR, and SSR.

(b) Integration of two individual maps to construct a composite intra-specific map of chickpea by using a single common parent Vijay.

(c) Map comparison with existing intra and inter-specific maps of chickpea.

Chapter 3: QTL mapping of agronomic traits in chickpea (*Cicer arietinum* L.)

(a) ANOVA analysis to identify the phenotypic variation within the population.

(b) Correlations studies among the agronomic traits.

(c) Single locus analysis (Composite Interval Mapping) for identification of QTL using QTL Cartographer v.2.5 (Basten et al., 1994; Wang et al., 2004).

(d) Two locus analysis to study the QTL interactions using QTL Network v.2.0 (http://ibi.zju.edu.cn/software/qtlnetwork).

Chapter 4: Molecular analysis of wilt resistance genes in chickpea (*Cicer arietinum* L.)

(a) Chickpea wilting analysis using pot culture method for the population Vijay x ICC-4958.

(b) Molecular analysis using PCR based markers and linkage map construction using JoinMap v.4.0 (Van Ooijen *et al*., 2001).

Chapter 5: Summary and future prospectives

Bibliography

Chapter II

Development of an integrated intra-specific map of chickpea (*Cicer arietinum* L.) using two recombinant inbred line populations

The contents of this chapter have been published as a full-length paper in Theoretical and Applied Genetics (2007)

Chapter II Development of an integrated intra-specific map of chickpea (*Cicer arietinum* L.) using two recombinant inbred line populations

Abstract

A composite intra-specific linkage map of chickpea was developed by integrating two individual maps developed from $F_{8:9}$ RIL populations with one common parent. Different molecular markers *viz*. RAPD, ISSR, RGA, SSR and ASAP were used in the mapping analysis. A total of 273 markers and 186 RILs were used to generate the map with eight linkage groups at a LOD score of ≥3.0 and maximum recombination fraction of 0.4. JoinMap v.4.0 was used to calculate marker order and recombination distances based on the consensus of the two maps. The map spanned 739.6 cM with 228 markers and an average marker density of 3.2 cM. Forty three markers were common to both the populations. The high similarity in marker order and in marker distance among the two maps enhanced the integration of the maps. The predominantly used SSR markers facilitated identification of homologous linkage groups from the previously published inter and intra-specific linkage maps of chickpea and confirmed conservation of the SSR markers across the maps as well as the variation in terms of marker distance and order. The map integration facilitated to map a large number of markers, to obtain a near-complete coverage of the chickpea genome, to fill the number of gaps and consolidate the linkage groups.

2.1. Introduction

Chickpea (*Cicer arietinum* L.) is a traditional low-input and low yielding crop in the farming systems of Indian subcontinent and Near East, where it is an integral part of the daily diet of the majority of the population. The low yield of chickpea is mostly due to its susceptibility to various biotic and abiotic stresses. Molecular marker based linkage maps have been useful in identifying and localizing important genes controlling both qualitatively and quantitatively inherited traits in a wide range of species (Tanksley *et al*., 1989). Marker assisted selection (MAS) of agronomically desirable traits such as yield, quality, biotic and abiotic stress resistance, etc. requires an intra-specific linkage map saturated with co-dominant and single locus PCR based markers like SSRs. The SSRs also enable transfer of linkage information among maps developed from different

populations and can be used as anchors to combine the maps and to develop a highly saturated consensus map.

Mapping of the chickpea genome has been of interest to identify genomic locations of disease resistance genes (Winter *et al*., 2000) and other yield related traits (Cho *et al*., 2002; Rajesh *et al.,* 2002a). However, due to very low polymorphism (Udupa *et al*., 1993; Labdi *et al*., 1996), progress in chickpea genomic research has been relatively slow, compared with legumes like soybean and *Medicago*. Nevertheless, a few genetic maps have been initially reported in chickpea based on morphological and isozyme markers (Gaur and Slinkard, 1990; Kazan *et al*., 1993; Simon and Muehlbauer, 1997) followed by DNA markers (Winter *et al*., 2000). Till date, seven intra-specific linkage maps have been reported in chickpea, constructed mainly based on SSRs (Cho *et al*., 2002, 2004; Flandez-Galvez *et al*., 2003a; Udupa and Baum, 2003; Taran *et al*., 2007a, 2007b) and RAPDs (Cobos *et al*., 2005). Even though there are common markers between these maps, map integration has not been completed and their usage has become limited. To further saturate the intra-specific linkage map of chickpea and to locate the disease resistance and yield related traits, an integrated linkage map was developed using SSRs as anchor markers from two RIL populations.

2.2. Materials and methods

2.2.1. Plant material

In the year 1993-94, at Pulses Research Center, Mahatma Phule Krishi Vidyapeeth (MPKV) Rahuri, Maharashtra, India eight genotypes of chickpea, namely ICCV-10, Phule G-12, ICC-4958, Vishal, Phule G-89219, Phule G-91028, Vijay and Bheema were selected based on their elite characteristics such as wide adaptability, seed size, 100 seed weight, wilt resistance and drought tolerance. These eight genotypes were used in 8 x 8 diallel mating and the best three genotypes namely Vijay, ICC-4958 and JG-62 with better and contrasting agronomically-important-characters were selected for cyclic crosses [JG-62 x Vijay (197 lines), JG-62 x ICC-4958 and Vijay x ICC-4958 (108 lines)]. Among these, the first and the third crosses were selected for the current study. The

selected crosses were advanced to F_8 by single seed descent method and subsequently were bulked for further generation advancement (Table 2.1). The current study was conducted with $F_{8:9}$ generation. Ninety-three RILs from each population were randomly chosen and used for marker analysis. These three genotypes differ for many agro-economically important traits and are listed in Table 2.2.

Table 2.1: Schematic details of various generations developed in different seasons

Place	Season	Cross/self	Stage of seed
P.G.I. Farm	Rabi (Oct to Jan)	P_1 x P_2	F_1
P.G.I. Farm	Early rabi (Sept to Dec)	Self of P_1 x P_2	F_2
Pulse Improvement Project, MPKV, Rahuri	Late rabi (Dec to Mar)	Self of F_2 Self of F_3 to F_9	F_3 F_4 to F_9

P_1 = JG-62, P_2 = Vijay, in JV population; P_1 = Vijay, P_2 = ICC- 4958, in VI population

2.2.2. Development of RIL populations

A uniform piece of land was selected for the experiment. It was ploughed, harrowed; stubbles of the previous crop were collected and brought to fine tilth. The experiment was conducted in randomized block design with two replications during rabi season of 2002-2003. Sowing was done in rows of 3m length placed 30cm apart, accommodating 20 plants at 15cm distance in a row. Fully developed single plumpy seeds were dibbled at 15cm distance in each row. Fertilizer dose was applied at uniform rate of 25kg N and 50kg P_2O_5/ha at the time of sowing. The operations like thinning, weeding, hoeing, irrigation and plant protection were carried out regularly as per the need and stage of the crop. An annual rainfall of 518mm and average day time temperature of 30-35˚C was

recorded at the field station during the investigational period. The experimental plots were surrounded by non experimental border rows of variety PG12, in order to avoid border effect.

Table 2.2: **Main features of the parental genotypes**

Parents	Pedigree	Origin	Special features
Vijay	P-1270 x Annigeri	Pulses Research Center, Mahatma Phule Krishi Vidyapeeth (MPKV) Rahuri, Maharashtra, India	Wilt resistant, drought tolerant, high yield, wider adaptability, high pod number
JG-62	Selection from germplasm	Jabalpur (Madhya Pradesh)	Wilt susceptible, twin pod, early mature, medium size seed
ICC-4958	GW-5/7	Gwalior (Madhya Pradesh)	Late wilt tolerant, drought tolerant and bold seed

2.2.3. DNA extraction and quantification

The parents (Vijay, JG-62 and ICC-4958) and all recombinant inbred lines were grown in pots in controlled condition at glass house of National Chemical Laboratory, Pune, India. The genomic DNA was extracted by using the leaves of 20 days old seedling by modified Sarkosyl method (Doyle and Doyle, 1987). One gram of each leaf sample from individual line was submerged in liquid nitrogen and then ground to a fine powder. The powder was quickly transferred to a tube containing 7.5 ml of ice cold extraction buffer (0.35 M Sorbitol, 0.1 M Tris, 5 mM EDTA, pH 7.5). The tube was briefly shaken and 7.5 ml of nucleic acid lysis buffer (2 M NaCl, 0.2 M Tris, 50 mM EDTA, 2% CTAB, pH 7.5) was quickly added, followed by 3 ml of 5% Sarkosyl solution. Sample sets were incubated in

a 65°C water bath for 20 min. After incubation the tubes were cooled for few minutes and 18 ml of Chloroform : Isoamylalchol ($CHCl_3$: IAA) (24:1) mixture was added to each tube. The tubes were then centrifuged at 5000 rpm for 15mins. The aqueous layer was removed and extracted again with 15 ml $CHCl_3$: IAA mixture. Finally, DNA was precipitated with two volumes of chilled ethanol and suspended in 1 ml of TE buffer (10mM Tris and 1mM EDTA; pH 8.0). To remove RNA from the samples, 100 µg/ml RNase A (DNase free) was added and incubated at 37˚C for 90 min.

Extracted genomic DNA (1µl) and two more dilutions of 1:5 and 1:10 were loaded on a 0.8% agarose gel in 0.5 X Tris acetate EDTA (TAE) (0.02M Tris-acetate, 0.0005M EDTA; pH 8.0) buffer, which contained ethidium bromide (EtBr, 10 mg/ml) . The DNA concentration of the sample was estimated by visual comparison of the band with known dilutions of bacteriophage λ DNA (50ng, 100ng and 200ng etc). Purity and concentration of the extracted DNA for each sample was also checked spectrophotometrically at 230nm, 260nm, 280nm and 320nm. The DNA quality was determined by calculating the ratio A260/A280 nm and it was ensured that the ratio ranged between 1.8 and 1.9. The A260 / A230 ratio denoted the contamination of DNA with organic compounds and the DNA quality was best, if the ratio was greater than 1.5. The absorption at 320 nm was recorded to ensure that the DNA solution was without any turbidity.

2.2.4. PCR analysis

The primers used (Table 2.3) in the present study included 800 Random Amplified Polymorphic DNA (RAPD), 100 Inter Simple Sequence Repeat (ISSR), 24 Resistance Gene Analog (RGA), one Allele Specific Associate Primer (ASAP) and 510 chickpea Simple Sequence Repeat (SSR). The primers, which gave clear and reproducible polymorphic patterns with the parents, were used for further analysis on the population. Optimal PCR conditions (Table 2.4) were established for each primer type and all the marker loci were scored at least twice to minimize interpretation errors. PCR amplifications were performed in PTC-200 thermocycler (MJ Research, USA).

RAPD analysis

RAPD assays were performed by using 800 random 10-mer oligonucleotide primers obtained from the UBC, Canada. Amplification reaction was carried out in 25 µl volumes containing 10 ng of genomic DNA, 1.5mM $MgCl_2$, 50 mM KCl, 0.1 mM dNTPs, 15pmoles primer and 0.6U *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd., India). The thermal cycling protocol was used as described by Winter *et al.* (2000).

ISSR analysis

A set of one hundred ISSR primers (UBC 801-900) were used for the analysis. The primers, which gave clear and reproducible polymorphic patterns, were used for further analysis. A 25 µl reaction mixture consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCL_2$, 0.1mM dNTPS, 0.4 mM spermidine, 15pM of primer, 1 unit of *Taq* DNA polymerase and 20 ng of genomic DNA. The thermal cycling protocol as described by Ratnaparkhe *et al.* (1998a) was followed.

Table 2.3 Primers used for the parental analysis

Primer	Source
1. RAPD (800)	University of British Columbia, Vancouver, Canada, UBC 1-800
2. ISSR (100)	UBC 801-900
3. RGA (24)	UBC
4. ASAP (1)	CS27; Mayer *et al*. (1997)
5. Chickpea SSRs	
5a. (22)	Huttel *et al*. (1999)
5b. (180)	Winter *et al.* (1999)
5c. (95) (NCPGR)	Sethy *et al.* (2003), (2006a), (2006b)
5d. (200) (H series)	Lichtenzveig *et al*. (2005)
5e. (13) (NCPGR)	Choudhury *et al.* (2006)

Numbers in parentheses ( ) total number of primers used

SSR analysis

The SSR analysis was carried out by using 510 chickpea SSR primers. The polymerase chain reaction (PCR) was performed as described by Huttel *et al.* (1999) and Winter *et al.,* (1999), with some modifications. In order to increase the screening efficiency, microsatellites with compatible annealing temperatures of primer pairs and no overlapping size of amplification products were multiplexed in the PCR. The total reaction volume, primer concentration and amount of DNA sample were optimized for each microsatellite combination as recommended by Winter *et al.* (2000).

RGA analysis

PCR amplifications were performed for 24 RGA primers in a total volume of 15 µl in 100 mM Tris-HCl, pH 8.3; 500 mM KCl; 0.01% (w/v) gelatin, 5 mM $MgCL_2$; 0.2 mM dNTP (each), 2.4 ng/μl of both forward and reverse primers, 40 ng of chickpea DNA and 0.4 U *Taq* polymerase (Rajesh *et al*., 2002).

ASAP analysis

PCR amplifications for one pair of ASAP primer was conducted using the method followed by Mayer *et al*. (1997).

2.2.5. Resolution of PCR products using various methods

Agarose gel electrophoresis

The amplified products were resolved on 2% agarose gels in 0.5 X TAE buffer, visualized and gel documented with Image Master VDS gel documentation system (Amersham Pharmacia Biotech, Denver, USA) under UV light. Agarose was dissolved in 0.5X TAE buffer by slow, circular motion and the mixture was boiled in microwave oven for 3 min. Proper care was taken to avoid over boiling / frothing of agarose. The agarose

solution was cooled to 40-50°C and poured on gel casting trays fitted with 24 well combs. About 4 µl of Bromophenol blue loading dye (0.25% bromophenol blue, 30% glycerol w/v) was added to 25 µl amplified PCR product and was loaded on the gel. The gel electrophoresis was carried at 100V, 50 mA for 45 min to 90 min and stained with 200 ml ethidium bromide staining solution with 5 µl of ethidium bromide stock (10 mg/ ml) for 10-15 min, with slow circular motion. Following staining, the gels were destained with plain ultrapure water gently for 5 min. The stained gels were visualized on gel documentation system (Amersham Pharmacia Biotech, USA) and digital images were stored in tiff format. All SSR amplified products were resolved initially on 3% metaphor agarose gels in 0.5x Tris borate EDTA (TBE) (0.045M Tris-Borate, 0.001M EDTA) buffer, visualized and gel documented.

Table 2.4: Details of the PCR reactions used for generating molecular markers

Marker	Template concentration	Primer concentration	Denaturation	Cycling conditions	Elongation
RAPD	20ng	15pM/20µl	94˚C-4min	5 cycles of 92˚C–30s, 36˚C-2min, 72˚C-90s followed by 35 cycles of 92˚C-5s, 40˚C-20s, 72˚C-90s	72˚C-5min

Marker	Template concentration	Primer concentration	Denaturation	Cycling conditions	Elongation
ISSR	20ng	15pM/20µl	94˚C-5min	40 cycles of 94˚C-1min, 50˚C-45s, 72˚C-2min	72˚C-5min
SSR	50ng	2µM/20µl	94˚C-5min	35 cycles of 94˚C-2min, 55˚C-50s, 60˚C-50s, 72˚C-2min	72˚C-5min
RGA	50ng	0.25µM/50µl	94˚C-2min	35 cycles of 93˚C-45s, 51˚C/49˚C/ 45˚C-45s, 72˚C-80s	72˚C-10min
ASAP	40ng	2µM/20µl	94˚C- 5min	40 cycles of 94˚C-20s, 62˚C-1min, 72˚C-3min	72˚C-8min

Polyacrylamide gel electrophoresis

The SSR primer gel products unable to resolve on Metaphor gels were resolved on 0.4 mm polyacrylamide using the sequencing gel unit from Life Technologies, USA. The

bind plate (larger glass plate, 33.3 x 41.9 cm) was treated with 4 ml of methacryloxypropyl-trimethoxysilane (Plus one Bind- Silane, Amersham Pharmacia Biotech), in 1 ml of acidic ethanol (0.5% glacial acetic acid in 95% ethanol) to covalently attach the gel onto the glass plate. The plates were dried for 5 min and the excess silane was removed using a paper tissue moistened with 95% ethanol. The Repel plate- smaller glass plate (33.3 x 39.4 cm) was treated with 1 ml of a 2% solution of dimethyldichlorosilane in octamethyl cyclo-octasilane (Plus one Repel-Silane ES, Amersham Pharmacia Biotech) for complete release of gel from the plate. The plates were dried for 5 min and excess silane was removed with a tissue paper moistened in distilled water. The gel solution was prepared by mixing 50 ml of the urea: acrylamide solution in 0.5x TBE with 200 µl of freshly prepared 10% ammonium per sulfate (APS) solution and 50 µL of Tetramethylethylenediamine (TEMED). The gel solution was poured into the assembled gel plates (0.4 mm thickness) using a pointed beaker. The gel was allowed to polymerize for 60 min.

Prerun and sample electrophoresis

The sequencing gel was run at 60 W (42 mA; 1500 V) for 60 min or until the gel temperature reaches 55 °C in 0.5x TBE buffer. The samples were denatured for 5 min at 94°C in the thermocycler and immediately placed on ice. About 8 µl was loaded to the gel as quickly as possible and the electrophoresis was performed at 60 W for 80 / 100 min at 50-55°C.

Silver staining

The gel bound to the binding plate was removed from repel plate and fixed with fixer solution (200 ml of crude alcohol and 10 ml of acetic acid in 1790 ml of double distilled water) for 20 min in gentle circular motion. The gel plate was drained and silver stained with staining solution (4 gm of silver nitrate in 2 liter of double distilled water) for 30 min. After staining, the plate was drained free of staining solution and developed using

developer solution (30gms of NaOH and 5 ml formaldehyde in 2 liter of double distilled water) for 5-10 min. After the appearance of sharp and dark bands, the gel was treated with stop solution (10% glacial acetic acid) to end the staining process. The gel was completely washed with ultrapure water and further dried for gel documentation.

2.2.6. Data analysis

The genotype of each sample in case of RAPD, ISSR and RGA (dominant) primers , was scored as presence or absence of amplified DNA locus and for SSR and ASAP (co-dominant) primers, was done based on the size variation of the alleles in the parents. The RILs with a genotype of Vijay were given score 'a', and of JG62/ICC-4958 'b', the heterozygous RILs 'h' and the missing data were given '-'.

The X^2 test was used to assess goodness-of-fit to the expected 1:1 segregation ratio for each marker. All markers including those with distorted segregation were used for linkage analysis using JoinMap v. 4.0 (Van Ooijen and Voorrips, 2001). The markers were classified into linkage groups (LGs) using the minimum LOD threshold of 3.0 and maximum recombination fraction of 0.4. Kosambi mapping function was used to estimate the map distances (Kosambi, 1944). *Combine groups for map integration function* were used to combine the data from the separate populations. Heterogeneity of recombination rate between common markers in the two populations was tested using χ^2 tests as implemented in JoinMap 4.0.

The images of the linkage groups and comparison of the present map with the inter-specific map developed by Winter *et al*. (2000) were created using MapChart v. 2.2 (Voorrips, 2002). In the comparison, the LGs of the present map were designated with Arabic numerals, whereas the LGs of the map of Winter *et al.* (2000) were designated with Roman numerals.

2.3. Results

2.3.1. Linkage maps of the two RIL populations

JV population

Marker analysis for both the population was performed separately. Of the 1,426 primers (800 RAPD, 100 ISSR, 510 chickpea SSRs, 15 *Medicago truncatula* SSRs and 1 ASAP) screened between the parents of JV population, only 116 (9.5%) primers revealed clear and consistent polymorphism generating 121 reproducible and segregating markers for linkage analysis. The 15 *Medicago* SSRs although produced amplifications, were not polymorphic with the parents. The linkage analysis revealed seven linkage groups with 105 markers (Table 2.5) at a LOD score of ≥3.0 and maximum recombination fraction of 0.4 (96 SSRs, 9 RAPDs and 1 ASAP). This map (Fig. 2.1) covered 484.9 cM, with an average marker density of 4.61 cM. Sixteen markers containing four RAPDs, one ISSR and eleven SSRs were unlinked. Though the total number of RAPD and ISSR primers used in the analyses were more, the polymorphism percentage they showed was very low, as compared to the SSR makers. LG-3 was the longest linkage group with 17 markers and spanned 123.5 cM with an average marker density of 7.26 cM (Fig.2.1). This LG corresponded to LG-II of the reference map. LG-1 was the densest linkage group with a marker density of 2.15 cM (Table 2.5) and had 36 markers spanning 77.3 cM. This group corresponded to LG-III and LG-V of the inter-specific map of Winter *et al.* (2000). The LG-2 corresponded mainly to LG-I and LG-IV of the reference map. The LG-4 had five markers spanning 45.4 cM and shared many markers from LG-II of the reference map. LG-5 had seven markers with an average marker interval of 6.48 cM. LG-6 spanned 89.6 cM with 16 markers and a marker density of 5.56 cM. (Fig 2.1) These two LGs corresponded to LG-VII and LG-VI of the inter-specific map respectively. LG-6 corresponded to LG-VI of Winter *et al.* (2000). The LG-7 had only 4 markers (RAPD) spanning 47 cM and 11.75 cM marker interval, forming the smallest LG among the seven LGs constructed. This LG did not have any common markers to be compared with the

reference map. Inversions were observed with respect to marker order in all the linkage groups between the present and the inter-specific map of Winter *et al.* (2000).

Table 2.5: M**ain features of chickpea intra-specific map JG-62xVijay (JV population)**

	LG	Total markers	Total length (cM)	Average marker density (cM)
1	(LG-**III** +LG-**V**)	36	77.3	2.15
2	(LG-**I** + LG-**IV**)	20	80.6	4.03
3	(LG-**II**)	17	123.5	7.26
4	(LG-**VII)**	5	24.4	4.88
5	(LG-**VII)**	7	45.4	6.48
6	(LG-**VI**)	16	89.6	5.56
7		4	47.0	11.75
	Total	**105**	**484.9**	**4.61**

Unlinked markers: 16; LG in parentheses () corresponds to LG of the Winter *et al*.

(2000); *Arabic numerals* corresponds to the current LG

VI population

The parents of the VI population (Vijay and ICC-4958) were screened with 1,435 primers (800 RAPDs, 100 ISSRs, 510 SSRs , 24 RGAs and 1 ASAP), which produced 166 segregating markers containing 113 SSRs, 35 RAPDs, 16 ISSR and two RGAs on seven LGs (Table 2.6) at a LOD value of >3.0 and maximum recombination fraction of 0.4. The map spanned 624 cM with a marker density of 3.67 cM (Fig. 2.2). The distribution of marker was uniform within most LGs, with the exception of LG-7 with two markers. LG-

1 was the longest and the densest LG with 136.3 cM total length and 63 markers. Small clusters and gaps were found on LG-2, LG-3 and LG-4. Common markers among homologous LGs, allowed the comparison of the marker order between the reference and the current map. LG-1 alone was comparable with 3 LGs of the reference map (LG-I, LG-V and LG-V). LG-2 was also equally long (110 cM) but with less number of markers (37) and corresponded to LG-I and LG-V (Table 2.6). Significant differences in marker order of the SSR markers; TS35, TS43, TR59, TR29, TA179 and TA5 were observed between LG-2 and the reference LG-V. LG-3, LG-4 and LG-5 had 24, 19 and 10 markers, respectively and had analogous reference LGs (Table 2.6). LG-6 and LG-7 lacked markers to be compared with the reference map. LG size was not correlated to the number of linked markers in the group. For instance, LG-6 had eleven markers and covered 125.9 cM while LG-2 had 37 markers but covered 110.7 cM. However, the size of a LG and number of markers together, provided an estimation of marker density. LG-1 had an average marker density of 2.12 cM, while LG-6 had a smaller marker density (11.44 cM). The only mapped RGA marker (Ptokin) revealed two alleles and was present on LG-1. LG-6 was constructed with only RAPD markers (eleven) spanning 125.9cM length. LG-7 was the smallest LG with two SSR markers and 15.4cM total length. Marker density was not affected by the marker type. All marker types were densely or sparsely located in different regions of the mapped genome. Similar to JV population, inversions of marker order were observed between the present and the inter-specific map of Winter *et al.* (2000). Eleven SSR, two RAPD and one ISSR remained unlinked among the 178 markers analyzed in the population.

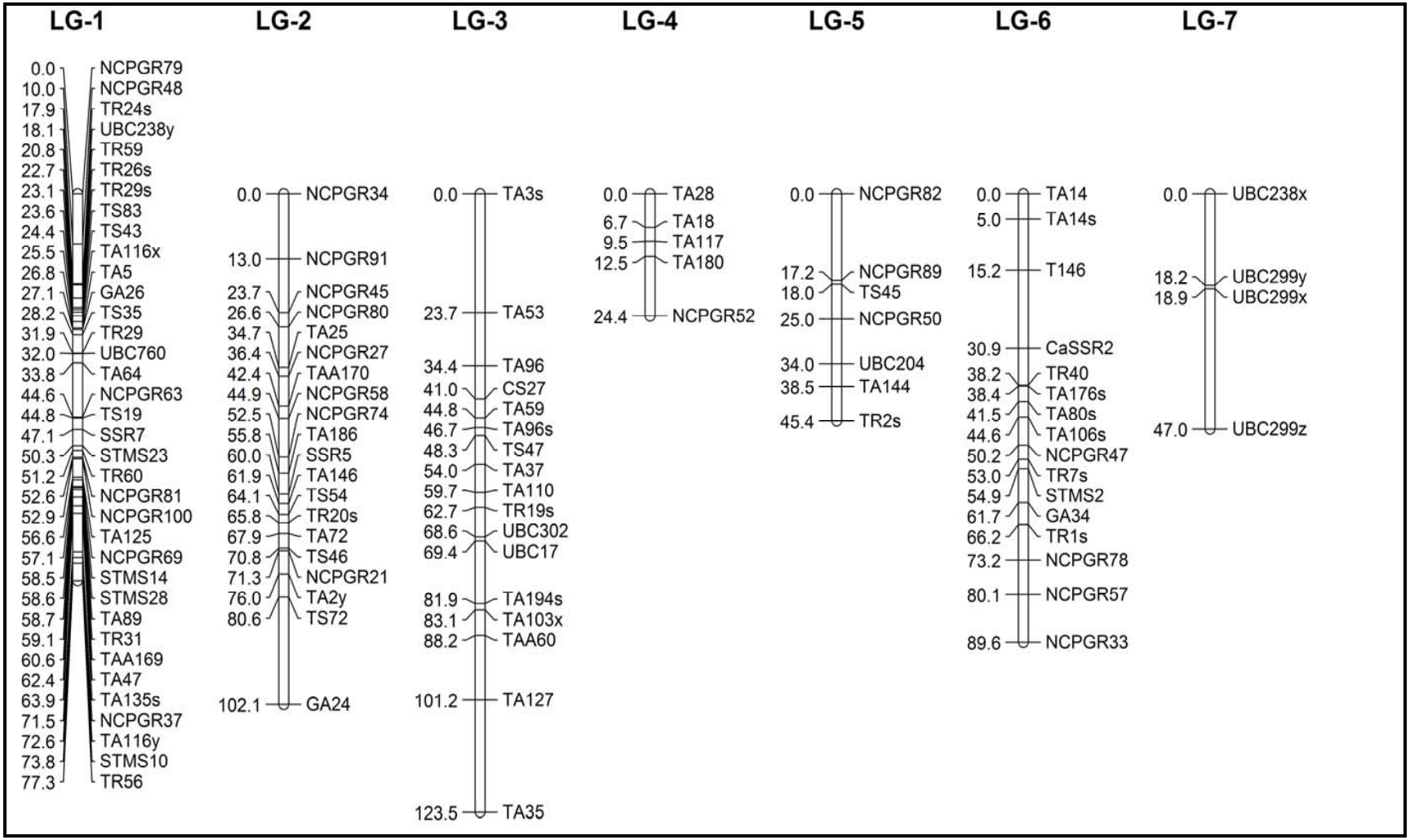


Figure 2.1: **Chickpea intra-specific map (JV population)**

Table 2.6: Main features of chickpea intra-specific map Vijay x ICC-4958 (VI population)

LG	Total Markers	Total Length (cM)	Average marker Density (cM)
1 (LG-**I**+LG-**IV**+LG-**V**)	63	136.3	2.16
2 (LG-**I** +LG-**V**)	37	110.7	3.0
3 (LG-**I** +LG-**VII**)	24	93.2	3.88
4 (LG-**VI**)	19	83.9	4.41
5 (LG-**VIII**)	10	58.6	5.86
6	11	125.9	11.44
7	2	15.4	7.7
Total	**166**	**624**	**3.76**

Unlinked markers: 11; LG in parenthesis () corresponds LG of the Winter *et al*. (2000)

Arabic numerals corresponds to the current LG

Integrated map

JoinMap v.4.0 (van Ooijen and Voorrips, 2001) was used for integrating the two individual maps along with 43 common markers on five LGs. The joint segregation analysis with a total of 247 markers and 186 RILs produced an integrated map with 228 markers (containing 44 RAPDs, 16 ISSRs, 165 SSRs, 2 RGAs and 1 ASAP (Fig. 2.3). The integrated intra-specific map, which covered 739.6 cM, had an average marker density of 3.2 cM. Relative to the estimated physical size of the chickpea genome (750 Mbp; Arumuganathan and Earle, 1991), 1 cM distance in the present map approximately equals 1 Mbp. LG-1 was the longest linkage group with 63 markers and spanned 132.4 cM with an average marker density of 2.1 cM (Table 2.7). It shared 17 markers from three LGs (LG-III, LG-V and LG-XIII) of the inter-specific map of Winter *et al*. (2000) (Fig. 2.3). LG-2 was the densest linkage group with an average marker density of 1.8 cM and 72 markers spanning 127.4 cM. The two RGA loci (Ptokin), RGA6x (1000 bp) and RGA6y (600 bp) were mapped on this LG. This group corresponded to LG-I and LG-IV of the inter-specific map (Fig 2.4).

The LG-3 corresponded mainly to LG-II but shared a single marker (TS12) from LG-VIII. The LG-4 had 26 markers spanning 97.3 cM and shared four markers from LG-VI. LG-5 spanned 65.9 cM with 25 markers and corresponded to LG-VII of the inter-specific map. However, it also shared the marker STMS25 from LG-XV. LG-6 had only seven markers and corresponded to LG-VIII of the map of Winter *et al.* (2000). The LG-6, LG-7 and LG-8 (Fig 2.3) were individual LGs from JV and VI populations, respectively. LG-7 and LG-8 comprised only RAPD markers. These LGs lacked common markers and could not be compared with the LGs of the reference map. Inversions were observed with respect to marker orders in all linkage groups between the present and the reference map as well as few existing intra-specific maps. By mapping multiple loci from the same markers, it was possible to identify putative homoeologous relationships between the linkage groups. Homoeologous chromosomes could be identified when multiple markers are mapped in the same linear order in different linkage groups. Three putative homoeologous pairs were identified; a) LG-1 and LG-8 connected by UBC238, b) LG-2 and LG-7 by UBC190 and UBC43. The maximum distance of 17.8 cM separating two markers occurred on LG-5 between SSR markers NCPGR52 and STMS21y and the minimum distance was 0.1 cM on LG-2 between markers UBC335x and SSR NCPGR58.

Table 2.7: Main features of the integrated map

LG	Total Markers	Total Length cM	Average marker Density (cM)	Corresponding LG of Winter *et al.* (2000)
1 (p1:**1** + p2: **3**)	63	132.4	2.1	**III, V, XIII**
2 (p1:**2** + p2: **1**)	71	127.4	1.8	**I, IV**

	LG	Total Markers	Total Length cM	Average marker Density (cM)	Corresponding LG of Winter *et al.* (2000)
3	(p1: **3** + p2: **5**)	22	98.3	4.46	**II**
4	(p1: **4** + p2: **3**)	25	97.3	3.89	**VI**
5	(p1: **6** + p2: **4**)	25	65.9	2.63	**VII, XV**
6	(p1: **5**)	7	45.4	6.48	NA
7	(p1: **7**)	11	125.9	11.44	NA
8	(p2: **6**)	4	47	11.75	NA
	Total	**228**	**739.6**	**3.24**	

Unlinked markers: 19. *Arabic numerals*: current LG, p1 and p2 = JV and VI population, *Roman numerals*: Winter *et al.* (2000) LGs

Table 2.8: Polymorphic markers generated for JV, VI and integrated map

JV					VI				
RAPD	**ISSR**	**SSR**	**RGA**	**ASAP**	**RAPD**	**ISSR**	**SSR**	**RGA**	**ASAP**
9	NA	95	NA	1	38	13	113	2	NA
1.125%		17.6%		100%	4.75%	13%	113%	8.33	

				%
INTEGRATED				
RAPD	**ISSR**	**SSR**	**RGA**	**ASAP**
44 (5.5)%	16 (16)%	165 (32.35)%	2 (8.33)%	1 (100)%

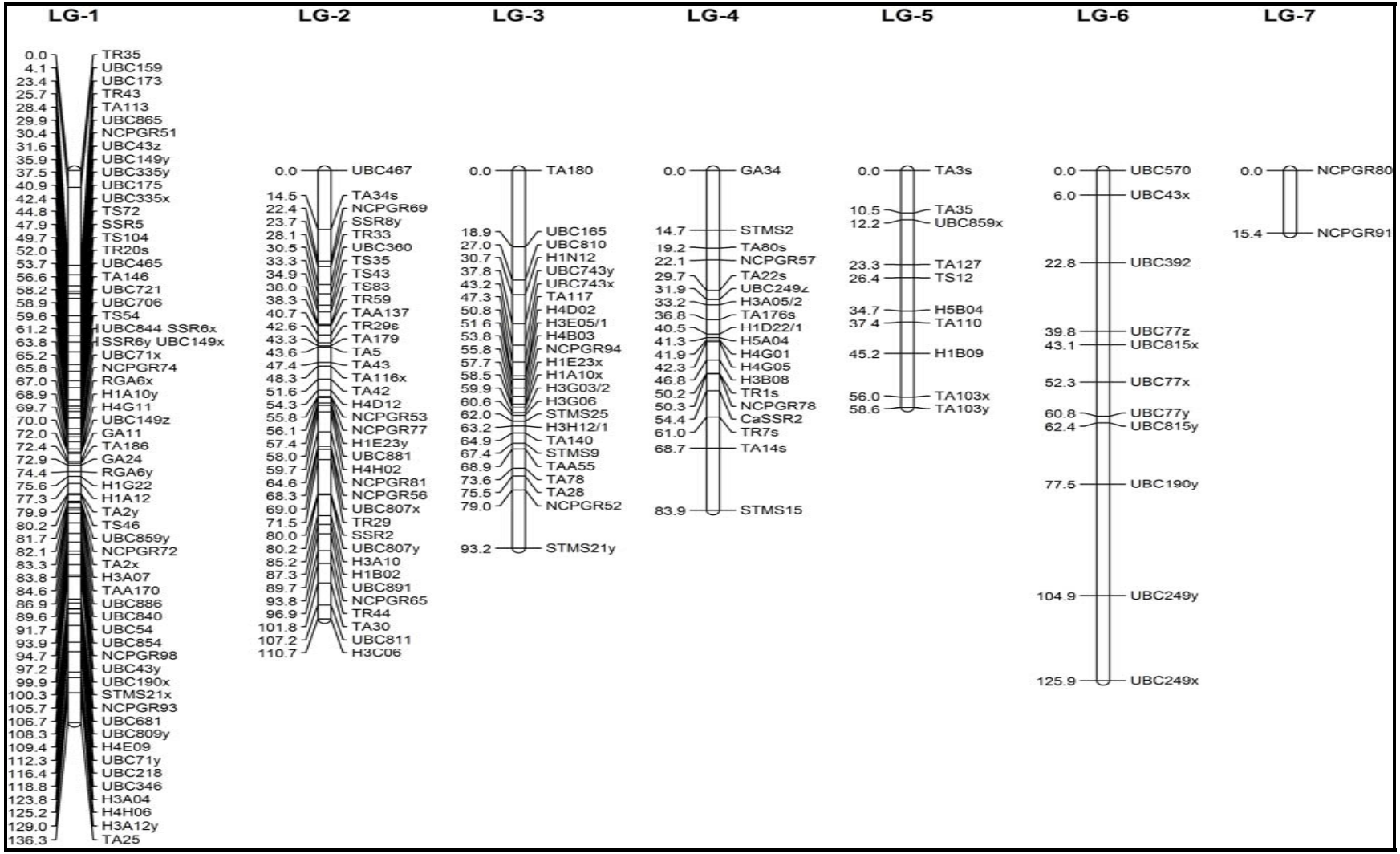


Figure 2.2: Chickpea intra-specific map (VI population)

2.3.2. Comparison of JV, VI and integrated maps

Total primers used in the three mapping processes (1426, 1435, 1435) gave an average 7.36%, 11.56% and 15.88% markers on the JV, VI and integrated maps, respectively. The correlation between number of markers on each LG and length of the respective LG gave an indication of the distribution of markers over the linkage groups. These correlation coefficients were 0.58, 0.72 and 0.70 ($P < 0.001$) for the maps of JV, VI and integrated map, respectively which indicated more random distribution of markers among the LGs in VI and integrated map than the JV map. A high number of skewed segregation of markers was observed in the RILs. General tendency towards the preferential inheritance of the alleles of a particular parent, as an extreme, resulted in distorted segregation at various levels among the three maps (Table 2.9) as judged from χ^2 tests ($P<0.05$). Of the 104 and 166 markers mapped in the JV and VI populations, respectively, 44 and 17 markers did not segregate according to the expected Mendelian ratio. However, only two markers (TA127 and TR29s) were distorted aberrantly in both the populations. Nonetheless, VI population had the least percentage of distortion (23.5%). LG-1 of JV and VI had maximum number of distorted markers. Different marker types exhibited different levels of skewdness however, SSRs were the most distorted. The skewed markers were indicated by asterisks on the integrated map (Fig. 2.3). Degree of polymorphism of the various types of markers for the three maps were different (Table 2.8). SSR markers showed more polymorphism than other type of markers used. The distribution of markers remained unvaried within most LGs of each mapping population, with the exception of LG-7 of JV and VI and LG-6, LG-7 and LG-8 of the integrated map. Clustering of markers at specific regions and sub-clustering of similar marker types was apparent among the LGs of all three maps. Nevertheless, one concrete evidance was observed on LG-4 of the integrated map. SSR marker H4G01 (36.2 cM) to TR40 (53.1 cM), a SSR cluster was identified excluding one single marker; UBC249z. Similarly on LG-5, SSR NCPGR52 to TA117, a distance of 27.0 cM was completely linked with eighteen SSR markers. LG-4 of VI population and the integrated map had a similarity by possessing majority of SSR markers and a single RAPD, UBC249z. LG-6 of JV and VI population had only SSR and RAPD marker respectively.

Table 2.9: Segregation distortion of the markers used in the population

JG-62 x Vijay			Vijay x ICC-4958			Integrated Map		
LG	**No. of markers**	**Distorted (P>0.05)**	**LG**	**No. of markers**	**Distorted (P>0.05)**	**LG**	**No. of markers**	**Distorted (P>0.05)**
LG 1	36	14 (38%)	**LG 1**	63 (20.63%)	13	**LG 1**	63	16 (25.39%)
LG 2	20	9 (45%)	**LG 2**	37 (16.21%)	6	**LG 2**	71	16 (22.53%)
LG 3	17	6 (35.2%)	**LG 3**	24 (20.83%)	5	**LG 3**	22	8 (36.36%)
LG 4	5	2(40%)	**LG 4**	19 (42.10%)	8	**LG 4**	25	11 (44.0%)
LG 5	7	3 (42.8%)	**LG 5**	10 (60.0%)	6	**LG 5**	25	5 (20.0%)
LG 6	16	9 (56.2%)	**LG 6**	11 (9.09%)	1	**LG 6**	7	3 (42.85%)
LG 7	4	Nil	**LG 7**	2	Nil	**LG 7**	11	1 (9.09%)
LG 8	NA	NA	**LG 8**	NA	Nil	**LG 8**	4	Nil
Total	**105**	**43 (41%)**	**Total**	**166**	**39 23.5%)**	**Total**	**228**	**60 (26.31%)**

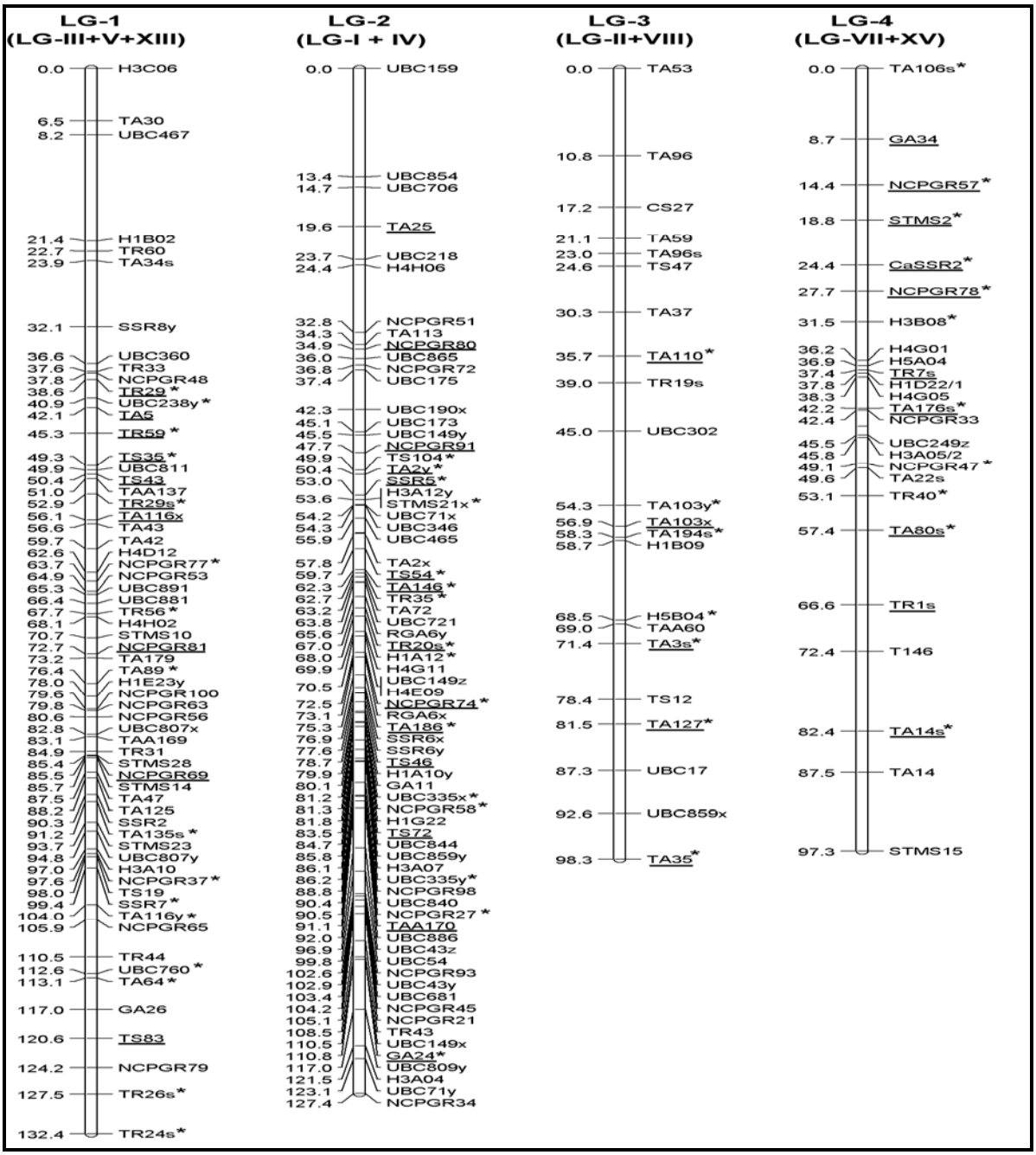


Figure 2.3: Composite map of the chickpea genome obtained by integrating the linkage maps of JV and VI RIL populations. The markers common between the two maps were *underlined* and those showing distorted segregation are indicated by *asterisks*. Corresponding LGs of Winter *et al.* (2000) reference map is indicated in *Roman numerals* in parentheses

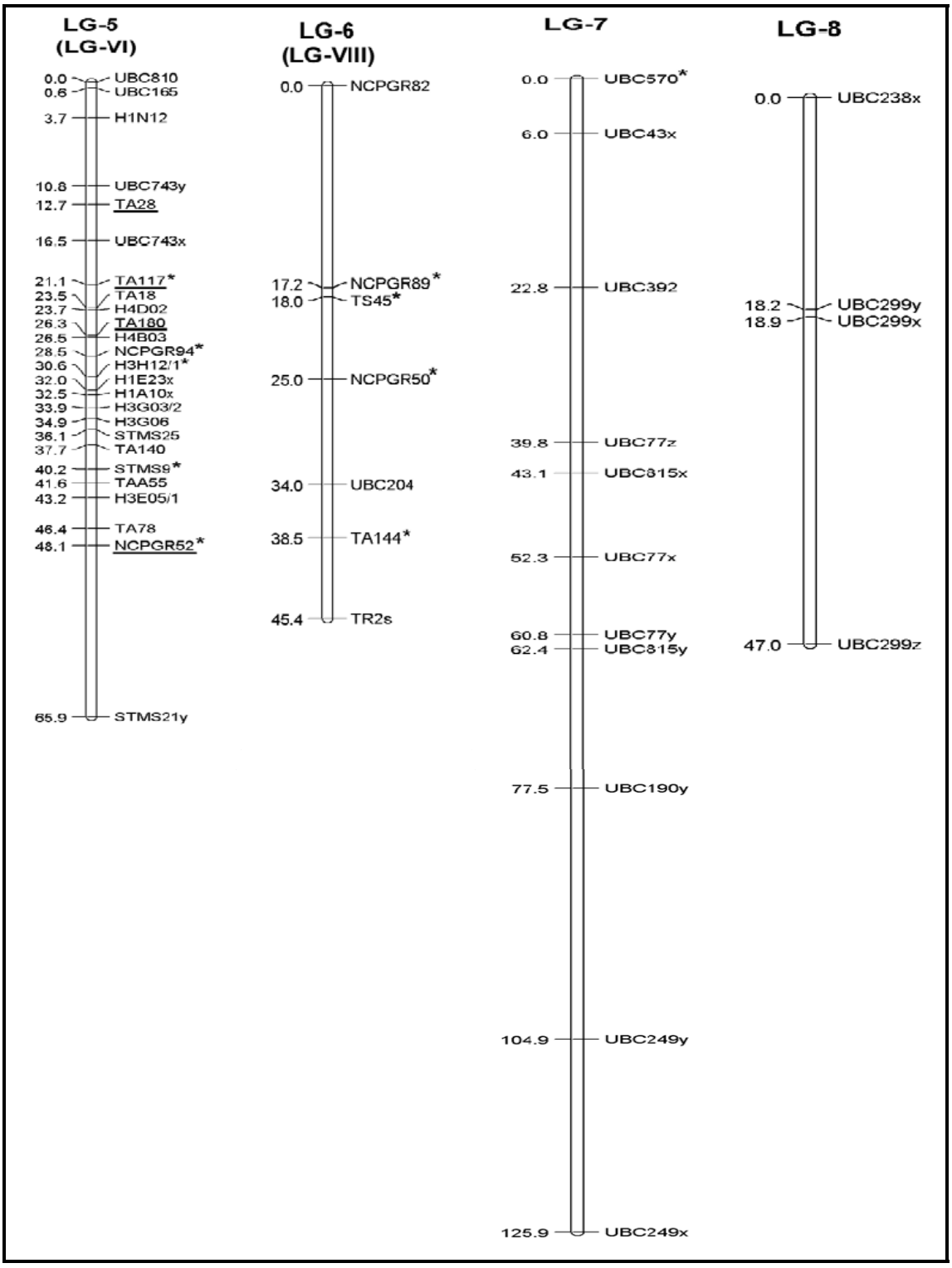


Figure 2.3: (Cont…)

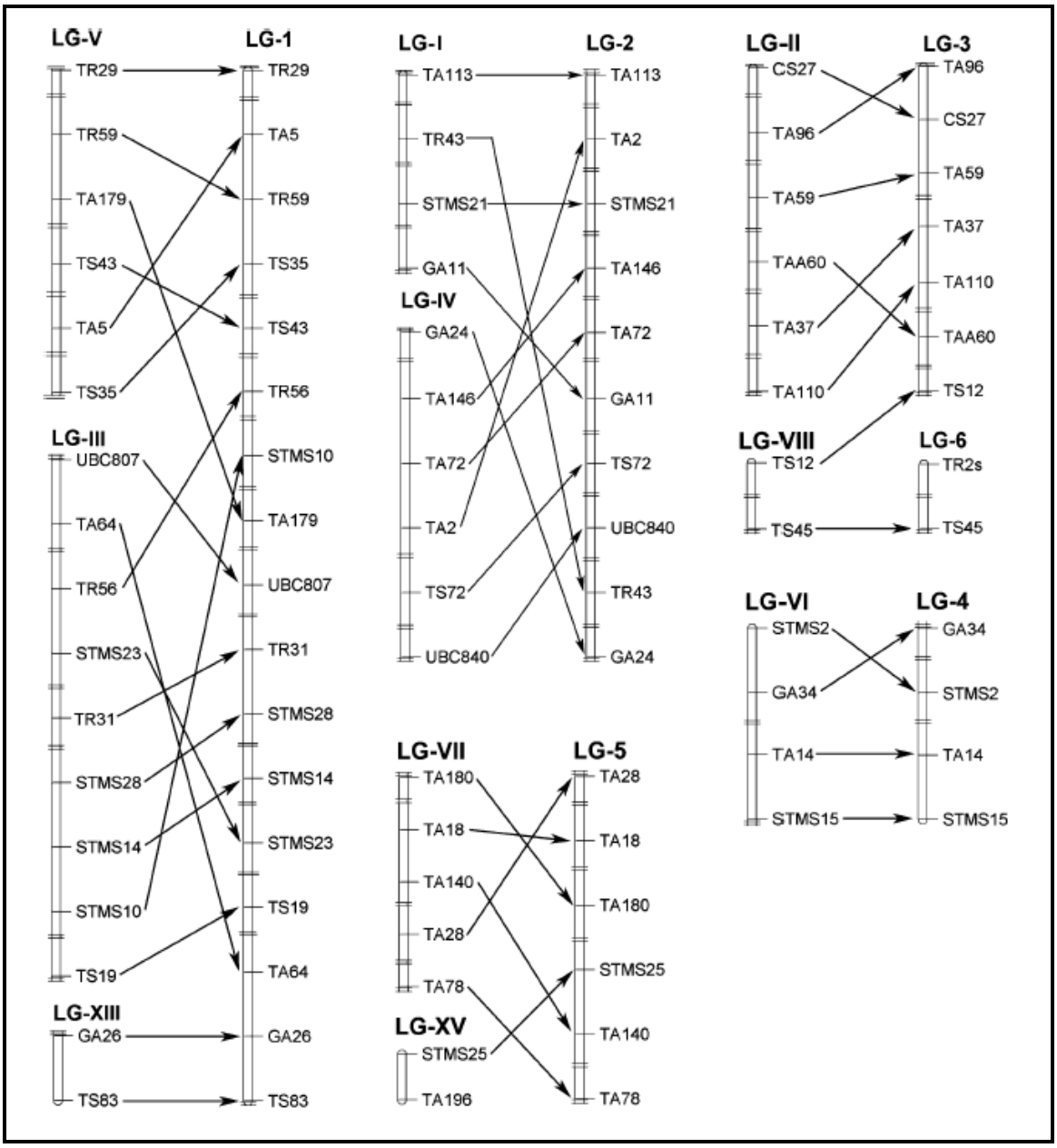


Figure 2.4: Comparison between the inter-specific map of Winter *et al.* (2000) and the present composite map. The LGs of Winter *et al*. (2000) map are designated with *Roman numerals*, whereas those of the present map are designated with *Arabic numerals.*

2.4. Discussion

The main objective of this study was to construct a saturated map of chickpea by using consensus-mapping approach. Rather than a high-density map with a fine order of markers, the purpose was to develop a framework consensus map with a general order of markers that could be used as a reference for more-precise genetic studies. This approach allows locating markers or genes for important traits, which might not segregate in one mapping population, but in the other. This is particularly important for crops like chickpea, where very low levels of polymorphism have initially been reported (Udupa *et al*., 1993; Labdi *et al.,* 1996). Such maps have been developed in pea, tomato, barley, etc. (Ellis *et al.,* 1992; Tanksley *et al*., 1992; Kleinhofs *et al.,* 1993). Availability of a saturated linkage map facilitates marker-assisted breeding as well as mapping of quantitative trait loci (QTLs). In *Vicia faba*, previously reported maps were used for identification of QTLs associated with seed weight and broomrape/ascochyta resistance (Vaz Patto *et al*., 1999; Roman *et al*., 2003, 2004). The establishment of two individual maps held great importance in the chickpea mapping studies due to intra-specific nature and possessing more number of SSR markers than the existing intra-specific maps. They not only facilitate map integration, but, could also be used in MAS for trait identification as the populations were having wilt resistance and late wilting parents.

2.4.1. JV population

One limitation of this chickpea linkage map was that, certain linkage groups were not adequately saturated with markers. Linkage groups 4, 5, and 7 comprised only four to seven markers. This could be due to a high chromosomal homology and thus, lack of recombination between the parental genotypes. This is often common when crossing two related lines of the same species, as demonstrated in cultivated chickpea, where minimal polymorphisms were observed for certain marker systems, thus making it difficult to construct genetic linkage maps (Huttel *et al*., 1999). The main feature of a genetic map

indicating completion is that the number of linkage groups obtained should be equal to the number of chromosomes in the organism (Skiba *et al*., 2003).

The present linkage map consists of seven linkage groups spanning 484.9 cM. The shortage of markers may have also been responsible for the map not completing into eight linkage groups, corresponding to the haploid chromosome number of chickpea. A difficulty associated with obtaining an equal number of linkage groups is that markers are not necessarily evenly distributed over the chromosome, but had clustered in some regions and be absent in others (Paterson, 1996). Additionally, the frequency of recombination is not equal along the chromosomes, (Young, 1994) therefore, identification of markers to these regions is more difficult. Clustering of markers at specific regions was observed for SSR and RAPD markers. Similar observation has been made by Becker *et al.* (1995) in an intra-species map of barley. In their map, AFLP markers rarely interrupted groups of RFLP markers, but were mostly clustered adjacent to them. Similarly in this current study, RAPD markers hardly disturbed SSR marker clusters. Markers that showed segregation distortions were indicated in the linkage map. Several distorted markers were clustered on LG-2 (nine) and LG-6 (nine). Different marker classes exhibited different levels of segregation distortion. Segregation distortion was more distinct for SSRs than RAPDs. Around 44 chickpea SSRs from seven LGs showed distortion. Since SSRs were ubiquitously found over the chickpea genome one should not expect high percentage of SSR marker distortion. Nevertheless, the extent of segregation distortion was not dependent on the marker type, but more on the overall segregation distortion of the region where they resided. Therefore, the difference in segregation behavior of different marker types may be attributed to the fact that they are located in different genomic regions. The average segregation distortion was 41%, which is equal to that reported for RILs (39.4±2.5%) by Xu *et al*. (1997), who surveyed 53 different populations with a known number of distortedly segregating markers. Most of the distorted loci in the JV population were skewed in favor of the parent Vijay. This might be due to accumulation of distorted alleles in the population with progressive cycles of selfing undergone in the development of the RILs (Flandez-Galvez *et al*., 2003).

2.4.2. VI population

High genome coverage was provided by this population (624 cM) comparing to the JV population (484.9 cM). This fact could be attributed to differences in linkage intensity as suggested by Laucou *et al*. (1998) in pea. The intra-specific and F_9 generation of the population reduced the biasness towards a single parent as well as heterozygosity. A significant higher recombination rate was found in this population. Such differences in recombination frequency have already been reported between male and female parents in loblolly pine (Sewell *et al*., 1999) and apple (Maliepaard *et al*., 1998). This feature had been prominent in LG-1, LG-2, LG-3 and LG-4. The high and significant correlation between the length of the LGs and the number of markers in each mapping population indicated that markers are randomly distributed among LGs. However, non-uniform marker distribution was observed for LG-5, LG-6 and LG-7 either due to clusters or gaps of markers. Non-uniformity has been reported in rapeseed by Uzunova *et al*. (1995), Foisset *et al*. (1996) and Parkin and Lydiate (1997), and in other crops such as tomato (Tanksley *et al.,* 1992). In chickpea, clusters of markers were mostly located in central regions of LGs, whereas marker density in distal regions was low. In this population except LG-1, other 6 LGs abide this hypothesis. Non-random distribution of markers, often with centrally located clusters, was also reported for sugar beet (Halldén *et al.,* 1996) and wheat (Langridge *et al*., 1995). In soybean, the random distribution of SSRs reported in the first study (Akkaya *et al*., 1995) vanished and clustering became visible when more markers were applied. Presence of homoeologous chromosomes becomes evident when multiple loci from the same markers tend to get mapped on different LGs. This evidence was proved by UBC43z of LG-1 whereas, UBC43x was found on LG-6. Like-wise UBC249x and y were on LG-6 and UBC249z on LG-4. The parental genotypes used in this study were 22.2% polymorphic at 113 microsatellite sites. Huttel *et al*. (1999) also observed 41% polymorphism using STMS markers among three

chickpea accessions. Nevertheless the level of DNA polymorphism within chickpea is quite low for high density linkage mapping in the *C. arietinum* genome, the use of chickpea SSR markers as anchor markers has provided a molecular insight into the genetic evolution of chickpea, which is a logical starting point towards intra-generic comparative mapping in *Cicer*.

2.4.3. Integrated map

Forty three common markers were used from the two separate maps as anchors to combine other markers from the maps into a composite map. By integrating the two maps, the length of the composite map increased by 18.5%, from 623.9 cM (VI map) to 739.6 cM, while the number of mapped markers increased from 166 (VI) to 228. This increase was due to the addition of molecular markers to the distal parts of the LGs (LG-2 of VI) and the filling of the gap between the two subgroups of LG-3 of VI population. A major part of the remainder was probably due to recombination-frequency differences between VI and integrated map. However, the integrated map probably has not covered non-dense regions of genes in the genome. Extensive micro satellites or random markers, such as AFLP and RAPD, would be helpful tools for filling in non-dense regions of the genome.

As the size of the two individual maps was small (93 RILs), the chances of positioning the markers accurately was lower than larger populations. The disagreements in marker order of closely linked markers between genetic maps and derivation of the most correct marker order can be facilitated by the construction of the consensus map. The consensus map provided a large number of markers along the length of the chromosome that can be used to genotype individuals for detecting recombinants, fixing loci, restoring a recurrent genetic background, or assembling complex genotypes in complex crosses (Gupta *et al.,* 1999; Huang *et al*., 2003). In addition, the consensus map is routinely used to perform bulked segregant analysis (Michelmore *et al*., 1991;

McCartney *et al*., 2003), QTL mapping (Somers *et al*., 2003a), and genome scanning. The marker density on the consensus map can provide a better choice of markers for specific breeding populations to ensure adequate polymorphic marker coverage in regions of interest. Further, the marker density on the consensus map is likely sufficient to perform association mapping and possibly linkage disequilibrium (LD) studies on germplasm collections. Some changes in marker order were observed during construction of consensus maps (Sewell *et al*., 1999; Lespinasse *et al*., 2000; Sebastian *et al*., 2000; Cervera *et al*., 2001; Jeuken *et al*., 2001; Lombard and Delourme, 2001). Small discrepancies in marker ordering may be due to mapping imprecision rather than to real rearrangements (Lombard and Delourme, 2001). In the individual maps clustering of SSR markers in certain regions has been observed. This feature allows choosing from several different markers for the same genomic region, one of which will almost certainly detect polymorphism in any given population (Cobos *et al.,* 2005). The highly significant correlation (0.70, $P < 0.001$) observed between the lengths of the LGs and the number of markers in the respective LGs, indicated random distribution of the markers in the map. However, non-uniform distribution of markers was observed in some linkage groups. This might be due to non-random sampling of the genome by the primers used, uneven distribution of recombination along the length of the LGs (Tanksley *et al*., 1992), or by clustering of some markers due to their preferential targeting of particular genomic regions (Castiglioni *et al*., 1999).

2.4.4. Comparison with inter-specific and intra-specific chickpea maps

Comparison of the present intra-specific map of chickpea with the inter-specific map developed by Winter *et al*., (2000) revealed high linkage conservation in at least five linkage groups. However, the map distances and marker orders of the common SSR markers differed, possibly due to the intra-specific nature of the mapping populations. The merging of more than one LGs of the inter-specific map with single LGs of the intra-specific map was observed. This might be due to homology and subsequent resolution of the sequences that joined the linkage groups in the intra-specific mapping populations (Flandez-Galvez *et al*., 2003a). By developing separate intra-specific maps for *C.*

arietinum and *C. reticulatum* using common SSR markers and comparing them might provide the molecular insight of the likely chromosomal rearrangements that led to the evolution of *C. arietinum* from *C. reticulatum via* inversions and minor intra and inter chromosomal translocations of DNA sequences.

In view of the intra-specific map comparison, VI became an ideal population due to marker saturation and more number of SSR markers mapped, especially the NCPGR and H series. The very recently published map of ICCV 96029 x CDC Frontier population by Taren *et al*. (2007a) was the most comparable one among the existing intra-specific maps. Except LG-6 and LG-7 of the VI population, remaining LGs showed high co-linearity with the Frontier population. Each comparable LG had same markers with the exception of minor marker re-arrangement. The current map could not be completely compared with the intra-specific map of Flandez- Galvez *et al*. (2003a), due to the less number of SSRs mapped in their population. Only LG-3 and LG-7 had same SSRs. With respect to Cobos *et al*. (2005) map, mapped SSRs were very much limited (fourteen in number) and the majority of mapped markers were RAPDs (Operon Technologies) which had not been used in the current study. Despite that, the SSRs TR43, TA113, TA42, TR59, TS35, TA5, TA3, and TA80 enhanced the co-linearity between the two maps. Lastly, Cho *et al*. (2003 and 2004) maps had similarity with LG-4 of VI population with respect to SSRs like TR54, TA146, TA2, TR20, and TA186.

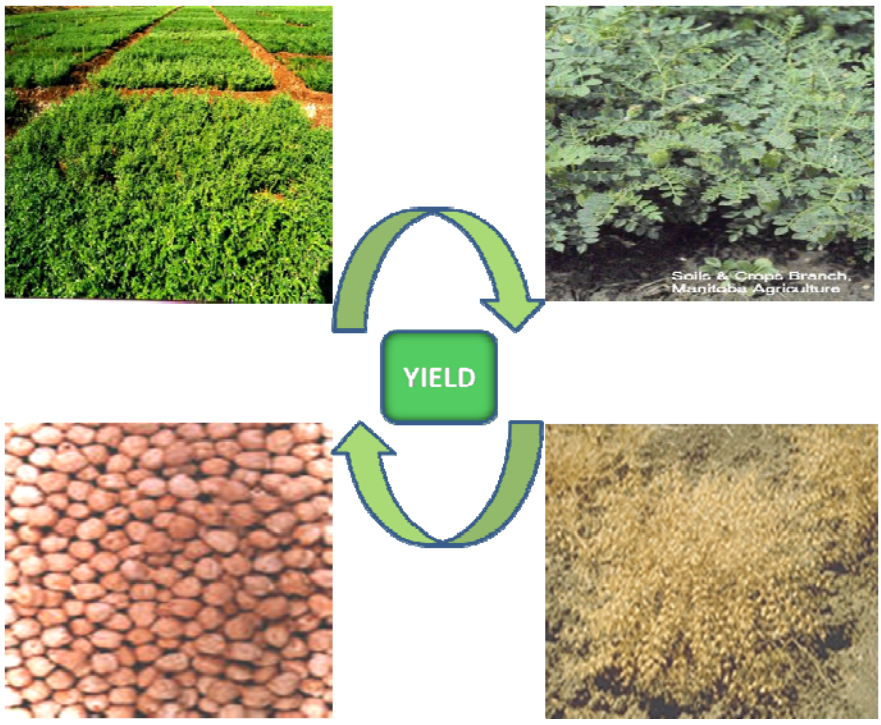
2.5. Conclusion

The integration of two genetic maps into a consensus map facilitated to acquire a general order and distances for more number of markers and less linkage gaps to get a near-complete coverage of the chickpea genome. The obtained framework consensus map can be used as a reference map to develop genetic studies in different genetic backgrounds. A map useful for marker-assisted selection requires saturation, especially in the regions harbouring agronomically important traits. There are still hundreds of microsatellites in the public domain that could be added to the present consensus map. Thus, the future aims to extend the genome coverage and attach all currently unassigned markers.

Considering all the limitations of the map integration process, this map could be seen as the best approximation, obtained with the present data. The map will be very useful for the future research since it would enable to preselect markers to saturate relevant genomic regions (where putative gene/QTL for specific traits would be located). Moreover, the work will continue in a way where the mapping of new major genes and QTLs of agronomic interest and refinement of their position in the map, to facilitate marker assisted selection in chickpea.

Chapter III

QTL mapping of agronomic traits in chickpea (*Cicer arietinum* L.)



Chapter III QTL mapping of agronomic traits in chickpea (*Cicer arietinum* L.)

3.1. Abstract

Chickpea is one of the most important leguminous cool season food crops, cultivated prevalently in the South Asia and Middle East. The improvement in chickpea has been primarily through conventional breeding, however, recent advances in biotechnology can significantly contribute for better sustainability. The main objective of this study was to identify quantitative trait loci (QTLs) associated with seven agronomic and yield traits in a F_9 derived recombinant inbred line populations having the parents; Vijay a wilt resistant variety and ICC-4958 a late wilting cultivar. Composite Interval Mapping (CIM) was conducted to identify single locus QTLs and Multiple Composite Interval Mapping (MCIM) for pleiotropic QTLs. Two-locus analysis was conducted to identify the main effect QTLs (M-QTLs), epistatic QTLs (E-QTLs) and QTL × environment interactions. A total of 42 significant QTLs and seven pleiotropic QTLs were identified for the seven traits. LG-1 possessed at least one QTL for each trait. Further studies of this LG might bring simultaneous improvement of these traits. The dissection of agronomic and yield traits in chickpea will improvise the future marker assisted selection (MAS) strategies and permit significant improvement across different environments.

3.2. Introduction

Chickpea (*Cicer arietinum* L.) is the most important legume in the Indian subcontinent in terms of the area under cultivation, production and consumption. However, in spite of its importance, chickpea productivity is very low (~850 kg/ha; FAOSTAT, 2008) in its intensively cultivated areas. The major causes of low yield of chickpea are: low yield potential and susceptibility of the present day cultivars to various biotic and abiotic stresses and lack of cultivars' response to intensive management practices like fertilizers, irrigation etc.

One of the major goals of plant breeders is to develop genotypes with high yield potential and the ability to maintain the yield across environments. With the development of molecular markers, breeders have a complimentary tool to traditional selection and

markers linked to a trait of interest could be used to assist the breeding programs. QTL mapping analysis has provided an effective approach for locating and subsequently manipulating the QTLs associated with various quantitative traits in plants (Rachid *et al*., 2004). Availability of DNA marker based maps for the genome of many crops facilitated mapping of QTLs of interest and marker-assisted selection (Winter and Kahl, 1995). However, a DNA marker map of sufficient density for use in QTL mapping of important traits is still lacking in chickpea.

Identification and mapping of the QTLs governing grain yield and other agronomic traits would increase our understanding of the genetic control of the characters and to use them effectively in breeding programs. Some of the agronomic and yield influencing traits like double-flower (Singh and van Rheenen, 1994; Kumar and Van Rheenen, 2000), flowering time (Or *et al*., 1999), chilling tolerance during flowering (Clarke and Siddique, 2003), flowers per axis (Srinivasan *et al*., 2006), double-podding and other morphological characters (Rubio *et al*., 1998, 2004; Cho *et al*., 2002; Rajesh *et al*., 2002; Lichtenzveig *et al*., 2006) and nutritional traits like β-carotene and lutein content (Abbo *et al*., 2005) have been extensively studied in chickpea. Similarly, Upadhyaya *et al*., (2002) studied the phenotypic diversity of flower color, plant color, dots on seed testa, plant width, days to maturity, pods per plant, 100-seed weight and plot yield in 1956 germplasm accessions of chickpea.

In the present study, genetic dissection of seven agronomic and yield related traits of chickpea had been carried out to identify the QTL contribution and their stability across two environments (Rahuri, Maharashtra; Dharwad, Karnataka). The two methodologies (single and two locus analysis) further confirmed the QTL accuracy. LG-1 and LG-2 were highlighted to possess more number of QTLs for the seven traits.

3.3. Materials and methods

3.3.1. Mapping population

A $F_{8:9}$ recombinant inbred line (RIL) population advanced by single seed descent method from F_2 as detailed in materials and methods of chapter II was used in the present study. The population was derived from an intra-specific cross; Vijay × ICC-4958 and comprised 108 lines. The two parental genotypes used in the present study belong to desi type, of which Vijay is a wilt resistance and drought tolerant, high pod number and wider adaptability cultivar. ICC-4958 is a late wilting, drought tolerant and bold seeded cultivar. The RIL population was grown in the field at Pulses Research Station, Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri, Maharashtra, India and University of Agricultural Sciences (UAS), Dharwad, Karnataka, India.

3.3.2. Phenotypic evaluation of agronomic and yield traits

The experiment was conducted in randomized block design with two replications during winter seasons of 2002-03, 2003-04, 2004-05, 2005-06 at MPKV, Rahuri and 2007-2008 at UAS, Dharwad. Sowing was done in 3 m rows placed 30 cm apart, accommodating 20 seeds each at 15 cm distance in a row. The two parental genotypes, Vijay and ICC-4958, also served as checks and were sown after every 10 rows of the RILs. Data of seven traits were recorded on 108 lines in the populations.

Plant height (Pht)

Plant height was obtained by measuring the height of the tallest tip of the plant from ground level at maturity and expressed in cm.

Plant spread (Psp)

Maximum horizontal spread of the plant was recorded at maturity.

Branches per plant (Brp)

Total number of branches per plant was recorded, which included both primary and secondary branches.

Pods per plant (Pdp)

The total number of pods from each observational plant was counted at the time of harvesting.

Seed weight (Swt)

Seed weight was the measure of the weight of the 100 seeds expressed in grams weighed on an electronic weighing balance.

Yield per plant (Yld)

The weight of the seeds from each observational plant was recorded in gram

Days to maturity (Dmt)

Number of days required from the date of sowing to complete maturity of the population in the plot.

Ten plants were randomly chosen from each row for trait evaluation, excluding the plants at the border or those in border rows.

3.3.3. DNA extraction and PCR analysis

The DNA extraction and PCR analysis was conducted as explained in chapter II.

3.3.4. Statistical analysis

The analysis of variance (ANOVA) of the phenotypic data was performed using "Cross site analysis module" of IRRISTAT v.5.0 (IRRI, 2005 for windows). The G×E interaction was deciphered using the AMMI (Additive Main Effects and Multiplicative Interaction) model of IRRISTAT. The sums of squares were partitioned into genotype, environment and G×E interaction. The sum of squares for G×E interaction was further

partitioned by principal components analysis (PCA) using the AMMI bi-plot model (Crossa *et al*., 1990; Gauch, 1992).

Broad sense heritability (H^2) was calculated using the formula $H^2 = 1- M2/M1$; where M1 is the mean sum of squares due to genotypes and M2 is the mean sum of squares due to genotype x environment (GXE). Correlation coefficients among the seven traits were analysed using Qgene v.2.0 (Nelson, 1998).

3.3.5. QTL analysis

The QTLs were identified through CIM (composite interval mapping) using Windows QTL Cartographer v.2.5 (Basten *et al*., 1994; Wang *et al*., 2004). For each trait, the analysis was carried out using the data from individual environments. The threshold LOD scores were calculated using 1000 permutations (Deorge and Churchill, 1996) and a LOD score of 3.0 and >3.0 was used for declaring presence of the major QTLs. In very few cases LOD >2.5 was used. Model 6 of the CIM with forward regression and backward elimination module of Windows QTL Cartographer with scanning intervals of 2.0 cM between the markers was used. The positional genetic effects and percentage of phenotypic variation of the QTLs were estimated at the significant LOD peaks in the region under consideration.

The QTLs identified using CIM for various traits were grouped as different LGs and QTL plots were drawn using QTL Mapchart (Voorrips, 2002). The QTLs were designated as Q*X.ncl-Y*, where X denotes the phenotypic trait abbreviation and Y represents the chromosome / linkage group on which the quantitative trait locus was located. Each QTL was defined on one line, along the linkage group map with the extent of QTL intervals and peak of the QTL. Different styles and colors of lines were used to represent various QTLs of different traits. Respective year, in which the data was collected is given in parentheses, 02 = 2002-2003; 03 = 2003-2004; 04 = 2004-2005; 05 = 2005-2006; 07 = 2007-2008 along with the QTL.

Multiple trait analysis involving MCIM was conducted using the module JZmap QTL available in Windows QTL Cartographer with an objective to detect pleiotropic QTLs. The confidence intervals were obtained by marking positions ± 1 LOD from the peaks. QTLs in the adjacent intervals and / or with overlapping confidence intervals were treated as a single QTL.

QTL interactions were studied by using QTL Network v.2.0 (http://ibi.zju.edu.cn/software/qtlnetwork). QTLs are mainly divided into main effective QTLs and epistatic QTLs based on their expression or interactions. P=0.05 was used as a threshold for detecting putative M-QTLs or E-QTLs. Main effective QTLs have their own genotypic effect and sometimes they showed interaction with environment also. Epistatic QTLs are usually involved in QTL x QTL interactions as well as QTL x QTL x Environment interactions.

3.4. Results

3.4.1. Trait variations and correlations

The parents, Vijay and ICC-4958, and their RIL progeny were analysed for seven quantitative traits contributing to yield and the yield related traits at two different locations. Mean values of traits for the parents and for the RILs are shown in (Table 3.1). The most significant difference has been described for the trait Swt. The parent ICC-4958 has a higher mean value of 40.6 compared to 18.2 of the other parent Vijay. The parents showed a marginal difference between them for the traits Psp (33.8-34.4) and Dmt (112-114), compared to other traits like Pht, Brp, Yld and Pdp (Table 3.1). Although difference between the mean values of the parents were small in the above mentioned traits, they were significant in the population. The population mean was higher than the parent for Pht in the population. Transgressive segregants were observed in all the environments and comparisons between the best parent and the best RIL showed a significant difference for all the traits except “days to maturity”. The phenotypic distribution for each trait in the population has been shown graphically in Figs. 3.1 to 3.4 and the

distribution analysis of all the seven quantitative traits from the five environments showed nearly a normal distribution. The analysis of variance (ANOVA) revealed significant differences among the parental genotypes for all the seven traits. Trait-wise ANOVA analysis is given in the Table 3.2 and 3.3. As depicted in Table 3.1, the index of broad-sense heritability showed a range of non-zero values from 36% to 84% for all the traits. Swt has the highest heritability value (84%). Next to that was plant height with 77% heritability while, Brp, Dmt and Pdp showed a value of range from 62% - 64%. Yield heritability was less (52%) compared to other traits whereas; plant spread was the least heritable trait (36 %).

Simple correlation coefficients among the traits were calculated and are presented in Table 3.4 and its continuations. Correlations among traits were evaluated at $P < 0.05$, $P < 0.01$ and $P < 0.001$. The correlations were analysed for four years ; 2002-2003, 2003-2004, 2004-2005 and 2005-2006 at MPKV, Rahuri and one year, 2007-2008 UAS, Dharwad. While considering the correlation between the seven traits, Yld was significantly and positively correlated with traits like Pht, Psp, Brp and Pdp in the year (2002-2003); however this phenomenon was not observed likewise in the next years (2003-2004, 2004-2005, 2005-2006 and 2007-2008). Similarly Swt was also correlated with Pht and Yld in a significant and positive manner. Dmt failed to show any promising correlation with the remaining six traits. The highest significant correlation value of 0.779 ($P < 0.001$) was exhibited between Pdp and Brp in the year 2003-2004. The least non-significant correlation was observed between Dmt and Pht in the year 2004- 2005 (0.001). A total of 76 significant and positive correlations were observed for all the seven traits in five years. Only two negatively significant correlations were seen between Pht: 2004-2005, Brp: 2002-2003 (-0.271) and Psp: 2004-2005, Swt: 2002-2003 (-0.205).

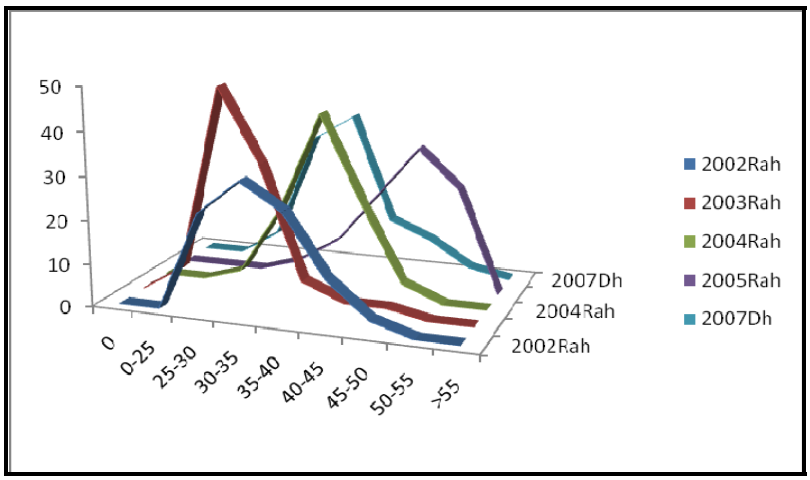
3.4.2. AMMI (Additive main effect and multiplicative interaction) analysis

Using AMMI model, the magnitude and significance of the effects of GE interaction and its interaction principal components relative to the effects of G and E was estimated. The stability and adaptability of specific cultivars were assessed by plotting their phenotypic

values at specific environments. The analysis of variance (ANOVA) for the seven traits with AMMI model is presented in Table 3.2 and 3.3. For all the traits AMMI model dissected the GEI into 4 principal components, the first interaction principal component axes (IPCA1) and the second component (IPCA II) score accounted for a large portion of the sum of squares with GEI for all the traits.

Table 3.1: Parental values and population distribution parameters of the agronomic and yield traits in the population

Traits	Parental lines		Population		Heritability
	Vijay	ICC-4958	Mean	Range	
Plant height (cm) (Pht)	40.2±4.5	44.6±4.3	47.6±6.3	31.7 – 63.0	77%
Plant spread (cm) (Psp)	33.8±2.5	34.4±5.2	29.6±8.1	8.7 - 50.0	36%
Number of branches per plant (Brp)	12.8±1.9	17.2±2.8	14.4±3.9	7.0 – 26.7	62%
Number of pods per plant (Pdp)	52.0±4.6	61.8±8.7	47.3±20.1	11.7 – 120.3	64%
Yield per plant (g) (Yld)	10.2±1.5	17.1±4.5	10.8±4.5	2.5 – 25.1	52%
100-seed weight (g) (Swt)	18.2±0.8	40.6±2.4	25.1±6.1	13.1 - 40.0	84%
Days to maturity (Dmt)	112.0±3.0	114.0±3.7	112.6±18.2	103.0 – 118.0	63%



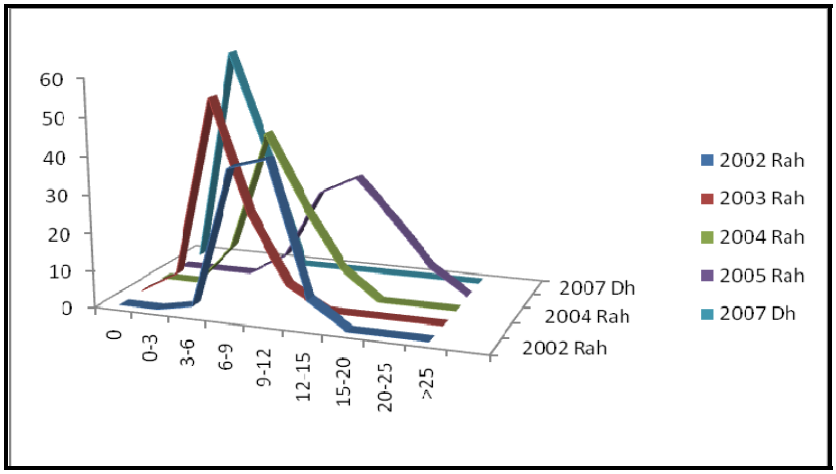
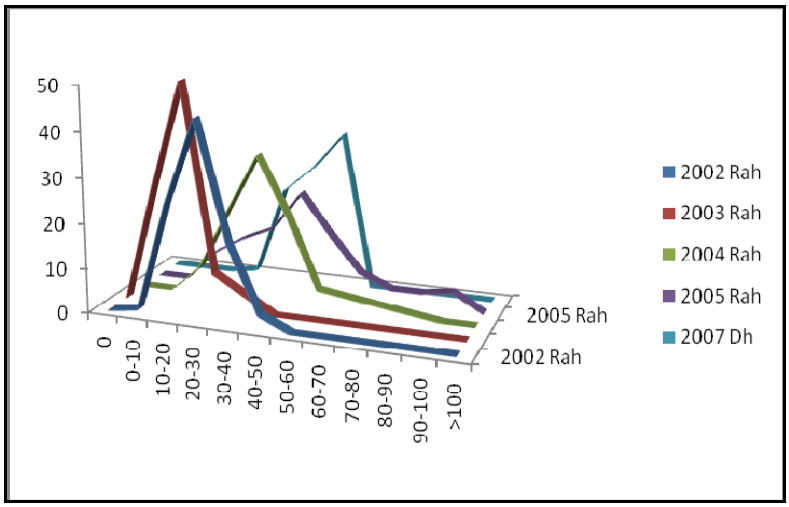


Figure 3.1: Pht and Brp frequency distribution in the population



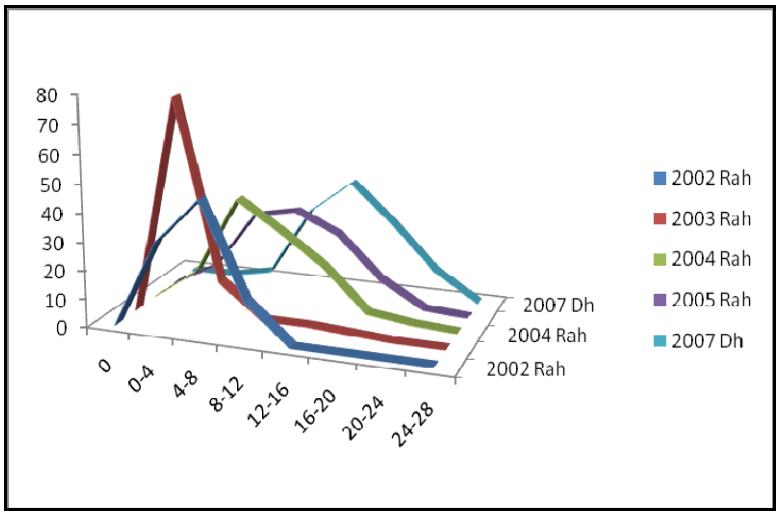
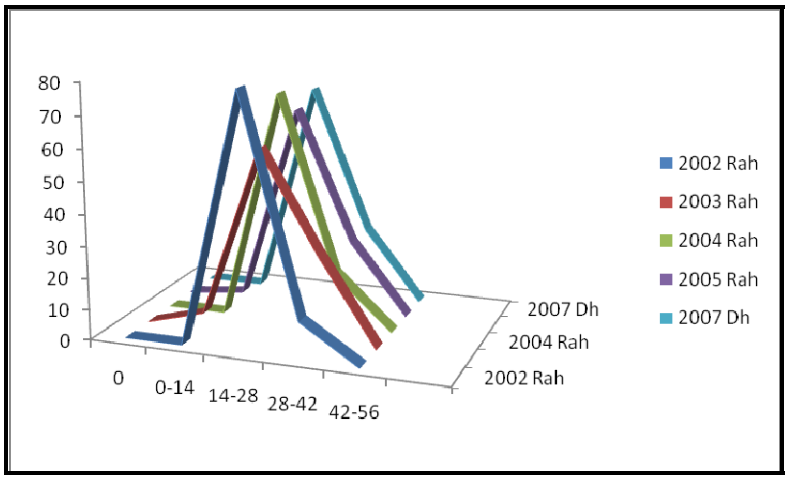


Figure 3.2: Pdp and Yld frequency distribution in the population



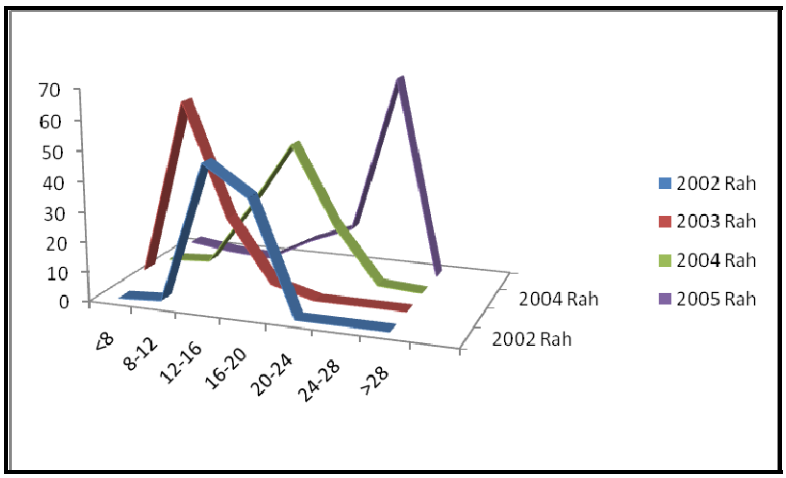


Figure 3.3: Swt and Psp frequency distribution in the population

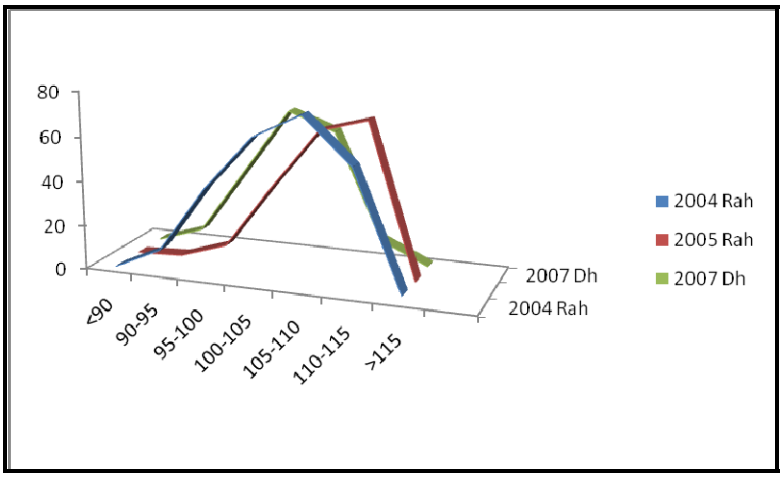


Figure 3.4: Dmt frequency distribution in the population

3.4.2.1. Plant height (Pht)

The AMMI analysis of variance of Pht tested in the five environments showed that 29.71% of the total sum of squares (TSS) was attributed to genotype x environmental effect, 15.19% to genotypic effect and a major part of 55.40% to the environment effect (Table 3.2). The 29.71% of GEI contribution was almost equally shared by the three IPC components. The AMMI2 bi-plot (Fig. 3.5) explained 64.1% of the GE interaction. Environments A, B and C (2002-2003, 2003-2004 and 2004-2005) showed similar type of interaction with the genotypes but environment D (2005-2006) comparatively showed

more (GEI) interaction. The environment E (2007-2008) was also slightly deviated from the patterns of A, B and C actions but, not to the extent of D.

Pht						Brp					
Source	**df**	**S.S.**	**M.S.**	**F**	**%explained**	**Source**	**df**	**S.S.**	**M.S.**	**F**	**%explained**
G	92	4106.90	73.6246		15.19	**G**	92	503.588	5.47379		6.03
E	4	14973.5	1051.81		55.40	**E**	4	5807.78	1451.95		69.56
GxE	363	8032.15	41.2199		**29.71**	**GxE**	363	2007.80	5.53114		**24.05**
IPCA 1	95	2952.30	82.8394	1.640***	10.92	**IPCA 1**	95	1207.41	12.7096	4.256***	14.46
IPCA 2	93	2193.00	32.948	1.429*	8.11	**IPCA 2**	93	417.405	4.48822	2.051***	4.99
IPCA 3	91	1832.51	31.0755	1.604***	6.78	**IPCA 3**	91	297.960	3.27429	3.235***	3.56
IPCA 4	89	1054.35	15.8105		3.90	**IPCA 4**	89	85.0251	0.955338		1.018
TOTAL	**459**	**27027.2**	**58.88**			**TOTAL**	**459**	**8348.39**	**18.18**		

Pdp						Swt					
Source	**df**	**S.S.**	**M.S.**	**F**	**%explained**	**Source**	**df**	**S.S.**	**M.S.**	**F**	**%explained**
G	92	10419.7	113.257		7.233	**G**	92	8506.87	94.4659		59.18
E	4	76992.7	19248.2		53.44	**E**	4	113.837	28.4593		0.79
GxE	363	56314.0	155.135		**39.09**	**GxE**	359	5759.79	16.0440		**40.07**

IPCA 1	95	34220.1	360.211	4.369***	23.75	**IPCA 1**	95	2338.63	24.6171	1.906***	16.27
IPCA 2	93	12043.1	129.495	2.255***	8.36	**IPCA 2**	93	1700.60	18.2861	1.817***	11.83
IPCA 3	91	5615.33	61.7069	1.169*	3.89	**IPCA 3**	91	1011.25	11.1126	1.253*	7.0357
IPCA 4	89	4435.58	49.8380		3.079	**IPCA 4**	89	709.311	7.96979		4.93
TOTAL	**459**	**144054.0**	**313.84**			**TOTAL**	**455**	**14373.1**	**31.58**		

Table 3.2: Analysis of variance for Pht, Brp Pdp and Swt

Significance of AMMI components was indicated with asterisk symbol ***P<0.001, *P<0.05; G= genotype; E= environment; IPCA1= first interaction principal component axes, IPCA2= second interaction principal component axes, IPCA3= third interaction principal component axes, IPCA4 = fourth interaction principal component axes; df = degree of freedom; S.S= sum of squares; M.S = mean sum of squares; F =statistical test of significance.

Table 3.3: Analysis of variance for Psp, Dmt and Yld

Psp						Dmt					
Source	**df**	**S.S.**	**M.S.**	**F**	**%explained**	**Source**	**df**	**S.S.**	**M.S.**	**F**	**%explained**
G	92	2092.64	22.7461		8.62	**G**	92	14489.1	157.49		16.4
E	3	16196.2	5398.73		66.72	**E**	3	33841.8	11280.6		38.4
GxE	271	5853.53	21.5997		**24.11**	**GxE**	276	39854	144.398		**45.2**
IPCA 1	94	4479.98	47.6593	6.142***	18.45	**IPCA 1**	94	37473.9	398.658	30.484	42.5
IPCA 2	92	746.520	8.11435	1.100*	3.07	**IPCA 2**	92	2301.06	25.0115	28.474	2.6
IPCA 3	90	627.028	6.96698		2.58	**IPCA 3**	90	79.0547	0.87839	******	0.9
TOTAL	**366**	**24274.7**	**66.32**			**TOTAL**	**371**	**88184.8**	**237.6**		

Yld					
Source	**df**	**S.S.**	**M.S.**	**F**	**%explained**
G	92	1188.86	12.9224		9.85
E	4	6757.51	1689.38		55.99
GxE	363	4098.04	11.2894		**33.95**

IPCA 1	95	1792.58	18.8693	2.193**	14.85
IPCA 2	93	1061.22	11.4109	1.605*	8.79
IPCA 3	91	806.785	8.86577	1.702*	6.68
IPCA 4	89	437.452	4.91519		3.625
TOTAL	**459**	**12067.5**	**26.2**		

Significance of AMMI components was indicated with asterisk symbol ***P<0.001, *P<0.05; G= genotype; E= environment; IPCA1= first interaction principal component axes, IPCA2= second interaction principal component axes, IPCA3= third interaction principal component axes, IPCA4 = fourth interaction principal component axes; df = degree of freedom; S.S= sum of squares; M.S = mean sum of squares; F =statistical test of significance

3.4.2.2. Plant spread (Psp)

Environment had played a major role in plant spread by showing 66.72% of total phenotypic variation (Table 3.3). Genotype x environment interaction contributed 24.11% and genotype effect was very less with 8.62% of the total variation. The AMMI2 bi-plot analysis (Fig. 3.5) showed that all the three environments were highly diverse, with 100% of total GE interactions.

3.4.2.3. Branches per plant

The results of AMMI analysis (Table 3.2) revealed that only 29.71% of total variability was justified by the GE interaction, whereas major 55.40% by the environment and 15.19% by the genotypic effect. The AMMI2 bi-plot analysis (Fig. 3.6) showed that, environments B and C had intense GEI. Nevertheless environment D (2005-2006) had the highest variation while A, (2002-2003) had very low GEI. The AMMI2 bi-plot explained 64.1% of the total GE interactions.

3.4.2.4. Pods per plant

Analysis of variance for Pdp showed that GE interaction had major influence, with 39.09% of the total phenotypic variation (Table 3.2). Another 53.44% was governed by environmental effect and remaining 7.233% was due to the genotypic effect. AMMI bi-plot analysis showed 82.2% (Fig. 3.6) interaction with the three environments. Environment D (2005-2006) and C (2004-2005) were involved in high GE interactions but environment E (2007-2008) showed the least interactions followed by B (2003-2004). RIL number 52 was noticed to be much adapted to an environment like C (2004-2005). Environment D (2005-2006) was specific for many RILs.

Table 3.4: Simple correlations among the yield and yield related traits

Trait	Pht02	Psp02	Brp02	Pdp02	Yld02	Swt02	Pht03	Psp03
Psp02	0.429***							
Brp02	0.395***	0.631***						
Pdp02	0.43***	0.582***	0.757***					
Yld02	0.583***	0.544***	0.658***	0.768***				
Swt02	0.508***	0.139	0.147	0.151	0.51***			
Pht03	0.412***	-0.052	0.038	-0.035	0.21*	0.389***		
Psp03	-0.019	-0.116	-0.098	-0.097	0.088	0.136	0.123	
Brp03	0.195	0.056	0.009	0.094	0.216*	0.177	0.308****	0.56***
Pdp03	0.096	0.091	-0.019	0.085	0.184	0.21	0.31***	0.491***
Yld03	0.175	0.17	0.063	0.137	0.211*	0.341***	0.503***	0.247***
Swt03	0.442***	0.043	-0.065	-0.016	0.356***	0.559***	0.421***	0.302***
Pht04	0.074	-0.186	-0.271**	0.371***	-0.175	0.197	0.227**	0.171
Psp04	-0.104	-0.101	-0.02	-0.099	-0.097	-0.205*	-0.122	0.137
Brp04	-0.067	-0.005	0.083	0.075	-0.017	-0.105	-0.107	0.13

Pdp04	-0.077	-0.04	-0.061	-0.123	0.001	-0.03	0.025	0.345***
Yld04	-0.064	-0.121	-0.036	-0.129	0.078	0.061	0.079	0.276***
Swt04	0.388***	0.024	-0.087	-0.064	0.351***	0.543***	0.37***	0.229**
Pht05	0.148	0.082	0.12	0.052	0.151	0.162	0.117	0.103
Psp05	-0.186	-0.148	-0.034	0.028	0.041	-0.049	-0.073	0.029
Brp05	-0.158	-0.057	0.07	-0.024	-0.062	0.005	-0.048	-0.11
Pdp05	-0.265**	-0.14	-0.104	-0.191	-0.28	-0.106	-0.057	-0.213
Yld05	0.03	-0.011	0.016	-0.064	-0.104	0.084	-0.037	-0.159
Swt05	0.268**	-0.047	-0.067	-0.095	0.169	0.325***	0.278**	0.157
Dmt04	0.013	-0.101	-0.093	0.035	0.006	0.058	0.095	0.116
Dmt05	-0.158	0.054	0.1	-0.117	-0.084	-0.177	0.058	-0.126

Significant at ***P<0.001, **P<0.01, *P<0.05 (environment 02=2002; 03=2003; 04=2004; 05=2005)

Table 3.4: Simple correlations among the yield and yield related traits (Contd……)

Trait	Brp03	Pdp03	Yld03	Swt03	*Pht04	Psp04	Brp04
Psp02							
Brp02							
Pdp02							
Yld02							
Swt02							
Pht03							
Psp03							
Brp03							
Pdp03	0.779***						
Yld03	0.456***	0.514***					
Swt03	0.214*	0.1	0.37***				
Pht04	0.08	-0.024	0.129	0.218*			
Psp04	-0.065	-0.167	-0.026	-0.035	0.2918**		
Brp04	0.055	-0.038	0.182	-0.099	0.208*	0.624***	
Pdp04	0.061	-0.056	0.075	0.091	0.248*	0.566***	0.582***
Yld04	0.07	-0.015	0.083	0.178	0.295**	0.469***	0.501***
Swt04	0.139	0.027	0.293**	0.742***	0.275**	0.059	-0.115
Pht05	0.196*	0.096	0.097	0.248*	0.179	0.007	-0.169
Psp05	0.034	-0.055	0.096	0.048	0.096	0.138	0.017
Brp05	-0.164	-0.159	-0.076	-0.04	0.073	-0.057	-0.083
Pdp05	-0.205*	-0.256**	-0.092	-0.046	0.189	-0.096	-0.138
Yld05	-0.104	-0.175	-0.057	0.083	0.198*	-0.081	-0.124
Swt05	0.205*	0.063	0.165	0.453***	0.318***	0.007	-0.196*
Dmt04	-0.003	0.073	0.016	0.179	-0.033	-0.193*	-0.191

Dmt05	-0.138	-0.144	-0.142	-0.137	0.001	0.018	-0.281**

Significant at ***P<0.001, **P<0.01, *P<0.05 (environment 02=2002; 03=2003; 04=2004; 05=2005)

Table 3.4:- Simple correlations among the yield and yield related traits (Contd……)

Trait	Pdp04	Yld04	Swt04	Pht04	Psp04	Brp04	Pdp04
Psp02							
Brp02							
Pdp02							
Yld02							
Swt02							
Pht03							
Psp03							
Brp03							
Pdp03							
Yld03							
Swt03							
Pht04							
Psp04							
Brp04							
Pdp04							
Yld04	0.643***						
Swt04	0.09	0.27**					
Pht05	-0.021	-0.027	0.295**				
Psp05	-0.012	0.202*	0.013	0.088			
Brp05	-0.095	-0.093	-0.096	0.113	0.428***		
Pdp05	-0.115	-0.125	-0.043	0.083	0.343***	0.663***	
Yld05	-0.079	-0.089	0.142	0.256**	0.111	0.538***	0.687***
Swt05	-0.05	0.059	0.584***	0.573***	-0.091	0.107	0.159
Dmt04	-0.151	-0.134	-0.021	0.082	-0.038	-0.071	-0.113

Dmt05	-0.048	-0.144	-0.101	0.379***	-0.161	0.139	0.18

Significant at ***P<0.001, **P<0.01, *P<0.05 (environment 02=2002; 03=2003; 04=2004; 05=2005)

Table 3.4: Simple correlations among the yield and yield related traits (Contd……)

Trait	Yld05	Swt05	Dmt04
Psp02			
Brp02			
Pdp02			
Yld02			
Swt02			
Pht03			
Psp03			
Brp03			
Pdp03			
Yld03			
Swt03			
Pht04			
Psp04			

Brp04			
Pdp04			
Yld04			
Swt04			
Pht05			
Psp05			
Brp05			
Pdp05			
Yld05			
Swt05	0.453***		
Dmt04	-0.107	0.125	
Dmt05	0.168	0.248*	-0.039

Significant at ***P<0.001, **P<0.01, *P<0.05 (environment 02=2002; 03=2003; 04=2004; 05=2005)

3.4.2.5. Yield per plant

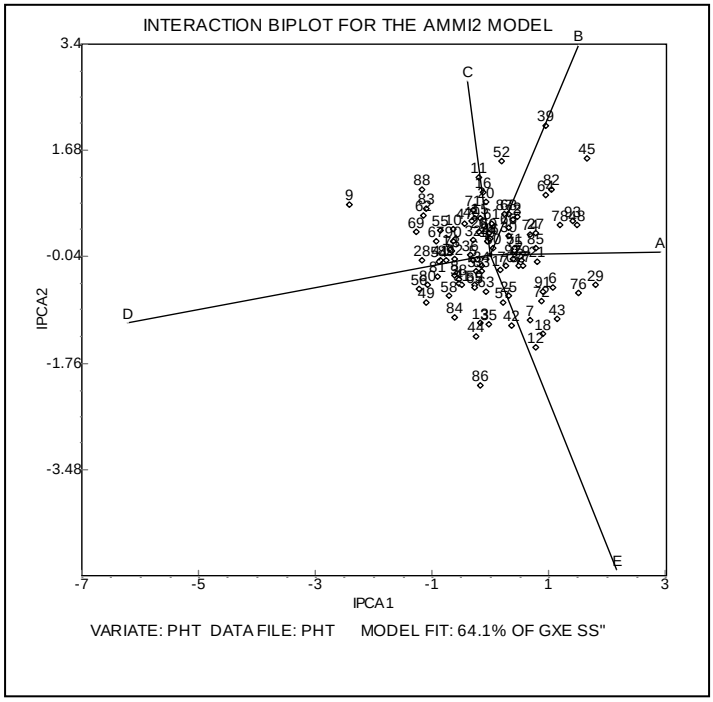
Most of the phenotypic variation for yield was explained by environment interaction; 38.4% and GxE interaction; 45.2% (Table 3.3). AMMI bi-plot (Fig 3.7) showed the first two components with a 69.6% of total interaction. Environments C and D showed high variation comparing to A, B and E. Excluding very few RILs, others are well adapted for the five environments.

3.4.2.6. Seed weight

Environment played the least role for 100 seed weight with a value of 0.79% of the total variation. Genotypic contribution had the highest value (59.18%) while GE interaction governed 40.07% of the total variation (Table 3.2). Bi-plot (Fig. 3.7) showed 70% variation. Environments B (2003-2004), D (2005-2006) had very high interaction while A and E were slightly less, however, environment C seems to be the best among the five environments. This trait was observed to be highly environment specific for many RILs.

3.4.2.7. Days to maturity

AMMI for Dmt showed that GE interaction influenced 45.2% of total variation followed by environmental effect with 38.4% and the least 16.4 % explained by genotypic effect (Table 3.3). The AMMI2 bi-plot showed that the two principal components explained 100% of the total GE interactions (Fig. 3.8). The environment A (2002-2003) and B (2003-2004) imposed equal effects on the genotypes except the RIL number 45.



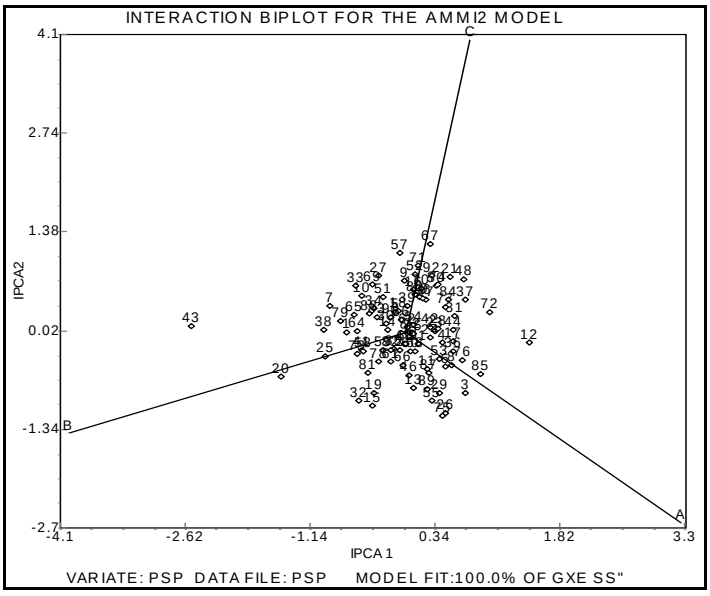
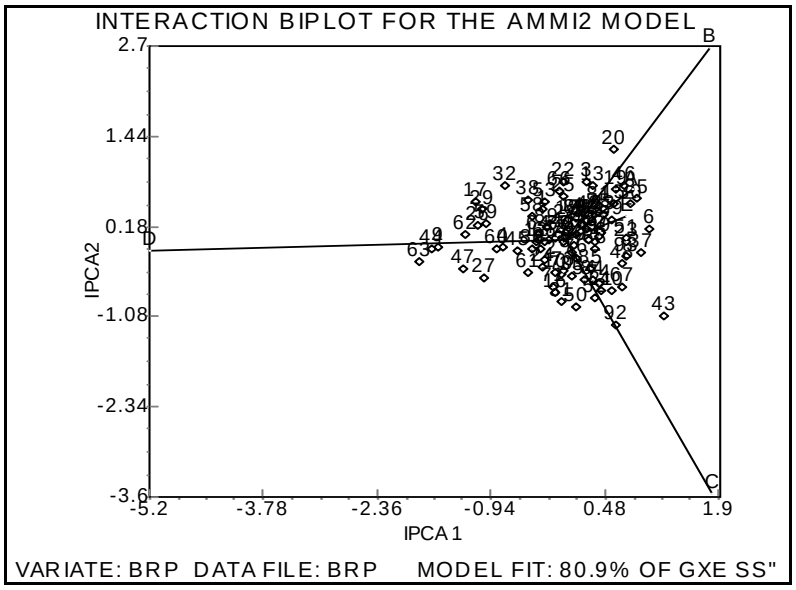


Figure 3.5: Bi-plot of Pht and Psp

A, B, C, D, E denotes the environments; A= 2002-2003, B= 2003-2004, C= 2004-2005, D= 2005-2006, E= 2007-2008; Genotypes: (1 to 93)



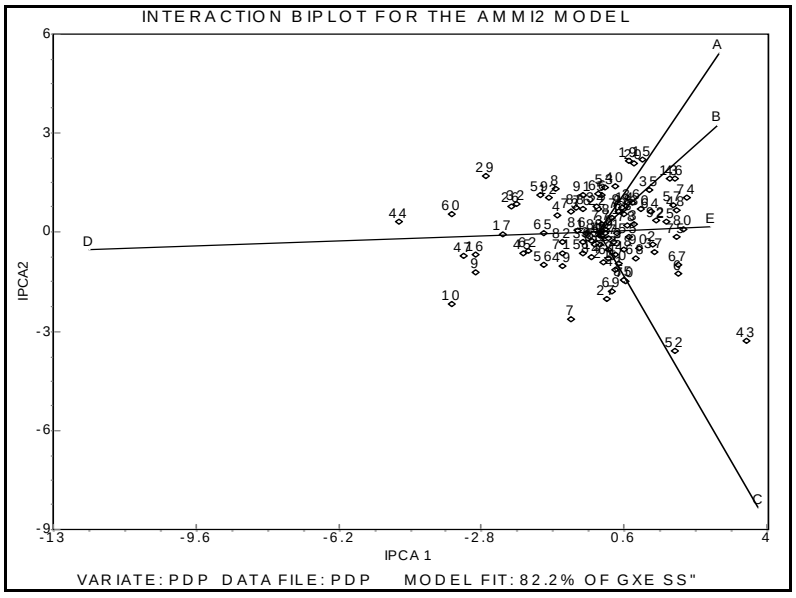


Figure 3.6: Bi-plot of Brp and Pdp

A, B, C, D, E denotes the environments; A = 2002-2003, B = 2003-2004, C = 2004-2005, D = 2005-2006, E = 2007-2008; Genotypes: (1 to 93)

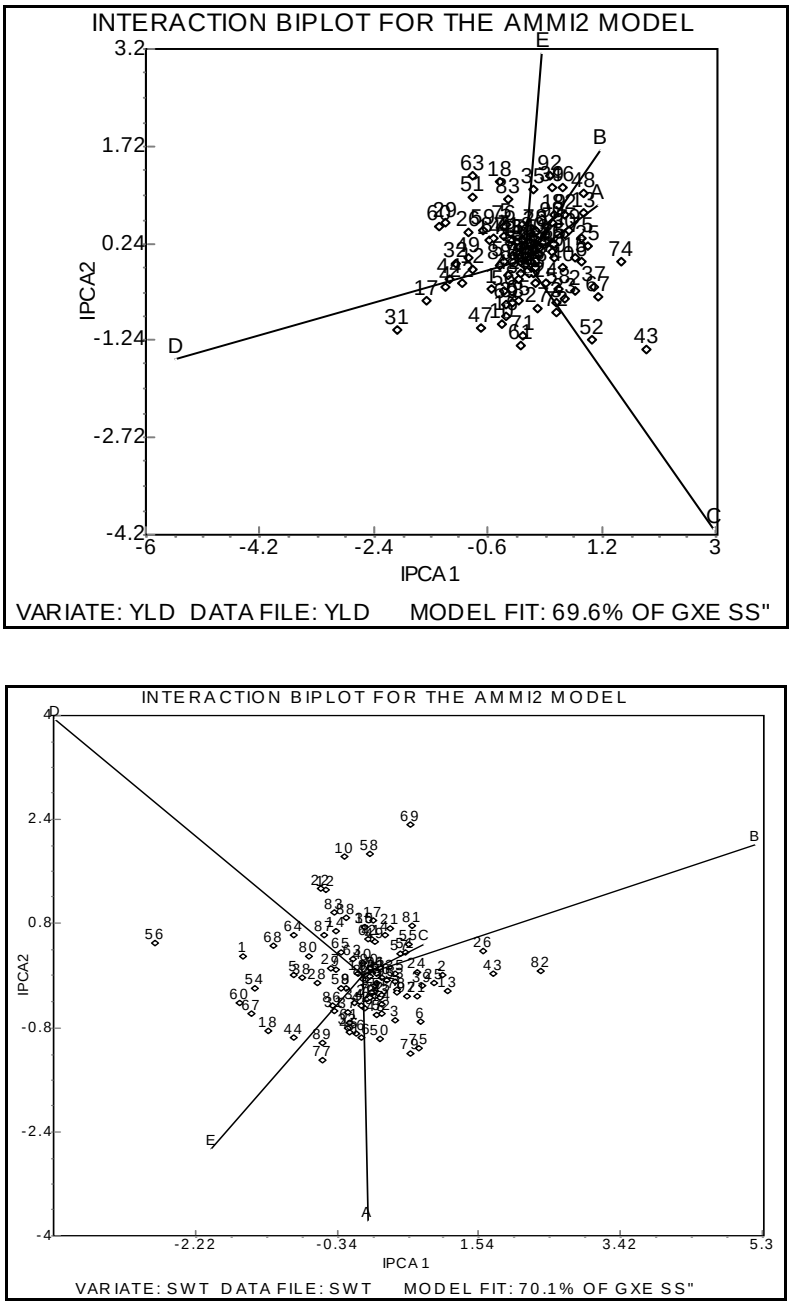


Figure 3.7: Biplot of Yld and Swt

A, B, C, D, E denotes the environments; A = 2002-2003, B = 2003-2004, C = 2004-2005, D = 2005-2006, E = 2007-2008; Genotypes: (1 to 93)

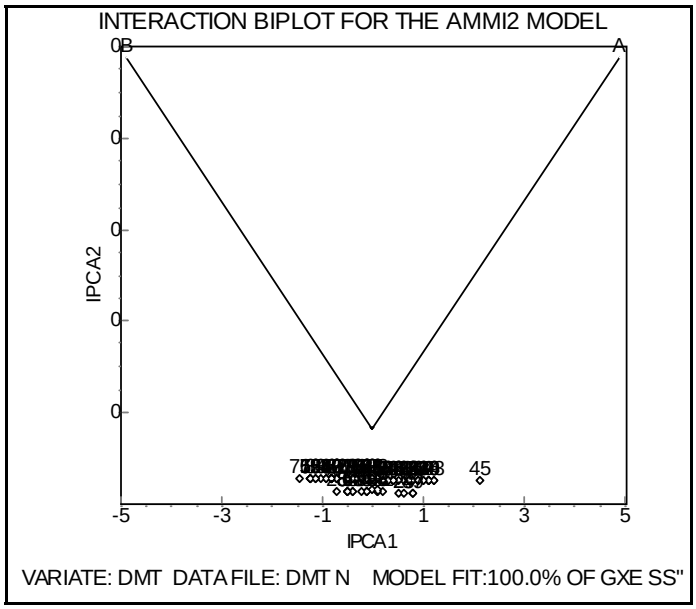


Figure 3.8: Biplot of Dmt

A, B, C, D, E denotes the environments; A= 2002-2003, B = 2003-2004, C = 2004-2005, D = 2005-2006, E = 2007-2008; Genotypes: (1 to 93)

3.4.3. Single locus QTL analysis [Composite Interval Mapping (CIM)]

A Total of 74 QTLs were detected for the seven traits. Among them 41 were significant QTLs (LOD ≥ 3.0) (Table 3.5) and 33 were suggestive QTLs (2.0 > LOD < 3.0). The QTLs were mapped on the respective 8 LGs of the chickpea genome (Fig. 3.9). The number of QTLs for individual traits ranged from three (Dmt and Brp) to sixteen (Psp). More number of QTLs were detected in the A (2002-2003) environment (21 QTLs) followed by 19 QTLS from environment B (2003-2004). Most of the QTLs were environment specific, however seven QTLs were stable and expressed in more than one environment. The QTL; *QBrp.ncl-1.1* accounted for the highest phenotypic variation (23.84%) while the lowest variation was exhibited by the QTL; *Swt.ncl-3.3* with 6.27% variation. Interestingly, the SSR marker, TAA170 was associated with QTLs of many traits, *viz.* Pht, Brp, Yld and Swt. This feature accounts for the pleiotropic effect in the population. LG-1 possessed the highest number of QTLs (30) and had at least one QTL

for each trait except for Dmt. LG-2 had 15 QTLs and LG 4 had 11 QTLs. Only one QTL was mapped on LG-8. LG-3, LG-5, LG-6 and LG-7 had 7, 4, 2 and 7 QTLs respectively. ICC-4958 allelic contribution was more than Vijay for all the seven traits (Table 3.5). Out of 74 QTLS, 47 had the contribution from ICC-4958 and 27 from Vijay alleles (Table 3.5). The traits Pht, Psp, Swt, Dmt, Pdp and Yld were positively influenced by ICC-4958 alleles.

3.4.3.1. QTLs of Pht

Fourteen QTLs were identified for Pht which were dispersed on six linkage groups (Table 3.6). Out of which, eight were significant QTLs. LG-2 and LG-6 did not possess any Pht QTLs. Single QTL (*QPht.ncl-1.4)* was detected in Dharwad location, and the remaining QTLs were specific for Rahuri. Contribution by the QTLs to the phenotypic variation ranged from 7.79 to 17.35%. Both Vijay and ICC-4958 contributed for increase as well as decrease of Pht through seven QTLs each (Table 3.5). Majority of QTLs were on LG-1 (ten QTLs) (Fig. 3.9) whereas QTLs; *QPht.ncl-1.2* and *QPht.ncl-1.7* were stable across two environments.

3.4.3.2. QTLs of Psp

Sixteen QTLs were identified on six different LGs for plant spread (Table 3.7). Among them, seven were significant QTLs. LG-3 and LG-8 totally lacked Psp QTLs. Contribution to the phenotypic variation ranged from 6.5 to 14.27%. Each parent gave equal contribution (eight) of QTLs. All the 16 QTLs were completely environment specific and failed to express stable QTL.

3.4.3.3. QTLs of Brp

For trait Brp, only three QTLs were identified on two LGs with two on LG-1 and one on LG-7 (Table 3.8). *QBrp.ncl-1.1* explained 23.84% (LOD 4.49) of total phenotypic variation. Vijay allele influenced the trait branches per plant. *QBrp.ncl-1.1* was stable across two environments.

Table 3.5: Number of significant QTLs identified and the contribution of each parent towards trait enhancing alleles of the QTLs

S. No.	Trait	No. of QTLs	No. of trait enhancing alleles contributed by each parent	
			Vijay	ICC-4958
1	Pht	14	7	7
2	Psp	16	8	8
3	Brp	3	2	1
4	Pdp	10	6	4
5	Yld	15	3	12
6	Swt	13	1	12
7	Dmt	3	1	2
	Total	**74**	**28**	**46**

LG-1

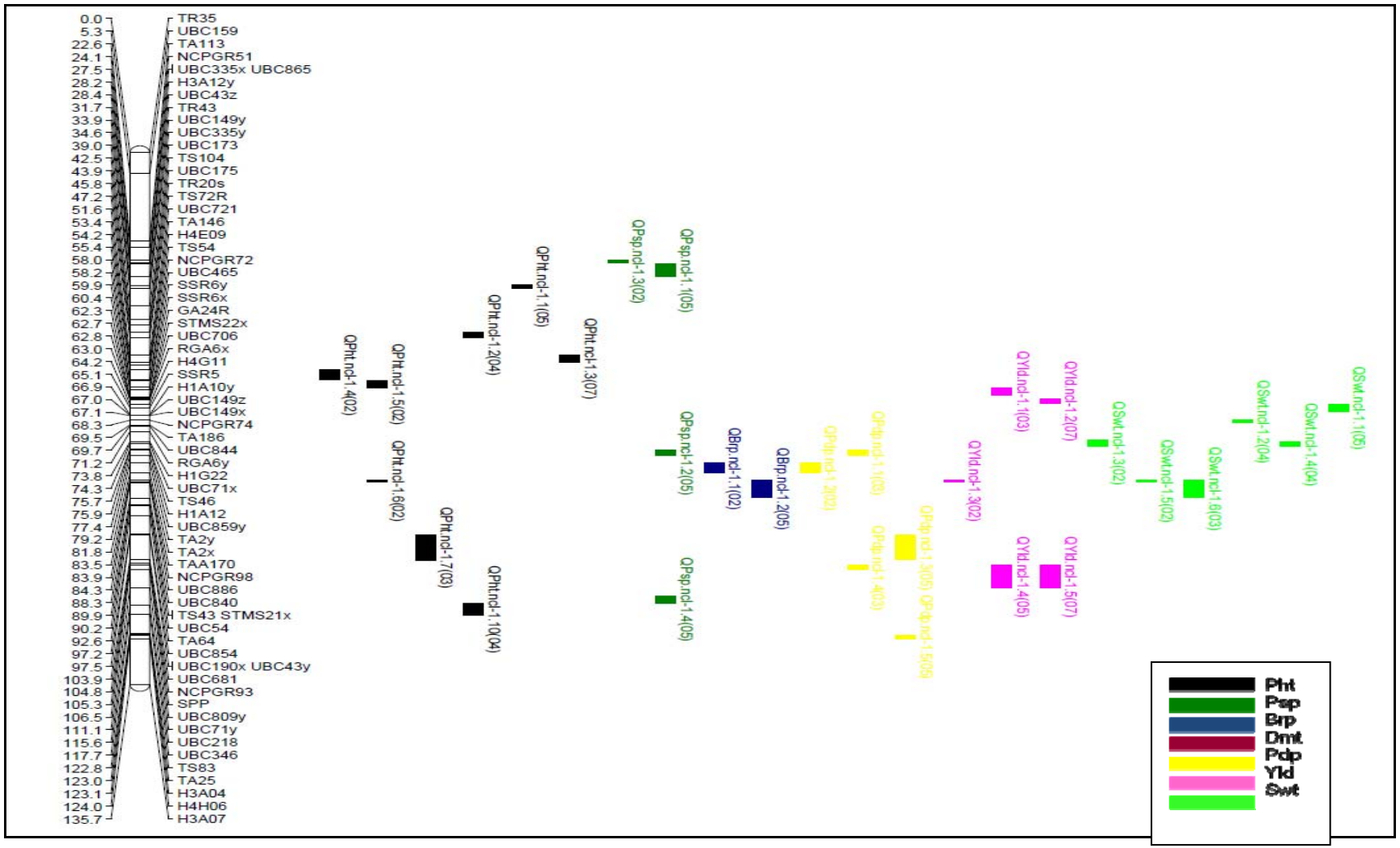


Figure 3.9: The linkage map showing QTLs for seven quantitative traits detected in the Vijay x ICC-4958 population

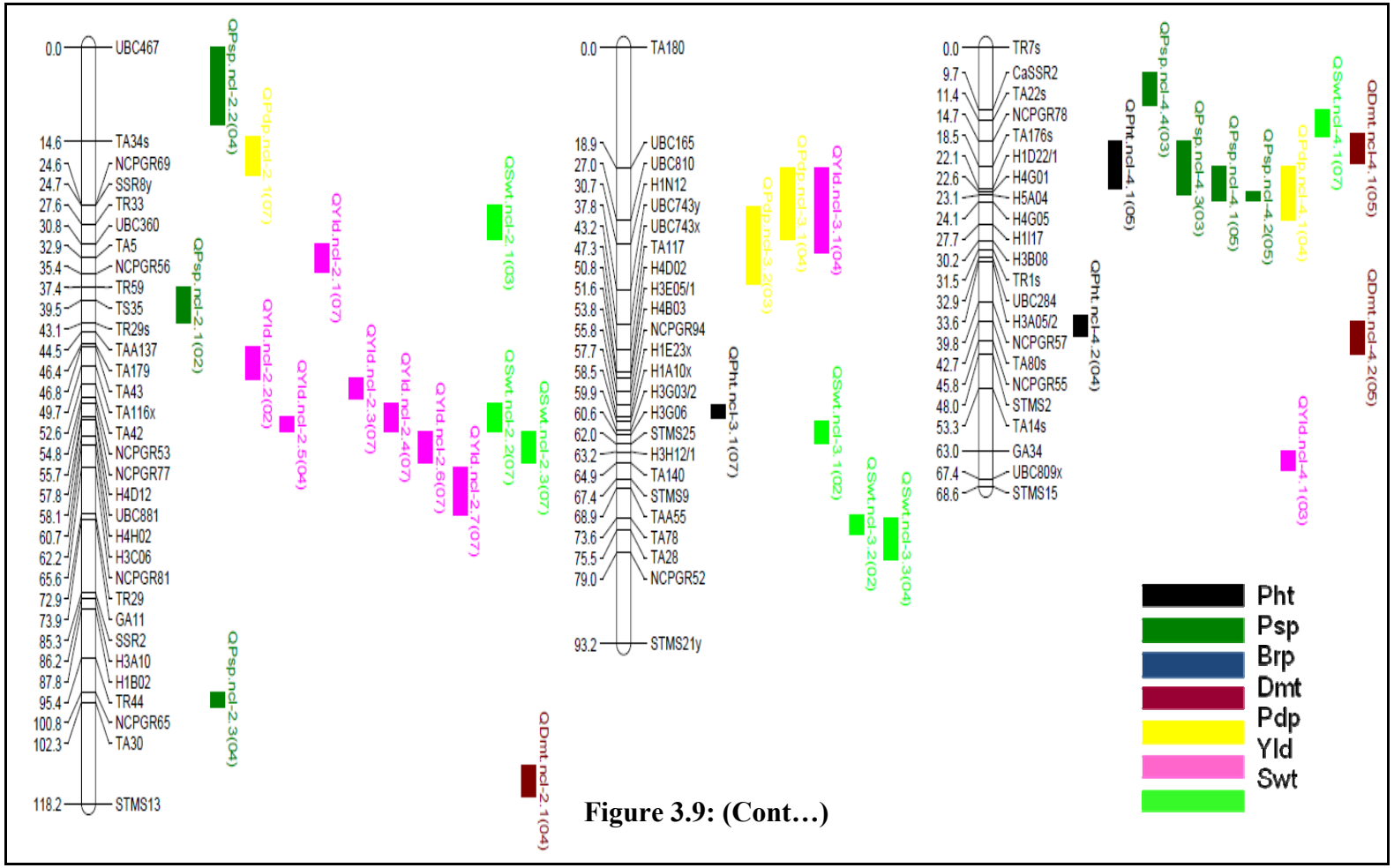


Figure 3.9: (Cont…)

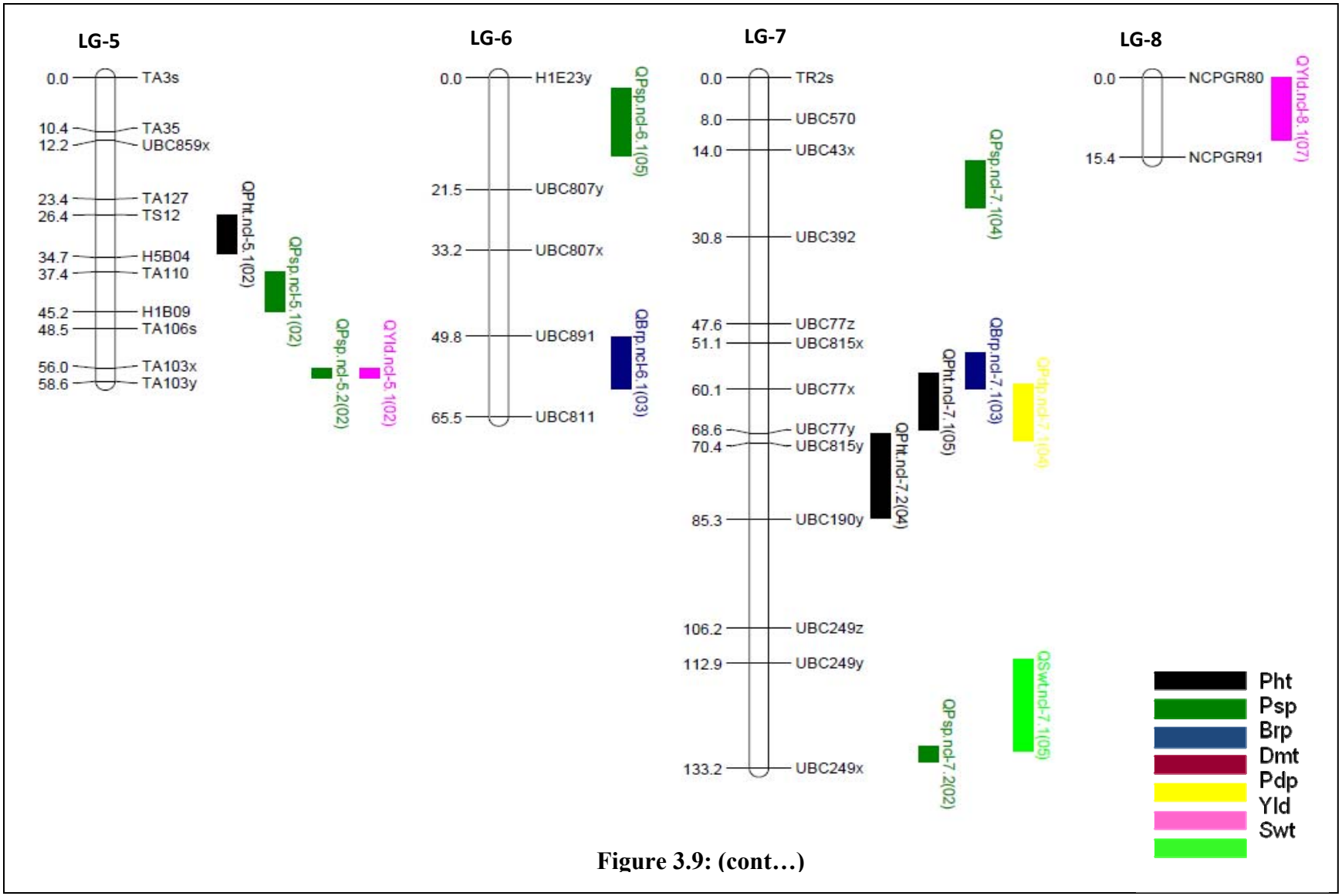


Figure 3.9: (cont…)

3.4.3.4. QTLs of Pdp

Ten QTLs were identified for Pdp, (Table 3.9) distributed in five LGs, except LG-5, LG-6 and LG-8. Among them three were significant and environment specific. Total phenotypic variation explained by individual QTLs ranged from 6.97 to 15.62%. Six QTLs were favored by Vijay and four by ICC-4958 for their expression.

Table 3.6: Composite interval mapping for Pht

LG	Marker	Position (cM)	LOD	Name	Additive	PVE%
1	TS54	55.4	3.199	*QPht.ncl-1.4*	-1.97	11
1	UBC465	58.2	2.685	*QPht.ncl-1.5*	1.608	9
1	TAA170	83.5	3.545	*QPht.ncl-1.6*	-1.89	10
1	UBC43y	97.6	2.652	*QPht.ncl-1.7*	1.368	7.79
1	UBC218	115	2.577	*QPht.ncl-1.8*	1.28	7.87
1	TR43	33.7	4.63	*QPht.ncl-1.1*	2.752	**17.35**
1	TR20	45.8	***4.64***	*QPht.ncl-1.2*	-2.53	13.81
1	TA146	51.6	3.908	*QPht.ncl-1.3*	2.65	12.34
3	H1A10x	55.8	2.742	*QPht.ncl-3.1*	1.56	7.98
4	NCPGR57	41.8	3.225	*QPht.ncl-4.2*	-2.02	15.96
4	NCPGR78	14.7	2.682	*QPht.ncl-4.1*	1.807	7.83
5	TS12	26.4	3.0	*QPht.ncl-5.1*	-1.55	7.2
7	UBC77y	68.6	3.603	*QPht.ncl-7.2*	-1.57	11.29

7	UBC815x	57.1	2.535	*QPht.ncl-7.1*	-2.09	10.85

(Markers underlined: QTLs contributing for more than one trait; bold in PVE – highest variability contribution; Italicized and bold in LOD – highest LOD)

Table 3.7: Composite interval mapping for Psp

LG	Marker	Position (cM)	LOD	Name	Additive	PVE%
1	UBC865	27.5	4.209	*QPsp.ncl-1.3*	-0.98	11.33
1	UBC43z	28.4	2.982	*QPsp.ncl-1.1*	2.538	8.08
1	H1A12	75.9	2.584	*QPsp.ncl-1.2*	2.748	8.65
1	UBC71y	113.1	2.892	*QPsp.ncl-1.4*	-3.17	14.26
2	TR59	37.4	2.737	*QPsp.ncl-2.1*	-0.71	7.34
2	UBC467	0	3.023	*QPsp.ncl-2.2*	-0.87	8.12
2	NCPGR65	100.8	***4.247***	*QPsp.ncl-2.3*	-1.07	13.08
4	TR7s	4	2.904	*QPsp.ncl-4.4*	1.204	13.91
4	NCPGR78	14.7	2.836	*QPsp.ncl-4.3*	0.985	9.25
4	TA176s	18.5	3.08	*QPsp.ncl-4.1*	-2.67	8.31
4	H4G01	22.6	2.984	*QPsp.ncl-4.2*	-3.11	8.76
5	TA110	37.4	2.634	*QPsp.ncl-5.1*	0.687	6.51
5	TA103x	56	3.643	*QPsp.ncl-5.2*	-0.86	9.83
6	H1E23y	2	2.9	*QPsp.ncl-6.1*	2.5	8.4
7	UBC249y	128.9	4.196	*QPsp.ncl-7.2*	0.973	**14.27**

LG	Marker	Position (cM)	LOD	Name	Additive	PVE%
7	UBC43x	16	3.276	*QPsp.ncl-7.1*	1.008	10.91

(Markers underlined: QTLs contributing for more than one trait; bold and Italicized in PVE –highest variability contribution, Italicized and bold in LOD – highest LOD)

Table 3.8 Composite interval mapping for Brp

LG	Marker	Position (cM)	LOD	Name	Additive	PVE%
1	TA2y	81.2	***4.492***	*QBrp.ncl-1.1*	1.32	**23.84**
1	TAA170	83.5	3.658	*QBrp.ncl-1.2*	1.46	13
7	UBC815x	53.1	2.638	*QBrp.ncl-7.1*	-0.67	10.12

(Markers underlined: QTLs contributing for more than one trait; bold and italicized in PVE – highest variability contribution, Italicized and bold in LOD – highest LOD)

Table 3.9 Composite interval mapping for Pdp

LG	Marker	Position (cM)	LOD	Name	Additive	PVE%
1	TA2Y	79.2	***4.265***	*QPdp.ncl-1.2*	3.793	**13.35**
1	H1A12	75.9	4.089	*QPdp.ncl-1.1*	-2.47	11.78
1	SPP	105.3	2.863	*QPdp.ncl-1.4*	1.916	7.51
1	UBC43Y	97.6	2.588	*QPdp.ncl-1.3*	5.424	6.97

1	H3A04	123.1	2.885	*QPdp.ncl-1.5*	-2.22	8.31
2	TA34s	14	2.884	*QPdp.ncl-2.1*	3.107	10.79
3	UBC165	24.9	3.765	*QPdp.ncl-3.2*	-2.83	15.62
3	UBC810	18.9	2.997	*QPdp.ncl-3.1*	3.96	9.34
4	TA176S	18.5	2.971	*QPdp.ncl-4.1*	4.47	8.88
7	UBC815X	59.1	2.905	*QPdp.ncl-7.1*	-4.07	10.63

(Markers underlined: QTLs contributing for more than one trait; bold and italicized in PVE – highest variability contribution, italicized and bold in LOD – highest LOD)

3.4.3.5. QTLs of Yld

Fifteen QTLs were identified for plant yield which were distributed on six different linkage groups (Table 3.10). LG-2 alone has seven QTLs followed by five on LG-1. All the QTLs were environment specific except, *QYld.ncl-1.4.* Interestingly another putative gene Spp (seeds per pod) controlled this stable QTL. The highest phenotypic variation, 14.72%, was contributed by the QTL *QYld.ncl-2.6***.** Twelve QTLs were influenced by alleles of parent ICC-4958 and three by Vijay alleles.

3.4.3.6. QTLs of Swt

A total of 13 QTLs were identified for Swt on five LGs, with majority mapped on LG-1 (four QTLs) (Table 3.11). Among these, eight QTLs were significant. Thirteen QTLs had negative additive effect, suggesting the contribution of alleles from the inferior parent ICC-4958. Three major and stable QTLs were identified and mapped on LG-1. The QTL, (*QSwt.ncl-1.5*) explained 20.56% of total phenotypic variation and expressed in more than one environment. *QSwt.ncl-1.3* contributed a higher LOD value of 6.249 and 18.42% phenotypic variation.

3.4.3.7. QTLs of Dmt

Three QTLs were mapped on two LGs (LG-2 and LG-4) (Table 3.12; Fig 3.9). All were environment specific. Two QTLs were influenced by alleles of ICC- 4958 and one QTL by Vijay allele. QTL, *QDmt.ncl-2.1*, explained 14.08% of total phenotypic variation. All three QTLs were expressed in Rahuri location.

3.4.4. Multiple Composite Interval Mapping (MCIM)

Single locus MCIM was conducted in the population and a total of 15 QTLs were identified. Some of these QTLs (eight) were similar to the QTLs detected through CIM. Among the similarity QTLs, three belonged to the trait Pht, two for Yld and three for Swt. QTLs, those were not detected through CIM were for the traits Psp (1), Pdp (1) and Swt (4). MCIM could not identify any QTLs for Brp and Dmt.

Table 3.10: **Composite interval mapping for Yld**

LG	Marker	Position (cM)	LOD	Name	Additive	PVE%
1	TAA170	83.5	***4.821***	*QYld.ncl-1.3*	-0.91	13.24
1	SSR6y	60	2.542	*QYld.ncl-1.1*	0.924	7.88
1	SPP	105.3	3.022	*QYld.ncl-1.4*	-1.04	9.77
1	UBC706	62.8	4.098	*QYld.ncl-1.2*	-1.21	10.91
2	TA43	46.8	3.021	*QYld.ncl-2.2*	3.021	7.38
2	H4D12	57.8	3.94	*QYld.ncl-2.5*	-1.75	13.9
2	UBC360	30.8	3.718	*QYld.ncl-2.1*	-1.11	9.15
2	TA116X	51.7	3.203	*QYld.ncl-2.3*	-1.15	10.34
2	NCPGR77	55.7	4.452	*QYld.ncl-2.4*	-1.32	13.06

LG	Marker	Position (cM)	LOD	Name	Additive	PVE%
2	UBC881	60.1	2.741	*QYld.ncl-2.6*	-1.35	**14.72**
2	NCPGR81	65.6	2.702	*QYld.ncl-2.7*	-1.01	8.35
3	UBC165	18.9	3	*QYld.ncl-3.1*	1.329	10.57
4	GA34	63	3.143	*QYld.ncl-4.1*	-0.8	8.57
5	TA103X	56	4.01	*QYld.ncl-5.1*	-0.79	10.66
8	NCPGR80	0	3.685	*QYld.ncl-8.1*	-1.09	9.75

(Markers underlined: QTLs contributing for more than one trait; bold and italicized in PVE – highest variability contribution, italicized and bold in LOD – highest LOD)

Table 3.11: Composite interval mapping for Swt

LG	Marker	Position (cM)	LOD	Name	Additive	PVE%
1	H4G11	64.2	3.303	*QSwt.ncl-1.1*	-1.98	10.01
1	RGA6y	73.2	4.384	*QSwt.ncl-1.4*	-2.97	15.5
1	TAA170	83.5	6.181	*QSwt.ncl-1.5*	-3.39	**20.56**
1	NCPGR74	68.3	2.621	*QSwt.ncl-1.2*	-1.51	6.54
1	H1G22	73.8	***6.249***	*QSwt.ncl-1.3*	-2.48	18.42
2	SSR8y	24.7	3.601	*QSwt.ncl-2.1*	-2.19	8.23
2	NCPGR77	55.7	3.168	*QSwt.ncl-2.2*	-1.58	9.45
2	UBC881	60.1	4.872	*QSwt.ncl-2.3*	-2.11	18.63

LG	Marker	Position (cM)	LOD	Name	Additive	PVE%
3	H1A10x	58.5	2.692	*QSwt.ncl-3.1*	-1.27	7.72
3	TAA55	73	2.792	*QSwt.ncl-3.2*	-1.32	8.46
3	TA78	73.6	2.814	*QSwt.ncl-3.3*	-1.42	6.27
4	CsSSR2	9.7	3.22	*QSwt.ncl-4.1*	1.417	8.57
7	UBC249z	112.2	3.293	*QSwt.ncl-7.1*	-2.07	9.63

(Markers underlined: QTLs contributing for more than one trait; bold and italicized in PVE – highest variability contribution, italicized and bold in LOD – highest LOD)

Table 3.12: **Composite interval mapping for Dmt**

LG	Marker	Position (cM)	LOD	Name	Additive	PVE%
2	TA30	112.3	2.711	*QDmt.ncl-2.1*	-1.3832	**14.08**
4	TA22s	13.4	***2.979***	*QDmt.ncl-4.1*	1.5186	10.89
4	TA80s	42.7	2.842	*QDmt.ncl-4.2*	-1.3331	7.31

(Markers underlined: QTLs contributing for more than one trait; bold and italicized in PVE – highest variability contribution, italicized and bold in LOD – highest LOD)

3.4.5. Pleiotropic QTLs

Since most of the yield traits showed correlation, it was possible to locate few pleiotropic QTLs in this population. The identified pleiotropic QTLs are given in the Table 3.13. The SSR marker TAA170 of LG-1 contributed for many traits like Pht, Brp, Yld, Swt and Pdp. Likewise SSR H1A12 of LG-4 (PSp+Pdp+Brp), NCPGR77 of LG-2 (Yld+Swt), NCPGR78 of LG-4 (Pht+Psp+Pdp) and TA103x of LG-5 (Psp+Yld) had interactions with the traits. The RAPD UBC815x and UBC165 were also involved in the pleiotropic interaction contributing for the traits (Pdp+Yld) respectively. One RGA marker Ptokin of LG-1 also contributed in trait interaction and this was the only pleiotropic QTL that was not revealed through CIM analysis. LG-1 of chickpea genome could be considered as the major region for the pleiotrophic action.

Table 3.13: **Pleiotropic QTLs based on CIM and MCIM**

Trait	LG	Marker interval	Position (cM)
A			
Pht, Brp, Yld, Swt, Pdp	LG-1	TAA170-Spp	83.5-105.3
PSp, Pdp, Brp	LG-4	H1A12-TA2y	75.9-79.2
Pht, Psp, Pdp	LG-4	NCPGR78-TA176s	14.7-18.5
Yld, Swt	LG-2	NCPGR77-UBC881	55.7-60.1
Pht, Pdp	LG-7	UBC815x	57.1
Psp, Yld	LG-5	TA103x	56.0

Trait	LG	Marker interval	Position (cM)
Pdp, Yld	LG-3	UBC165	24.9
B			
Pht, Swt	LG-1	Ptokin	71.2

A: CIM analysis; B: MCIM analysis

3.4.6. Two locus analysis

QTL interactions were studied by using QTL Network software. The results are summarized in Tables 3.15 and 3.16. A total of 23 QTLs (11 M-QTL and 12 E-QTLs) were identified which were involved in QE and QQE interactions. Eight of the M-QTLs have been already identified through single locus CIM analysis in the same and / or adjacent marker intervals. The minor differences associated with the position and the marker interval of the QTLs could be attributed to different approaches and software used. Brp, and Swt are the traits that had only QE interactions and Pdp, Psp and Yld expressed both the QQ and QQE interactions with respect to all the environments. The traits Pht and Dmt failed to illustrate any interactions through this two way analysis.

Among the identified three Brp QTLs, (Table 3.15) two were confirmed by CIM, (*QBrp.ncl-2.1* and *QBrp.ncl-1*.5). The parent ICC-4958 favored both of these M-QTLs with a significant additive effect and they interacted at both the locations, Rahuri and Dharwad, during the five year period. All the three QTLs displayed significant epistatic main effect (QE). The third Brp QTL; *QBrp.ncl-1.3* is contributed by the parent Vijay allele. This QTL had its high environment effect during the year (2004-2005) with an AE (additive X environment) value of 2.015. QQE action was totally absent in Brp. Thus, the established CIM QTLs and M-QTLs independently controlled their QTL action. The total

number of QTLs observed for Brp was less, compared to other traits. The marker interval TA2y-TAA170 of LG-1 turned out to be a major location for Brp QTLs.

Similar to Brp, three M-QTLs were detected for Pdp (Table 3.15). Only one QTL differed from the CIM. All the five environments interacted with this trait. It was noticed that the environment (2004-2005) had a high interaction with both the traits, Brp as well Pdp. To support this, a very high value of AE (-6.3480) was obtained to the QTL *QPdp.ncl-1.4*. Interestingly, the Spp locus was interlinked with QTL *(QBrp.ncl-1.5*) and a Pdp (*QPdp.ncl-1.4)* on LG-1. The marker interval TAA170-Spp was strongly linked with these QTLs. Definition of the graphic meta system for genetic architecture presentation based on QTL Network analysis is described in Table 3.14. The graphic presentation for Brp (Fig. 3.10) and Pdp (Fig. 3.12) explained the epistatic interaction and their major actions. The trait Pdp obtained four QTLs for QQE interaction. All these QTLs were confirmed by CIM analysis. The E-QTL *QPdp.ncl-7.1* became a common interactive QTL for the remaining two E-QTLs, *QPdp.ncl-3.2* and *QPdp.ncl-1.2*. All these four E-QTLs had significant (QxQ) as well as QQE interaction. All the five environments (Rahuri, Dharwad) played a crucial role over these QTLs.

Psp had two M-QTLs detected, which were comparable with already recognized CIM QTLs (Fig. 3.11, Table 3.15). Both the QTLs were mapped on LG-1. Among the four environments, 2002-2003, 2003-2004 and 2004-2005 showed an equal QE interaction but 2005-2006 was much significant, contributing a highest AE value (6.4375) by the QTL; *QPsp.ncl-1.1*. Six E-QTLs were obtained for this trait and were confirmed by CIM except for the QTL *QPsp.ncl-3.1*. Many LGs (LG-4, LG-2, LG-6, LG-5 and LG-3) were engaged in the QQE interactions. Excluding the fifth environment, all the four environments contributed to the QxE interaction.

Only one main effective QTL (*QYldncl-1.4*) (Fig. 3.14, Table 3.15) was recognized for the trait Yld. The main effective QTL showed both, additive and additive x environment, interactions. The marker interval UBC891-Spp controlled this M-QTL. This QTL was located on LG-1. An additive value of 0.5294 confirmed the role of Vijay

allele. Environment 2004-2005 had a significant interaction with this M-QTL with a negative additive effect contributed by the poor parent suggesting the importance of alleles from ICC-4958 (AE4= -1.2674). The E- QTLs; *QYld.ncl-1.1* and *QYld-ncl.4.1* interacted in QQ and QQE interaction through the SSR markers SSR6x and STMS13 of LG-1 and LG-4 (Fig. 3.14, Table 3.15and 3.16).

The two M-QTLs of Swt were located on LG-1 and LG-7 (Fig. 3.13, Table 3.15). The negative additive effect of both the QTLs shows the importance of ICC-4958 alleles over the trait Swt. Both of these QTLs showed negligible amount of QE interaction. The SSR marker TAA170 controlled the M-QTL *QSwt.ncl-1.5* also.

In the present study, the relative merits of two-locus and single-locus analyses for QTL detection were assessed. This kind of comparison revealed that, in an individual population, some of the QTLs were detected in both the analyses but not all. In an individual environment, QTLs may often escape detection at the threshold value of LOD score due to Q × E interactions that lead to variable expressions of QTLs. Thus, it was obvious that QTL effects detected using CIM are often confounded by the fact that these analyses do not resolve the network interactions involving QQ, QE or QQE. The present study in chickpea, thus, confirmed the role of interacting QTLs in controlling quantitative traits of economic importance.

Table 3.14: Definition of the graphic meta system for genetic architecture presentation based on QTL Network analysis

Graphic meta system	Line (Epistasis)	Circle (Shape)
Red	with only epistatic main effect (I)	with only additive effect (A)
Green	..... with only epistasis ×environment interaction effect (IE)	with only additive × environment interaction effect (AE)

Blue	---------- with both I and IE	● with both A and AE
Dark	Not available	● with no additive related effect

Table 3.15: **QTLs with main effects and environment interactions for yield traits by two locus analysis**

Marker interval	QTL	LG	A	AE
NCPGR69-NCPGR72	*QBrp.ncl-2.1*	LG-2	-0.7931	AE (I) = 0.6083 AE (II) = 0.5953 AE (III) = 0.4141 AE (IV) = -2.2209 AE (IV) = 0.6338
TAA137-TAA170	*QBrp.ncl-1.3*	LG-1	0.5148	AE (I) = -0.2957 AE (II) = 0.7557 AE (III) = 0.5398 AE (IV) = 2.0151 AE (V) = -0.382
UBC891-Spp	*QBrp.ncl-1.5*	LG-1	-0.2871	AE (I) = 0.0426 AE (II) = 0.7295 AE (III) = 0.2429 AE (IV) = -1.2339 AE (V) = -0.2310

Marker interval	QTL	LG	A	AE
TAA137-TAA170	*QPdp.ncl-1.2*	LG-1	1.1626	AE (I) = -0. 8727 AE (II) = -0.9205 AE (III) = -1.8086 AE (IV) = 4.0728 AE (V) = -0.4403
UBC467-UBC570	*QPdp.ncl-2.3*	LG-2	-1.8757	AE (I) = 3.7705 AE (II) = 2.4194 AE (III) = -1.7117 AE (IV) = -4.7237 AE (V) = 0.2297
UBC891-Spp	*QPdp.ncl-1.4*	LG-1	-1.3365	AE (I) = 1.7187 AE (II) = 2.6845 AE (III) = 1.3973 AE (IV) = -6.3480 AE (V) = 0.6539
TS54-TS72	*QPsp.ncl-1.1*	LG-1	1.76	AE (I) = -2.0415 AE (II) = -2.2599 AE(III) = -2.0028 AE (IV) = 6.4375 AE (V) = NA
TA2y-TA3s	*QPsp.ncl-1.2*	LG-1	-0.9617	AE (I) = 0. 2674 AE (II) = 1.5960 AE (III) = 2.2368 AE (IV) = -4.0686

Marker interval	QTL	LG	A	AE
				AE (V) = NA
UBC891-Spp	*QYldncl-1.4*	LG-1	0.5294	AE (I) = 0. 4917 AE (II) = 0.7331 AE (III) = 0.3562 AE (IV) = -1.2674 AE (V) = -0.3044
TAA170-TR1s	*QSwt.ncl-1.5*	LG-1	-1.8378	AE (I) = 0. 001 AE (II) = 0.0347 AE (III) = 0.0048 AE (IV) = 0.0120 AE (V) = -0.0168
UBC249y-UBC249z	*QSwt.ncl-7.1*	LG-7	-2.3902	AE (I) = 0.0 AE (II) = 0.0 AE (III) = -0.0001 AE (IV) = -0.0001 AE (V) = -0.0

A: additive effect; AE (I), AE (II), AE (III), AE (IV) and AE (V): QTL × environment interaction effects for environments; I=2002-2003, II=2003-2004, III=2004-2005, IV=2005-2006 and V=2007-2008, respectively

Table 3.16: QTL interactions involving (Q × Q or Q × Q × E) for yield traits by two locus analysis

Marker interval	QTL	LG	Marker interval	QTL	LG	AA	AAE
TA127-TA140	*QPdp.ncl-3.2*	LG-3	TR2s-TR7s	*QPdp.ncl-7.1*	LG-7	1.397	AAE (I) = -0.6835 AAE (II) = -2.5058 AAE (III) = -3.9401 AAE (IV) = -8.4675 AAE (V) = -2.5162
TAA137-TAA170	*QPdp.ncl-1.2*	LG-1	UBC467-UBC570	*QPdp.ncl-7.1*	LG-7	1.008	AAE (I) = 0.6595 AAE (II) = -2.0339 AAE (III) = 4.706 AAE (IV) = -3.1352 AAE (V) = 1.104
TA78-TA80s	*QPsp.ncl-4.2*	LG-4	UBC807y-UBC809x	*QPsp.ncl-6.1*	LG-6	4.4374	AAE (I)= -4354 AAE (II) = -153 AAE (III) = -2.491 AAE (IV) = 8.1194 AAE (V) = NA

Marker interval	QTL	LG	Marker interval	QTL	LG	AA	AAE
NCPGR 77- NCPGR78	*QPsp.ncl-2.1*	LG-2	H1G22-H1I17	*Qpsp.ncl-4.2*	LG-4	-4.3797	AAE (I) = -3.1446 AAE (II) = -3.4037 AAE (III) = 1.0169 AAE (IV) = -7.5172 AAE (V) = NA
H5A04-H5B04	*QPsp.ncl-5.2*	LG-5	*UBC165-UBC173	*Qpsp.ncl-3.1*	LG-3	-1.637	AAE (I) =0.9014 AAE (II) =1.1355 AAE (III) = 1.8881 AAE (IV) = -.9568 AAE (V) =NA

Marker interval	QTL	LG	Marker interval	QTL	LG	AA	AAE
SSR6x-SSR6y	*QYld.ncl-1.1*	LG-1	STMS13-STMS15	*QYld.ncl-4.1*	LG-4	-1.1341	AAE (I) =0.6387 AAE (II) = 0.0182 AAE (III) = 3.685 AAE (IV) = -2.7233 AAE (V) = -1.6116

AA additive effect; AAE (I), AAE (II), AAE (III), AAE (IV), AAE (V): epistasis associated with environment; I=2002-2003, II=2003-2004, III=2004-2005, IV=2005-2006 and V=2007-2008, respectively

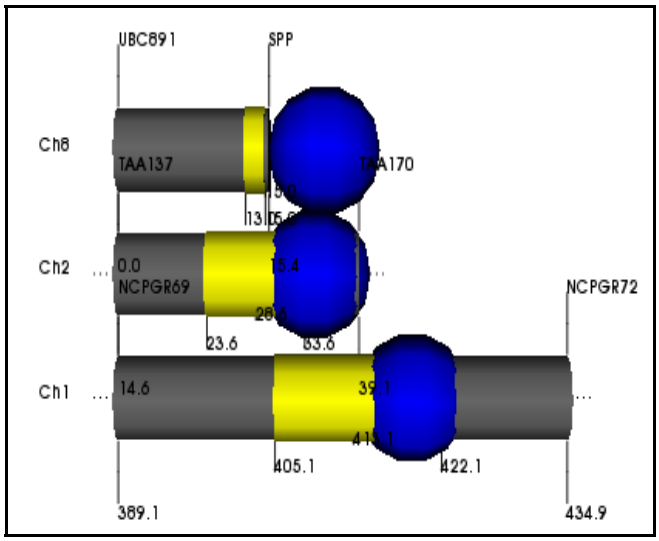
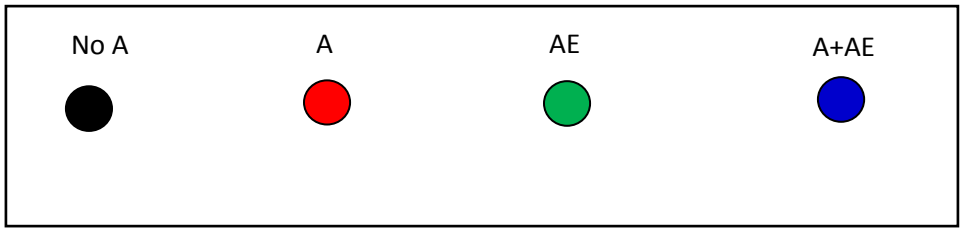


Figure 3.10: QTL x QTL x E interaction for Brp

Ch1, Ch2, Ch8 represents the linkage group- LG-1, LG-2 and LG-8



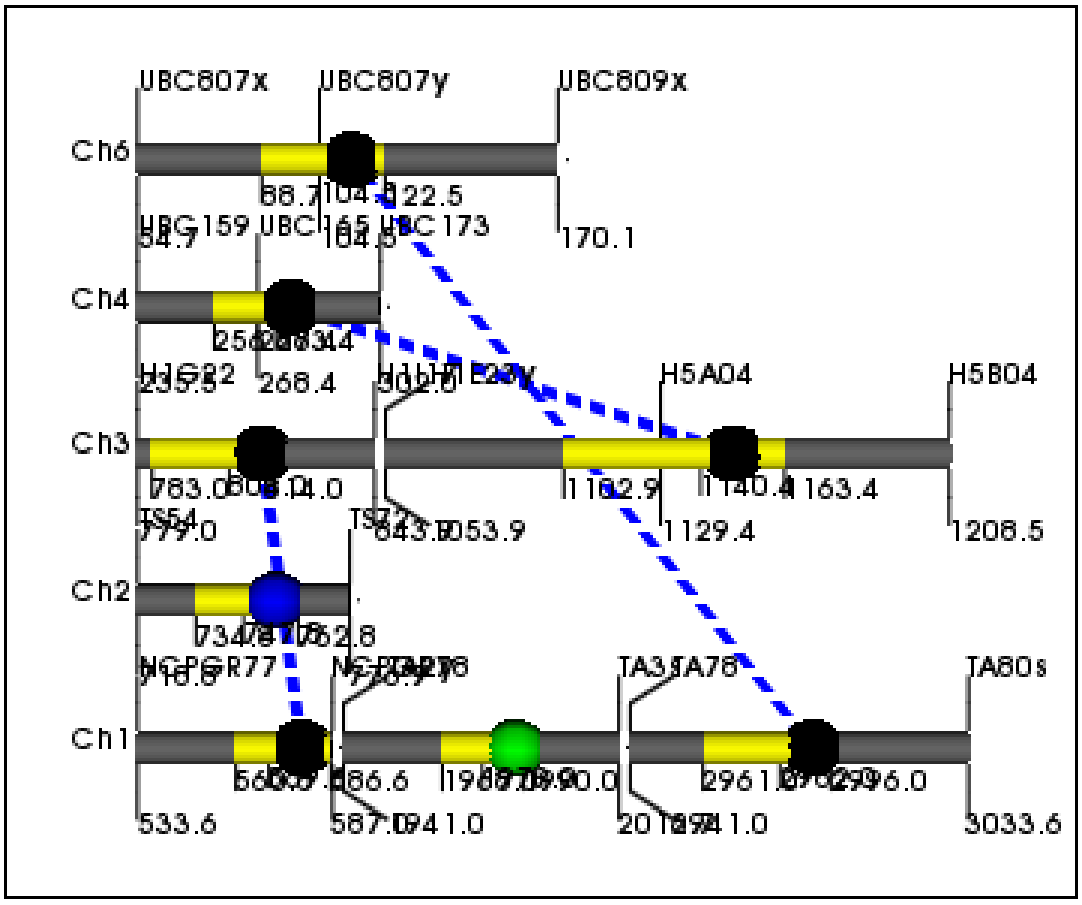
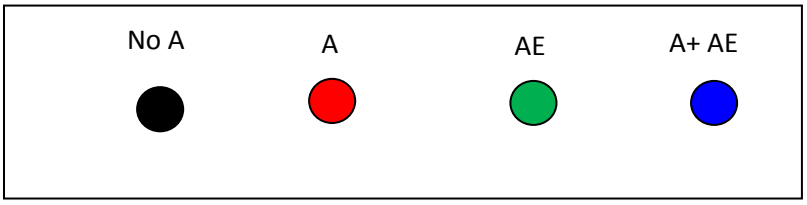


Figure 3.11: QTL x QTL x E interaction for Psp

Ch1, Ch2 Ch3, Ch4, Ch6 represents the linkage group- LG-1, LG-2, LG-3, LG-4 and LG-6



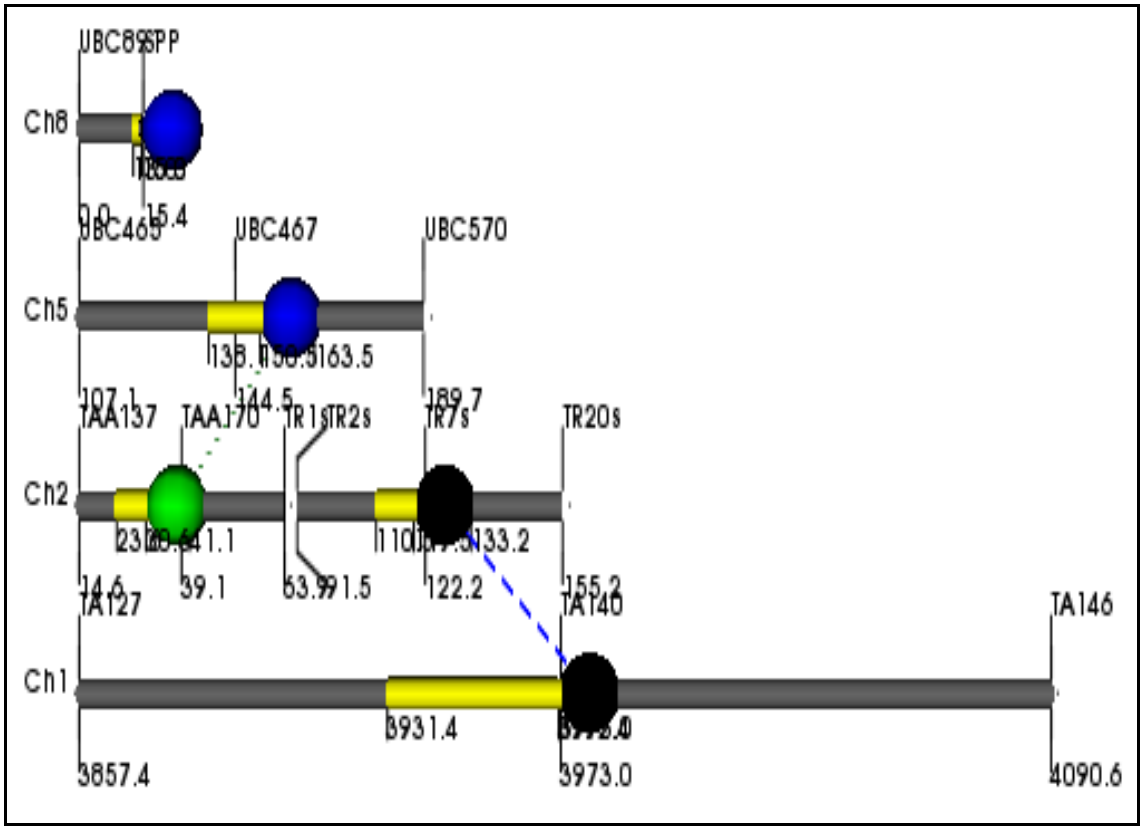
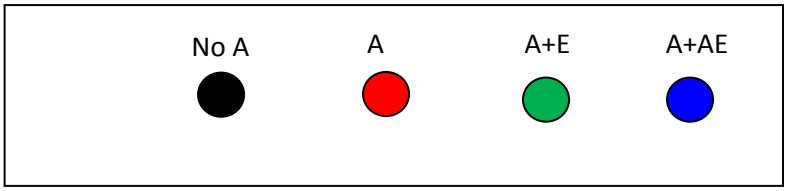


Figure 3.12: **QTL x QTL x E interaction for Pdp**

Ch1, Ch2 Ch5, Ch8 represents the linkage group- LG-1, LG-2, LG-5 and LG-8



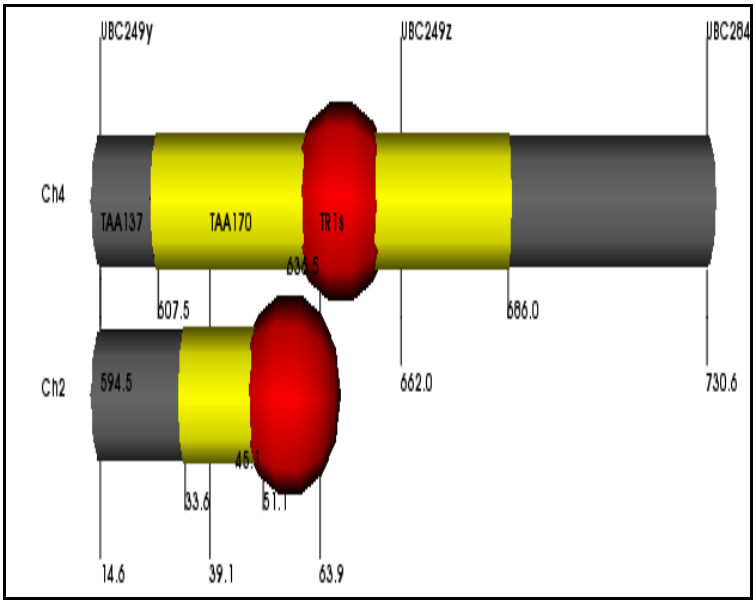
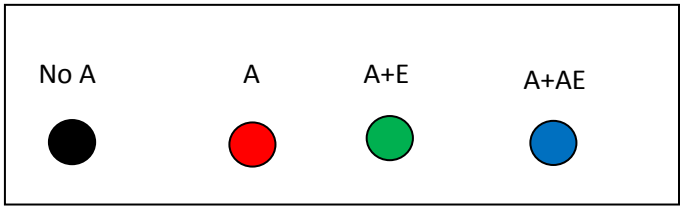


Figure 3.13: QTL x QTL interaction of Swt

Ch2, Ch4 represents the linkage group- LG-2 and LG-4



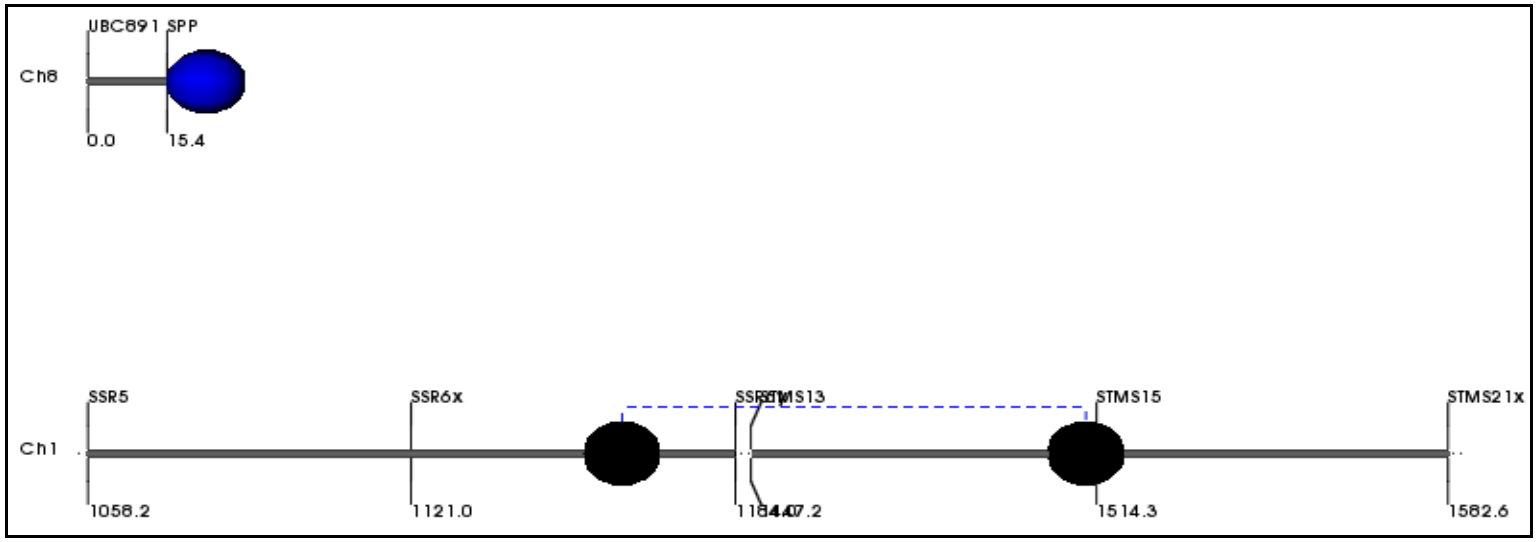
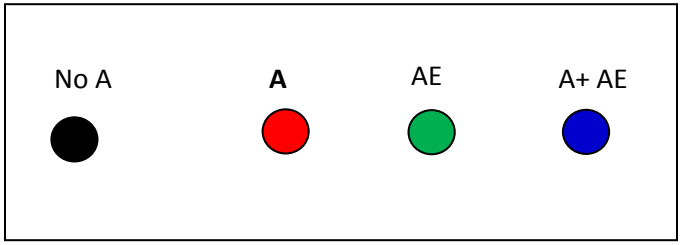


Figure 3.14: QTL x QTL x E interaction of Yld

Ch1, Ch8 represents the linkage group- LG-1 and LG-8



3.5. Discussion

To scrutinize the yield trait, one intra-specific F_9 RIL population with contrasting seven traits *viz*. plant height (Pht), plant spread (Psp), number of branches per plant (Brp), number of pods per plant (Pdp), yield per plant (Yld), 100-seed weight (Swt) and days to maturity (Dmt) was selected. The experiment was conducted for five years at two locations in order to minimize the over estimation of the genetic component. The results of the present study reconfirmed that the genetics of yield and yield components are complex in nature and are controlled by large number of major and minor QTLs

3.5.1. Importance of phenotypic evaluation of the yield traits

All the yield contributing traits were subjected to heritability analysis to verify the heritable viability. The broad-sense heritability estimated for the seven traits ranged from 36% (Psp) to 84% (Swt). These values agreed with those reported earlier in chickpea (Eser, 1976; Muehlbauer and Singh, 1987; Singh, 1991; Abbo *et al*., 2005; Cobos *et al*., 2007). Eser (1976) observed 13% narrow-sense heritability for single plant yield, 25% for number of pods per plant, 30% for plant height and 78% for seed weight in chickpea, and also concluded that seed weight was the least influenced trait by the environment. Singh (1991) studied pure lines of chickpea and estimated the heritability values for several traits *viz*. seed yield, plant height, number of pods per plant, days to flowering, days to maturity and 100-seed weight that ranged from 49% to 91%. Abbo *et al*. (2005) and Cobos *et al*. (2007) also reported high heritable values for seed weight (71%) and seed size (90%), respectively. In the present study, we also observed high heritability (84%) for seed weight, followed by plant height (77%).

The population means for the seven traits in five environments depicted a normal distribution, without skewing towards either of the parents, suggesting least epistatic effects between the QTLs (Blanco *et al.*, 2006). The RIL population developed for the present study described both positive and negative transgressive segregants, suggesting the possibility of finding positive alleles in the poor parent while negative alleles in the

parent with better yield traits. Grain yield and related traits of chickpea (*Cicer arietinum* L.) are quantitative in nature, affected by many genetic factors as well as environment fluctuations (Muehlbauer and Singh, 1987).

Determination of correlation between yield and yield related traits is important to select favorable plant types for effective chickpea breeding. Therefore, correlations between all the traits were analysed to confirm the yield stability. Yld was significantly and positively correlated with traits like Pht, Psp, Brp and Pdp in the year 2002-2003. However, this phenomenon was not alike in the next consecutive years. One or the other trait influenced the yield positively or negatively. This indicates that if not all the related traits, but support from one or two related traits also would be sufficient for yield enhancement. Early maturity usually escapes any unfavorable conditions leading to minimize the yield loss. This was applicable to drought tolerant variety like ICC-4958. But in the current study this did not occur, instead Dmt failed to show any noteworthy correlation with yield in any of the environments. Generally, Swt and Pdp are accepted as the most important characters due to its close relationship with grain yield. Higher the pod numbers, higher would be the grain yield. Furthermore, the number of pods and seed weight were found as the most important selection criteria in grain yield because of the fact that they had the significant positive direct effect (Singh *et al*., 1990). To stabilize these evaluations, we observed a similar condition between Swt 2004-2005 / Yld 2002-2003; Yld 2003-2004 / Pdp 2003-2004; Yld 2004-2005 / Pdp 2004-2005 with significantly high correlation values (0.351, 0.514, 0.643; $P<0.001$).

3.5.2. G x E interaction of the yield QTLs

Beyond the correlation studies, the phenotypic data was further analysed to reconfirm the QTL stability over the presently studied environments. The results of combined analysis of variance showed significant differences for environments and the genotype × environment interaction. The results of AMMI analysis were not similar for all the traits. Traits like Pht, Psp, Brp and Pdp seem to be lined by the environment impact rather than the G or GxE interaction. Whereas Swt was the only trait controlled by the genetic

impact. Yld seems to be completely under the influence of GxE for the total variation and thus indicating the low level of heritability. This condition can be circumvented by partioning the environment into subsets of environment that have either low or non-crossover GxE interactions; and stable QTL for each subset of environment be developed and used in MAS. Additionally, AMMI bi-plot analysis identified the best performing genotype for the trait of interest over a specific environment. This model combines ANOVA and PCA (principal component analysis) into a single analysis. A bi-plot represents a versatile graphical approach for analyzing METs (multiple environment trials) (Yan, 2001; Yan and Kang, 2003). Previous studies revealed that AMMI with only two interaction principal component axes (IPCA1 and IPCA2) was the best predictive model (Zobel *et al.*, 1988). In the present study the contribution of IPCA1 to the GE interaction was greater than that of IPCA2 and IPCA3 for all the traits. Similar results were found in barley (Monica *et al*., 2008). It was observed that most of the genotypes and environments were dispersed around the bi-plot for all the traits. Most of the RILs were clustered in center indicating all these RILs are stable genotypes and all the environments are very much diverse. Among the yield traits analysed, Psp and Dmt shared high GxE interactions (100%). The second highest category comes out with traits like Brp (80%), Pdp, (82%) and Swt (70%). Somewhat moderate amount of GE actions were given by the traits Yld and Pht (69.6%, 64%), respectively. In a previous study on barley, RILs showed moderate GE interactions compared to landraces (Monica *et al.*, 2008) for yield. As chickpea is an annual crop, another presumption was to possess, high GE index during multi-locational, multi-year practice which has been supported by Mohammadi *et al.* (2007). There are two strategies for developing genotypes with low GE interactions. The first is sub-division or stratification of a heterogeneous area into smaller, more homogeneous sub-regions, with breeding programs aimed at developing genotypes for specific sub-regions. The second strategy involves selecting genotypes with better stability across a wide range of environments in order to predict better behavior (Eberhart and Russell, 1966; Tai, 1971). Various methods use GE interaction to facilitate genotype characterization, and as a selection index together with the mean yield

of the genotypes. Accordingly, genotypes with minimal variance for yield across environments are considered stable.

3.5.3. Effect of epistasis on yield QTLs of chickpea

Supplementary evidences that came through two locus analysis further stabilized the identified QTLs. As these evidences are more interactive, the omitted facts on individual QTLs came to the limelight with much ease. QTL analysis with a provision for detecting these interactions would generally avoid the biased estimate of main effect QTLs and increase the success rate in marker assisted selection (MAS) (Kumar *et al.*, 2007). Majority of the previous reports on QTL analysis in chickpea had not performed any interaction (QE, QQ and QQE) studies. When the single and two locus analysis were compared, it was noted that eight out of eleven M-QTLs detected were similar to the already identified CIM QTLs originated either in the same or adjacent marker intervals for all the traits. Only three M-QTLs had escaped detection through CIM. This may be due to the saturated nature of the LG-1 and LG-2 where majority of the QTLs are positioned. The clear evidence shown in this analysis is the QTLs involved in QE interactions were mainly M-QTLs; while the QTLs involved in QQ/QQE interactions were the QTLs, which had no main effects. They exercise only through interactions with other M-QTLs or E-QTLs. Among the 11 M-QTLs, two were involved in QE interactions, which were detected in two environments. Some of the QTLs were stable across the environments and were not involved in QE interactions. Of the 12 E-QTLs detected, all QTLs were involved in either QQ or QQE interactions. In addition to these QQ interactions, there was one instance (for Pdp) where the same QTL (*QPdp.ncl-7.1*) was involved in two epistatic interactions.

3.5.4. Yield related QTLs spread all over the chickpea genome also show pleiotropism

After resolving all the preliminary components the major part of this study was initiated. QTL identification and QTL mapping was the target of this entire study. The principle of

using genetic markers to study quantitative trait loci (QTL) is well established (Lander and Botstein, 1989; Zeng, 1993, 1994). In recent years, the advent of fine-scale molecular genetic marker maps for various organisms by molecular biology techniques has greatly facilitated the systematic mapping and analysis of individual QTL.

In order to dissect the yield and yield related QTLs, I used a RIL population of 93 lines which could be considered as a smaller population for QTL mapping of complex traits. However, Price (2006) postulated that QTL positions identified using small populations were nearly same as that of large mapping population. The QTL analysis for 100 seed weight, days to flowering and number of seeds per plant (76 lines and 80 lines) (Cho *et al.*, 2002; Cobos *et al.*, 2007) has been already reported with small population.

A total of 74 QTLs has been identified through single locus CIM analysis (QTL Cartographer) and 23 QTLs by two locus analysis (QTL Network). This kind of dual estimation refined the QTLs more precisely and also had initiated the identification of some new trait locative regions which were missed out in one or the other method of identification. There were eight significant (LOD>3) QTLs mapped for Pht which were distributed on six linkage groups. All the eight Pht QTLs of LG-1are clearly clustered together forming a single group. This region should be a major genomic region for this trait. The QTLs *QPht.ncl-1.2* and *QPht.ncl-1.7* were stable across two environments. Three QTLs namely *QPht.ncl-1.7*, *QPht.ncl-4.1* and QPht.ncl-7.1 were engaged in pleotropic act with other traits like Psp, Brp, Pdp,Yld and Swt. The SSR marker TAA170 seems to be playing a major interactive role between all these traits. The coincidence of QTL association congruently supports the observed positive significant phenotypic correlation over different environments. Both the parental alleles contributed almost equally for Pht. In spite of this the total phenotypic variation contributed by the parent Vijay seems to be higher than the other parent ICC-4958. As many of these yield traits were correlated, MCIM analysis was performed to detect pleiotropic QTLs. Although many QTLs were detected in CIM, most of them were not detected in the MCIM analysis. Genes of high pleiotropic actions are expected to be under strong stabilizing selection because they can affect multiple traits. Moreover, these pleiotropic genes are

believed to be evolutionarily conserved and can lead to beneficial mutation (Fisher 1958). All these facts explain the need to use wild types in the pleiotropic studies, so that modifications they underwent during domestication can be made clear.

Seven significant Psp QTLs were identified and among them three pleiotropic QTLs; *QPsp.ncl-1.2, QPsp.ncl-4.3* and *QPsp.ncl-5.2* were clustered with QTLs for Brp, Pdp, Pht and Yld in LG-4 and LG-5. These results do not abide the phenotypic correlation, rather showed inverse relationship. This may be related to the decline of vegetative growth following onset of flowering. All these QTLs were highly environment specific.

Brp possessed the least number of QTLs amongst the seven traits. This indicates the need for new marker addition to the existing map to dissect this trait. LG-1 and LG-7 played a role in the identification of three QTLs. Amidst one QTL was stable across two environments. Besides, the QTL *QBrp.ncl-1.2* showed the highest phenotypic variation (23.84%) at a higher LOD value (4.492). Thus this small genomic region constituted by the SSR marker TA2y seems to be highly significant if mutually correlated with other related traits.

Pods per plant had direct influence to improve the grain yield. Ten QTLs were identified for Pdp with one of them contributing up to 15.62% of the total phenotypic variation. The five environments had equal impact on these QTLs. The phenotypic relationship with Yld seems to be high and positive. The correlation coefficient values were highly significant ($P<0.001$). For instance Pdp 2002-2003 / Yld 2002-2003 showed a correlation value of 0.768. On the other hand an equally significant correlation with the trait Brp 2003-2004 was observed. This fact can be related to more the branches higher the pod number.

The QTL for seed size (*QSwt.ncl-2.2*) and another QTL for yield (*QYld.ncl-2.4*) in the same genomic region in LG-2 might explain the correlation between these two traits. These results agree with those reported by Cho *et al.* (2002), who found a QTL for seed

size and seed number per plant (an important yield component) and Cobos *et al*. (2007) for Yld and Swt in same LG-2. Similar type of clustering of QTLs was observed in LG-1 for these two traits. Further analysis should be made in the future to saturate this major QTL region in the LG-1 and LG-2 of chickpea map, using robust markers such as SSR (Winter *et al.*, 2000; Tekeoglu *et al.*, 2002; Cho *et al.*, 2004). The detection of these new QTLs for seed size would help to overcome the selection problem for larger seeds in chickpea.

3.5.6. Comparison with previous studies

The results of the present study can also be compared with the previous studies performed in chickpea. Cho *et al*. (2002) mapped a pleiotropic QTL for seed number per plant and 100-seed weight on LG-IV. In the current study six Swt QTLs were identified on LG-1 which corresponds to LG-IV of Winter *et al*. (2000). Hence, it was possible that one or two of the current Swt QTLs might be the same as the seed weight QTLs detected by Cho *et al*, (2002). However, as the marker orders between the two maps are different, it is difficult to determine the similiarity of the QTLs. Similarly, Abbo *et al*, (2005) detected three seed weight QTLs on LGs I, IV and VI. The "Seed weight QTL 1" of Abbo *et al*. (2005) was linked to the STMS marker GA24 on LG-IV. In the present study one stable QTL (*Swt.ncl-1.1*) across two environments was located very close to GA24 on LG-1. This QTL might correspond to the "Seed weight QTL 1" of Abbo *et al*., (2005). A comparable inference that has been noted was instead of Swt QTL, one Yld QTL (*QYld.ncl-4.1*) was identified exactly associated with another SSR marker CaSSR2 on LG-4. Similarly, Cobos *et al*, (2007) identified three tightly linked QTLs for seed size and yield on LG-IV. This position can be allied with the two QTLs identified on LG-4 for Swt and Yld in the current studies.

3.5.7. Importance of LG-1 and LG-2 for yield enhancement

LG-1 and LG-2 could be considered as interesting genomic regions for yield traits in chickpea. LG1 and LG-2 had 28 and 15 QTLs, respectively representing at least one QTL

for each trait. Two QTLs for resistance to ascochyta blight have been already reported in the same LG-2. (Santra *et al.*, 2000; Tekeoglu *et al.*, 2002; Collard *et al.*, 2003; Flandez-Galvez *et al.*, 2003; Rakshit *et al.*, 2003; Udupa and Baum, 2003; Cho *et al.*, 2004; Iruela *et al.*, 2006). Such kind of clusters of yield related traits have been reported in several species such as rice (Mei *et al.*, 2003; Thomson *et al.*, 2003), common bean (Tar'an *et al.*, 2002) and pea (Timmerman-Vaughan *et al.*, 2005). SSR marker TAA170 of LG-1 which was closely linked to QTLs like Pht, Brp, Pdp, Swt and Yld might be useful in the selection of these traits. Similarly another SSR marker, NCPGR78 also had made significant interactive pleiotropism among the traits; Pht, Psp and Pdp in-order to enhance yield competence. Based on the linked markers, LG-1 which corresponds to the LGs I, IV and V of the chickpea reference map (Winter *et al*., 2000), contained QTLs for many traits, *viz*. plant height, plant spread, number of branches per plant, number of pods per plant, yield, seed weight (current study); seed number per plant, 100-seed weight, days to 50% flowering (Cho *et al*., 2002); blight resistance (Tekeoglu *et al*., 2002; Cho *et al*., 2004; Lichtenzveig *et al*., 2005); seed weight and lutein concentration (Abbo *et al*., 2005) and seed size, yield and days to 50% flowering (Cobos *et al*., 2007). Hence, concentrating on this LG through breeding programs might bring a futuristic prospect of the aforementioned traits.

3.6. Conclusion

The development and implementation of MAS for agronomic and yield traits is a high future priority as it will allow identification of relevant recombination events at an early stage without the need for costly and repetitive pheno-typing, especially when genetic markers are displayed concurrently for a number of traits. At the same time, the knowledge about genetic variation and estimates of breeding value are important for achieving higher response in MAS (Li *et al.*, 2003). This study demonstrates, how the environment influences the QTLs to effect yield and related traits. The inheritance of alleles of ICC-4958 also helped to complete the picture why it has been selected and released as a cultivar in spite of its late wilting nature. Evidence from the characterization of cloned QTLs from plants (Morgante and Salamini 2003; Paran and Zamir 2003)

suggested that many QTLs do not directly contribute to the variation in the trait, but instead may be either directly involved in interaction with coding sequences or else their gene products may be involved in such interactions. Co-location of many QTLs in the present study provides confirmation for the genetic basis of such correlations and suggests that selection for a small number of target genomic regions may achieve gains for multiple characters. Inclusion of the QTLs expressed in a particular environment or physiological conditions along with the stable QTLs could be a breeding strategy for developing genotypes adapted to a wide range of environments. The LGs (LG-5 LG-6 and LG-8) with sparingly located QTLs can be further saturated with more appropriate SSR markers to dissect additional QTLs. In conclusion the genetic control of grain yield and associated agronomic traits of chickpea has been dissected into QTLs and confined to the appropriate genomic regions. With further validation, the identified QTLs should be allowed in MAS practices with appropriate strategies that center on multi-trait selection for desirable characters with coincident QTL locations and on breaking unfavorable linkages between negatively correlated

Chapter IV

Molecular analysis of wilt resistant genes in chickpea (*Cicer arietinum* L.)

Chapter IV Molecular analysis of wilt resistant genes in chickpea (*Cicer arietinum* L.)

4.1. Abstract

PCR based molecular markers linked to late wilting resistant genes of race1 and race4 of *Fusarium oxysporum* Schlechtend: Fr f.sp. *ciceri* in chickpea *(Cicer arietinum*) were identified and linkage between the genes was estimated at LOD 3.5. Ninety three RILs developed from an $F_{8:9}$ population of chickpea cultivars with contrasting disease reactions to Fusarium wilt (Vijay - resistant and ICC-4958 - late wilting, cultivars) was used in the study. The SSR markers TS72 and TA02x flanked the *Foc1* resistant gene at 2 cM interval while the *Foc4* resistant gene was identified at a distal end of LG-4 and was flanked by two RAPD markers $UBC43_{550}$ and $UBC173_{600}$ with 3 and 10 cM distance, respectively while an SSR marker NCPGR51 was co-located with *Foc4* gene. The two resistant genes were located on the same LG at 19 cM apart, along with seven more markers. Segregation ratio of 1:1 for resistance: susceptibile confirmed the monogenic inheritance of both the genes in this population. Further evaluation of the markers linked to both the resistant loci would facilitate the pyramiding of the resistant genes through marker assisted selection (MAS).

4.2. Introduction

The wilt of chickpea (*Cicer arietinum L)* caused by *Fusarium oxysporum* f.sp. c*iceri* is estimated to cause up to 90% loss in the annual yield in India. The pathogen is seed and soil borne and numerous screening techniques for evaluating Fusarium wilt resistance have been developed and standardized; however, they are expensive in terms of time and effort. Screening of wilt can be achieved biochemically or genomically. It has been proved that wilting mechanism upon pathogen infection in field is biochemical. *In vitro* assay using Fusaric acid (FA), a phytotoxin produced by strains of *Fusarium oxysporum* could differentiate resistant and susceptible genotypes by causing wilt early in susceptible genotypes (Ravikumar and Babu, 2007). In another study, the production of medicarpin and maackiain (phytoalaexins) was induced in chickpea roots by exposure to *F. oxysporum* f.sp. *ciceri*, with greater induction occurring in resistant cultivars than in susceptible cultivars (Stevenson *et al.,* 1997). Apart from this, naturally infested field

plots have been used for large scale screening under natural conditions. The use of these sick plots may be hampered by unsuitable inoculum level of the pathogen, lack of uniformity of infestation across sites and the occurrence of multiple infections by several pathogens existing in the soil. The progress and understanding in breeding for resistance to wilt would be enhanced by the use of artificially infested field plots, in which a single pathogen is established at a convenient inoculum concentration. Alternatively, DNA-based diagnostics assays, which are fast, do not need screening of differential lines, not influenced by environment, are being developed for the pathogen and its races (Kelly *et al*., 1994; Barve *et al*., 2000; Gurjar *et al*., 2009). Random amplified polymorphic DNA (RAPD) markers have been used successfully to detect the pathogen in soil (Gracia-Pedrajas *et al*., 1999) and to distinguish between yellowing and wilting pathotypes either from isolated cultures (Kelly *et al.,* 1994) or from infected chickpea plants without fungal isolation (Kelly *et al.,* 1998). The utility of these assays to replace the traditional method based on host reaction for identification of the pathogen and its races is still to be confirmed. These assays need further refinements before being routinely used by the pathologists or breeders.

Despite the conventional chemical control methods available today, which are expensive and impractical, resistant cultivars are the most effective means of disease control (Jiménez-Diaz *et al*., 1993). Nevertheless, the effectiveness of resistant cultivars is often challenged by the appearance of new pathogenic races. Based on host differentials, eight pathogenic races (0, 1A, 1B/C, 2, 3, 4, 5 and 6) of the fungus have been reported worldwide (Sharma and Muehlbauer, 2007). The races 1, 2, 3, and 4 have been reported from India and races 0, 5 and 6 from Spain (Landa *et al.*, 2004). Different cultivars of chickpea show wilting at different growth stages and influence the loss in yield with different degrees of severity (Haware and Nene, 1982). Generally, wilting at early growth stages causes greater loss than that at later stages. A highly susceptible cultivar, under favorable conditions, may wilt within three to four weeks of sowing in a wilt-infested field whereas late wilting (tolerant) cultivars show general yellowing and drying of the lower leaves with wilting at a later stage, about five to six weeks of sowing

(Sharma *et al*., 2005) causing less yield damage. Identifying fusarium wilt race-specific resistant genes and transferring them to adapted backgrounds are the major challenges of chickpea breeding. Substantial amount of work has been conducted to discover the inheritance of Foc resistant genes in varied inter and intra-specific populations using resistant x susceptible or tolerant chickpea genotypes (Sharma *et al.,* 2005). The genes controlling resistance to Foc races 1, 2, 3, 4 and 5 have been identified on LG-2 forming a cluster of genes (Sharma and Muehlbauer, 2007). Recently Gowda *et al*. (2009) identified resistance genes of *Foc1, Foc2* and *Foc3* on LG-2, using an intra-specific *C. arietinum* population. Earlier, over 150 resistance sources were identified, of which some were resistant to more than one race (Nene *et al*., 1981) and it is possible that the resistance in these lines involved distinct genes. Combining resistance to more than one race in a commercial cultivar i.e., pyramiding of resistant genes is expected to provide durable resistance against the disease. The present work aimed to identify the markers closely linked to resistance genes to Foc1 and Foc4 in chickpea (*Cicer arietinum*) genome. Various marker systems like RAPD, ISSR, SSR and RGA were employed to identify closely linked markers for the resistant genes using an $F_{8:9}$ RIL mapping population. Co-linearity studies between related chickpea maps of intra and inter-specific population further confirmed the marker stability of LG-4 across related populations.

4.3. Materials and methods

4.3.1. Plant material

A set of ninety three F_9 recombinant inbred lines (RILs) from a cross between two *Cicer arietinum* cultivars, Vijay and ICC4958, were used to map the fusarium wilt resistant genes (*Foc1* and *Foc4*). Vijay is completely resistant to Foc races 1-5 and ICC-4958 is a late wilting cultivar [wilt symptoms within 30-45 days after infection (DAI)]. The RIL population was evaluated for reaction to the disease of race1 and race4 in wilt sick pots under controlled conditions at Pulses Research Station, Mahatma Phule Krishi Vidyapeeth, (MPKV) Rahuri, India.

The pot culture method as described by Barhate and Dake (2006) was followed. Interaction between the two genotypes of chickpea (Vijay and ICC-4958) and two races of *Fusarium oxysporum* f.sp. c*iceri* (Foc1 and Foc4) was evaluated in pot culture experiment. These isolates were multiplied on crushed cotton seed separately for 15 days at 27˚C. One hundred gram of inoculum was mixed per kg of autoclaved soil (soil + farm yard manure (FYM) mixture in 3:1 proportion) in 30 cm diameter earthen pots surface sterilized with 5% copper sulfate solution. The pots were watered lightly and incubated for seven days to allow the fungus become established before sowing of the seeds. The seeds were surface disinfected with 2.5% solution of sodium hypochlorite for 2-3 minutes before sowing. The experiment was conducted with three replications in a completely randomized design with ten plants per RIL in each replication with a specific innoculum. Some pots without the pathogen inoculum were used as control to grow plants of JG-62 (wilt susceptible, Gowda *et al*., 2009). For each Foc reaction (Foc1 and Foc4) separate pot analysis was carried out for the ninety three RILs. Numbers of wilted and healthy plants of each RIL were noted at five days interval from 20 to 80 days. The RILs were evaluated separately for Foc1 and Foc4 reactions. RILs upto 30% dead plants were considered resistant while others susceptible.

4.3.2. DNA extraction and PCR analysis

The DNA extraction and PCR analysis were carried out as explained in chapter II.

4.3.3. Statistical analysis

The data generated by different markers were recorded in binary fashion. The disease reactions of each RIL to different races of the pathogen and each marker data were analyzed by chi-square to determine goodness of fit to the expected segregation ratio of one resistant: one susceptible. Linkage between the markers and resistance genes was established using JoinMap v.4.0 (van Ooijen and Voorrips, 2001) at LOD 3.5 and Kosambi (1944) mapping function. The chi-square values were calculated using the following formula implemented in JoinMap v.4.0.

$$X^2 = \sum \frac{(\text{Observed frequency} - \text{Expected frequency})^2}{\text{Expected frequency}}$$

Data analysis was made as explained in chapter II. The image of the LG-4 and comparison of the present LG with the reported LGs were produced using MapChart v. 2.2 (Voorrips, 2002).

4.4. Results

4.4.1. Disease scoring

Wilting data with Foc1 and Foc4 were analysed in the parental genotypes, Vijay and ICC-4958 as well as in the population. Initially, the late wilting parent ICC-4958 did not demonstrate any wilting symptoms but as the disease progressed (40 DAI) the wilting symptoms appeared and sustained till the end of the period (80 DAI). At the same time, the resistant parent Vijay did not depict any wilting symptoms during the entire experimental period (till 80 DAI). The late wilting phenomenon on the RILs appeared to be analogous for both Foc1 and Foc4 races. However, the resistant and susceptible RILs took different time to develop wilt symptoms.Of the 93 RILs tested for the reaction to race1 of *Fusarium oxysporum* f.sp. *ciceri;* 49 were resistant and 44 were susceptible. Chi-square analysis indicated a good fit to the 1:1 (resistance:susceptible) segregation ratio probable for a monogenic trait ($P<0.05$) (Table 3.1). The RILs also segregated in the ratio of 1:1 (resistance (50): susceptible (43) for race 4 as well.

4.4.2. Molecular analysis

The 1,434 primers screened between the parents to identify polymorphic markers yielded 167 segregating loci. Linkage analysis of marker segregation was conducted using JoinMap V.4.0 at a LOD value of 3.5 (Fig 4.1). Linkage analysis confined both the Foc resistance genes to the same linkage group. Initially this LG was compared with the LGs of Winter *et al*. (2000) and co-linearity of the markers of LG-IV designated the current LG as LG 4. Thirty one markers (9 RAPD, 3 ISSR, 17 SSR and 2 RGA) showed linkage

with the two Foc resistance genes (Table 4.1). The SSR markers TS72 (Fig. 4.2A) and TA02x (Fig. 4.2B) flanked the *Foc1* late wilting gene at a distance of 2 cM and 1 cM, respectively. The *Foc4* late wilting gene was located between $UBC43_{550}$ (3 cM) (Fig 4.2D) and $UBC173_{600}$ (10 cM) while NCPGR 51 (Fig 4.2C) was co-located with *Foc4* gene. The two resistant loci were 18 cM apart from each other. Besides the flanking markers of the *Foc1* loci, two additional SSR markers *viz*. NCPGR6 and TS46 were observed in close proximity at a distance of 3 cM and 9 cM, respectively. Additionally, one RGA locus, Ptokinx of the two RGA loci (Ptokin) separated by 4 cM distance, was located 19 cM away from the *Foc1* resistance gene. Chi-square analysis revealed that three out of 31 loci deviated significantly ($P>0.05$) from the expected Mendelian segregation ratio of 1:1. One distorted locus (UBC335y) was observed in the near vicinity of *Foc4* resistance gene while the remaining two distorted loci (NCPGR7x and NCPGR7y) were located away from the two Foc resistant loci.

4.4.3. Co-linearity of linkage groups

The current LG showed similarity with the map of Winter *et al*. (2000) (Fig. 4.3A) Cho *et al*. (2002, 2004) (Fig. 4.3B,C), Tekeoglu *et al.* (2002) (Fig. 4.3D); and Anbessa *et al*. (2009) (Fig. 4.3E). The SSR markers TS72, TS54, TA2, TR20s, TA146, TA186, TAA170, NCPGR7, NCPGR6 and the RAPD UBC721 were the common markers identified between the current LG and the above mentioned maps of LG-4. Until now, markers have been reported to be linked with genes for resistance to races 1, 2, 3, 4 and 5 of Fusarium wilt on LG-2. Differences between the marker order of the current study and those of others were only minor, which indicates that the SSR regions are conserved in chickpea genome. To validate the identified closely linked markers of Foc1 and Foc4 on LG-4; a map established for an intra-specific population (JG-62 x Vijay; resistant x susceptible; JV population; Thesis. M.J.Gowda, 2009) was compared (Fig. 4.3F). Phenotypic data for the reactions to race1 and race4 were also obtained from this population. Eight SSR markers showed co-linearity between the current and the compared JV map of LG-4, however, none of the markers were linked to either *Foc1* or

Foc4 resistance genes. *Foc1* resistance gene has already been identified on LG-2 (Gowda *et al.,* 2009) of this population.

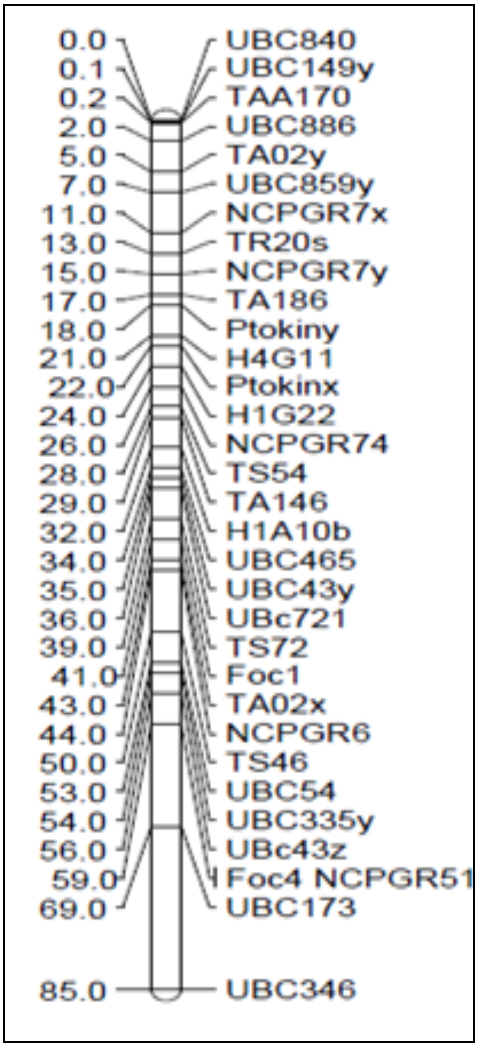


Figure 4.1: Mapping of the *Foc1* and *Foc4* loci imparting resistance to the races 1 and 4 of *Fusarium oxysporum* f.sp. *ciceri* in chickpea. Based on the common markers, the present linkage group corresponds with LG-4 of the reference map of Winter *et al*. (2000). The markers are arranged on the right side, while the map distances on the left side. UBC: RAPD and ISSR, Ptokin (x, y): Resistance gene analogs; Others: SSR markers

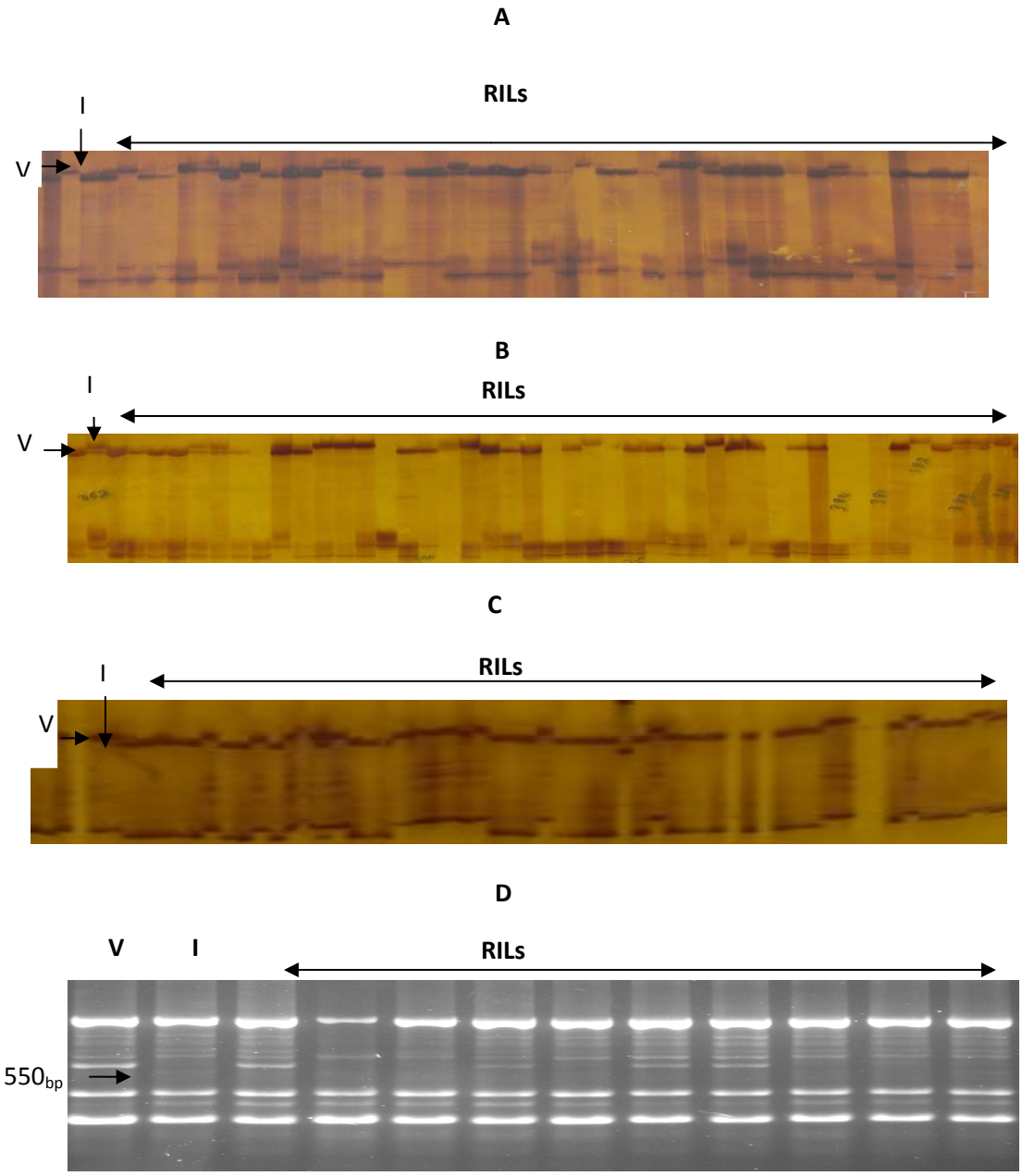


Figure 4.2: Segregation of flanking markers of *Foc1* and *Foc4* resistance genes. A: SSR TS72, B: SSR TA02x, C: SSR NCPGR51, D: UBC43z_{550}; VI: parents; Vijay, ICC-4958; RILs: Recombinant inbred lines

Table 4.1: **Disease reaction of the RILs to race1 and race4 of *Fusarium oxysporum* f.sp. *ciceri* (*Foc*) and frequencies of marker genotypes**

Gene/Marker	Resistant	Susceptible	X^2 (1:1)	Gene/Marker	Resistant	Susceptible	X^2 (1:1)
Foc1	49	44	0.3	NCPGR74	44	34	0.8
Foc4	50	43	0.5	TS54	53	31	5.8
UBC840	49	39	1.1	TA146	53	32	5.2
UBC149y	52	33	4.3	H1A10b	36	43	0.6
TAA170	36	34	0.1	UBC465	42	42	0.0
UBC886	48	38	1.2	UBC43y	45	48	0.1
TA02y	51	38	1.9	UBC721	44	46	0.0
UBC859y	48	35	2.0	TS72	42	50	0.7
NCPGR7x	52	36	2.9	TA02x	46	41	0.3
TR20s	58	35	5.7	NCPGR6	44	40	0.2
NCPGR7y	47	37	1.2	TS46	45	37	0.8
TA186	41	47	0.4	UBC54	32	45	2.2
Ptokiny	37	44	0.6	UBC335y	29	64	13.2*
H4G11	36	41	0.3	$UBC43z_{550}$	58	35	5.7
Ptokinx	38	43	0.3	NCPGR51	38	44	0.6

H1G22	45	37	0.8	$UBC173_{600}$	42	51	0.9
UBC346	58	35	5.7				

$\mathbf{X^2}$ = P< 0.05 * distorted markers (p>0.05) Resistant and susceptible refers to reaction of the RILs to Foc races 1 and 4

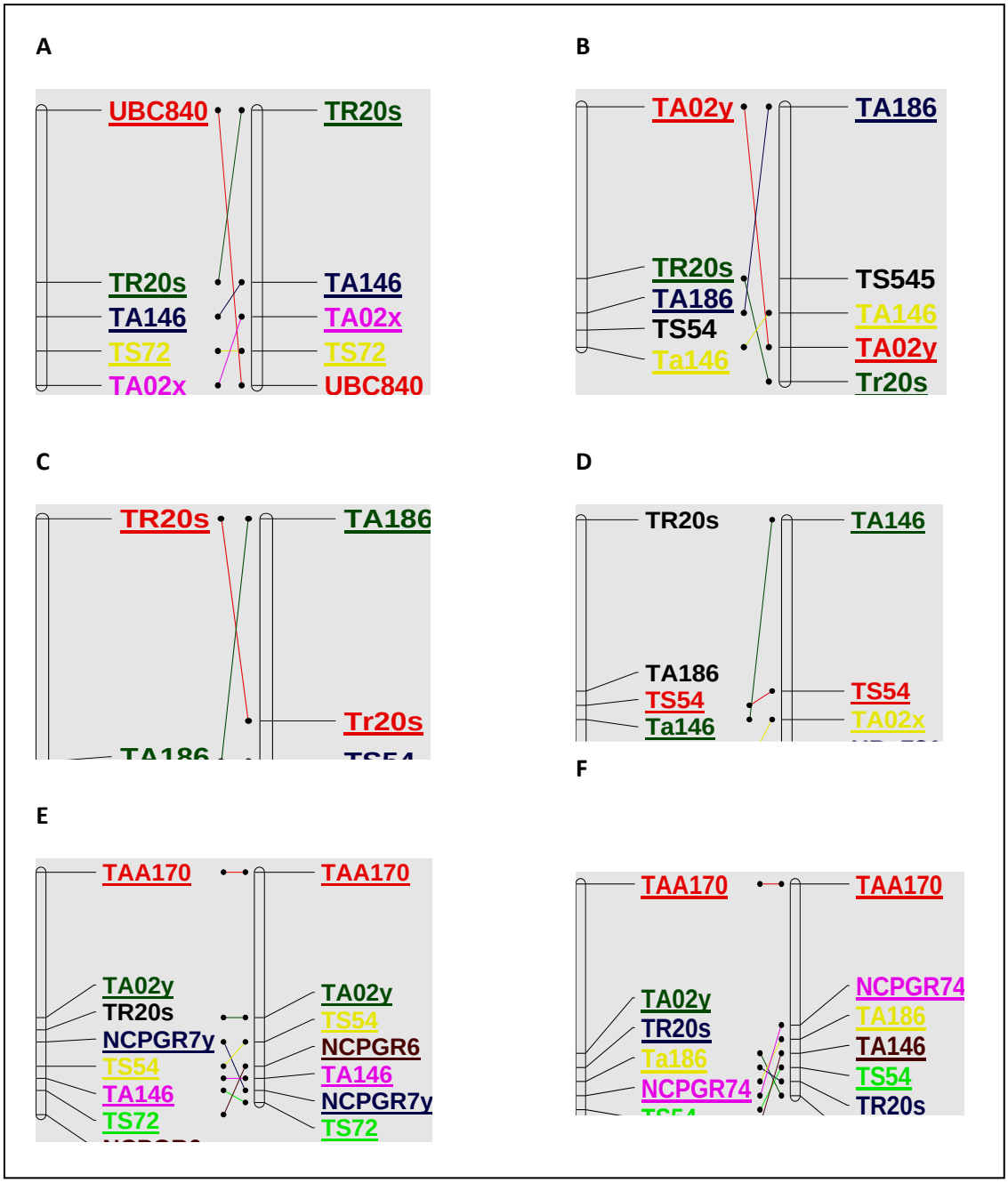


Figure 4.3: Comparison of current LG-4 with various reported LG-4A: Winter *et al*., 2000; B: Cho *et al*., 2002; C: Cho *et al*., 2004; D: Tekeoglu *et al*., 2002; E: Taren *et al*., 2007a; F: M.J. Gowda Thesis, 2009, Pune University. Left side current LG, right side others

4.5. Discussion

An important goal of genetic mapping in chickpea in the present study is to tag genes for resistance to important fungal pathogen *Fusarium oxysporum* f.sp. *ciceri.* The identification of molecular markers linked to various traits in many other crops has accelerated plant breeding in a number of areas including disease and insect resistance (Melchinger, 1990).

4.5.1. Inheritance of wilt resistance genes in Vijay x ICC-4958 population

Monogenic inheritance observed for the race1 and race4 in the present study is in agreement with the results of Sharma *et al*. (2004), Mayer *et al.* (1997) and Tullu *et al*. (1998). However the parent C-104 used in Sharma *et al*. (2004) was a complete susceptible cultivar for races 1 and 4, whereas ICC-4958 used in the current study was a late wilting cultivar. This clearly indicates the presence of a control gene that delays the onset of disease symptoms (Upadhyaya *et al.,* 1983b; Singh *et al.,* 1987). This further signifies that the resistant and the late wilting parents disagree only with respect to this single dominant gene for late wilting each for both the races. In a cross between a resistant and a late wilting cultivar (WR315 x K850) all the F_1 plants were late wilting, indicating late wilting to be dominant over resistance (Kumar, 1998). This study also confirms the existence of differences among chickpea cultivars for days to wilting, as reported by Haware and Nene (1980) and Upadhyaya *et al*. (1983a) for race 1. Late wilting could be of greater practical value than a high level of resistance, which, because of high selection pressure, forces the pathogen to evolve into a more aggressive race. The identification of a single gene for late wilting , reported in this study, opens up the possibility of using late wilting cultivars in the chickpea improvement programme to obtain complete resistance, as demonstrated in the cross K850 x GW5/7 (Kumar, 1998. Singh *et al.* (1987) also obtained resistant plants in a cross of two tolerant cultivars for race1. A much wider range of parents can, therefore, be available for breeding wilt resistance. This also underlines the importance of recording the wilting of germplasm

lines to distinguish highly susceptible lines from the late-wilting ones because any delay in disease development is important for reducing yield loss.

4.5.2. Mapping of wilt resistance genes in chickpea

Until now, the resistance genes for Foc1 and Foc4 were mapped only on LG-2 in close proximity (*foc-1,* Mayer *et al.*, 1997; Gowda *et al.,* 2009; *foc-4,* Ratnaparkhe *et al.,* 1998a, b; Tullu *et al.,*1998; Tekeoglu *et al.*, 2000; Winter *et al.*, 2000; Benko-Iseppon *et al.*, 2003; Sharma *et al.*, 2004b; Sharma and Muehlbauer, 2005; the second resistance gene for race 4, Tullu *et al.*, 1999). In the present study the genes for Foc1 and Foc4 late wilting were identified on LG-4. The SSR and RAPD markers TS72, TS54, TA2, TR20s, TA146 and UBC721 identified in the present study have also shown co-linearity with LG-4 in some of the other genetic maps of the *Cicer* genome (Winter *et al.,* 1999, 2000; Cho *et al.*, 2002; Tekeoglu *et al.*, 2002 (Fig 3.4); Udupa and Baum 2003; Cho *et al.,* 2004 and Anbessa *et al*., 2009) but not in linkage with any wilt resistance genes. However, this region indicates clustering of disease resistant genes since some of the identified SSR markers remained as indicative markers of the blight QTLs on LG-4. (TR20- Collard *et al.,* 2003, Rakshit *et al.,* 2003; TS 72- Udupa and Baum 2003; TA-146 –Millan *et al.,* 2003, Iruela *et al.,* 2006). Thus, position of genes for resistance and other traits observed in various populations can be assigned to a particular region of the chickpea genome (Sharma *et al.,* 2003). However, the differences observed in the order of mapped SSR and RAPD markers might be due to the different parental combinations used. Similarly different methods of linkage analysis performed in different studies could also affect the order or distance between mapped markers even in an intra-specific population as observed with the report of Cho *et al.* (2002, 2004) and Tekeoglu *et al.* (2002). Population size could be another determining factor, since larger populations are expected to give more accurate estimates of the marker distance and order.

The Mendelian inheritance of the 28 markers indicated that most lines are relatively unbiased and show low heterozygosity. Using an advanced generation RIL population (F_{8-9}) has facilitated the homozygosity of the RIL population. The lines would

thus appear to constitute a suitable population for the mapping of the major disease resistance genes.

Close linkage of microsatellites with resistance genes has been documented for many other crops and the traits, e.g. bacterial leaf blight in rice (Blair and McCouch, 1997), fusarium wilt resistant genes in chickpea (Gowda *et al*., 2009) and soybean cyst nematode resistance in *Glycine soja* (Shawn *et al*., 2007). Recently, mapping of the chickpea genome advanced considerably due to the availability of SSR markers (Taran *et al*., 2007, Anbessa *et al.,* 2009) and the integration of different marker types into a single map (Winter *et al*., 2000). For practical use in chickpea breeding, and especially marker-assisted selection, the close linkage of genes for resistance to Fusarium wilt and several SSR markers is of great importance as it allows the use of at least a couple of the highly polymorphic markers for analysis of the segregation of wilt resistant genes in a wide range of germplasm.

In the current study, a cluster of markers were closely linked to the *Foc1* resistant locus, whereas only very few markers segregated with the *Foc4* locus at considerable distance. Possible explanation for the discrepancy between marker densities around the two resistant loci could be that, the region surrounding the Foc1 locus could be more polymorphic than the one around the Foc4 locus, and the region around Foc4 locus could be a gene rich region.

4.5.3. Clustering of disease resistant genes

Although there are no other reports of wilt resistant genes on LG-4, genes associated with ascochyta blight resistance have been identified. Udupa and Baum (2003) attempted to map the genes for pathotype-specific blight resistance on an intra-specific linkage map from the cross of ILC 1272 × ILC 3279. They identified two QTLs for resistance to pathotype II of *Ascochyta rabiei*. Two important QTLs related to ascochyta blight resistance were also validated by Iruela *et al.* (2006, 2007). The *QTL 3* identified by Santra *et al.* (2000) for ascochyta blight and the QTL identified by Tekeoglu *et al.* (2002) using an inter-specific cross were also located on LG-4. This particular QTL co-localized

with another QTL identified by Cho *et al.* (2004) for resistance to pathotype II of ascochyta blight. It was concluded that the QTL on LG-4 is the major locus for resistance to *A. rabiei* in chickpea (Cho *et al.,* 2004).

In addition to the QTLs of ascochyta blight resistance, Flandez-Galvez *et al.* (2003b) identified RGA marker clusters on LG-4 adjacent to several QTLs of *A. rabiei*. Many studies have found that RGA markers have a tendency to locate at specific regions in the chickpea genome (Flandez–Galvez *et al.*, 2003a). Moreover, RGA markers clustered on LG-4 might suggest that this region contains a conserved repertoire of different but functionally related R-genes of chickpea (Flandez –Galvez *et al*., 2003a). The linkage of two RGA (Ptokin) loci with the two wilt resistance genes in this study supports the view that disease resistance genes tend to be clustered in plant genomes and RGAs identified using this approach are frequently located close to previously identified resistant loci or QTLs (Radwan *et al.*, 2003; Rossi et al., 2003; McIntyre *et al.*, 2005). Clustering of resistant genes for different races of a pathogen and also for different pathogens has been demonstrated in several plants including legumes (Kanazin *et al.*, 1996; Yu *et al.,* 1996) and may be the result of gene duplication, exon shuffling and recombination processes that are thought to have generated different resistant genes from one or a few ancestral progenitor genes (reviewed by Michelmore, 1996; Hammond-Kosack and Jones, 1997). The results presented here highly coincided with that of Sharma *et al.* (2003) and those by Udupa and Baum (2003) where resistance against two diseases (fusarium wilt and ascochyta blight) resided on the same LG.

4.6. Conclusion

Even though in the present study, there are some makers identified linked to resistance genes, it is still necessary to saturate different genomic areas in order to more accurately locate the genes or QTLs involved in fusarium wilt (Millan *et al.*, 2006). An ideal situation can be where the marker is the part of the gene. The current study has revealed close linkage of genes for resistance to fusarium wilt along with adjacent SSR markers, which are of great importance as it allows the use of at least one of the highly

polymorphic markers for analysis of the segregation of wilt resistant genes in a wide range of germplasm. The flanking RAPD markers of the *Foc4* resistant gene need to be converted into more precise SCAR or ASAP marker to increase the reproducibility of the identified marker. Further genomic analysis and validation of the markers in a compatible population is essential. None of the presently available markers were located close enough to respective loci to allow map-based cloning of the respective genes. There are eight different races of *Fusarium* causing wilt in chickpea, and resistance to some of them is governed by more than one gene. Allelic tests need to be performed and genetic stocks need to be developed for all of them as a basis for complete understanding of the complex genetics of resistance, which is a prerequisite for pyramiding of resistant genes to obtain durable resistant varieties.

Chapter V

Summary and future prospectives

Chapter V Summary and future prospective

5.1. Summary

In the present work a consensus framework map for chickpea (*Cicer arietinum* L.) was constructed from the integration of two mapping populations derived from crosses between wilt resistant, susceptible and late wilting cultivars. One of these map, was used to dissect the genomic regions controlling QTLs for yield and yield related traits as well as to position the genes of Fusarium wilt resistance *(Foc1 and Foc4*).

5.2. Construction of individual maps

Linkage analysis was established using ninety-three randomly selected RILs of the F_9 population derived from crosses, JG-62 x Vijay and Vijay x ICC-4958. The primers used in the present study included 800 RAPDs (UBC1-800), 100 ISSRs (UBC801-900), 24 RGAs, 1 ASAP (CS27) and 504 chickpea SSRs. Optimal PCR conditions were established for each polymorphic primer type. PCR products were resolved on either 2% agarose gel (RAPD and ISSR products) or metaphor gel or PAGE gels (SSR and RGA) depending on their allelic size difference. All the marker loci were scored at least twice to minimize interpretation errors. The linkage analysis for the JV population revealed seven linkage groups with 104 markers and a genomic coverage of 484.9 cM at an average marker density of 4.61 cM. While the VI population produced 166 segregating markers, 624 cM total length and a marker density of 3.67 cM on seven LGs. Linkage analysis was conducted using JOINMAP ver 4.0 (van Ooijen and Voorrips, 2001). A recombination frequency of 0.4 and a LOD value 3.0 were used as threshold limits for linkage group construction. Of the 104 and 166 markers mapped in the JV and VI populations, respectively, 44 and 17 markers did not segregate according to the expected Mendelian ratio.

5.2.1. Construction of integrated map

JoinMap Ver. 4.0 (Van Ooijen and Voorrips, 2001) was used for integrating the two individual maps along with 43 common markers on five LGs. The LG-6 and LG-7 of the individual maps lacked common markers, therefore integration was not established. Thus,

the LG-6, LG-7 and LG-8 of the integrated map possessed LGs from JV and VI populations, respectively. The joint segregation analysis with 228 markers (44 RAPDs, 16 ISSRs, 165 SSRs, 2 RGAs and 1 ASAP) and 186 RILs produced an integrated map that covered 739.6 cM with an average marker density of 3.2 cM. The correlation coefficients 0.58, 0.72 and 0.70 ($P < 0.001$) for the maps JV, VI and integrated map, indicated random distribution of markers over the LGs. All the three maps showed co-linearity with the existing intra and inter-specific chickpea linkage maps.

5.3. QTL mapping of agronomic traits in chickpea

Quantitative trait loci (QTLs) associated with seven agronomic traits (plant height, plant spread, branches per plant, days to maturity, pods per plant, seed weight and yield per plan) in a F_9 derived recombinant inbred line populations was studied in detail. Analyse performed were single locus QTL analysis through composite interval mapping (CIM) for individual traits and multiple-trait composite interval mapping (MCIM) for pleiotropic QTLs. Two-locus analysis was conducted to identify the main effect QTLs (M-QTLs), epistatic QTLs (E-QTLs) and QTL × environment interactions.

A Total of 74 QTLs were detected for all the seven traits. Among them 41 were significant QTLs (LOD ≥ 3.0) and 33 were suggestive QTLs (2.0 > LOD < 3.0) (including seven pleiotropic QTLs). The parental genotypes used to develop the mapping populations were distinct for all the traits. These traits showed good fit to the normal distribution in the population. Transgressive segregants were observed in all the environments. Comparisons between the best parent and the best RIL showed a significant difference for all the traits. Correlations among traits were evaluated at $P < 0.05$, $P < 0.01$ and $P < 0.001$. Yld was significantly and positively correlated with traits like Pht, Psp, Brp and Pdp. The number of QTLs for individual traits ranged from three (Dmt and Brp) to sixteen (Psp). The QTL, *QBrp.ncl-1.1* accounted for the highest phenotypic variation (23.84%). The lowest variation had been exhibited by the QTL, *Swt.ncl-3.3* with 6.27% variation. The SSR marker, TAA170 was observed to be associated with QTLs of many traits, *viz.* Pht, Brp, Yld and Swt. This feature accounts for

possible pleiotropic effect in the population. ICC-4958 allelic contribution has been more than Vijay allele for all the seven traits. Out of the 74 QTLS, 46 had the contribution from ICC-4958 and 28 from Vijay. The traits Pht, Psp, Swt, Dmt, Pdp and Yld QTLs were positively influenced by ICC-4958 alleles. However, in case of Pht and Psp QTLs, contribution seems to be equal. LG-1 contained at least one QTL for each trait. Further studies on these LG would bring simultaneous improvement of these traits.

QTL interactions were studied by using QTL Network software. A total of 23 QTLs (11 M-QTL and 12E- QTLs) were identified which were involved in QE and QQE interactions. Eight of the M-QTLs had been already identified through single locus CIM analysis in the same and / or adjacent marker intervals. The minor differences associated with the position and the marker interval of the QTLs may be attributed to the different approaches and software used. Brp, and Swt are the traits that had only QE interactions and Pdp, Psp and Yld expressed both the QQ and QQE actions with respect to all the environments. The traits Pht and Dmt failed to illustrate any interactions through this two way analysis.

5.4. Molecular mapping of Fusarium wilt resistance genes in chickpea

PCR based molecular markers linked to *Fusarium oxysporum* Schlechtend.: Fr f.sp. *ciceri* races Foc1 and Foc4 late wilting resistance genes in chickpea *(Cicer arietinum*) were identified and linkage between the genes were estimated at LOD 3.5. Ninety three RILs developed from an $F_{8:9}$ population of chickpea cultivars with contrasting disease reactions to Fusarium wilt was used in the study. The SSR markers TS72 and TA02 flanked the *Foc1* resistance gene at 2 cM interval while the *Foc4* gene was identified at a distal end of the LG-4 flanked by two RAPD markers $UBC43z_{550}$, $UBC173_{600}$ and a SSR marker NCPGR51 with 3 and 10 cM distance, respectively. The two resistance genes were located on the same LG at 19 cM apart, along with seven more markers. 1:1 segregation for resistance: susceptibility confirmed the monogenic inheritance of both the genes in this population.

5.5. Future prospectives

Owing to the advances in the area of molecular genetics and automation, dense molecular genetic maps are now available for the major cereal species, however, there is still the need to integrate more markers in the genetic maps of important pulses like chickpea, pigeon pea and soybean. This provides molecular breeding strategies with more choice in the quality of markers and more probability of polymorphic markers in an important chromosome interval. Accordingly the currently developed maps need to be saturated with more appropriate marker systems like SSRs, EST-SSRs and CAPs etc. From the currently developed seven LGs, the individual maps should possess the near complete genome coverage with eight LGs. The integrated map has many linkage gaps to be filled up with suitable markers. The up-coming intra-specific maps can be integrated with this integrated map. This is done by mapping several common microsatellites on each LG in different mapping populations, such that this subset of markers anchor the linkage maps from different populations. This approach derives a single genetic map constructed by the consensus of marker order and recombination distances.

QTL dissection has yielded, QTLs with high phenotypic variation (*QBrp.ncl-1.1,* 23.84%), stable QTLs (*QPht.ncl-1.2*, *QPht.ncl-1, QBrp.ncl-1.1*) and pleiotropic QTLs (*QPht.ncl-1.6, QBrp.ncl-1.2*). All these QTLs directly or indirectly contribute for yield improvement, hence few more multi-locational trials and validation of identified major QTLs using larger population would be necessary to establish better confidence in these loci for MAS.

The currently used chickpea cultivars (Vijay and ICC-4558) had been identified with two late wilting genes of Fusarium wilt (race1and race4). Efforts need to be taken to identify the markers linked with other important genes for the races race 2, 3 and 5 in the same population or introgress these valuable genes into other elite chickpea varieties. The marker density around the mapped Fusarium wilt resistant genes can be increased by further saturation of the linkage map. The identified tightly linked markers can be

screened with the available chickpea BAC libraries to confirm their position on the chickpea genome.

5.6. Conclusion

Besides, high-throughput approaches combined with automation, availability of sequence data in the public domain and enhanced bioinformatic techniques can be utilized for chickpea crop improvement. Not only the genomic changes, but also proteomic and metabolomic changes can be studied in order to elucidate the chickpea molecular evolution. Thus, integrating various 'omics' into the field of molecular breeding is expected to bring in more fundamental revolutions in plant breeding. It would be crucial to recognize the complementarities between molecular technologies and conventional breeding, and harness this vital synergy into research strategies aimed towards more efficient crop improvement in future.

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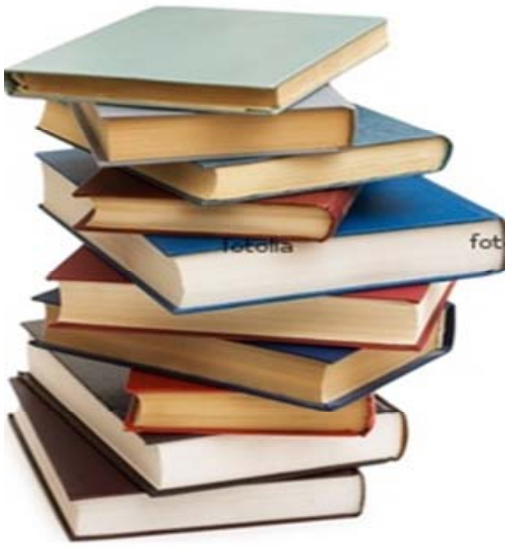

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Publications arising from the current research work

1. **Radhika P**, Gowda M, Kadoo NY, Mhase LB, Jamadagni BM, Chandra S, Sainani MN, Gupta VS (2007) Development of an integrated intraspecific map of chickpea (*Cicer arietinum* L.) using two recombinant inbred line populations. *Theoretical and Applied Genetics*, 115(2): 209-216.

2. Gowda S.J.M, **Radhika P**, Kadoo N.Y, Mhase L.B, Gupta V.S (2009) Molecular mapping of wilt resistance genes in chickpea. Mol. Breed 24: 177-183.

3. **Radhika P**, Gowda M, Kadoo NY, Mhase LB, Jamadagni BM, Gupta VS (2009) Mapping of wilt resistant genes (late wilting *Foc1* and *Foc4*) in chickpea (*Cicer arietinum*.L) (To be communicated).

4. Gowda M, **Radhika P**, Kadoo NY, Mhase LB, Jamadagni BM, Chandra S, Sainani MN, Gupta VS (2009) Mapping of yield and related QTLs in chickpea (*Cicer arietinum* L.) using two recombinant inbred line populations. (To be communicated).

5. **Radhika P** Gowda M, Kadoo NY, Mhase LB, Jamadagni BM, Chandra S, Sainani MN, Gupta VS (2009) Mapping of stable QTLs for yield and related traits in chickpea (*Cicer arietinum* L.) (Manuscript under preparation).

Review articles

1. Gupta VS, Gowda M, Kadoo NY, **Radhika P** and Sainani MN (2005) Quantitative Trait Loci (QTL) Mapping and Marker Assisted Selection (MAS) in Food Legumes. International Food Legumes Research Conference -IV, held at IARI, New Delhi from Oct 18-22, 2005

Posters presented

1. **Radhika P**, Gowda M, Kadoo NY, Jamdagni BM, Mhase LB and Gupta, VS (2009) Mapping of agronomic traits in chickpea (Cicer arietinum L). Poster presented in the Science day celebrations, National Chemical Laboratory, Pune, on February 25, 2009.

2. Gowda M, **Radhika P**, Kadoo NY, Jamdagni BM, Mhase LB and Gupta, VS (2005) Mapping of QTLs for yield traits in chickpea (*Cicer arietinum* L.). Poster presented in International conference on New Horizons in Biotechnology (NHBT), held at NIIST, Trivendrum, Kerala, India, from Nov 26-29, 2007.

3. Gowda M, **Radhika P**, Kadoo NY, Jamdagni BM, Mhase LB and Gupta, VS (2005) Mapping of agro-economically important traits in chickpea (*Cicer arietinum* L.). Poster presented in International Food Legumes Research Conference -IV, held at Indian Agricultural Research Institute, New Delhi, India, from Oct 18-22, 2005.