

**IDENTIFICATION OF QTLs
CONTROLLING GRAIN IRON AND ZINC
CONCENTRATION IN SORGHUM
*(Sorghum bicolor (L.) Moench)***

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M.Sc. (Ag.)

**DOCTOR OF PHILOSOPHY IN AGRICULTURE
(GENETICS AND PLANT BREEDING)**



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**BY
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M.Sc. (Ag.)**

**THESIS SUBMITTED TO THE PROFESSOR JAYASHANKAR
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IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE AWARD OF THE
DEGREE OF**

**DOCTOR OF PHILOSOPHY IN AGRICULTURE
(GENETICS AND PLANT BREEDING)**

CHAIRPERSON: Dr. Farzana Jabeen



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AGRICULTURAL UNIVERSITY
2016**

DECLARATION

I, **PHUKE RAHUL MADHAVRAO**, hereby declare that the thesis entitled "**IDENTIFICATION OF QTLs CONTROLLING GRAIN IRON AND ZINC CONCENTRATION IN SORGHUM (*Sorghum bicolor* (L.) Moench.)**" Submitted to the **Professor Jayashankar Telangana State Agricultural University** for the degree of **DOCTOR OF PHILOSOPHY IN AGRICULTURE** is the result of original research work done by me. I also declare that no material contained in this thesis has been published earlier in any manner.

Place: Rajendranagar

(PHUKE RAHUL MADHAVRAO)

Date:

I. D. No. RAD/12-35

CERTIFICATE

Mr. **PHUKE RAHUL MADHAVRAO** has satisfactorily prosecuted the course of research and that thesis entitled "**IDENTIFICATION OF QTLs CONTROLLING GRAIN IRON AND ZINC CONCENTRATION IN SORGHUM (*Sorghum bicolor (L.) Moench.*)**" Submitted is the result of original research and is of sufficiently high standard to warrant its presentation to the examination. I also certify that neither the thesis nor its part thereof has been previously submitted by him for a degree of any University.

Place: Rajendranagar

Date:

(FARZANA JABEEN)

Chairperson

CERTIFICATE

This is to certify that the thesis entitled "**"IDENTIFICATION OF QTLs CONTROLLING GRAIN IRON AND ZINC CONCENTRATION IN SORGHUM (*Sorghum bicolor (L.) Moench.*)"** Submitted in partial fulfilment of the requirements for the degree of 'Doctor of Philosophy in Agriculture' of the Professor Jayashankar Telangana State Agricultural University, Hyderabad is a record of the bonafide original research work carried out by Mr. **PHUKE RAHUL MADHAVRAO** under our guidance and supervision.

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

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Date:

(Rahul M. Phuké)

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Per cent
<	:	Less than
>	:	Greater than
\leq	:	Less than or equals to
\geq	:	More than or equals to
Σ	:	Summation
AAS	:	Atomic Absorption Spectrophotometry
ANOVA	:	Analysis of variance
BLUPs	:	Best Linear Unbiased Prediction
C TAB	:	Cetyl Trimethyl Ammonium Bromide
CGIAR	:	Consultative Group on International Agricultural Research
CIM	:	Composite Interval mapping
cM	:	centiMorgan
cm	:	centimetre
DArT	:	Diversity Array Technology
DTF	:	Days to 50% Flowering
<i>et al.</i>	:	and others
FAO	:	Food and Agriculture Organization
Fe	:	Iron
G \times E	:	Genotype \times Environment Interaction
GY	:	Grain Yield
ha	:	hectares
ICP-OES	:	Inductively Coupled Plasma Optical Emission Spectrometry

ICRISAT	:	International Crops Research Institute for Semi-Arid Tropics
i.e.	:	that is
IIMR	:	Indian Institute of Millets Research
kg ha^{-1}	:	Kilograms per hectare
LG	:	Linkage Group
LOD	:	Logarithm of Odds
Mbp	:	Mega Base pair
mg kg^{-1}	:	Milligram per kilogram
NILs	:	Near Isogenic Lines
P1	:	Parent 1
P2	:	Parent 2
PH	:	Plant Height
QTL	:	Quantitative trait Loci
R^2	:	Phenotypic Variance
RIL	:	Recombinant Inbreed Line
SNPs	:	Single Nucleotide Polymorphism
SSR	:	Single Sequence Repeat
$t \text{ ha}^{-1}$:	tons per hectare
TW	:	Test Weight (100 Seed Weight)
<i>viz.,</i>	:	Namely
VNMKV	:	Vasantrao Naik Marathwada Krushi Vidhypeeth
WHO	:	World Health Organization
XRF	:	X-ray Fluorescence Spectrometry
Zn	:	Zinc

Abstract

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ABSTRACT

The grain iron and zinc are well documented problems in food crops, causing decrease of crop nutritional quality especially in cereals crops. The sorghum is major source of energy and micronutrients for majority of population in Africa and central India. The understanding of genetic variation, genotype \times environment interaction, correlation between these traits and QTLs mapping for grain iron and zinc concentration is expected to contribute for better improvement in cultivars. A total of 336 RILs (Recombinant Inbred Lines) of F₆ generation developed from contrasting parents 296 B and PVK 801 were evaluated for grain iron and zinc concentration along with other agronomic traits for two consecutive years at three locations. Analysis showed that large variability exists in the population for both nutritional and agronomic traits. The genotype \times environment interaction (GEI) for both micronutrients (Iron and Zinc) was highly significant. Compared to zinc, iron showed greater variation across the environments. The iron and zinc content were positive and correlated indicating the possibility of simultaneous effective selection for both the traits. The result suggested that GE was substantial for grain iron and zinc, hence greater efforts for taking care of GE interaction are needed to breed iron and zinc rich sorghum lines. The RIL population showed good variability and heritability for all studied trait. From preliminary observation of histogram of frequency distribution of all phenotypic traits using across environments BLUPs means indicated continuous distribution of traits as expected from quantitative traits and also transgression beyond the parents value indicating large variation among 336 RIL, hence supersite to map QTL for studied trait using present RIL population.

Parental polymorphism involving 271 SSR markers spanning all ten sorghum chromosomes was carried out. Among 271 SSR markers screened, forty five markers were found polymorphic, in addition to that a set of 6126 (70.15%) polymorphic DArT clones were identified in total of 8732 clones, also 3331 (91.51%) polymorphic SNP clones were identified out of 3640 clones, on the array of 296 B and PVK 801. The linkage map was developed using 24 SSR, 1184 DArT and 950 SNPs markers as remaining markers showed certain problems like dominant inheritance, lack of linkage, high distortion towards parents and having more than 10% missing data points. Out of total 2158 markers (SSR, DArT and SNPs) used for mapping, only 2088 (96.7%) markers assigned in 10 linkage groups with a LOD score 10.0 to construct the genetic linkage map. The total length of map was 1356 cM (Kosambi), which represent on an average one marker for every 0.64 cM. The individual Linkage Group (LGs) ranged from 180.6 cM for LG 3 with total number of 257 markers to 102.06 cM for LG 10 with lowest number of markers 79 markers. The average linkage group length was 135.5 cM with an average of 208.7 loci.

The QTL mapping experiment was carried for all individual trial data of total six trials and for across environment data (Pooled data of six trials) using software QTL Cartographer. For grain iron concentration In E₁ (ICRISAT 12-13) total 22 QTLs were detected on linkage group LG 1, LG 4, LG 6, LG 7, LG 8 and LG 9. In E₂ (IIMR 12-13) only three QTLs were identified which were located on LG 4 accommodating two QTLs and single QTL on LG 8, In E₃ (VNMV 12-13) total 10 QTLs were identified one on LG 4, four on LG 7 and five on LG 9; in E₄ (ICRISAT 13-14) total seven QTLs were identified for grain iron concentration, three QTLs on LG 1 and single QTL from each LG 4, LG 5, LG 6 and LG 7. In E₅ (IIMR 13-14) total five QTLs were identified in which LG 3 and LG 5 were accommodating two QTLs each, while single QTL on LG 7 and in E₆ (VNMKV 13-14) nine QTLs were observed from four different linkage groups *viz.*, LG 2, LG 3, LG 4 and LG 7.

For grain zinc concentration; in E₁ (ICRISAT 12-13) total 22 QTLs were identified located on five different linkage groups *viz.*, LG 4, LG 6, LG 7, LG 9 and LG 10; in E₂ (IIMR 12-13) only two QTLs were identified, single QTL each on LG 4 and LG 7; in E₃ (VNMKV 12-13) total 17 QTLs were identified on five different linkage group *viz.*, LG 4, LG 5, LG 7, LG 9 and G 10. In E₄ (ICRISAT 13-14) total seven QTLs were identified on four different linkage groups single QTL on LG 4, two QTLs on each LG 5, LG 7 and LG 9; in E₅ (IIMR 13-14) only one QTL was identified on LG 3 and in E₆ (VNMKV 13-14) a total of 12 QTLs were identified one each on LG 2, LG 4, LG 5 and LG 10, three QTLs on LG 6 and five QTLs on LG 7.

A total of 21 QTLs controlling grain Fe and Zn were found across six environments pooled analysis. Nine QTLs for grain Fe and 12 QTLs for grain Zn concentration were identified with the phenotypic variance (R^2) ranging from 2.82% to 6.66% and 0.30% to 5.74% respectively. In both individual environment and across environment analysis, Linkage Group 7 accommodated most of the QTLs for grain iron and zinc with the highest LOD score and also with same phenotypic variance. Moreover LG 7 accommodate Co-Localized QTL

for grain Fe and Zn concentration, hence LG 7 can be most preferred linkage group for improving of sorghum genotypes rich in grain iron and zinc concentration. Also in individual environment analysis and across environment analysis it was observed that multiple minor QTLs for both iron and zinc were located within the distance of 10 cM, which together can act as major QTL to give maximum phenotypic expression, moreover much of the variation for both the micronutrient trait was covered by multiple minor QTLs which indicates that Marker Assisted Recurrent Selection (MARS) will play the crucial role in improving these traits through molecular breeding.

Introduction

Chapter I

INTRODUCTION

Dietary deficiency of micronutrient, leading to hidden hunger, has been recognized by the World Health Organization as a serious human health problem worldwide, especially in population having limited access to fruits, vegetables and livestock products. Three micronutrients *viz.*, iron (Fe), zinc (Zn) and provitamin ‘A’ are widely deficient, especially among low socio-economic group in developing countries. Micronutrient malnutrition arising from Zn and Fe deficiencies alone affects over 3 billion people around the world (<http://www.unscn.org>). Nearly 500,000 children (<5 years of age) die annually because of the deficiencies of these two micronutrients (Black *et al.*, 2008). Among the 26 major risk factors of the global burden of disease estimates, iron deficiency ranks 9th, while zinc and vitamin A deficiencies occupy 11th and 13th positions, respectively (Ezzati *et al.*, 2002).

The disease burden associated with iron deficiency in India could be reduced to an extent of 19% – 58% by crop biofortification (Stein *et al.*, 2008). More recently Meenakshi *et al.* (2010) concluded that overall biofortification can make a significant impact on reducing the burden of micronutrient deficiencies in the developing world in a highly cost-effective manner. An alternative (or complement) to the above approaches is to use plant breeding to naturally fortify commonly consumed staple crops with micronutrients, a process known as genetic biofortification (Bouis, 2003). Biofortification is being used to improve micronutrient intake of populations in many parts of the world and was recently ranked as the fifth most cost-effective solution for the world’s greatest problems by the Copenhagen Consensus Centre (2008). Biofortified crops offer a rural-based intervention that, by design, initially reaches these more remote population, which comprise a majority of undernourished population and then penetrates to urban populations as production surpluses are marketed.

Sorghum (*Sorghum bicolor*) is one among the top ten crops that feed the world (Goldschein, 2011). Sorghum is a predominantly self-pollinated diploid ($2n=2x=20$) C₄ grass with a high photosynthetic efficiency. Its small genome size (730 Mbp, about 25% of the size of maize or sugarcane) is fully sequenced and makes sorghum an attractive model for functional genomics of C₄ grasses. Sorghum is one among the few resilient crops that can adapt well to future climate change conditions, particularly the increasing drought, soil salinity

and high temperatures. It is one of the cheapest sources of energy and micronutrients and a vast majority of population in Africa and central India depends on sorghum for their dietary energy and micronutrient requirement (Parthasarathy Rao *et al.*, 2006). Grain sorghum is used for flour, porridges and side dishes, malted and distilled beverages and specialty foods such as popped grain. Micronutrient malnutrition, primarily the result of diets poor in bio-available vitamins and minerals, causes blindness and anemia (even death) in more than half of the world's population, especially among women of reproductive age, pregnant and lactating women and pre-school children (Underwood, 2000; Sharma, 2003 and Welch and Graham, 2004) and efforts are being made to provide fortified foods to vulnerable groups of the society. Biofortification, where possible, is the most cost-effective and sustainable solution for tackling micronutrient deficiencies as intake of micronutrients is on a continuing basis with no additional cost to the consumer in developing countries of arid-tropical and sub-tropical regions. Biofortification of sorghum by increasing mineral micronutrients especially iron and zinc in grain is of widespread interest (Pfeiffer and McClafferty, 2007; Zhao, 2008 and Ashok Kumar *et al.* 2009). The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) has been working for a decade on sorghum biofortification for enhancing grain Fe and Zn concentrations.

Improving iron and zinc densities of staple crops by breeding offers a cost-effective and sustainable solution to reduce micronutrient malnutrition in resource poor communities. In order to realize the potential impact of the micronutrient-dense cultivars, the high micronutrient density trait must be delivered in high-yielding backgrounds with farmer's preferred traits (Ashok Kumar *et al.*, 2010). In broad terms, three things must happen for successful biofortification program. First, the high nutrient density must be combined with high yield and high profitability. Second, efficacy must be demonstrated in improved micronutrient status of human must be shown when biofortified varieties are consumed. Thus sufficient nutrients must be retained in processing and cooking and these nutrients must be sufficiently bioavailable. Third, the biofortified crop must be adopted by farmers and consumed by those suffering from micronutrient malnutrition in significant numbers. Inheritance studies showed that grain Fe and Zn are quantitatively inherited and grain Zn is predominantly under control of additive gene action, while both non-additive and additive gene action are important in conditioning grain Fe concentration (Ashok Kumar *et al.*, 2013).

Quantitative or complex traits exhibit continuously distributed phenotypic variation in natural populations due to genetic complexity and environmental sensitivity. Genetic complexity arises from segregating alleles at multiple loci. The effect of each of these alleles on the trait phenotype is often relatively small and their expression is sensitive to the environment and in most cases their effects were approximately additive (Kearsey and Pooni, 1996). Quantitative Trait Loci (QTLs) are very complex and challenging to the breeder to undertake quality improvement in large scale breeding programmes through conventional breeding approaches. However, two major developments during 1980s changed the scenario: i) the discovery of extensive, yet easily visualized, variability at DNA level that could be used as markers and ii) development of statistical packages that can help in analysing variation in quantitative traits in congruence with molecular marker data generated in segregating population. The identification of QTLs using DNA markers was a major breakthrough in characterization of quantitative traits (Paterson *et al*, 1988). Salient requirements of QTL mapping are i) saturated mapping population generated from phenotypically contrasting parents. ii) A saturated linkage map based on molecular markers. iii) Reliable Phenotypic screening of mapping population and iv) Appropriate statistical package for QTL detection.

Random mapping populations are more difficult for QTL mapping, because linkage disequilibrium is a key to detecting QTLs with markers. It is essential to develop a suitable experimental mapping population using parental lines that are highly contrasting phenotypically for the target trait. Another requirement is that these parental lines should be genetically divergent; this is important to enhance the possibility of identifying large set of polymorphic markers that are well distributed across the genome. The ability to detect QTLs or information contained in RILs (Recombinant Inbreed Lines) are relatively higher than other populations. The advantage through RILs is ability to perform large experiments at several locations and even in multiple environment.

A number of molecular marker systems are now available for use in QTL analysis, and several laboratories in India are working on molecular markers for crop improvement (Gupta, *et al.*, 1996). Similar to gene, a QTL merely indicate a region on the genome comprised of one or more functional genes. However, the genetic architecture of complex trait consists of not only the actions of genes in single locus, but also the inter-locus interaction and gene \times environment interactions. More and more evidence indicating that the complexity of genetic architecture can be largely attributed to epistasis has been proved, which play significant role in heterosis, inbreeding depression, adaptation, reproductive isolation and

speciation. In addition, the property, that QTL effects are environmentally sensitive results in phenotypic plasticity or the ability of the organism to take an alternative developmental fates, depending on environmental cues (Lukens and Doebley, 1999). With strong epistasis and genotype \times environment interactions for fitness traits, local adaptation can be readily achieved (Li *et al.*, 2001). Realizing the importance of epistasis and QTL \times Environment (QE) interaction, the multilocation and multiseasonal analysis of quantitative trait is prerequisite in QTL mapping study. In a process of QTL mapping, association between observed trait values and presence/absence of alleles of markers, that have been mapped on linkage map is analysed. When it is significantly cleared that the correlation that is observed did not result from same random process, it is proclaimed that QTL is detected. Exploiting molecular markers in breeding involves finding a subset of markers associated with one or more QTLs that regulate the expression of complex traits. Many QTL mapping studies conducted in the last two decades identified QTLs that generally explained a significant proportion of the phenotypic variance, and therefore, gave rise to an optimistic assessment of the prospects of markers assisted selection. Previously, it was assumed that most markers associated with QTLs from preliminary mapping studies were directly useful in marker assisted selection (MAS). However, in recent year it has become widely accepted that QTL conformation, QTL validation and fine mapping may be required (Langridge *et al.*, 2001).

The basic objective of QTL mapping studies is to detect QTL, while minimizing the occurrence of false positive (Type 1 error, which is detecting an association between a marker and QTL when in fact one does not exist). Test for QTL/Trait association are often performed by several approaches like single marker analysis, single interval mapping (SIM), composite interval mapping (CIM) and multiple interval mapping (MIM). However, the composite interval mapping (CIM) (Zeng, 1994) has several advantages over single marker analysis and single interval mapping (SIM) as i) Mapping of multiple QTLs can be accomplished by the search in one direction ii) By using linked markers as a cofactors, the test is not affected by QTL outside the region, thereby increasing the precision of QTL mapping and iii) By eliminating much of the genetic variance by other QTL, the residual variance is reduced, thereby increasing the power of detection of QTL.

QTL mapping studies have been reported in most crop plants for diverse traits including yield, quality, disease and insect resistance, abiotic stress tolerance and environmental adaptation. Molecular mapping experiments for grain iron and zinc concentration in maize, rice, wheat and pearl millet have been conducted. However, not much

effort has been made to locate the QTLs responsible for iron and zinc concentration in sorghum. Therefore, keeping all these points in view, the present investigation was undertaken with the following objectives:

- To phenotype the available mapping (RIL) population (296 B x PVK 801) for grain iron and zinc along with other agronomic traits.
- To genotype the available mapping (RIL) population (296 B x PVK 801) using SSR and DArT markers.
- To identify the QTLs conditioning grain Fe and Zn concentration.

Review of Literature

Chapter II

REVIEW OF LITERATURE

2.1 Sorghum area and Production

Sorghum (*Sorghum bicolor* (L.) Moench) is a climate smart nutri-rich grain cereal and the fifth most important cereal crop in the world in terms of production and acreage after wheat, maize, rice and barley. It is grown on 47 m ha in 104 countries, it is cultivated as a staple cereal in Eritrea and Sudan and as a major food crop in most of South Asia and Africa, primarily to ease food security and in the USA, Australia and South America for animal feed. It is the fourth most important food crop in India after rice, wheat and pearl millet and is a staple food in central India. It is also the staple food for large tribal populations across the country. India is one of the largest sorghum growers in the world with an area of 6.18 m ha and production of 5028 m t with an average productivity of 854 kg ha (FAO, 2013).

2.2 Sorghum as food, feed and nutraceutical crop

Sorghum grain contains 11.3% protein, 3.3% fat and 56–73% starch. It is relatively rich in iron, zinc, phosphorus and B complex vitamins. Tannins, found particularly in red-grained types, contain antioxidants that protect against cell damage, a major cause of diseases and aging. The protein and starch in sorghum grain are more slowly digested than those from other cereals, and slower rates of digestibility are particularly beneficial for people with diabetes. Sorghum starch is gluten-free, making sorghum a good alternative to wheat flour for individuals suffering from celiac disease.

Sorghum is the second cheapest source of energy and micronutrients (after pearl millet); and a vast majority of the population in Africa and central India depends on sorghum for their dietary energy and micronutrient requirement (Parthsarthy Rao *et al.*, 2006; Ashok Kumar *et al.*, 2011). Sorghum stover is the major source of dry fodder for urban and peri-urban dairy production in India (Tesfaye, 1998). Importance of sorghum as a fodder crop is increasing in many regions of the world due to its high productivity and ability to utilize water efficiently even under drought conditions. Sorghum is highly adaptable in terms of genetic resources and germplasm that allows the breeding and development of new cultivars adapted to different agro-ecological regions around the globe (Zhang *et al.*, 2010). Its fodder contains 7 to 12% protein, 70% carbohydrates, minerals, crude fat and nitrogen free extract.

2.3 Need of Biofortification to overcome micronutrient malnutrition

Experts estimate that two billion people, mostly in poorer countries, suffer from micronutrient malnutrition, also known as hidden hunger (WHO and FAO 2006). This is caused by a lack of critical micronutrients such as vitamin A, zinc, and iron in the diet. Hidden hunger impairs the mental and physical development of children and adolescents and can result in lower IQ, stunting and blindness, women and children are especially vulnerable. Hidden hunger also reduces the productivity of adult men and women due to increased risk of illness and reduced work capacity. Worldwide, more than three billion people, who live on staple crops, suffer from micronutrient malnutrition (Welch and Graham, 2004). Iron deficiency is the most common nutritional disorder in the world affecting over four billion people, with more than two billion people mainly in developing countries, actually being anemic (WHO, 2011). According to WHO (2008) an estimated 41% of pregnant women and 27% of preschool children worldwide have anaemia. It often leads to impaired physical growth, mental development, and learning capacity (Cakmak *et al.*, 2008). Malnutrition is the most common cause of zinc deficiency and 25% of the world's population is at risk of zinc deficiency (Maret and Sandstead, 2006).

Traditional efforts to solve the problem of micronutrient deficiency have focused on micronutrient supplementation and food fortification (White and Broadley, 2005). However, these methods have not proven to be sustainable, especially in developing countries, where, people cannot afford animal and fish products with a high micronutrient content. Instead, most people in these regions consume cereals as their staple food, which provide only a small amount of the micronutrients and do not meet human nutrition needs (Shi *et al.*, 2008). Also associated costs and small number of primary health care programs in developing countries makes micronutrient supplementation and food fortification as difficult task. Hence there is an urgent and compelling need to develop varieties with improved content of micronutrients using biofortification. Agriculture is the primary source of nutrients necessary for a healthy life, but agricultural policies and technologies have focused on improving profitability at the farm and agroindustry levels, not on improving nutrition (Bouis and Welch, 2010). Given the prevalence of hidden hunger, there is growing interest for agriculture to play a role in improving nutrition, in particular by paying more attention to the nutritional quality of food. Biofortification is a scientific method for improving the nutritional value of foods already consumed by those suffering from hidden hunger.

Plant breeding is an excellent ‘tool’ for micronutrient nutritional enhancement in combating the problem of malnutrition. The micronutrient density traits are stable across

environments and it is possible to improve the content of several limiting micronutrients together. Another approach, referred to as agronomic fortification, seeks to improve the mineral content of food crops through fertilizer applications, which are applied to the soil or directly to the leaves by foliar spray. The harvest zinc fertilizer project has found that foliar application of zinc fertilizers to wheat can significantly increase zinc concentration in the grain (Cakmak , 2008), but it is short-term approach compare to breeding strategy which is most sustainable and cost-effective approach useful in improving micronutrient concentrations. Transgenic approaches can be used to improve the nutrient content of crops where natural variation in germplasm is limited. However, transgenic crops also face more regulatory hurdles compared to their conventionally breed counterparts. Breeders can utilize molecular biology technique such as quantitative trait locus (QTL) maps and marker assisted selection to accelerate the identification of high mineral cultivars.

Based on research predictions that examined the cost-effectiveness of a variety of staple crops biofortified with provitamin A, iron and zinc in Africa, Asia, and Latin America it was found that biofortification could be highly cost effective to deal with micronutrient malnutrition, especially in Asia and Africa (Meenakshi *et al.*, 2010). Because, this strategy relies on foods which people already eat habitually and it is sustainable (Copenhagen Consensus, 2008). Harvest Plus, a component of the CGIAR Research Program on agriculture for improved nutrition and health, leads a global effort to develop and deliver biofortified staple food crops with one or more of three most limiting nutrients in the diets of the poor: vitamin A, zinc and iron.

2.4 Phenotyping of RIL population

2.4.1 Genetic variation for grain iron and zinc in sorghum

Several results indicated the existence of large amount of variability for grain Fe and Zn in various type of genetic materials (cultivars, hybrid parental lines and germplasm lines) of sorghum. For example, the variation for grain Fe and Zn ranged from 6.3 to 168.1 mg kg⁻¹ and from 3.6 to 91.3 mg kg⁻¹, respectively (Table 2.1).

Preliminary studies at Patancheru, India have shown large variation for Fe and Zn in different genetic materials. Reddy *et al.* (2010) found wide range of variation for Fe and Zn in germplasm core collection and also the accessions with white grains were showing marginally higher Fe and Zn than those with coloured grains and the Fe and Zn concentration of accessions with testa and without testa were comparable, when the grain Fe and Zn compared between different categories of genetic material by Reddy *et al.*

(2005) the mean grain Fe and Zn in germplasm lines were significantly higher than those in other categories of genetic materials (B- lines and varieties/ R lines), while mean grain Fe content was slightly higher in B- lines compared to that in varieties/ R- lines, there were no significant differences in grain Zn content between B-lines and varieties/ R- lines. Ashok Kumar *et al.* (2010) reported considerable variability for grain Fe and Zn concentration in the elite commercial sorghum cultivars grown in India for food purpose and also in hybrids developed by private sector.

Table 2.1. Mean and Range for grain iron and zinc concentration in sorghum

Entry	Mean	Range	References
Fe concentration (mg kg^{-1})			
84	28.00	20.1- 37.0	Reddy <i>et al.</i> (2005)
76	59.00	30.0 – 113.0	Kayode <i>et al.</i> (2006)
20	38.8	29.8 – 44.2	Ashok Kumar <i>et al.</i> (2010)
1394	42.2	7.7 – 132.6	Reddy <i>et al.</i> (2010)
14	44.0	28.0 – 63.00	Nguni <i>et al.</i> (2012)
74	-	21.5 – 55.5	Ashok Kumar <i>et al.</i> (2013)
222	-	6.3 – 168.1	Hariprasanna <i>et al.</i> (2014)
Zn concentration (mg kg^{-1})			
84	19.00	13.4 – 31.0	Reddy <i>et al.</i> (2005)
76	24.00	11.0 – 44.0	Kayode <i>et al.</i> (2006)
20	27.2	22.2 – 32.9	Ashok Kumar <i>et al.</i> (2010)
1394	33.5	15.1 – 91.3	Reddy <i>et al.</i> (2010)
14	33.0	23.0 – 55.0	Nguni <i>et al.</i> (2012)
74	-	21.5 – 55.5	Ashok Kumar <i>et al.</i> (2013)
222	-	3.6 – 87.0	Hariprasanna <i>et al.</i> (2014)

2.4.2 Genotype × Environment interaction for grain iron and zinc concentration

The most challenging problem in the quantitative genetics is to understand how genetic and environmental factors influence complex polygenic traits. Moreover, the genetic background of the plants is also responsible for variation in response to environmental signals. This differential relationship between environmental factors and various genetic materials are manifested as genotype × environment interaction. This, genotype × environment interaction (GEI) exists when two or more genotypes respond differently to different environmental conditions. The assessment of environmental

stability of genotype for micronutrient concentrations in grain is important for reliable and useful enhancement of the nutritional quality of cereal crop (Gomez-Becerra *et al.*, 2010). Differential response of genotypes to varying environment is evidence because of significant genotype \times environment interaction (GEI) for grain Fe and Zn. Kayode *et al.* (2006) found that grain iron and zinc in seventy six farmer's varieties of sorghum varied significantly across the field location, similarly Ashok Kumar *et al.* (2010) found significant genotype \times year interaction over two years for different sorghum landraces and commercial cultivars. Hariprasanna *et al.* (2012) also reported significant GEI in ten sorghum genotypes grown across six location. In pearl millet significant genotype \times environment interaction (GEI) was reported by (Gupta *et al.*, 2009; Velu *et al.*, 2011b and Govindaraj *et al.*, 2013), In wheat Peleg *et al.*, 2008 reported significant G \times E interaction for iron and zinc under water sufficient and water stress conditions. Moreover, Joshi *et al.* (2010) reported higher influence of environment on zinc than iron concentration. Whereas, in maize Prasanna *et al.* (2011) reported higher environmental influence on iron than zinc. For significant increase in statistical power of QTL detection and accuracy of the estimates of QTL position and effect one should conduct multi-environment trials for testing the hypothesis of QTL \times E interaction (Jansen and Stam, 1994). Unless the population size is large enough, the lines or families are uniform and the evaluation is consistent through evaluators, the study of QTL \times E interaction is not relevant (Asins, 2002). It is now well understood by plant breeders that G \times E interactions exist for many QTLs, implying that general conclusions about small effect QTL detected on the basis of single environment, could lead erroneous decision. Thus, for efficient breeding, knowledge about the genetics of the observed variation and insight into the genotype by environment (G \times E) interaction is important.

2.4.3. Heritability for grain iron and zinc concentration in sorghum and other cereals

Heritability is measured by estimating relative contribution of genetic and non-genetic differences to the total phenotypic variation. Heritability is an important concept in quantitative genetics. It is widely accepted that QTL mapping is model selection procedure, and accurate estimation of heritability is essential to avoid false positive and false negative errors in QTL mapping. Studies in sorghum found that broad sense heritability for grain Fe and Zn densities in diverse sorghum genotypes was 95.5% and 96.5% respectively (Susmita and Selvi , 2014). Similarly Hariprasanna *et al.* (2014) found

that broad sense heritability varied from 71 % to 87 % for grain Fe and 65 % to 84 % for Zn density in grains of adopted cultivars, parental lines, advance breeding lines and selected germplasm accession of sorghum.

Study of 85 near isogenic lines (NILs) of rice showed high (72.8%) to moderate (40.6%) heritability for grain Fe and Zn concentration respectively (Garcia-oliveria *et al.*, 2009). In wheat, across 10 environments data broad sense heritability for Fe and Zn was 37% and 25% respectively (Joshi *et al.*, 2010). In pearl millet, broad sense heritability for Fe ranges from 65.3-71.2% and for Zn 64.8 -79.7% in S₁ progenies from two different population across two seasons (Gupta *et al.*, 2009).

2.4.4. Association between grain Fe and Zn concentration and other agronomic traits

To explore the relevant molecular genetic mechanism of iron and zinc and other agronomic traits, the association study using large RIL population will be supportive to identified co-localized QTLs. The significant positive association between two traits indicates the common genomic region or genes or biochemical pathway involved in expression of the trait. Hence the knowledge of phenotypic association between the traits gives basic idea for QTLs relationships. Earlier studies showed significant positive association between grain iron and zinc concentration (Table 2.2) in sorghum (Reddy *et al.*, 2005; Ashok Kumar *et al.*, 2010, 2013; Reddy *et al.*, 2010; Nugni *et al.*, 2012 and Hariprasanna *et al.*, 2014) in pearl millet (Velu *et al.*, 2007, 2008a, 2008b; Gupta *et al.*, 2009; Rai *et al.*, 2012 and Govindraj *et al.*, 2013), in wheat (Garvin *et al.*, 2006; Velu *et al.*, 2011a; Morgounov *et al.*, 2007), in rice (Marr *et al.*, 1995 and Chadel *et al.*, 2010), in maize (Brkic *et al.*, 2003; Oikeh *et al.*, 2003; long *et al.*, 2004 and Lungaho *et al.*, 2011) and finger millet (Upadhyaya *et al.*, 2011). The observed high positive correlation between grain Fe and Zn in different cereals crops indicate the common genes or biochemical pathways for expression of these traits.

Association of grain Fe and Zn with agronomic traits has studied in many crops (Table 2.3). In sorghum, Reddy *et al.*, 2005 and Reddy *et al.*, 2010 found significant negative albeit, low association of both the micronutrient with grain yield. Whereas, Ashok Kumar *et al.* (2010) found non- significant association between grain micronutrient and grain yield. In pearl millet (Rai *et al.*, 2012), in wheat (Garvin *et al.*, 2006; Morgounov *et al.*, 2007; Shi *et al.*, 2008 and Zhao *et al.*, 2009) and in maize (Banziger and Long, 2000) reported low to moderate negative relationships between micronutrients and grain yield (but not always significant).

Reddy *et al.*, 2005; Reddy *et al.*, 2010 and Nguni *et al.*, 2012 reported negative association of grain micronutrient with 100-seed weight which is low in magnitude and not always significant. Whereas, plant growth factors like days to 50% flowering and plant height showed significant positive (Reddy *et al.*, 2010) to no association with both the micronutrient (Reddy *et al.*, 2005 and Ashok Kumar *et al.*, 2010).

Table 2.2. Nature and magnitude of correlation coefficient between grain Fe and Zn Concentration

Crop	Correlation Coefficient (r)	Reference
Sorghum	0.55**	Reddy <i>et al.</i> (2005)
	0.17	Kayode <i>et al.</i> (2006)
	0.85**	Ashok Kumar <i>et al.</i> (2010)
	0.60**	Reddy <i>et al.</i> (2010)
	0.49**	Nguni <i>et al.</i> (2012)
	Positive sig.	Ashok Kumar <i>et al.</i> (2013)
	0.2 - 0.5**	Hariprasanna <i>et al.</i> (2014)
Maize	0.69**	Brkic <i>et al.</i> (2003)
	0.71**	Oikeh <i>et al.</i> (2003)
	0.44**	Lungaho <i>et al.</i> (2011)
Rice	0.31**	Marr <i>et al.</i> (1995)
	0.71**	Chandel <i>et al.</i> (2010)
Wheat	0.55** to 0.71**	Garvin <i>et al.</i> (2006)
	0.79**	Morgounov <i>et al.</i> (2007)
	0.81**	velu <i>et al.</i> (2011a)
	0.39*	Hussain <i>et al.</i> (2012)
Pearl Millet	0.82** to 0.80**	Gupta <i>et al.</i> (2009)
	0.49** to 0.71**	Rai <i>et al.</i> (2012)
	0.63** to 0.87**	Govindaraj <i>et al.</i> (2012)
	0.86** to 0.90**	Govindaraj <i>et al.</i> (2013)

*, ** = significant at $P \leq 0.05$ and $P \leq 0.01$, respectively; ns = non-significant.

Table 2.3. Nature and magnitude of correlation coefficient of grain Fe and Zn with agronomic characters

Crop	Correlation Coefficient (r)	Reference
Grain Fe and Flowering		
Sorghum	0.18 ^{ns}	Reddy <i>et al.</i> (2005)
	0.09 ^{ns}	Ashok kumar <i>et al.</i> (2010)
	0.09**	Reddy <i>et al.</i> (2010)
Maize	0.01 ^{ns}	Chakraborti <i>et al.</i> (2009)
Pearl millet	-0.35*	Velu <i>et al.</i> (2008a)
Grain Fe and 100 seed weight		
Sorghum	-0.18 ^{ns}	Reddy <i>et al.</i> (2005)
	0.42**	Kayode <i>et al.</i> (2006)
	0.28 ^{ns}	Ashok Kumar <i>et al.</i> (2010)
	-0.13**	Reddy <i>et al.</i> (2010)
	-0.33 ^{ns}	Nguni <i>et al.</i> (2012)
Maize	-0.12 ^{ns}	Lungaho <i>et al.</i> (2011)
Wheat	-0.29 ^{ns}	Hussain <i>et al.</i> (2012)
Pearl Millet	-0.15 to 0.32 ^{ns}	Rai <i>et al.</i> (2012)
Grain Fe and grain yield		
Sorghum	-0.32**	Reddy <i>et al.</i> (2005)
	0.02 ^{ns}	Ashok kumar <i>et al.</i> (2010)
	-0.16**	Reddy <i>et al.</i> (2010)
Maize	-0.25*	Chakraborti <i>et al.</i> (2009)
Wheat	-0.15 ^{ns}	Zhao <i>et al.</i> (2009)
Pearl millet	-0.58** to -0.13 ^{ns}	Rai <i>et al.</i> (2012)
Grain Zn and Flowering		
Sorghum	0.12 ^{ns}	Reddy <i>et al.</i> (2005)
	0.16 ^{ns}	Ashok kumar <i>et al.</i> (2010)
	0.21**	Reddy <i>et al.</i> (2010)
Maize	-0.278	Chakraborti <i>et al.</i> (2009)
Pearl millet	-0.22 ^{ns}	Velu <i>et al.</i> (2008a)
Grain Zn and 100 seed weight		
Sorghum	-0.11 ^{ns}	Reddy <i>et al.</i> (2005)
	0.09 ^{ns}	Ashok kumar <i>et al.</i> (2010)
	0.22 ^{ns}	Reddy <i>et al.</i> (2010)
	-0.14**	Chakraborti <i>et al.</i> (2009)
Maize	-0.05 ^{ns}	Lungaho <i>et al.</i> (2010)
Wheat	-0.44**	Hussain <i>et al.</i> (2012)

Table 2.3. (Cont.)

Grain Zn and Grain yield		
Sorghum	-0.54**	Reddy <i>et al.</i> (2005)
	-0.05	Ashok kumar <i>et al.</i> (2010)
	-0.20**	Reddy <i>et al.</i> (2010)
Maize	0.17 ^{ns}	Chakraborti <i>et al.</i> (2010)
Wheat	-0.49**	Hussain <i>et al.</i> (2012)
Pearl millet	-0.32 to 0.17 ^{ns}	Rai <i>et al.</i> (2012)

* , ** = significant at $P \leq 0.05$ and $P \leq 0.01$, respectively; ns = non significant.

2.5 Types of molecular markers

The molecular markers can be classified into different groups based on mode of transmission, mode of gene action and method of analysis. For hybridization-based markers, DNA profiles are visualized by hybridizing restriction endonuclease digested DNA fragments, to a labelled probe, which is a DNA fragment of known sequence. In contrast, PCR-based markers involve *in vitro* amplification of particular DNA sequences with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme. The amplified DNA fragments are separated by electrophoresis (Sharma *et al.*, 2008).

There are various types of DNA-based molecular markers such as RFLP, RAPD, AFLP, STS, SSR, expressed sequence tag (EST), inter-simple sequence repeat (ISSR), SNP, and DArT (reviewed by Semagn *et al.*, 2006a). These may differ in a variety of ways, such as in the technical requirements, the amount of time, money and labour needed, the number of genetic markers that can be detected throughout the genome and the amount of genetic variation found at each marker in a given population (Choudhary *et al.*, 2008). RFLP is the most widely used hybridization-based molecular marker. RAPD, AFLP, ISSR, EST, SSR, STS etc. are PCR-based markers and SNPs are sequence-based DNA markers. SSRs are mostly co-dominant markers and are indeed excellent for studies of population genetics and mapping (Jarne and Lagoda, 1996 and Goldstein and Schlotterer, 1999), QTL analysis, forensics and diagnostics (Powell *et al.*, 1996; Schlotterer, 2004 and Varshney *et al.*, 2005). The use of fluorescent primers in combination with automatic capillary or gel-based DNA sequencers has been adapted in most advanced laboratories and SSRs are excellent markers for fluorescent techniques, multiplexing and high-throughput analysis. SNPs have enjoyed massive popularity for their high density within the genome and their ease of characterization. However, their identification requires access to reliable DNA sequence from the complete range of plants strains/varieties or ecotypes that will subsequently be used. Further, the sequential nature of the above gel-based marker systems reduces throughput, increasing costs per assay. DArT is one of the recently developed microarray hybridization-based DNA marker technique that enables simultaneous genotyping of several hundred polymorphic loci across the genome (Jaccoud *et al.*, 2001 and Welch and Graham, 2004). No molecular markers are available yet that fulfil all requirements needed by researchers. According to the kind of study to be undertaken, one can choose among the variety of molecular techniques that combines at least some desirable properties.

2.6 Diversity Arrays Technology (DArT)

The DArT technology was originally developed by Jaccoud *et al.*, 2001 at the Centre for Application of Molecular Biology to International Agriculture (CAMBIA) at the inventors promote it as an open source (nonexclusive) technology with a great potential for genetic diversity and mapping studies in a number of crops. It is a novel, solid-state, microarray-based, open- platform method for genome wide discovery and genotyping of genetic variation. A DArT marker is a segment of genomic DNA, the presence of which is polymorphic in a defined genomic representation. DArT markers are biallelic and behave in a dominant (present *vs* absent) or co-dominant (2 doses *vs* 1 dose *vs* absent) manner. DArT operates on the principle that the genomic representation contains two types of fragments: constant fragments, found in any representation prepared from a DNA sample from an individual belonging to a given species, and variable (polymorphic) fragments called molecular markers, only found in some but not all of the representations. The variable fragments called DArT markers are informative because they reflect sequence variation that determines the fraction of the original DNA sample that is included in the representation. DArT allows simultaneous scoring of hundreds of restriction site based polymorphisms between genotypes and does not require DNA sequence information or site-specific oligonucleotides. To identify the polymorphic markers, a complexity reduction method is applied on the meta genome, a pool of genomes representing the germplasm of interest. The genomic representation obtained from this pool is then cloned and individual inserts are arrayed on a microarray resulting in a “discovery array”. Labelled genomic representations prepared from the individuals can be genotyped by hybridisation to the discovery array. Polymorphic clones (DArT markers) show variable hybridization signal intensities for different individuals. The hybridization signal for each marker is measured and converted into a score. DArT fingerprints will be useful for accelerating plant breeding, and for the characterisation and management of genetic diversity in domesticated species as well as in their wild relatives. Wenzl *et al.* (2004), Syvanen (1999) and Xia *et al.* (2005) reported simultaneous analysis of hundreds of markers at once, with the added advantage of much lower cost per marker than other technologies like SNPs and microsatellites (Huttner *et al.*, 2005). In contrast to current SNP technologies, DArT performs well in polyploid species such as wheat (Akbari *et al.*, 2006; Wenzl *et al.*, 2007), banana (Kilian, 2007) and sugarcane (Heller-Uszynska *et al.*, 2011).

DArT offers low cost, quick, high throughput, electrophoresis-independent, highly reproducible and sequence-independent genotyping. The other advantages include fast data acquisition and analysis, detection of single-base changes as well as insertions/deletions, detection of differences in DNA methylation depending on the enzyme used to generate the fragments, generation of sequence-ready clones, minimal DNA sample requirement, good transferability of markers among breeding populations, and high quality markers. The same platform is used for both discovery and scoring of markers, therefore, no assay development is required after the initial marker discovery. The system is highly automated and the data generated will have increasing value with continuing advances in bioinformatics, particularly if polymorphic clones are sequenced. The genetic scope of DArT analysis is defined by the user and easily expandable. This technique, however, has also its own limitations as DArT markers are primarily dominant and the microarray-based technique includes several steps, preparation of genomic representations for the target species, cloning, and data management and analysis. The latter requires dedicated software such as DArTsoft and DArTdb. The establishment of a DArT system, therefore, demands extensive investment both in laboratory facilities and skilled manpower. Intellectual property constraints and process variation also limit its widespread use.

2.6.1 Applications of DArT

Potential applications of DArT include genome profiling and genome background screening, rapid construction of high-density genetic linkage maps (Akbari *et al.*, 2006; Alsop *et al.*, 2007 and Mace *et al.*, 2008), identification of QTLs (Alsop *et al.*, 2007; Pozniak *et al.*, 2007 and Rheault *et al.*, 2007), association mapping (Bouchet *et al.*, 2007), rapid introgression of genomic regions in accelerated backcrossing programs, simultaneous marker-assisted selection for several traits, microbial diagnostics, evaluation of genetic diversity, rapid germplasm characterization and tracking genome methylation changes in a cost-effective and high-throughput manner (Jaccoud *et al.*, 2001 and Wenzl *et al.*, 2004). The types of polymorphism detected by DArT (single nucleotide polymorphisms, insertion-deletions and methylation changes) expand the potential of traditionally used markers, increasing power to ascertain the structure of germplasm collections. Wenzl *et al.* (2007) used a DArT platform for quantitative bulk segregant analysis (BSA) in barley and found that DArT-BSA identifies genetic loci that influence phenotypic characters in barley with at least 5 cM accuracy and should prove useful as a generic tool for high-throughput, quantitative BSA in plants irrespective of their ploidy level.

2.6.2 Availability of DArT for different species/crops

As the DArT technique proved to be efficient for marker discovery and screening in various species, it has been applied in number of plant, animal, microbial and fungal species. It has most widely been used in plants and has proven to be successful in the various species like rice (Xie *et al.*, 2006), cassava (Xia *et al.*, 2005), barley (Wenzl *et al.*, 2004, 2006), wheat (Akbari *et al.*, 2006; Semagn *et al.*, 2006b and White *et al.*, 2008), pigeonpea (Yang *et al.*, 2006), sorghum (Mace *et al.*, 2008), *Arabidopsis* (Wittenberg *et al.*, 2005), banana (Risterucci *et al.*, 2009; Kilian, 2007), eucalyptus (Lezar *et al.*, 2004), fern and moss (James *et al.*, 2006). In addition, DArT platforms have been developed for other crop species such as lupin, potato, quinoa, rice, ryegrass, coconut, apple, lily and tomato (Wang *et al.*, 2006 and Kilian *et al.*, 2005). Currently DArT Private Limited provides DArT genotyping services for a number of these species. Further, Triticarte Private Limited, a joint venture between the Value Added Wheat CRC Limited and DArT Private Limited has been established to deliver genotyping services for barley and wheat breeders (Huttner *et al.*, 2006).

Xie *et al.* (2006) used and validated DArT for rice genotyping in a high throughput manner and 1152 clones were re-arrayed on a slide and used to fingerprint 17 of 24 germplasms. Xia *et al.* (2005) used DArT for high-throughput genotyping of cassava and its wild relatives and detected nearly 1,000 candidate polymorphic clones using two arrays. The genetic relationships among the samples analyzed with DArT were consistent with existing information on these samples. Hurtado *et al.* (2008) compared SSR and DArT markers for assessing genetic diversity in cassava and suggested that SSR markers, while low throughput in comparison with DArTs, are relatively better at detecting genetic differentiation in cassava germplasm collections. Wenzl *et al.* (2004) used DArT for whole-genome profiling of barley and constructed a genetic map between cultivars Steptoe and Morex. Most of the DArT markers (98.8%) were incorporated into a linkage map whose quality was superior to that of an RFLP-based framework map (Wenzl *et al.*, 2004). The resulting map included 385 unique DArT markers and spanned 1,137cM. A polymorphism-enriched *PstI/BstNI* array was produced from 1,920 candidate polymorphic clones. Wenzl *et al.* (2006) built a high-density consensus map of barley linking DArT markers to SSR, RFLP and STS loci, this comprised 2,935 loci (2,085 DArT and 850 other loci) and spanned 1,161 cM. A similar study in *Arabidopsis* confirmed the Mendelian behavior of DArT markers, and also established perfect co-linearity between the genetic and the physical maps (Wittenberg *et al.*, 2005). Hearnden *et al.* (2007) developed a high-density genetic map in wide barley cross between cultivated barley

(*Hordeum vulgare*) and wild subspecies *H. vulgare* ssp. *spontaneum*. The map comprises 1,000 loci, including 558 SSR (detected by 536 primer pairs) and 442 DArT markers. To incorporate novel alleles into cultivated barley, Alsop *et al.* (2007) crossed a wild barley accession possessing multiple disease resistance (Damon) with a malting cultivar (Harrington) and constructed a DArT marker-based linkage map for qualitative/quantitative trait analysis of disease resistance loci. A genotyping array was developed for sorghum representing approximately 12,000 genomic clones using *PstI+BanII* complexity with a subset of clones obtained through the suppression subtractive hybridization method. Over 500 markers detected variation among 90 accessions used in a diversity analysis and an integrated linkage map was also constructed with DArT markers, which spanned 1431.6 cM (Mace *et al.*, 2008). Yang *et al.* (2006) reported the development of DArT for pigeonpea using a *PstI/HaeIII* array, which revealed low levels of genetic diversity in cultivated pigeonpea compared to its wild relatives. A total of nearly 700 markers were identified with the average call rate of 96% and the scoring reproducibility of 99.7%. Semagn *et al.* (2006b) compared the utility of DArT with AFLP and SSR markers, in a genetic linkage map of a doubled-haploid hexaploid wheat population. The map contains a total of 624 markers with 189 DArTs, 165 AFLPs and 270 SSRs and spans 2595.5 cM. It has been successfully used to identify novel QTLs for resistance to *Fusarium* head blight and powdery mildew. Akbari *et al.* (2006) used DArT for high-throughput profiling of the hexaploid wheat genome and generated a large number of high-quality markers in wheat (99.8% allele-calling concordance and approximately 95% call rate). Mantovani *et al.* (2008) developed a DArT platform for durum wheat. The integrated DArT-SSR map included 554 loci (162 SSRs and 392 DArT markers) and spanned 2022 cM. White *et al.* (2008) analyzed the genetic diversity of UK, US and Australian cultivars of *Triticum aestivum* measured by DArT markers. Risterucci *et al.* (2009) used DArT for high-throughput DNA analyses in *Musa* and found that DArT markers revealed genetic relationships among *Musa* genotypes consistent with those provided by the other marker technologies, but at a significantly higher resolution and speed, and reduced cost. Bonin *et al.* (2008) used a new miniature inverted repeat transposable element (MITE) based genome complexity reduction method taking advantage of the abundance of MITEs in the genome of mosquito *Aedes aegypti* and constructed a library comprising more than 6,000 DArT clones.

2.7 Molecular markers in Sorghum

2.7.1 Sorghum SSR markers

Simple sequence repeat-(SSR) containing clones isolated from both bacterial artificial chromosome (BAC) and enriched genomic DNA (gDNA) libraries and database sequences that contains SSRs were the source for the sorghum SSRs mapped by Bhatramakki *et al.* (2000). Targeted isolation of SSR loci using BAC clones as proposed by Cregan *et al.* (1999) is likely to be the most efficient method for placing SSR loci in specific target genomic region. BTx623 (Frederiksen and Miller, 1972) is the reference genotype used for sorghum molecular marker genotyping and it was the source of DNA used to construct the enriched libraries and two sorghum BAC libraries that are currently available (Bhatramakki *et al.*, 2000). PCR primers for the amplification of DNA fragments containing SSRs from sorghum were successfully developed through three different approaches by Brown *et al.*, (1996) and it was reported that sorghum fragments can be amplified using at least some maize SSR primers.

Bhatramakki *et al.*, (2000) reported map location of 46 SSR loci based on previously reported primer sequences (Taramino *et al.*, 1997; Tao *et al.*, 1998a and Kong *et al.*, 2000) 113 SSR loci (including four SSR containing gene loci) based on novel primer sequences. These SSR marker loci were incorporated into pre-existing RFLP based map of Xu *et al.*, (1994) (Kong *et al.*, 1997) and Peng *et al.* (1999). First complete genetic linkage map of sorghum, comprised of ten linkage group putatively corresponding to ten gametic chromosome of *Sorghum bicolor* and *Sorghum propinquum*. The map includes 276 RFLP loci, predominantly detected by *pstI* digested *Sorghum bicolor* genomic probes, segregating in 56 progenies of a cross between *Sorghum bicolor* and *Sorghum propinquum*. The remarkable level of a DNA polymorphism between these species will facilitate development of high density genetic map (Chittenden *et al.*, 1994). Ramu *et al.*, (2009) aligned all publically available SSR markers on sequence based physical map for sorghum, linking this physical map with already existing linkage maps to provide better potential for applied molecular breeding programs.

2.7.2 Sorghum DArT markers

The current molecular marker technologies have characteristics which additionally affect the level of genome coverage, their discrimination ability, reproducibility and technical and time demand. A number of the limitations associated with the different marker technologies can be overcome by utilizing specialized hardware such as high throughput capillary electrophoresis machines, which can impact on

discrimination ability, reproducibility and speed. Diversity arrays technology (DArT) can overcome these limitations and has been developed as a hybridization-based alternative to the majority of gel-based marker technologies currently in use. Mace *et al.* (2008) first used DArT technology in sorghum and found that sorghum DArT markers are of high quality, as assessed by their call rate, scoring reproducibility and PIC values and also constructed a genetic linkage map for a cross between R931945-2-2 and IS 8525 lines, integrating DArT and other marker types with an average DArT marker density of 1/3.9 cM.

2.8. Linkage maps in sorghum

Genetic studies of morphological traits in sorghum began early this past century. Doggett (1988) summarized genetic linkage of morphological and physiological mutants involving 49 loci. To date over 200 morphological and agronomically important markers have been identified (Berhan *et al.*, 1993) however, only nine linkage groups could be established with these markers and these consisted of only 2- 10 loci (Pereira *et al.*, 1994). The biggest linkage group consisted of ten linked morphological marker loci (Doggett, 1988). Sorghum genome mapping based on DNA markers began in the early 1990s and since then several genetic maps of sorghum have been developed with large numbers of DNA-based markers including RFLPs, AFLPs and SSRs. Where opportunities have permitted, morphological marker loci have been integrated into these molecular marker based genetic linkage maps. Presently consensus maps based on SSRs, RFLPs and DArT markers are available, these maps will be useful in advanced breeding and genetic studies. The construction of the first DNA-based sorghum linkage map was done using the RFLP technique with heterologous maize probes (Hulbert *et al.*, 1990). Later several more RFLP-based linkage maps of *S. bicolor* have been constructed (Binelli *et al.*, 1992; Whitkus *et al.*, 1992; Berhan *et al.*, 1993; Chittenden *et al.*, 1994; Pereira *et al.*, 1994; Ragab *et al.*, 1994; Xu *et al.*, 1994; Dufour *et al.*, 1997; Tao *et al.*, 1998a; Peng *et al.*, 1999, Haussmann *et al.*, 2002 and Bowers *et al.*, 2003). Similarly, the RFLP maps of Xu *et al.* (1994) and Peng *et al.* (1999) have been improved with addition of over 100 SSR markers (Kong *et al.*, 1997 and Bhatramakki *et al.*, 2000), while that of Dufour *et al.*, (1997) has been augmented with AFLP markers (Boivin *et al.*, 1999). High-density genetic maps using AFLP, RFLP and SSR markers (Menz *et al.*, 2002) and RFLP probes (Bowers *et al.*, 2003) have been reported. These high-density integrated maps will accelerate genome mapping and comparative mapping activity in sorghum and other related grass species. Mace *et al.*, (2009) constructed six component maps independently using six different mapping populations and finally developed single consensus map that

contain commonly utilized SSR's, AFLP's and high-throughput DArT markers which gives more complete coverage of sorghum genome and to fill number of gaps on individual maps. The characteristics of different sorghum genetic maps are given in Table 2.4

2.9. QTL mapping in Sorghum

With rapid advancement of molecular technology, it is now possible to use molecular marker information to map major QTLs on chromosomes (Paterson *et al.*, 1988, 1991; Hilbert *et al.*, 1991; Jacob *et al.*, 1991 and Stuber *et al.*, 1992). Mapping is putting markers in order, indicating the relative genetic distances between them, and assigning them to their linkage groups on the basis of the recombination values from all their pair wise combinations. QTL mapping provides a means to dissect complex phenotypic characters into their component traits (QTLs), and allows the identification of molecular markers linked to desirable QTLs, so that these can be directly used in marker-assisted selection (Tanksley *et al.*, 1989; Lee, 1995; Schneider *et al.*, 1997; Mohan *et al.*, 1997 and Paterson, 1996). The theory of QTL mapping was first described in 1923 by Sax and was further elaborated by Thoday (1961). QTLs are identified *via* statistical procedures that integrate genotypic and phenotypic data. QTL mapping studies have been reported in most crop plants for diverse traits including yield, quality, disease and insect resistance, abiotic stress tolerance and environmental adaptation. QTL mapping requires a suitable mapping population generated from phenotypically contrasting parents, a saturated linkage map based on molecular markers, reliable phenotypic screening of mapping population, appropriate statistical packages to analyse the genotypic information in combination with phenotypic information for QTL detection. A number of methods for mapping QTL and estimating their effects have been suggested and investigated (Edwards *et al.*, 1987; Haley and Knott, 1992; Jiang and Zeng, 1995; Lander and Botstein, 1989; Jansen and Stam, 1994; Utz and Melchinger, 1994 and Zeng, 1994). Methods for QTL mapping range from the simplest method of single-marker analysis (Sax, 1923) to more sophisticated methods such as interval mapping (Lander and Botstein, 1989 and Haley and Knott, 1992) multiple regression (Wright and Mowers, 1994 and Whittaker *et al.*, 1996), and composite interval mapping (Zeng, 1994). Software packages for mapping include MAPMAKER/QTL (Lincoln *et al.*, 1993), JoinMap (Stam, 1993), QTL Cartographer (Basten *et al.*, 1994), PLABQTL (Utz and Melchinger, 1996), QGene (Nelson, 1997), and TASSEL (Buckler, 2007).

Table 2.4. Characteristics of different sorghum genetic linkage maps published to date

Reference	Parents	Size and type of population	Markers	Genome length	Linkage Group	Probe Source
Hulbert <i>et al.</i> , 1990	Shanqui Red x M91051	55 F ₂	37 RFLPs	283 R	8	Maize
Binelli <i>et al.</i> , 1992	IS 18792 x IS 24756	149 F ₂	21 RFLPs	440U	5	Maize
Whitkus <i>et al.</i> , 1992	IS2482C x IS 18809	81 F ₂	85 RFLPs, 7 isozymes	949H	13	Sorghum Maize
Berhan <i>et al.</i> , 1993	Shanqui Red x M91051	55 F ₂	96 RFLPs	709R	15	Maize
Pereira <i>et al.</i> , 1994	CK60 x PI229828	78 F ₂	RFLPs	1530U	10	Sorghum, Maize
Chittenden <i>et al.</i> , 1994	BTx623 x S. propinquum	56 F ₂	276 RFLPs	1445U	10	Sorghum, Rice, Oat
Xu <i>et al.</i> , 1994	IS 36200 x BTx631	50 F ₂	190 RFLPs	1789K	14	Sorghum, Maize
Ragab <i>et al.</i> , 1994	BSC35 x BTx631	93 F _{2:3}	71 RFLPs	633H	15	sorghum, Maize
Lin <i>et al.</i> , 1995	BTx623 x S. propinquum	370 F ₂	202 RFLPs	935K	11	Sorghum
Tuinstra <i>et al.</i> , 1996	Tx7078 x B35	98 RILs	150 RAPDs, 20 RFLPs	Ca150R	17	Maize, Sorghum
Dufour <i>et al.</i> , 1997	IS 2807 x 379	110 F ₅ RILs	145 RFLPs 4 cloned gen	977H	13	Maize, Sugarcane
Taramino <i>et al.</i> , 1997	CK60 x PI229828	68 F ₂	201 RFLPs, 7 SSRs	1575U	10	maize, Sorghum

Table 2.4. (Cont.)

Reference	Parents	Size and type of population	Markers	Genome length	Linkage Group	Probe Source
Tao <i>et al.</i> , 1998a	QL39 x QL41	120 F ₅ RILs	155 RFLPs, 8 SSRs	1400U	21	Sorghum cereals
Ming <i>et al.</i> , 1998	BTx623 x S.propinquum	56 F ₂	328 RFLPs	1750K	10	Sorghum cereals
Bovin <i>et al.</i> , 1999	IS 2807 x 379	110F ₃ RILs	298 RFLPs, 137 AFLPs	1899H	11	Sorghum cereals
Crasta <i>et al.</i> , 1999	B35 x RTx430	96 F _{6:7}	142 RFLPs	1602K	14	sorghum cereals
Peng <i>et al.</i> , 1999	BTx623 x Is 3620C	137 F ₆₋₈ RILs	323 RFLPs	1347 K	10	Sorghum cereals
Kong <i>et al.</i> , 2000	BTx623 x IS 3620C	137 F ₆₋₈ RILs	11 RFLPs, 33 SSRs	1287K	10	Sorghum cereals
Klein <i>et al.</i> , 2001b	RTx x Sureno	125 F ₅ RILs	44 SSRs, 85 AFLPs	970	10	Sorghum
Menz <i>et al.</i> , 2002	BTx623 x IS 3620C	137 F ₆₋₈ RILs	336RFLPs, 136 SSRs, 2454 AFLPs	1713K	10	Sorghum cereals
Bowers <i>et al.</i> , 2003	BTx623 x S.propinquum	65 F ₂	2512 RFLPs	1059K	10	Saccharum, Arabidopsis
Bian <i>et al.</i> , 2006	Early Folger x N32B	207 F _{2:3}	31 RFLPs, 254 AFLPs	983.5 K 42 SSRs	10	Sorghum cereals

*H, K= Map distances estimated using the mapping functions of Haldane (1919) and Kosambi (1994), respectively; U= mapping function not specified; R= Recombination frequency (%)

Numerous studies to identify QTLs for agronomically important traits have been conducted in sorghum and QTLs have been identified for a wide array of important traits (Table 2.5) Genetic linkage map in sorghum have been constructed and QTL's are identified for several traits like ergot resistance (Parh *et al.*, 2008) using set of 303 markers including 36 SSR, 117 AFLP and 148 DArT. Klein *et al.* (2001a) identified five QTL for grain mold resistance each accounting for between 10 and 23% of phenotypic variation. Tao *et al.* (1998b) identified genomic region with largest effect on rust resistance on linkage group 10, which account for 40% of total phenotypic variation. Haussmann *et al.* (2002) mapped QTL for stay green in two different recombinant inbreed line population, the number of QTL's detected for three traits (Green leaf area at 15, at 30 and 45 days after flowering) by composite interval mapping, expressing 31% to 42% of genetic variation. Sanchez *et al.*, (2002) identified four genomic regions associated with stay green trait using RIL population developed from B3J x Tx7000 and these four QTL accounted for 53.5% phenotypic variation.

2.9.1. QTL mapping for Grain Iron and Zinc in Cereals

Molecular mapping experiment for grain iron and zinc concentration in maize, rice, wheat and pearl millet have been conducted and presented in table 2.6. Tiwari *et al.* (2009) analysed the QTL for Fe and Zn in RILs (Recombinant Inbred Lines) of *Triticum boeoticum* (pau5088) and *Triticum monococcum* (pau14087). The QTL analysis led to the identification of two QTL for grain Fe on chromosomes 2A and 7A and one QTL for grain Zn on chromosome 7A. Singh *et al.* (2010) analysed recombinant inbred line (RILs) population from the cross of A genome species (pau5088) *Triticum monococcum* (pau14087) this population led to the identification of two QTL for grain Fe on chromosomes 2A and 7A and one QTL for grain Zn on chromosome 7A. Anuradha *et al.* (2012) mapped the QTLs for iron and zinc in RIL population of Madhukar × Swarna unpolished rice grains, 14 QTLs were identified for these two traits. QTLs for iron were co-located with QTLs for zinc on chromosome 7 and 12. Simic *et al.* (2012) analysed grain samples by coupled plasma- optical emission spectrometry in 294 F₍₄₎ lines of a

Table 2.5. Summary of qualitative and quantitative trait loci identified in Sorghum

Trait	References
Drought tolerance Pre and Post-anthesis	Tuinstra <i>et al.</i> (1996,1997), Crasta <i>et al.</i> (1999) Subudhi <i>et al.</i> (2000); Tao <i>et al.</i> (2000), Xu <i>et al.</i> (2000), Coulibaly (2002) and Huassmann <i>et al.</i> (2003)
Anthracnose resistance	Boora <i>et al.</i> (1998) and Mehta (2002)
Rust Resistance	Tao <i>et al.</i> (1998b)
Head smut resistance	Oh <i>et al.</i> (1994)
Downy mildew resistance	Gowda <i>et al.</i> (1995) and Oh <i>et al.</i> (1996)
Maturity	Lin <i>et al.</i> (1995) and Childs <i>et al.</i> (1997)
Height	Lin <i>et al.</i> (1995) and Pereria and Lee (1995); Klein <i>et al.</i> (2001a), Brown <i>et al.</i> (2008)
Yield and components	Pereria <i>et al.</i> (1995), Tuinstra <i>et al.</i> (1997) and Rami <i>et al.</i> (1998)
Quality and mold	Franks (2003)
Fertility restoration	Klein <i>et al.</i> (2001b)
Striga resistance	Haussmann <i>et al.</i> (2004)
Shoot Fly resistance	Folkertsma <i>et al.</i> (2005)
Seed size and dispersal	Paterson <i>et al.</i> (1995)
Midge resistance	Tao <i>et al.</i> (2003)
Stay Green	Harris <i>et al.</i> (2007b)
Sugar related trait	Shiringani <i>et al.</i> (2009)
Early season cold Tolerance	Knoll and Ejata (2008)

Table 2.6. List of QTLs identified for Grain Iron and Zinc in cereals

Crop	Cross	mapping pop	Size of mapping population	No. of QTLs Identified	Reference
Rice	Madhukar × Swarna	RIL	168	14 (Both Iron and Zinc)	Anuradha <i>et al.</i> (2012)
Rice	Zhenshan97 × Min ghui63	RIL	241	2 (Iron), 3 (Zinc)	Kaiyang <i>et al.</i> (2009)
Diploid Wheat	Tb5088 × Tb4087	RIL	93	3(Iron), 2 (Zinc)	Tiwari <i>et al.</i> (2009)
Barley	Clipper × Sahara 3771	DH	150	5 (Zinc)	Lonergan <i>et al.</i> (2009)
Maize	Inbreed line 178 × P53	F2:3	218	10 MQTLs (Both iron and zinc)	Jin <i>et al.</i> (2013)

biparental population taken from field trials of over 3 years. QTL analysis detected 32 significant QTLs for 7 traits, of which some were co-localized. The additive-dominant model revealed highly significant additive effects, suggesting that biofortification traits in maize were generally controlled by numerous small-effect QTLs. Three QTLs for Fe/P, Zn/P, and Mg/P were co-localized on chromosome 3, coinciding with simple sequence repeats marker bnlg1456, which resides in close proximity to previously identified phytase genes (ZM phys1 and phys2).

2.10. Statistical techniques for QTL analysis

QTL analysis is predicated on looking for associations between the trait and the marker alleles segregating in the quantitative traits and the marker alleles segregating in mapping population. It has two essential stages: the mapping of the markers and association of the trait with the markers. Both of these require accurate data and statistical software (Kearsey and Farquhar, 1998). The basic theory underlying marker mapping has been available since the 1920s (Mather, 1938). But has to be extended to handle hundreds of markers simultaneously. The availability of computer software packages has made this much easier (Young, 2001).

The traditional approach (Soller and Brody, 1976; Tanksley *et al.*, 1982 and Edwards *et al.*, 1987) for detecting a QTL in the vicinity of a marker involves studying single genetic markers one at a time. However, if the QTL does not lie at the marker locus, its phenotypic effect diminishes relative to the true effect of the QTL, as the distance (recombination frequency) increases between the marker locus and the QTL (Edwards *et al.*, 1987 and Lander and Botstein, 1989). To overcome this, Knapp (1989) developed an approach that utilizes pairs of markers in a sequential manner and estimates the phenotypic effect of the QTL and its significance in the region bracketed by the two markers in each pair. Lander and Botstein (1989) reported development of such a method of mapping QTLs interval mapping using LOD scores. Intervals between adjacent pairs of markers along a chromosome are scanned and the likelihood profile of a QTL being at any particular point in each interval is determined; or to be more precise. The log of the ratio of the likelihood (LOD) of there being one QTL *vs* no of QTL at a particular point is determined (Lander and Botstein, 1989). An alternative approach using multiple regression was developed by Haley and Knott (1992). It often

produces very similar results to LOD mapping both in terms of accuracy and precision, but has the advantages of speed and simplicity of programming. Tests of significance and confidence intervals can be obtained. Tanksley and Nelson (1996) advise that the statistical detection of QTLs is likely to depend not only on the type of population utilized, but is also likely to depend on the intra-locus and inter-locus interactions of the segregating QTLs.

For most mapping projects the most widely used genetic mapping software is MAPMAKER (Lander *et al.*, 1987). MAPMAKER is based on the concept of the LOD scores, "the log of odds ratio" (Morton, 1955). The popularity of MAPMAKER is based on the ease with which it performs multipoint analysis of many linked loci (Young, 2001). The computer program JOINMAP is especially suited to relate one's map to those derived from other mapping populations (Stam, 1993).

To apply linkage maps to QTL analysis. MAPMAKER/QTL has been written to carry out simple interval mapping (SIM) QTL analysis using mathematical models and interfaces very much like the original MAPMAKER program (Lander and Botstein. 1989). Other programs like QTL Cartographer (Basten *et al.*, 1998) provide very much the same type of analysis. QTL analysis can also be performed by using composite interval mapping (CIM) with the PLABQTL software as described by Rami *et al.* (1998) or with QTL Cartographer. For large-scale use of linkage information in a marker-assisted breeding, a program like Map Manager (Manley and Cudmore, 1998) helps to keep track or marker data in the population of interest. Hypergene (Young and Tanksley, 1989) or Graphical Genotype (GGT) can help to display graphical genotypes. The programme qGENE seeks to bring all of these important DNA marker tools together into single package (Nelson, 1997).

Materials and
Methods

Chapter III

Materials and Method

The present investigation was carried out principally to study the variation for grain iron (Fe) and zinc (Zn) densities and to identify QTLs for these two traits in RIL population developed from parents 296B and PVK 801 selected based on their contrast nature for the traits under study. The phenotypic trials were conducted for 2 years (October 2012 to march 2014) at three different locations *viz.*, International Crops Research Institute for the Semi-arid Tropics (ICRISAT), Indian Institute of Millet Research (IIMR) and Vasantrao Naik Marathwada Krishi Vidhyapeeth (VNMKV) Parbhani. All the experiments were conducted during post-rainy seasons. Also RIL population was genotyped with SSR, DArT and SNP markers, and Linkage Map was constructed for QTLs based on genotypic and phenotypic data for grain iron and zinc densities.

The present investigation was carried out objective-wise under three separate experiments as mentioned below.

- To phenotype the available mapping (RIL) population (296 B x PVK 801) for grain iron and zinc along with other agronomic traits.
- To genotype the available mapping (RIL) population (296 B x PVK 801).
- To construct the linkage map and identify the QTLs for grain iron and zinc densities

3.1 Experimental Material

Plant Material: The RIL population consists of 336 individuals of F₆ generation, developed from contrasting parents 296 B and PVK 801 was used for QTL mapping. The details of parents are presented in table 3.1.

Chemicals: During the course of investigation, precaution were taken to use chemicals of high purity only, all the enzymes used in the present study were from New England Biolabs (NEB), UK or Sibenzymes Ltd., Russia. Analytical grade chemicals from Qualigens, India; Sigma chemicals Company, USA; Hi-Media, India; USB Corporation, USA; Amersham, UK and Life technologies, USA were used for carrying out all the experiments (Appendix I).

Glassware's and plastic wares

All the glassware used was of the borosilicate quality obtained from Borosil India. Disposables like, micropipette tips, centrifuge tubes, 96 and 384-well PCR plates and 384well library storage plates were from Tarsons Products Private Ltd., India; Axygen Scientific, USA and Genetix, India respectively.

Table 3.1 Information on Parents used in mapping population development

Sr. No	Variety	Pedigree	DTF	Fe (mgKg ⁻¹)	Zn (mgKg ⁻¹)	Grain Mold reaction	Plant Height (cm)
1	296 B	[(IS 3922 (Kafir-durra) x Karad Local (Kharif local)]	85	32	20	Susceptible	110
2	PVK 801	[(IS 23528 x SPV 475) x(PS 29154)]-4-2-2-4 4	80	42	30	Resistance	140

3.2 METHODS

3.2.1 Field Evaluation

Recombinant Inbred Line (RIL) population consisting of 336 individuals were evaluated separately for two years at three different locations. During first year (Post- rainy 2012-13) based on availability of seeds, only 309 RILs along with parents (296 B and PVK 801) were used for sowing at three different locations, similarly during second year (Post- rainy 201314) based on availability of seed 334 RILs at ICRISAT and 325 RILs at IIMR and VNMKV were used for sowing along with parents (Table 3.2) All the trials were conducted using Alpha Latice design with three replications, plots of each replication were randomized independently using GenStat statistical package. For each entry equal quantity of seed was packed in sowing packets labelled with number of rows of each plot and randomized plots number were arranged according to planned field layout.

Table 3.2. Information for field layouts and sowing for each location

Year	Location	No. of Plots	RIL + Parents	Design
Rabi 12-13	ICRISAT (E1)	320	309 RIL + P1 (6 times) + P2 (5 times)= 320	10 entries / block x 32 blocks / rep
	IIMR (E2)	320	309 RIL + P1 (6 times) + P2 (5 times)= 320	10 entries / block x 32 blocks / rep
	VNMKV (E3)	324	309 RIL + P1 (8 times) + P2 (7 times)= 324	9 entries / block x 36 blocks / rep
Rabi 13-14	ICRISAT (E4)	360	334 RIL + P1 (13 times) + P2 (13 times)= 360	10 entries /block x 36 blocks /rep
	IIMR (E5)	360	325 RIL + P1 (17 times) + P2 (18 times)= 360	10 entries /block x 36 blocks /rep
	VNMKV (E6)	360	325 RIL + P1 (17 times) + P2 (18 times)= 360	18 entries /block x 20 blocks /rep

3.2.2 Agronomic practices

Sowing was done by tractor-mounted 2-cone planter (7100 US model) at ICRISAT and hand sowing at IIMR and VNMKV, with each entry planted in two rows of 2 m length, spaced at 75 cm between rows at ICRISAT and 60 cm between rows at IIMR and VNMKV, Overplanted plots were thinned 15 days after planting to single plant, spaced 10 cm apart within each row. The crop was supplied with a fertilizer dose of 80 kg N and 40 kg P₂O₅ per hectare and nitrogen was applied in two split doses. Trials were irrigated as needed, to ensure no moisture stress. All the recommended agronomic practices were followed for good crop growth.

3.2.3 Observations recorded and harvesting

Observations were recorded in RIL populations for days to 50% flowering, plant height, 100grain weight, grain yield per plot and grain Fe and Zn densities, detailed measurement of these traits are given in subheads 3.3. The entries of all plots were harvested at physiological maturity (black spot observed at hilum of grain). During harvest, main panicles of five random plants from each plot were harvested and stored separately in a cloth bag to produce clean grain samples for micronutrient analysis. The remaining panicles of the plot were harvested as a bulk. These panicles were sundried for 10 to 15 days. While threshing, five panicles were harvested separately, harvested panicles were manually threshed and approximately 20 g of grains were collected for Fe and Zn analysis, and the left over grains from these panicles were added to the bulk grain produced by threshing in a multi head

machine thresher. The grain yield including the 20 g sample taken for micronutrient analysis was recorded for each plot and converted to tonnes per hectare.

3.3 OBSERVATIONS RECORDED

3.3.1 Agronomic observations

Days to 50% flowering (days): The number of days from the day of sowing to flowering of 50 per cent of plants in a plot were counted and recorded as days to 50 per cent flowering.

Plant height (cm): It was measured at maturity stage. The height of the plant was measured by using centimetre scale from base of plant to the tip of the panicle.

Seed weight of 100 fully developed grains (g): The weight of 100 grains drawn randomly from each of the five randomly selected plants was recorded and expressed in grams.

Grain yield ($t\ ha^{-1}$): After threshing, grains obtained from all the plants of a plot were weighed. Plot yield was converted into $t\ ha^{-1}$ by using following formula.

$$\text{Grain yield} = \frac{\text{plot yield (kg)} \times 10000}{1000 \times \text{plot size (m}^2)}$$

3.3.2 Grain micronutrient analysis

3.3.2.1 Grain samples collection

In all the experiments, self-pollinated (SP) grain samples were produced and used to estimate grain Fe and Zn densities expressed in mg kg^{-1} . At the time of harvesting, 5 representative main panicles from each plot were harvested at physiological maturity. The harvested panicles were stored directly in a separate cloth bag to avoid soil contamination and was dried in the sun light up to < 12% post-harvest grain moisture content. While threshing, the separately harvested panicles were manually threshed first and approximately 20 g of grains were collected for Fe and Zn analysis. Grains were cleaned from glumes, panicle chaff and debris and transferred to new non-metal foldable envelops and stored in cold temperature. Care was taken at each step to avoid contamination of the grains with dust particles and any other extraneous matter (Stangoulis and Sison, 2008).

3.3.2.2 Analysis of grain iron and zinc densities

Grain Fe and Zn densities were analyzed at the Charles Renard Analytical Laboratory, ICRISAT, Patancheru, India following the method described by Wheal *et al.* (2011). The ground samples were digested in closed tubes; and Fe and Zn in the digests were analyzed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Briefly, grain samples were finely ground and oven dried at 60°C for 48 hrs before analyzing them for Fe and Zn densities. Ground sample (0.2 g) was transferred to 25 ml polypropylene PPT tubes; digestion was initiated by adding 2.0 ml of concentrated nitric acid (HNO_3) and 0.5 ml of 30% hydrogen peroxide (H_2O_2). Tubes were vortexed to ensure that entire sample was wetted, and then pre-digested overnight at room temperature. Tubes were vortexed again before placing them into the digestion block and initially heated at 80°C for 1 hour, followed by digesting at 120°C for 2 hours. After digestion, the volume of the digest was made up to 25 ml using distilled water; and the content was agitated for 1 minute by vortex mixer. The digests were filtered and Fe and Zn densities were determined using ICP-OES, the details of which were briefed below:

Principle of ICP-OES:

Inductively Coupled Plasma (ICP), is a method of optical emission spectrometry. When plasma energy is given to an analysis sample from outside, the component elements (atoms) are excited. When the excited atoms return to low energy position, emission rays (spectrum rays) are released and the emission rays that correspond to the photon wavelength are measured. The element type is determined based on the position of the photon rays, and the concentration of each element is determined based on the rays' intensity. To generate plasma, first, argon gas is supplied to torch coil, and high frequency electric current is applied to the work coil at the tip of the torch tube. Using the electromagnetic field created in the torch tube by the high frequency current, argon gas is ionized and plasma is generated. This plasma has high electron density and temperature (10000K) and this energy is used in the excitation-emission of the sample. Solution samples are introduced into the plasma in an atomized state through the narrow tube in the center of the torch tube.

ICP-OES determination:

Determinations were carried out using A Prodigy High Dispersion Inductively Coupled Plasma (ICP) Spectrometer equipped with a dual view torch and 60 position auto sampler. The Prodigy is a compact bench-top simultaneous ICP-OES featuring an 800 mm focal length Echelle optical system coupled with a mega-pixel Large Format Programmable Array Detector (L-PAD). At 28 × 28 mm, the active area of the L-PAD is significantly larger than any other solid-state detector currently used for ICP-OES. This combination allows Prodigy to achieve significantly higher optical resolution than other solid-state detector based ICP systems. The detector also provides continuous wavelength coverage from 165 to 1100 nm permitting measurement over the entire ICP spectrum in a single reading without sacrificing wavelength range or resolution. This detector design is inherently anti-blooming and is capable of random access, non-destructive readout that results in a dynamic range of more than 6 orders of magnitude.

3.3.3 Soil micronutrient analysis

3.3.3.1 Soil samples collection

At the time of planting of each experiment, four well-spread representative soil samples were collected from the experimental fields from 0-30 cm top layer. The soil samples were air-dried, crushed with a wood mallet and sieved through a 6 mm nylon screen. Precautions were taken to avoid contamination during sampling, drying, crushing and storage. A representative sub-sample of each soil sample was further pulverized with a wooden rolling pin and screened through a 1 mm stainless sieve and used for the laboratory analyses.

3.3.3.2 Analysis of soil iron and zinc contents

The soil Fe and Zn contents were analyzed by DTPA extractable method at Charles Renard Analytical Laboratory, ICRISAT, Patancheru, and expressed as mg kg⁻¹ (ppm). Ten grams of air-dried soil was placed in a 125 ml conical flask and 20 ml of the DTPA extracting solution was added. Each flask was covered with screw cap and placed on a horizontal shaker with a stroke of 8.0 cm and with a speed of 120 cycles min⁻¹. After 2 hours of shaking, the suspensions were filtered by gravity through Whatman no. 42 filter paper. The filtrates were analyzed for Fe and Zn contents using Atomic Absorption Spectrophotometry (AAS). These

Fe and Zn contents in the soil were in the sufficient range for normal plant requirements (2.6 to 4.5 mg kg⁻¹ for Fe; 0.6 to 1.0 mg kg⁻¹ for Zn) (Tisdale *et al.*, 1993)

3.4 STATISTICAL ANALYSIS

3.4.1 General statistics

3.4.1.1 Best Linear Unbiased Predictions (BLUPs)

The phenotypic variance was partitioned using the residual maximum likelihood (ReML) algorithm with a mixed model, for each environment separately where, replication and block were considered to be fixed effects, while genotypes were considered to be random effects, to obtain the best linear unbiased predictions (BLUPs) of the performance of genotypes for each observed trait (Patterson and Thompson, 1971). These BLUPs means were used for QTL analysis.

3.4.1.2 Mean

Mean value (X) of each character was determined by dividing the sum of the observed values with the corresponding number of observations.

$$\bar{X} = \frac{\sum_{i=1}^N X_i}{N}$$

Where,

X_i - Observation of the ith treatment

N - Total number of observations.

3.4.1.3 Standard error (SE)

The standard error is a measure of the precision of sample mean. To know how widely they are scattered some measurements like standard deviation is used and to indicate the uncertainty around the estimate of mean measurement, the standard error of the mean is quoted (Altman and Bland, 2005 and Biau, 2011).

$$SE = \sqrt{\frac{MSE}{n}} \quad \text{or} \quad \frac{SD}{\sqrt{n}}$$

3.4.1.4 Range

Lowest and highest values for each character were recorded.

3.4.1.5 Heritability

Broad-sense heritability on plot-mean basis was computed from the estimates of genetic (σ_g^2) and residual (σ_e^2) variances using progeny means across RILs in each environment for all traits. Heritability (h^2) was estimated as explained by Falconer (1989) $h^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_e^2/r)$ where, σ_g^2 = genotypic variance σ_e^2 = error variance r = no of replications.

Heritability (h^2) estimates across the environments were estimated by the formula-

$$H = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_{yg}^2 / y + \sigma_{eg}^2 / e + \sigma_{yeg}^2 / ye + \sigma_e^2 / rye}$$

where, σ_g^2 = genotypic variance

σ_e^2 = error variance

σ_{yg}^2 = Genotype \times Year interaction variance

σ_{eg}^2 = Genotype \times Environmat interaction variance

σ_{yeg}^2 = Genotype \times Environmat \times Year interaction variance

y = no of years

e = no of enviroments

r = no of replication.

3.4.2 Analysis of variance (ANOVA)

Combined analysis

Four variance components σ_g^2 (Genotype), σ_{gy}^2 (Genotype \times Year), σ_{gl}^2 (Genotype \times Location), σ_{gyl}^2 (Genotype \times Year \times Location) were estimated for each of the six traits using restricted maximum likelihood (Patterson and Thompson, 1971) estimation as implemented in the ASREML software (Gilmour *et al.*, 2009). In these analyses, trial (a combination of location and year) was fitted as a fixed effect. A three-replicate α -lattice design was used in the analysis. Experimental design factors, genotype, and its interactions with location and year, were fitted as random effects. The phenotypic observations z_{ijklm} on accession m in replicate k of block l of location j and year i was modelled as:

$$z_{ijklm} = \mu + y_i + e_j + ye_{ij} + (y/e/r)_{ijk} + (y/e/r/b)_{ijkl} + g_m + (yg)_{im} + (eg)_{jm} + (yeg)_{ijm} + \varepsilon_{ijklm}$$

Where μ is the grand mean; y_i is the fixed effect of year i ; e_j is the fixed effect of location j ; ye_{ij} is the fixed effect of interaction between year i and location j ; g_m is the random effect of accession m and is $\sim NID(0, \sigma_g^2)$; $(y/e/r)_{ijk}$ is the random effect of replication in location j and year i and is $\sim NID(0, \sigma_r^2)$; $(y/e/r/b)_{ijkl}$ is the random effect of block l nested with replication k in location j and year i and is $\sim NID(0, \sigma_b^2)$; $(yg)_{im}$ is the random effect of the interaction between accession m and year i and is $\sim NID(0, \sigma_{yg}^2)$; $(eg)_{jm}$ is the random effect of the interaction between accession m in location j and $\sim NID(0, \sigma_{eg}^2)$; $(yeg)_{ijm}$ is the random effect of the interaction effect of the accession m in year i and location j and $\sim NID(0, \sigma_{yeg}^2)$; and ε_{ijklm} is the random residual effect and $\sim NID(0, \sigma_\varepsilon^2)$.

Combined Analysis of Variance was carried out at three locations across two years by modelling individual error variances using mixed model procedure. The components of variance and standard error of random terms were estimated using REML procedure of GenStat software, 17th edition (VSN International, Hemel Hempstead, UK).

Individual environment analysis

Analyses of variance were also conducted using data from each environment for all six traits. The model used was the same as in combined analysis (above) without the terms $\sigma^2 gy$ (Genotype \times Year), $\sigma^2 gl$ (Genotype \times Location), $\sigma^2 gyl$ (Genotype \times Year \times Location).

3.5 Assessment of association of grain iron and zinc concentration with agronomic traits

3.5.1 Covariance

Covariance was calculated to estimate the relationship between pair of traits. Covariance is always measured between two dimensions. If the covariance was calculated between one dimension and itself, the variance is obtained.

$$cov(X, Y) = \frac{\sum_{i=1}^n (X_i - \bar{X})(Y_i - \bar{Y})}{n - 1}$$

\bar{X} – Mean of X variable

\bar{Y} – Mean of Y variable

n- Number of observations or sample size.

3.5.2 Correlation coefficient

The Pearson correlation coefficient was calculated to estimate the linear relationship between pair of traits by the following formula:

$$r_{(XY)} = \frac{\text{COV}(X,Y)}{S_X S_Y}$$
$$r_{(XY)} = \frac{\sum_{i=1}^n (X_i - \bar{X})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^n (x_i - \bar{X})^2} \sqrt{\sum_{i=1}^n (Y_i - \bar{Y})^2}}$$

Where,

$r_{(X,Y)}$ is the phenotypic correlation between the variables X and Y,
 $\text{COV}(X, Y)$ is the phenotypic covariance of the variables X and Y,
 S_x is the sample standard deviation of the random variable X and
 S_y is the sample standard deviation of the random variable Y.

The observed value of correlation coefficient is compared with the tabulated value for $n-2$ degrees of freedom, where n is number of observations.

$$t = r \sqrt{\frac{n-2}{1-r^2}}$$

r- Correlation coefficient,

n- Total number of observations

3.5.3 Principal Component Analysis (PCA)

Associations among the traits were also determined by Principal Component Analyses (PCA) (Hatcher, 1994) using R version 3.0.2 (R Project for Statistical Computing, <http://www.r-project.org/>).

3.6 Genotyping of RIL population

3.6.1 Seed germination and DNA isolation

For genotyping of RIL population, the seeds of 336 RIL individuals of sorghum were sown in pots in glass house and after 20-25 days young leaves were collected for DNA isolation. DNA was extracted using the C TAB buffer method described below:

Procedure for DNA isolation

Approximately 100 mg of young tender leaves were taken and 450 µl of C TAB buffer was added and the leaves were ground in a Genogrinder for 10 minutes at the speed of (350 strokes /2 minute). The ground sample was kept in water bath maintained at 65°C for 20-30 minutes and the contents were mixed 2-3 times. 400 µl of chloroform + IAA (Isoamyl Alcohol) (24:1) mixture was added to it and mixed gently. It was centrifuged at 6000 rpm for 12 minutes. Supernatant was collected and equal volume of chilled isopropanol was added to it and mixed gently. Then, it was kept at -20°C for 20 minutes and centrifuged at 6000 rpm for 12 minutes. Supernatant was discarded and the pellet was collected. To the pellet, 200 µl of low salt TE (T₅₀E₁₀) + 3 µl of RNase (10 mg/ml) was added and pellet was disturbed by tapping gently. Then, it was kept in 37°C incubator for 40 minutes. 200 µl of phenol + chloroform + IAA (24:24:1) was added to it and mixed gently. Centrifugation was done at 5000 rpm for 15 minutes. Supernatant was collected and equal volume of chloroform + IAA was added to it and mixed gently and centrifuged at 5000 rpm for 5 minutes. Supernatant was collected and 300 µl of absolute ethanol (100%) and 15 µl of 3 M sodium acetate (pH 5.2) was added per sample. It was mixed gently and kept at -20°C for 10 minutes. It was centrifuged at 6200 rpm for 10 minutes. It was decanted and then 200 µl of 70% ethanol was added and centrifuged at 6000 rpm for 5 minutes. It was decanted again and vacuum dried for 5-10 minutes. 100 µl of T10 E was added per sample and kept at room temperature for 30 minutes and then transferred to 4° C.

3.6.2. Quantification and dilution of DNA

DNA was quantified on 0.8% agarose gel.

Gel loading orange dye (for 100 ml)

0.5M EDTA (pH=8.0)	10 ml
5M NaCl	1 ml
Glycerol	50 ml
MQ H ₂ O	39 ml

After adding the above components Orange G dye powder was added until the colour was sufficiently dark and mixed properly.

Procedure

To prepare gel, 0.8 g of agarose was melted in 100 ml of 1X TBE buffer in microwave oven and cooled to 50°C by keeping at room temperature for 5-10 minutes. Now, 5 µl of 10 mg/ml stock of ethidium bromide was added to it. The gel plate was sealed on both sides with tape and a comb was inserted and the gel solution was poured in it gently. Then, it was allowed to solidify for 30 minutes. After setting, the seal tapes were removed from both sides and comb was removed gently. The gel plate was placed in an electrophoresis unit and filled with 1X TBE buffer. The DNA sample was prepared by adding 3 µl of gel loading dye, 3 µl of sterile distilled water and 1 µl of the DNA sample and then it was loaded on agarose gel with diluted uncut λ DNA as standard and electrophoresis unit was run at 80 V for 20 minutes and then gel was visualized under UV and photographed on the gel documentation system (Appendix II). After quantification of DNA, it was diluted to 100 ng/µl.

3.6.3 Sorghum genotyping using SSR markers

Testing parental polymorphism using SSR primers

To identify SSR primer pairs detecting polymorphism between parents, initial screening of parental lines was conducted. For this, DNA from 296 B (taken as first parent *i.e.*, P₁) and PVK 801 (taken as second parent *i.e.*, P₂) were subjected to PCR amplification with SSR primers with two repeats for each parent. A total of 271 SSR markers (Xtxp 94, Xisep 76, Xiabtp 62, XmbCIR 12, Xgap 11, Xcup 10 and Xgpsb 6) were used to screen the parents for variation (Appendix III). These SSR markers were selected based on uniform distribution across the 10 sorghum chromosomes.

3.6.3.1 PCR using SSR markers

Multiplex PCR was carried out to amplify SSR loci for RIL mapping population based on cross 296 B \times PVK 801, consisting of 336 F₆ RILs. Fluorescently labelled and M13labelled SSRs were used Table 3.3. For M13-labelled primers a three-primer strategy was used with 1:15:15 ratio for forward primer with M13 tail, regular reverse primer and universal fluorescent labelled M13 primer, respectively. The various components used for PCR reaction are as follows:

Component	Concentration used	Volume/reaction (μ l)
10 X PCR buffer	1X	1
50 mM MgCl ₂	1.0mM	0.5
2 mM dNTPs	0.2mM	0.5
Primers (2 pm)	0.4mM	2.0
Taq polymerase (NEB 5 U/ μ l)	0.2 U	0.05
MQ H ₂ O	-	4.00
DNA (3 ng/ μ l)	3ng	2
Total		10

Cycling conditions used for PCR

Forty cycles of amplification were carried out in a thermocycler (Applied Biosystems) using a Touch down programme having the following conditions:

Initial denaturation	3 min. at 94 ⁰ C
	1 min. at 94 ⁰ C
Touchdown step	1 min. at 60 ⁰ C
	1 min. at 72 ⁰ C
No. of cycles	5
Denaturation	1 min. at 94 ⁰ C
Annealing	1 min. at 51 ⁰ C
Extension	1 min. at 72 ⁰ C
No. of cycles	40
Final extension	20 min. at 72 ⁰ C

The amplified product was analysed on 1.5% agarose gel as described in section 3.6.2.

3.6.3.2. Analysis of PCR product on ABI 3730 DNA analyzer

To increase the throughput, after checking the amplification, based on the amplicon sizes and fluorescent dyes used, the post PCR multiplexing of amplicons from each fluorescent dye (FAM/NED/PET/VIC) were pooled to facilitate multiplex capillary electrophoresis. While pooling, 2 μ l PCR product was taken from each marker of the multiplex set (markers labelled with different dyes) and pooled together for simultaneous detection of the amplified alleles. 7 μ l of formamide and 0.2 μ l of fragment size standard GeneScan TM 500 LIZ were

Table 3.3. Details of SSR markers used for genotyping of RIL population

Sr	locus name	Cho No	Motif	Size	Forward	Reverse
1	Xiabtp278	1	NA	227	CACGACGTTGTAAAACGACGCCAAGAGCAGACTGACCTC	TGAAGTCGGTGTTCACGAAG
2	Xiabtp450	1	NA	224	NA	NA
3	Xtxp302	1	(TGT)8	179	TAGGTTCTGGACCACTTCTTTGTGTT	GAATCAACTATGTGCTTGCATTGTGCT
4	Xtxp357	1	(GT)10	272	CGCAGAAATACGATTG	GCTATCTGGAGTAACGTGTT
5	Xiabtp346	2	NA	171	CACGACGTTGTAAAACGACCCGTCTCCACAAGCTTCTC	GACTGTGCCAGCTGTCTCC
6	Xisep0841	2	GCA(10)	215	CACGACGTTGTAAAACGACTAGGAATGACGACACCAACCA	CAAAGGCAAGGGTTTGCTA
7	Xtxp013	2	(TG) ₁₃	119	TCTTCCCAGGAGCCTAG	GAAGTTATGCCAGACATGCTG
8	Xtxp304	2	(TCT)42	302	ACATAAAAGCCCCCTTTC	CTTTCACACCCCTTATTCA
9	Xisep824	3	CCG(4)	196	CACGACGTTGTAAAACGACTCCTGAAAGAACGCACACA	GAGGAGGGTGTGGAGGTGTA
10	Xcup28	4	(TGAG)5	163	GGTGTGAGACTGTGAGCAGC	TATAGCACGGTTGTGCTGCC
11	Xiabtp481	4	GCA(10)	436	CACGACGTTGTAAAACGACTAGGAATGACGACACCAACCA	CAAAGGCAAGGGTTTGCTA
12	Xisep0210	4	GA(8)	187	CACGACGTTGTAAAACGACACGAGACACGACTCCTCCAT	CGAGGAGGTCGAGTAGAACG
13	Xiabtp420	5	NA	216	CACGACGTTGTAAAACGACACATGCATGCTTGGAAAGTTG	CTCTAGCATGGACCTGCACA
14	Xisep1107	5	GCA(6)		CACGACGTTGTAAAACGACGGATAATCTGCAGGCGACTT	CCATCTGCTGCTCTGACTTG
15	Xtxp015	5	(TC)16	214	CACGACGTTGTAAAACGACCAACAAACACTAGTGCCTTATC	CATAGACACCTAGGCCATC
16	Xtxp017	6	(TC)16(AG)12	163	CGGACCAACGACGATTATC	ACTCGTCTCACTGCAATACTG
17	Xtxp145	6	(AG)22	238	CACGACGTTGTAAAACGACGTTCCCTGCCATTACT	CTTCCGCACATCCAC
18	Xiabtp360	7	NA	99	CACGACGTTGTAAAACGACCAACACTCATCAACAGCCAAC	ACGTCGACCTTCATCGTCTC
19	Xisep0831	7	AAAAG(3)	199	CACGACGTTGTAAAACGACTCCATGACCTTGAGGAGGAG	TTGAAGCAGGACAACACACC
20	Xtxp525	7	NA	211	NA	NA
21	Xgap034	8	[(AC)/(CG)]15	203	AACAGCAGTAATGCCACAC	TGACTTGGTAGAGAACTTGTCTTC
22	Xiabtp399	8	NA		CACGACGTTGTAAAACGACACACCGCTCCCTGTTCTCAC	CATGCCTGCCTGTTTCTTA
23	Xiabtp415	8	NA	401	CACGACGTTGTAAAACGACCCATACCCCTCATGGTGTGTC	CCTGCTTCCCTTCCACTG
24	Xiabtp458	9	NA	180	CACGACGTTGTAAAACGACGGACTGCTGACTGGTTCTCC	GTGCTGGTCCATCAGGATCTG
25	Xtxp010	9	(CT)14	144	CACGACGTTGTAAAACGACATACTATCAAGAGGGGAGC	AGTACTAGCCACACGTAC
26	Xtxp289	9	(CTT)16(AGG)6	289	AAGTGGGTGAAGAGATA	CTGCCTTCCGACTC
27	Xiabtp178	10	NA	267	CACGACGTTGTAAAACGACCATGTCGTTGGAGTACG	GAGACTAGGCGTCACGGAAC
28	Xiabtp261	10	NA	203	CACGACGTTGTAAAACGACACATGAGCCAGCTCCACTG	GATGCTGCCTCAACAAGTGA

added to the pooled and denatured at 95°C for 5 mins followed by cooling on ice. Capillary electrophoresis of denatured pooled products was performed using ABI 3730 DNA/Genetic analyzer (Applied Biosystems).

3.6.3.3. Data collection and analysis

The data was collected automatically by the detection of the different fluorescences and analyzed by GeneMapper v 4.0 software (Applied Biosystems).

3.6.4. Sorghum genotyping using DArT markers

The high-throughput genome analysis method called Diversity Arrays Technology (DArT) (Akbari *et al.*, 2006) has been used in both populations to develop the saturate genetic map. For DArT genotyping, DNA samples ($20\text{ ng }\mu\text{L}^{-1}$) was submitted to the Diversity Arrays Technology Private Limited Australia, where RIL population was genotyped using an array of 8732 DArT clones developed by *PstI/BamII* complexity reduction. DArTsoft, a software package developed at DArT P/L, was used to automatically analyze the output data. The DArTsoft generated 0–1 scores of the polymorphic DArT markers detected among the inbred lines and parents of the particular RIL population were converted to A–B scores and used as input for mapping. DArTseq generates two types of data:

1. scores for “presence/absence” (dominant) markers, called Silico DArTs as they are analogous to microarray DArTs, but extracted “in silico” from sequences obtained from genomic representations
2. SNPs in fragments present in the representation.

3.6.5. Genetic Linkage Mapping

The score of all polymorphic SSR, DArT and SNP markers were converted into genotype codes ('A','B') according to the score of parents after genotyping mapping population with DArT array and SSR markers. In linkage analysis markers were deleted that had high segregation distortion ($P < 0.5$), call rate less than 85%, reproducibility less than 90% and marker having more than 10% missing data points. To construct the map (17) RILs (5% of the population) were discarded from the original set of the 336 lines because they were contaminated or had more than 10 % loci with non parental alleles. Further, only 323 remaining RILs were used for mapping of SSR, DArT and SNP markers. Linkage analysis

was performed by using Join Map 3.0 programme (Van Ooijen and Voorrips, 2001). The critical logarithm of odds (LOD) score for the test of independence of marker pairs was set at 10.0 and maximum recombination fraction (Θ) of 0.25. The distance between marker loci calculated using the Kosambi mapping function. A graphical representation of map was drawn using Map Chart software (Vorrips, 2002). DArT markers were named with the prefix ‘Dr’ and SNP with ‘SN’ followed by numbers corresponding to unique clone ID, whereas SSRs were named as it is without any prefix. The consensus map developed by Mace *et al.* (2009) was considered as reference map for grouping of DArT and SNP markers and to construct the map in present study.

3.6.7. QTL Mapping

To identify QTL, genetic linkage map developed by Join Map 3.0 software and phenotypic data (BLUPs) obtained from RIL mapping population for Iron and Zinc concentration separately from six environments individually and polled data (across six environment data) were used. The positions and effects of QTL were determined following composite interval mapping (CIM) using the software QTL CARTOGRAPHER v. 2.5 (Wang *et al.* 2007). The significant threshold logarithm of odds (LOD) score for detection of the QTL were calculated based on 1000 permutations at $p < 0.05$ (Churchill and Doerge 1994). The proportion of observed phenotypic variation explained due to particular QTL was estimated by the coefficient of determination (R^2) using maximum likelihood for CIM. The putative QTLs detected for each of the respective traits were assigned to linkage groups based on the map position of their flanking markers.

Results and
Discussion

Chapter IV

RESULTS AND DISCUSSION

The link between food and health is long and well documented as humans requires at least 49 nutrients for their normal growth and development, and the demand for most nutrients is supplied by food (Welch and Graham, 2004). Deficiencies or insufficient intakes of these nutrients especially vitamins or minerals leads to several dysfunctions and diseases in humans leads to micronutrient malnutrition or hidden hunger. The World Bank estimates that India is ranked 2nd in the world among number of children suffering from malnutrition, after Bangladesh in 1998. According to the report of Sengupta (2009), about 42.5% of the children in India suffer from malnutrition. Malnutrition especially due to Fe and Zn has been recognized as a serious health problem in developing countries (Welch and Graham, 1999). In India, out of 6,000 deaths under the age of five, more than half of these are due to malnutrition-mainly the lack of Vitamin A, iron, iodine, zinc and folic acid. Moreover, about 80% of the pregnant women and 52% of the ever-married women in India suffer from iron deficiency induced anemia coupled with Zn deficiency (Chakravarty and Ghosh, 2000). Thus micronutrient deficiency is a serious public health related social problem in India and other developing countries. Targeted strategies available to alleviate micronutrient deficiencies include dietary diversification, food fortification, and supplementation but with the exception of iodised salt, the success of above interventions in developing countries has been mixed (Stein *et al.*, 2005). More recently breeding of agricultural crops for higher nutrient levels (or ‘bio-fortification’) is an emerging approach that complements the existing “toolbox” of interventions. This mineral diversity for Fe and Zn concentration in grain is reported by various researchers previously. The knowledge of correlation among various traits is important when the target is to pyramid or combine the traits/genes of interest and prerequisite to plan a meaningful breeding program.

Genetic and genomic resources for sorghum are rapidly developing. These genetic and genomic resources in combination with breeding technologies offer opportunities to exploit the genetic diversity of crop to formulate the mineral level in grain. Diversity in mineral concentrations for bio-fortification has been reported in various studies. With the development of molecular markers especially SSRs it is possible to study the quantitative traits by considering them as individual Mendelian factors (Paterson *et al.*, 1991). Development of markers, genetic linkage map and QTL mapping in various crops like Arabidopsis (Vreugdenhil *et al.*, 2004; Harada and Leigh, 2006 and Waters and Grusak,

2008), bean (Guzman-Maldonado *et al.*, 2003), wheat (Shi *et al*, 2008; Distelfeld *et al*, 2007 and Peleg *et al*, 2009) and rice (Gregorio *et al* 2000; Stangoulis *et al.*, 2007) brought large information about molecular breeding of various quantitative traits. Association of markers with seed mineral variation has been identified. QTL mapping for grain mineral could be carried out with existing mapping populations, or specific populations could be constructed by crossing contrast parents based on wide variation in minerals. The QTL information could be used for marker assisted selection breeding programs, or for positional cloning studies to identify specific genes that affect grain mineral traits. Thus, the present study was primarily carried out to phenotype the RIL mapping population, to develop linkage maps and characterizes the QTLs associated with grain micronutrient (Fe and Zn) concentration. The results of study are discussed here.

4.1 Phenotypic Analysis

There is large variation for grain Fe and Zn concentration and agronomic traits in sorghum. The phenotypic characterization of material was carried out with the objective to get additional information on variation at genetic levels. The phenotypic characterization helps in broadening the genetic understanding of the quantitative traits. The explanatory power of trait detection and QTL analysis largely depend on the reliable assessment of the phenotypic variance of the trait under study. Variance cannot be detected in single individual but can be estimated for whole population. Experimental mapping populations are basic resources to elucidate the genetic basis of the quantitative multigenic traits. Therefore the Recombinant Inbred Line (RIL) population was evaluated for agronomic traits and grain Fe and Zn concentration to detect the genomic regions responsible for the selected trait expression and to identify non-genetic effect (such as environmental influence) on phenotypes. The RIL population derived from cross 296 B × PVK 801 having 336 RILs of F₆ generation were phenotyped for agronomic traits and grain Fe and Zn concentration along with parents to obtain means and variances. The phenotypic data collected from the population for two seasons during post-rainy 2012-13 and postrainy 2013-14 at three different locations were analysed statistically to obtain variance components, hereafter, referred to as E₁ (ICRISAT 2012-13), E₂ (IIMR 2012-13), E₃ (VNMKV 2012-13), E₄ (ICRISAT 2013-14), E₅ (IIMR 2013-14) and E₆ (VNMKV 2013-14). The component of variance has been used to estimate the heritability of traits. A high value for heritability was observed, which is a prerequisite for effective QTL mapping and marker assisted selection. The details are given below.

4.1.1 Mean Performance

The means, standard deviation, ranges and significance of genotypes for the traits measured in RILs were compared with parental means in all the environments separately and summarized in table 4.1.

Except days to 50% flowering (DTF), the parent 296 B exhibited ignorantly lower means as compared to another parent PVK 801 for all agronomic traits in all the six environments. But parental means difference for DTF in E₃ was non-significant, similarly these differences for 100 seed weight and grain yield for E₂ were non-significant, whereas for all the remaining traits both the parents significantly differed in all six environments. The mean performance for grain iron and zinc concentration in both the parents were the highest in E₃ and the lowest in E₄. Both the parents exhibited wider range of variation for grain iron and zinc concentration in different environments under study. The mean performance of parents for other agronomic traits like plant height, 100 seed weight and grain yield was higher in E₁.

The mean performance of RILs for grain iron and zinc concentration was the highest in E₃ whereas, the lowest in E₄. The performance of the QTLs for other agronomic traits like plant height, 100 seed weight and grain yield was high in E₁. The mean performance of RILs for 100 seed weight and grain yield was the lowest in E₃, while, it was lowest for plant height in E₄. The mean performance of RIL population for grain iron and zinc was significantly different from the parent 296 B over all the six environments. The mean performance of RILs for grain iron and zinc was non-significant compared to the parent PVK 801 except in E₂ and E₅ for zinc, for 100 seed weight and grain yield the RIL population differed non significantly from 296 B, whereas, the RIL population was significantly differed for parent PVK 801 for 100 seed weight and grain yield except in E₂ for 100 seed weight.

The present study showed that parents had substantial differences for micronutrients, iron and zinc, in all the environments. The average iron and zinc was highest in location VNMKV Parbhani followed by IIMR and ICRISAT, respectively (Fig 4.1) as VNMKV Parbhani soils are deep black and nutrient rich compared to IIMR and ICRISAT. This clearly indicated the role of Genotype × Environment interaction and effect of environment in mineral uptake, translocation and distribution, similar results were obtained by Vreugdenhil *et al.*(2004) indicating that nutrient content of plant seeds depends on environment factors. Alteration in environment or physiology of plant can affect the accumulation of different multiple elements simultaneously (Buescher *et al.*, 2010). Variation in mineral uptake in different environments has been described in

Table 4.1. Descriptive statistics of phenotypic values in RILs derived from cross 296B X PVK 801 in SIX different Environments

Trait	Environment	296B (P1)		PVK 801 (P2)		RILs		ST DEV	P1 vs P2	P1 vs RIL	P2 vs RIL
		MEAN	RANGE	MEAN RANGE	MEAN RANGE	Pr > F	Pr > F		Pr > F	Pr > F	Pr > F
DTF	(E1) ICRISAT 12-13	82.00	80-85	78.00	75-80	80.00	70-92	3.12	**	**	**
	(E2) IIMR 12-13	78.00	72-89	73.00	66-78	76.00	65-88	4.79	*	NS	NS
	(E3) VNMKV 12-13	84.00	81-88	82.00	81-84	84.00	77-91	2.23	NS	*	NS
	(E4) ICRISAT 13-14	90.00	85-97	83.00	77-89	87.00	74-101	3.89	**	*	**
	(E5) IIMR 13-14	91.00	85-99	88.00	79-86	90.00	77-100	3.78	**	*	NS
	(E6) VNMKV 13-14	88.00	83-92	84.00	80-87	86.00	78-97	3.26	**	**	**
PH (cm)	(E1) ICRISAT 12-13	119.44	100-140	163.33	150-180	146.27	90-230	26.98	**	**	**
	(E2) IIMR 12-13	106.05	97-114	156.20	142-169	133.28	75-208	26.31	**	**	**
	(E3) VNMKV 12-13	94.56	87-102	152.76	140-170	126.57	66-195	24.82	**	**	**
	(E4) ICRISAT 13-14	105.47	95-122	128.73	117-143	121.34	65-198	21.51	**	**	**
	(E5) IIMR 13-14	105.93	85-133	142.11	121-162	131.00	57-196	22.95	**	**	**
	(E6) VNMKV 13-14	99.40	85-110	145.11	134-156	125.32	68-200	25.06	**	**	**
TW (g)	(E1) ICRISAT 12-13	3.07	2.7-3.5	3.77	3.09-4.5	3.22	2.0-4.9	0.42	**	NS	**
	(E2) IIMR 12-13	2.60	2.0-3.5	2.58	2.0-3.5	2.53	1.2-4.2	0.47	NS	NS	NS
	(E3) VNMKV 12-13	2.24	1.8-2.6	2.77	2.2-3.4	2.27	1.3-3.2	0.34	**	NS	**
	(E4) ICRISAT 13-14	2.90	2.0-4.2	3.26	2.5-4.2	2.77	1.2-4.3	0.5	*	NS	**
	(E5) IIMR 13-14	2.90	2.1-3.9	3.61	2.4-4.7	2.92	1.3-4.9	0.52	**	NS	**
	(E6) VNMKV 13-14	2.76	2.2-3.8	3.13	2.0-3.7	2.73	1.4-3.9	0.42	**	NS	**

DTF = Days to 50% flowering, PH= Plant Height (cm), TW= 100 seed weight (g), Fe= Iron (mg kg^{-1}), Zn =Zinc (mg kg^{-1}) and GY= Grain Yield (t/ha)

* Significant at 5% level; ** Significant at 1% level

Table 4.1 cont...

Trait	Environment	296B (P1)		PVK 801 (P2)		RILs		ST DEV	P1 vs P2	P1 vs RIL	P2 vs RIL
		MEAN	RANGE	MEAN RANGE	MEAN RANGE				Pr > F	Pr > F	Pr > F
Fe (mg kg⁻¹)	(E1) ICRISAT 12-13	28.00	24-32	33.4	29.4-41.0	33.60	16.5-65.2	5.6	**	**	NS
	(E2) IIMR 12-13	28.50	22-35	33	26.0-36.7	33.00	19.3-56.3	6.34	*	**	NS
	(E3) VNMKV 12-13	46.33	38-49	49.4	44.0-66.0	49.26	33-76.4	6.93	*	**	NS
	(E4) ICRISAT 13-14	26.00	20-31	28.2	20.8-41.3	28.00	15.0-47.6	4.9	**	*	NS
	(E5) IIMR 13-14	30.80	21-40	35.9	30.2-52.4	35.85	19.8-50.1	5.08	*	*	NS
	(E6) VNMKV 13-14	27.24	19-37	33.6	22.3-44.3	34.00	10.8-67.3	7.89	*	*	NS
Zn (mg kg⁻¹)	(E1) ICRISAT 12-13	21.32	19-26	24.33	21.0-30.9	24.63	13.6-54.2	4.78	**	**	NS
	(E2) IIMR 12-13	21.00	17-24	22.00	20.6-23.6	24.76	13.9-44.3	5	**	**	*
	(E3) VNMKV 12-13	26.44	20-36	30.43	23.0-37.0	31.43	17.3-58.7	6.46	*	**	NS
	(E4) ICRISAT 13-14	14.63	10.0-18	16.46	14.0-21.7	17.33	10.2-33.0	3.5	**	**	NS
	(E5) IIMR 13-14	21.19	13-29	24.82	18.3-40.0	25.66	11.8-41.2	4.03	**	*	**
	(E6) VNMKV 13-14	19.69	14-24	24.06	19.0-33.6	24.72	11.8-46.5	5.27	*	**	NS
GY (t/ha)	(E1) ICRISAT 12-13	4.13	2.5-5.6	5.85	5.0-7.3	3.7	0.7-7.0	0.11	**	NS	**
	(E2) IIMR 12-13	3	1.7-1.4	3.6	2.8-4.5	2.7	0.4-6.5	0.1	NS	NS	**
	(E3) VNMKV 12-13	1.4	0.8-2.0	2.39	1.8-3.0	1.5	0.3-3.0	0.05	**	NS	**
	(E4) ICRISAT 13-14	2.8	1.4-3.8	4.16	2.2-6.2	3.1	0.4-5.4	0.07	**	NS	**
	(E5) IIMR 13-14	2.14	1.0-3.8	5.19	1.8-3.0	3.3	0.25-0-6.4	0.12	**	NS	**
	(E6) VNMKV 13-14	2.69	1.4.3	3.99	2.2-7.8	2.5	0.4-5.1	0.09	**	NS	**

DTF = Days to 50% flowering, PH= Plant Height (cm), TW= 100 seed weight (g), Fe= Iron (mg kg⁻¹), Zn =Zinc (mg kg⁻¹) and GY = Grain Yield (t/ha)

* Significant at 5% level; ** Significant at 1% level

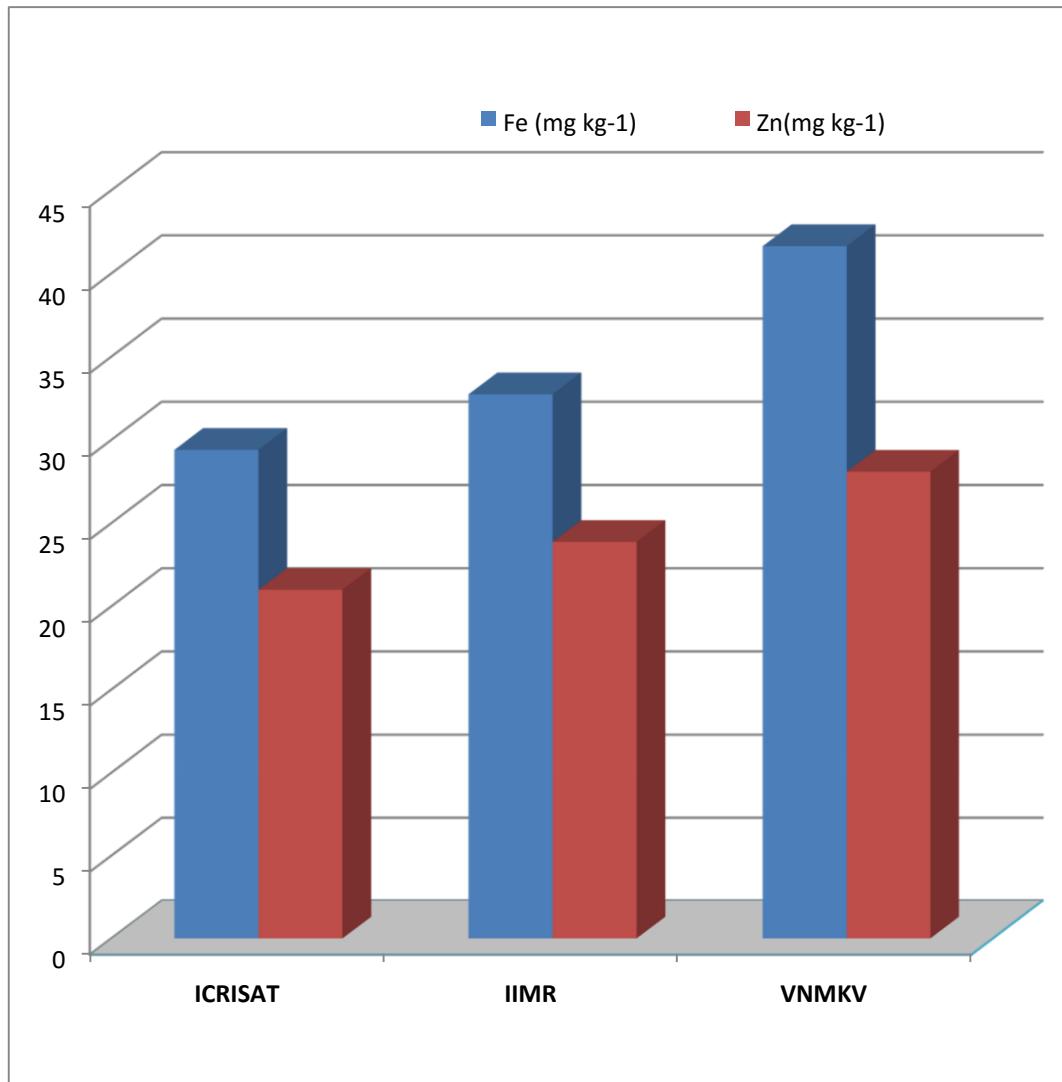


Fig. 4.1. Grain Fe and Zn concentration of RIL population at three different locations.

A. thaliana (Ghandilyan *et al.*, 2009a, 2009b and Loudet *et al.*, 2007) and *Silene vulgaris* (Ernst *et al.*, 2000). Moreover, the nutrient availability in the environment not only affects the nutrient concentration of the vegetative part, but also of the economic parts of a plant and different soil types also results in micronutrient variability (Ernst *et al.*, 2000). Sankaran *et al.* (2009) also detected the variation in mineral content in seed between environments emphasizing the importance of environmental factors on quantitative traits.

There have been concerns about the possibility that high grain mineral concentrations may result from “concentration effects” (Gomez-Becerra *et al.*, 2010) as a consequence of small seeds and /or low yield capacity. In the present study, RILs with high grain iron and zinc concentration had lowest 100-seed weight. This demonstrated that accession with higher nutrient concentration (especially iron and zinc) have lesser grain weight, when grain size increased , there is more accumulation of starch but grain Fe and Zn are not increased proportionally suggesting that higher grain Fe and Zn are related to smaller grain size or weight. In a nutshell, the results indicated the existence of sufficient variability between parents over all individual environments and the greater opportunities for recovering desirable recombination using them. Wide range of variation within population for grain micronutrient concentration has also been reported for numerous other crops, for instance in sorghum (Reddy *et al.*, 2005) and Maize (Banziger and Long, 2000). Thus the availability of wide range for both kind of nutritional and productive traits indicated the scope for development of iron and zinc rich genotypes in high yielding background of sorghum through the exploitation of variability within population variability. Thus crop improvement depends on the magnitude of genetic variability present in the base population. This variability can be exploited if heritability of the traits is high.

4.1.2 Frequency Distribution

By using immortalized mapping populations such as Recombinant Inbred Lines (RIL), derived from a divergent biparental cross, individual genes can be resolved into homozygous progenies. Plotting a histogram using the phenotypic data on such a population, the number and size of phenotypic classes obtained is directly related to the number of genes influencing the trait (Rao *et al.*, 2007). From the preliminary observation of histograms the approximate number of genes responsible for each trait could be predicted. If histogram is normally distributed it indicates the presence of additive effect with mid-parental value as the mean of population, while the presence of asymmetry in distribution or skewness in plot with transgressive segregation leads to epistatic

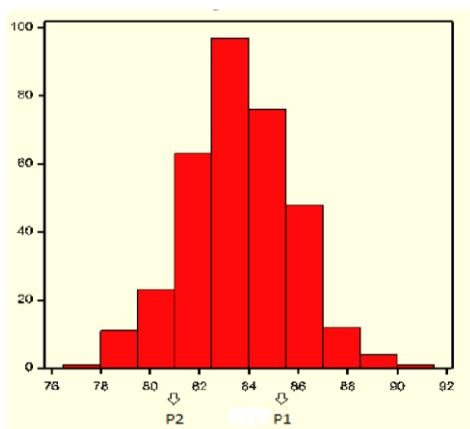
interactions (Pooni *et al.*, 1987). Presence of transgressive segregation also indicates the occurrence of genetic recombination (Falconer 1989), which points out that both favourable and unfavourable alleles for trait studies are scattered in the parents.

A segregating population with heterozygosity can show effects of additive gene action with over-dominance and epistasis (Cho *et al.*, 2002) therefore superiority of transgressive individuals will not be maintained in successive generations (Kuczynska *et al.*, 2007). But the populations used for this study were nearly homozygous inbred lines as they were developed with six generations of selfing.

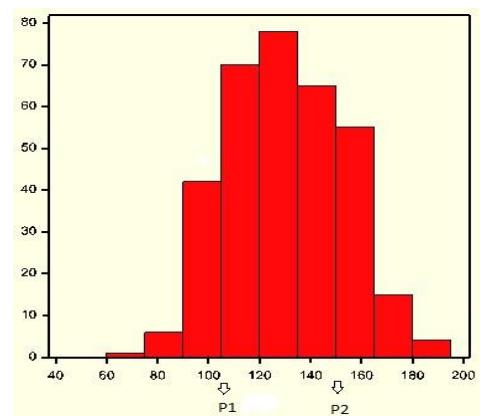
The frequency distribution of all six traits based on across environments BLUPs (Best Liner Unbiased Predication) data, is given in figure 4.2. The measurements were grouped into equally spaced classes on X axis and the frequency of individuals falling in each class was plotted on Y axis. Continuous distribution of phenotypic frequency of different traits supports the quantitative inheritance of traits as expected for quantitative traits. The arrow mark in diagrams on X axis indicates mean values of traits for each parent. Transgression beyond the parent's values was observed for all traits. Considerable transgressive segregation and significant differences in traits was obtained between two extreme RILs, indicating the occurrence of large variation among the 336 RILs under study. This large genetic variation indicates that QTL mapping was likely to reveal QTLs for the studied traits.

4.1.3 Analysis of genotypic and G × E variances

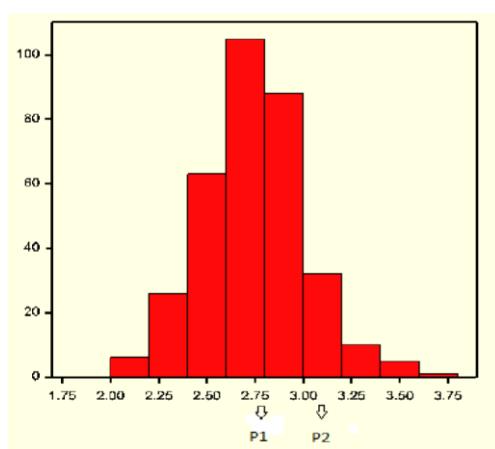
Reliability of QTL mapping also depends upon the variation and heritability of trait (Kearsey and Farquhar, 1998). Hence valid interpretations of mechanisms of inheritance as well as predictions of performance of breeding programs depend on accurate assessment of genotypic values which include both genetic and non-genetic influences (Moll and Stuber, 1974). A study conducted in natural populations by Kruuk (2004) indicated that the BLUP / REML methodology is a powerful way to estimate the components of variance and to predict additive genetic values. Among the three components of phenotypic variability (G (genotype), E (environment), and G × E interaction), the greatest attention has been given to genotypic variance. Exploration of the ratio between the G × E variance components to the genotypic variance component also gives insights about the magnitude of the G × E interaction. Interplay between genetic and environmental factors (G × E interactions) affect phenotypes of complex traits which results in reduction in heritability value. G × E interactions also results in different patterns of genetic associations across environments (Ye *et al.*, 2006). Allard and Bradshaw (1964)



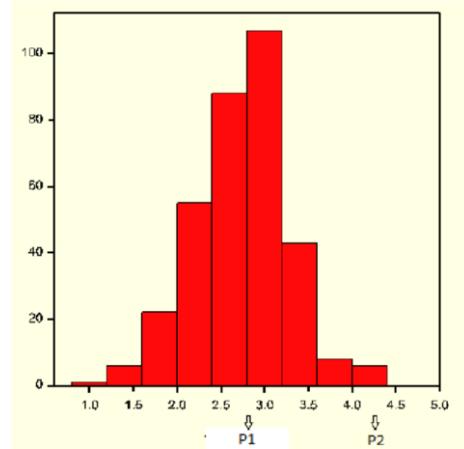
Days to 50% Flowering



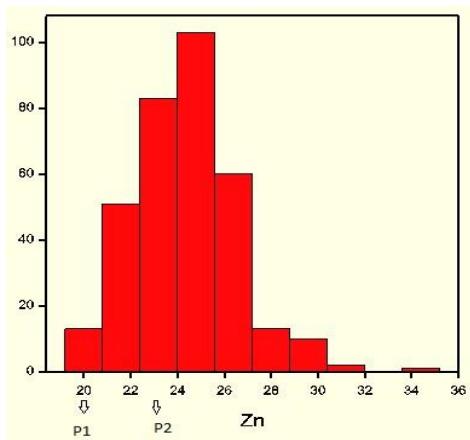
Plant Height (cm)



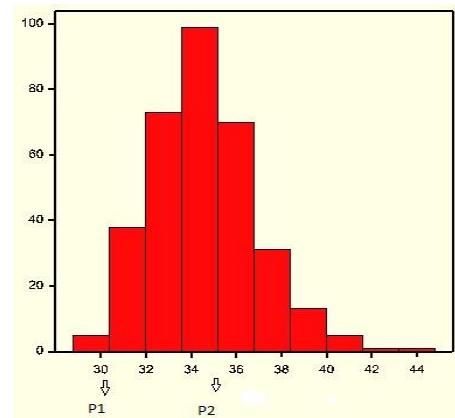
100 Seed Weight (TW) (g)



Grain Yield (t/ha)



Zinc (mg kg⁻¹)



Iron (mg kg⁻¹)

➡ Arrow indicates mean of P₁ (296 B) and P₂ (PVK 801)

Figure 4.2.Frequency distribution of six different traits in RIL population across six environments BLUPs.

indicated that nature of $G \times E$ interactions was extremely complex. Detecting $G \times E$ interaction requires that the same genotypes must be grown in multiple environments. The quantitative genetic theory has made major contributions to separate the genetic effects from environmental effects. High genetic variance indicates less influence of environmental factors on quantitative traits and traits are under genetic control. A partition of genotypic variance in RIL population into additive and epistatic variance components provides a basis to better understand the genetic inheritance properties of a quantitative trait from a parental population to their progeny population. The genetic variance components also play a crucial role in study of heritability. The magnitude of heritability is largely governed by the amount of genetic variance present in the population. High heritability denotes less effect of environment on the trait.

The analysis of variance from our experimentation showed highly significant differences among genotypes (RILs) for all the six traits in all the individual environments (Table 4.2) as well as across the environments (Table 4.3). Agronomic traits such as days to 50 % flowering, plant height, 100-seed weight and grain yield showed highly significant genotypic variances (σ^2g) in all the environments and same trend was continued for combined analysis (across environments). The highest genetic variance was found for days to 50 % flowering and 100-seed weight in E_2 and for plant height and grain yield in E_1 and E_6 , respectively. All the agronomic traits showed non-significant genotype \times year (σ^2gy) interactions. While, the genotype \times location (σ^2gl) interaction were non-significant for all agronomic traits except grain yield. The genotype \times year \times location (σ^2gyl) interactions were highly significant, but in less magnitude compared to genotypic variance (σ^2g) for all the agronomic traits, except grain yield.

The grain micronutrient (iron and zinc) concentrations showed highly significant genotypic variances in all the individual environments, grain iron concentration showed the highest genotypic variance in E_6 and grain zinc concentration in E_3 . Same trend was continued across environments, both the traits showed highly significant genotypic variances, whereas genotype \times year (σ^2gy) interaction for zinc was significant, but less in magnitude compared to genotypic variance. For iron, genotype \times year (σ^2gy) interaction was non-significant. Genotype \times location (σ^2gl) interactions were found to be non-significant for both the micronutrients and genotype \times year \times location (σ^2gyl) interactions were highly significant for both the traits and the magnitude of variances was more than genotypic variances.

Table 4.2. Genotypic variance(σ^2g), standard error (SE) and heritability in broad-sense (h^2) for traits in 296 B x PVK 801-derived RIL population at six different environments.

Trait	ICRISAT 12-13 (E ₁)			IIMR 12-13 (E ₂)			VNMKV 12-13 (E ₃)		
	σ^2g	SE	h^2	σ^2g	SE	h^2	σ^2g	SE	h^2
DTF	6.34**	0.60	0.85	14.46**	1.36	0.90	2.7**	0.26	0.83
PH	667.3**	55.41	0.97	658.21**	53.90	0.98	590.11**	47.86	0.99
TW	0.1**	0.01	0.83	0.15**	0.01	0.88	0.09**	0.01	0.95
Fe	15.51**	1.68	0.78	20.79**	2.17	0.81	27.44**	2.85	0.80
Zn	10**	1.12	0.74	12.72**	1.33	0.80	23.41**	2.43	0.80
GY (t/ha)	0.02**	0.00	0.90	0.014**	0.00	0.95	0.003**	0.00	0.94

Trait	ICRISAT 13-14 (E ₄)			IIMR 13-14 (E ₅)			VNMKV 13-14 (E ₆)		
	σ^2g	SE	h^2	σ^2g	SE	h^2	σ^2g	SE	h^2
DTF	10.48**	0.91	0.89	7.34**	0.75	0.81	7.97**	0.68	0.91
PH	407.21**	33.20	0.95	486.6**	39.51	0.97	618.32**	48.87	0.99
TW	0.12**	0.01	0.78	0.13**	0.01	0.78	0.14**	0.01	0.93
Fe	8.72**	1.05	0.68	10.84**	1.30	0.68	31.36**	3.23	0.77
Zn	5.48**	0.60	0.73	4.6**	0.68	0.56	12.71**	1.40	0.74
GY(t/ha)	0.013**	0.00	0.89	0.02**	0.00	0.93	0.027**	0.00	0.80

DTF = Days to 50% flowering, PH= Plant Height (cm), TW= 100 seed weight (g), Fe= Iron (mg kg⁻¹), Zn =Zinc (mg kg⁻¹) and GY= Grain Yield (t/ha)

*Significant at 5% level; ** Significant at 1% level

Table4.3. Genotypic variance(σ^2g), Genotype \times Year ($\sigma^2g \times y$), Genotype x Location($\sigma^2g \times l$) Genotype \times Year \times Location($\sigma^2g \times y \times l$) interactions, standard error (SE) and operational heritability's (h², broad-sense) for traits in 296 B \times PVK 801-derived RIL population

Trait	Pooled								
	σ^2g	SE	$\sigma^2g \times y$	SE	$\sigma^2g \times l$	SE	$\sigma^2g \times y \times l$	SE	h ²
DTF	4.33**	0.41	-0.115 NS	0.15	-0.016 NS	0.198	3.49**	0.279	0.86
PH	500.54**	40.08	5.74 NS	2.92	-0.03 NS	3.07	63.15**	4.35	0.97
TW	0.06**	0.01	0.00 NS	0.00	0.006 NS	0.00	0.06**	0.00	0.79
Fe	4.18**	0.69	-0.17NS	0.66	-0.7 NS	0.80	14.32**	1.18	0.58
Zn	4.17**	0.51	0.71**	0.35	-0.14 NS	0.37	5.22**	0.54	0.69
GY (t/ha)	0.22**	0.02	0.0043 NS	0.01	0.05**	0.017	0.3003*	0.212	0.73

DTF = Days to 50% flowering, PH= Plant Height (cm), TW= 100 seed weight (g), Fe= Iron (mg kg^{-1}), Zn =Zinc (mg kg^{-1}) and GY= Grain Yield (t/ha)

* Significant at 5% level; ** Significant at 1% level

The analysis of variances for all the traits revealed that genotypic variances were highly significant in individual environments as well as across environments (combined analysis) indicating high degree of genotypic variance for the traits studied. For agronomic traits, the genotype \times year \times location (σ^2_{gyl}) interactions values were significant but, lower than genotypic variances, suggesting that the traits are predominantly under genetic control and influenced by environments to a limited extent which implies there is no need for G \times E partitioning. Whereas, for both micronutrients, genotype \times year \times location (σ^2_{gyl}) interactions were significant and also higher than genetic variances indicating that environment played a significant role in the accumulation of micronutrients in grain. Assessment of the environmental stability of micronutrients is important in crop improvement program aimed at enhancing the nutritional quality of food crop plants (Oikeh *et al.*, 2004). The genotype \times year \times location (σ^2_{gyl}) interactions for iron were more in magnitude than those for zinc concentration, similar results were recorded by Prasanna *et al.* (2011) in maize, Gomez-Becerra *et al.* (2010) in wheat. Suwarto and Nasrullah (2011) found that the proportion of G \times E interaction was three times higher than genotypic variances for grain iron concentration across eight environments in rice. High G \times E interaction for grain iron and zinc concentrations, which affect the rank of genotypes across the environments have been reported in many cereal crops (Banziger and Long, 2000; Oikeh *et al.*, 2003; Oury *et al.*, 2006; Morgonuov *et al.*, 2007 and Gomez-Becerra *et al.*, 2010). The iron and zinc concentration in wheat grain depends largely on environmental conditions, particularly soil availability. Therefore iron and zinc concentration in grain show variation according to micronutrients concentration in soil and their availability to plants. Another reason for greater G \times E interaction for iron and zinc concentration could be their quantitative inheritance as reported in maize and rice (Gregorio, 2002 and Long *et al.*, 2004), though progress in the genetic analysis of these traits are expected to be slower than that of many traits. However, in spite of these challenges there is evidence that breeding for increased levels of micronutrient is feasible (Ortiz-Monasterio *et al.*, 2007).

4.1.4 Heritability of traits

Heritability estimates are always unique to the population under study, the growing conditions and the experimental design used. It is a good index of transmission of traits from parents to their offspring (Falconer, 1989). The estimation of heritability helps the breeders to make decisions for selection of elite genotypes. In the present study all the traits were highly heritable (>0.60) in individual environments except for grain zinc concentration in E₅ (Table 4.2) Agronomic traits like days to 50 % flowering, plant height,

100-seed weight and grain yield were found to be more heritable than grain micronutrients in all the environments. However, a partitioned genotype by environment interaction component reduced the heritability across the environments (pooled analysis). Broad sense heritability for all the traits was high (0.30 – 0.60) across six environments (Table 4.3). Broad sense heritability using pooled data ranged from 0.58 for grain iron concentration to 0.96 for plant height which was the most heritable trait in all the environments. For grain iron and zinc concentrations, the heritability was high in first year (postrainy 2012-13) compared to second year (postrainy 2013-14), whereas environment wise E₅ showed the lowest value for iron and zinc heritability. Similar range of heritability for grain micronutrient concentrations based on across season data was obtained by Gupta *et al.*, (2009) in pearl millet. Based on the knowledge of G × E variances and heritability of the traits in this study, there is good prospect for selection of grain iron and zinc rich sorghum genotype and for molecular mapping of these traits.

4.1.5 Association of grain iron and zinc concentration with agronomic traits

Knowledge of correlation among different plant traits is required to obtain the expected response of other traits when selection is applied to the trait of interest in a breeding program (Falconer 1989). Development of sorghum cultivars with high levels of grain iron and zinc concentration can make significant contribution to reducing widespread deficiencies of these micronutrients in populations heavily dependent on sorghum for their dietary energy and nutritional requirements. It is imperative that breeding of such cultivars must not compromise on grain yield and farmer-preferred traits. With this view, the present investigation was focused to examine the association of grain iron and zinc concentration with grain yield and other agronomic traits (days to 50% flowering, 100-seed weight and plant height) in RIL population of 296 B × PVK 801 in multiplications trials. Pearson's correlation for phenotypic correlation among traits were computed for each individual environment and across the environments based on BLUPs of each environment and pooled environments, respectively and results were summarized in tabular form (Table 4.4). Significant positive and negative correlations were observed between the traits. Some of the traits were highly correlated while many of them had weak correlations.

Table 4.4. Genotypic correlation coefficients between traits in RIL population derived from 296 B x PVK 801 in sorghum at all six individual environments and across six environments

E1 (ICRISAT 12-13)	DTF	PH	TW	Fe	Zn	GY(t/ha)
DTF	1					
PH	0.05	1				
TW	-0.21**	0.29**	1			
Fe	0.04	0.28**	0.28**	1		
Zn	-0.06	0.25**	0.3**	0.79**	1	
GY (t/ha)	-0.22**	-0.01	-0.13**	-0.31**	-0.26**	1

E2 (IIMR 12-13)	DTF	PH	TW	Fe	Zn	GY(t/ha)
DTF	1					
PH	0.05	1				
TW	0.077	0.32**	1			
Fe	-0.23**	0.002	0.06	1		
Zn	-0.1	0.12*	0.3**	0.69**	1	
GY (t/ha)	0.23**	0.46**	0.26**	-0.27**	-0.16**	1

E3 (VNMKV 12-13)	DTF	PH	TW	Fe	Zn	GY(t/ha)
DTF	1					
PH	0.03	1				
TW	-0.13*	0.11*	1			
Fe	0.01	0.29**	-0.014	1		
Zn	-0.02	0.3**	-0.023	0.70**	1	
GY (t/ha)	-0.01	0.16**	0.045	-0.21**	-0.29**	1

E4 (ICRISAT 13-14)	DTF	PH	TW	Fe	Zn	GY(t/ha)
DTF	1					
PH	0.17**	1				
TW	-0.13*	0.25**	1			
Fe	-0.139*	0.08	0.2**	1		
Zn	-0.11*	0.22**	0.4**	0.77**	1	
GY (t/ha)	0.13*	0.28**	-0.12*	-0.28**	-0.23**	1

E5 (IIMR13-14)	DTF	PH	TW	Fe	Zn	GY(t/ha)
DTF	1					
PH	0.13*	1				
TW	-0.003	0.36**	1			
Fe	-0.077	0.06	0.06	1		
Zn	-0.11*	0.03*	0.02	0.68**	1	
GY (t/ha)	0.12*	0.34**	-0.01	0.06	0.06	1

E6 (VNMKV 13-14)	DTF	PH	TW	Fe	Zn	GY(t/ha)
DTF	1					
PH	0.172**	1				
TW	-0.132*	0.25**	1			
Fe	-0.139*	0.08	0.2**	1		
Zn	-0.11*	0.22**	0.4**	0.72**	1	
GY (t/ha)	0.13*	0.28**	-0.12*	-0.28**	-0.23**	1

pooled 6 environment	DTF	PH	TW	Fe	Zn	GY(t/ha)
DTF	1					
PH	0.12*	1				
TW	-0.12*	0.35**	1			
Fe	-0.12*	0.31**	0.34**	1		
Zn	-0.12*	0.33**	0.36**	0.79**	1	
GY (t/ha)	0.19**	0.31**	-0.12*	-0.34**	-0.34**	1

DTF = Days to 50% flowering, PH= Plant Height (cm), TW= 100 seed weight (g), Fe= Iron (mg kg^{-1}), Zn =Zinc (mg kg^{-1}) and GY= Grain Yield (t/ha)

* Significant at 5% level; ** Significant at 1% level

Based on BLUPs the individual environments, highly significant and high positive association between grain iron and zinc concentration in all the environments was observed ($E_1 = 0.79$, $E_2 = 0.69$, $E_3 = 0.70$, $E_4 = 0.77$, $E_5 = 0.68$ and $E_6 = 0.72$; $p < 0.01$) and this trend was consistent in pooled analysis (across environment = 0.79; $p < 0.01$) (Fig.4.3). Similar relationships between these micronutrients have been reported in earlier studies on sorghum (Ashok Kumar *et al.*, 2010 and 2013; Reddy *et al.*, 2005 and 2010 and Nguni, 2012) and in other cereals, such as pearl millet (Velu *et al.*, 2007, 2008a and 2008b; Gupta *et al.*, 2009; Rai *et al.*, 2012, 2013 and 2014; Govindaraj *et al.*, 2013 and Kanatti *et al.*, 2014), maize (Oikeh *et al.*, 2003 and 2004), rice (Stangoulis *et al.*, 2007 and Anandan *et al.*, 2011), wheat (Garvin *et al.*, 2006; Peleg *et al.*, 2009; Zhang *et al.*, 2010 and Velu *et al.*, 2011a), and finger millet (Upadhyaya *et al.*, 2011). These positive associations between iron and zinc densities may likely due to common and overlapping Quantitative Trait Loci (QTL) as reported in wheat (Peleg *et al.*, 2009 and Singh *et al.*, 2010), rice (Stangoulis *et al.*, 2007), common bean (Cichy *et al.*, 2009 and Blair *et al.*, 2009) and pearl millet (Kumar, 2011) implying that simultaneous selection for both micronutrients is likely to be highly effective. This may point to common molecular mechanism controlling the uptake and metabolism of these minerals in grains or common transporters controlling for the minerals (Vreugdenhil *et al.*, 2004 and Ghandilyan *et al.*, 2006). Co-segregation of genes controlling these traits might be the reason for strong association between the minerals. The direction and intensity of association suggested a good possibility of simultaneous genetic improvement of both the micronutrients (Velu *et al.*, 2008a) by co-transferring these traits into the genetic background of elite lines.

Based on BLUPs in the individual environment iron density had significant negative, albeit low, correlation with grain yield in all the environments ($E_1 = -0.31$, $E_2 = -0.27$, $E_3 = -0.21$, $E_4 = -0.28$ and $E_6 = -0.23$; $p < 0.01$) except ($E_5 = 0.06$; non-significant), similar results was obtained for pooled BLUPs across the environments ($AE = -0.34$; $p < 0.01$). The correlation between grain zinc density and grain yield was also significant and negative, except in one environment ($E_5 = 0.06$, NS) but was smaller in magnitude compared to the association between grain iron concentration and grain yield except in one environment (E_3). The associations of grain iron and zinc concentration with grain yield were found to be of same magnitude in across environment analysis. Such patterns of relationships of grain iron and zinc concentration with grain yield are not unexpected considering the high positive correlation between iron and zinc concentration and larger $G \times E$ interaction effect relative to genotypic effect.

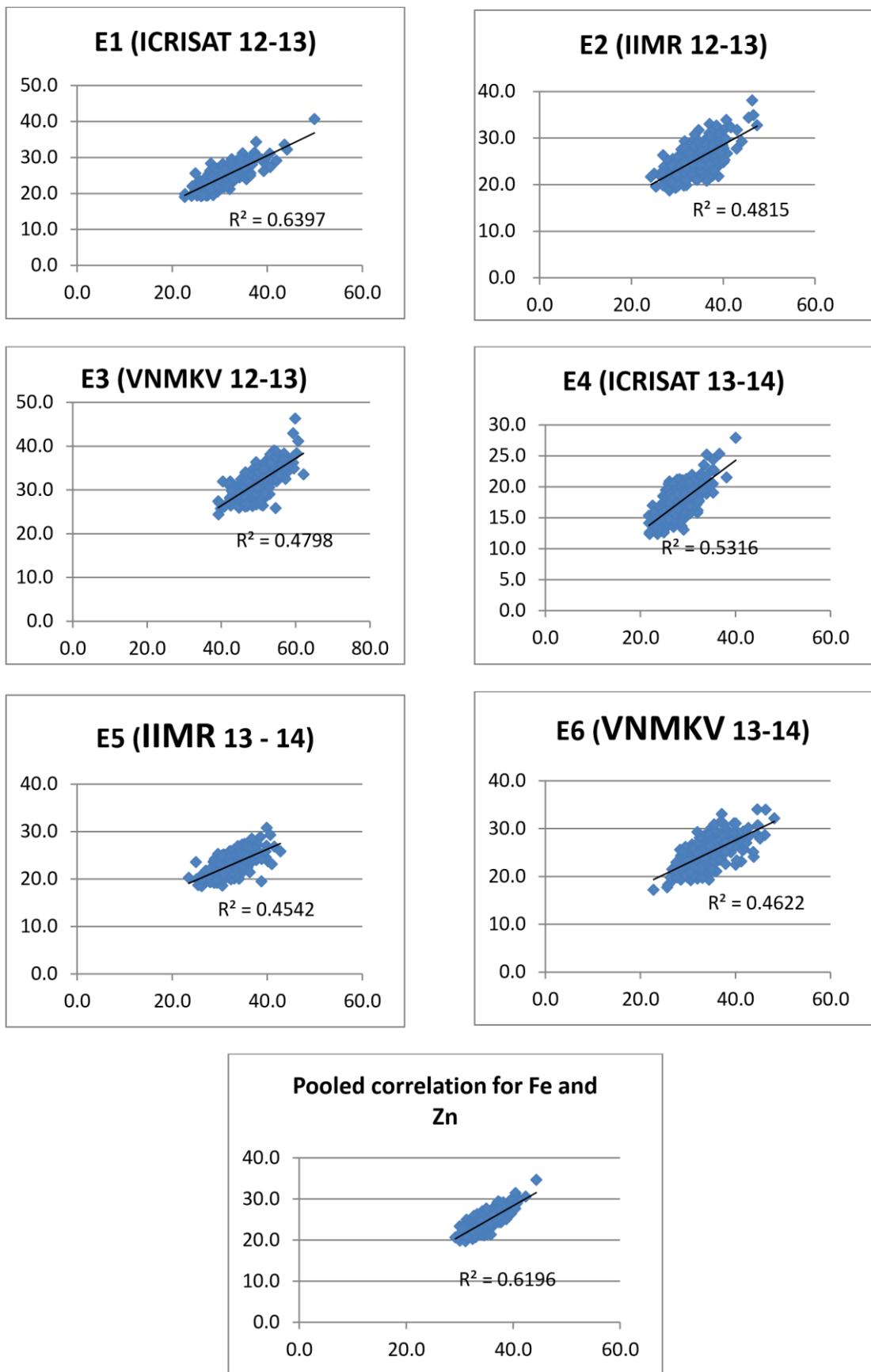


Fig 4.3. Association between grain iron and zinc densities in all six Individual environments and pooled over all environment

Earlier studies in sorghum (Reddy *et al.*, 2005), pearl millet (Rai *et al.*, 2012), wheat (Garvin *et al.*, 2006; Morgounov *et al.* 2007; Shi *et al.*, 2008 and Zhao *et al.*, 2009) and Maize (Banziger and Long, 2000) also revealed significant negative relationship between these micronutrients and grain yield. Though statistically significant (negative) a rather weaker correlation of grain iron concentration ($E_3 = -0.21$ to $E_1 = -0.31$) and grain zinc concentration ($E_2 = -0.16$ to $E_1 = -0.26$) with grain yield indicates the possibility of breeding for high grain iron and zinc concentration in high yielding backgrounds. This could call for the application of genetic tools for selective introgression of only those genes and genomic regions by using marker assisted selection in to the parental lines with high yielding background.

Based on BLUPs performance across the locations *viz.*, ICRISAT, IIMR and VNMKV Parbhani the difference in the location for two seasons was reflected for grain Fe concentration. The correlation for grain Fe ($r=0.47$) was lower in magnitude indicating low consistency in ranking of RILs across the three locations. Whereas, the correlation for grain Zn ($r=0.77$) indicated high level of consistency in the ranking of RIL across the locations. The differential ranking of RILs for grain Fe concentration across the locations necessitated the G \times E and stability analysis for developing Fe rich cultivars in sorghum.

The trait 100 seed weight, out of six environments showed significant positive association with grain iron in only three environments ($E_1=0.28$, $E_4=0.2$ and $E_6 = 0.2$; $p<0.01$), while in the remaining three environment iron density did not show any significant association with 100 seed weight. Whereas, in across environment (AE) analysis, iron density showed significant positive ($AE= 0.34$; $p<0.01$) association with 100 seed weight. In case of grain zinc density four locations ($E_1=0.3$, $E_2=0.3$, $E_4=0.4$ and $E_6=0.4$; $p<0.01$) exhibited significant positive association and remaining two environment (E_3 and E_5) showed non-significant association, whereas in across environment (AE) analysis zinc concentration showed significant positive ($AE= 0.36$; $p<0.01$) association with 100 seed weight. This non-significant or significant positive association of 100-seed weight with both micronutrients implies that higher grain iron and zinc densities are not necessarily related to small grain size, and in general, hybrids with higher micronutrient (iron and zinc) concentration also had greater grain weight. Thus, the enhancement of micronutrients would not affect 100-seed weight of the genotypes. This relationship could be more advantageous for selecting simultaneously or independently for the combination of bold grain and high micronutrient traits during genetic improvement program. Similar non-significant association in sorghum between these two micronutrients and 100 seed weight was found by Reddy *et al.* (2005) and Ashok Kumar *et al.*(2010), whereas significant

negative but lower magnitude of correlation of grain iron and zinc contents with 100 seed weight was found by Reddy *et al.*(2010) in 1394 core germplasm lines, while in pearl millet (Velu *et al.*, 2007, 2008a and 2008b) found significant positive association between grain micronutrient concentration and 100 seed weight.

The association between grain iron concentration and days to 50 % flowering ranged from $E_2 = -0.23$; $p < 0.01$ to $E_1 = 0.04$ and non-significant), while across the environments, significant negative ($AE = -0.12$; $p < 0.05$) association was observed between grain iron concentration and days to 50 % flowering. The association of zinc concentration with days to 50 % flowering was ranged from $E_4 = E_5 = E_6 = -0.11$; $p < 0.05$ to $E_1 = -0.06$; non-significant, while across the environments, it showed significant negative ($AE = -0.12$; $p < 0.05$) association between grain zinc concentration and days to 50 % flowering. Reddy *et al.* (2010) found that both the micronutrients had significant positive association with days to 50% flowering in sorghum, and most studies did not show significant association between micronutrients and days to 50 % flowering in sorghum (Reddy *et al.*, 2005 and Ashok Kumar *et al.*, 2010), pearl millet (Velu *et al.*, 2007 and 2008b., Gupta *et al.*, 2009 and Rai *et al.*, 2012) and wheat (Morgounov *et al.*, 2007).

The association between plant height and grain iron concentration varied from ($E_2 = 0.002$) and non-significant to $E_3 = 0.29$; $p < 0.01$), while across environment it had significant positive ($AE = 0.31$; $p < 0.01$) association. In case of grain zinc concentration, the correlation with plant height ranged from ($E_5 = 0.03$; $p < 0.05$ to $E_3 = 0.30$; $p < 0.01$), while across environments it recorded significant and positive ($AE = 0.33$; $p < 0.01$). It suggested that association between grain micronutrient and plant height was positive with lower magnitude. Earlier studies reported significant positive association between iron concentration and plant height in sorghum (Reddy *et al.*, 2010) and wheat (Hussain *et al.*, 2012) as well as between zinc concentration and plant height in sorghum (Reddy *et al.*, 2005 and Reddy *et al.*, 2010) and wheat (Zhao *et al.*, 2009) contrary to this, some earlier reports also indicated that both the micronutrients had significant negative association with plant height in wheat (Margounov *et al.*, 2007) and non-significant association with plant height in sorghum (Ashok Kumar *et al.*, 2010).

Though the correlations of grain iron and zinc concentration with days to 50 % flowering and plant height were significantly associated in negative and positive direction respectively, but the lower magnitude of correlation suggests near-independence of crop growth traits and grain micronutrient traits. The results indicate that sorghum grain iron and zinc concentration can be improved in different maturity and plant stature backgrounds.

Based on BLUPs performance across the environments, grain yield had significant positive association with days to 50 % flowering ($AE=0.19$; $p<0.01$) and plant height ($AE=0.31$; $p<0.01$), this trend was largely consistent in individual environment also, except E_1 for plant height and E_1 and E_3 for days to 50% flowering. The 100 seed weight (TW) showed significant positive association with plant height ($AE= 0.35$; $p<0.01$) same trend was consistent in individual environments also, but 100 seed weight exhibited significant negative ($AE= -0.12$; $p<0.05$), albeit weak, correlation with grain yield across the environments.

4.1.6 Principal Component analysis

Based on BLUPs performance over the six environments principal component analysis (PCA) was performed to understand how agronomic and grain micronutrient traits contributed to variability among RIL population (Fig.4.4). The first two principal components (PCs) accounting more than 50 % of total variance *i.e.* 63.6%. The PC1 represented 39.61% and PC2 was 23.99%.The distribution of traits based on PC1 and PC2 showed the phenotypic variation and how widely were dispersed they are along axes. The grain iron and zinc concentration formed one group (G1) grain yield and days to 50 % flowering formed another group (G2) while, plant height and 100 seed weight formed separate group (G3) and these traits had significant positive association among themselves. Both gain iron and zinc concentration had significant positive association with 100 seed weight (TW). Grain yield and days to 50 % flowering showed low to moderate negative association with both the micronutrients.

From this study, it could be suggested that, cultivars with high grain iron and zinc concentration can be developed without compromising on 100 seed weight, while selecting for high grain iron and zinc concentration with high grain yield, large number of parental combinations or large segregating population has to be screened than that for yield alone.

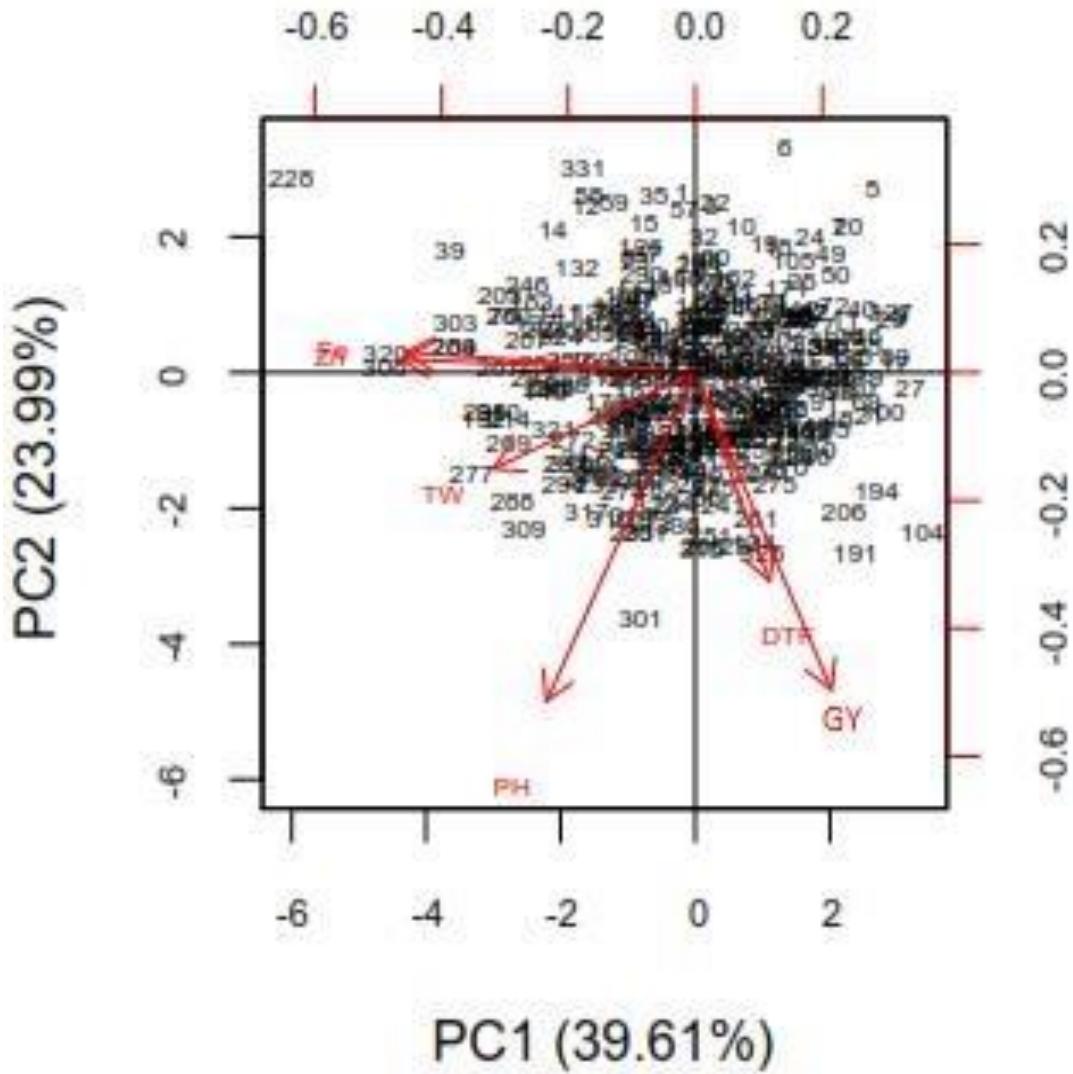


Fig 4.4. Principal component analysis for Grain Iron (Fe) and Zinc (Zn) densities, Days to 50%Flowering (DTF), Plant height (PH), 100 seed weight (TW) and Grain yield (GY) in set of RIL population over six environments BLUPs.

4.2 MOLECULAR ANALYSIS

The pioneer work of Nilsson-Ehle (1909) more than 100 years ago gave an idea that continuous variation in trait performance is due to the joint segregation of several genes and interaction of environment and all of genes with a small but quasi-additive effect together produce a phenotype. The genes responsible for such traits were originally called polygenes by Mather (1941), but are now generally referred to as QTL (Gelderman, 1975). Till the birth of molecular markers in late 1980s (Lander and Botstein, 1989), the determination of number, position and arrangement of genomic region/QTL controlling a polygenic trait and their effects with interaction was difficult to findout. QTL can only be mapped by following their co-segregation with molecular markers which are responsible for much of the progress in the area of polygenic controlled expression of traits over recent year. But with the advent of marker system to construct the linkage map using segregating population with powerful biometric methods has led to considerable progress in QTL mapping in plants as well as other animals.

Compared to better-studied cereals such as rice, wheat, maize and barley there has been relatively little research on the development and application of molecular genetic maps of sorghum (Boora *et al.*, 1998; Subudhi *et al.*, 2000; Tao *et al.*, 2000; Xu *et al.*, 2000; Klein *et al.*, 2001b; Mehta, 2002; Haussmann *et al.*, 2003, 2004; Harris *et al.*, 2007b; Knoll and Ejacta, 2008). Therefore, in the present study SSR, DArT and SNP markers were used to construct linkage maps on RIL population and QTLs for studied traits were mapped. The results of molecular marker analysis are discussed here.

4.2.1 Parental polymorphism

A total of 271 SSR markers (94 Xtxp, 76 Xisep, 62 Xiabtp, 12 XmbCIR, 11 Xgap, 10 Xcup and 6 Xgpsb) were used for polymorphism survey of 2 parental lines. A total 45 (16.60%) SSRs, detected polymorphism between 296 B and PVK 801 RIL parental lines, (table 4.5). A set of 6126 (70.15%) polymorphic DArT clones were identified in total of 8732 clones. Whereas, 3331(91.51%) polymorphic SNP clones were identified in total of 3640 clones on the array of 296 B and PVK 801. The large number of available DArT and SNP markers, their cost-effectiveness and relatively high polymorphism content are ideal characteristics for their application in extensive genome-wide screening for QTL discovery, recurrent parent background recovery in marker-assisted backcrossing, isolation of genes via map-based cloning, comparative mapping and genome organization

studies (Varshney *et al.*, 2007). Marker-assisted breeding is generally more efficient when molecular maps are well saturated, due to an increased chance of finding polymorphic markers in any genetic background in any genomic region of interest. While, in the course of genotyping with SSR markers we could detect only 28 SSR markers showing good amplification and scorable polymorphism in RIL population (Fig 4.5.)

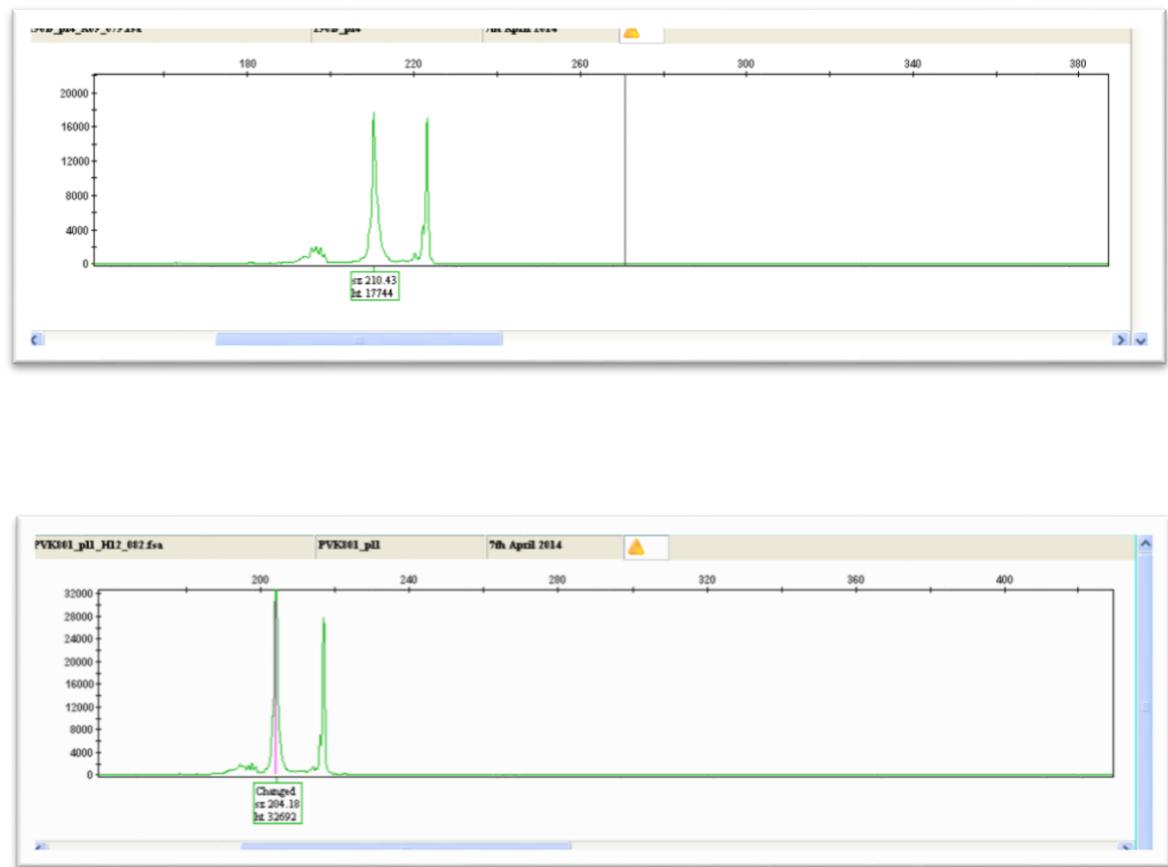


Fig. 4.5. Gene Mapper profile of amplified SSR marker showing polymorphism between two parents.

Table 4.5. List of polymorphic SSR markers between two parental lines (296B × PVK801)

Sr no	Marker name	Chromosome no	position	Marker size
1	Xtxp302	1	9.0	179
2	Xiabtp450	1	50.7	224
3	XmbCIR286	1	57.4	129
4	Xtxp357	1	23.8	272
5	Xgap342	1	1.3	187
6	XmbCIR286	1	57.4	116
7	Xiabtp278	1	6.5	227
8	Xisep0841	2	70.9	215
9	Xisep0733	2	70.7	330
10	Xcup63	2	59.1	145
11	Xtxp013	2	55.9	119
12	Xtxp304	2	5.7	302
13	Xtxp461	3	17.0	211
14	Xtxp336	3	55.4	166
15	Xisep824	3	73.9	196
16	Xtxp266	3	2.8	196
17	Xisep0210	4	2.0	187
18	Xtxp327	4	59.3	156
19	Xisep0202	4	4.7	184
20	Xiabtp 481	4	8.2	436
21	Xgap 121	4	0.9	219
22	Xcup28	4	51.2	163
23	Xtxp303	5	5.7	158
24	Xtxp23	5	54.5	182
25	Xtxp015	5	42.0	214
26	XmbCIR248	5	4.7	90
27	Xisep0443	6	55.7	188
28	Xiabtp230	6	59.4	251
29	Xisep0831	7	62.9	199
30	Xtxp312	7	4.6	191
31	XmbCIR 300	7	58.3	109
32	XmbCIR 246	7	56.3	100
33	Xiabtp360	7	55.9	99
34	Xtxp525	7	2.3	211
35	Xgap034	8	54.6	203
36	Xiabtp128	8	40.3	176
37	Xiabtp160	8	0.2	202
38	Xtxp010	9	47.9	144
39	Xcup02	9	8.1	197
40	Xgap206	9	59.2	125
41	Xiabtp417	9	2.3	231
42	Xisep0621	10	46.5	208
43	Xtxp320	10	55.4	289
44	Xcup42	10	1.2	139
45	Xisep0603	10	8.9	241

4.2.2 Linkage Map Construction

A genetic map or linkage map, an essential tool for QTL studies, is a map of the frequencies of recombination that occur between markers on homologous chromosomes during meiosis. Being a path to link a genetic region to a trait of interest it is also an important resource for fine mapping and cloning of genes. The present study sets out to construct genetic linkage maps in RIL population. Therefore, it was important to develop a reliable map from a large segregating population that provided good genome coverage. The published genetic maps in sorghum have in general been created with a high amount of RFLP and SSR markers. However, the linkage map constructed in present investigation was generated with SSRs, DArTs and SNPs which span 1356 cM accommodating 2088 markers. Most of the SSRs in present study are published which made current study easy because of the ease in use and their reproducibility. In general, different map length distances as reported previously in sorghum was a result of the variation in recombination frequencies of different population structures, number of mapped markers, population generation and population size.

Out of the 28 polymorphic SSR markers we could map only (5 Xtxp, 7 Xiabtp and 1 Xisep) 13 SSRs on 309 selected RILs. Rest of the polymorphic markers could not be mapped due to certain problem like dominant inheritance, lack of linkage, very high distortion towards parents. On the other hand, out of 6126 polymorphic DArT only 1184 DArT clones were used as many clones showed distortion towards one parent and having more than 10% missing (non-parental) data points. Similarly out of 3331 SNPs only 950 SNPs were used for linkage mapping as high distortion towards one parent and having more than 10% missing data points. All DArT and SNPs showing segregation distortion were not used for mapping. Hence the results showed out of 2158 total markers used for mapping only 2088 (96.7%) markers assigned in 10 linkage groups with a LOD score 10.0 to construct the genetic linkage map (Fig. 4.6). The assigned markers were 13 SSR, 1148 DArT and 927SNPs (Table 4.6).

The total length of map was 1356 cM (Kosambi), which represent on an average one marker for every 0.64 cM, taking in to consideration of genome size of sorghum (730 Mbp) the current map span 538.34 kb per cM. The individual LGs ranged from 180.6 cM for LG 3 with total number of 257 markers to 102.06 cM for LG 10 with lowest number of markers (79 markers). The linkage map constructed in this study was more highly

saturated, include more markers and have smaller marker intervals than any previously constructed map with RFLPs and/or SSRs. The genome coverage achieved makes the present maps particularly useful to select markers for use in whole genome breeding strategies and to saturate genomic regions of interest in other mapping populations. The maps showed a high level of genome coverage and distribution of markers was reasonably uniform including the distal regions of all chromosome arms (Fig. 4.5) this resulted largely from the inclusion of DArT and SNP. These markers typically show improved genome coverage compared to anonymous (non-coding) SSRs or AFLPs which are characteristically clustered around the centromeric regions (Ramsay *et al.*, 2000).

Table 4.6. Linkage group wise summary of the markers in 296 B × PVK 801 genetic linkage map

Linkage Group	Markers				Map Length (cM)	Avg. Marker interval (cM)
	DArT	SNP	SSR	Total		
1	138	121	2	261	133.11	0.51
2	104	141	Nil	245	157.74	0.64
3	92	165	Nil	257	180.60	0.70
4	157	Nil	Nil	157	125.29	0.80
5	155	82	3	240	122.50	0.51
6	139	122	2	263	124.87	0.47
7	111	81	2	194	130.78	0.67
8	88	63	1	152	136.47	0.90
9	122	116	2	240	142.10	0.59
10	42	36	1	79	102.06	1.29
Total	1148	927	13	2088	1355.52	

The average linkage group length was 135.5 cM with an average of 208.7 loci. The average adjacent marker interval length ranged from 0.47 (LG 6) to 1.29 cM (LG 10) followed by 0.90 cM (LG 8) and 0.0023 % of the intervals (5 out of 2087) were more than 5 cM (Fig 4.5). Linkage groups were named according to Kim *et al.* (2005) who developed a karyotypic map based on FISH (Fluorescent *In Situ* Hybridization) technique where short arm of the chromosome are placed at the top and long arm at the bottom of linkage groups and named as SBI-01, -02, -03, -04, -05 -06, -07, -08, -09 and -10 corresponding to LG A, B, C, D, J, I, E, H, F and G of Menz *et al.* (2002), respectively.

The details of 10 linkage groups is as follows

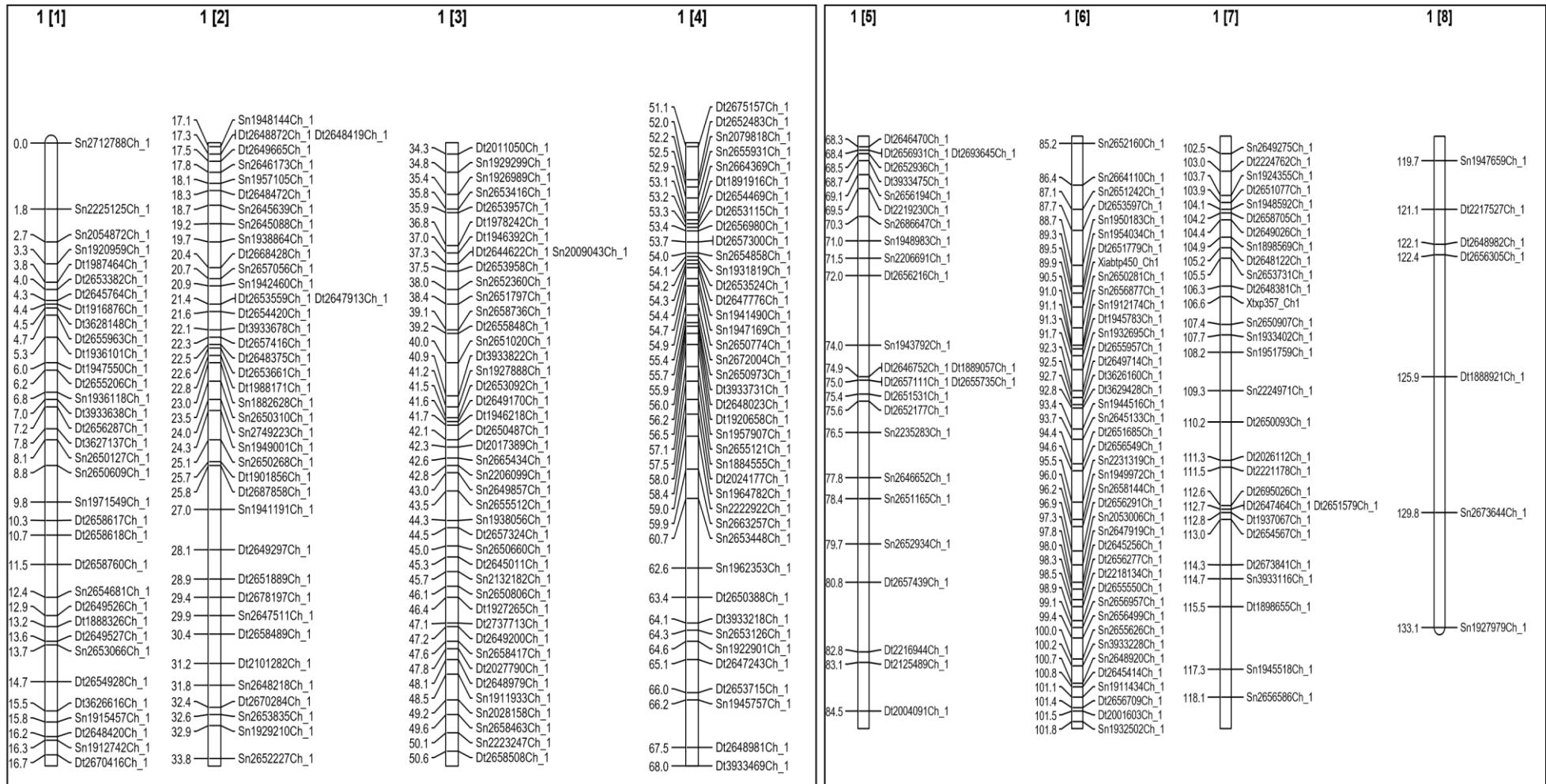


Fig 4.6 (a). Linkage group 1

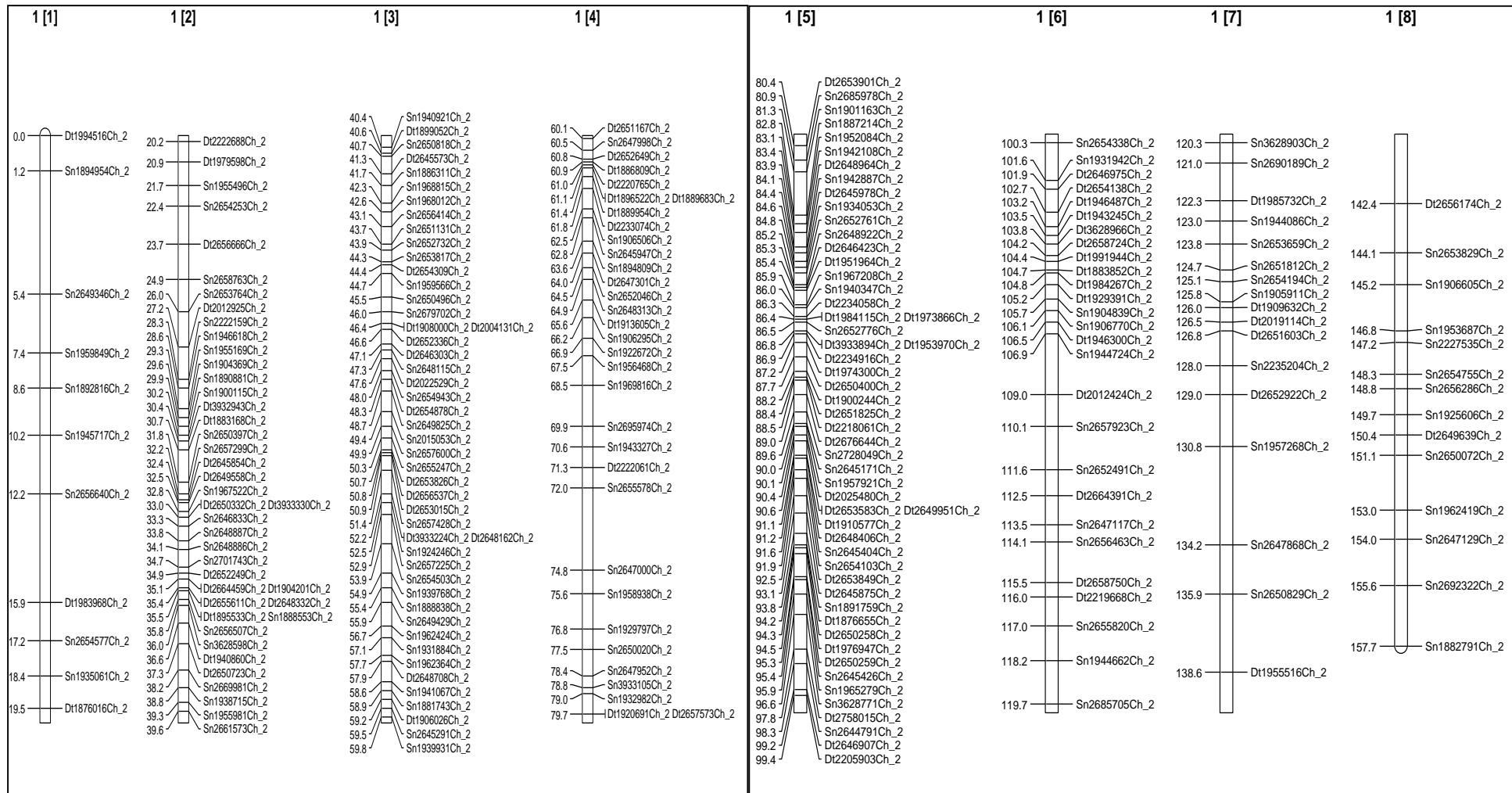


Fig 4.6(b). Linkage group 2

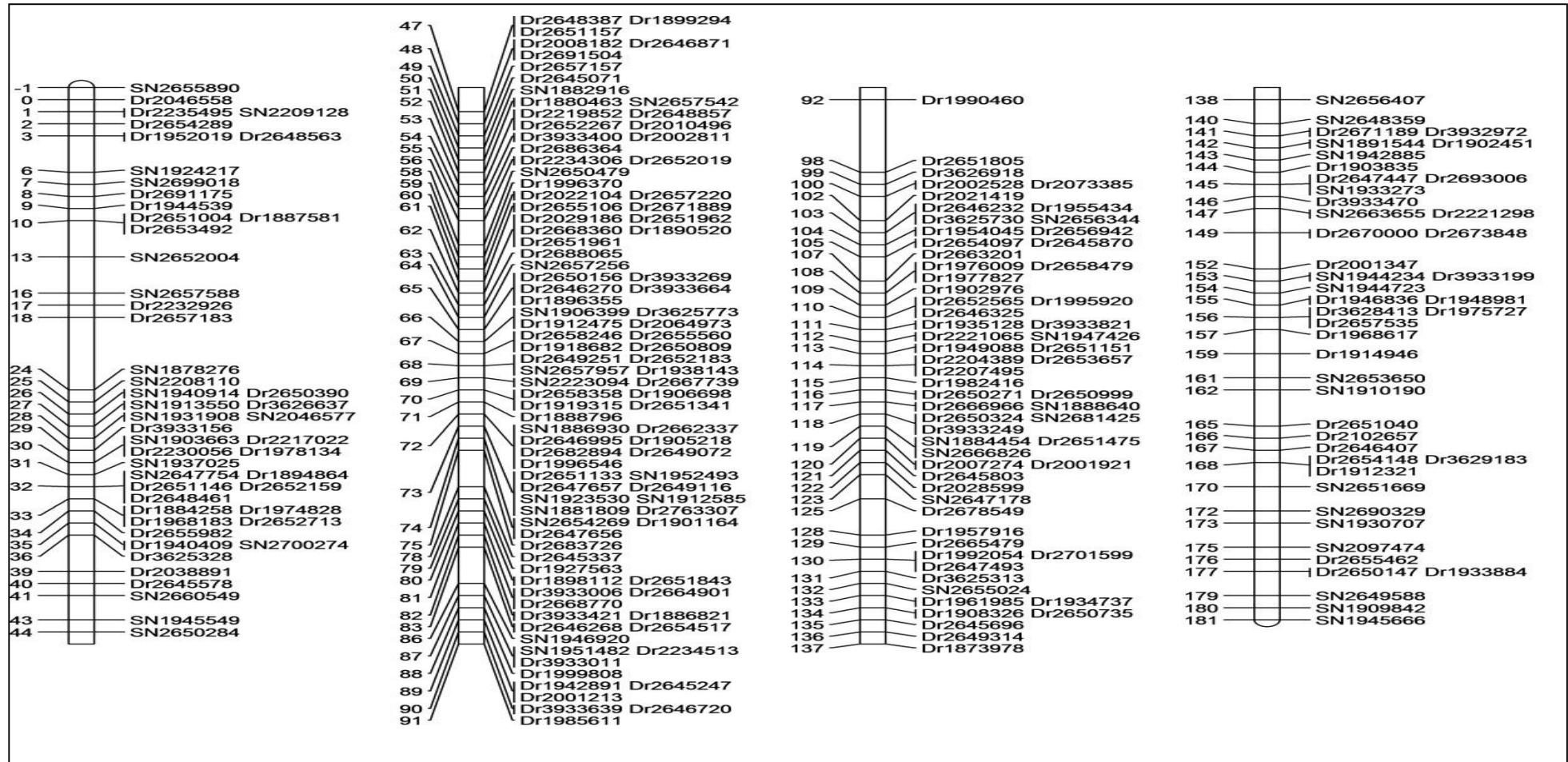


Fig 4.6(c). Linkage group 3

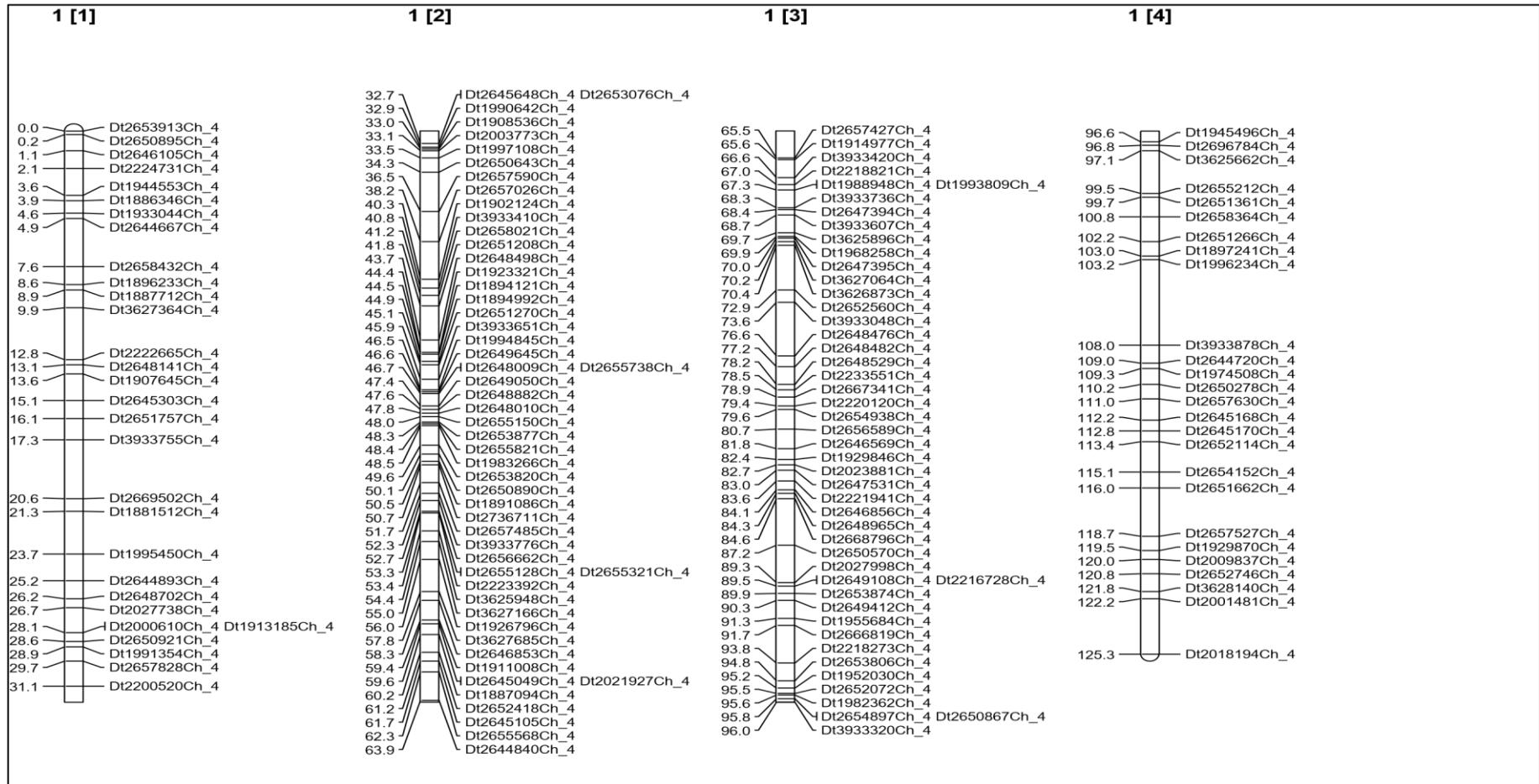


Fig 4.6(d): Linkage group 4

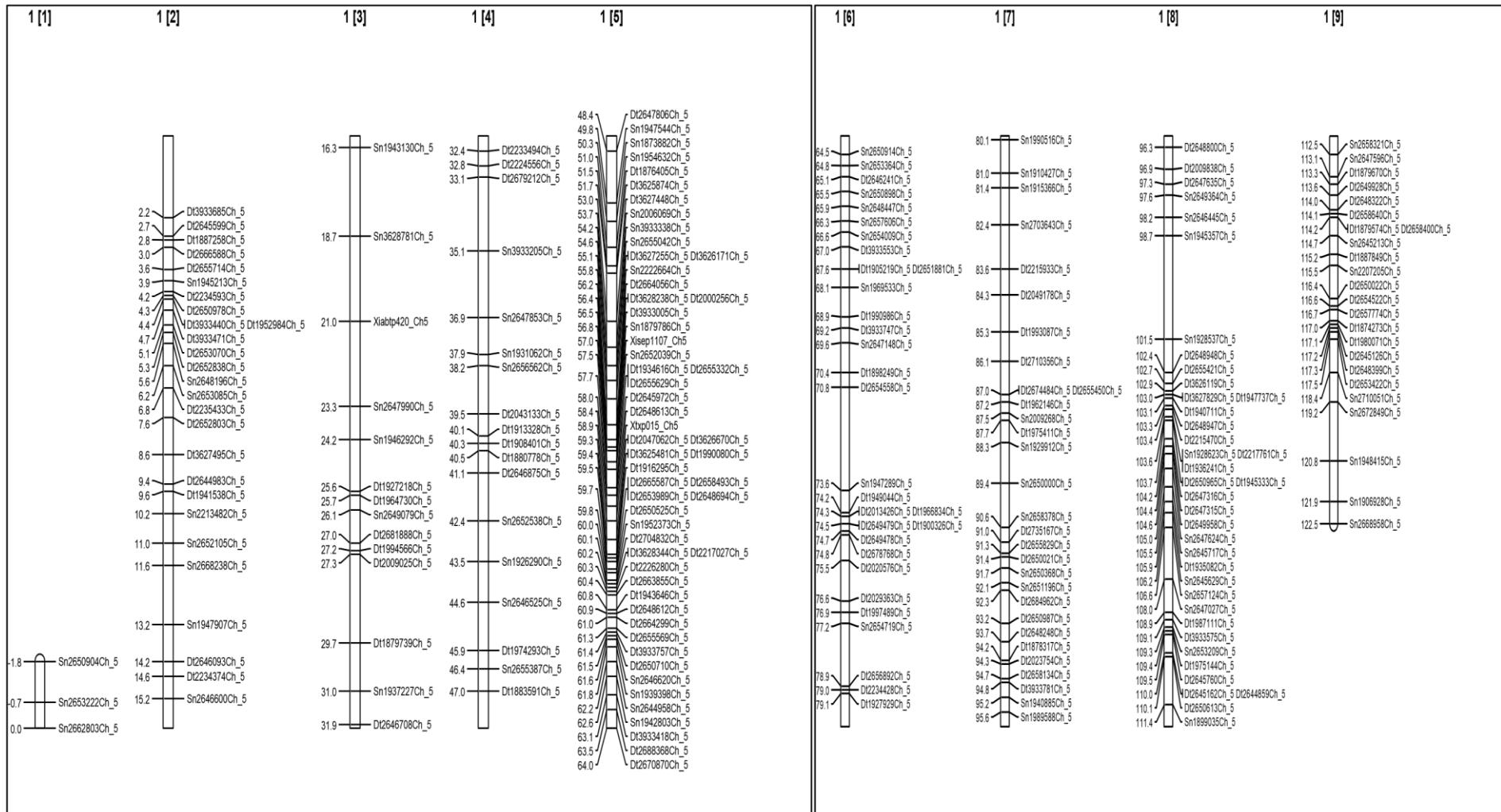


Fig 4.6(e): Linkage group 5

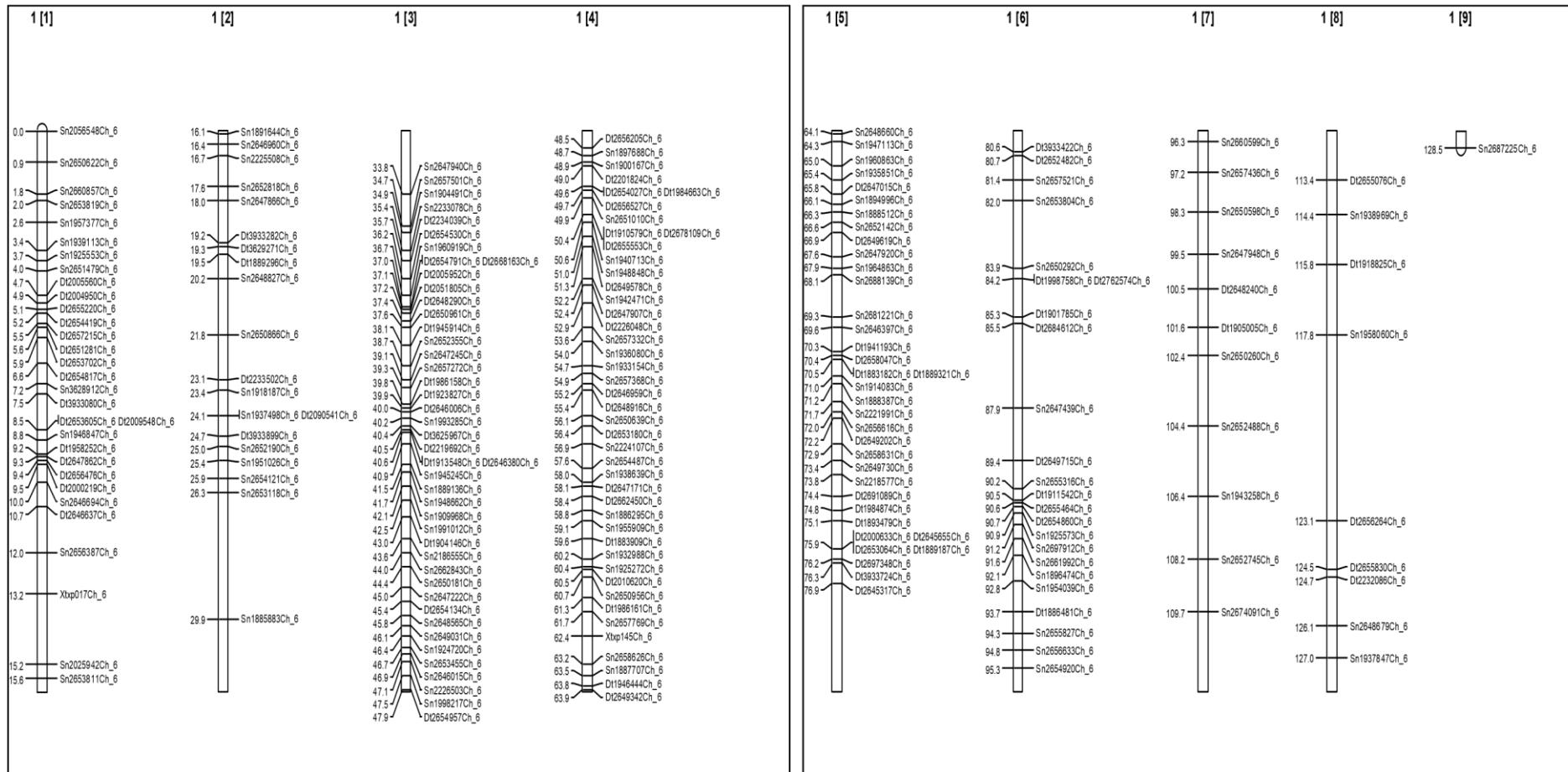


Fig 4.6(f): Linkage group 6

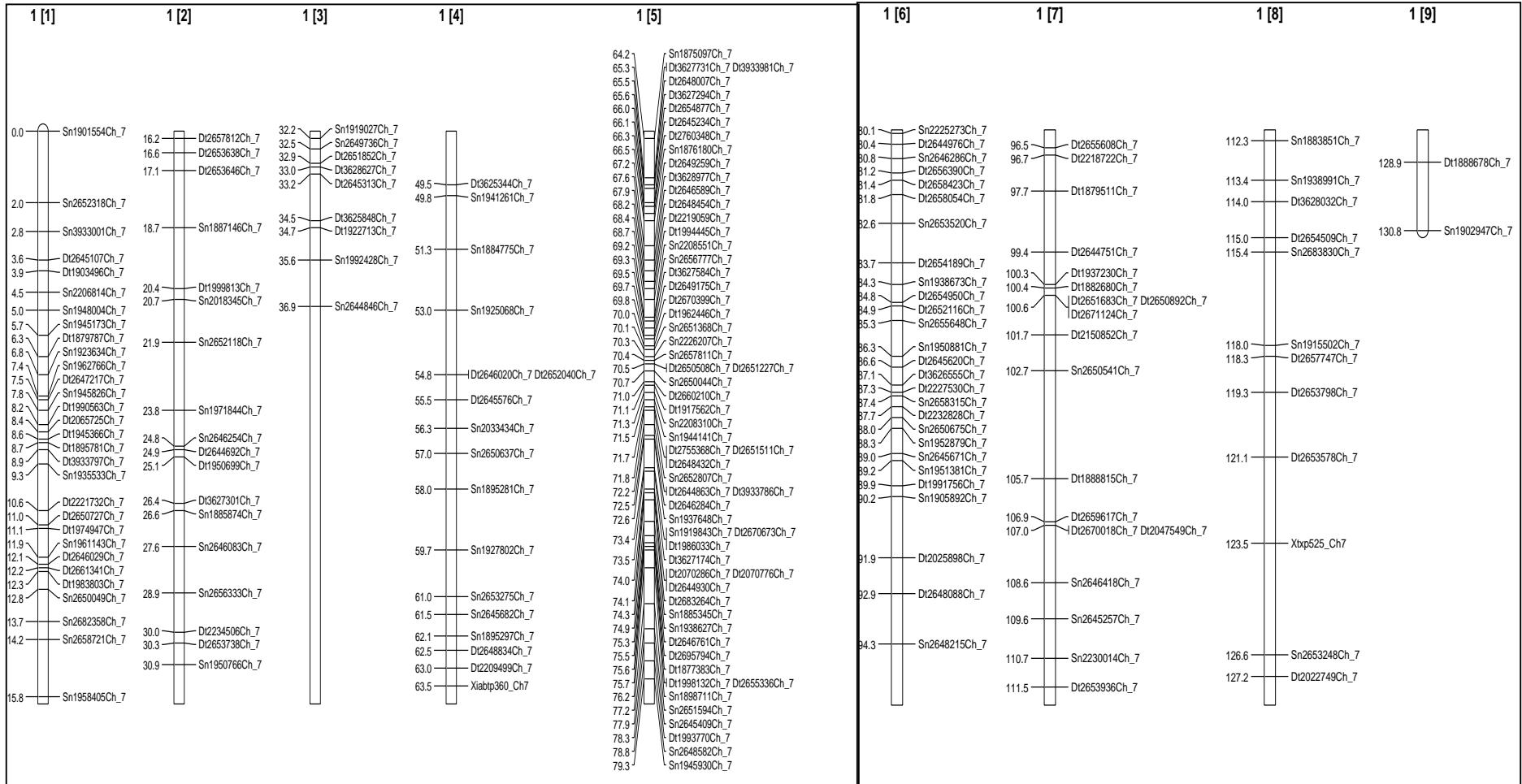


Fig. 4.6(g). Linkage group 7

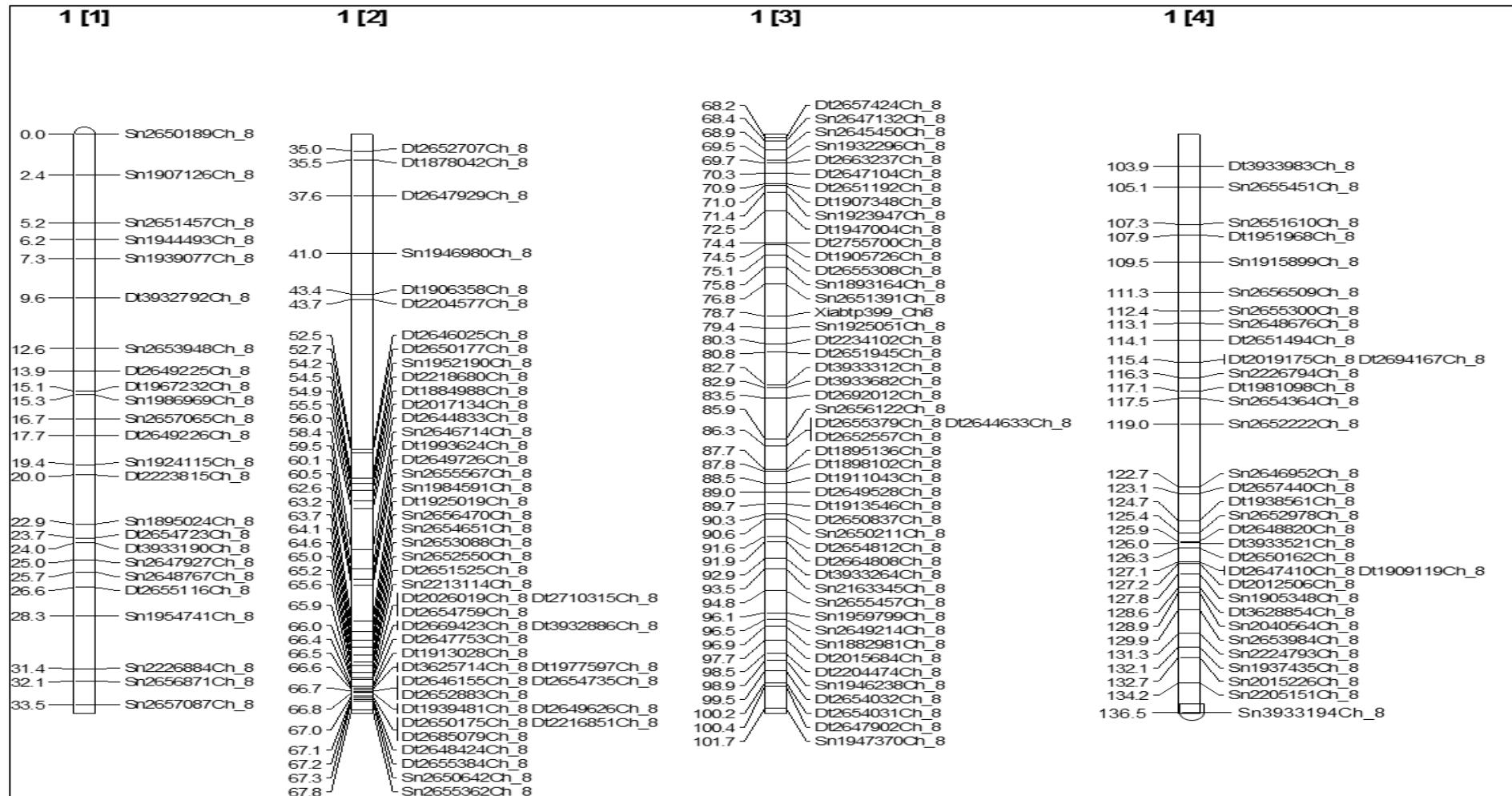


Fig 4.6(h). Linkage group 8

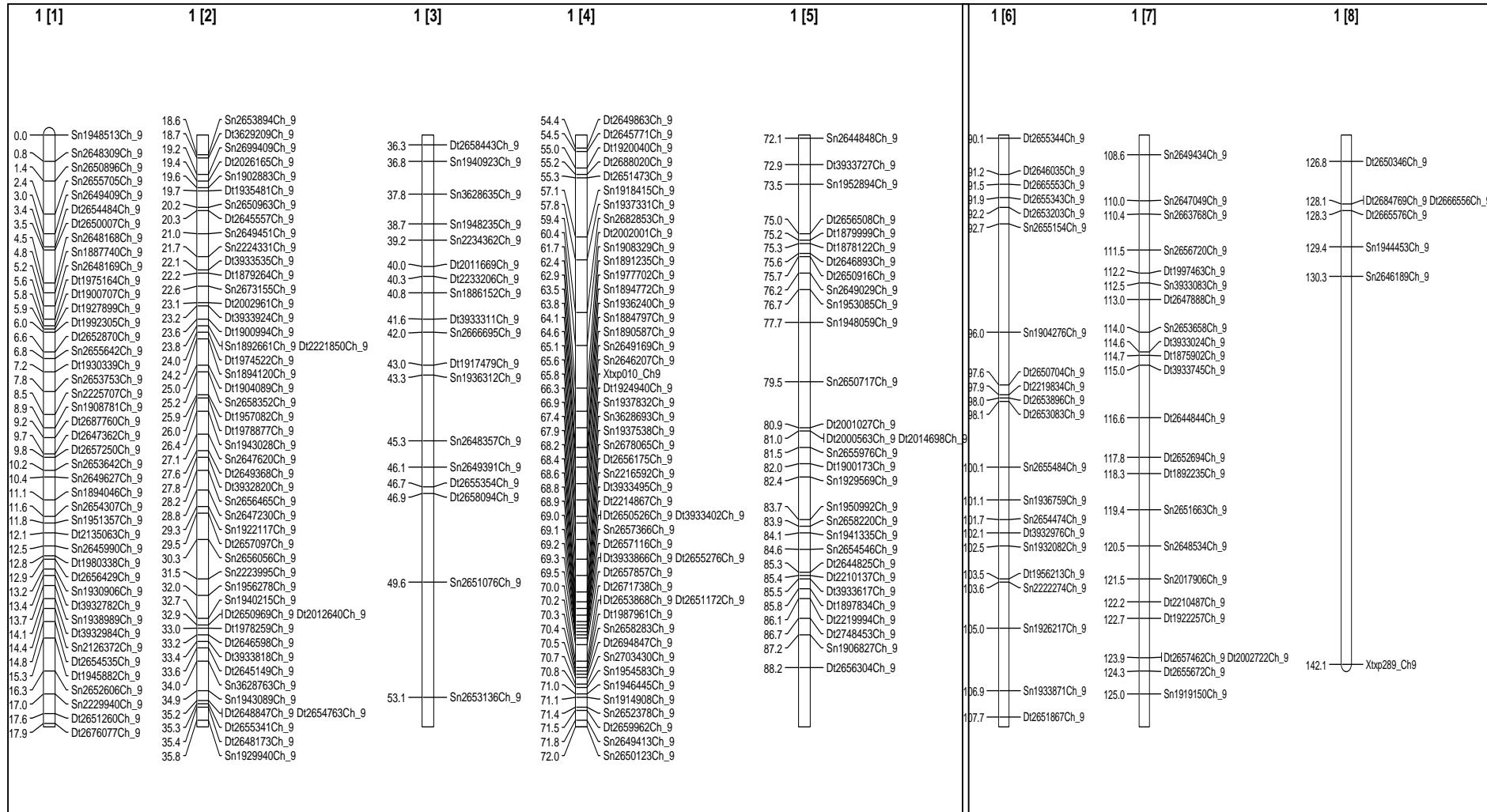


Fig.4.6(i). Linkage group 9

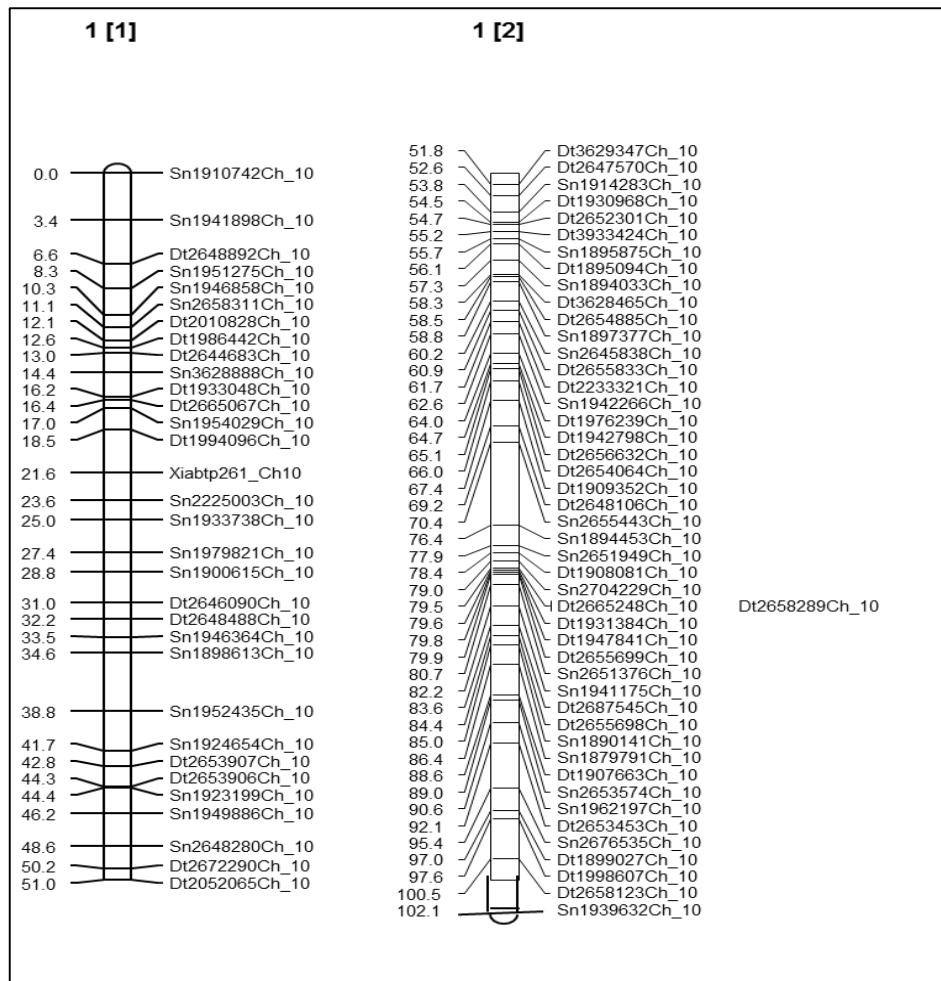


Fig.4.6(j). Linkage group 10

SBI-01

The map length of LG1 was 133.11 cM which accommodates 261 markers. Map distance between 2 consecutive markers varied from 0.001 to 3.93 cM, with an average adjacent marker interval length of 0.51 cM.

SBI-02

The map length of LG2 was 157.74 cM and it was second largest group for this RIL population had 245 markers. Map distance between 2 consecutive markers varied from 0.006 to 4.158 cM, with an average adjacent marker interval length of 0.64 cM.

SBI-03

LG 3 accommodate 257 markers with largest map length of 180.6 cM. Map distance between 2 consecutive markers varied from 0.001 to 4.695 cM, with an average adjacent marker length of 0.70 cM.

SBI-04

The map length of LG4 was 125.28 cM, and had 157 markers. Map distance between 2 consecutive markers varied from 0.001 to 4.80 cM with the average adjacent marker interval length of 0.80 cM.

SBI-05

The map length of LG5 was 122.5 cM and had 240 markers. Map distance between 2 consecutive markers ranged from 0 to 2.792 cM, with the average adjacent marker interval of 0.51 cM.

SBI-06

The map length of LG6 was 124.87 cM had 263 markers. Map distance between 2 consecutive markers ranged from 0.008 to 5.12 cM, with the average adjacent marker interval of 0.47 cM.

SBI-07

LG 7 accommodates 194 markers within its map length of 130.78 cM. The adjacent marker distance ranged from 0.002 to 12.563 cM, with an average marker interval of 0.67cM.

SBI-08

152 markers were placed in LG8, which had map length of 136.47 cM. The average adjacent marker interval ranged from 0.001 to 8.807 cM, with an average adjacent marker interval of 0.90 cM.

SBI-09

LG9 accommodate 240 markers within its map length of 142.10 cM. The adjacent marker distance ranged from 0.002 to 11.808 cM, with an average marker interval of 0.59 cM.

SBI-10

The map length of LG10 was 102.06 cM, it was smallest group for this RIL population and had lowest number of marker 79. Map distance between 2 consecutive markers varied from 0.039 to 6.02 cM, with an average marker interval of 1.29 cM.

In general, the 13 SSR markers were distributed evenly throughout the chromosomes except chromosome 2, 3 and 4, where, two or more SSRs were mapped, the marker order and position were in good agreement with the published map of Bhatramakki *et al.*, 2000 and Ramu *et al.*, 2009. Genomic SSR markers which were already mapped, served as anchor markers to assign the newly developed Linkage groups to respective chromosomes. The present map is longer than sorghum linkage maps previously reported by several authors (Klein *et al.*, 2001b; Dufour *et al.*, 1997; Kong *et al.*, 2000 and Bowers *et al.*, 2003). The greater map distance can be attributed to the increase in the recombination frequencies with increase in the population size included in the analysis, and also because of increase in marker density. Whereas, the present map is shorter than previously developed maps by (Pereira *et al.*, 1994; Chittenden *et al.*, 1994; Xu *et al.*, 1994; Taramino *et al.*, 1997; Ming *et al.*, 1998; Boivin *et al.*, 1999; Crasta *et al.*, 1999; Menz *et al.*, 2002 and Mace *et al.*, 2009) the shorter map length can be attributed to number and type of markers used to compare with other studies.

Most existing maps in sorghum were developed using RFLP and SSR markers (Bhatramakki *et al.*, 2000 and Haussmann *et al.*, 2004) or developed exclusively with SSRs such as for mapping green bug resistance (Wu and Huang, 2006 and 2008) and shootfly resistance (Folkertsma *et al.*, 2003). The first linkage map of sorghum using DArT and other marker was developed by Mace *et al.* (2009) which accommodated 2029 (1190 DArT + 839 non DArT) markers within its map length of 1603.5 cM with average marker density of 0.79 cM. In the present linkage map the highest marker distance between 2 consecutive markers is 11.80 cM followed by 8.8 cM. Out of total intervals, only one inter marker interval has more than 10 cM distance and remaining all were less than 10 cM, this indicate that good genome coverage has been achieved in present study using SSRs and DArT markers and the addition of more molecular markers would not serve to increase the map length.

4.2.3 QTL Mapping

Majority of quantitative traits in crops are controlled by polygenes, most of them have minor effect and only occasionally some of the genes have major effect (Falconar 1989). These gene loci are described as quantitative trait loci (QTL) and can be detected with the help of molecular markers, which should also segregate in a Mendelian manner. A QTL actually describes a region of chromosome define either by linkage to an individual molecular marker or by two flanking markers which may or may not be linked with QTL (Melchinger 1998). The association between marker and trait is used to discover genetic location of genes controlling the traits (Winter *et al.*, 2002). The association of marker trait helps breeder to construct beneficial allelic combination and accelerate breeding programme for cultivar development. The number of QTLs detected in any of the study depends upon genetic diversity among parents, environment conditions, number of markers in map and type and size of mapping population (Brondani *et al.*, 2002). The population used in this study was 309 RIL genotypes for creating genetic linkage map covering QTL. The QTL mapping experiment was carried, trial wise and pooled data over environments (6 environment pooled data). However, the data was received from same RIL population for three location, the existing variation between locations may give different QTL mapping results. The architecture of a trait characterized by the number of effective factors (Wright, 1968) has an impact on both the power of QTL detection and magnitude of the bias when estimating QTL effects (Melchinger *et al.*, 1998).

The mapping population derived from cross 296 B × PVK 801 (309 RILs) were used for identification of QTLs for traits under study. In order to identify the QTLs the phenotypic data from different locations over two years was used along with available genotypic data of SSR, DArT and SNPs in QTL Cartographer using composite interval mapping (CIM).

4.2.3.1 Individual Environment QTL Analysis for grain Iron Concentration

In E₁(ICRISAT 12-13), a total of 22 QTLs were detected on linkage group (LG) 01, 04, 06, 07, 08 and 09 (Table 4.7a), All the QTLs were minor or modifiers with phenotypic variance (R^2) ranging from 2.77% to 5.82% and LOD score ranging from 2.5 to 4.6, Out of 22 QTLs seven QTLs showed LOD less than 3. Maximum ten QTLs were detected on single LG 07, most of the QTLs were situated within length of 10 cM and

also inherited from same parents. On LG 07, QTLs were observed across the chromosome on both the arms but QTL number *qfe7.2* to *qfe7.9* were located very close to each other and inherited from same parent PVK 801, which explain these QTLs collectively could be worked as major QTL to give more phenotypic expression. In E₂ (IIMR 12-13) only three QTLs were identified which are located on LG 04 and LG 08 (Table 4.7b). All three QTLs were inherited from female parent 296 B, but QTL *qfe8.1* showed LOD score less than 3. In E₃ (VNMKV 12-13) a total 10 QTLs were identified one on LG 04, four on LG 07 and five on LG 09 (Table 4.7c). All the four QTLs of LG 07 were situated in an interval of 12 cM and all were inherited from PVK 801, also all the QTLs on LG 09 were located within an interval of 10 cM inherited from same parent 296 B, whereas all QTLs of LG 09 found with LOD score less than 3. The Flanking marker interval for all QTLs of this trial was less than 1 cM.

In E₄ (ICRISAT 13-14), total of seven QTLs were identified for grain iron concentration, three QTLs on LG 01 and single QTL from each LG 04, 05, 06 and 07 (Table 4.7d). The all three QTLs from LG 01 were located within region of 10 cM and all were inherited from male parent PVK 801 also all QTLs of LG 06 and LG 07 were inherited from same parent PVK 801, whereas, the QTLs on LG 04 and LG 05 were coming from female parent 296 B. But, all QTLs on LG 05, LG 06 and LG 07 were observed at LOD score less than 3. In E₅ (IIMR 13-14) total of five QTLs were identified, LG 03 and LG 05 were accommodating two QTLs each, while, single QTL on LG 07 (Table 4.7e) was identified. The each two QTLs located on LG 03 and LG 05 were situated within the range of 10 cM and are inherited from same parent 296 B, however only one QTL *qfe3.1* out of five QTLs showed LOD score more than 3, rest of all QTLs have LOD score less than 3. In E₆ (VNMKV 13-14) nine QTLs were observed from four different Linkage groups viz., LG 02, LG 03, LG 04 and LG 07 (Table 4.7f). The single QTL was located on each linkage group LG 02, LG 03 and LG 04, but at LOD score less than 3, whereas six QTLs were located on LG 07 itself and all were within interval of 10 cM, also inherited from same parent PVK 801, out of six QTLs on LG 07 only one QTL *qfe7.5* showed LOD score less than 3.

Table 4.7a. List of QTLs identified for grain iron concentration in E1 (ICRISAT 12-13)

Trait	QTL name	QTL Position(cM)	Flanking Marker	Support interval	Marker interval (cM)	LOD	Additive_effect	R2(%)
Iron (E1) ICRISAT 12-13	<i>qfe1.1</i>	1/109.21	Sn2224971 - Sn1951759	109.27 - 108.21	1.05	3.0	0.70	3.62
	<i>qfe1.2</i>	1/112.81	Dt1937067 - Dt2654567	112.83 - 112.74	0.09	3.4	0.72	3.94
	<i>qfe4.1</i>	4/1.11	Dt2646105 - Dt2224731	1.06 - 2.08	1.02	3.8	0.76	4.39
	<i>qfe4.2</i>	4/11.91	Dt3627364 - Dt2222665	9.89 - 12.84	2.95	3.2	0.73	4.10
	<i>qfe6.1</i>	6/80.11	Sn2647222 - Sn2650181	80.07 - 80.38	0.31	3.1	0.70	3.74
	<i>qfe6.2</i>	6/87.01	Sn2233078 - Dt2650961	87.02 - 87.96	0.95	3.3	0.73	3.95
	<i>qfe6.3</i>	6/88.61	Dt2005952 - Dt2648290	88.61 - 88.84	0.23	3.5	0.73	4.14
	<i>qfe6.4</i>	6/90.41	Sn2657501 - Sn2647940	90.38 - 91.59	1.21	4.6	0.84	5.44
	<i>qfe6.5</i>	6/97.21	Sn1885883 - Sn2653118	95.21 - 98.27	3.05	2.7	0.73	4.14
	<i>qfe6.6</i>	6/99.51	Sn1951026 - Sn1937498	99.49 - 100.05	0.56	3.6	0.73	4.25
	<i>qfe7.1</i>	7/24.91	Dt2644692 - Dt1950699	24.91 - 25.14	0.23	3.4	0.69	3.71
	<i>qfe7.2</i>	7/42.91	Sn2644846 - Dt3625344	36.9 - 49.47	12.56	3.6	0.87	5.82
	<i>qfe7.3</i>	7/49.81	Sn1941261 - Sn1884775	49.77 - 51.27	1.50	3.2	0.70	3.55
	<i>qfe7.4</i>	7/54.01	Sn1925068 - Dt2646020	53.04 - 54.79	1.74	2.8	0.68	3.51
	<i>qfe7.5</i>	7/57.01	Sn2650637 - Sn1895281	57.04 - 57.99	0.95	2.7	0.63	2.96
	<i>qfe7.6</i>	7/61.01	Sn2653275 - Sn2645682	60.98 - 61.47	0.48	2.8	0.67	3.14
	<i>qfe7.7</i>	7/65.21	Dt3933981 - Sn1875097	65.35 - 64.21	1.13	2.9	0.67	3.23
	<i>qfe7.8</i>	7/67.61	Dt3628977 - Dt2646589	67.62 - 67.95	0.33	2.6	0.63	2.90
	<i>qfe7.9</i>	7/72.61	Sn1937648 - Sn1919843	72.62 - 73.36	0.74	2.5	0.62	2.77
	<i>qfe7.10</i>	7/123.51	Xtxp525 - Sn2653248	123.48 - 126.57	3.10	4.3	0.81	5.09
	<i>qfe8.1</i>	8/78.71	Xiabtp399 - Sn1925051	78.7 - 79.39	0.69	3.7	0.73	4.28
	<i>qfe9.1</i>	9/83.71	Sn1950992 - Sn1929569	83.73 - 82.37	1.36	3.5	0.71	3.89

Table 4.7b. List of QTLs identified for grain iron concentration in E2 (IIMR 12-13)

Trait	QTL name	QTL Position(cM)	Flanking Marker	Support interval	Markar interval (cM)	LOD	Additive_effect	R2(%)
IRON (E2) IIMR 12-13	<i>qfe4.1</i>	4/13.61	Dt1907645 - Dt2645303	13.6 - 15.06	1.46	5.7	1.05	7.30
	<i>qfe4.2</i>	4/23.31	Dt1881512 - Dt1995450	21.32 - 23.71	2.39	3.4	0.82	4.64
	<i>qfe8.1</i>	8/91.61	Dt2654812 - Dt2664808	91.57 - 91.87	0.30	2.7	0.70	3.42

Table 4.7c. List of QTLs identified for grain iron concentration in E3 (VNMKV 12-13)

Trait	QTL name	QTL Position(cM)	Flanking Marker	Support interval	Markar interval (cM)	LOD	Additive_effect	R2(%)
IRON (E3) VNMKV 12-13	<i>qfe4.1</i>	4/41.21	Dt2658021 - Dt2651208	41.16 - 41.76	0.60	3.1	0.78	4.08
	<i>qfe7.1</i>	7/61.01	Sn2653275 - Sn2645682	60.98 - 61.47	0.48	3.4	0.83	4.48
	<i>qfe7.2</i>	7/65.51	Dt3627294 - Dt2648007	65.62 - 65.48	0.14	3.9	0.88	5.19
	<i>qfe7.3</i>	7/67.21	Dt2649259 - Dt3628977	67.17 - 67.62	0.45	3.5	0.85	4.81
	<i>qfe7.4</i>	7/72.61	Sn1937648 - Sn1919843	72.62 - 73.36	0.74	3.0	0.78	3.97
	<i>qfe9.1</i>	9/61.71	Sn1908329 - Sn1891235	61.68 - 62.41	0.73	2.7	0.74	3.57
	<i>qfe9.2</i>	9/64.61	Sn1890587 - Sn2649169	64.57 - 65.07	0.49	2.9	0.76	3.82
	<i>qfe9.3</i>	9/66.91	Sn1937832 - Sn3628693	66.92 - 67.39	0.46	2.6	0.72	3.45
	<i>qfe9.4</i>	9/69.11	Dt3933866 - Dt2655276	69.29 - 69.32	0.03	2.6	0.77	3.33
	<i>qfe9.5</i>	9/72.21	Sn2644848 - Sn2649413	72.15 - 72	0.15	2.5	0.74	3.28

Table 4.7d. List of QTLs identified for grain iron concentration in E4 (ICRISAT 13-14)

Trait	QTL name	QTL Position(cM)	Flanking Marker	Support interval	Markar interval (cM)	LOD	Additive_effect	R2(%)
IRON (E4) ICRISAT 13-14	<i>qfe1.1</i>	1/96.21	Sn2658144 - Dt2656291	96.18 - 96.86	0.67	4.5	0.75	5.58
	<i>qfe1.2</i>	1/103.91	Dt2651077 - Sn1948592	103.87 - 104.11	0.24	5.1	0.77	6.34
	<i>qfe1.3</i>	1/107.41	Sn2650907 - Sn1933402	107.37 - 107.69	0.32	5.5	0.79	6.80
	<i>qfe4.1</i>	4/11.91	Dt3627364 - Dt2222665	9.89 - 12.84	2.95	3.4	0.65	4.46
	<i>qfe5.1</i>	5/117.51	Dt2653422 - Sn2710051	117.51 - 118.36	0.85	2.6	0.54	3.14
	<i>qfe6.1</i>	6/123.51	Sn2660857 - Sn2056548	123.46 - 124.41	0.96	2.7	0.56	3.34
	<i>qfe7.1</i>	7/108.71	Sn2646418 - Sn2645257	108.64 - 109.62	0.97	2.5	0.53	3.02

Table 4.7e. List of QTLs identified for grain iron concentration in E5 (IIMR 13-14)

Trait	QTL name	QTL Position(cM)	Flanking Marker	Support interval	Markar interval (cM)	LOD	Additive_effect	R2(%)
IRON (E5) DSR 13-14	<i>qfe3.1</i>	3/100.21	Dt2664901 - Sn1880647	100.21 - 100.55	0.34	3.7	0.68	4.89
	<i>qfe3.2</i>	3/106.61	Dt2645975 - Sn1886930	106.54 - 107.03	0.49	2.8	0.60	3.73
	<i>qfe5.1</i>	5/107.61	Sn2657124 - Sn2647027	106.57 - 108.05	1.48	2.6	0.63	4.23
	<i>qfe5.2</i>	5/117.31	Dt2648399 - Dt2653422	117.26 - 117.51	0.25	3.6	0.67	4.75
	<i>qfe7.1</i>	7/84.31	Sn1938673 - Dt2654950	84.27 - 84.85	0.58	2.8	0.59	3.72

Table 4.7f. List of QTLs identified for grain iron concentration in E6 (VNMKV 13-14)

Trait	QTL name	QTL Position (cM)	Flanking Marker	Support interval	Marker interval (cM)	LOD	Additive_effect	R ² (%)
IRON (E6) VN MKV (13-14)	<i>qfe2.1</i>	2/64.51	Sn2652046 - Sn2648313	64.53 - 64.89	0.36	2.5	-0.76	3.16
	<i>qfe3.1</i>	3/19.11	Sn2135762 - Sn1910190	19.15 - 17.83	1.32	2.4	-0.72	3.08
	<i>qfe4.1</i>	4/29.71	Dt2657828 - Dt1991354	29.69 - 28.94	0.75	2.6	-0.76	3.33
	<i>qfe7.1</i>	7/61.01	Sn2653275 - Sn2645682	60.98 - 61.47	0.48	4.4	0.99	5.66
	<i>qfe7.2</i>	7/62.51	Dt2648834 - Dt2209499	62.53 - 62.95	0.43	3.4	0.88	4.51
	<i>qfe7.3</i>	7/64.21	Sn1875097 - Xiabtp360	64.21 - 63.55	0.66	3.1	0.83	4.03
	<i>qfe7.4</i>	7/66.11	Dt2760348 - Dt2645234	66.27 - 66.12	0.15	3.4	0.87	4.41
	<i>qfe7.5</i>	7/69.21	Sn2656777 - Sn2208551	69.32 - 69.18	0.14	2.9	0.82	3.85
	<i>qfe7.6</i>	7/70.51	Dt2651227 - Sn2650044	70.49 - 70.71	0.22	3.0	0.82	3.85

4.2.3.2 Individual Environment QTL Analysis for grain Zinc Concentration

In E₁ (ICRISAT 12-13) a total of 22 QTLs were identified for zinc located on five different linkage groups *viz.*, LG 04, LG 06, LG 07, LG 09 and LG 10 (Table 4.8a). Two QTLs were on LG 04 situated within interval of 10 cM and inherited from female parent 296 B with LOD score more than 3. Total of eight QTLs were identified on LG 06 among which, QTLs *qzn* 6.1, 6.2, 6.3, 6.4, 6.5, 6.6 and *qzn* 6.7 were located within the interval of 10 cM with LOD score more than 3 and QTL *qzn* 6.8 situated away from this block with LOD less than 3, all QTLs of LG 06 were inherited from PVK 801. Five QTLs were located on LG 07 all were inherited from same parent PVK 801 and having LOD score more than 3, except QTL *qzn* 7.1 remaining four QTL were situated within interval of 10 cM. Six QTLs were identified on LG 9, among those QTL *qzn* 9.6 having LOD less than 3, remaining five QTLs of these linkage group were showing LOD score more than 3 and inherited from female parent 296 B. Single QTL was also identified on LG 10 inherited from 296 B with LOD more than 3. In E₂ (IIMR 12-13) only two QTL were identified, single QTL each on LG 04 and LG 07 (Table 4.8b). On LG 04 it was inherited from 296 B, whereas on LG 07 it was from PVK 801, both the QTLs observed at LOD score more than 3. In E₃ (VNMKV 12-13) total of 17 QTLs were identified on five different linkage group *viz.*, LG 04, LG 05, LG 07, LG 09 and LG 10 (Table 4.8c). Two QTLs on LG 04 and both are having LOD less than 3. Three QTLs were on LG 5 within range of 10 cM all inherited from male parent PVK 801 and showed LOD score nearly equal to 3. Six QTLs were identified on LG 07 all were inherited from same parent PVK 801 and LOD more than 3. On LG 09 four QTLs were identified all were inherited from 296 B but QTL *qzn* 9.1 and *qzn* 9.4 are having LOD less than 3. On LG 10 two QTLs are identified both were from 296 B and QTL *qzn* 10.2 was having LOD less than 3. In E₄ (ICRISAT 13-14) total of seven QTLs were identified on four different linkage groups, single QTL on LG 04, two QTLs on each LG 05, LG 07 and LG 09 (Table 4.8d) only QTL *qzn* 9.2 showed LOD more than 3 all other are non-significant QTLs having LOD less than 3. In E₅ (IIMR 13-14) only one QTL was identified on LG 03 (Table 4.8e) but with LOD less than 3 and inherited from female parent 296 B.

Table. 4.8a. List of QTLs identified for grain zinc concentration in E1 (ICRISAT 12-13)

Trait	QTL name	QTL Position(cM)	Flanking Marker	Support interval	Marker interval (cM)	LOD	Additive effect	Phenotypic Variance
ZINC (E1) ICRISAT 12-13	<i>qzn4.2</i>	4/12.91	Dt2222665 - Dt2648141	12.84 - 13.13	0.29	4.9	0.71	5.65
	<i>qzn4.3</i>	4/19.31	Dt3933755 - Dt2669502	17.28 - 20.57	3.29	3.0	0.57	3.90
	<i>qzn6.1</i>	6/80.11	Sn2647222 - Sn2650181	80.07 - 80.38	0.31	4.8	0.71	5.75
	<i>qzn6.2</i>	6/81.21	Dt1904146 - Sn2186555	81.16 - 81.7	0.53	4.4	0.67	5.20
	<i>qzn6.3</i>	6/82.21	Sn2647245 - Dt2648262	82.23 - 82.49	0.25	4.8	0.71	5.61
	<i>qzn6.4</i>	6/83.71	Sn1889136 - Dt2646380	83.71 - 84.5	0.79	4.2	0.65	5.02
	<i>qzn6.5</i>	6/87.01	Sn2233078 - Dt2650961	87.02 - 87.96	0.95	3.8	0.63	4.47
	<i>qzn6.6</i>	6/88.61	Dt2005952 - Dt2648290	88.61 - 88.84	0.23	5.0	0.71	5.94
	<i>qzn6.7</i>	6/90.41	Sn2657501 - Sn2647940	90.38 - 91.59	1.21	5.6	0.75	6.57
	<i>qzn6.8</i>	6/99.51	Sn1951026 - Sn1937498	99.49 - 100.05	0.56	2.9	0.54	3.41
	<i>qzn7.1</i>	7/42.91	Sn2644846 - Dt3625344	36.9 - 49.47	12.56	5.6	0.88	9.42
	<i>qzn7.2</i>	7/54.01	Sn1925068 - Dt2646020	53.04 - 54.79	1.74	6.8	0.86	8.80
	<i>qzn7.3</i>	7/57.01	Sn2650637 - Sn1895281	57.04 - 57.99	0.95	5.9	0.77	6.96
	<i>qzn7.4</i>	7/61.01	Sn2653275 - Sn2645682	60.98 - 61.47	0.48	5.7	0.76	6.76
	<i>qzn7.5</i>	7/63.61	Xiabtp360 - Sn1875097	63.55 - 64.21	0.66	5.5	0.75	6.79
	<i>qzn9.1</i>	9/73.51	Sn1952894 - Dt2656508	73.51 - 75.02	1.51	3.4	0.58	3.97
	<i>qzn9.2</i>	9/75.21	Dt1878122 - Dt1879999	75.31 - 75.22	0.09	3.1	0.58	3.71
	<i>qzn9.3</i>	9/81.01	Dt2014698 - Sn2655976	81.01 - 81.53	0.52	4.1	0.64	4.85
	<i>qzn9.4</i>	9/83.71	Sn1950992 - Sn1929569	83.73 - 82.37	1.36	5.4	0.73	6.29
	<i>qzn9.5</i>	9/88.21	Dt2656304 - Sn1906827	88.24 - 87.2	1.04	3.4	0.60	4.13
	<i>qzn9.6</i>	9/91.61	Dt2665553 - Dt2655343	91.55 - 91.94	0.39	2.6	0.52	3.11
	<i>qzn10.1</i>	10/91.61	Sn1962197 - Dt2653453	90.61 - 92.1	1.49	3.7	0.63	4.68

Table 4.8b. List of QTLs identified for grain zinc concentration in E2 (IIMR 12-13)

Trait	QTL name	QTL Position(cM)	Flanking Marker	Support interval	Markar interval (cM)	LOD	Additive effect	Phenotypic Variance
ZINC (E2) IIMR 12-13	<i>qzn4.1</i>	4/12.91	Dt2222665 - Dt2648141	12.84 - 13.13	0.29	6.0	0.85	7.65
	<i>qzn7.1</i>	7/24.91	Dt2644692 - Dt1950699	24.91 - 25.14	0.23	3.7	0.65	4.63

Table 4.8c. List of QTLs identified for grain zinc concentration in E3 (VNMKV 12-13)

Trait	QTL name	QTL Position(cM)	Flanking Marker	Support interval	Markar interval (cM)	LOD	Additive effect	Phenotypic Variance
ZINC (E3) PARBHANI 12-13	<i>qzn4.1</i>	4/32.81	Dt1908536 - Dt2653076	32.97 - 32.75	0.21	2.4	0.55	3.04
	<i>qzn4.2</i>	4/41.21	Dt2658021 - Dt2651208	41.16 - 41.76	0.60	2.8	0.59	3.49
	<i>qzn5.1</i>	5/37.01	Sn2647853 - Sn1931062	36.94 - 37.89	0.95	3.7	0.68	4.69
	<i>qzn5.2</i>	5/39.31	Sn2656562 - Dt2043133	38.24 - 39.55	1.31	3.1	0.64	4.10
	<i>qzn5.3</i>	5/43.41	Sn1926290 - Sn2652538	43.52 - 42.36	1.16	2.9	0.64	3.98
	<i>qzn7.1</i>	7/55.51	Dt2645576 - Sn2033434	55.46 - 56.27	0.81	4.1	0.72	5.41
	<i>qzn7.2</i>	7/62.11	Sn1895297 - Dt2648834	62.06 - 62.53	0.47	4.1	0.72	5.29
	<i>qzn7.3</i>	7/64.21	Sn1875097 - Xabtp360	64.21 - 63.55	0.66	4.9	0.78	6.29
	<i>qzn7.4</i>	7/65.31	Dt2648007 - Dt3933981	65.48 - 65.35	0.14	4.1	0.74	5.32
	<i>qzn7.5</i>	7/67.21	Dt2649259 - Dt3628977	67.17 - 67.62	0.45	4.3	0.75	5.83
	<i>qzn7.6</i>	7/72.61	Sn1937648 - Sn1919843	72.62 - 73.36	0.74	3.1	0.62	3.98
	<i>qzn9.1</i>	9/62.41	Sn1891235 - Sn1977702	62.41 - 62.92	0.51	2.5	0.56	3.17
	<i>qzn9.2</i>	9/64.61	Sn1890587 - Sn2649169	64.57 - 65.07	0.49	3.1	0.62	3.92
	<i>qzn9.3</i>	9/69.11	Dt3933866 - Dt2655276	69.29 - 69.32	0.03	3.0	0.64	3.72
	<i>qzn9.4</i>	9/72.21	Sn2644848 - Sn2649413	72.15 - 72	0.15	2.6	0.60	3.35
	<i>qzn10.1</i>	10/77.91	Sn2651949 - Sn2704229	77.89 - 78.99	1.10	3.1	0.62	3.87
	<i>qzn10.2</i>	10/83.61	Dt2687545 - Dt2655698	83.58 - 84.41	0.84	2.5	0.56	3.15

Table. 4.8d. List of QTLs identified for grain zinc concentration in E4 (ICRISAT 13-14)

Trait	QTL name	QTL Position(cM)	Flanking Marker	Support interval	Markar interval (cM)	LOD	Additive effect	Phenotypic Variance
ZINC (E4) ICRISAT 13-14	<i>qzn4.1</i>	4/34.31	Dt2650643 - Dt1997108	34.32 - 33.48	0.84	2.5	0.43	3.13
	<i>qzn5.1</i>	5/68.91	Dt1990986 - Dt3933747	68.94 - 69.25	0.31	2.7	0.44	3.47
	<i>qzn5.2</i>	5/74.81	Dt2678768 - Dt2649478	74.83 - 74.74	0.09	2.4	0.42	3.11
	<i>qzn7.1</i>	7/55.51	Dt2645576 - Sn2033434	55.46 - 56.27	0.81	2.4	0.43	3.17
	<i>qzn7.2</i>	7/64.21	Sn1875097 - Xiabtp360	64.21 - 63.55	0.66	2.9	0.47	3.75
	<i>qzn9.1</i>	9/83.71	Sn1950992 - Sn1929569	83.73 - 82.37	1.36	2.6	0.44	3.28
	<i>qzn9.2</i>	9/93.71	Sn2655154 - Sn1904276	92.68 - 95.97	3.29	3.1	0.51	4.44

Table. 4.8e. List of QTLs identified for grain zinc concentration in E5 (IIMR 13-14)

Trait	QTL name	QTL Position (cM)	Flanking Marker	Support interval	Markar interval (cM)	LOD	Additive effect	Phenotypic Variance
ZINC (E5) IIMR 13-14	<i>qzn3.1</i>	3/107.01	Sn1886930 - Dt2645975	107.03 - 106.54	0.49	2.5	-0.37	3.44

Table. 4.8f. List of QTLs identified for grain zinc concentration in E6 (VNMKV 13-14)

Trait	QTL name	QTL Position(cM)	Flanking Marker	Support interval	Markar interval (cM)	LOD	Additive effect	Phenotypic Variance
ZINC (E6) VNMKV 13-14	<i>qzn2.1</i>	2/77.61	Sn2650020 - Sn2647952	77.55 - 78.36	0.81	2.4	-0.50	2.94
	<i>qzn4.1</i>	4/19.31	Dt3933755 - Dt2669502	17.28 - 20.57	3.29	2.7	-0.57	3.93
	<i>qzn5.1</i>	5/53.71	Sn2006069 - Sn3933338	53.71 - 54.24	0.54	2.5	0.52	3.17
	<i>qzn6.1</i>	6/87.01	Sn2233078 - Dt2650961	87.02 - 87.96	0.95	2.4	0.53	3.08
	<i>qzn6.2</i>	6/88.61	Dt2005952 - Dt2648290	88.61 - 88.84	0.23	3.3	0.59	4.15
	<i>qzn6.3</i>	6/90.01	Dt2234039 - Sn2657501	89.99 - 90.38	0.39	3.1	0.58	4.01
	<i>qzn7.1</i>	7/16.21	Dt2657812 - Sn1958405	16.23 - 15.79	0.44	3.6	0.63	4.53
	<i>qzn7.2</i>	7/55.51	Dt2645576 - Sn2033434	55.46 - 56.27	0.81	3.7	0.64	4.75
	<i>qzn7.3</i>	7/58.01	Sn1895281 - Sn1927802	57.99 - 59.7	1.72	2.5	0.53	3.20
	<i>qzn7.4</i>	7/67.21	Dt2649259 - Dt3628977	67.17 - 67.62	0.45	3.7	0.64	4.87
	<i>qzn7.5</i>	7/72.61	Sn1937648 - Sn1919843	72.62 - 73.36	0.74	2.7	0.54	3.44
	<i>znl0.3</i>	10/88.41	Dt1907663 - Sn1879791	88.56 - 86.36	2.19	2.9	-0.60	3.64

In E₆ (VNMKV 13-14) total of 12 QTLs were identified one each on LG 02, LG 04, LG 05 and LG 10, three QTLs on LG 06 and five QTLs on LG 07 (Table 4.8f), all three QTLs of LG 03 were situated within a region of 10 cM and inherited from same parent PVK 801, also QTL *qzn7.2* to *qzn7.5* on LG 07 were situated very close to each other and also inherited from male parent PVK 801. However all the QTLs on LG 2, LG 4, LG 5, QTL *qzn 6.1* on LG 6, QTLs *qzn 7.3* and *qzn 7.5* on LG 7 and QTL *qzn 10.3* on LG 10 are non-significant as having LOD less than 3.

The QTL analysis for grain iron and zinc in individual environment showed different QTLs in different environment, which proves the effect of environment on expression of traits, the soil type and soil iron and zinc concentration in all the location was different. Occurrence of multiple QTLs nearly in all the environment with small phenotypic variance, explains the cumulative effect of different genomic regions contributing to small percentage of phenotypic variation on expression of QTLs. This suggests that the transport and accumulation of mineral in seed is a complex processes and highly influence by environment also significant G × E is detected for both grain iron and zinc in this study. Occurrence of several QTLs was also detected in various previous studies in different crops like rice (Lu *et al.*, 2008), Medicago (Sankaran *et al.*, 2009) and wheat (Peleg *et al.*, 2009). Most QTLs mapping studies reported that quantitative traits were regulated by many genes, each explaining small portion of the total trait variation. For example, Laurie *et al.* (2004) reported about 50 QTLs that explained approximately 50% of the genetic variance for oil concentration in the maize kernel. Buckler *et al.* (2009) evaluated nearly a million maize plants in eight environments and found no evidence for any single large effect QTL for flowering time. However, the genetic variation of most quantitative traits likely involves a small number of major genes or QTLs, a larger number of loci with moderate effects and a very large number of loci with minor effects (Robertson, 1967; Kearsey and Farquhar, 1998). The effects of the major genes can be studied *via* segregation analysis as well as evolutionary and selection history. However, the present QTL mapping results for grain iron and zinc showing numerous genes with small effects, cannot be investigated individually. The detection of few QTLs with large phenotypic effect, often result of small population size (Beavis, 1998; Melchinger *et al.*, 1998; Utz *et al.*, 2000; Schon *et al.*, 2004) or can be the artifact of the strong direction selection often used to create phenotypically divergent lines that are used for mapping (Lande, 1983), whereas in the present study the mapping population used was a large size (309 RILs) and the variation in parents of RIL mapping population is nearly 4 mg/kg⁻¹ for both the mineral traits iron and zinc, which is lesser than variation used to create

phenotypically divergent parental lines using strong directional selection. Over estimation of the effect of QTLs is often a major problem because QTLs whose effects are over estimated and are more likely to be detected above the necessary stringent threshold than are those whose effects are correctly estimated or underestimated. Spurious QTLs (false positives) are occasionally detected and this represents an over estimation of an effect that is actually zero. Furthermore, most primary or coarse QTL mapping studies using small population size and low marker density allows only for an approximate mapping of the chromosomal region. Therefore, identification of reliable QTL is a preliminary step in developing a marker assisted selection programs for genetic improvement. To utilize QTLs in selective breeding or to identify functional genes, the identified major QTLs should be fine mapped to a higher level of resolution for QTL position and verified or validated in additional genetic backgrounds and environments by developing advanced segregating populations with large numbers of recombination's in the region of interest. QTL verification is defined as the repeated detection of the same marker alleles at a similar position on the genetic map of a chromosome, of a QTL controlling a trait under more than one set of experimental conditions (Brown *et al.* 2003). Verification of QTL is necessary to substantiate a biological basis for observed marker-trait associations, to provide precise estimates of the magnitude of QTL effects, and to predict QTL expression at a given age or in a particular environment.

Overall in individual environment analysis for grain iron concentration LG 4 and LG 7 were most common particularly, QTL on LG 7 at 61.01 cM flank by markers Sn2653275 – Sn2645682 was consistently present in three different environment hence this QTL can be targeted for improving iron concentration, also LG 4 and LG 7 can be targeted for multiple QTL improvement. In case of grain zinc concentration maximum contribution for QTL in most of the environment showed by LG 7, particularly QTL on LG 7 at 55.51 cM flanked by markers Dt 2645576 – Sn 2033434 was consistently present in three environments, hence it can be target for zinc improvement in Sorghum.

4.2.3.3. Across environment QTL analysis

Across environment QTL analysis has been done by using BLUPs mean from across six environment with linkage map of 2088 markers of present RIL population. A total of 21 QTLs controlling grain iron and zinc were found across six environments. Nine QTLs for grain iron and 12 QTLs for grain zinc concentration were identified with the phenotypic variance (R^2) range from 2.82% to 6.66% and 0.30% to 5.74% respectively. Among nine QTLs identified for grain iron concentration single QTL on LG 01, three

QTLs on each LG 04, LG 07 and two QTLs on LG 06 (Table 4.9a). QTL *qfe* 7.3 on LG 07 at 123.51 cM flanked by markers Xtxp525 – Sn2653248 has showed maximum LOD (5.61) and phenotypic variance (6.6 %), out of nine QTLs five QTLs (*qfe* 1.1, *qfe* 4.2, *qfe* 4.3, *qfe* 6.1 and *qfe* 7.1) showed LOD score less than 3, hence these QTLs are consider as non-significant QTLs, whereas remaining four QTLs (*qfe* 4.1, *qfe* 6.1, *qfe* 7.2 and *qfe* 7.3) are showing LOD more than 3, the QTLs on LG 4 are contributed by female parent 296 B and rest of all QTLs are inherited from male parent PVK 801, also out of nine QTLs only three QTLs (*qfe* 1.1, *qfe* 4.1 and *qfe* 7.3) are repeated from individual environment analysis remaining five QTLs are novel QTLs.

In case of zinc among 12 identified QTLs in across environment analysis, two each on LG 04 and LG 06, seven QTLs on LG 07 and single QTL on LG 09 (Table 4.9b). The QTLs on LG 04 and LG 09 are contributed from female parent 296 B, whereas rest all QTLs are contributed from male parent PVK 801. The QTLs *qzn* 4.1, *qzn* 6.1, *qzn* 6.2, *qzn* 7.1, *qzn* 7.6 and *qzn* 7.7 showed LOD score less than 3 indicating non-significant QTLs. The QTL *qzn* 7.2 at 55.51 cM flanked by markers Dt 2645576 – Sn 2033434 was also observed in three different environments, hence this QTL considered as most important QTL for zinc improvement, three novel QTLs were also observed (*qzn* 4.1, *qzn* 4.2 and *qzn* 7.6) which were not present in any individual environment analysis. In candidate gene analysis study for grain iron and zinc in sorghum by Anuradha *et al.*, 2014, with other cereals homologous genes, the maximum number of hits were found on chromosome 1, ten hits were pertaining to *Nicotianamine synthase* (NAS) and also on chromosome 6 many hits were for *YSL* (yellow strip like gene) which act as metal chelator responsible for easy absorption and translocation of iron and zinc from soil to plant, hence QTLs analysed on chromosome 1 and 6 in present study be confirmed by expression analysis and along with chromosome 7 which showed maximum QTLs for both the traits could be used for improvement of both the minerals, iron and zinc in sorghum.

Overall QTL analysis in individual environment and across environment analysis showed different QTLs, however the genotypic data used for different environment is similar, the existing variation between environments may give different QTL mapping results. The genetic architecture of traits characterized by the number of effective factors (Wright, 1968) has an impact on both the power of QTL detection and the magnitude of bias when estimating QTL effects (Melchinger *et al.*, 1998). The inconsistency in present study due to appearance (loss of QTL across environments) could be explained by the altering gene expression in environments pressure (Sankaran *et al.*, 2009). The ability of

genotype to alter phenotypic expression in response to different environment condition is referred as phenotypic plasticity (Ungerer *et al.*, 2003). Phenotypic plasticity of quantitative traits arises in nature from interactions between QTL and environments at molecular levels. Inconsistent QTL across environments is common (Paterson *et al.*, 1991). This, QTL detected in one environment but not in another indicates QE interaction.

4.2.3.4. Co-localize markers

Quantitative traits affected by pleiotropism and linkage tends to reveal a correlation among these traits. This in turn generally leads to detection of co-mapped QTL's. However, it is not easy to differentiate between linkage and pleiotropy until QTN (Quantitative Trait Nucleotide) responsible for phenotypic variation of each trait is identified (Mackay, 2001). In the present study, in E₁ (ICRISAT 12-13) co-localized QTL for iron and zinc (QTL mapped at same position and having LOD more than 3 for both trait) were mapped on LG 06, LG 07 and LG 09 for individual environment analysis. Similarly in E₃ (VNMKV 12-13) on LG 07 (Table 4.10). Whereas a pair of QTLs for both the minerals were detected very close to each other in E₁ and E₂ on LG 04, in E₃ on LG 07 and LG 09, in E₅ on LG3 and in E₆ on LG 07 (Table 4.11 and Fig.4.7). The co-localization of QTLs for multiple elements was previously observed in wheat (Wu *et al.*, 2008; Chatzav *et al.*, 2010), *Brassica oleracea* (Broadley *et al.*, 2008), *Arabidopsis* (Vreugdenhil *et al.*, 2004), rice (Shimizu and Purugganan, 2005; Ishikawa *et al.*, 2010 and Anuradha *et al.*, 2012) and *Medicago* (Sankaran *et al.*, 2009). These results pointed out that many of the QTL regions appear to affect multiple traits. The co-mapped QTLs demonstrated the existence of genes or gene clusters with major effect. These loci might indicate to a common transporter such as ZIP gene family member, which is capable of transporting Zn in addition to Fe or to a synthetic protein for nicotinamide, a metal chelator involved in Fe, Zn, Cu and Mn homeostasis (Delhaize *et al.*, 2003; Sankaran *et al.*, 2009).

Table. 4.9a. List of QTLs for grain iron concentration in across environment analysis.

Trait	QTL name	QTL Position(cM)	Flanking Marker	Support interval	Markar intervel (cM)	LOD	Additive_effect	R2(%)
IRON (Pooled)	<i>qfe1.1</i>	1/112.81	Dt1937067 - Dt2647464	112.83 - 112.74	0.093	2.84	0.42	3.12
	<i>qfe4.1</i>	4/1.11	Dt2646105 - Dt2224731	1.06 - 2.08	1.016	3.86	-0.51	4.46
	<i>qfe4.2</i>	4/34.31	Dt2650643 - Dt1997108	34.32 - 33.48	0.84	2.65	-0.41	3.05
	<i>qfe4.3</i>	4/40.81	Dt3933410 - Dt2658021	40.77 - 41.16	0.40	2.45	-0.39	2.82
	<i>qfe6.1</i>	6/100.31	Dt2090541 - Dt2657314	100.24 - 100.94	0.70	3.25	0.45	3.85
	<i>qfe6.2</i>	6/101.41	Dt2233502 - Sn1918187	101.4 - 101.66	0.26	2.75	0.42	3.19
	<i>qfe7.1</i>	7/16.21	Dt2657812 - Dt2653638	16.23 - 16.58	0.35	2.57	0.41	2.97
	<i>qfe7.2</i>	7/24.81	Dt2644692 - Sn2646254	24.91 - 24.77	0.15	3.16	0.44	3.62
	<i>qfe7.3</i>	7/123.51	Xtp525 - Sn2653248	123.48 - 126.57	3.10	5.61	0.60	6.66

Table. 4.9b. List QTLs for grain zinc concentration in across environment analysis.

Trait	QTL name	QTL Position(cM)	Flanking Marker	Support interval	Markar intervel (cM)	LOD	Additive_effect	R ² (%)
ZINC (Pooled)	<i>qzn4.1</i>	4/2.11	Dt2224731 - Dt1944553	2.08 - 3.65	1.57	0.25	-0.43	0.30
	<i>qzn4.2</i>	4/13.61	Dt1907645 - Dt2645303	13.6 - 15.06	1.46	4.36	-0.53	5.39
	<i>qzn6.1</i>	6/88.61	Dt2005952 - Dt2648290	88.61 - 88.84	0.23	2.49	0.39	3.06
	<i>qzn6.2</i>	6/90.41	Sn2657501 - Sn2647940	90.38 - 91.59	1.21	2.64	0.41	3.24
	<i>qzn7.1</i>	7/16.21	Dt2657812 - Dt2653638	16.23 - 16.58	0.35	2.57	0.40	3.17
	<i>qzn7.2</i>	7/55.51	Dt2645576 - Sn2033434	55.46 - 56.27	0.81	4.49	0.54	5.66
	<i>qzn7.3</i>	7/57.01	Sn2650637 - Sn1895281	57.04 - 57.99	0.95	3.76	0.49	4.66
	<i>qzn7.4</i>	7/62.11	Sn1895297 - Dt2648834	L4:T16	0.47	4.16	0.51	5.14
	<i>qzn7.5</i>	7/67.21	Dt2649259 - Dt3628977	67.17 - 67.62	0.45	4.43	0.53	5.74
	<i>qzn7.6</i>	7/69.51	Dt3627584 - Dt2649175	69.47 - 69.74	0.26	2.84	0.43	3.63
	<i>qzn7.7</i>	7/72.61	Sn1937648 - Sn1919843	72.62 - 73.36	0.74	2.91	0.43	3.65
	<i>qzn9.1</i>	9/83.71	Sn1950992 - Sn1929569	83.73 - 82.37	1.36	3.58	-0.47	4.38

Table 4.10. Co-localized QTLs for grain iron and zinc in individual environments

Environment	Linkage Group	QTL position (cM)	Flanking Marker
E ₁ (ICRISAT 12-13)	6	80.11	Sn2647222-Sn2650181
	6	87.01	Sn2233078- Dt2650961
	6	88.61	Dt2005952 – Dt2648290
	6	90.41	Sn2657501- Sn2647940
	7	42.91	Sn2644846 – Dt 3625344
	9	83.71	Sn1950992 – Sn1929569
	7	67.21	Dt2649259 - Dt3628977
E ₃ (VNMKV 12-13)	7	72.61	Sn1937648 – Sn1919843

Table 4.11. Closely linked QTLs pair for grain iron and zinc in individual Environments

Environment	Linkage Group	Iron QTL Position (cM)	Zinc QTL Position (cM)
E ₁ (ICRISAT 12-13)	LG 4	11.91	12.91
E ₂ (IIMR 12-13)	LG 4	13.61	12.91
E ₃ (VNMKV 12-13)	LG 7	65.51	64.21
	LG 7	61.01	62.11
	LG 9	61.71	62.41
E ₄ (ICRISAT 13-14)	LG 4	29.71	34.31
E ₅ (IIMR 13-14)	LG 3	106.61	107.01
E ₆ (VNMKV 13-14)	LG 7	66.11	67.21

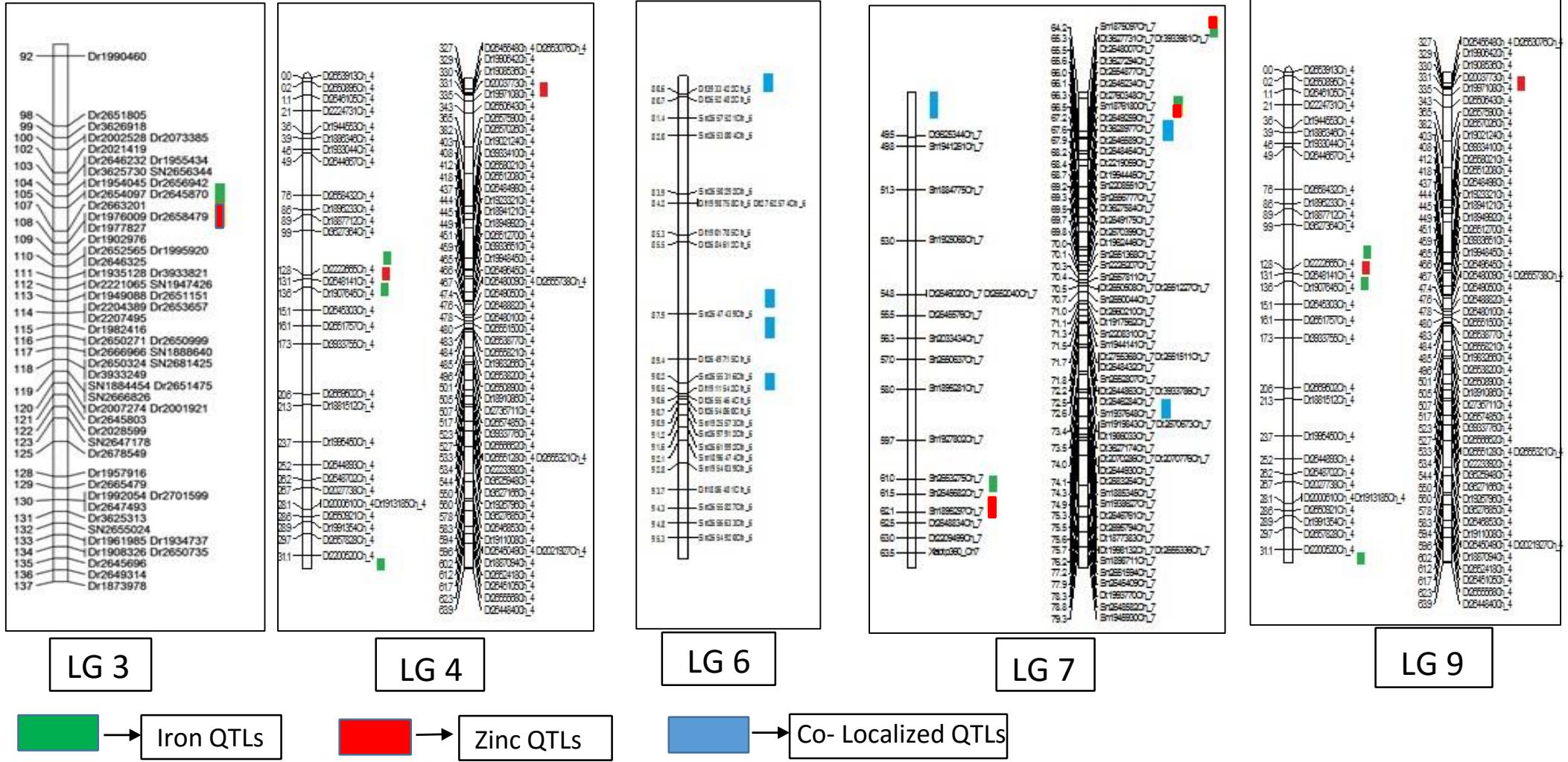


Fig. 4.7. Positions of Co-Localized and Linked QTLs for Iron and Zinc Concentration

Chapter V

SUMMARY AND CONCLUSIONS

The present research was carried out to understand the genetics, Genotype × Environment interaction, association between grain Iron (Fe) and Zinc (Zn) and mapping of QTL for these two traits in sorghum which have a direct bearing on devising effective strategies to breed sorghum cultivars with enhanced levels of grain iron and zinc densities and indirect selection of these complex traits using identified linked molecular markers. The specific objectives of research were: to phenotype the available RIL population at three different locations for two consecutive years, to genotype this available RIL population using SSR, DArT and SNP markers and finally mapping QTLs for grain Fe and Zn using available phenotypic and genotypic data. The findings are summarized objective wise hereunder:

5.1 Mean performance of RIL population for selected traits

The means, standard deviation, range and significance of genotypes for the selected traits measured in RILs were compared with parents means in all the environments separately. The study showed that parents has substantial differences for micronutrients iron and zinc in all the environments. The average grain iron and zinc were highest in location VNMKV (Parbhani) followed by IIMR and ICRISAT respectively, this was because VNMKV (Parbhani) soils are deep black and nutrient rich compared to other locations. This clearly indicates the role of G × E interaction and effects of mineral uptake, translocation and distribution.

5.2 Frequency Distribution

The observation of frequency distribution for selected trait showed transgression beyond the parents and significant differences in traits was observed between two extreme RILs, indicating the occurrence of large variation among RILs under study. The large genetic variation indicates that QTL mapping was likely to reveals QTLs for studied traits.

5.3 Analysis of Genotypic and Genotypic × Environment Variances

The analysis of variance from our experimentation showed highly significant differences among genotypes (RILs) for all the six traits in all the individual environments as

well as across the environments. Agronomic traits such as days to 50 % flowering, plant height, 100-seed weight and grain yield showed highly significant genotypic variances (σ^2g) in all the environments and same trend was continued for combined analysis (across environments) For all the agronomic traits, the genotype \times year (σ^2gy) interactions were found non-significant, while the genotype \times location (σ^2gl) interaction were non-significant for all agronomic traits except grain yield. For all the agronomic traits, the genotype \times year \times location (σ^2gyl) interactions were highly significant, but in less magnitude compared to genotypic variance (σ^2g) except for grain yield.

The grain micronutrient (iron and zinc) concentrations showed highly significant genotypic variances in all the individual environments. Same trend was continued across environments, both the traits showed highly significant genotypic variances, whereas genotype \times year (σ^2gy) interaction for zinc was significant, but less in magnitude compared to genotypic variance. For iron, genotype \times year (σ^2gy) interaction was non-significant. Genotype \times location (σ^2gl) interactions were found to be non-significant for both the micronutrients and genotype \times year \times location (σ^2gyl) interactions were highly significant for both the traits and the magnitude of variances was more than genotypic variances.

The analysis of variances for all the traits revealed that genotypic variances were highly significant in individual environments as well as across environments (combined analysis) indicating high degree of genotypic variance for the traits studied for both micronutrients, genotype \times year \times location (σ^2gyl) interactions were significant and also higher than genetic variances indicating that environment played a significant role in the accumulation of micronutrients in grain.

5.4 Heritability and association between different traits

In the present study all the traits were highly heritable (>0.60) as per scale of Robinson, (1966) However, a partitioned genotype by environment interaction component reduced the heritability for across the environments (pooled analysis). Broad sense heritability for all the traits was high (0.30 – 0.60) across six environments (Table 4.3). Broad sense heritability using pooled data ranged from 0.58 for grain iron concentration to 0.96 for plant height which was the most heritable trait in all the environments. For grain iron and zinc concentrations, the heritability was high in first year (postrainy 2012-13) compared to second year (postrainy 2013-14). Pearson's correlation among phenotypic traits were computed for

each individual environment and across the environments based on BLUPs of each environment and pooled environments, respectively. Based on BLUPs from the individual environments, there was highly significant and high positive association between grain iron and zinc concentration in all the environments ($E_1 = 0.79$, $E_2 = 0.69$, $E_3 = 0.70$, $E_4 = 0.77$, $E_5 = 0.68$ and $E_6 = 0.72$; $p < 0.01$) and this trend was consistent in pooled analysis ($AE = 0.79$; $p < 0.01$). Co-segregation of genes controlling these traits might be the reason for strong association between the minerals. The direction and intensity of association suggested a good possibility of simultaneous genetic improvement of both the micronutrient by co-transferring these traits into the genetic background of elite lines. Based on BLUPs performance across the locations *viz*, ICRISAT, IIMR and VNMKV the difference in the location for two seasons was reflected for grain Fe concentration. The correlation for grain Fe ($r=0.47$) was lower in magnitude indicating low consistency in ranking of RILs across the three locations. Whereas, the correlation for grain Zn ($r=0.77$) indicated high level of consistency in the ranking of RIL across the locations. The differential ranking of RILs for grain Fe concentration across the locations necessitated the G × E and stability analysis for developing Fe rich cultivars in sorghum.

5.5 Parental Polymorphism and Linkage Mapping

Parental polymorphism involving 271 SSR markers spanning all ten sorghum chromosomes was carried out. Out of 271 SSR markers screened, forty five markers were found polymorphic. A set of 6126 (70.15%) polymorphic DArT clones were identified in total of 8732 clones. Whereas, 3331(91.51%) polymorphic SNP clones were identified in total of 3640 clones on the array of 296 B and PVK 801. the linkage map constructed in present investigation was generated with SSRs, DArTs and SNPs which span 1356 cM accommodating 2088 markers. The total length of map was 1356cM (Kosambi), which represent on an average one marker for every 0.64 cM. The individual LGs ranged from 180.6 cM for LG 3 with total number of 257 markers to 102.06 cM for LG 10 with lowest number of markers 79 markers. The average length of linkage group was 135.5 cM with an average of 208.7 loci. The linkage map constructed in this study is more highly saturated in terms of more markers and smaller marker intervals than any previously constructed map involving either RFLPs and/or SSRs. The genome coverage in the present study particularly useful to

select markers for use in whole genome breeding strategies and to saturate genomic regions of interest in other mapping populations.

5.6 QTL Mapping

The QTL mapping experiment was carried out for individual location / season data of total six trials across environment (6 environment pooled data). In order to identify the QTLs the phenotypic data from different locations over two years was used along with available genotypic data of SSR, DArT and SNPs in QTL Cartographer using composite interval mapping (CIM) as mapping function. In E₁ (ICRISAT 12-13) a total of 22 QTLs were detected on linkage group (LG) viz., LG 1, LG 4, LG 6, LG 7, LG 8 and LG 9 for grain iron concentration. In E₂ (IIMR 12-13) only three QTLs were identified which are located on LG 4 and LG 8, In E₃ (VNMKV 12-13) total of 10 QTLs were identified, one on LG 4, four on LG 7 and five on LG 9 and in E₄ (ICRISAT 13-14) total of seven QTLs were identified for grain iron concentration, three QTLs on LG 1 and single QTL on each LG 4, LG 5, LG 6 and LG 7. In E₅ (IIMR 13-14) total of five QTLs were identified, LG 3 and LG 5 were accommodating two QTLs each, while single QTL was identified on LG 7. In E₆ (VNMKV 13-14) nine QTLs were observed from four different Linkage groups *viz.*, LG 2, LG 3, LG 4 and LG 7. For grain Zinc concentration in E₁ (ICRISAT 12-13) total of 22 QTLs were identified and located on five different linkage groups *viz.*, LG 4, LG 6, LG 7, LG9 and LG 10, in E₂ (IIMR 12-13) only two QTLs were identified, single QTL each on LG 4 and LG 7, In E₃ (VNMKV 12-13) total of 17 QTLs were identified on five different linkage groups *viz.*, LG 4, LG 5, LG 7, LG 9 and LG 10. In E₄ (ICRISAT 13-14) total of seven QTLs were identified on four different linkage groups single QTL on LG 4, two QTLs each on LG 5, LG 7 and LG 9. In E₅ (IIMR 13-14) only one QTL was identified on LG 3 and in E₆ (VNMKV 13-14) 12 QTLs were identified one each on LG 2, LG 4, LG 5 and LG 10, three QTLs on LG 6 and five QTLs on LG 7. A total of 21 QTLs controlling grain Fe and Zn were found across six environments pooled analysis. Nine QTLs for grain Fe and 12 QTLs for grain Zn concentration were identified with the phenotypic variance (R^2) ranging from 2.82% to 6.66% and 0.30% to 5.74% respectively. Large variation for QTL analysis was observed in different environments indicating the significant role of environment in the expression of grain iron and zinc, also much variation for both the trait was covered by multiple number of minor QTL, hence to use these QTLs, Marker Assisted Recurrent Selection (MARS) will be the future strategy.

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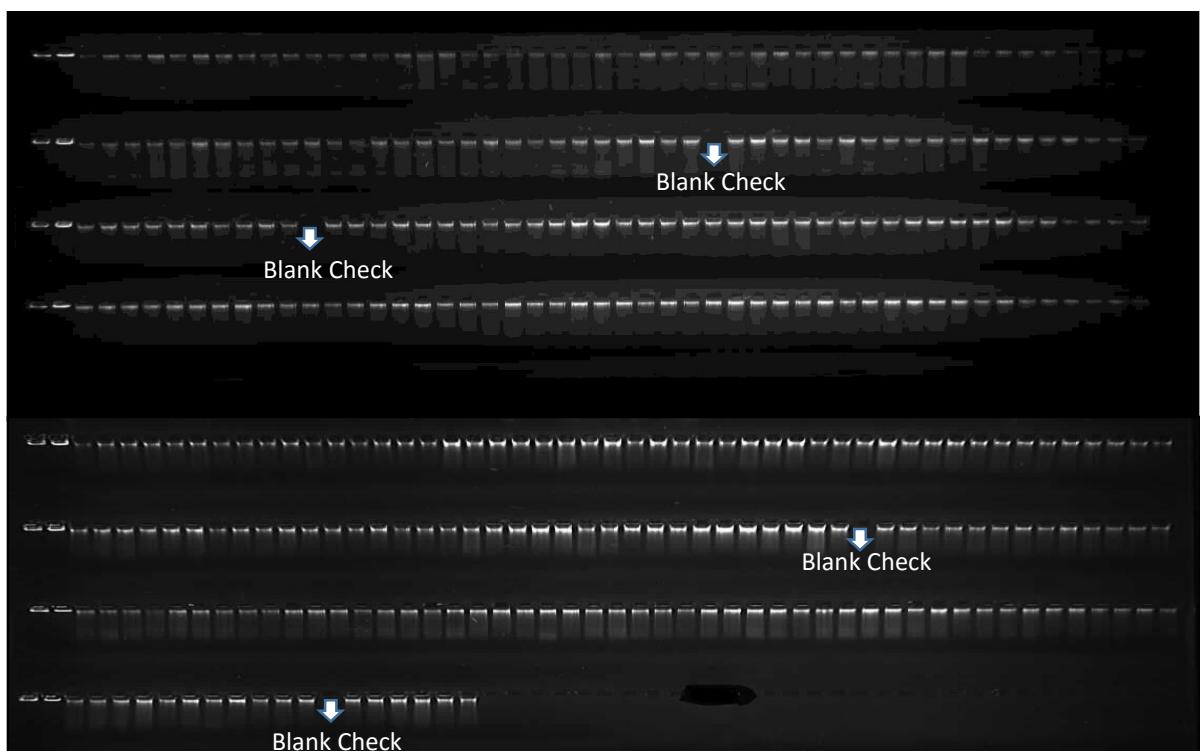
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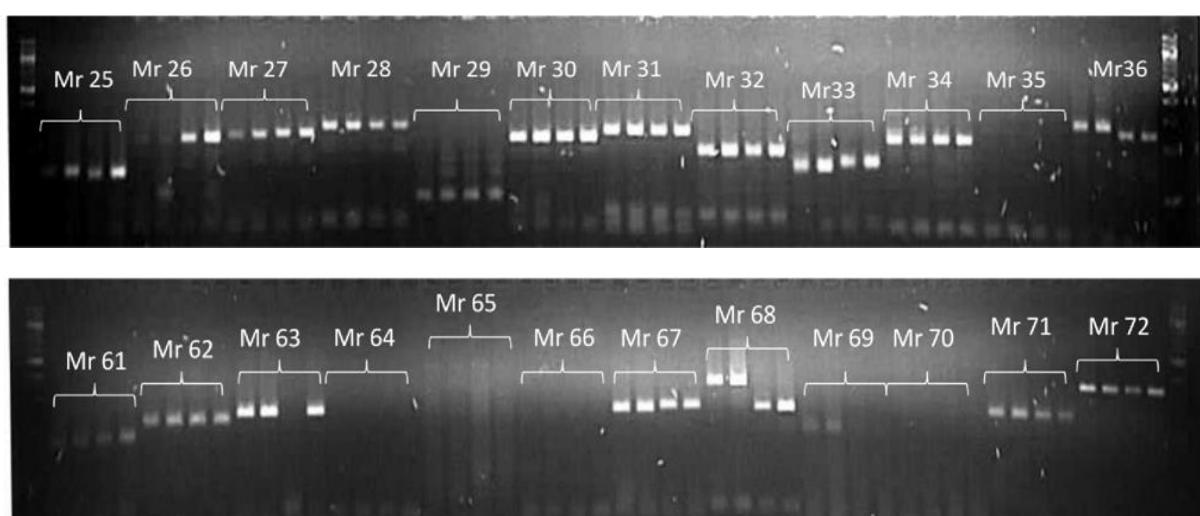
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APPENDIX II

IMAGES



DNA of RIL Population derived from cross 296 B × PVK 801



Survey of parental polymorphism with SSR marker



Phenotypic expression of RIL population after 30 days of germination



Phenotypic expression of RIL population at grain filling stage

APPENDIX I

LIST OF CHEMICALS

1. Agarose (Lonza, USA)
2. Bromophenol blue (sigma)
3. Chloroform (Qualigens)
4. Cetyl Trimethyl Ammonium Bromide (CTAB)(Sigma)
5. dNTPs (Deoxy Nucleoside Triphosphate) (Jonaki, BRIT)
6. Ethylene Diamine Tetra Acetic Acid (EDTA)
7. Ethidium bromide (10 mg / ml)
8. Chilled isopropanol
9. Isoamyl alcohol(Merck)
10. Liquid Nitrogen
11. 2- Mercapto Ethanol (Merck)
12. NaOH pellets(Qualigens)
13. NaCl
14. Nitric Acid (HNO₃ 65%)
15. Phenol(Bangalore Genei)
16. PolyVinyl Pyrrolidone(Sigma)
17. Proteinase K
18. RNase H
19. TaqPolymerase (Sigma)
20. Sodium acetate
21. Sodium chloride(Sigma)
22. Tris base
23. 50 bp ladder(Genei step uptm)
24. 100 bp ladder (Genei step uptm)
25. 70 %Ethanol

BUFFERS AND STOCK SOLUTIONS

CTAB buffer 100ml

2.0 g CTAB (HexadecetylTrimethyl- Ammonium Bromide)

10.0 ml 1M Tris pH 8.0

4.0 ml 0.5 M EDTA pH 8.0 (EthyleneDiamine Tetra Acetic acid)

28.0 ml 5 M NaCl

40.0 ml H₂O

1g PVP 40 (Poly Vinyl Pyrrolidone homopolymer, MW 40,000)

Adjusted all to pH 8.0 with HCl and made the vol. to 100 ml with H₂O

0.5 M Tris Buffer (pH 8.0)

Dissolved 60.55 g of Tris base in 400 ml of distilled water. Adjusted the pH to 8.0 by adding HCl. Adjusted the volume to 500 ml with H₂O. Sterilized using an autoclave.

1M EDTA (Ethylene Diamine Tetra acetic acid)

Dissolved 186.1 g of EDTA, free acid in about 200 ml of distilled water. Adjusted the pH to 8.0 with NaOH and made the vol to 500 ml with distilled water. Sterilized by autoclaving.

Ethidium Bromide

Stock 20mg / ml was prepared by dissolving 1gm of Ethidium Bromide in 50 ml of water.

Chloroform: Isoamyl alcohol (24:1)

Chloroform: isoamyl alcohol (24:1) were mixed and stored at room temperature.

Phenol: Chloroform: Isoamyl alcohol (25:24:1)

Phenol: Chloroform: isoamyl alcohol (25:24:1) were mixed and stored at room temperature.

TAE buffer (Tris / acetate / EDTA) 50X stock solution

242 g Tris base

57.1 ml Glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

Adjusted the pH to 8.3 with acetic acid and made to the volume to 1 lit with distilled water. Sterilization is done by autoclaving.

TE buffer (pH 8.0)

10 mM Tris HCl

1 mM EDTA.

2 ml of 0.5 M Tris-Cl pH 8.0 was mixed with 0.2 ml of 0.5 EDTA, make up to the vol to 100 ml with sterile distilled water.

6X Gel loading buffer

0.25% (W/V) Bromo phenol blue

40% (W/V) sucrose in water

Dissolved 0.25 g of Bromo phenol blue was mixed with 40g of sucrose, make up the vol to 100ml with distilled water.

RNase preparation**RNase buffer**

A. 1M Tris (pH 7.5)

B. 5M NaCl

0.5ml of 1M Tris (final concentration 10mM) and 75 μ l of 5M NaCl (final concentration 15mM) was taken and made the volume to 50 ml. Weighed 25 mg of ribonuclease H into a tube and added RNase buffer to make a final volume of 5 ml. Kept the tube in a boiling water bath for 10 min, cool and made aliquots of 1 ml in 1.5 ml Eppendorf tubes and stored at -20°C.

APPENDIX III

List of SSR markers used for Parental Polymorphism study

Sr no	marker name	Chr	Position	Product size
1	gpsb 089	1	43.88	168
2	Xcup 62	1	68.74	189
3	Xcup44	1	66.88	217
4	Xgap 342	1	1.31	187
5	Xgap256	1	5.43	168
6	Xiabtp183	1	68.51	235
7	Xiabtp255	1	5.37	269
8	Xiabtp278	1	6.55	227
9	Xiabtp450	1	50.67	224
10	Xisep0504	1	24.77	191
11	Xisep0839	1	4.44	192
12	Xisep0949	1	5.37	99
13	Xisep728	1	-	-
14	Xisepl025	1	19.72	201
15	Xisepl028	1	52.06	199
16	Xisepl032	1	-	-
17	Xisepl039	1	27.50	203
18	Xisepl213	1	22.33	197
19	XmbCIR286	1	57.40	116
20	XmSbCIR286	1	57.45	129
21	XmSbCIR306	1	71.04	121
22	Xtxp043	1	50.27	170
23	Xtxp061	1	-	-
24	Xtxp088	1	50.71	143
25	Xtxp208	1	-	-

Sr no	marker name	Chr	Position	Product size
26	Xtxp248	1	72.03	238
27	Xtxp302	1	9.05	179
28	Xtxp340	1	69.74	197
29	Xtxp357	1	23.81	272
30	Xtxp524	1	71.48	249
31	Xcup 63	2	59.10	145
32	Xcup64	2	1.99	220
33	Xiabtp182	2	19.86	141
34	Xiabtp334	2	3.94	243
35	Xiabtp444	2	55.46	240
36	Xiabtp76	2	-	-
37	Xisep0310	2	-	-
38	Xisep0522	2	77.75	331
39	Xisep0701	2	77.62	203
40	Xisep0733	2	70.75	330
41	Xisep0747	2	0.55	205
42	Xisep0835	2	19.89	206
43	Xisep0841	2	70.89	215
44	Xisep0938	2	63.44	205
45	Xisep1145	2	1.99	175
46	Xisepl013	2	9.17	201
47	XmSbCIR238	2	14.75	74
48	XSbAGB03	2	58.13	144
49	XSbAGH04	2	-	-
50	Xtxp 304	2	5.70	302

Sr no	marker name	Chr	Position	Product size
51	Xtxp004	2	13.21	172
52	Xtxp013	2	55.95	119
53	Xtxp050	2	5.08	297
54	Xtxp055	2	47.31	208
55	Xtxp072	2	27.85	122
56	Xtxp080	2	3.86	285
57	Xtxp084	2	4.85	209
58	Xtxp096	2	1.02	197
59	Xtxp201	2	-	-
60	Xtxp211	2	4.99	212
61	Xtxp296	2	71.11	168
62	Xtxp297	2	4.24	219
63	Xtxp304	2	5.71	302
64	Xtxpl97	2	1.45	160
65	XtxplOO(kaf)	2	69.64	116
66	Xcup14	3	72.46	208
67	Xiabtp093	3	15.99	462
68	Xiabtp218	3	17.05	211
69	Xiabtp449	3	51.18	293
70	Xiabtpl64	3	62.98	157
71	Xisep 1248	3	8.38	488
72	Xisep 824	3	73.92	196
73	Xisep0102	3	-	-
74	Xisep0107	3	3.21	198
75	Xisep0132	3	16.20	201

Sr no	marker name	Chr	Position	Product size
76	Xisep0138	3	72.84	-
77	Xisep0843	3	72.15	200
78	Xisep1218	3	4.80	199
79	Xisep1031	3	-	-
80	XisepOllO	3	1.47	290
81	XisepOlOI	3	7.10	210
82	Xtxp033	3	51.37	216
83	Xtxp038(lg)	3	68.16	436
84	Xtxp266	3	2.78	196
85	Xtxp266	3	2.78	196
86	Xtxp336	3	55.39	166
87	Xtxp336	3	55.39	166
88	Xtxp444	3	55.93	185
89	Xtxp457	3	1.91	209
90	Xtxp460	3	17.05	165
91	Xtxp461	3	17.05	211
92	Xtxp461	3	17.05	211
93	Xtxp492	3	3.60	190
94	Xtxp494	3	1.73	213
95	Xtxp494	3	1.08	213
96	Xtxp501	3	14.19	149
97	Xtxp507	3	55.32	194
98	Xtxp518	3	2.66	200
99	XtxplI4	3	60.79	233
100	Xcup28	4	51.25	163

Sr no	marker name	Chr	Position	Product size
101	Xgap 10	4	51.72	250
102	Xgap121	4	0.86	219
103	Xgap1O	4	-	-
104	Xiabtp167	4	9.75	326
105	Xiabtp220	4	50.66	167
106	Xiabtp364	4	10.44	133
107	Xiabtp453	4	48.07	248
108	Xiabtp481	4	8.24	436
109	Xiabtpl44	4	-	-
110	Xisep 224	4	0.70	208
111	Xisep0202	4	4.75	-
112	Xisep0210	4	1.97	-
113	Xisep0228	4	9.99	204
114	Xisep0948	4	0.97	195
115	Xispl0229	4	61.91	26S
116	Xtxp 24	4	60.49	224
117	Xtxp 60	4	60.49	224
118	Xtxp012	4	48.58	192
119	Xtxp012	4	48.58	192
120	Xtxp027	4	67.09	331
121	Xtxp041	4	-	-
122	Xtxp097	4	66.13	129
123	Xtxp104	4	66.13	186
124	Xtxp212	4	61.21	149
125	Xtxp327	4	59.27	156

Sr no	marker name	Chr	Position	Product size
126	Xtxp343	4	48.80	157
127	gpsb 017	5	4.91	186
128	msbCIR 248	5	4.75	90
129	msbCIR 329	5	0.21	116
130	Xiabtp23	5	60.56	245
131	Xiabtp251	5	58.02	234
132	Xiabtp432	5	6.91	140
133	Xiabtp454	5	3.44	155
134	xisep 1129	5	44.02	192
135	Xisepll07	5	10.62	198
136	Xisepll27	5	46.09	211
137	Xisepll33	5	17.52	193
138	Xispl0258	5	-	-
139	XmbCIR 329	5	0.21	116
140	Xtxp 23	5	54.51	182
141	Xtxp014	5	42.27	148
142	Xtxp015	5	42.05	214
143	Xtxp053	5	11.29	364
144	Xtxp303	5	5.73	158
145	Xtxpl23(Kaf2)	5	57.86	278
146	Xgap072	6	41.44	190
147	Xiabtp230	6	59.45	251
148	Xiabtp304	6	3.83	132
149	Xiabtp424	6	58.27	168
150	Xiabtp430	6	60.28	271

Sr no	marker name	Chr	Position	Product size
151	Xiesp0402	6	-	-
152	Xisep0417	6	57.60	272
153	Xisep0422	6	-	-
154	Xisep0432	6	45.91	217
155	Xisep0435	6	62.20	199
156	Xisep0443	6	55.68	188
157	Xisep0444	6	-	-
158	Xtxp 06	6	3.17	119
159	Xtxp 06	6	3.17	119
160	Xtxp 145	6	49.29	238
161	Xtxp057	6	57.42	249
162	Xtxp265	6	51.18	209
163	Xtxp274	6	51.18	333
164	Xtxp521	6	0.45	177
165	Xtxp521	6	0.45	177
166	Xtxpl76	6	55.89	161
167	msbCIR 246	7	56.28	100
168	msbCIR 300	7	58.29	109
169	Xiabtp 280	7	25.70	160
170	Xiabtp032	7	4.76	239
171	Xiabtp280	7	25.70	160
172	Xiabtp297	7	4.69	168
173	Xiabtp360	7	55.97	99
174	Xiabtp379	7	2.90	175
175	Xiabtp92	7	8.35	206

Sr no	marker name	Chr	Position	Product size
176	Xisep 0328	7	5.44	310
177	Xisep0131	7	0.17	-
178	Xisep0439	7	62.91	253
179	Xisep0704	7	63.92	202
180	Xisep0829	7	59.43	176
181	Xisep0831	7	62.91	199
182	Xisep0844	7	62.91	253
183	Xtxp 295	7	61.17	168
184	Xtxp040	7	0.86	137
185	Xtxp278	7	51.12	248
186	Xtxp312	7	4.59	191
187	Xtxp414	7	0.89	174
188	Xtxp525	7	2.34	211
189	Xgap034	8	54.57	203
190	Xgpsb067	8	13.75	179
191	Xiabtp 128	8	40.33	176
192	Xiabtp 128	8	40.32	176
193	Xiabtp 169	8	41.00	220
194	Xiabtp 35	8	6.02	245
195	Xiabtp0349	8	-	-
196	Xiabtp160	8	0.16	202
197	Xiabtp354	8	45.63	159
198	Xiabtp372	8	43.45	226
199	Xiabtp394	8	38.98	329
200	Xiabtp439	8	45.77	284

Sr no		Chr	Position	Product size
201	Xiabtp45	8	3.09	188
202	Xiabtp487	8	16.24	238
203	Xiabtp525	8	16.24	238
204	Xisep0632	8	1.87	189
205	Xisep0809	8	55.44	188
206	Xtxp018	8	50.51	238
207	Xtxp047	8	-	-
208	Xtxp273	8	-	-
209	Xtxp483	8	2.35	242
210	Xcup02	9	8.14	197
211	Xgap 32	9	55.09	179
212	Xgap206	9	59.16	125
213	Xgap206	9	59.16	125
214	Xiabtp174	9	9.07	258
215	Xiabtp307	9	3.67	298
216	Xiabtp321	9	50.57	227
217	Xiabtp417	9	2.33	231
218	Xiabtp425	9	7.60	191
219	Xiabtp475	9	52.17	269
220	Xiabtp51	9	1.19	212
221	Xisep0125	9	59.37	-
222	Xisep0506	9	3.18	208
223	Xisep0506	9	3.18	208
224	Xisep0513	9	-	-
225	Xisep0523	9	47.37	217

Sr no	marker name	Chr	Position	Product size
226	Xisepl014	9	2.59	217
227	Xisepl241	9	8.36	182
228	Xisepll28	9	-	-
229	Xtxp010	9	47.92	144
230	Xtxp10	9	47.92	144
231	Xtxp324	9	45.09	190
232	Xtxp410	9	2.17	158
233	Xtxp412	9	2.99	163
234	Xtxp527	9	7.12	196
235	Xgap 1	10	54.51	251
236	gpsb 027	10	4.00	172
237	msbCIR 262	10	55.32	213
238	msbCIR283	10	18.10	142
239	Xcup 07	10	60.57	270
240	Xcup42	10	1.22	139
241	Xcupl3	10	3.50	199
242	Xiabtp196	10	9.46	198
243	Xiabtp256	10	1.04	199
244	Xiabtp337	10	2.48	112
245	Xiabtp351	10	52.90	114
246	Xiabtp392	10	14.71	178
247	Xiabtp392	10	14.71	178
248	Xiabtp495	10	48.66	204
249	Xiabtp534	10	60.65	149
250	Xiabtpl04	10	6.57	200

Sr no	marker name	Chr	Position	Product size
251	Xisep0603	10	8.92	241
252	Xisep0608	10	2.59	212
253	Xisep0608	10	2.59	212
254	Xisep0614	10	2.11	208
255	Xisep0621	10	46.55	208
256	Xisep0624	10	8.59	187
257	Xisep0625	10	48.10	167
258	Xisep0649	10	59.16	196
259	Xisep648	10	-	-
260	Xispl0263	10	49.67	330
261	XmSbCIR283	10	18.10	142
262	Xtxp129	10	47.14	180
263	Xtxp129	10	47.14	180
264	Xtxp129	10	47.14	180
265	Xtxp217	10	-	-
266	Xtxp309	10	11.00	482
267	Xtxp320 (phyB)	10	55.38	289
268	Xtxp337	10	2.48	112
269	Xtxpl41	10	58.25	162
270	Xisep0327	-	6.79	194
271	Xtxp037	-	55.12	187