“IDENTIFICATION, CHARACTERIZATION AND VALIDATION OF QTL’S SPECIFIC MARKERS RELATED TO GRAIN NUTRITIVE VALUE IN RICE (*Oryza sativa* L.)”

M. Sc. (Ag.) THESIS

by

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DEPARTMENT OF BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
INDIRA GANDHI KRISHI VISHWAVIDYALAYA
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“IDENTIFICATION, CHARACTERIZATION AND VALIDATION OF QTL’S SPECIFIC MARKERS RELATED TO GRAIN NUTRITIVE VALUE IN RICE (*Oryza sativa* L.)”

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CERTIFICATE – I

This is to certify that the thesis entitled **"IDENTIFICATION, CHARACTERIZATION AND VALIDATION OF QTL'S SPECIFIC MARKERS RELATED TO GRAIN NUTRITIVE VALUE IN RICE (*Oryza sativa* L.)"**submitted in partial fulfilment of the requirements for the degree of **"MASTER OF SCIENCE IN AGRICULTURE"** of the Indira Gandhi Krishi Vishwavidyalaya, Raipur, is a record of the bonafide research work carried out by **Mr. VINAY PREMI** under my guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma (Certificate awarded etc.) or has been published/ Published part has been fully acknowledged. All the assistance and help received during the course of the investigations have been duly acknowledged by him.

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CERTIFICATE – II

This is to certify that the thesis entitled **"IDENTIFICATION, CHARACTERIZATION AND VALIDATION OF QTL'S SPECIFIC MARKERS RELATED TO GRAIN NUTRITIVE VALUE IN RICE (*Oryza sativa* L.)"** Submitted by **Mr. VINAY PREMI** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur in partial fulfilment of the requirements for the degree of **M.Sc. (Ag.)** in the **DEPARTMENT OF BIOTECHNOLOGY** has been approved by the external examiner and Student's Advisory Committee after an oral examination.

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Date:

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LIST OF ABBREVIATIONS

%	- per cent
°C	- degree Celsius
µl	- microlitre
AFLP	- Amplified Fragment Length Polymorphism
bp	- Base pair
dATP	- deoxy adenosine 5’ triphosphate
dGTP	- deoxy guanosine 5’ triphosphate
dCTP	- deoxy cytidine 5’ triphosphate
dNTPs	- deoxynucleotide triphosphates
dTTP	- deoxy thymidine 5’ triphosphate
EDTA	- ethylene diamine tetra acetic acid
DNA	- deoxyribo nucleic acid
et al.	- and others
Gm	- gram
h.	- hours
ID	- Iron Deficiency
i.e.	- that is
Kg	- Kilogram
M	- molar
N	- Nitrogen
mg	- milligram
ml	- milliliter
ng	- nanogram
nm	- nanometer
PCR	- polymerase chain reaction
RAPD	- random amplified polymorphic DNA
RFLP	- restriction fragment length polymorphism
rpm	- rotations per minute
SSR	- simple sequence repeats

CHAPTER I

INTRODUCTION

The primary objective of modern agriculture and breeding programmes over the past 50 years has been to increase productivity by increasing yields. However, equally important but often overlooked objectives in breeding crops are the nutrient composition and enhancing nutritional value (Grusak and Dellapenna, 1999). Our food system is failing us globally by not providing enough balanced nutrient output to meet all the nutritional needs of every person, the consequences of which are affecting human health, well being, productivity and livelihood and contributing to stagnating national development efforts in many developing nations. The reflections of which are clearly seen in the estimates of about 800 million people suffering from protein energy malnutrition (PEM) world wide (Graham and Welch, 1999). Further approximately 40% of the world's population suffers from micronutrient deficiencies (hidden hunger) including iron and zinc deficiency, specially women and preschool children (UN SCN,2005) as more than 60% population is deficient in iron and 30% are having zinc deficiency (Philip and Martin, 2009). Poor quality diets, characterized by high intake of staple foods and low consumption of animal and fish products, fruits, legumes, and vegetables is the primary cause of Fe/Zn malnutrition among population having cereal based diets. To overcome micronutrient deficiencies through diet supplementation and food fortification, new approaches are needed to expand the reach of food-based intervention to the rural poor and contribute to sustainable micronutrient deficiency alleviation (Pfeiffer and McClafferty, 2007). Biofortification is an approach that relies on conventional plant breeding and modern biotechnology to increase micronutrient density of staple crops and upholds great promises for adequate attainment of essential dietary nutrients to poor and rural people and in turn improving health of mass (*Harvest Plus, 2008*). Utilization of genetically enriched food with these micronutrients has been suggested as the most cost- effective means of managing malnutrition. Recently, a sustainable solution to mineral malnutrition termed as 'Biofortification" has been proposed of crop plants for enhanced nutrients in the edible portions of crop plants through agronomic intervention or genetic selection. Molecular mechanisms affecting the accumulation of iron and zinc are being investigated in rice, which is accelerating the efforts at genetic manipulation of crop plants. Candidate gene approach is becoming a widespread method for characterizing QTLs (quantitative trait loci) as well as Mendelian traits in both the animal and plant systems. Candidate genes for economically important traits have been potentially useful in plant breeding. Candidate genes have been successfully used in genetic and association mapping, molecular marker-assisted selection and development of transgenic plants for various traits in crop plants.

Rice is not only a major staple food for the world's population but it is also a model species for a major group of flowering plants, the monocotyledonous plants, belonging to the family of grasses, Gramineae. Asia is the leader in rice production accounting for about 90% of the world's production. Over 75% of the world supply is consumed by people in Asian countries and thus rice is of immense importance to food security of Asia. The demand for rice is expected to increase further, keeping in view the expected increase in the population. Rice is also an important vector to be developed as functional food because even small improvements in increasing the nutritional value of rice could have a substantial additive impact (Yassir, 2007). Researchers have reported wide genetic variation for grain Fe and Zn content in genus *Oryza* ranging from 2 -24 µg/g and 4.45 -58.4 µg/g respectively. Yet narrow yet genetic base for grain Fe/Zn trait has been reported in cultivated *O. sativa* species.

Biofortification is a new approach that complements the existing "toolbox" of interventions. It refers to breeding staple food crops which form the mainstay of the diet for large numbers of at-risk people for higher micronutrient levels. Thus the strategy is targeted at those who cannot afford a diet adequate in fruits, vegetables and meats, which are better sources of micronutrients. In principle, once such micronutrient-rich crops are developed and successfully disseminated, they automatically form part of the food chain. Hence, for a largely one-time investment this strategy can produce a constant stream of future benefits to consumers of these crops. Grain Fe/Zn content is known to be a polygenic trait with cumulative phenotypic effect of several genes (Ghandilyan *et. al.,* 2006). The complex polygenic traits are governed by Quantitative trait loci (QTLs) thus identification as well as characterization of QTLs controlling grain micronutrient contents in rice harbors great potential for Markers assisted selection (MAS) and QTLs introgression based breeding approaches to develop nutrient rich rice. In this context the present study entitled "Identification, characterization and validation of QTL's specific markers related to grain nutritive value in rice (*Oryza sativa* L.)." was undertaken with the following major objectives-

1. Phenotyping of parents and mapping population for grain protein and micronutrient

   contents.

2. *In silico* characterization of QTL's controlling grain protein and Fe, Zn content in rice.

3. Genotyping of the mapping population using QTL's specific DNA markers.

CHAPTER II

REVIEW OF LITERATURE

Rice is an important crop which feeds nearly 50 % of the world population. Because of its importance as food crop and its smaller genome size, rice has been extensively used for molecular genetic studies. The genetics of rice facilitates the study of complex traits that are controlled by several genes with minor effects. It has also been important in comparative mapping in graminae species such as wheat, barley, rye, oat and maize. Hence, mapping of genes/QTLs of agronomic importance in rice can be expected to have positive impact on several of the most important cereal crops in the world (McCouch and Doerge, 1995). Most of the economically important traits are polygenic in nature and environment dependent, making the study more difficult. Molecular marker technology has facilitated in dissecting the complex nature of these traits by QTL analysis (Wang *et al.*, 1999). An important goal in genetics and breeding is to identify and characterize QTL for the target traits. QTL mapping and the number of QTLs detected, which mainly depends on the accuracy of the experimental data (both phenotypic and marker data), also depends on other important factors including the model used for its analysis. Thus, locating significant and common QTLs detected across models proves to be an important work in this aspect.

Protein content in rice grain (*Oryza sativa* L.) is an important trait for health of people whose main food in daily life is rice. In order to improve the efficiency of breeding for rice nutrient quality, understanding variation of gene expression in different environments is necessary. Studies have shown that protein content of rice is a quantitative trait (Singh & Singh 1982; Shenoy *et al.*, 1991). HilleRisLambers *et al.*,

(1973) pointed out that the heritability for protein content was 0.130–0.372 due to genotype x environment (GxE) interaction effect. Results by Shenoy *et al.*, (1991) indicated that heritability of protein content was about 58.8%. Lang *et al.*, (2005) found that protein content was controlled by genetic effects with heritability of 25.9%. Through inheritance studies carried out earlier, we noticed that difference in GPC between two parents was due to two major genes, even though a number of QTLs per se may control the trait. In recent years, the potential of molecular marker-assisted selection in plant breeding has been demonstrated in several crops (Huang *et al.,* 1997; Reddy *et al.,* 1997, Lang *et al.,* 1999, 2000, 2001, 2004). However, its utility in the hands of plant breeders has not demonstrated yet in protein, even though molecular markers associated with dozens of genes controlling several traits of economic importance have been developed in this crop. The improvement in grain protein content (GPC) and its composition in protein has been a major concern of plant breeders. It has been difficult to achieve for effective selection criteria and because selection is expensive and time consuming. In this repots, development of one or more molecular markers to be used for indirect selection for protein content / composition should be a convenient alternative.

2.1 Qualities and nutritional properties of rice grain

High grain yield is an important consideration for commercial and private producers, but demand for superior grain quality is increasingly a priority for international export markets in all rice producing areas of the world (Juliano *et al.,* 1990; Unnervehr *et al.,* 1992; Tan *et al.,* 2000). The primary components of rice grain quality include appearance, eating, cooking, milling and nutritional qualities. Each of these components consists of attributes whose values are determined by their physical-chemical properties and other socio-cultural factors such as the history and traditions of the localities where rice is grown. Appearance quality is determined by grain dimension as stipulated by grain length, width, width-length ratio, grain size and shape, and translucency of the endosperm (Unnevehr *et al.,* 1992; Juliano and Villareal 1993). Grain dimension can be used to classify rice into so-called short, medium and long grain types. Medium and short grain cultivars tend to exhibit low amylose content, high gelatinization temperatures, and moist, chewy cooking properties. Long grains generally possess high amylase content and remain separate after cooking with a dry, fluffy consistency (McKenzie and Rutger 1983).

Eating and cooking qualities are primarily influenced by the physical properties of starch in the endosperm, composed of amylose and amylopectin. The amylose content (AC) of rice, recognized as one of the most important determinants of eating and cooking qualities (Bao *et al.,* 2002; Juliano 1985; Webb 1980; Zhou *et al.,* 2003), has been reported to be governed by the waxy (Wx) locus and mapped to chromosome 6 (Tan *et al.,* 1999; Zhou *et al.,* 2003). Other studies have reported AC to be specified by single major gene with modifications by minor genes (Bollich and Webb 1973; IRRI 1976; McKenzie and Rutgers 1983; Okuno *et al.,* 1983; Kumar and Khush 1988). Additional reports have indicated that AC exhibits a complex genetic basis due to the triploid nature of the endosperm that results in additional cytoplasmic and epistatic effects (Mo 1993; Pooni *et al.*, 1993). Three independent studies reported that the waxy locus was linked to a gene for alkali spreading score, a measure of the temperature at which the rice grain becomes gelatinous during cooking (McKenzie and Rutgers 1983; Ghosh and Govindaswamy 1972; Sano 1984).

Milling quality is assessed using three principal characteristics, namely brown rice, milled rice, and head milled rice. Brown rice consists of grains from which the bran has not been removed by milling. Milled rice is made up of whole and broken rice grains that have the bran removed. Head rice, or the proportion of whole kernel including broken kernels that are 75-80 percent of the whole kernel, is a major factor determining rice market value and is one of the most important criteria for milled rice.

Rice is known to be unique among cereals by having a storage protein primarily made of glutelin, which has a more balanced amino acid profile than the prolamine-rich storage proteins found in most cereals (Juliano 1985). Increasing the protein content of rice may increase and balance the protein intake of people who depend on rice as a staple food. Protein content has been shown to exert a direct impact on the chemical and physical properties of cooked rice (Hamaker and Griffin 1990, 1991; Marshal *et al.*, 1990; Juliano 1993; Hamaker 1994).

Rice grain consists of the starchy endosperm, the bran including the embryo and the outer grain layers, and the inedible fibrous hull. The endosperm i.e. the inner part of the grain contains mostly starch and around 4 to 10% protein. The bran is more diverse in its composition and contains protein, lipid, fiber, vitamins, and minerals. The major vitamins present in the rice bran are vitamin E (alpha- tocopherol) and the B-Vitamins (thiamin, riboflavin, niacin). The mineral fraction is mainly composed of phosphorous, potassium, and magnesium (Juliano, 1993). Nutritional composition of rice is shown in (Table: 2.1).

Rice proteins are encapsulated in discrete protein bodies distributed throughout the endosperm. viz large spherical bodies (PB I) which contain lysine poor prolamin protein are found in centre of endosperm (Lookhart and Scott, 2000), they constitute about 20% of milled rice protein and small crystalline protein bodies (PB II) about 60-65% of rice protein having lysine rich glutelin protein, (Klopfenstein, 2000). Rice is considered to have "one of the highest quality proteins among cereals" because of its low prolamin content, even though the 10-kDa prolamin polypeptide in rice is rich in cysteine and methionine (both are sulphur containing amino acids), proline and threonine. Other cereals that have higher quantities of prolamin are considered to be less nutritious than rice because of low nutritious value of prolamin. Prolamin is rich in hydrophobic and uncharged amino acids, which includes glutamine (Chen *et al*., 1995). However, it is poor in essential charged amino acids such as lysine and tryptophan (Coffman and Juliano, 1987).

Table 2.1: Chemical composition of rice grains (100gm)

Rice fraction	Crude protein (gm N x 5.95)	Crude Fat (gm)	Available carbohydrate (gm)	Thiamine (mg)	Riboflavin (mg)	Lysine (mg)	Iron (mg)	Zinc (mg)
Rough rice	5.8-7.7	1.5-2.3	64-73	0.26-0.33	0.06-0.11	3.2-4.7	1.4-6.0	1.7-3.1
Brown rice	7.1-8.3	1.6-2.8	73-87	0.29-0.61	0.04-0.14	3.7-4.1	0.2-5.2	0.6-2.8
Milled rice	6.3-7.1	0.3-0.5	77-89	0.02-0.11	0.02-0.06	3.2-4.0	0.2-2.8	0.6-2.3
Rice bran	11.3-14.9	15.0-19.7	34-62	1.20-2.40	0.18-0.43	4.8-5.4	8.6-43	4.3-25.8
Rice Hull	2.0-2.8	0.3-0.8	22-34	0.09-0.21	0.05-0.07	3.8-5.4	3.9-9.5	0.9-4.0

2.2 Malnutrition

An estimated 2 billion people in the developing world is suffering from the effects of micronutrient malnutrition, in particular iron deficiency anemia, vitamin A deficiency, and zinc deficiency. Micronutrient malnutrition lowers disease resistance, damages cognitive development among children, and increase risks of mortality and morbidity among mothers during childbirth (HarvestPlus 2008). Much of the world's population relies on a few staple foods (rice, maize, wheat, and cassava) that are poor sources of essential nutrients (Jeeyon and Guerinot 2008). Traditional strategies to deliver these minerals to susceptible populations have relied on supplementation or food fortification programs. Unfortunately, these interventions have not always been successful (Philip and Martin 2005). The primary underlying cause of micronutrient malnutrition is poor quality diets. Most of the undernourished are those who cannot afford to purchase high-quality, micronutrient-rich foods. Biofortification of staple food crops is new and powerful approach for alleviating the micronutrient deficiencies. By developing crops whose edible portions are denser in bioavailable minerals and vitamins, biofortification can serve as an additional instrument to existing nutrition interventions in reducing micronutrient malnutrition, and improve human health (HarvestPlus 2008). Rice is the staple food for more than half of the world's population thus; even small improvements in increasing the nutritional value of rice could have a substantial impact on the health of millions of people suffering from micronutrient malnutrition (Yassir 2007). Identification of germplasm with high grain micronutrients and understanding the genetic basis of their accumulation are the prerequisites for manipulation of these micronutrients (Tiwari *et. al.,* 2009). Many researchers have already studied genetic variation for mineral elements in cereal grains such as rice, wheat and maize and reported the narrow genetic base.

However, at the molecular level information on cereals is limited. (Kaiyang *et. al.*, 2008). The identification of alleles and genes involved in the translocation and accumulation of these minerals within plants is of primary importance for the marker assisted selection and biofortification of plants. Quantitative Trait Loci (QTL) analysis is an important starting point for the identification of these genes. It also allows the study and manipulation of the complex agronomic traits. The integration of biometrical and molecular techniques have made it possible to explore the nature of quantitative traits by mapping QTLs. With the advances in genomic research and computer simulation techniques, it is now possible to identify, locate and clone gene groups, constituting a QTL (Sofi and Rather 2007). In this chapter an attempt is made to review the available literature on biofortification and QTL analysis with respect to improve human nutrition.

2.2.1 The burden of iron deficiency in India

The starting point for quantifying the burden of ID is information on the prevalence of anaemia. For maternal mortality it was assumed that 5% of total maternal mortality, which was also taken from IIPS, is due to IDA. The group sizes, i.e. the number of people in the target groups, are based on Census of India data (online at www.censusindia.net). The average remaining life expectancies used are taken from an Indian life table (WHO 2001). Applying these data to the DALY framework yields the current burden of ID in India: an annual loss of 4m healthy life years (3.7m of which are lost through morbidity and 240,000 are lost due to mortality). Even though ID is already an acknowledged public health problem in India, this figure underlines the severity of this deficiency.

2.2.2 Status of malnutrition in Chhattisgarh

In Chhattisgarh about 90 % of children (both boys and girls) of 4-6 years of age suffer by PEM derived underweight (<-2 SD Weight for age), which was comparatively lower in 7-9 and 10-12 years age group children. About 85 % of boys suffered by stunting (<-2 Height for age), which was much higher than girls (47.54%) in 4-6 year age group. Similarly 80% of 4-6 years age group children were affected by wasting (Weight for height) of body mass. The consumption of energy and protein were also much lower among 'Kamar' (a tribal population) children than the RDA (Recommended dietary allowances) of India throughout the age.

2.2.3 Effects of Malnutrition

Malnutrition increases the risk of infection and disease; for example, it is a major risk factor in the onset of active tuberculosis. Malnutrition kills many times more from not getting enough micronutrients, such as iron and zinc, rather than from simply starving to death, as is commonly imagined (Wikipedia, the free encyclopedia). According to Jean Ziegler (the United Nations Special Reporter on the Right to Food for 2000 to March 2008), in 2006, more than 36 millions died of hunger or diseases due to deficiencies in micronutrients this account for 58% of the total mortality in 2006. According to the World Health Organization, malnutrition is by far the biggest contributor to child mortality. Underweight births and inter-uterine growth restrictions cause 2.2 million child deaths a year. Poor or non-existent breastfeeding causes another 1.4 million (Wikipedia, the free encyclopedia). In developing countries under nutrition contributes to 53 percent of the 9.7 million deaths of children under five each year. This means that one child dies every six seconds from malnutrition and related causes (UNICEF, 2006).

More than one-third of the world's population suffer from anaemia; half of it caused by iron deficiency. Endemic infectious diseases exacerbate the incidence of iron deficiency anaemia in developing countries. Iron deficiency adversely affects cognitive development, resistance to infection, work capacity, productivity, and pregnancy. Children of anaemic mothers have low iron reserves, requiring more iron than is supplied by breast milk, and suffer from growth impairment (WHO/UNICEF/UNU, 2001). It is estimated that 800,000 deaths are attributable to iron deficiency anaemia annually (Mayer *et. al.*, 2008). An estimated 684,000 child deaths worldwide could be prevented by increasing access to vitamin A and zinc (WFP Annual Report, 2007*)*. According to the UN Standing Committee on Nutrition Vitamin A deficiency affects approximately 25 percent of the developing world's pre-schoolers. It is associated with blindness, susceptibility to disease and higher mortality rates. It leads to the death of approximately 1-3 million children each year. Worldwide, 1.9 billion people are at risk of iodine deficiency. Iodine deficiency is the greatest single cause of mental retardation and brain damage (UN Standing Committee on Nutrition, 2005). Even moderate iodine deficiency, especially in pregnant women and infants, lowers intelligence by 10 to 15 I.Q. points, shaving incalculable potential off a nation's development (Wikipedia, the free encyclopedia).

Table2.2: Number of undernourished people (million) in 2001-2003

Country	Number of Undernourished (million)
India	217.05
China	154.0
Bangladesh	43.45
Democratic Republic of Congo	37.0
Pakistan	35.2
Ethiopia	31.5
Tanzania	16.1
Philippines	15.2
Brazil	14.4
Indonesia	13.8
Vietnam	13.8
Thailand	13.4
Nigeria	11.5
Kenya	9.7
Sudan	8.8
Mozambique	8.3
North Korea	7.9
Yemen	7.1
Madagascar	7.1
Colombia	5.9
Zimbabwe	5.7
Mexico	5.1
Zambia	5.1
Angola	5.0

Source: FAO, 2007.

2.2.4 Strategies to overcome micronutrient deficiency

Different strategies have been developed to alleviate or prevent micronutrient malnutrition are dietary diversification, supplementation, post-harvest processing, fortification and biofortification (Slingerland *et. al*., 2003).

Dietary diversification represents a combination of actions. One action is the identification of food items (wild and cultivated) with high micronutrient content and bioavailability, and to promote their consumption. When the supply of these foods is low, interventions may aim to increase their availability by promoting cultivation of specific crops or keeping livestock, presuming that the produce is locally consumed (Slingerland *et. al*., 2003).

Supplementation is periodic administration of pharmacological preparations to target groups by way of injection, capsules or tablets (Lotfi *et al.*, 1996; WHO, 1997; International_Life_Sciences_Institute, 1998). For example for iron, it may involve daily consumption of iron containing pills (UNICEF, 2002).

Post-harvest food processing aims to transform primary products into edible, enjoyable, nutritious dishes. In addition it preserves food for storage, distribution, etc. by killing pathogens and by providing an unfavorable environment for pathogen multiplication and growth in case of contamination. Soaking, heating, fermenting etc. lead to chemical and physical changes and inactivation of specific anti-nutritional factors, and can increase micronutrient bioavailability (Slingerland *et. al*., 2003).

Fortification is defined as the addition of (pro) nutrients to foods that are regularly consumed by most of the population. Examples are iodized salt and vitamin A and D enriched margarine. A prerequisite for successful fortification is the availability of basic foods or ingredients that undergo centralized processing so that the fortificant can be added in a controlled and safe manner. These food items must be generally consumed by the target population in such quantities that the risk of excessive intake of the fortificant is negligible (Slingerland *et. al.*, 2003).

However the diets of the poor in developing countries, who live mostly in rural areas, consist largely of inexpensive staple foods, such as rice or maize that provide insufficient nutrition. Furthermore, rising food prices have meant that the poor are less able to afford more nutritious foods that could provide them with needed micronutrients

(Yassir I., 2009). While supplementation and fortification have been the mainstay of strategies to reduce micronutrient malnutrition, the political will, and thus funding, to eliminate hidden hunger through these means has been insufficient. Furthermore, as strategies that work best in urban areas, they are not as effective in reaching the rural poor (Bouis, 2008). Different interventions are although used to control micronutrient malnutrition, and a limited progress has been made to control micronutrient deficiencies in some countries through these strategies, but their overall success remains limited and new approaches are needed, especially to reach the rural poor (Nestel *et. al.,* 2006; Qaim *et. al.,* 2007; Stein *et. al.,* 2008).

Breeding crops to be naturally higher in nutrients through a process called **biofortification**, is a promising new strategy to reduce hidden hunger that has several advantages (Qaim *et. al.,* 2007; Ramaswami, 2007; Yassir I., 2009). First, as a food-based intervention, biofortification uses the very staple foods that the poor are already eating to deliver necessary micronutrients to them. Therefore, biofortified foods are more easily integrated into the livelihoods and diets of the poor. Second, it is an agricultural intervention targeted to rural areas where more than seventy-five percent of the poor in developing countries live, and where access to supplements, fortified foods and other urban-based interventions are limited. Third, a one-time investment in breeding biofortified crops would provide micronutrients far more cost-effectively than through conventional means, which have high annual recurring costs (Yassir I., 2009). For seventy-five million dollars we can provide iron to about thirty percent of South Asia's population – through fortification – for one year. Or, for the same amount of money, we could develop, and distribute, iron-biofortified wheat and rice varieties that could help alleviate iron deficiency in many more people, year after year (Bouis, 2008).

2.2.5 Calculating the impact of biofortification

By increasing the iron content of rice and wheat, biofortification is expected to increase iron intakes, and thereby to decrease the prevalence of ID and IDA. However, before making an assessment of the likely impact of an intervention such as biofortification, it is useful to review dietary patterns in India: cereal consumption in India is high; it averaged 12.7 kg per capita per month (pcpm) in rural areas, and 10.4 kg pcpm in urban areas. Rice and wheat constitute the bulk of the cereals consumed: in rural areas rice consumption averaged 6.8 kg pcpm and that of wheat averaged 4.5 kg pcpm (India 2001). This is why in this study the focus is on rice yet, there are distinct geographical patterns in dietary intakes: in some regions rice predominates, in others wheat does, and in yet others both are consumed. Given this background, biofortifying rice and wheat can be expected to contribute significantly to increased iron intakes. However, iron-rich crops are not yet out on the farmers' fields and on the consumers' plates. Therefore any assessment of a potential impact of such an intervention must necessarily be *ex-ante* in nature. It also depends critically on the assumptions made, *inter alia,* about the likely micronutrient content of the new wheat and rice varieties, and their acceptance by farmers and consumers, under both optimistic and pessimistic scenarios, so as to capture the range of possible outcomes. The scenarios reflect the expert input of breeders, agronomists, agricultural economists and other scientist at the centers of the Consultative Group on International Agricultural Research (CGIAR), who are working on biofortification research.6 According this information, an increase in iron content of

100-167% for rice compared to current varieties is deemed to be a reasonable assumption; for wheat the assumed range is 20-60%. As the additional iron will be bred into the biofortified varieties by means of non-transgenic methods, the iron compounds will be the same as in existing varieties and there is no reason to assume that the bioavailability of this additional iron changes; it is simply more of the same. For the coming years it is assumed that the "iron trait" will be bred into more and more varieties and the estimates of the experts indicate that after 20 years iron-rich rice can reach a share of 20-50% in total rice production and iron-rich wheat can reach a share of 30-50% in total wheat production.7 Note that the biofortification strategy explicitly involves breeding nutrient-dense *and* agronomically-superior varieties to facilitate adoption among farmers. As these are expected to be developed in collaboration with national agricultural research systems as part of ongoing research efforts, seed prices should be unaffected and hence not be a deterrent to adoption.

2.3 QTL mapping of grain quality traits

The genetic basis of segregation distortion in rice was first reported to be associated with the occurrence of a gametophytic gene (ga-1) on chromosome six (Iwata *et al.*, 1964). Nakagahra (1972; 1986) further reported the presence of ga-2 and ga-3 on chromosome three. Lin *et al.*, (1992) showed that segregation distortion may be due to abortion of male and female gametes or selective fertilization in certain interspecific or inter-sub specific crosses. The work of Nakagahra (1972) led to the suggestion that the gametophytic loci were responsible for the partial or total elimination of gametes carrying one of the parental alleles. Segregation distortion at a marker locus would occur as a result of linkage between the marker and the gametophyte gene (ga) which would confer low pollinating ability (Nakagahra 1972). Xu *et al.,* 1997 reviewed the occurrence of segregation distortion across a wide range of species and mapping populations. Recombinant inbred lines (RILs) were found to display the highest frequencies of distorted markers (40 percent) compared to F2, doubled haploid (DH) or backcross populations (20-30 percent). In the RILs there are more cycles of meiosis compared to DH lines and F2, thus giving more opportunity for segregation distortion in RILs than F2s or DH lines.

Segregation distortion has been identified as a problem often encountered in mapping that can produce deviations of single locus segregation ratios from expected frequencies. (Lyttle 1991; Zivy *et al.,* 1992). Several authors have discussed methods to test linkage or estimate recombination frequencies between genes showing aberrant segregation ratios (Bailey 1949; Garcia-Dorado and Gallego 1992; Lorieux *et al.,* 1995). The classical estimate of linkage (Garcia- Dorado and Gallego 1992) is defined as the ratio of the number of recombinant individuals over the total number of individuals in a population. In this case, the proportionality between the expected frequencies of the parental and recombinant classes remains the same. The Bailey's estimate takes into consideration the viability of the dominant allele relative to the recessive and uses maximum likelihood procedures to estimate linkage. (Lorieux *et al.,* 1995) examined various models developed for estimating recombination fractions for backcrosses and found that Bailey's estimate was more consistent and efficient than the classical estimate of Garcia-Dorado (1992). Bailey's method is used for statistical software like Mapmaker (Lander *et al.*, 1987), Joinmap (Stam 1993), QTL Cartographer (Zeng 1994), and others have been designed for constructing genetic maps. However, these programs are designed for markers that exhibit Mendelian segregating ratios. There is no option in these programs to adjust for genetic markers which show deviations from the expected Mendelian frequencies. The software program “Mapdisto” was developed by Lorieux *et al.*, (2000) for the analysis of molecular marker data showing aberrant segregating ratios.

Sterility barriers between *O. sativa and O.glaberrima* in early hybrid generations have limited the transfer of useful genes between these species (Jones *et al.*, 1996; Second 1984). Heuer and Miezan (2003) recently assessed hybrid sterility in an *O. glaberrima* x *O. sativa* crosses using a segregating BC2F3 population, and microsatellite markers mapped to the waxy promoter region of chromosome six. These researchers found that fertile plants were homozygous for the microsatellite marker while semi-sterile plants were heterozygous, suggesting the presence of a gene causing hybrid sterility. Despite the sterility barriers that hinder *O. sativa* x *O. glaberrima* crosses, natural gene flow between *O. glaberrima* and *O. sativa* or *O. longistaminata* have been reported and analyzed by several workers (Takeoka 1965; Second 1984 and De Kochko 1987). Several useful genes from *O. glaberrima* germplasm have been introgressed into adapted *O. sativa* cultivars. For example, Jones *et al.*, 1997 characterized 1,130 accessions of *O. glaberrima* that harbored several desirable morphological and agronomic traits including seedling vigor, growth duration, plant height, panicles m-2, and grain shape, a determinant of milling quality. Substantial levels of transgressive segregation were detected for these traits among progenies of *O. sativa* and *O. glaberrima* crosses. Good milling and eating quality traits were also found in this population (WARDA 1998). Moreover, accessions of *O. glaberrima* often have shown superior attributes for several agronomic traits under poor management conditions as well as resistance to biotic (viruses, nematodes and insects) and abiotic (acidity, iron toxicity, drought) stresses (Attere and Fatokun 1983; Reversat and Destombes 1998). The recently released NERICA varieties for West Africa, derived from *O. sativa* x *O. glaberrima* hybrids, show increased resistance to several pests, enhanced levels of protein, and a three-fold increase in grain yield over traditional *O. glaberrima* varieties (Linares 2002).

In most rice breeding programs, a small core of adapted lines are used repeatedly to develop varieties. Consequently, the genetic base of rice in most rice producing countries is narrow. There is need to widen this genetic base through introgression of genes from wild relatives of rice. These studies suggest that interspecific hybridization of the African (*O. glaberrima*) and Asian rice (*O. sativa*) can help provide useful variation for future advances in rice grain quality. In this study, a molecular marker-based analysis of QTL for traits that determine milling and grain quality was carried out using DH lines derived from the cross between *O. sativa* and *O. glaberrima*. These six traits include milling quality, protein content, amylose content, alkali spreading score, grain length, grain width and length/width ratio. The primary objective of this research was to map the QTLs for milling, eating and cooking qualities of rice using a population of DH lines derived from the interspecific cross between African (*O. glaberrima,* IRGC 103544) and Asian rice (*O. sativa* cv. Caiapo). Information about molecular markers tightly linked to QTLs that control these traits will facilitate breeding strategies to improve rice milling, eating and cooking qualities. In addition, detection and assessment of segregation distortion are needed for accurate QTL mapping involving the DH material. Finally, detection of *O. glaberrima*-derived QTLs and their comparison with previous studies will shed light on the potential of African rice as new sources for enhanced grain quality traits.

2.3.1 QTL based approach to develop micronutrient rich rice

Many agriculturally important traits such as yield, quality and some forms of disease resistance are controlled by many genes and are known as quantitative traits (also 'polygenic,' 'multifactorial' or 'complex' traits). The regions within genomes that contain genes associated with a particular quantitative trait are known as quantitative trait loci (QTLs) (Collard *et. al.*, 2005). A major breakthrough in the characterization of quantitative traits that created opportunities to select for QTLs was initiated by the development of DNA (or molecular) markers in the 1980s (Collard *et. al.,* 2005). One of the main uses of DNA markers in agricultural research has been in the construction of linkage maps for diverse crop species. Linkage maps have been utilized for identifying chromosomal regions that contain genes controlling simple traits (controlled by a single gene) and quantitative traits using QTL analysis (Mohan *et al.*, 1997). The process of constructing linkage maps and conducting QTL analysis–to identify genomic regions associated with traits–is known as QTL mapping.

2.3.2 Methods of QTL identification

Three widely-used methods for detecting QTLs are single-marker analysis, simple interval mapping and composite interval mapping (Liu, 1998; Tanksley, 1993). Single marker analysis (also single-point analyses) is the simplest method for detecting QTLs associated with single markers. The statistical methods used for single-marker analysis include *t*-tests, analysis of variance (ANOVA) and linear regression. Linear regression is most commonly used because the coefficient of determination ($R2$) from the marker explains the phenotypic variation arising from the QTL linked to the marker. This method does not require a complete linkage map and can be performed with basic statistical software programs. However, the major disadvantage with this method is that the farther a QTL is from a marker, the less likely it will be detected. This is because recombination may occur between the marker and the QTL. This causes the magnitude of the effect of a QTL to be underestimated (Tanksley, 1993). The use of a large number of segregating DNA markers covering the entire genome (usually at intervals less than 15 cM) may minimize both the problems (Tanksley, 1993).

The simple interval mapping (SIM) method makes use of linkage maps and analyses intervals between adjacent pairs of linked markers along chromosomes simultaneously, instead of analyzing single markers (Lander & Botstein, 1989). The use of linked markers for analysis compensates for recombination between the markers and the QTL, and is considered statistically more powerful compared to single-point analysis (Lander & Botstein, 1989; Liu, 1998). More recently, composite interval mapping (CIM) has become popular for mapping QTLs. This method combines interval mapping with linear regression and includes additional genetic markers in the statistical model in addition to an adjacent pair of linked markers for interval mapping (Jansen, 1993; Jansen & Stam, 1994; Zeng, 1993, & 1994). The main advantage of CIM is that it is more precise and effective for mapping QTLs compared to single-point analysis and interval mapping, especially when linked QTLs are involved (Collard *et. al.*, 2005).

2.3.3 QTL to gene approach

Efficiency of marker-aided selection in breeding programs depends on the strength of linkage between molecular markers and the target trait. QTL to gene approach is a method to identify a novel gene from the QTL sequence of specific traits. Traditionally, anonymous molecular markers are used to establish linkage with a phenotype. However, even for tightly linked markers, the effectiveness of marker aided selection is greatly diminished by the occasional uncoupling of the marker from the trait during many cycles of meiosis in a breeding program. With the availability of large genome databases, it is now possible to predict putative function of a gene based on sequence information, thus enabling the identification of candidate genes involved in a particular biochemical pathway. These candidate genes, or DNA sequences with predicted function, are used as molecular markers to associate with phenotypes expressed in segregating populations or genetic stocks (Thorup *et al.*, 2000).

To systematically associate function with available candidate gene sequences from multiple species, it would be useful to locate them onto a frame map with a maximal amount of phenotypic information. Simply locating candidate genes to chromosomal regions with mapped phenotypes does not confirm the function of the gene; however, this approach provides an efficient way to narrow down a few candidate sequences that can be tested by detailed genetic analyses using appropriate mapping populations and mutants (Collins *et al.*, 1998).

2.3.4 Computational genomics based primer designing

In the last 10 to 15 years the computer has become an essential companion for cell and molecular biologists. Bioinformatics is an emerging scientific discipline that uses information technology to organize, analyze, and distribute biological information in order to answer complex biological questions. Bioinformatics is an interdisciplinary research area, which may be broadly defined as the interface between biological and computational sciences (Singh and Kumar, 2001). It involves the solution of complex biological problems using computational tools and systems. It also includes the collection, organization, storage and retrieval of biological information from databases. Selection of oligonucleotide primers is useful for polymerase chain reaction (PCR), oligo hybridization and DNA sequencing. Various bioinformatics programs are available for selection of primer pairs from a template sequence. The plethora programs for PCR primer design reflects the central role of PCR in modem molecular biology. The use of software in biological applications has given a new dimension to the field of bioinformatics. Many different programs for the design of primers are now available (Abd-Elsalam 2003) like batch primer 3 (*www.Batchprimer3.org*), primer 3 etc.

DNA Template and oligonucleotide primers must be considered in greater detail (Linz *et al*., 1990). Efficacy and sensitivity of PCR largely depend on the efficiency of primers. The ability for an oligonucleotide to serve as a primer for PCR is dependent on several factors including: a) the kinetics of association and dissociation of primer-template duplexes at the annealing and extension temperatures; b) duplex stability of mismatched nucleotides and their location; and c) the efficiency with which the polymerase can recognize and extend a mismatched duplex. The primers which are unique for the target sequence to be amplified should fulfill certain criteria such as primer length, GC%, annealing and melting temperature, 5' end stability, 3' end specificity etc. The primer sequence determines several things such as the length of the product, its melting temperature and ultimately the yield. A badly designed primer can result in little or no product due to non-specific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation (Dieffenbach *et al*., 1995). Following guidelines needed to be considered before designing gene and QTL specific primers.

Primer length: Since both specificity and the temperature and time of annealing are at least partly dependent on primer length, this parameter is critical for successful PCR (Wu *et al.,* 1991). For broad-spectrum studies, primers of typically 18-30 nucleotides in length are the best (Abd-Elsalam 2003). **Melting Temperature (Tm):** The optimal melting temperatures for primers in the range 52-58^{o}C, generally produce better results than primers with lower melting temperatures. Primers with melting temperatures above 65^{o}C should also be avoided because of potential for secondary annealing (Abd-Elsalam 2003).

GC Content: GC% is an important characteristic of DNA and provides information about the strength of annealing. Primers should have a GC content between 45 and 60 percent (Dieffenbach et al., 1995). **Dimers and false priming cause misleading results:** Primers should not contain complementary (palindromes) within themselves; that is, they should not form hairpins (Breslauer *et al.,* 1986).

2.3.5 BatchPrimer3: A high throughput web application for PCR and sequencing primer design

BatchPrimer3 is a new web primer design program, developed based on Primer3. BatchPrimer3 adopted the Primer3 core program as a major primer design engine to choose the best primer pairs. A new score based primer picking module is incorporated into BatchPrimer3 and used to pick position-restricted primers. BatchPrimer3 v1.0 implements several types of primer designs including generic primers, SSR primers together with SSR detection, and SNP genotyping primers (including single-base extension primers, allele-specific primers, and tetra-primers for tetra-primer ARMS PCR), as well as DNA sequencing primers. DNA sequences in FASTA format can be batch read into the program. The input sequences can be pre-processed and masked to exclude and/or include specific regions, or set targets for different primer design purposes as in Primer3Web and primer3Plus. BatchPrimer3 program produces four parts of outputs: a main HTML page containing the primer design summary of all input sequences, an HTML table page listing all designed primers and primer properties, a tab-delimited text file with the same contents in the HTML table page, and a detailed primer view page for each sequence with successfully designed primers. A simple click on the links on the main HTML page or HTML table page will display the primer view. The primer list can be directly saved as a text file or an Excel file for further editing or primer ordering. All primer design results can be downloaded as a zipped file. Thousands of primers, including wheat conserved intron-flanking primers, wheat genome-specific SNP genotyping primers, and *Brachypodium* SSR flanking primers in several genome projects have been designed using the program and validated in several laboratories (You, 2008).

2.4 Molecular Markers and their use

The basic theory of using genetic markers to manipulate loci controlling plant traits was introduced by Sax in 1923 (Mazur and Tingey, 1995) who reported the association of quantitatively inherited seed size with simply inherited genetic markers that governed seed coat pigmentation and pattern in common bean (*Phaseolus vulgaris* L.). Subsequent reports of linkage between single gene markers and quantitative trait loci (QTLs) used morphological mutations as genetic markers, the nature of which posed major limitations for the study of quantitative variation (Rasmusson 1953; Thoday 1961). In these studies, only a few such markers were available in any given cross, and the effect of marker genes on quantitative traits was often larger than that of the linked QTLs, making it difficult to effectively study quantitatively inherited traits extensively (Tanksley *et al.*, 1998). Application of these association studies was therefore limited by the lack of available segregating genetic markers.

Recent advances have produced segregating genetic markers in many crop species including rice. Earlier studies on linkage of simply inherited genes wit quantitative traits provided the insight into the potential of markers. However, the slow progress made in the use of morphological markers was due to limited number of markers available and the undesirable effects on phenotype of many of the morphological markers.

Later development led to the use of isozymes as genetic marker, which are multiple forms of enzymes arising from genetically determined differences in primary structure. .Isozymes were used successfully as markers to identify QTLs in maize (Stuber and Edwards 1986). However, the low number of markers available reduced the utility of isozymes as markers.

DNA markers have been used in a number of crops to determine the number of genes controlling trait inheritance and for gene tagging (Anderson *et al.*, 1993). The utility of DNA based markers is determined to a large extent by the technology that is used to reveal DNA polymorphism (Mazur and Tingey, 1995). The available assays fall into two broad categories, *viz*. restriction enzyme based assays and DNA amplification based assays. The first category comprises restriction fragment length polymorphism (RFLP) which detects DNA polymorphism through restriction endonuclease digestion followed by visualization via DNA blot hybridizations. Many researchers still prefer to use RFLP markers because each polymorphic co-dominant allele at a locus is detected in the assay. Moreover, they have been found useful for detecting locus-specific polymorphisms across species boundaries (Liu *et al.*, 1994). Restriction fragment length polymorphisms (RFLPs) have been useful for constructing saturated genome maps in rice (Panaud 1992; Causse *et al.*, 1994). However, these markers require relatively large amounts of DNA for the assay, are time consuming, labor intensive, and therefore only low-resolution maps have been developed (O'Brien, 1993; Liu *et al.*, 1994).

Different types of DNA amplification-based markers have been developed over the years for use in rice studies, including Randomly Amplified Polymorphic DNA (RAPD) (Virk *et al.*, 1995), Amplified Fragment Length Polymorphism (AFLP) and microsatellites (McCouch *et al.*, 1997). The discovery that short primers (usually 10-mers) of an arbitrary nucleotide sequence could be used to amplify segments of genomic DNA from a wide variety of species led to the application of RAPD technology. RAPD markers are faster and easier to assay than RFLPs, and only a very small amount of DNA template is required for RAPD assays. Nevertheless, the major limitation of RAPD is that it shows dominant inheritance and marker/marker homozygotes cannot be distinguished from marker/null heterozygotes. AFLP has been recognized as a reliable and efficient DNA marker system compared to RFLP and RAPD methods. AFLP markers have been found useful in the study genetic diversity of different plant species due to high reproducibility and throughput.

The AFLP technology is not species specific and so can be used as an alternative to microsatellites markers. Moreover, AFLP has a high number of polymorphic marker fragments amplified by PCR in one reaction, resulting in a sufficient genome coverage using only a small number of primer combinations. However, AFLP markers are dominant, and this is a major drawback for genetic analysis, unless a high number of loci are investigated.

Microsatellite markers, also known as simple sequence repeats (SSR), are short, tandemly-repeated DNA sequences that are very abundant in the genome of eukaryotes including rice. SSR markers are codominant, require only a small DNA template, and are technically simple to assay (Wang *et al.*, 1994; McCouch *et al.*, 1997). Microsatellites are evaluated using PCR primers targeted to unique sequences flanking a microsatellite motif. The resulting PCR products are separated according to size by gel electrophoresis using either agarose or acrylamide gels or more recently by capillary electrophoresis systems. Because microsatellites are randomly distributed throughout the rice genome, saturated genome coverage would be possible using these markers. A major limitation of the microsatellite marker is the tendency for physical breakage within the extended repeat motif which may lead to smaller length of some motifs (Yang *et al.* 1994). A positive relationship between the length of microsatellite motif and allelic diversity in rice can occur, so small sized, broken motifs would be of limited utility in the study of allelic diversity. Moreover, microsatellites are species specific.

The use of molecular markers to enhance rice breeding efforts has great potential due to the availability of different types of polymorphic markers, genetic maps and on-line database resources (examples: Ricegenes (http://stein.cshl.org/ricegenes.html) and Gramene (http://www.gramene.org)). Molecular markers may be effectively utilized in a breeding program depending on the value, ease and cost of measurement and, the nature of the genetic control of the quantitative trait. Various markers have been developed and used recently to identify loci within the rice genome affecting agronomic traits (Paterson et al. 1988; Paterson et al. 1996; Moncada et al., 2001; Yu *et al.,* 2002; Hua *et al.*, 2002)..

Moreover, reported QTLs have not been adequately tested under different environments, so a more comprehensive combination of useful markers and genetic approaches are needed to accurately and predictably identify quantitative traits. Non-parametric methods, discussed below, do not strictly demand normal distribution of variables, and have been developed to analyze both quantitative and categorical data (Gnanadesikan, 1987; Hand, 1997). These methods and the advances in computer technology leading to the availability of fast and very powerful computers have increased the opportunities for application of multivariate classification techniques.

2.4.1 Identification of co-localized ESTs

Genomic sequences underlying OsFRO2 and OsZIP9 genes were analyzed for co-localization of identified ESTs. PASA (Program to Assemble Spliced Alignments) program tool available at TIGR rice genome browse (http://www.tigr.org/tdb/e2k/osa1/dnav/) and EST database at RGP website (http://rgp.dna.aafrc.go.jp/E/publicdata/estmap2001/) were used to identify co-localized ESTs. It resulted in incorporation of high quality ESTs by transcript alignment in a genomic and full length cDNA (fl-cDNA). ESTs identified for each gene were further characterized for respective expression tissue library using digital northern and anatomy viewer search tools. ESTs corresponding to a tissue library provided information about putative site of expression of the metal related genes in which it was identified. The minimal alignment allowed by the PASA program is 95% identity over 90% length of the transcript (Chandel *et al.*, 2010).

CHAPTER III

MATERIALS AND METHODS

The present investigation entitled "**Identification, characterization and validation of QTL's specific markers related to grain nutritive value in rice (*Oryza sativa* L.)"** was carried out at Department of Biotechnology, Indira Gandhi Krishi Vishwavidyalaya, Raipur. The details of the experiment are explained below.

3.1. Materials

The plant material used for this study includes rice genotypes Kranti, E2035 and the F_3 population of 50 lines derived from the cross between Kranti and E2035. Seeds of both parents and population were shown in pots under green house condition for 2-3 weeks, in the Department of Biotechnology.

3.2 Methods

3.2.1 Processing of rice grains.

Before analyzing the rice samples for total grain protein, iron and zinc content, the seeds of both parent and populations were subjected to dehusking.

3.2.1.1. Dehusking

Around 50 gm seeds of each samples were hand dehulled using polyurethane coated hand dehulling unit to avoid metal contamination.

3.2.2 Estimation of protein

Total protein content of brown rice grains of all samples were estimated by modified micro-Kjeldahl method (Johri *et al.,* 2000). The details of the procedure are as under:

3.2.2.1 Digestion process

About 0.5 gm of rice grain was transferred into the digestion tube and 5-7 gm of K_2SO_4 and C_uSO_4 mixture was added. 10 ml of concentrated sulphuric acid was added and digestion tubes were placed on the digestion block with temperature set at 360 $^{\circ}$C and then increased to 400 ºC. After 2 to 3 hours when the samples color turned light green, the digestion tubes were taken out of digestion block. The tubes were allowed to cool at room temperature.

3.2.2.2 Distillation process

Digested samples were subjected to Pelican make distillation unit and Distillation of samples was carried using 4% Boric acid and 40% Sodium hydroxide. 10 ml of Boric acid was then taken in conical flask, to which 2-4 drops of mixed indicator dye was added. The flask was beneath the condenser with the delivery tip immersed in the solution. The digested samples were transferred to distillation apparatus and 8-10 ml of 40% Sodium hydroxide was added to it. Around 20 ml of distillate was collected in a conical flask. A blank was always run containing the same quantities of the entire reagent but without the sample for every set of nitrogen determination.

3.2.2.3 Titration process

The distilled samples were titrated against the 0.05 N Sulfamic acid until the first appearance of violate color as the end point. The titer value was used to calculate percent Nitrogen, which is then used to estimate total protein content by using conversion factor 5.95 (Julliano, 1993).

$$\text{Nitrogen \%} = \frac{(Vol.\ of\ Sulfamic\ acid - Vol.\ of\ blank)\ X\ Normality\ X\ 14\ X\ 100}{Sample\ weight\ (gm)\ X\ 1000}$$

Protein % = % N X 5.95

3.2.3 Estimations of iron and zinc

Whole brown grains were subjected to di-acid mixture based digestion as described below in triplicate (Fig. 3.2). Iron and zinc content was estimated by using standard method described under HarvestPlus, (2006) guidelines using Atomic absorption spectrophotometer (AAS200). The protocol for step-wise procedure is as follows.

1. 1 gm of rice grain of each rice genotype under study was placed in a 250 ml digestion tube and 10 ml of di-acid mixture (HNO_3, $HClO_3$) was added and the content mixed by gentle shaking.
2. Digestion was carried out at temperature 280^0C in a digestion chamber.
3. Completion of digestion was confirmed when the liquid turns colorless.
4. After cooling, the solution was filtered through a Watsman's No. 40 filter paper and volume of the filtrate was made up to 50 ml using double distilled water.
5. Aliquots of this solution were used for determination of Fe and Zn using atomic absorption spectrophotometer.

3.2.3.1 Reagents and solutions for iron and zinc estimation

1. Acid mixture

   900 ml Nitric acid (HNO_3),

   400 ml Perchloric acid (70%) ($HclO_3$) (All glass distilled or A.R. grade)

2. Double distilled water (for volume make up)

3.2.3.2 Formula for calculation of Iron and Zinc in ppm

ppm= $$\frac{(sample\ concentration - blank\ concentration) X\ Dilution\ factor}{Sample\ weight\ (g)}$$

Sample conc.: iron /zinc concentration as determined for each sample by AAS200

Blank conc.: iron/zinc concentration of blank solution

Dilution factor: 50

Sample weight: weight of grains in g (1g)

3.2.4. Statistical analysis

The data obtained in present study was statistically analyzed using randomized block design, for checking genetic differences within these advanced breeding lines. The formulas for different parameters are given below:

3.2.4.1 Standard deviation (SD)

Standard deviation is the root of sum of squares of deviation divided by their number, Calculated by the formula.

Standard deviation = $\sqrt{\frac{\Sigma d^2}{n}}$

3.2.4.2 Coefficient of variation (CV)

Coefficient of variation in percentage was calculated by the formula;

CV (%) = $\frac{standard\ deviation\ x\ 100}{Mean}$

3.2.4.3 Coefficient of correlation

Coefficient of correlation is the numerical measure of the amount of correlation existing between the two variables and is calculated by the formula

r = $\frac{\Sigma xy}{n\ \sigma x\ \sigma y}$

3.2.4.4 Standard error (SE)

Standard error = $\frac{S}{\sqrt{n}}$

3.2.5.1 Characterization of QTLs controlling grain protein content in rice

Four QTLs namely qPRO-1, qPRO-2, qPRO-6, and qPRO-11 reported to govern high grain protein content in rice by (Parveaz Sofi and A.G. Rather 2007) were selected based on their higher phenotypic variance and lower LOD value. These four QTLs are located on chromosome 1, 2, 6, and 11 of rice respectively (Table 3.1).

Table 3.1 List of QTLs present between the markers.

S. n.	Name of QTLs	Markers Interval
1	qPRO-1	RM 226 –RM 297
2	qPRO-2	RM 6 – RM 112
3	qPRO-6	RM 190 – RM 253
4	PRO-11	RM 209 – RM 229

The positions of markers flanking the QTL region were taken from the TIGR (The institute of Genomic Research) genome browser (*www.tigr.org.in*)and Gramene database (*http://www.gramene.org*). The region underlying QTLs between two markers in ordered BAC clones were downloaded in FASTA format. The sequences were further analyzed for the *in silico* identification of novel SSR loci (*http://www.gramene.org/db/searches/ssrtool*).

3.2.5.2 Identification of putative SSRs

Simple sequence repeats (SSRs) loci were identified in the genomic region of QTL controlling grain protein content. The query genomic DNA sequences were analyzed using Batchprimer3 website (*www.Batchprimer3.org*) for SSR Screening primers.

3.2.6 Designing of primers

SSR primers were designed with 12 selected putative SSR motifs using BatchPrimer-3 software (*www.Batchprimer3.org*). Primer designing was done by putting the nucleotide sequences in FASTA format with desired specifications for GC content, annealing temperature (Tm values), primer length and length of amplified fragments with other parameters as default setting (Table 3.2).

Table 3.2 Specifications for primer designing

S. n.	Criteria	Optimum	Range
1	Length of target sequence to be amplified	150 bp	100-300bp
2	Tm	55^0C	50-60^0C
3	GC content	55%	50-60%
4	Length of primer	20bp	18-22bp

3.2.7 SSR based genotyping of mapping population

The 50 lines belonging to F_3 population derived from cross between Kranti and E2035 were used to obtain SSR genotypic data.

3.2.7.1 Genomic DNA extraction

Total rice genomic DNA was extracted from four-week old plants of the parental lines i.e., Kranti and E2035 as well as the cross population by the method described by Dellaporta *et al*., (1983). The protocol was as follows:

1. About 2-5g of fresh leaves were selected and cut into small pieces by sterile scissors and crushed in presence of liquid nitrogen using mortar and pestle. The leaf powder (approx. up to 15 ml level in tarson tube) was transferred immediately into a 50 ml centrifuge tube.

2. 15-25 ml of extraction buffer (preheated to 65° C and added with 3.8 gm of SDS per 1000 ml) was added in each tube and incubated at 65° C for 15-20 minutes with gently mixing in every 5 min.
3. 10 ml of 5M potassium acetate was added to the sample and mixed vigorously and incubated on ice for 45 minutes with shaking.
4. The samples were centrifuged for 15 minutes at 3000 rpm. and then supernatant was filtered through Mira cloth into another 50 ml fresh centrifuge tube.
5. 2/3rd volume of pre-chilled isopropanol (17 ml for 25 ml supernatant) was added and incubated at 4 ºC for overnight.
6. The samples were centrifuged for 10 minutes at 3000 rpm. and the DNA pellet was collected and washed with 70% ethanol and air dried.
7. The pellet was resuspended in 5 ml of TE buffer and allowed to dissolve completely.
8. 5 µl of RNase A (10 mg/ml) was added and incubated at 37º C for 40 minutes.
9. 500 µl (1/10 volume) of 3M sodium acetate and 10 ml of chilled absolute ethanol was added, gently mix and incubate at 4 ºC for overnight.
10. The samples were centrifuged for 8-10 minutes at 3000 rpm. DNA was collected by discarding the supernatant and washed with 70% ethanol, and air dried under laminar air flow until last traces of ethanol was removed.
11. DNA was re-suspended in 200-500 µl of TE buffer, transferred in 1.5ml of microcentrifuge tubes and finally stored at -20° C.

3.2.7.2 Quantification of DNA

For quantification, 3 µl of the DNA samples isolated from each line, along with standards of known quantity of DNA, was loaded on 0.8% agarose gel. The electrophoresis was performed at 60 volts for 45 minutes. The gel was stained with ethidium bromide 2.5 µl/100 ml. and observed under UV transilluminator. The amount of fluorescence is directly proportional to the total amount of DNA. The quantity of samples was known by comparing with fluorescence of the standards.

3.2.7.3 Dilution of DNA samples

After quantification, the DNA samples were diluted in TE buffer such that the final concentration of DNA was approximately 40 ηg/µl, for PCR analysis.

3.2.7.4 PCR analysis to detect parental polymorphism and validation of molecular marker

PCR analysis was done using the selected SSR and designed markers to identify the polymorphic loci between the parental lines, Kranti and E2035 and their F_3 population.

Table 3.3: PCR components with their quantity for microsatellite analysis

S. n.	Components	Concentration	Quantity
1	PCR buffer with $MgCl_2$	10X	2.0 µl
2	dNTPs	2 mM	2.0 µl
3	Primer (Forward)	10 µM	1 µl
4	Primer (Reverse)	10 µM	1 µl
5	Taq DNA Polymerase	5 U	0.25 µl
6	Sterile water	-	11.75 µl
7	Template DNA	40 ηg/µl	2.0 µl
8	Total		20.0 µl

The mixture was overlaid with a drop of mineral oil before the amplification was carried out for 36 cycles of PTC 100 (Programmable Thermal Cycler) of MJ Research Pvt. Ltd., USA.

Amplified products were resolved by electrophoresis on agarose gel along with 100 bp DNA ladder as molecular weight marker in 1X TAE buffer, and visualized with UV. Where bands of interest were observed, replicate PCR was performed to confirm amplification patterns. Gels were photographed by using digital camera under photodyne.

Table 3.4: Temperature profile used for PCR amplification

Steps	**Temperature (°C)**	**Duration**	**Cycles**	**Activity**
1	94	3 min.	1	Initial denaturation
2	94	1 min.		Denaturation
3	50-55	45 sec.	35	Annealing
4	72	2 min.		Extension
5	72	7 min.	1	Final Extension
6	4	9 hrs	1	Storage

3.2.7.5 Gel Electrophoresis

PCR amplified SSR products were mixed with 2-4 µl of loading dye and loaded in the wells of 2.5% agarose gel prepared in 1X TAE buffer, electrophoresis was carried out at 100 volts for 1 h. The banding patterns were observed under U. V. transilluminater and photographed.

3.2.7.6 Detection of parental polymorphism using simple sequence repeats (SSR) primers

Twenty two previously designed primers and five random rice microsatelites were used to detect polymorphism between the two parents Kranti and E2035**.** The parental polymorphism generated by SSR primers was detected on the basis of length/ size differences of the amplicons between the parents. The primers used for this purpose are presented in table 3.5.

Table 3.5:- List of Primers used for parental polymorphism analysis.

S. n.	Markers	Primer Forward	Primer Reverse
1	gRM 1-1	CTAGCATTTCCCACATTCATA	TCTAGCATTTTCCACATTCAT
2	gRM 1-4	ATGTGGCTAGATTCATTAACAT	AAAACTTTCTAGCATTGCTCAT
3	gRM 9-3	TCCAAAATTAGTTATGGCATC	ACCAGTATCCAAAGTGTACCA
4	gRM 9-7	ATCTACGGAGTTATGCATGTG	CAGAATTGAAGTATTCGCTTG
5	gRM 7-1	TATTTTTCATCCCTCCTCTTT	AATTTTGTGCATTTGAAACC
6	gRM 7-2	TCTCTAGGCGTTTATCTTTTG	ATTAGTTGTCCCCTTTCTCAC
7	gRM 33-2	AACGACATGCAAAATGAGAG	AAGAGGAGATTCCATGTTCA
8	cRM 34-1	ATGTCTAACATGGTGGCTTG	CGCTTTGAAGGATTTGAATA
9	gRM 37-1	TCACGGAGCTCGTACTTG	ACCTTCCGATCTGGAGTC
10	gRM 9-4	TAGTGTGTGTGTGTGTGTGTG	GTGGCAAGAAGTTCCTAATTT
11	gRM 9-5	AGTTGGCTTAGTCTTTGAGGT	ACGCAAAAGATAGGGTTAAGT
12	gRM 9-6	AGGGTGAAGAACCTCACTTAG	TAACATGTTTGTGAACCGATA
13	gRM 33-1	TCGTTCTGACATGTTGAGG	CCCGACAAAGTCAACGTC
14	gRM 34-1	ATAGTATGCCCAGCATTAGC	TGTTCTCTCTGCACTTGTTG
15	gRM 39-2	GTGATGTGATGTGATGGAAA	CACCTCCAGGATCTCGTC
16	eRM 39-1	GAGCCAAGAGATGAGTTTCA	AGGACGAATCAGACAAACAG
17	cRM 40-1	CTTGTGTTTTGGACTGCTTC	CCACTTTCTGCTGACAACTC
18	gRM 7-3	TATATAGCGATTCTGCCACTT	GGAGCACCAAACAAATTTAC
1	gRM 9-	ATATGTTTATGCCCAAGTGG	TTTGGGATGAGATATGCTTT

9	2	T	A
20	eRM 33-1	GCCGACGTTGACTTTGTC	AAAGCGAGACACCTTTTCTT
21	gRM 7-4	GTCACCTAATGCTTTTGCTT	AGCATATGAAATACGGAGAGA
22	gRM 9-1	TATGTGTGTGTGTGTGTGTGT	GGAGTTGGATGTTTGAAAATTA

3.2.7.7 Analysis of genotypic data

The primer exhibiting polymorphism on parents were further screened against population of 50 lines. Genotypic data were generated with a set of polymorphic primers.

3.2.7.8 Scoring of data

The banding pattern of population developed by each set of SSR primer were scored separately as described in Table 4.8.

Table 3.6:- Scoring SSR banding pattern in population

S.N.	Type of band	Code
1	Kranti like allele	A
2	E2035 like allele	B
3	Both alleles	H

3.2.7.9 QTL analysis

In selected lines, single marker analysis was used to estimate association between marker and trait by using ‘t’- test formula given below:

$$t = \frac{\bar{X} - \bar{Y}}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where,

X = Mean of lines having A (Swarna) allele
Y = Mean of lines having B (Moroberekan) allele

n_1 and n_2 = number of Swarna and Moroberekan alleles respectively
S= Population Variance

3.3. Reagents and solutions

3.3.1 Reagents for PCR

a. Primers: Microsatellite markers from Imperial Life Sciences (p) Ltd. were used.

b. dNTPs: (dATP/dCTP/dGTP/dTTP)

100 mM stock of each dNTP was diluted to 10 mM of dNTP (i.e., 10 µl of each dNTP + 460 µl of sterile water).

c. PCR buffer (10X) (Stored at -20^{0}c)

Table 3.7: Components of PCR buffer

Components	Stock Concentration	Final Concentration	For 10 ml
Tris (pH 8.3)	1 mM	200 mM	2.0 ml
KCl	1 mM	500 mM	2.0 ml
$MgCl_2$	150 mM	15 mM	1.0 ml
Gelatin	-	0.01%	1.0 mg
H_2O	-		2.0 ml

d. Taq polymerase

3.3.2 Stock solutions

a. DNA extraction buffer

Trizma base- 12.11 gm

EDTA disodium salt- 18.07 gm

NaCl- 29.22 gm

SDS (10%) - 12.05 gm

SDS was added after autoclaving when the solution was hot. The pH was adjusted to 8.0 and final volume was adjusted to one liter.

b. 5M Potassium Acetate

490.7 gm Potassium Acetate was dissolved in 350 ml of distilled water and the Final volume was made up to one liter and autoclaved.

c. 3M Sodium Acetate

204.12 gm of Sodium Acetate was dissolved in 350 ml of distilled water and final volume was made up to 500 ml and autoclaved.

d. TE buffer

Trizma base 1.21 gm

EDTA disodium salt 0.372 gm

pH was adjusted to 8.0; Final volume was adjusted to one liter and autoclaved.

e. RNase A Stock solutions

1. 10 mM Tris HCl (pH 7.5)
2. 15 mM NaCl

10 mg of RNase A was added per ml of above solution, mixed, boiled and Allowed to cool at room temperature and stored in freezer.

f. 1M Tris (pH 8.3 at 25° C)

30.28 gm of Trizma base was dissolved in 200 ml of distilled water. The pH was set to 8.3 using concentrated HCl. The solution was allowed to cool at room temperature before making a final adjustment of pH. The final volume was adjusted to 250 ml with distilled water and sterilized by autoclaving.

g. 1M KCl

18.64 gm of Potassium Chloride was dissolved in 200 ml of distilled water and the final volume was made to 250 ml with distilled water and sterilized by autoclaving.

h. 15 mM $MgCl_2$

1.43 gm of Magnesium Chloride was dissolved in 80 ml of distilled water. Final volume was adjusted to 100 ml with distilled water and sterilized by autoclaving.

i. Isopropanol (pre-chilled)

j. Absolute alcohol (pre-chilled)

k. 70% Ethanol (pre-chilled)

3.3.3 Solutions for electrophoresis

a. 50X TAE buffer

Trizma base-	121.00 gm
Glacial Acetic Acid-	28.55 gm
0.5M EDTA (pH 8.0) -	100.00 ml
Distilled water-	50.45 ml
Total-	300.00 ml

b. Tank buffer (1X TAE)

20 ml 50X TAE + 980 ml of distilled water.

c. Orange loading dye

CHAPTER- IV
RESULT AND DISCUSSION

The present study entitled **"identification, characterization and validation of QTL's specific markers related to grain nutritive value in rice (*Oryza sativa* L.)"** was carried out in the Department of Biotechnology, COA, IGKV, Raipur to identify novel SSRs markers associated to QTLs related to grain micronutrient (iron zinc and protein) concentration in rice. The major objectives of the study included phenotyping of mapping population for grain Fe, Zn and protein contents, identification of novel SSRs markers within known QTLs (for grain Fe, Zn and protein content) and validation of the identified putative SSRs markers based on co-segregation with high grain Fe, Zn and protein contents. The findings of the present study are presented and discussed below in this chapter.

4.1 Assessment of grain nutritive content in parents and mapping population

On the basis of previous screening for grain protein content in rice by Chandel *et al*., 2007, cross derived from two genotypically different rice varieties, E2035 (having high protein content) and Kranti (having low protein content) were selected for the estimation of grain protein and Fe and Zn contents in rice.

4.1.1 Grain micronutrient (Fe and Zn) contents in parents and mapping population

The Fe and Zn content in the whole brown grains of rice as analyzed by Atomic Absorption Spectrophotometer (AAS200) as per *HarvestPlus*, (2006) protocol showed that the two rice genotypes, Kranti and E2035 are nutritionally different from one another. E2035 showed high Zn content of 30.06 µg/g and low grain Fe content of 13.55 µg/g whereas reverse was the case for the parent Kranti which showed high iron content (17.48 µg/g) and low zinc content (19.43µg/g). The results of elemental analysis for the cross population of 50 lines revealed that the brown rice grain iron content ranged from 8.16 to 16.86 µg/g with an average of 12.70 µg/g and brown grain zinc content ranged from 12.41 to 23.29 µg/g with an average of 17.01 µg/g the cross population studied (Table:4.1). The coefficient of variation (CV) for grain Fe and Zn content was found to be 14.42 % and 5.46 %, respectively. The analysis of variance using Randomized Block Design (RBD) indicated significant variation in grain Fe and Zn content among 50 rice lines belonging to Kranti X E2035 cross population at 5% level of significance (table 4.2 and 4.3). Similar observations have been reported by Banerjee *et al.*, (2010) where grain Fe and Zn concentration were found to be in the range of 4.82-22.69 µg/g and 13.95-41.73 µg/g respectively in the whole grains of a set of 12 diverse rice genotypes including both cultivated and wild genotypes of rice.

Efforts have been made since more than a decade to analyze genetic variability in several crops for iron (Fe) and zinc (Zn) contents (Gregorio *et al*., 2000) which has revealed that micronutrient contents in major crops varied from about 5 µg/g to 150 µg/g (Pfeiffer and McClafferty 2007). Studies conducted so far to evaluate rice grain Fe and Zn content have revealed that grain Fe content varies from 2 to 24 µg/g while grain Zn content varies from 4.45 to 58.4 µg/g in brown and polished rice grains (Bouis & Ruel, 1998; Graham *et al.,* 1999; Gregario 2000; Gregorio, 2002; Welch and Graham 2004; Chandel *et al.*, 2005; Martinez *et al.,* 2006; Virk *et al.,* 2007; Graham, *et al.*, 2007; Kaiyang *et al.,* 2008; Garcia *et al.,* 2009).

4.1.2 Grain protein content in parents and mapping population

Our protein estimation results also suggest that the two parents are nutritionally different from each other. E2035 showed 11.30% protein content and considered as high protein content variety where as Kranti showed 9.51% grain protein and was referred as low protein content genotype. Population derived from cross between these two genotypes showed an unexpected range of protein content from 5.07% to 7.01% with an average protein content of 6.21% which was lower than either of the parents. The coefficient of variation (CV) was found to be 4.03. (Table: 4.4). Similar unexpected observations have been reported by Moon *et al.,* (2001) where lower performance for yield related traits have been observed in the population. The yield per plant recorded in breeding population was even lower than the parent showing lowest yield per plant values. Riza *et al*. (2003) have reported grain protein content range from 6.3% to 9.1% in a set of 438 rice genotypes. They reported the genotypes PR-27423-MS6 (6.3%) containing lowest and PR-31595-PSC101 with the highest (9.1%) grain protein. Similar results were observed in a study conducted by Banerjee *et al*. (2010) for the estimation of protein content in a set of 12 diverse rice genotypes including both cultivated and wild genotypes of rice which showed a range of 6.19-10.75 μg/g protein in whole grains. Abbas (2000) showed significantly higher protein level of 9.0%, 8.8% and 7.4% for Basmati type rice varieties, Rachna Basmati, Basmati-370, and Basmati-385, respectively. Hamaker (1993) reported wide variation in gross protein content of milled rice ranging from 6.0- 9.0%, 6.1-11.4% and 7.7-10.0% among the rice genotypes grown in India, Philippines, and the United states respectively. Wide variation for protein concentration in milled grains level from 2.8 to 9.9% of rice germplasm lines of Chhattisgarh have been reported by Chandel *et al.,* 2005.

Table 4.1 Mean whole grain iron and zinc concentration in µg/g of 50 rice lines with parents kranti and E 2035

S. No.	Genotypes	Mean iron	SEm	Genotypes	Mean zinc	SEm
1	KRANTI	17.48	2.28	KRANTI	19.43	1.20
2	E 2035	13.55	0.32	E 2035	30.06	6.52
3	K X E1	10.85	1.02	K X E1	15.09	0.95
4	K X E2	9.05	1.92	K X E2	14.41	1.29
5	K X E3	11.65	0.62	K X E3	15.94	0.53
6	K X E4	10.32	1.29	K X E4	23.29	3.14
7	K X E5	12.52	0.19	K X E5	14.40	1.30
8	K X E6	16.87	1.98	K X E6	14.56	1.22
9	K X E7	13.58	0.33	K X E7	16.65	0.18
10	K X E8	12.33	0.28	K X E8	14.33	1.33
11	K X E9	12.97	0.03	K X E9	14.75	1.13
12	K X E10	13.83	0.46	K X E10	15.85	0.58
13	K X E11	14.12	0.60	K X E11	14.61	1.19
14	K X E12	9.74	1.58	K X E12	22.81	2.90
15	K X E13	8.16	2.37	K X E13	20.31	1.65
16	K X E14	10.23	1.33	K X E14	18.40	0.69
17	K X E15	13.93	0.51	K X E15	17.23	0.11
18	K X E16	16.85	1.97	K X E16	17.28	0.13
19	K X E17	15.09	1.09	K X E17	17.91	0.45
20	K X E18	16.75	1.92	K X E18	17.80	0.39
21	K X E19	14.25	0.67	K X E19	17.78	0.38
22	K X E20	15.30	1.19	K X E20	17.95	0.46
23	K X E21	16.58	1.83	K X E21	18.95	0.96
24	K X E22	15.20	1.14	K X E22	18.11	0.55
25	K X E23	13.43	0.26	K X E23	15.70	0.65
26	K X E24	9.72	1.59	K X E24	12.89	2.05

27	K X E25	13.24	0.16	K X E25	17.01	0.00
28	K X E26	10.63	1.13	K X E26	15.62	0.69
29	K X E27	11.11	0.89	K X E27	16.02	0.49
30	K X E28	13.59	0.34	K X E28	20.07	1.53
31	K X E29	11.28	0.81	K X E29	16.74	0.13
32	K X E30	12.98	0.03	K X E30	15.01	0.99
33	K X E31	12.10	0.40	K X E31	14.90	1.05
34	K X E32	11.28	0.81	K X E32	15.30	0.85
35	K X E33	14.65	0.87	K X E33	19.05	1.01
36	K X E35	13.05	0.07	K X E35	17.21	0.10
37	K X E36	12.23	0.33	K X E36	18.80	0.89
38	K X E37	11.23	0.83	K X E37	19.01	1.00
39	K X E38	13.35	0.22	K X E38	18.43	0.71
40	K X E39	11.75	0.57	K X E39	20.93	1.96
41	K X E40	13.12	0.10	K X E40	21.05	2.01
42	K X E41	13.10	0.09	K X E41	13.28	1.86
43	K X E42	12.88	0.08	K X E42	18.20	0.59
44	K X E43	12.78	0.06	K X E43	15.00	1.00
45	K X E44	10.87	1.01	K X E44	14.03	1.48
46	K X E45	12.65	0.12	K X E45	14.80	1.10
47	K X E46	9.70	1.60	K X E46	16.01	0.49
48	K X E47	9.32	1.79	K X E47	12.41	2.29
49	K X E48	13.43	0.26	K X E48	13.78	1.61
50	K X E49	12.17	0.36	K X E49	14.25	1.38
51	K X E50	11.32	0.79	K X E50	14.11	1.44

Variance = 6.66 (iron) and 9.66 (zinc); SEm 10.74 (iron) and 5.36 (zinc); CV = 14.42% (iron) and 5.46% (zinc)

Table 4.2: ANOVA for grain Fe content

Source of variation	Degree of freedom	Sum of square	Mean Sum of Square	F-cal	F-tab (5%)
Replication	2	7.02059	3.5102	1.013066	3.0872
Treatment*	50	1000.274	20.005	5.77355*	1.4772
Error	100	346.50	3.4650		

* Significant at 5% level of significance and 50 degrees of freedom

Table 4.3: ANOVA for grain Zn content

Source of variation	Degree of freedom	Sum of square	Mean Sum of Square	F-cal	F-tab (5%)
Replication	2	4.039233	2.019616	2.3359354	3.0872
Treatment*	50	1449.986	28.99973	33.54176*	1.4772
Error	100	86.45857	0.864586		

* Significant at 5% level of significance and 50 degrees of freedom

Table 4.4 Mean whole grain protein concentration in % of 50 rice lines with parents Kranti and E 2035

S. No.	Genotypes	Mean protein	SEM
1	KRANTI	9.51	1.63
2	E 2035	11.30	2.53
3	K X E1	6.28	0.02
4	K X E2	6.46	0.11
5	K X E3	5.93	0.14
6	K X E4	5.40	0.41
7	K X E5	6.43	0.09
8	K X E6	6.65	0.21
9	K X E7	6.33	0.04
10	K X E8	6.16	0.03
11	K X E9	5.69	0.26
12	K X E10	6.21	0.01
13	K X E11	7.01	0.38
14	K X E12	6.58	0.17
15	K X E13	6.33	0.04
16	K X E14	6.58	0.17
17	K X E15	6.45	0.10
18	K X E16	6.60	0.18
19	K X E17	6.48	0.12
20	K X E18	5.94	0.14
21	K X E19	5.96	0.13
22	K X E20	5.94	0.14
23	K X E21	6.34	0.05
24	K X E22	5.81	0.20
25	K X E23	5.07	0.57
26	K X E24	5.43	0.40
27	K X E25	5.27	0.48
28	K X E26	5.61	0.31

29	K X E27	5.67	0.28
30	K X E28	5.65	0.29
31	K X E29	5.92	0.15
32	K X E30	6.34	0.05
33	K X E31	6.16	0.03
34	K X E32	6.22	0.00
35	K X E33	6.29	0.02
36	K X E35	5.91	0.15
37	K X E36	5.95	0.14
38	K X E37	5.79	0.21
39	K X E38	5.82	0.20
40	K X E39	6.26	0.01
41	K X E40	6.40	0.08
42	K X E41	6.27	0.02
43	K X E42	6.11	0.06
44	K X E43	5.36	0.43
45	K X E44	5.31	0.45
46	K X E45	5.74	0.24
47	K X E46	5.86	0.18
48	K X E47	6.27	0.02
49	K X E48	6.29	0.03
50	K X E49	6.23	0.00
51	K X E50	6.30	0.03

Variance = 0.91; SEm = 1.45; CV = 4.03%

Table 4.5: ANOVA for grain protein content

Source of variation	Degree of freedom	Sum of square	Mean Sum of Square	F-cal	F-tab (5%)
Replication	2	0.284906	0.142453	2.252614268	3.087296
Treatment*	50	137.9449	2.758898	43.62656665*	1.477231
Error	100	6.323894	0.063239		

***** Significant at 5% level of significance and 50 degrees of freedom

Twelve classes of grain Fe, Zn and protein content were made to characterize phenotypic variation in the mapping population. The frequency of the number of plants falling in each class given in Fig. 4.1. The distribution pattern of the population for grain Fe and Zn content suggested its suitability for polymorphism and co-segregation analysis for marker loci and trait of interests (Gupta, 2002).

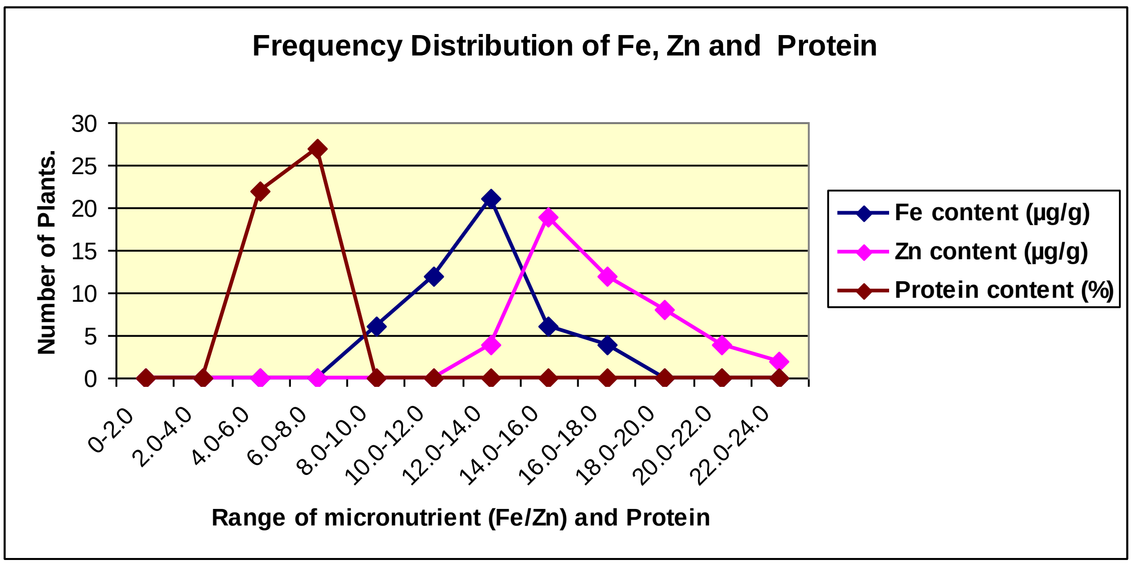

Fig. 4.1: Frequency distribution of grain iron, zinc and Protein content in cross population

The rice line KXE 8 showed maximum grain Fe content (16.87µg/g) which was more than the better parent E2035. Whereas maximum grain Zn content of 23.29 µg/g was observed in rice line KXE 6 which is higher than Kranti. But in grain protein content in population showed lower than both of the parent table (4.4). The genetic variability evaluated and reported till date in different *indica*, *japonica*, aromatic and wild rice genotypes harbors great potential to be used in intensive molecular marker based breeding programmes for development of iron and zinc rich rice.

4.2 *In silico* identification of simple sequence repeats (SSRs) in the genomic region encompassing QTLs governing grain protein content in rice

Grain protein content is a polygenic quantitative trait governed by several genes or QTLs. Closely linked molecular markers related to loci contributing major variation for the trait will improve the efficacy of marker assisted selection (MAS). The closely linked molecular markers will facilitate selection of most favorable genotypes in the mapping populations without going for a relatively complex, destructive and expensive assay of grain Fe/Zn content in generations of breeding population.

DNA markers based linkage mapping has been used since long to map and identify a number of genes or QTLs related to a segregating trait. Recent advanced in rice genomics research and availability of finished rice genome sequence in public databases has accelerated identification and development of novel molecular markers as evident by the development of about 500 SSR marker by the year 2000 which increased by 2200 in the year 2002 (Collard *et al.*, 2008). These new generation sequence specific molecular markers such as SSRs, SNPs, ESTs-SSRs *etc*. are useful tools for saturation mapping, high resolution mapping of QTLs or positional cloning of genes. Parallel improvisation in user friendly bioinformatics tools has enabled analysis of a sequence in question for identification of thousands of new generation DNA markers, putative candidate genes, putative expression pattern and known co-localized markers. Mining of putative genes and DNA markers linked to or within genomic region underlying known QTLs has opened up unprecedented opportunities to identify tightly linked markers.

Genomic region encompassing four known QTLs namely qPRO-1, qPRO-2, qPRO-6, and qPRO-11 governing high grain protein content in rice (table 4.4) identified in rice (Kaiyang *et al.*, 2008), were selected based on their higher phenotypic variance and lower LOD value and were analyzed for co-localized SSRs loci. Genomic sequence underlying these QTLs was downloaded from TIGR (The Institute of Genomic Research) Genome browser (*http://www.tigr.org/tdb/e2k1/osa1/*), GRAMENE (*http://www.gramene.org*) and used for identification of novel putative SSRs.

4.3 Identification of novel SSRs markers

The sequence of four QTLs, qPRO-1, qPRO-2, qPRO-6, and qPRO-11 selected for high grain protein content and were analyzed for identification of SSRs using Batchprimer3 SSR search tool (*http://www.Batchprimer3*). The ubiquity of SSRs in eukaryotic genome and their usefulness as genetic markers have led to the development of gene and QTL specific SSRs. (McCouch S. R., 2002; Yang *et al*., 2006; Ghneim 2008; Maia *et al*., 2008; Sharma and Chauhan, 2008). So SSR screening primers were designed from the genomic DNA sequences following the strict specification criteria are like, product size, Tm value, GC % content *etc* to avoid any non specific amplifications.

A total of 1562 putative SSRs were identified within genomic region encompassing the Four QTLs including qPRO-1, qPRO-2, qPRO-6, and qPRO-11. Occurrence of di and tri-nucleotide repeats occurred more commonly in all identified SSRs. The tri-nucleotides repeats has been reported to be more common in eukaryotes especially cereals and legumes plant genomes. The potential of putative SSR to be used as a marker depends on repeat motif, number of repeats and position in gene. The putative SSRs identified in the present study belonged to Class II SSRs and Class I SSRs.

Out of 1562 putative SSRs, 195 class I SSRs (more than 20 nt long) were selected such that they covered each 100-200 Kb fragment of the QTLs in each question. The selected Class I SSRs were found to be present in immediate vicinity of a cross membrane transporter family protein or hypothetical protein encoding genes and were used to design ~ 20 nt primers using BatchPrimer-3 software. The newly designed primers from 12 novel SSRs loci were used to fine map the genomic region encompassing four known QTLs controlling grain Protein.

In silico approaches offers great opportunity to search for the specific, tightly associated SSRs in the region underlying a QTL governing important traits as well as to design specific markers from the sequences in the vicinity of candidate genes. This also enables us to look for and target the expressed regions viz. ESTs and Fl-cDNA in the genome for marker development. 16 novel class I SSRs and 176 candidate SNPs have been identified within gene sequences of 21 metal homeostasis related genes using SSRIT and *Oryza* SNP search tool by Banerjee *et al.*, (2010). Wan *et al*., (2006) also designed 11 SSR markers for the fine mapping of an identified QTL (*gl-3*) related to the rice grain length and narrowed down the candidate genomic region, and provides a basis for map based cloning of the target region and for marker aided QTL pyramiding for quality rice breeding. Similarly Nguyen *et al.*, (2004) also developed 5 SSR markers for the saturation mapping of the QTL region and identification of putative candidate genes for drought tolerance in rice.

Table 4.6 Details of SSRs within QTLs

S. n.	QTLs	Class I SSRs	Total no. of SSRs
1	qPRO-1	60	388
2	qPRO-2	62	483
3	qPRO-6	43	432
4	qPRO-11	30	259
5	Total	195	1562

Table 4.7 List of designed QTLs specific SSR markers

Marker	Primer Forward	Primer Reverse	Tm (°C)
gRM 6-1	AGTTACGACCAATGATACGC	GACTAGCAGCTCACGATCTAA	55
gRM 6-2	CTTGTGATCAAGTCGTCGTA	CTGCAGTATCATCATCGACA	55
gRM 6-3	CATCACACGACATCATTCC	GAGCTACAAGAGATCCTCTCC	55
gRM 11-1	CCTCCTACTCACCTGTGTTA	GAGTTTAGGGGAGGTTCTG	53
gRM 11-2	GGAGCTTACATCTCTTGGAC	TCTATCTCTGCTCTCAGTGG	53
gRM 11-3	GTTCTTCACCTTCCAGCTAC	CCCTCATCTAATCCTACTCC	53
gRM 1-1	GTGGGTCGTGTGTGATTA	CACGATCGACATCAGTTC	53
gRM 1-3	ACACACACACACACACACAC	CTGGAGCCTACTGAATACTG	53
gRM 1-4	GGTGATCGATCTCACTCG	TCACCTAGACTGAGTTGTGG	54
gRM 1-5	TCCTCGTACCTCTCGAAC	CACCTCCACGCTACTACTC	53
gRM 1-6	CGTCAGTCGCGTATAGAA	GATCCACATGTCACTCACAC	53
gRM 1-7	CTGTGCTCCGTGTACATA	TCGATCGACTTGGAGTAG	51

4.4. Validation of identified SSR markers

4.4.1 Parental polymorphism analysis using SSR primers

In this study we have cross validated 22 previously designed SSR primers and 5 known random rice microsatelite (RM SSRs) markers and tested them in KrantiX E2035 F3 population to detect any polymorphism between the parents Kranti and E2035. These markers were designed from the genomic, ESTs and cDNA sequences underlying the QTL regions. Out of a total of 27 markers screened, only 4 markers showed polymorphism between the two parents (Fig. 4.6), whereas rests of the primers were monomorphic in nature. The monomorphic or polymorphic amplification of the 22 new SSRs generated have cross validated them as new generation genomic DNA based markers in the population under study. Occurrence of only four polymorphic loci out of a total of 27 SSR loci represents a low level of polymorphism. This can be because the population used in the study is developed from the cross between the two *indica* parents. The lower polymorphism rate of markers is indicative of lower genetic variation between the two parental genotypes. Lower polymorphism rates have been observed for certain repeat motifs in *Oryza sativ*a (Temnykh *et al*., 2001). These 4 polymorphic primers were used for co segregation studies in the F3 cross population.

4.4.2 SSR based genotyping of mapping population

The Four SSRs showing polymorphism with parents were selected for genotyping of the mapping population. The genotypic data thus generated (Fig 4.5, Fig. 4.6, Fig. 4.7, and Fig. 4.8) was analyzed for segregation of Kranti and E2035 like alleles in the population. The Scoring of bands was done by designating the Kranti parent as A allele, E2035 parent as B allele. A perusal of gel pictures/ genotyping data indicated amplification of either Kranti like or E2035 like allele in the breeding lines, while some of the lines showed both Kranti and E2035 like alleles and thus were considered as heterozygous. It was found that the Kranti contributed about 42.85% of its trait (on the mean basis) whereas the E2035 contributed about 29.8% of its trait on similar basis. The variation in trait was represented departure from the theoretically expected ratio of 1:1 i.e. equal contribution from both the parents. The rice lines showing Kranti like allele were found to carry high Fe and low Zn, low Fe and high Zn content while those showing kranti and like allele showed comparatively lower grain Fe and higher Zn content. Yet many rice lines of mapping population having E2035 like allele were found to contain higher and lower Zn grain micronutrient contents. All the 4 polymorphic SSRs markers show a significant deviation from the expected 1:1 ratio. The genotypic data generated is presented in Table 4.8 and 4.9.

Table 4.8: Scoring of banding pattern of population derived from cross between Kranti and E2035

S No	Genotypes	gRMm9-6	gRMm7-2	gRMm39-2	eRMm33-1
1	Kranti	A	A	A	A
2	E2035	B	B	B	B
3	1	A	H	M	B
4	2	A	A	A	B
5	3	A	H	B	B
6	4	A	A	B	A
7	5	A	A	M	A
8	6	A	A	B	M
9	7	A	H	B	B
10	8	A	A	B	A
11	9	A	H	H	B
12	10	A	A	H	B
13	11	A	H	B	B
14	12	A	A	M	M
15	13	H	H	A	B
16	14	H	A	A	A
17	15	M	H	M	A
18	16	A	A	A	B
19	17	A	H	M	B
20	18	A	H	B	A
21	19	A	H	B	A
22	20	A	H	M	H
23	21	A	H	M	A
24	22	M	H	A	B
25	23	M	H	A	B
26	24	A	A	B	A
27	25	B	A	H	B
28	26	B	A	M	A
29	27	B	A	A	B

30	28	B	A	H	A
31	29	B	H	H	B
32	30	B	A	H	B
33	31	B	H	H	B
34	32	B	A	A	M
35	33	A	A	A	B
36	35	B	A	A	A
37	36	B	A	M	B
38	37	B	A	A	A
39	38	B	A	B	A
40	39	M	M	B	A
41	40	B	B	B	B
42	41	B	H	H	A
43	42	M	A	M	A
44	43	M	A	A	B
45	44	B	A	A	M
46	45	M	A	A	B
47	46	A	A	H	B
48	47	M	A	A	B
49	48	A	A	A	B
50	49	B	B	M	A
51	50	B	B	A	M
	TOTAL	A-22 B-17	A-28 B-3	A-17 B-12	A-17 B-25

Overall percentage of A allele-42.85% of allele B-29.08% and H-28.06%

Table 4.9: Individual analysis of markers with the population

Parameter	gRMm9-6	gRMm7-2	gRMm-39-2	eRMm 33-1
% of allele A	44.89%	57.14%	34.69%	34.69%
% of allele B	34.69%	6.12%	24.48%	51.02%
% of heterozygous	20.40%	36.73%	40.81%	34.69%

The marker gRMm7-2 produces maximum no. of Kranti like allele (A) with a frequency of 57.14% whereas the lowest percentage of Kranti like allele was observed for two markers gRM39-2 and eRM 33-1 with a value of 34.69% and the marker eRM33-1 produces maximum no. of E2035 like allele (B) with a frequency of 51.02%, whereas the lowest percentage of E2035 like allele was observed for marker gRM7-2 with a value of 6.12%.

4.4.3 Association mapping

Single marker Association mapping technique was used to identify the association of SSRs markers to either iron or zinc contents in brown rice grains. ‘t’ value was determined for each of the polymorphic primer to analyze its significant association to grain micronutrient and protein contents which is presented in the following Table 4.9 and 4.10. The calculated‘t’ value for each markers was compared with table ‘t’ value (2.0) at 50 degree of freedom and 5% level of significance. The table below shows that polymorphic SSRs marker gRMm7-2 exhibited a significant association with the grain zinc content with the population under study. Determination of association of a marker with a trait is the basic principal of association mapping. The mapping population is partitioned into different phenotypic classes based on the variability for the trait. The correlative statistical analysis of the genotypic data of the marker locus for individual genotype is performed with the phenotypic classes and forms the basis of association mapping. The failure of independent segregation of marker loci with the phenotypic class is said to display “linkage disequilibrium” (Prasnna *et al.,* 2005) and QTLs identification is based on linkage disequilibrium. Several statistical methods and softwares have been developed to determine association of marker loci with a trait including Single Marker Analysis, interval mapping, MAPMAKER, QTL mapper and Q Gene *etc* (Zeng 1994, Wang *et al*. 2006).

Table 4.10:‘t’ –test for the polymorphic primers for Iron and Zinc

S.No.	**Primer**	**t-value (iron)**	**t-value (zinc)**	**Association with grain iron content**	**Association with grain zinc content**
1	gRMm9-6	0.492	0.129	Not Associated	Not Associated
2	gRMm7-2	0.100	2.433*	Not Associated	Associated*
3	gRMm39-2	0.455	0.498	Not Associated	Not Associated
4	eRMm33-1	0.353	0.539	Not Associated	Not Associated

Table 4.11:‘t’ –test for the polymorphic primers for Protein

S.No.	**Primer**	**t-value (Protein)**	**Association with grain protein content**
1	gRMm9-6	4.793	Associated*
2	gRMm7-2	3.029	Associated*
3	gRMm39-2	0.996	Not Associated
4	eRMm33-1	0.974	Not Associated

CHAPTER V

SUMMARY, CONCLUSION AND SUGGESTION FOR FUTURE RESEARCH WORK

Nutritional well being and food habits have coevolved with human civilization where crop plants served as the critical component of human foods. The primary objective of modern agriculture and breeding programmes over the past 50 years has been to increase food grain production for growing population. The quest for increased yield is no doubt a primary concern and should be continued with vigor but has led to ignorance of nutritional quality traits in breeding programs for staple food crops (Grusak and Dellapenna, 1999). The daily diet not diverse in terms of components and their respective concentrations is the primary cause of widespread mineral malnutrition among low income groups of populations (WHO, 2003). Developing nutrient rich rice i.e. biofortification is an upcoming approach which will ensure adequate attainment of important dietary elements and will add to improve health of poor people especially those residing in developing countries of Asia and Africa. Haas *et al.,* (2005) reported that consumption of bio-fortified rice, without any other changes in diet, is efficacious in improving iron stores in the non-anemic Filipino women with iron-poor diets. Micronutrient rich staple rice varieties are being developed by using conventional breeding and modern biotechnological approaches (*HarvesetPlus,* 2006). Plant breeding along with biotechnological interventions upholds great promise for developing a significant, low cost, sustainable contribution to reduce micronutrient malnutrition. Grain protein and micronutrient (iron and zinc) contents are polygenic trait having additive effect of multiple genes. Quantitative trait locus/loci (QTL) analysis is a powerful approach to unravel polygenic characters or to identify the different genes responsible for a quantitative trait thus identification and characterization of QTLs governing grain protein and micronutrient content provides an opportunity to understand not only the genetics of inheritance these traits but also to develop nutrient rich rice. Reliable estimation and selection of a mapping population for a QTL depends on the heritability of the trait and linkage between markers and the QTLs. As the QTLs refer to the larger genetic region, often 1 to several centimorgan (corresponding to thousands of Kilobases) having several genes. Moreover estimation of protein and Fe/Zn content of grains is an indirect destructive and time consuming method, thus to truly understand the quantitative variation and dissect the genes affecting grain micronutrient content related QTLs, fine mapping or high resolution mapping of QTLs is necessary. The present study **"Identification, characterization and validation of QTL's specific markers related to grain nutritive value in rice (*Oryza sativa* L.)**" was thus undertaken to identify novel microsatellites markers within known QTLs controlling grain protein and micronutrients and to determine their association with high grain protein, Fe and Zn contents. Co-segregation analysis was performed in the F_3 mapping population derived from cross between Kranti and E 2035 rice genotypes.

Total grain protein and Fe and Zn estimation of Kranti and E2035 showed that Fe content (17.48 μg/g) is high in Kranti and low in E2035 (13.55 μg/g), whereas Zn content is low in Kranti (19.43μg/g) and high in E2035 (30.06 μg/g) rice genotype. In this study grain iron and zinc content of F_3 mapping population developed from Kranti, a popular *indica* rice cultivar and E2035 which has been previously identified as high protein variety, were analyzed by using Atomic Absorption Spectrophotometer (AAS200) as per *HarvestPlus*, (2006) protocol and protein content was analyzed by modified micro-Kjeldahl method (Johri *et al.*, 2000). The results of elemental analysis revealed that the brown rice grain iron content ranged from 8.16 to 16.86 μg/g with an average of 12.70 μg/g and brown grain zinc content ranged from 12.41 to 23.29 μg/g with an average of 17.01 μg/g in F_3 cross population (Table:4.1). The coefficient of variation (CV) for grain Fe and Zn content was found to be 14.42 % and 5.46 %, respectively. The analysis of variance using Randomized Block Design (RBD) indicated significant variation in grain Fe and Zn content among 50 rice lines belonging to Krant X E2035 mapping population at 5% level of significance. The protein estimation results showed that the brown rice grain protein content was unexpectedly found to be in the range of 5.07 to 7.01 with an average of 6.21 (Table:4.1). The coefficient of variation (CV) for grain protein content was found to be 14.4 %. The analysis of variance using Randomized Block Design (RBD) indicated significant variation in grain protein content among 50 rice lines belonging to Krant X E2035 population at 5% level of significance.

In order to identify within QTLs novel SSR markers, four QTLs, qPRO-1, qPRO-2, qPRO-6, and qPRO-11 were analyzed *in-silico* for putative SSRs and co-localized genes. A total of 1562 novel SSRs loci were identified in these four QTLs located with in genomic sequences with 2-3 nt long repeat motifs and 12 – 80 nt total repeat length. Out of these 195 Class I SSRs sequences were selected which were further categorized on the basis of their location in vicinity to the metal transporter or membrane transporter genes. A total of 12 novel SSRs were used to design primers which can be used to generate SSR profile of all genotypes of the cross population analyzed for total grain protein and Fe/Zn content in brown rice. Further we have cross validated 27 SSR markers designed previously from the regions of various QTLs controlling grain protein and micronutrient contents in rice by performing co-segregation analysis for segregation of allele with total grain protein and micronutrient (Fe/Zn) content in brown rice grains. Of these 27 SSRs primers 23 were found to be monomorphic in population while only 4 primers were polymorphic. These 4 polymorphic markers were used to generate SSR profile of the cross population. The genotypic data was then scored for segregation of Kranti like allele ‘A’ and E2035 like alleles ‘B’ in 50 rice lines derived from Kranti X E2035 cross. The allelic segregation revealed that on an average the parent Kranti contributed about 42.85% of total amplified alleles whereas the parent E2035 contributed about 29.08 % alleles indicating departure from the theoretically expected ratio of 1:1 or equal contribution from the parents. The polymorphic SSRs markers were then analyzed for their association with grain protein and Fe/Zn content in rice. The genotypic and phenotypic data obtained for the F_3 population in the present study was then used to study the marker trait association i.e. the association between the 4 polymorphic markers and brown grain protein and micronutrient (iron and zinc) contents in rice. SSR marker gRMm 7-2 was found to be co-segregated with grain Zn content and gRMm 9-6 and gRMm 7-2 were found to be co-segregated with grain Protein hence markers were recorded to be associated with rice grain micronutrient and protein content in the F_3 population studied. While no significant association wad found between the novel SSR markers and grain Fe contents in rice. Thus, it can be concluded that more population with significant differences in the grain Fe content needs to be analyzed for identification of tightly linked markers and fine mapping of known QTLs.

Conclusions:

- The total grain iron and zinc content of the different rice lines of population derived from a cross between Kranti and E2035 ranged from 5.14 to14.1 µg/g with an average of 9.55 µg/g for iron and from 12.60 to 29.61 µg/g with an average of 19.0 µg/g for zinc. The coefficient of variation (CV) for grain Fe and Zn content was found to be 11.84 % and 6.13 %, respectively and also showed significant difference in grain micronutrient level at 5% level of significance. The analysis of variance using Randomized Block Design (RBD) indicated significant variation in grain Fe, Zn and protein content among 50 rice lines belonging to Kranti X E2035 mapping population at 5% level of significance.
- The genomic region underlying four QTLs (Parveaz Sofi and A.G. Rather 2007) analyzed for putative genes and novel SSRs markers showed the presence of a total of 1562 SSRs loci and twelve novel SSR primers have been designed from the selected Class I SSR loci which are needed to be experimentally validated in the mapping population.
- Occurrence of only 4 polymorphic markers out of a total of 27 previously designed primers screened, between the parents Kranti and E2035 indicates lower genetic variations between the two *indica* parental genotypes. The phenotypic and genotypic data generated from the F_3 population analyzed for co-segregation analysis of Kranti and E2035 like allele revealed that 42.85% alleles were Kranti like and 29.08% were E2035 like.
- The association analysis between the markers and trait revealed that out of four polymorphic markers, two markers (gRMm 7-2 and gRMm 9-6) showed significant associations to grain nutritive value. One novel QTL specific SSR marker, gRMm 7-2 was associated to grain Zn content. Two markers namely gRMm 7-2 and gRMm 9-6

were associated to grain protein contents, while no significant association was found between the novel SSR markers and grain Fe contents in rice for the QTL analyzed in the study.

Suggestions for future research work:

- The high Fe, Zn and protein lines identified in the study should be further analyzed at multi locations to assess the environmental effect on grain micronutrients and protein contents.
- Novel SSR markers identified must be cross validated in other mapping populations to assess their cross transferability among different populations. The markers showing association to different nutritive content should also be cross validated in larger populations derived from cross between genotypes with wider difference in nutritive contents.
- Primers for the remaining SSR loci identified can be designed and screened against parents and mapping population to study their polymorphism and association to grain nutritive contents and thus to saturate and fine map the target QTL region.

"Identification, characterization and validation of QTL's specific markers related to grain nutritive value in rice (*Oryza sativa* L.)."

By
Mr. Vinay Premi

ABSTRACT

Biofortification of crop plants requires the identification of candidate genes involved in micronutrient uptake distribution and accumulation in the grains**.** Nutritional deficiencies (e.g. iron, zinc and protein) account for almost two-thirds of the childhood deaths worldwide. Most of those afflicted are dependent on staple crops such as Rice and Wheat for their sustenance, who cannot afford the fortified foods to meet out the micronutrient requirements. Thus micronutrient enrichment, i.e. biofortification (genetic enhancement) of staple food crops has been considered a sustainable strategy to tackle the problem of micronutrient deficiencies. In this study **"Identification, characterization and validation of QTL's specific markers related to grain nutritive value in rice (*Oryza sativa* L.)"** 50 rice lines of F_3 mapping population derived from cross Kranti and E2035 were analyzed for their grain nutritive value. Further known QTLs related to Protein, Iron and Zinc content have been analyzed. Micronutrient estimation results showed wide variation for grain nutritive traits rise Fe and Zn content. The brown rice grain iron content ranged from 8.16 to 16.86 µg/g with an average of 12.70 µg/g and zinc content ranged from 12.41 to 23.29 µg/g with an average of 17.0 µg/g. The value for GPC varied from 5.0 % to 7.01 % with an average of 6.21 % among the fifty rice lines of the mapping population.

The four QTLs identified for GPC such as qPRO-1, qPRO-2, qPRO-6, and qPRO-11 were analyzed *in-silico* for putative SSRs and co-localized genes. A total of 1562 novel SSRs loci were identified in the genomic region underlying these four QTLs. Out of these 1562 SSR, 195 Class I SSRs sequences were selected and were further categorized on the basis of their location, and PHD-finger domain containing protein, putative expressed, retrotransposon protein, transmembrane amino acid transporter protein, etc candidate genes were found. A total of 12 novel SSRs were used to design primers which can be used to generate SSR profile of all genotypes of the cross population analyzed for total grain protein and Fe/Zn content.

The cross validation and co-segregation analysis out of 27 SSR markers related with GPC, Fe, and Zn contents, 4 are found to be polymorphic in nature in the mapping population. The genotypic data was then scored for segregation of Kranti like alleles 'A' and E2035 like alleles 'B' in 50 rice lines derived from Kranti X E2035 cross. The allelic segregation revealed that on an average the parent Kranti contributed about 42.85% of total amplified alleles whereas the parent E2035 contributed about 29.08 % alleles indicating departure from the theoretically

expected ratio of 1:1 or equal contribution from the parents. SSR marker gRMm 7-2 was found to be co-segregated with grain Zn content and gRMm 9-6 and gRMm 7-2 were found to be co-segregated with grain Protein hence markers were recorded to be associated with rice grain micronutrient and protein content in the F_3 population studied. While, no significant association was found between the novels SSR markers and grain Fe contents in rice.

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http://www.tigr.org.in

“IDENTIFICATION, CHARACTERIZATION AND VALIDATION OF QTL’s SPECIFIC MARKERS RELATED TO GRAIN NUTRITIVE VALUE IN RICE (*Oryza sativa* L.)”

Major Advisor: Dr. Girish Chandel

Speaker: Vinay Premi

DEPARTMENT OF PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY INDIRA GANDHI KRISHI VISHWAVIDYALAYA, RAIPUR (C.G.)

INTRODUCTION

Why Rice….

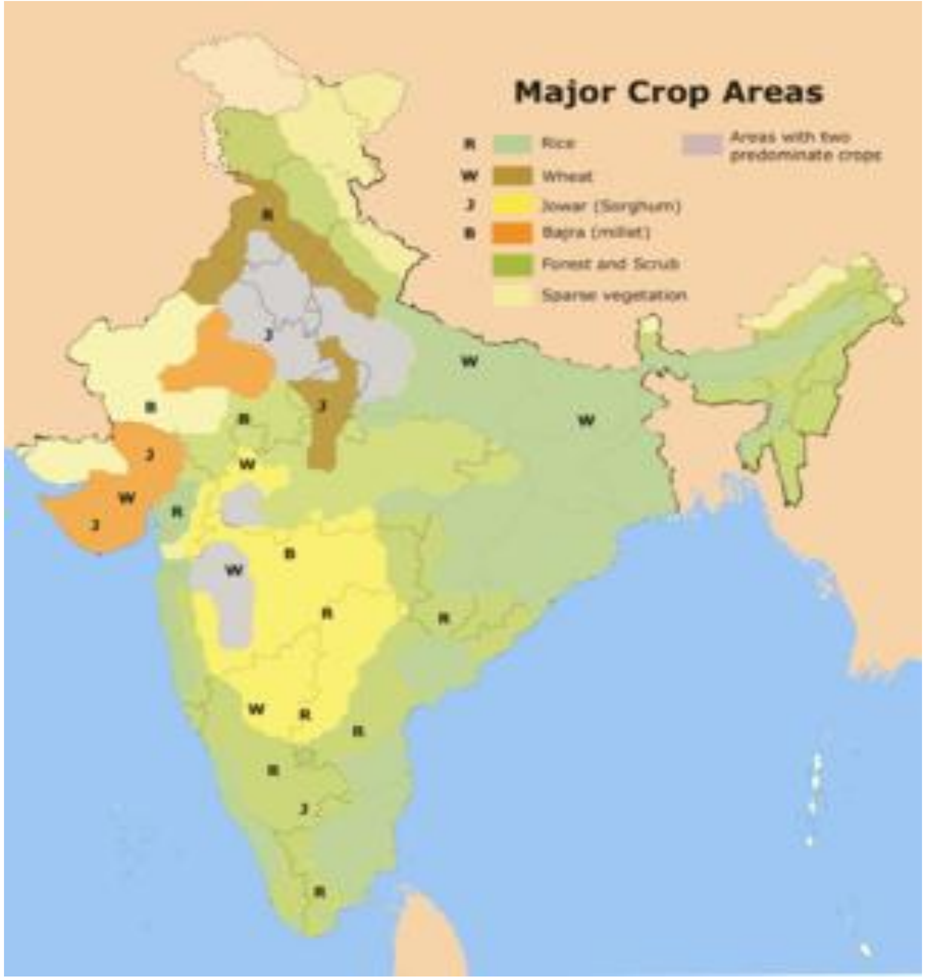
- Asia is the leader in rice production accounting for 90% of the world's production.
- Rice in not only a major staple food for the world's population but it is also a model species for a major group of flowering plants.
- India is a major centre of diversity

Crop	Iron (µg/g)	Zinc (µg/g)	Protein(%)
Rice grain	2-24	4.45-58.4	5.5-11.4

(Singh *et al*., 2000, Chandel *et al*., 2005; Graham *et al*., 1990)

Cont….

MAJOR CROP AREAS COVERED BY DIFFERENT CROPS IN INDIA

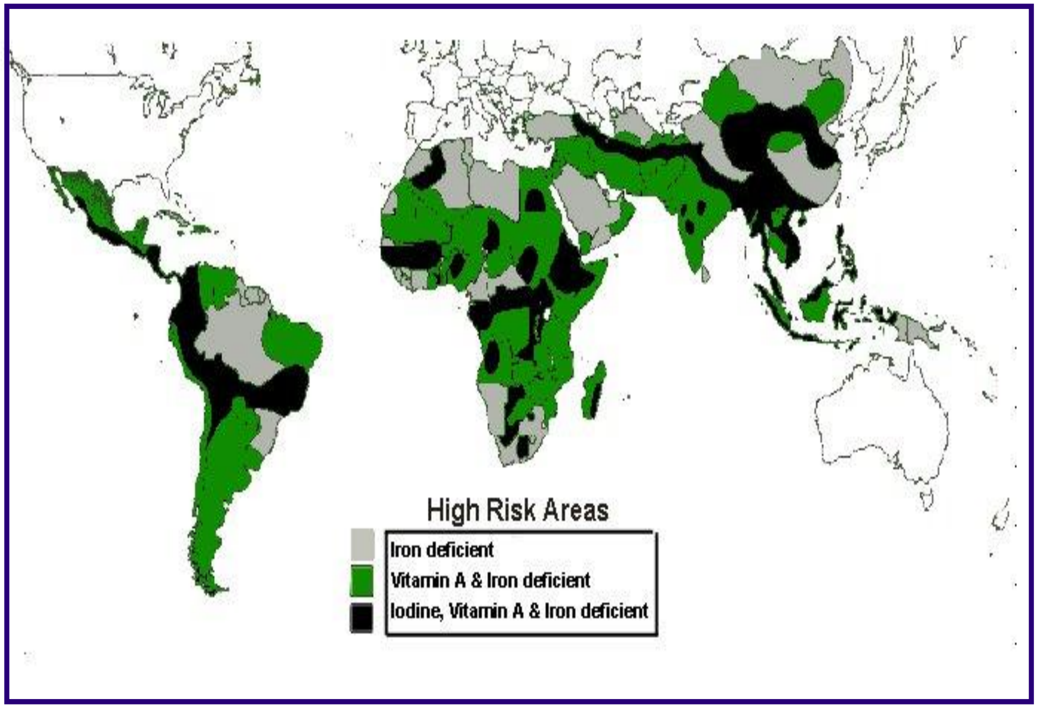


(India.png)

MALNUTRITION

❑ Malnutrition in the world

GLOBAL MICRONUTRIENT DEFICIENCIES



(Map from USAID 2003)

Country	No. of undernourished People (million)
India	217.05
China	154.0
Bangladesh	43.45
Pakistan	35.2
Ethiopia	31.5
Tanzania	16.1
Philippines	15.2
Brazil	14.4
Indonesia	13.8
Vietnam	13.8

FAO,2007

> 3 billion people afflicted

Cont…

❑ Malnutrition in India

- ❑ India is the second rank in the world of the no. of children suffering from malnutrition after Bangladesh.
- ❑ Children exhibit a degree of malnutrition is 47%.

1. Iron malnutrition – 4.5 billion
2. Zinc malnutrition – 2.7 billion
3. Protein Energy malnutrition (PEM) – 2 billion

BIOFORTIFICATION

- Recently, a sustainable solution to mineral malnutrition termed as “Biofortification”.

- Biofortification is a method to increase nutritional values of food crops:

  Conventional selective breeding.

  Genetic engineering.

- The complex polygenic traits are governed by QTLs thus identification as well as characterization of QTLs controlling grain micronutrient contents in rice harbors great potential for Marker Assisted Selection (MAS).

OBJECTIVES

1. Phenotyping of parents and mapping population for grain protein and micronutrient contents.

2. *In silico* characterization of QTL’s controlling grain protein, Fe and Zn content in rice.

3. Genotyping of the mapping population using QTL’s specific DNA markers.

MATERIALS AND METHODS

MATERIALS

The plant material used for this study includes rice genotypes Kranti, E2035 and the F_3 population of 50 lines derived from the cross between Kranti and E2035.

Sr. No.	Name of genotypes	Generation	Sr. No.	Name of genotypes	Generation
1	K X E1	F_3	26	K X E26	F_3
2	K X E2	F_3	27	K X E27	F_3
3	K X E3	F_3	28	K X E 28	F_3
4	K X E4	F_3	29	K X E 29	F_3
5	K X E5	F_3	30	K X E 30	F_3
6	K X E6	F_3	31	K X E 31	F_3
7	K X E7	F_3	32	K X E 32	F_3
8	K X E8	F_3	33	K X E 33	F_3
9	K X E9	F_3	34	K X E 34	F_3
10	K X E10	F_3	35	K X E 35	F_3
11	K X E11	F_3	36	K X E 36	F_3
12	K X E12	F_3	37	K X E 37	F_3
13	K X E13	F_3	38	K X E 38	F_3
14	K X E14	F_3	39	K X E 39	F_3
15	K X E15	F_3	40	K X E 40	F_3
16	K X E16	F_3	41	K X E 41	F_3
17	K X E17	F_3	42	K X E 42	F_3
18	K X E18	F_3	43	K X E 43	F_3
19	K X E19	F_3	44	K X E 44	F_3
20	K X E20	F_3	45	K X E 45	F_3
21	K X E21	F_3	46	K X E 46	F_3
22	K X E22	F_3	47	K X E 47	F_3
23	K X E23	F_3	48	K X E 48	F_3
24	K X E24	F_3	49	K X E 49	F_3
25	K X E25	F_3	50	K XE 50	F_3

METHODS

- **Processing of rice grains.**
- **Dehusking:** Around 30 gm seeds of each samples were hand dehulled using polyurethane coated hand dehulling unit.

ESTIMATION OF TOTAL PROTEIN

Total protein of brown rice grains was estimated by modified micro-kjeldal method for rice as described by Johri *et al*., (2000).

- **Digestion process:**

  - **0.5g rice grain + 4-5g salt mixture (K_2SO_4 and $CuSO_4$ in ratio 5:1)**
  - **10 ml conc. H_2SO_4**
  - **Digestion temperature 360 ºC- 410 ºC**
  - **Sample turns ice blue in color on completion.**

Cont…

❑ Distillation Process:

- Using 4% Boric acid and
- 40% sodium hydroxide solution

❑ Titration:

- Carried out against 0.05 N sulfamic acid
- % N calculated & converted to % protein

$$\% \text{ Nitrogen} = \frac{(\text{Vol. of Sulfamic acid} - \text{Vol. of blank}) \times \text{Normality} \times 14 \times 100}{\text{Sample weight (gm)} \times 1000}$$

Protein (%) = % N × 5.95

(Julliano,1993)

ESTIMATIONS OF IRON AND ZINC

- Estimation of iron and zinc content in brown rice grains was done as standard method described under Harvest Plus (2006).

- 1 g of rice grain sample was taken in digestion tube.
- 10 ml acid mixture (HNO_3, $HClO_3$) in the ratio 9:4 was added.

- Digestion was done at 290ºC temp. for about two hours till the acid mixture became colorless.
- The volume was made up to 50 ml using double distilled water.
- Determination of iron and zinc content by AAS.

Iron /Zinc conc. (µg/g) = Absorbance reading X (Dilution factor)

Phenotyping of parent and mapping population for grain Iron, Zinc and Protein content.

Mean whole grain iron, zinc and protein concentration in µg/g of rice lines with parents kranti and E 2035

S. No.	Genotypes	Mean iron (µg/g)	Mean Zinc (µg/g)	Mean Protein (%)
1	KRANTI	17.48	19.43	9.51
2	E 2035	13.55	30.06	11.30
3	K X E	16.87(6)	23.29 (4)	7.01 (13)

Formula	Iron (µg/g)	Zinc (µg/g)	Protein (%)
Variance	6.66	9.66	0.91
Sem	10.74	5.36	1.54
CV	14.42	5.46	4.03

ANOVA for grain Fe content

Source of variation	Degree of freedom	Sum of square	Mean Sum of Square	F-cal	F-tab (5%)
Replication	2	7.02059	3.5102	1.013066	3.0872
Treatment*	50	1000.274	20.005	5.77355*	1.4772
Error	100	346.50	3.4650		

*** Significant at 5% level of significance and 50 degrees of freedom**

ANOVA for grain Zn content

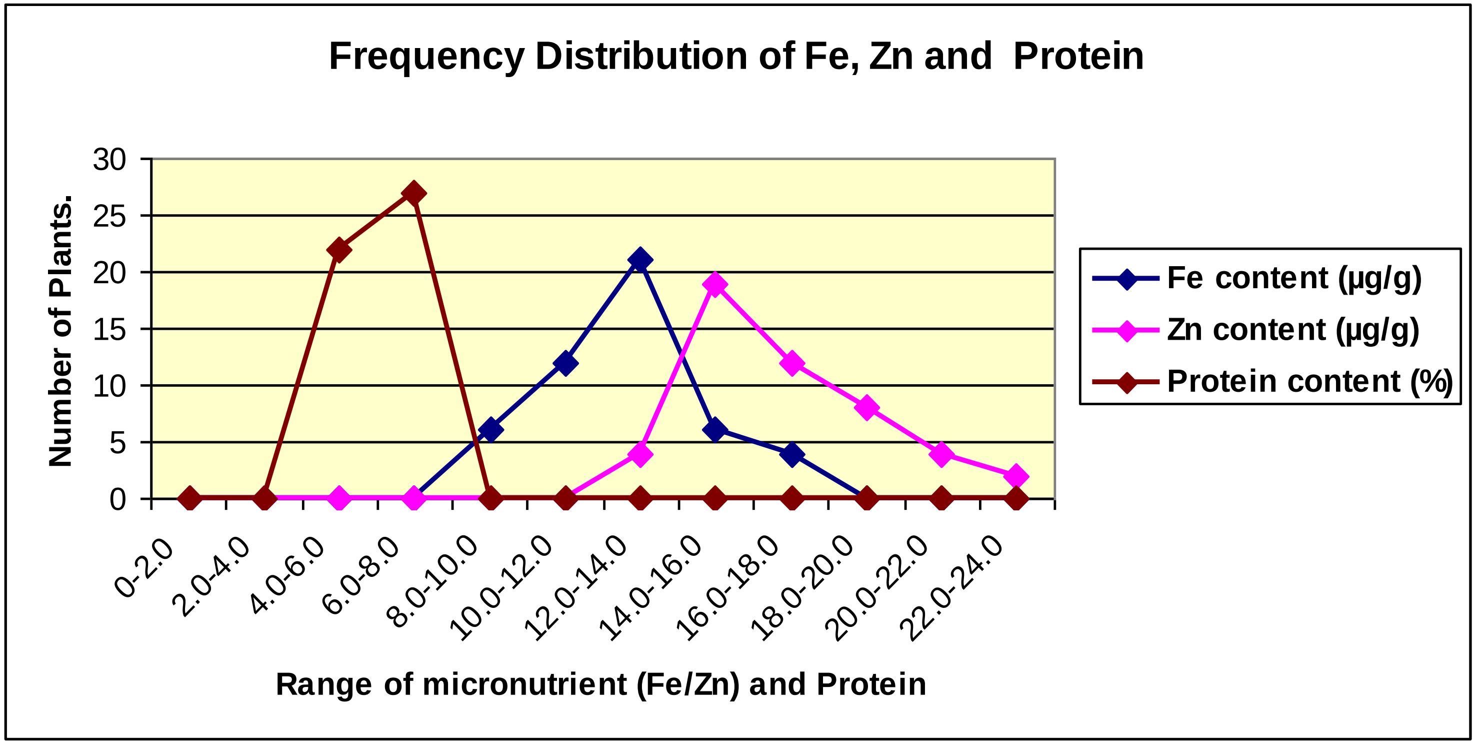
Source of variation	Degree of freedom	Sum of square	Mean Sum of Square	F-cal	F-tab (5%)
Replication	2	4.039233	2.019616	2.3359354	3.0872
Treatment*	50	1449.986	28.99973	33.54176*	1.4772
Error	100	86.45857	0.864586		

*** Significant at 5% level of significance and 50 degrees of freedom**

ANOVA TABLE FOR GRAIN PROTEIN CONTENT

Source of variation	Degree of freedom	Sum of square	Mean Sum of Square	F-cal	F-tab (5%)
Replication	2	0.284906	0.142453	2.252614268	3.087296
Treatment*	50	137.9449	2.758898	43.6265666*	1.477231
Error	100	6.323894	0.063239		

*** Significant at 5% level of significance and 50 degrees of freedom**



Frequency distribution of grain iron, zinc and Protein content in cross population

In silico characterization Studies

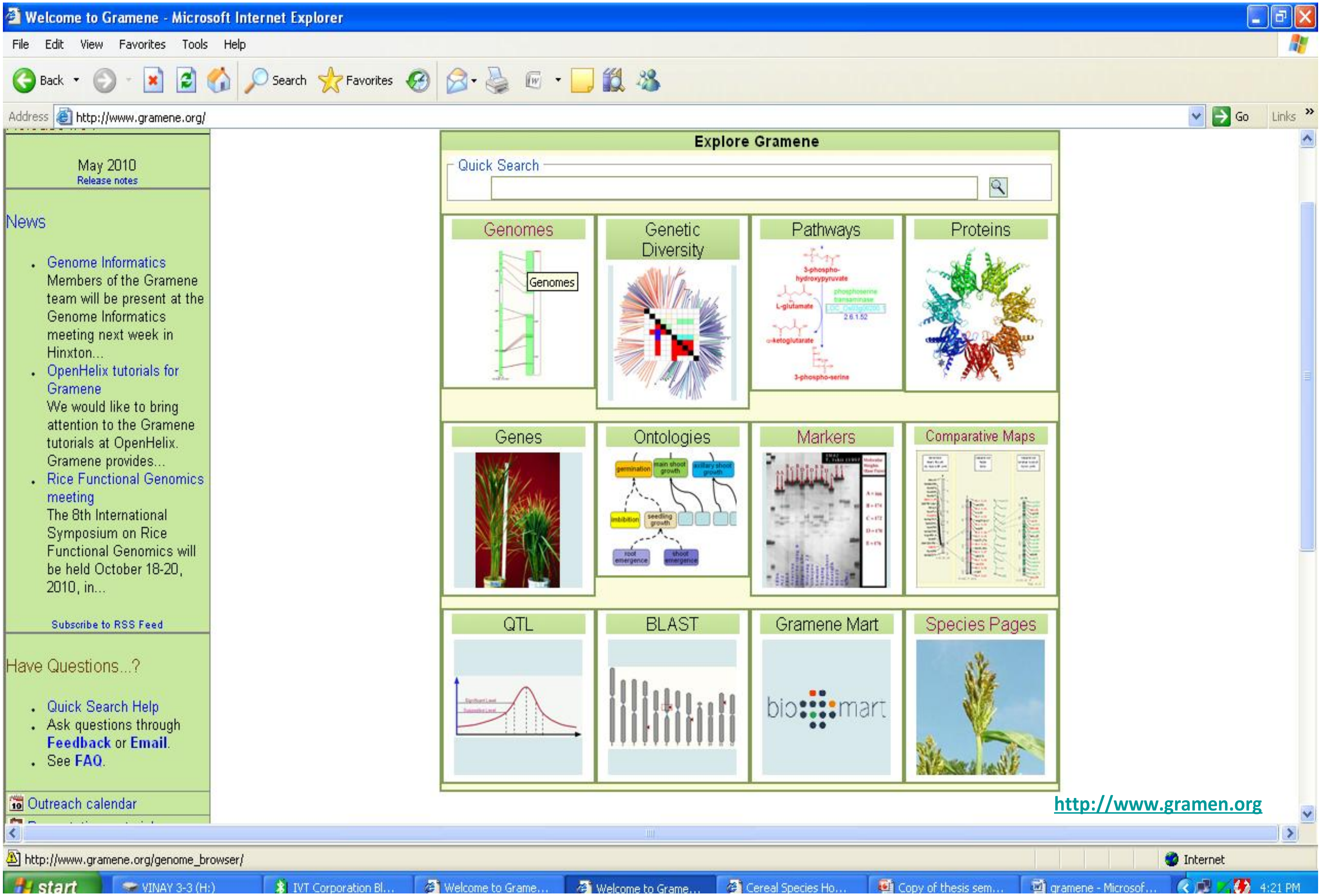
Freely available sites:

http://www.gramen.org

http://www.tigr.org.in

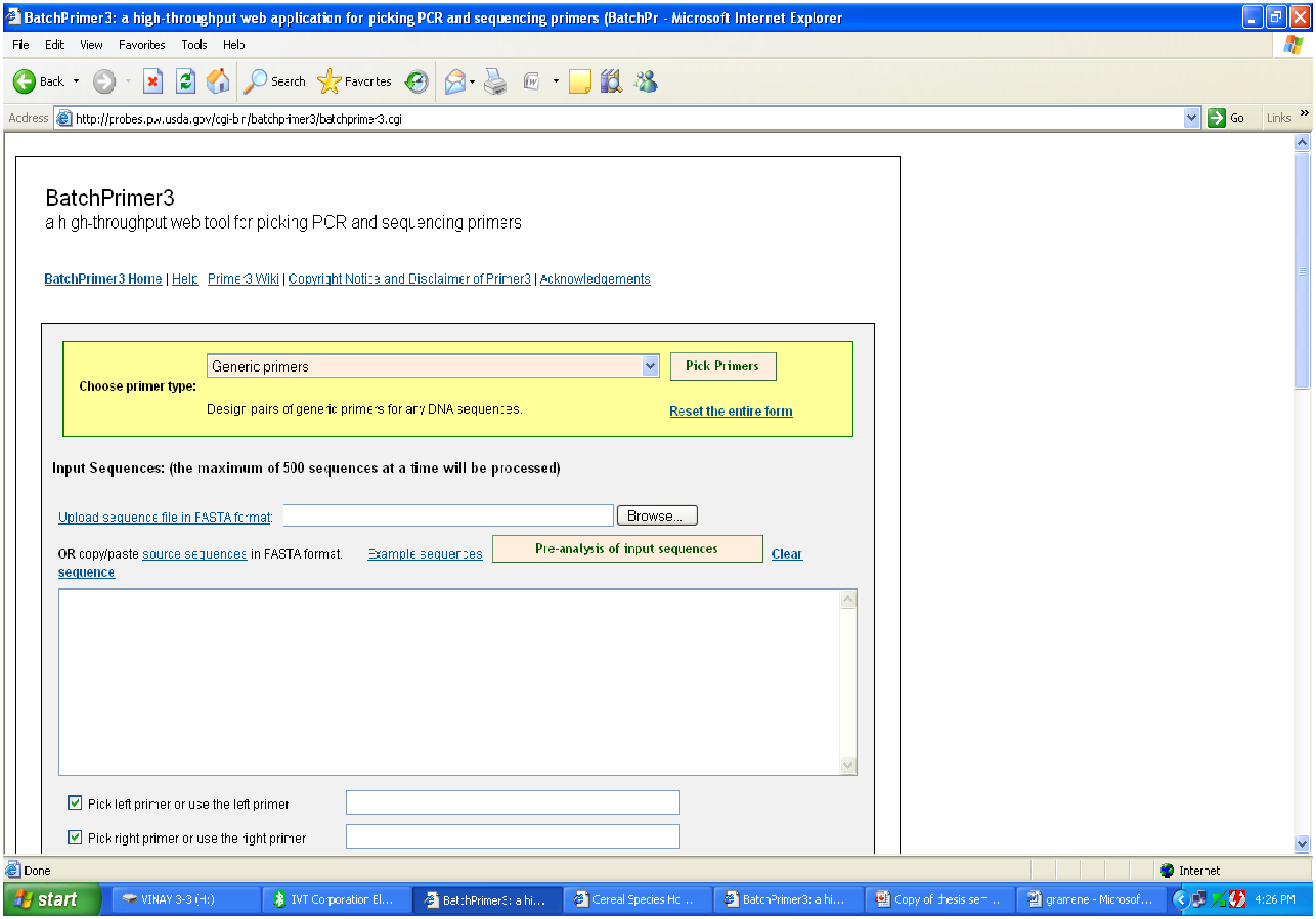
http://www.batchprimer3.org

GRAMENE HOME : Online databases for gramene family



http://www.gramen.org

Batchprimer3: Online software tool for Primer designing.



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QTL Analysis in Rice Improvement: Concept, Methodology and Application

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Abstract: Plant breeding is the management of genetic variability. A large number of traits of economic importance are quantitative in nature and are characterized by continuous variation owing to large number of genes governing them. Advances in molecular biology have helped to dissect these traits into finer details but we are still far from precise location and enumeration of the genes conditioning a quantitative trait. Various aspects of study of quantitative traits are number of genes, chromosomal locations, allelic and non-allelic interactions, G x E interaction and pleiotropy effects. QTL mapping is an integration of linkage mapping and traditional statistical and quantitative genetic approaches. Various methods of QTL mapping such as single marker analysis, interval mapping, composite interval mapping and multi trait mapping have been standardized using several mapping populations such as F2, back cross, RIL's, NIL's and double haploids and various software packages. A large number of QTL's have been identified for various economic traits which account for a sizeable proportion of the genetic variation and as such is turning out to be more than just a statistical inference. Advances in QTL mapping will help in genetic analysis of complex traits, plant genomics, germplasm enhancement, improved selection efficiency through MAS and studying gene expression along growth and developmental phases of plant life.

Key words: Rice, quantitative trait, QTL mapping, mapping populations, single marker analysis, interval mapping, composite interval mapping, marker aided selection

INTRODUCTION

The success of plant breeding operations exclusively relies on genetic variation. In fact plant breeding uses selection for improving plant architecture for traits of economic and agronomic importance by using genetic variability. In crop plants a greater proportion of such traits are governed by a large number of genes with smaller contributions to the trait resulting in continues rather than discrete variation (Liu, 1998). Even traits considered to be more simply inherited, such as disease resistance, may be actually quasi-qualitative for which trait expression is governed by several genes i.e., major genes plus several modifiers (Stuber *et al*., 1999). Thus the trait values are measured rather than counted. The analysis of such quantitative variation especially its potential genetic basis is of prime importance to a plant breeder (Asins, 2002). Because of their features such a large number of genes, small effects and greater vulnerability to environmental influences. Their phenotype does not provide ample insight into their genotype as against simple monogenic traits (Kearsey, 2002).

Fisher (1918) was first to provide an understanding of quantitative traits and their measurement. Even upto 1980's, the genetics of such traits was studied by using simple statistical techniques (means, variances, co-variances, heritabilities etc.). The assumption underlying such techniques was that there are several genes segregating in a given population and that these genes would share individual allelic contributions which are slight relative to environmental contribution. Even on such a minimalistic or black box concept, considerable progress was made in advancing our knowledge of nature and effect of quantitative inheritance. Considerable theoretical and experimental progress has been made in understanding various aspects of quantitative traits such as heritability, direct and correlated response to selection and subsequently optimizing the breeding methodologies for improving upon a crop species (Kearsey, 2002). There are, however, obvious limitations to understanding of nature of QT's because of lack of discrete phenotypic segregation and because genotype effects of each gene associated with a complex trait are relatively small.

The advances in biometrics has made it possible to study QT's in finer details. However, these advances only

IN SILICO CHARACTERIZATION OF QTLs CONTROLLING GRAIN PROTEIN CONTENT IN RICE

- ❑IRCG 103544 X Caiapo DH
- ❑Trait- Grain Protein (%)

List of QTLs selected for the study and their flanking markers

S. n.	Name of QTLs	Markers Interval
1	qPRO-1	RM 226 –RM 297
2	qPRO-2	RM 6 – RM 112
3	qPRO-6	RM 190 – RM 253
4	qPRO-11	RM 209 – RM 229

(Parveaz Sofi and A.G. Rather 2007)

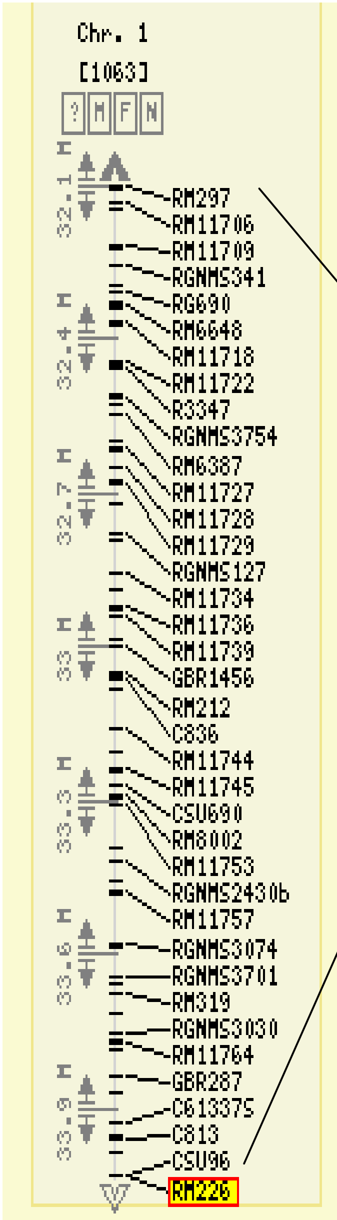
DETAILS OF SSRs WITHIN QTLS

- SSRs with 2-5 nt. Repeat motifs were found and total repeat length varied from 12 to 76 bp.

- All types repeat motifs at, ag, tgg, tcta, tg, cga, atgc etc. were found.

- Out of these class I SSRs with ≥ 20 bp repeat length were selected for primer designing.

S. n.	QTLs	Class I SSRs	Total no. of SSRs
1	qPRO-1	60	388
2	qPRO-2	62	483
3	qPRO-6	43	432
4	qPRO-11	30	259
	Total	**195**	**1562**

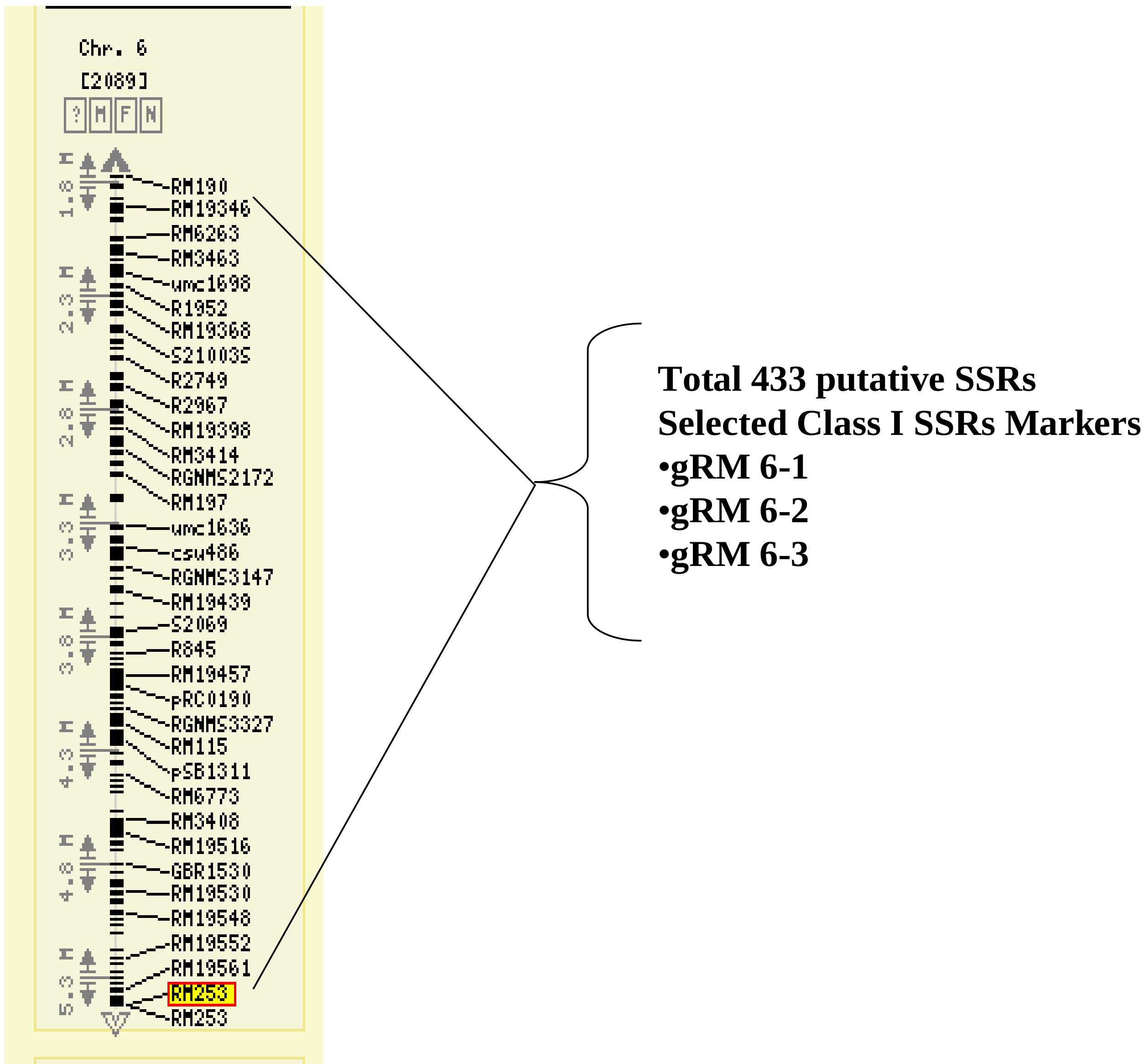


Total 388 putative SSRs

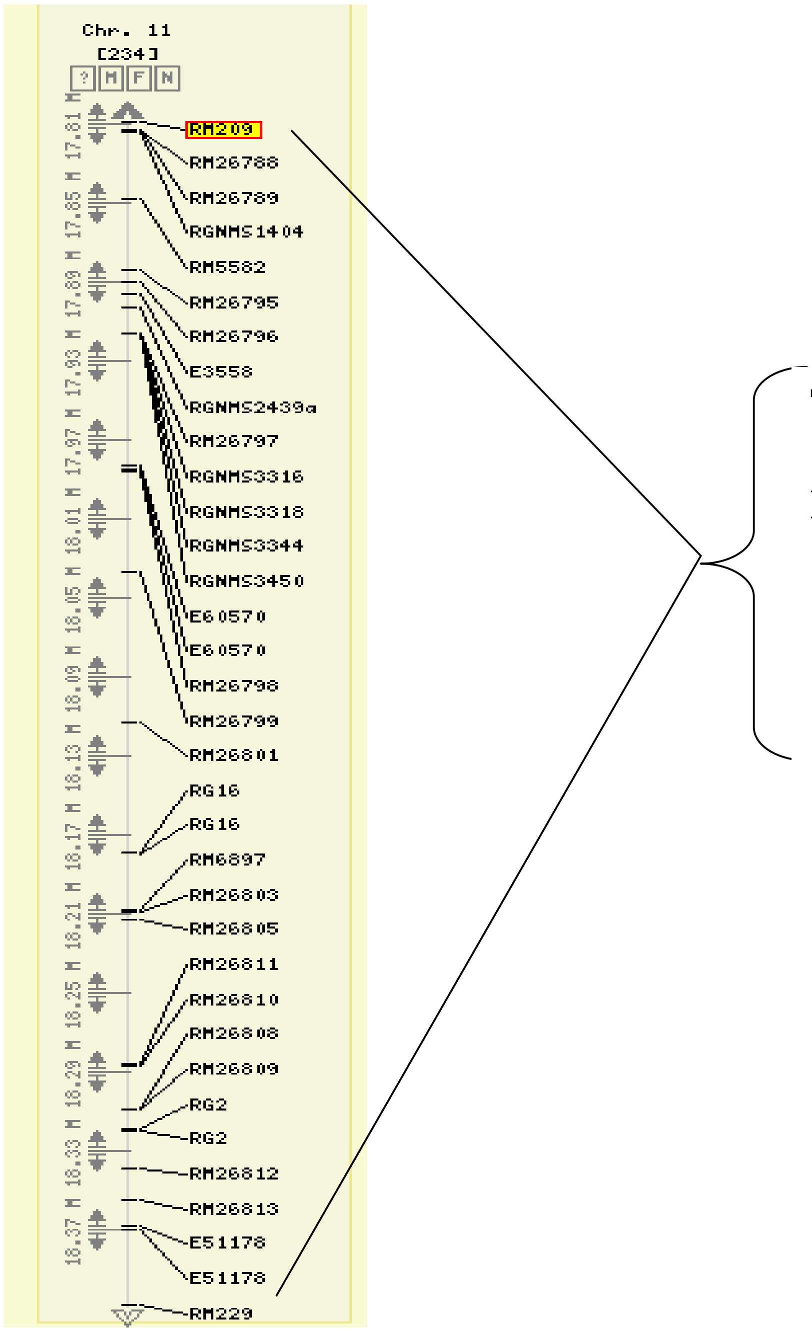
Selected Class I SSRs Markers

- **gRM 1-1**
- **gRM 1-2**
- **gRM 1-3**
- **gRM 1-4**
- **gRM 1-5**
- **gRM 1-6**

Map position of QTL qPRO-1 on ch #1 along with co-localized Putative SSR markers identified.



Map position of QTL qPRO-6 on ch #6 along with co-localized Putative SSR markers identified.



Total 259 putative SSRs
Selected Class I SSRs
Markers
•gRM 11-1
•gRM 11-2
•gRM 11-3

Map position of QTL qPRO-11 on ch #11 along with co-localized Putative SSR markers identified

LIST OF DESIGNED QTLs SPECIFIC SSR PRIMERS

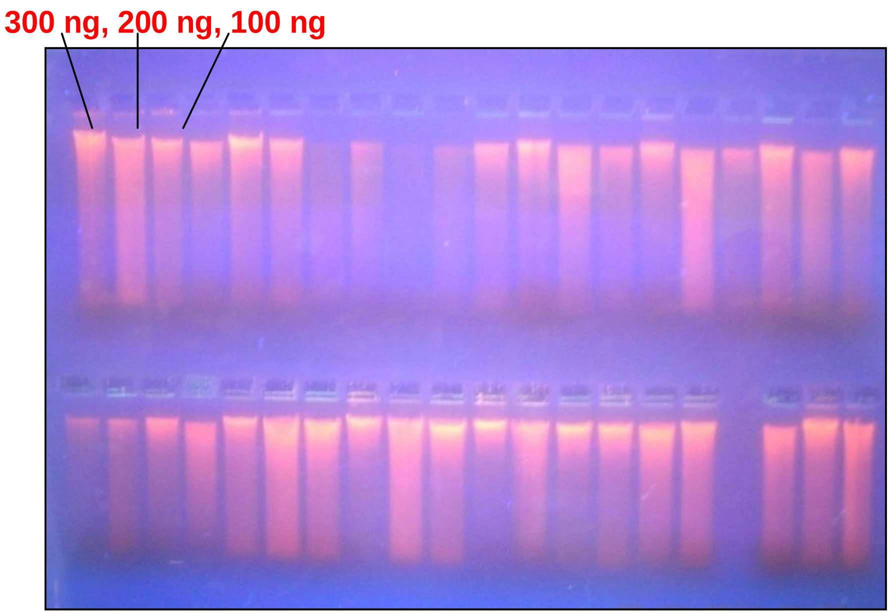
Marker	Primer Forward	Primer Reverse	Tm (°C)
gRM 6-1	AGTTACGACCAATGATACGC	GACTAGCAGCTCACGATCTAA	55
gRM 6-2	CTTGTGATCAAGTCGTCGTA	CTGCAGTATCATCATCGACA	55
gRM 6-3	CATCACACGACATCATTCC	GAGCTACAAGAGATCCTCTCC	55
gRM 11-1	CCTCCTACTCACCTGTGTTA	GAGTTTAGGGGAGGTTCTG	53
gRM 11-2	GGAGCTTACATCTCTTGGAC	TCTATCTCTGCTCTCAGTGG	53
gRM 11-3	GTTCTTCACCTTCCAGCTAC	CCCTCATCTAATCCTACTCC	53
gRM 1-1	GTGGGTCGTGTGTGATTA	CACGATCGACATCAGTTC	53
gRM 1-3	ACACACACACACACACACAC	CTGGAGCCTACTGAATACTG	53
gRM 1-4	GGTGATCGATCTCACTCG	TCACCTAGACTGAGTTGTGG	54
gRM 1-5	TCCTCGTACCTCTCGAAC	CACCTCCACGCTACTACTC	53
gRM 1-6	CGTCAGTCGCGTATAGAA	GATCCACATGTCACTCACAC	53
gRM 1-7	CTGTGCTCCGTGTACATA	TCGATCGACTTGGAGTAG	51

Genotyping of the mapping population using QTL's specific DNA markers

DNA ISOLATION AND QUANTIFICATION

- Approximately 15 seeds of kranti, E2035 and their populations were sown in green house. the leaves are used for DNA isolation by the method described by dellaporta *et al,.* (1983).

- For quantification:
  - 3 µl of the DNA samples.
  - 0.8% agarose gel.
  - The electrophoresis was performed at 60 volts for 45 minutes.
  - Ethidium bromide 2.5 µl/100 ml.
  - Observed under UV transilluminator



PCR COMPONENTS WITH THEIR QUANTITY FOR MICROSATELLITE ANALYSIS

S. n.	Components	Concentration	Quantity
1	PCR buffer with $MgCl_2$	10X	2.0 μl
2	dNTPs	2 mM	2.0 μl
3	Primer (Forward)	10 μM	1 μl
4	Primer (Reverse)	10 μM	1 μl
5	Taq DNA Polymerase	5 U	0.25 μl
6	Sterile water	-	11.75 μl
7	Template DNA	40 ηg/μl	2.0 μl
8	**Total**		**20.0 μl**

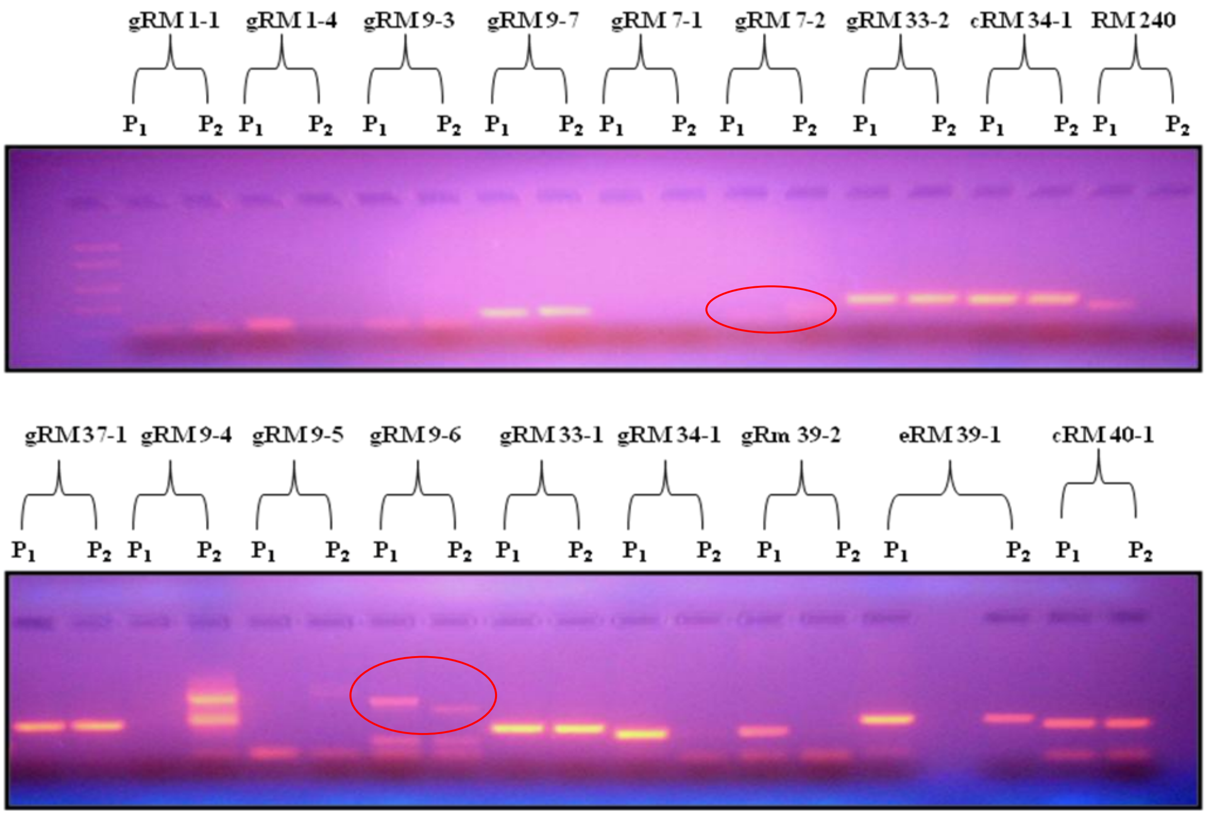
TEMPERATURE PROFILE USED FOR PCR AMPLIFICATION USING MICROSATELLITE MARKERS

Steps	Temperature (°C)	Duration	Cycles	Activity
1	94	3 min.	1	Initial denaturation
2	94	1 min.	↑	Denaturation
3	50-55	45 sec.	35	Annealing
4	72	2 min.	↓	Extension
5	72	7 min.	1	Final Extension
6	4	9 hrs	1	Storage

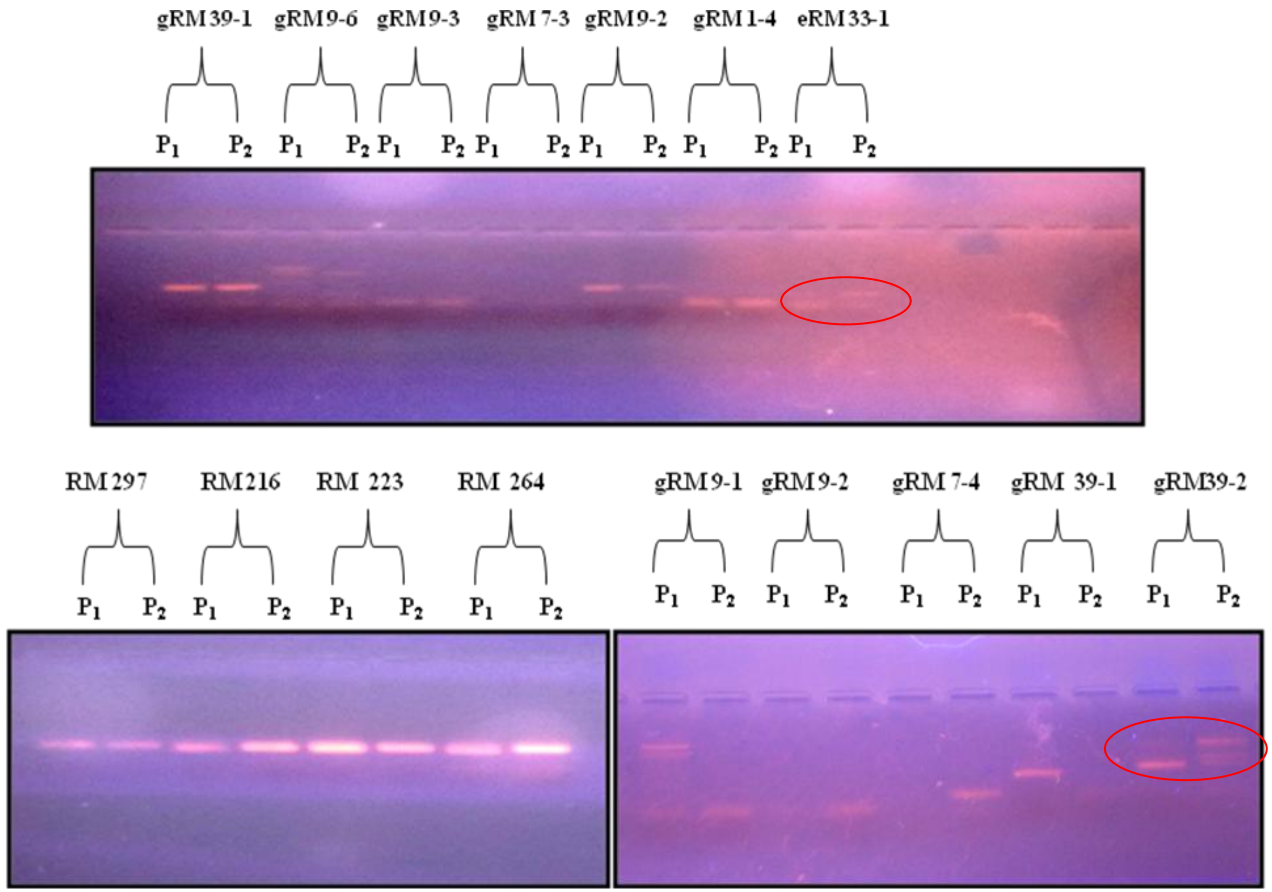
List of Primers used for parental polymorphism analysis

S. n.	Markers	Primer Forward	Primer Reverse
1	gRM 1-1	CTAGCATTTCCCACATTCATA	TCTAGCATTTTCCACATTCAT
2	gRM 1-4	ATGTGGCTAGATTCATTAACAT	AAAACTTTCTAGCATTGCTCAT
3	gRM 9-3	TCCAAAATTAGTTATGGCATC	ACCAGTATCCAAAGTGTACCA
4	gRM 9-7	ATCTACGGAGTTATGCATGTG	CAGAATTGAAGTATTCGCTTG
5	gRM 7-1	TATTTTTCATCCCTCCTCTTT	AATTTTGTGCATTTGAAACC
6	gRM 7-2	TCTCTAGGCGTTTATCTTTTG	ATTAGTTGTCCCCTTTCTCAC
7	gRM 33-2	AACGACATGCAAAATGAGAG	AAGAGGAGATTCCATGTTCA
8	cRM 34-1	ATGTCTAACATGGTGGCTTG	CGCTTTGAAGGATTTGAATA
9	gRM 37-1	TCACGGAGCTCGTACTTG	ACCTTCCGATCTGGAGTC
10	gRM 9-4	TAGTGTGTGTGTGTGTGTGTG	GTGGCAAGAAGTTCCTAATTT
11	gRM 9-5	AGTTGGCTTAGTCTTTGAGGT	ACGCAAAAGATAGGGTTAAGT
12	gRM 9-6	AGGGTGAAGAACCTCACTTAG	TAACATGTTTGTGAACCGATA
13	gRM 33-1	TCGTTCTGACATGTTGAGG	CCCGACAAAGTCAACGTC
14	gRM 34-1	ATAGTATGCCCAGCATTAGC	TGTTCTCTCTGCACTTGTTG
15	gRM 39-2	GTGATGTGATGTGATGGAAA	CACCTCCAGGATCTCGTC
16	eRM 39-1	GAGCCAAGAGATGAGTTTCA	AGGACGAATCAGACAAACAG
17	cRM 40-1	CTTGTGTTTTGGACTGCTTC	CCACTTTCTGCTGACAACTC
18	gRM 7-3	TATATAGCGATTCTGCCACTT	GGAGCACCAAACAAATTTAC
19	gRM 9-2	ATATGTTTATGCCCAAGTGGT	TTTGGGATGAGATATGCTTTA
20	eRM 33-1	GCCGACGTTGACTTTGTC	AAAGCGAGACACCTTTTCTT
21	gRM 7-4	GTCACCTAATGCTTTTGCTT	AGCATATGAAATACGGAGAGA
22	gRM 9-1	TATGTGTGTGTGTGTGTGTGT	GGAGTTGGATGTTTGAAAATTA

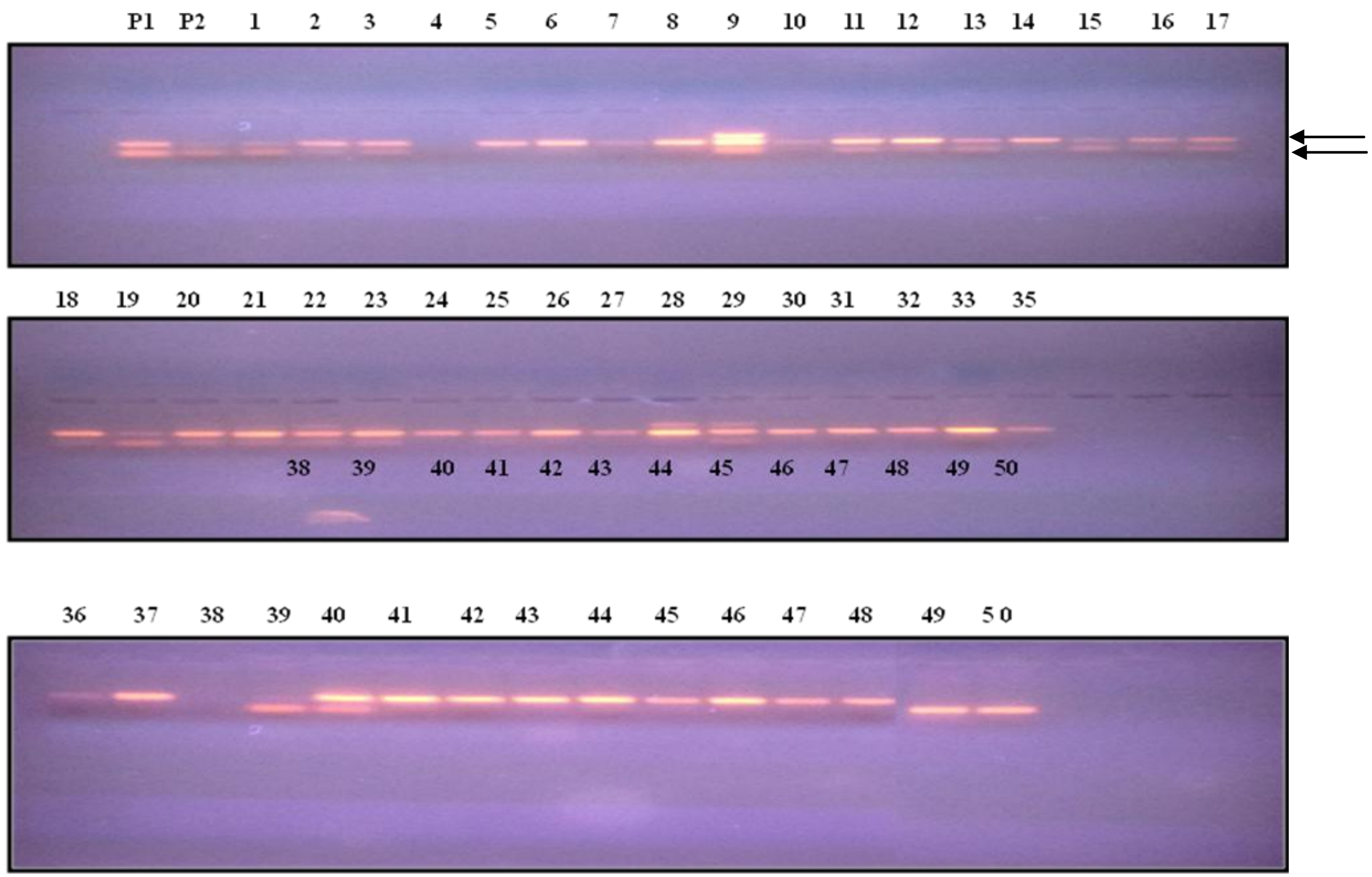
Parental polymorphism using SSR primers (p_1- Kranti, p_2- E2035)



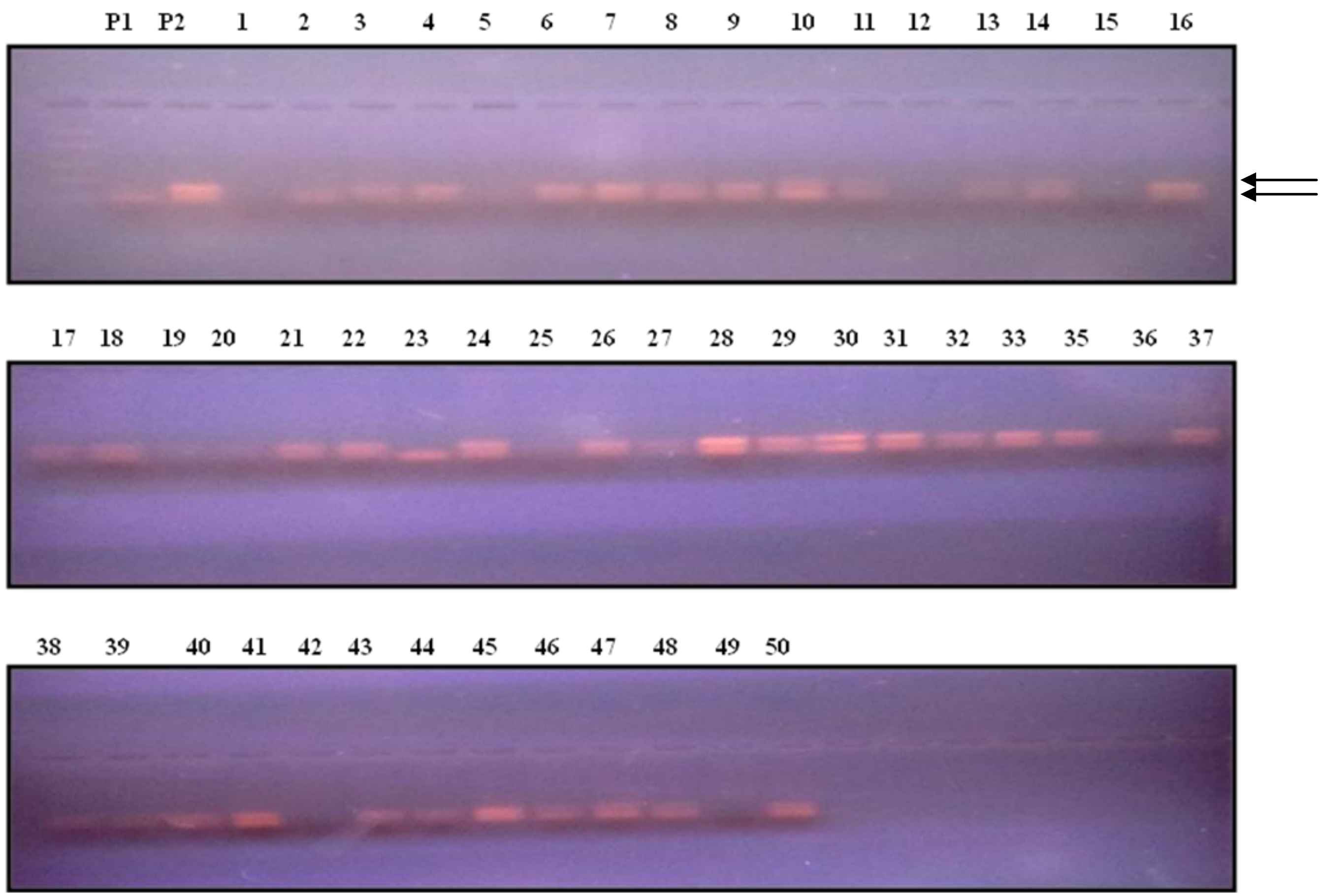
Parental polymorphism using 16 SSR primers (P_1- Kranti, P_2- E2035)



SSR PROFILE OF 50 RICE LINES DERIVED FROM A CROSS BETWEEN KRANTI AND E2035 USING gRM 7-2 SHOWING POLYMORPHISM (P_1-KRANTI, P_2 - E2035)



SSR profile of 50 rice lines derived from a cross between Kranti and E2035 using gRM 39-2 showing polymorphism (P_1-Kranti, P_2- E2035)



Scoring of banding pattern of population derived from cross between Kranti and E2035

S. N.	Genotypes	gRMm9-6	gRMm7-2	gRMm39-2	eRMm33-1
1	Kranti	A	A	A	A
2	E2035	B	B	B	B
3	1	A	H	M	B
4	2	A	A	A	B
5	3	A	H	B	B
6	4	A	A	B	A
7	5	A	A	M	A
8	6	A	A	B	M
9	7	A	H	B	B
10	8	A	A	B	A
11	9	A	H	H	B
12	10	A	A	H	B
13	11	A	H	B	B
14	12	A	A	M	M
15	13	H	H	A	B
16	14	H	A	A	A
17	15	M	H	M	A
18	16	A	A	A	B
19	17	A	H	M	B
20	18	A	H	B	A
21	19	A	H	B	A
22	20	A	H	M	H
23	21	A	H	M	A
24	22	M	H	A	B
25	23	M	H	A	B

Cont…

S. No.	Genotypes	gRMm9-6	gRMm7-2	gRMm39-2	eRMm33-1
26	24	A	A	B	A
27	25	B	A	H	B
28	26	B	A	M	A
29	27	B	A	A	B
30	28	B	A	H	A
31	29	B	H	H	B
32	30	B	A	H	B
33	31	B	H	H	B
34	32	B	A	A	M
35	33	A	A	A	B
36	35	B	A	A	A
37	36	B	A	M	B
38	37	B	A	A	A
39	38	B	A	B	A
40	39	M	M	B	A
41	40	B	B	B	B
42	41	B	H	H	A
43	42	M	A	M	A
44	43	M	A	A	B
45	44	B	A	A	M
46	45	M	A	A	B
47	46	A	A	H	B
48	47	M	A	A	B
49	48	A	A	A	B
50	49	B	B	M	A
51	50	B	B	A	M
	TOTAL	A-22 B-17	A-28 B-3	A-17 B-12	A-17 B-25

Overall percentage of A allele-42.85% of allele B-29.08% and H-28.06%

ASSOCIATION ANALYSIS

- Association analysis was perform to establish markers trait association i.e. between polymorphic SSR marker and grain nutritive trait (Iron, Zinc and Protein)

- The analysis was performed by simple 't' test.

Individual analysis of markers with the population

Parameter	gRMm9-6	gRMm7-2	gRMm-39-2	eRMm 33-1
% of allele A	44.89%	57.14%	34.69%	34.69%
% of allele B	34.69%	6.12%	24.48%	51.02%
% of heterozygous	20.40%	36.73%	40.81%	34.69%

‘t’ –test for the polymorphic primers for Iron and Zinc

S.No.	Primer	t-value (iron)	t-value (zinc)	Association with grain iron content	Association with grain zinc content
1	gRMm9-6	0.492	0.129	Not Associated	Not Associated
2	gRMm7-2	0.100	2.433*	Not Associated	Associated*
3	gRMm39-2	0.455	0.498	Not Associated	Not Associated
4	eRMm33-1	0.353	0.539	Not Associated	Not Associated

‘t’ –test for the polymorphic primers for Protein

S.No.	Primer	t-value (Protein)	Association with grain protein content
1	gRMm9-6	4.793	Associated*
2	gRMm7-2	3.029	Associated*
3	gRMm39-2	0.996	Not Associated
4	eRMm33-1	0.974	Not Associated

SUMMARY AND CONCLUSION

- **Total grain Fe, Zn and protein estimation of Kranti and E2035 showed that:**

- Fe content (17.48 µg/g) is high in Kranti and low in E2035 (13.55 µg/g) rice genotype.

- The brown rice grain Fe content ranged from 8.16 to 16.86 µg/g with an average of 12.70 µg/g in F_3 cross population and the coefficient of variation (CV) was found to be 14.42 %. (IR8-12.3, IR36-11.8,IR74-11.2, Gregorio *et al*.,2000)

- Zn content is low in Kranti (19.43µg/g) and high in E2035 (30.06 µg/g) rice genotype.

- The Brown grain Zn content ranged from 12.41 to 23.29 µg/g with an average of 17.01 µg/g in F_3 cross population and the coefficient of variation (CV) was found to be 5.46 %. (IR8-17.3, IR36-23.1, Gregorio *et al*.,2000)

- The Protein content is low in Kranti (9.51 %) and high in E2035 (11.30 %) rice genotype. (PR-27423-MS6- 6.3%, Riza *et al*.,)

- The brown rice grain protein content was found to be in the range of 5.07 to 7.01 with an average of 6.21 and the coefficient of variation (CV) for grain protein content was found to be 14.4 %.

SUGGESTIONS FOR FUTURE RESEARCH WORK

- The high Fe, Zn and protein lines identified in the study should be further analyzed at multi locations to assess the environmental effect on grain micronutrients and protein contents.

- Novel SSR markers identified must be validated in suitable mapping populations to assess their cross transferability among different populations.

- Primers for the remaining SSR loci identified can be designed and screened against parents and mapping population to study their polymorphism and association to grain nutritive contents .

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Thank You