IDENTIFICATION OF DNA MARKERS ASSOCIATED WITH GRAIN FE, ZN AND PROTEIN CONTENTS IN RICE (*oryza sativa* L.)

M. Sc. (Ag.) THESIS

By

Ajay Kumar

DEPARTMENT OF PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
INDIRA GANDHI KRISHIVISHWA VIDYALAYA,
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IDENTIFICATION OF DNA MARKERS ASSOCIATED WITH GRAIN FE, ZN AND PROTEIN CONTENTS IN RICE (*oryza sativa* L.)

Thesis

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Ajay Kumar

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CERTIFICATE-I

This is to certify that the thesis entitled **"Identification of DNA Markers Associated with Grain Fe, Zn and Protein Contents in Rice (*Oryza sativa* L.)"** submitted in partial fulfillment of the requirement for the degree of **"Master of Science in Agriculture (Plant Molecular Biology and Biotechnology)"** of the Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.), is a record of the bonafide research work carried out by **Ajay Kumar** under our guidance and supervision. The subject of the thesis has been approved by 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 investigation have been duly acknowledged by him.

Chairman

Date: 21.7.17.

THESIS APPROVED BY THE STUDENT'S ADVISORY COMMITTEE

Chairman: Dr. Girish Chandel

Member: Dr. (Smt.) Shubha Banerjee 21/7/17

Member: Dr. R. R. Saxena

Member: Dr. L.k.Srivastava

CERTIFICATE-II

This is to certify that the thesis entitled **"Identification of DNA Markers Associated with Grain Fe, Zn and Protein Contents in Rice (*Oryza sativa* L.)"** submitted by **Ajay Kumar** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur in partial fulfillment of the requirements for the degree of **Master of Science** in the Department of Plant Molecular Biology and Biotechnology has been approved by the external examiner and Student's Advisory Committee after oral examination.

A Dubey 29/8/17

External Examiner

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(Name Dr Amit Dubey)

Major Advisor _____

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Dean/ Dean Faculty _____

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"Education plays fundamental role in personal and social development and teacher play a fundamental role in imparting education. Teachers have crucial role in preparing young people not only to face the future with confidence but also to build it with purpose and responsibility. There is no substitute for teacher-pupil relationship."

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Ajay Kumar

Ajay kumar

Department of PMBB
College of Agriculture, IGKV
Raipur (CG),

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LIST OF ABBREVIATIONS

%	Percent
°C	degree Celsius
µl	Microlitre
AFLP	Amplified fragment length polymorphism
Approx.	Approximately
Bp	Base pair
Ch.	Chromosome
Cm	Centimeter
Cm	Centimorgon
DNA	Deoxyribo nucleic acid
DAS	Days after sowing
DAT	Days after transplanting
EDTA	Ethylene diamine tetra acetic acid
EtOH	Ethanol
Etbr	Ethedium bromide
et al	and others
gm	gram
H_2O	water
Ha	Hectares
HCL	Hydrocloric acid
i.e.	that is
m^{-2}	Per square meter
M	Molar
Min	Minutes
ml	Milliliter
Nacl	sodium chloride
P^+/P1	Sufficient phosphorus
P-/P0	Deficient Phosphorus
PCR	polymerase chain reaction
Pup1	Phosphorus uptake 1
PUE	Phosphorus use efficiency
RT-PCR	Reverse Transcriptase Polymerase chain Reaction
PHT	Plant height
NOT	Number of tillers
NOP	Number of panicle
FLL&W	Flag leaf length & width
QTL	Quantitative trait loci
GY	Grain yield

THESIS ABSTRACT

Title of the Thesis	:	"Identification of DNA Markers Associated, with Grain Fe, Zn and Protein Contents in Rice (*Oryza sativa* L.)"
Full Name of the Student	:	Ajay kumar
Major Subject	:	Plant Molecular Biology and Biotechnology
Major Advisor	:	Dr. Girish chandel (Professor) Department of Plant Molecular Biology and Biotechnology
Degree to be awarded	:	Master of Science

Ajay Kumar
Signature of the Student

Signature of major Advisor

Signature of Head of the Department

Date: 21.7.17.

ABSTRACT

Rice is the primary or secondary staple food for 50 % of the world population. Rice contributes major on source of calories for rice eating population. Over 800 million people in developing world are undernourished suffering from either protein energy or micro-nutrient deficiency. There is a serious need to redesign the way that will ensure the balance nutrient supply of major stable food for people in adequate and affordable amount. To improve nutritive value of rice the preliminary step is to characterized genetic variability for iron, zinc and grain protein content in germplasm and then to use this variability for breeding nutrient rich. Looking to this present study entitled **"Identification of DNA Markers Associated with Grain Fe, Zn and Protein Contents in Rice (*Oryza sativa* L.)"** was conducted at the department of Plant Molecular Biology and Biotechnology, Indra Gandhi Krishi Vishwavidyalaya, Raipur. Molecular markers have proven to be powerful tools in the assessment of genetic variation and in the elucidation of genetic relationships within and among species (Shivapriya and Hittalmani, 2006).Hence the present study was undertaken in

order to identify the molecular markers associated with micronutrient and grain protein of rice along with Initially 96 rice were characterized for 17 morphological trait, phenotyping for major 8 quantitative and 9 qualitative traits. A set of 96 rice genotypes including landraces, breeding lines and cultivars were screened for the micronutrient and protein contents wide genetic variables for all trait i.e. Fe, Zn and protein were recorded among the tested rice genotypes, which ranged from14.3ppm (Bisni) to 4.7ppm (IR681444*Moro) Fe, 34.8ppm (Dullar) to13.6ppm (GP-145-138) for Zinc and 10.28% (Kalam Gurmatia) to 5.78% (CGR-1539) for grain protein content. The rice genotypes processing higher grain Fe, Zn and protein contents were identified as donor rice genotypes to be useful in breeding.

Molecular markers, a powerful tool for assessing the genetic variability were also used to characterization the rice genotypes out of total 42 SSR marker used in this study 18 were found to be polymorphic in nature, and only 8 were found to be associated with grain nutritive value i.e. Fe, Zn and protein .In which 8 marker RM5, RM19, RM154, RM234, RM279, RM490, RM225, and Crm33-1are to be associated with Iron, Zinc and grain protein content of rice present on chromosome number 1,12,2,7,2,1,6 and 3.the associated markers identified for grain Fe, Zn and protein content can be further useful in marker assisted selection and speedy development of nutri-rich rice varieties to compact malnutrition.

KEYWORDS: Microsattelite Marker, Rice, Iron, Zinc, Protein, Association Analysis.

शोध सारांश

अ)	शोध का शीर्षक	:	''डीएनए मार्कर्स ऐसोसिएटेड की पहचान, अनाज, लोह, जस्ता और प्रोटीन के साथ चावल (ओरीजा सैटिवा एल।) ''
ब)	छात्र का पूरा नाम	:	अजय कुमार
स)	प्रमुख विशय	:	पादप आणविक जीव विज्ञान एवं जैव प्रौद्योगिकी
द)	प्रमुख सलाहकार का नाम एवं पता	:	डॉ. गिरीश चंदेल (प्राध्यापक) पादप आणविक जीव विज्ञान एवं जैव प्रौद्योगिकी विभाग, कृषि महाविद्यालय, इं. गा. कृ. वि. रायपुर (छ.ग.), 492012
इ)	डिग्री से सम्मानित किया जाना ळे	:	एम. एससी. (एजी.)

Ajaykumar
छात्र के हस्ताक्षर

प्रमुख सलाहकार के
हस्ताक्षर

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विभागाध्यक्ष के हस्ताक्षर

सारांश

चावल विश्व के 50 प्रतिशत आबादी के लिए प्राथमिक व माध्यमिक भोजन का स्त्रोत है। चावल कैलोरी का मुख्य स्त्रोत है। विकासशील देश में 800 मिलियन से अधिक लोग प्रोटीन,उर्जा और सूक्ष्म पोषक तत्व की कमी से ग्रसित है। आवश्यकता यह है कि लोगो को संतुलित एवं पौष्टिक पोषक तत्व की कमी से कैसे पूर्ति कि जाये। इसके लिए नई तकनीक विकसित की जाये, कि मूलतः लोहा,जस्ता और अनाज प्राटीन आनुवांशिक परिवर्तनशील के लिए जर्मप्लाजम लाईन द्वारा सुधार किया जाये, वर्तमान अध्ययन से चावल में लौहा,जस्ता और प्रोटीन के साथ डी एन ए मार्कर की पहचान की गई हैं। आणविक मार्कर आनुवांशिक परिवर्तन के मुल्यांकन में शक्तिशाली उपकरण साबित हुए है। कम आणविक सूक्ष्म पोषक तत्व के साथ जुडे मार्कर की पहचान की गई है। 96 सेट चावल के अध्ययन में पता चला कि, 17 आकारिकी लक्षण एवं प्रमुख 8 मात्रात्मक व

9 गुणात्मक लक्षण का पता लगा। लोहा,जस्ता और प्रोटीन का औसत मान 14.3 पीपीएम से 4.7 पीपीएम लोहा में,34.8 पीपीएम से 13.6 पीपीएम जस्ता एवं 10.28% से 5.78% प्रोटीन पाया गया। उच्च किस्म के प्रजनन क्षमता के रुप में इन जिनोटाईप का भविष्य में उपयोंग किया जाये ।

आणविक मार्कर आनुंवांशिक परिवर्तनशीलता का आंकलन करने के लिए एक शक्तिशाली उपकरण का पता किया गया जिनमेंसे 42 मार्कर में 18 मार्कर बहुरुपता पाया गया और उसमें केवल 8 एसोशियसन मार्कर मिलें आरएम 5, आरएम 19, आरएम 154, आरएम 234 ,आरएम 279 , आरएम490, आरएम 225 और सी आरएम 33–1 जो गुणसूत्र क्रमांक 1,12,2,7,2,1,6 व 3 के पहचान की गई जो चावल में पोषक तत्वों की समृद्ध व उच्च किस्म की प्रजाति के विकास में तेजी लाने में उपयोगी साबित होगा।

कीवर्डः माइक्रोसेटेलाइट,चावल,लोहा,जस्ता एवं एसोशियसन एनालाइसिस।

CHAPTER-1
INTRODUCTION

Rice, *Oryza sativa* (2n = 24) belonging to the family *Graminae* and subfamily *Oryzoidea* is the most important cereal and a source of calories. Rice is an indispensable staple food crop for more than half of the world's population, supplies adequate energy in the form of calories. However, rice is a poor source of proteins, minerals such as iron and zinc that are essential to human health. These deficiencies are concentrated in the semi-arid tropics, particularly in South and Southeast Asia and sub-Saharan Africa (Reddy *et al.,* 2005). More than 800 million people in developing world are undernourished suffering from either protein energy or micro-nutrient (Vitamin A, Iron and Zinc) deficiency (ACC/ SCN 2004 WHO 2002). Also, the micronutrient malnutrition associated health risks have become a major hindrance in achieving the Millennium Development Goals (MDG) such as reducing poverty and hunger, improved maternal health status, and less child mortality (Cakmak 2008; White and Broadley 2011; Wessells and Brown 2012) and these are also important sustainable development goals (SDGs) to be achieved by 2035 (https://sustainabledevelopment.un.org). So there is a serious need to re-design the global food system and change in the way that that will ensure the balance nutrient supply of major staple food for people in adequate and affordable amount. Attempts have been made to alleviate these deficiencies through dietary diversification, food fortification, supplementation with limited sustainable success, but these strategies do not reach most of those suffering from deficiency. Biofortification has emerged as one possible solution to alleviate malnutrition with the development of new cultivars with elevated concentration of protein, Fe and Zn (Zimmermann and Hurrell, 2002). Biofortification is the most economical and sustainable strategy to alleviate malnutrition (White and Broadley, 2005; Brar *et al*., 2011, Gregorio *et al*., 2000).

Approximately 11% of the world's arable land is planted annually to rice, and it ranks next to wheat. The world's rice production has doubled during last 25 years, largely due to the use of improved technology such as high yielding varieties

and better crop management practices (Byerlee, 1996). Rice is an ideal model plant for the study of grass genetics and genome organization due to its diploid genetics, relatively small genome size 430 Mb (Causse *et al*., 1994; Kurata *et al*., 1994), significant level of genetic polymorphism (McCouch *et al*., 1998; Tanksley, 1989; Wang *et al*., 1992), large amount of well conserved genetically diverse material (approximately 100,000 accessions of rice germplasm worldwide) and the availability of widely collected, compatible wild Species. There is wide genetic variability available in rice among and between wild relatives and varieties leaving a wide scope for future crop improvement. Diversity based on phenological and morphological characters usually varies with environments and evaluation of these traits requires growing the plants to full maturity prior to identification.

Now, the rapid development of biotechnology allows easy analysis of a large number of loci distributed throughout the genome of plants. Grain nutrient content is governed by many genes, which makes the accumulation of minerals in seeds a complex polygenic phenomenon (Grusak, 2004, Jiang 2008). Significant Genetic X Environment effect has been observed for the trait as it is affected by soil conditions, application of fertilizer, biotic and abiotic stresses (Gregario, 2002; Virk *et al.,* 2007; Graham, *et al.,* 2007). QTL's and genes controlling above traits has been identified and mapped in rice and several efforts have been made to improve grain protein/Fe/Zn content in rice which led to a significant understanding of the physiological, genetic, and molecular basis of Fe/ Zn/protein accumulation in grains, and also the influence of agronomic management and environmental factors on uptake, translocation and loading into grains (Wilson et al; 1999; Impa and Johnson-Beebout 2012). To improve nutritive value of rice the preliminary step is to characterize genetic variability for grain protein/Fe/ Zn content in germplasm lines and then to use this variability for breeding nutrient rich rice (Frei and Becker, 2002). Molecular marker assisted breeding is thus most suitable for intensive screening of large populations for identification of environment stable high grain protein iron and zinc rice genotypes. Molecular markers have proven to be powerful tools in the assessment of genetic variation and in the elucidation of genetic relationships within and among species. Several molecular markers *viz.,* Restriction fragment length polymorphism (Becker *et al*.,

1995; Paran and Michelmore, 1993), Random amplified polymorphic DNA (Tingey and Deltufo, 1993; Williams *et al*., 1990), Simple sequence repeats (Levinson and Gutman, 1987), ISSRs (Albani and Wilkinson, 1998; Blair *et al*., 1999), Amplified fragment length polymorphism (Mackill *et al*., 1996; Thomas *et al*., 1995; Vos *et al*., 1995; Zhu *et al*., 1998) and Single nucleotide polymorphism (Vieux, *et al*., 2002) are presently available to assess the variability and diversity at molecular level (Joshi *et al*., 2000). Information regarding genetic variability at molecular level could be used to help, identify and develop genetically unique germplasm that compliments existing cultivars.

Association or linkage disequilibrium (LD) mapping, which revolutionized genetic mapping in humans (Donnelly, 2008) and is increasingly being applied to plants (Nordborg and Weigel, 2008), and is considered an efficient way of determining the genetic basis of complex traits. Comparing traditional linkage mapping which depends on restricted allelic variation with a small number of recombination events, using the association mapping there is no need to develop segregating populations such as F2, double haploid (DH) or back-cross populations, instead a natural collection of inbred lines or varieties can be used. AM involves searching for genotype-phenotype correlations among unrelated individuals. Its high resolution is accounted for by the historical recombination accumulated in natural populations and collections of landraces, breeding materials and varieties. By exploiting broader genetic diversity, AM offers three main advantages over linkage mapping: mapping resolution, allele number and time saving in establishing a marker-trait association and its application in a breeding program (Flint-Garcia *et al*., 2003). Association mapping is therefore feasible, and potentially very useful for rice.

Exploiting the genetic variation in crop plants for protein, iron and zinc content is one of the most powerful tools to change the nutrient balance of a given diet on a large scale. Keeping in view the present study entitled **"Identification of DNA Markers Associated with Grain Fe, Zn and Protein Contents in Rice (*Oryza sativa* L.)"** was undertaken with the following major objectives,

1. Characterization of a set of rice landraces, popular varieties and advance breeding lines for Iron (Fe), Zinc (Zn) and grain protein content.
2. SSR based genotyping of rice landraces, popular varieties and advance breeding lines.
3. Identification of DNA markers associated with grain Fe, Zn and grain protein content.

CHAPTER-II
REVIEW OF LITERATURE

The success of green revolution has been instrumental in averting hunger. Despite the substantial progress made in food production, post green revolution serious nutritional challenges continue to threaten its progress in human resource development. The deficiency of nutrition is mainly due to undiversified and deficient food habits often referred as hidden hunger. Much of the world population relies on staple food crops such as rice, maize, wheat and cassava for their sustenance, who cannot afford the fortified foods to meet out the micronutrient requirements. More than 800 million people in developing world are undernourished suffering from either protein energy or micro-nutrient (Vit.A, Vit.C, Iron and Zinc) deficiency (ACC/ SCN 2004 WHO 2002). Globally, malnutrition, including both overt nutrient deficiencies as well as diet related chronic diseases (e.g. heart disease, cancer stroke and diabetes) is responsible for more deaths than any other cause accounting for over 20 million mortalities annually. Malnutrition also contributes to increased morbidity, disability, stunted mental and physical growth and reduced national socio-economic development (Nestle *et al.,* 2006). So there is a serious need to re-design the global food system and change in the way that will ensure the balance nutrient supply of major staple food for people in adequate and affordable amount. The high nutritional rice landraces can form a solid basis for changing priorities in rice breeding, putting more emphasis on the grain nutritional value for combating the protein energy and micronutrient malnutrition in human populations (Kennedy and Burligame *et al.,* 2003). Therefore, developing protein enriched cereals and improving their bioavailability (biofortification) using genetics and genomics tools are considered promising and cost effective approaches for diminishing malnutrition (Bouis, 2003, Cakmak, 2002). Recent development in the field of DNA technology has resulted in the development of several molecular markers, which are linked to many traits that are used in characterizing, true species and genera.

2.1 Rice crop

The genus *Oryza* belongs to the tribe *Oryzae* of the family *Poaceae* and subfamily *oryzoideae. Oryza* has two cultivated species, *Oryza* sativa and *Oryza glaberrima*. *Oryza,* the common cultivated rice is grown worldwide. Rice (*Oryza sativa* L.) is a true diploid (2n=24) with twelve chromosome pairs and contains 5.8x105 kb/haploid genome (Bennet and Smith, 1976). There is sample polymorphism in rice DNA and it is highly recombinogenic compared to other plants. One centimorgan of rice equals approximately 250 kb, compared to more than 500 kb in tomato and 750 kb in potato (Tanksley *et al.*, 1989). Most of the species in the genus *Oryza* have been characterized in terms of their chromosome number, genome symbols, phenotypic characters and geographical distribution (Khush and Kinoshita, 1991). The DNA content per map unit in rice, the most important food crop in the world is only 2-3 times greater than *Arabidospsis thaliana,* the ideal plant for molecular genetics. There is a vast reservoir of germplasm (2, 00,000 Landracess) of rice worldwide. Rice is considered as the most ideal monocot for molecular mapping and map based cloning of agriculturally important genes.

2.2 Global food and nutritional problem

Global food and Nutrition aims to provide current knowledge of different food and nutrition related topics around the world. The diet-related nutrition and health challenges such as undernutrition, micronutrient deficiencies, obesity and diabetes in the era of globalization and showcases various nutrition interventions focusing on dietary behaviouralchanges as well as long term food security and sustainable food system.The success of green revolution has been instrumental in averting hunger called for millions of in the world. Despite the substantial progress made in India’s food production post green revolution serious nutritional challenges continue to threaten its progress in human resource development. The deficiency of nutrition often referred as hidden hanger is mainly due to undiversified and deficient food habits. The majority of the undernourished population in the world subsists on diets based on cereals. Human beings require at least 49 nutrient elements to meet their metabolic needs, inadequate consumption

of even one of these nutrients will result in adverse metabolic disturbances leading to sickness, poor health and impaired development in children and large economic costs of society (Bronca and Ferrari, 2000; Ramakrishanan *et al*., 1999).

2.3 Effects of malnutrition

2.3.1 Protein

The World Health Organization (WHO) defines malnutrition as "the cellular imbalance between the supply of nutrients and energy and the body's demand for them to ensure growth, maintenance, and specific functions." The term protein-energy malnutrition (PEM) applies to a group of related disorders that include marasmus, kwashiorkor and intermediate states of marasmus-kwashiorkor The most common symptom of undernutrition is unintentional weight loss (losing 5-10% or more of your body weight over three to six months).Other signs can include like weak muscles, feeling tired all the time, low mood, an increase in illnesses or infections The main sign of overnutrition is being overweight or obese. However, people with undernutrition can also be overweight if they eat a diet high in energy (calories), but low in other nutrients.Signs of malnutrition in children can include failure to grow at the expected rate and changes in behaviour, such as appearing unusually irritable, sluggish or anxious.The World Health Organizationestimates that currently 150 million children under 5 years of age (26.7% of the world’s children in this age group) are malnourished when measured in terms of weight for age, and 182 million are stunted. This global burden of malnutrition is rooted in poverty, underdevelopment, and inequality. But in some areas rapid population growth is an important contributing factor. In Africa natural disasters, wars, population displacement and civil disturbances have also contributed to the continuous increase in the prevalence of malnutrition. However, geographically it is Asia (especially South Asia) that is home to more than two thirds of the world’s malnourished children compared with the 25.6% in Africa and 2.3% in Latin America (Asia Pacific J Clin Nutr 2002)

2.3.2 Iron

Iron deficiency is the most common and widespread nutritional disorder in the world. According to the World Health Organization approximately two billion people suffer from iron deficiency they tire easily, experience problems metabolizing harmful substances, and eventually suffer from anemia. Based on the recorded incidence of anemia, most preschool children and pregnant women in developing countries and at least 30 – 40% in industrialized countries are iron deficient. In developing countries where rice is the major staple food, children are particularly affected, as well as women during their fertile life period. Peeled rice, also called polished rice, does not have enough iron to satisfy the daily requirement, even if consumed in large quantities. Rice actually has a lot of iron, but only in the seed coat. Because unpeeled rice quickly becomes rancid in tropical and subtropical climates, the seed coat along with precious iron must be removed for storage. For many people, a balanced diet or iron supplements are often unaffordable. Moreover, iron is the most difficult mineral for food fortification, since the most soluble and absorbable iron compounds (e.g.,$FeSO_4$) are unpalatable, and less soluble iron compounds are poorly absorbed (Christof and Wilhelm *et al., 2010*).

2.3.3 Zinc

Dietary deficiency of zinc (Zn) is a substantial global public health and nutritional problem (Krishnaswami, 1998; Myers *et al.*, 2014). One third of the world population is at risk due to low dietary intake of Zn (Hotz and Brown, 2004; Myers *et al*., 2015), including 2 billion people in Asia and 400 million in sub-Saharan Africa (Institute, 2006).Zinc deficiency is a well-documented problem in food crops, causing decreased crop yields and nutritional quality. Generally, the regions in the world with Zn-deficient soils are also characterized by widespread Zn deficiency in humans. Recent estimates indicate that nearly half of world population suffers from Zn deficiency (Cakmak *et al.*, 2016). Zn is essential for gene regulation and expression under stress conditions and is therefore required for protection against infections and diseases (Todd *et al.*, 2011). Though rice is the predominant source of energy and micronutrients for more than half of the world

population, it does not provide enough protein, iron and zinc to match human nutritional requirements. Therefore, rice biofortification has been recognized as a key target to increase the grain Zn concentration to address global Zn malnutrition. Major bottlenecks for protein, iron and zinc biofortification in rice are identified as low Zn uptake, transport and loading into the grain. However, environmental and genetic contributions to grain protein, Fe/Zn accumulation in rice have not been fully explored. Growing conditions and the analytical methods employed (Sugimoto *et al*., 1986; Ogawa *et al.*, 1987; Huebner *et al*., 1990). Nutritional composition of rice is shown in (Table: 2.1)

Table2.1 Micronutrient status of rice vis-à-vis other cereals

Crop	Protein (%)	Iron(ppm)	Zinc(ppm)
Rice	6-7	2-34	10-33
Wheat	13-14	25-55	25-65
Maize	8-11	10-63	13-58
Sorghum	10-15	10-65	14-55
Pearl Millet	6-21	30-146	25-85
Small Millet	8-20	37-142	5-60

(Ravindra Babu V. 2013) DRR, Hyderabad, A.P, India

Table 2.2:-Global food and nutrition problems.

Type	Causes	people affected
Hunger	Deficiency of calories and protein	0.9 billion
Underweight	Inadequate intake of food and frequent disease	126 million
Micronutrient deficiency	Deficiency of vitamins and minerals	More than 2 billion
Overweight to chronic	Unhealthy diets, lifestyle	Increasing also among the poor

Source:-Based on data from FAO, 2005, UNICEF, 2005

2.4 Losses of essential mineral nutrients by polishing of rice

The effect of different polishing techniques on loss of mineral elements from rice grains was quantified using a panel of *indica* and tropical *japonica* genotypes, previously classified as differing in ease of polishing. Gradients in mineral elements across the bran-endosperm interface were quantified using microscaled precision abrasive polishing in combination with inductively coupled

plasma mass spectrometry and synchrotron X-ray fluorescence microscopy. Frictional polishing, similar to that of commercial mills, i.e. 8-10% loss of grain weight, reduced the concentration of Fe, Mg, P, K and Mn by 60-80% in all genotypes. Following gentler polishing (35% weight loss), genotypes classified as difficult to polish showed smaller decreases in Fe, Mg, P, K and Mn compared to genotypes classified as easy to polish. The concentration of other elements, e.g. Zn, S, Ca, Cu, Mo and Cd, showed comparable reductions (<30%) irrespective of polishing technique or ease of polishing. The different patterns of polishing losses of minerals reflected their distribution within the grain. Five-fold differences in the reduction of Zn concentration during polishing were observed for different genotypes which started with similar Zn concentrations in the unpolished grain, thus showing clear potential for selecting genotypes with reduced polishing losses of Zn. (Thomas H. *et al.*, 2012)

2.5 Elemental Analysis

Humans require a suite of mineral elements in varying amounts for proper growth, health maintenance and general well-being [National Research Council (US), Food and Nutrition Board, 1989; Linder, 1991]. Plant-derived foods have the potential to serve as dietary sources for all human-essential minerals (US Department of Agriculture, Agricultural Research Service 2001) and with a well-balanced diet that includes mixed sources of grains, fruits and vegetables, plant foods can make a significant contribution to daily mineral needs at all stages of the life cycle (Dwyer, 1991; Dwyer, 1994; American Dietetic Association 2002). There is no gene which allows a plant (or any other organism) to synthesize minerals. All mineral elements must be acquired from the external environment (Grusak, 2002). Plants can absorb and thus provide a number of different mineral elements from soil this includes the 14 mineral elements defined as essential for plant growth and reproductive success (Marschner, 1995). These are N, S, P, K, Ca, Mg, Cl, Fe, Zn, Mn, Cu, B, Mo, and Ni. Because of their essentiality, all plant foods contain some level of each of these elements, and it should come as no surprise that plants have developed various forms of molecular machinery (i.e., membrane transporters) to acquire these mineral nutrients from their soil

environment (Kochian, 1991; Fox and Guerinot, 1998; Maathuis and Sanders, 2002). Of these 14 elements, human essentiality has been confirmed for all (Nielsen, 1996). Na, Cr, I and Se also are required by humans, but not by plants. Fortunately for humans, however, plants can acquire these other elements through non-specific influx processes using existing transporters localized to their roots (Kabata and Pendias, 1992). The overall uptake of these plant non-essential elements depends on their availability in the soil, in conjunction with the extent of their influx through non-specific transporters. In fact, a wide range of plant non-essential elements (both benign and detrimental) have been measured in plant tissues, with concentrations sometimes reaching dramatic levels if soil availability is high (e.g., Cr, Se, Ar) (Kabata and Pendias, 1992). A number of these elements also referred to as the ultratrace elements, have been demonstrated to provide various health benefits in humans (Nielsen, 1996).

2.6 Approaches to combat malnutrition

The most widely recognized different strategies have been developed to alleviate or prevent protein and micronutrient (Fe, Zn) malnutrition are dietary diversification, post-harvest processing, supplementation, Bio-fortifications, and marker assisted breeding (Slingerland *et al.,* 2003).

2.6.1 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).

2.6.2 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)

2.6.3 Supplementation is a technical approach in which nutrient are delivered directly by means of syrup or pills. Micro-nutrient (Fe, Zn) and Protein supplementation has also been useful in developing countries for rapid improvement of nutritional status in deficient individual. This programme are used only as a short term measure and are then replaced with long term and sustainable food based measures such as fortification and dietary modification, usually by increasing food diversity (Swaminathan., 2004).Iron supplementation and fortification were successfully practiced in industrialized countries (Hurrell., 1997). Protein supplementation has also been useful in developing countries for rapid improvement of nutritional status in deficient individual.

2.6.4 Biotechnological approaches

2.6.4.1 Biofortification:-

Biofortification is defined as the enhancement of micronutrient levels of staple crops through biological processes, such as plant breeding and genetic engineering (Bouis *et al.,* 2002). Biofortification hold great promise for making a significant low-cost and sustainable improvement in the intake of Fe, Zn and protein in populations. They are the addition of (pro) nutrients to foods that are consumed by most of the population. It is the more common intervention to tackle malnutrition. It could be effective in reducing the problem of malnutrition as part of a strategy that includes dietary diversification, supplementation, commercial fortification and other aspects. Harvest Plus is a CGIAR initiative which started "biofortification" umbrella through which international agricultural and research centers have made efforts to develop new breeds of staple foods that are rich in vitamins and minerals. Biofortification has multiple advantages, including the fact that it capitalizes on the regular daily intake of a consistent amount of staple food by all family members. In comparison with other cereals, rice contains low nutritional value. Therefore, rice alone cannot meet the recommended daily allowance (RDA). Healthy and productive populations require adequate amounts of essential vitamins and minerals. As staple foods are eaten in large quantities

everyday by malnourished poor, addition of even small quantities of micronutrients is unbeneficial. High zinc seeds are more vigorous and better able to withstand weed competition, and pathogen and pest attack (Gregorio *et al.,* 2000) Deficiencies of zinc, iron and vitamin A in human population of developing countries were noticed and particularly, zinc deficiency is the fifth major cause of diseases and deaths in these countries.

2.7 Marker assisted breeding

Development of DNA Markers and their use in molecular marker assisted breeding since 1990s have been instrumental in inferring phenotypic and genotypic data for breeding material and accelerating breeding of rice varieties. Markers are heritable entities that are associated with economically important traits and can be used by plant breeders as selection tools. Markers may be morphological, biochemical and molecular markers from which molecular markers or DNA markers are the most widely used type of markers predominately due to their abundance. The potential and efficiency and molecular marker is widely accepted at present and marker assisted breeding (MAB) has been demonstrated by numerous examples as commercial varieties and ongoing breeding programs for various traits such as tolerance to drought, cold, salinity, diseases and insect pest etc. The use of molecular markers for identifying and introgressing favorable genes and gene combinations within the rice species, and the use of transgenic technologies to incorporate traits for herbicide tolerance, biotic-stress resistance, abiotic-stress resistance, and nutritional value into rice were recently summarized by Coffman *et al.,* (2004).Molecular markers are discrete co-dominant or dominant non-deleterious characters that are unaffected by the environment and free of epistatic interactions. They can be used in breeding programs to follow the inheritance of important genes and can further be useful in pyramiding multiple genes for resistance to varying insects, fungi, viruses and bacteria in order to achieve the durable resistance. Till date more than 10,000 molecular markers have been developed in rice Causse *et al.,* (1994), Kurata *et al.,* (1994), Harushima *et al.,* (1998) and Wu *et al*., (2002). DNA based markers are generally classified as hybridization based markers and polymerase chain reaction (PCR) based markers.

Restriction Fragment Length Polymorphism (RFLP) is the most widely used hybridization based molecular marker in which, the DNA profiles are visualized by hybridizing the restriction enzyme digested DNA, to a labeled probe, which is a DNA fragment of known origin or sequence. On the other hand, PCR based markers involve in vitro amplification of particular DNA sequences or loci, with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermo stable DNA polymerase enzyme. Owing to its sensitivity and high speed, PCR has opened up a multitude of new possibilities in molecular biology research. These markers include Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR), Amplified Fragment Length Polymorphism (AFLP), Sequence Tagged Sites (STS),Cleaved Amplified Polymorphic Sequence (CAPS), Sequence Characterized Amplified Region (SCAR), Expressed Sequence Tags (EST) etc. which find huge application in genetics and plant breeding research. Development of molecular markers that are tightly linked to the gene of interest has improved the efficiency of conventional plant breeding (Huang *et al.,* 1997; Hittalmani *et al.,* 2000). Among the several molecular markers available, the SSR markers have several advantages over other markers and hence have received more attention in molecular marker studies (Fjellstorm *et al.,* 2006). SSR markers are reliable, co-dominant, multi-allelic, chromosome specific and highly informative (Swarup *et al.,* 2006). The markers based on known genomic sequence information, like SSR, EST, SNP are extremely effective and appropriate tools for molecular breeding as they are based on simply protocols yet readily provide reliable high quality data (Juliano *et al.,* 2002)

2.8 Microsatellite markers

Microsatellites are also known as Simple Sequence Repeats (Hearne *et al.,* 1992) or short Tandem Repeats (Edwards *et al.,* 1996).

Microsatellites are simple tandemly repeated di to tetra nucleotide sequence motifs flanked by unique sequences and are found mostly confined to telomeres. (McCouch *et al.,* 1997).Microsatellite sequences are abundant, dispersed throughout the genome, and are highly polymorphic in plant genomes even among

closely related cultivars, due to mutations causing variation in the number of repeating units in genomes (Condit and Hubbell, 1991; Akkaya *et al.,* 1992; Morgante and Oliveri, 1993). A number of strategies have been designed to exploit microsatellite sequences for the study of DNA polymorphism in eukaryotes. They involve both hybridization a nd PCR based approaches. Oligonucleotide fingerprinting, a hybridization based approach represents polymorphism due to variation in the length of the restriction fragments that carry the microsatellites while PCR based approaches detect variation in the length of microsatellites. Microsatellite markers have become available in several individual crops due to production of genomic libraries enriched for microsatellites (Ostrander *et al.,* 1992; Edwards *et al.,* 1996; Fisher *et al.,* 1996). The frequencies of microsatellites vary significantly among different organisms (Morgante and Oliveri, 1993; Wang *et al.,* 1994, Gupta *et al.,* 1996). In a survey of published DNA sequences in 54 plant species, Wang *et al.,* (1994) observed that the (AT)n sequences are the most abundant in plants. Microsatellites are abundant and occur frequently and randomly in all eukaryotic nuclear DNAs (Gupta *et al.,* 1996). The microsatellites are valued highly as genetic markers because they are co-dominant, detect high levels of allelic diversity and are easily and economically assayed by the PCR (McCouch *et al.,* 1997).

2.9 Microsatellite markers in rice

Microsatellite primers have been developed in a number of crops. In rice, they are commercially available as "Rice Map Pairs" (RM pairs) through Research Genetics, AL 35801, USA. Reports of rice microsatellite linkage maps show a range of 1-300 mapped microsatellite loci (Yang *et al.,* 1994, Akagi *et al.,* 1996; Panaud *et al.,* 1996; McCouch *et al.,* 1997; Cho *et al.,* 2000; Temnykh *et al.,* 2000). Panaud *et al.,* (1995) investigated the relative frequency of 13 different SSR motifs in rice based on the screening of both genomic and cDNA libraries and the results suggested that there are 5,700-10,000 microsatellites in rice. In the same study it was also seen that 1360 poly (GA)n and 1230 poly (GT)n occurred in rice genome and the frequency of repeats decreased with increasing size of the motif. Reports by Akagi *et al.,* (1996) showed that 35 per cent of the rice chromosomes

were covered by 56 microsatellite markers. It has been found that genetically mapped microsatellite markers cover the entire rice genome with at least one microsatellite for every 16 to 20 cM (Chen *et al.,* 1997). A map consisting of 120 microsatellite markers demonstrates that they are well distributed throughout the 12 chromosomes of rice. The current level of genome coverage provided by SSLPs in rice is sufficient to be useful for genotype identification, gene and QTL analysis, and marker assisted selection in breeding (McCouch *et al.,* 1997). Use of microsatellite polymorphisms for the identification of Australian breeding lines of rice (*Oryza sativa* L.) was investigated and most of the cultivars could be uniquely identified by at least one microsatellite marker (Garland *et al.,* 1999). Rice microsatellites have been demonstrated to be polymorphic between rice varieties (Yang *et al.,* 1994; Panaud *et al.,* 1996; Akagi *et al.,* 1997; Chen *et al.,* 1997; Olugowote *et al.,* 1997; Bligh *et al.,* 1999). The allelic diversity of microsatellite markers in cultivated rice varieties has been reviewed by Gupta and Varshney (2000) as 2-25 alleles per microsatellite locus. A total of 312 microsatellite markers provide whole genome coverage in rice with an average density of one SSLP per 6 cM (Temnykh *et al.*, 2000).

2.10 Association analysis

Association analysis, or linkage disequilibrium mapping, is a notable strategy used for identifying genes controlling important traits. It is already being successfully applied for identifying genes related to human diseases. Research in humans has turned to association analysis, since linkage analysis has not been successful in the fine-scale mapping of disease loci, due to the impossibility of undertaking controlled-breeding crosses (Flint-Garcia *et al.*, 2003). Unlike humans, in most plant species, the identification of those genomic regions which contribute to important characteristics has been mostly achieved through linkage analysis within segregating populations, the result of crosses between genitors with contrasting phenotypes and genotypes (Buntjer *et al.*, 2005; Skot *et al.,* 2005)

According to Zondervan and Cardon (2004), the main purpose in linkage analysis, as in association mapping, is the detection of correlations between phenotypic variation and genotypes through linkage disequilibrium. However,

association analysis has the advantage of contemplating all the meiotic and recombination events that may occur in the evaluated population (Ferreira and Grattapaglia, 2006). Furthermore, this form is highly dependent on the extent of linkage disequilibrium (LD), a higher degree implying the use of less markers per chromosome, without the loss of genetic resolution for marker assisted selection (MAS) (Rostoks *et al.,* 2006). One of the great advantages of association mapping lies in the fact that no mapping population needs to be developed. Furthermore, association analysis can benefit by including data collected over years of experimental analysis with genotypes of breeding programs, with the additional possibility of analyzing several traits simultaneously.

Choudhury *et al.,* (2001), RAPD profiling was employed using 58 random decamer primer for identification and classification of aromatic rice genotype. Most of these primers (96.5%) detected polymorphism among the genotype. The dendrogram based on 58 primers was highly similar to that based on 10 and 15 primer with matrix correlation (r) of 0.88 and 0.91 respectively. This suggested that a set of 10 primers (OPA-13, 16, 17, 19; OPB-8, 11, 13, 18; OPN-1 and 17) can be employed for an initial assessment of genetic diversity in large number of collection.

Riza *et al*., (2004) have also reported grain protein content range from 6.3% to 9.1% in a set of 438 rice genotype. Similar results were observed in a study conducted by Banerjee *et al.,* (2010) on estimation of protein content in a set of 12 diver's rice genotype including both cultivated and wild genotype of rice result showed a range of 6.19-10.75% protein in whole grains. Wide variation for protein concentration in milled grain level from 2.8% to 9.9% of rice germplasm lines of Chhattisgarh have been reported by Chandel *et al.,* (2005).

Meena *et al.,* (2008) used 25 SSR primers which generated 72 SSR alleles to assess the genetic diversity among 35 rice varieties released from TRRI, Aduthurai. A wide range of morphological diversity was noticed for 7 quantitative and 20 qualitative traits in rice varieties released from TRRI, Aduthurai. Polymorphism information content value ranged between 0.382 (RM-420) and 0.711 (RM-4955). Highest diversity was found between ADT-35 (Bhavani/ Jaya) and ADT-6 (Pure line) with similarity level 12% and lowest diversity was found

between ADT-22 (Pure line selection) and ADT-20 (ADT-3/ ADT-2) with similarity level 68%.

Rehman *et al.,* (2009) reported three rice microsatellite markers RM-11, RM-151, RM-153 for the identification and discrimination of 17 HYVs and 17 local rice cultivar including two wild rice cultivars. All analyzed microsatellite markers were able to found to be polymorphic with an average of 6.33 alleles per locus. A total of three varieties specific alleles, RM-11/147, RM-151/289, RM-153/178 were identified for BR-11, Badshabhog and BR-19 cultivars respectively.

Garcia-Oliveria *et al.,*(2009)measured Fe and Zn contents of 85 introgression lines (ILs) derived from a cross between an elite indica cultivar, Teqing and wild rice (Oryza rufipogon) by inductively coupled argon plasma (ICAP) spectrometry. Among micro-elements, Zn was observed in highest quantities with a combined mean value of 27.1 µg g-1, whereas Fe was found in the lowest quantities with a mean performance of 9.6 µg g-1. The back cross lines used in the present investigation showed mean iron of 18.5 µg g-1 and mean zinc concentration of 26.9 µg g-1. These results clearly indicated that the population used in the present study exhibited high iron concentration and slightly lower zinc concentration than the lines developed by Garcia-Oliveria *et al.,* (2009).

Sperotto *et al*., (2010) In an expression analysis study with 25 metal-related genes revealed that nine genes such as OsYSL6, OsYSL8, OsYSL14, OsNRAMP1, OsNRAMP7, OsNRAMP8, OsNAS1, OsFRO1 and OsNAC5 were specifically over expressed in the flag leaves and showed significant correlations with Fe and Zn concentrations in the seeds.

Borba *et al*., (2010) applied association analysis to a panel of Landracess of Embrapa Rice Core Collection (ERiCC) with 86 SSR and field data from two experiments. The association of yield and grain-quality traits with SSR was undertaken with a mixed linear model, with markers and subpopulation as fixed factors, and kinship matrix as a random factor. Eight markers from the two appraised panels showed significant associfation with four different traits, although only one (RM190) maintained the marker-trait association across years and cultivation.

Banerjee *et al.,* (2011) have also reported the protein content of milled grains ranged from 4.91% to 12.08% with the mean of 6.63%.The rice genotypes identified with higher grain protein content can serve as donor lines for this trait for the improvement of elite and popular rice cultivar poor in grain protein. Efficient breeding programs planned with high grain protein content genotype identified in this study will be beneficial in improvement of local cultivars of Chhattisgarh helpful in eradicating protein malnutrition from the state.

Lee and Johnson *et al., (2011)* reported Several studies have shown that the over expression of OsNAS genes improved the grain Fe and Zn concentrations by several folds, OsNAS2 and OsNAS3 over expression showed increased accumulation of Fe and Zn. OsIRO2 increases Fe content in rice plants grown in calcareous soils (Ogo *et al.,* 2011). The ferritin gene OsFer2 over expressed in a basmati rice (Pusasugandh II) accumulated higher levels of Fe and Zn (Paul *et al.*, 2012). Several transcription factors such as OsNAC, NAM-B1, OsIDEF1, OsIDEF2 and OsIRO2 also play an important role in up regulating the genes involved in metal homeostasis (Ogo *et al.*, 2006, 2007, 2008; Waters *et al.*, 2009; Banerjee *et al.*, 2010; Ogo *et al.*, 2011; Gande *et al.*, 2014).

Banerjee and Chandel *et al.,* (2011) tested the range of iron and zinc concentration (µg g-1) in brown rice within 11 rice genotypes and found 8.5 to 18.6 µg g-1 of iron and 13.9 to 39.3 µg g-1 zinc.

Banerjee and Chandel *et al.,* (2011) reported Similarly, transcriptome analysis of 25 metal homeostasis genes in different tissues of 12 rice genotypes showed expression of highest number of genes (24) in flag leaf, while genes such as OsZIP4, OsZIP11, OsNRAMP5, OsNRAMP7, OsYSL2, OsYSL4, OsYSL6, OsYSL9, OsNAAT1, OsNAC, OsFER1, OsVIT1, OsFRO2, OsIRT1, OsFER2, OsZIP7, OsZIP8, OsZIP9, OsNRAMP4, OsNRAMP6 and OsYSL12 were expressed in roots. Expression of OsNAC, OsYSL2, OsYSL9, OsZIP4, OsVIT1, OsNAAT1 and OsNRAMP7 genes in the flag leaf was highly correlated with the high grain Zn content

Rajendran *et al.,* (2012) used hundred SSR markers for molecular fingerprinting and genetic distance analysis of twelve commercial hybrid rice parental lines, among the marker screened sixty two were polymorphic and

generating 203 alleles with an average of 3.2 alleles per primers. Commercially rice hybrid KRH-2 can be detected using its male parent KMR-3 specific marker RM-297, RM-442, RM-541, RM-584, RM-107 and female parent IR58025A specific marker RM-529, RM-489, RM-589, RM-533 and RM-182 are identified.

Zhou *et al*., (2012) used a natural population comprising 128 japonica rice varieties were investigated during two years, for eleven important agronomic traits. The population was genotyped using 152 microsatellite markers across the whole genome. A unified mixed linear model was used to identify marker-trait associations, taking into account population structure and kinship. A total of 16 significant marker-trait associations were identified.

Sajib *et al.,* (2012) in this study a total of 24 SSR markers were used for characterization and discrimination of 12 elite aromatic rice genotypes. Among these 24 markers 9 microsatellite markers were showed polymorphism. The number of alleles per locus ranges from 2 alleles (RM-510, RM-244 and RM-277) to 6 alleles (RM-163) with an average of 3.33 alleles across 9 loci. The PIC value ranged from 0.14 (RM-510) to 0.71 (RM-163) in all 9 loci with an average of 0.48. RM-163 was found the best marker for the identification of 12 genotypes as revealed by PIC value. Highest genetic distance was obtained between Basmati PNR 346 and Deepa; Basmati PNR and Patnai-23; Dolargura and Sugandha; Bhogganijia and Sugandha; and finally between Dolargura and Chinikani (88.89%). Opchaya, Basmati PNR 346 and Sugandha had close similarity among them but showed wide dissimilarity with other genotype.

ROJA V *et al*., (2013) reported 128 lines of BC4F4 population derived from the backcross between an indica cultivar, Samba Mahsuri and wild rice, The iron and zinc concentration of 128 rice lines ranged from 6.4 to 106.6 ìg g-1 and 15.5 to 52.05 ìg g-1 respectively. The top ten lines had high iron concentration ranging from 24 to 106.6 ìg g-1 and that of zinc concentration ranged from 31.5 to 52.05 ìg g-1. The lines that had high iron concentration also had the high zinc concentration but the lines with high zinc concentration did not have high iron levels. In case of iron concentration the maximum number of lines i.e. 81 % were falling in between the range 13 to 24 ìg g-1. In case of zinc concentration majority (53 %) of lines were falling in between the range 26 to 30 ìg g-1 and 30 % were

falling in between the range 21 to 25 ìg g-1.All the top ten lines with high iron concentration (24 to 106.6 ìg g-1) had iron concentration more than O.rufipogon (21.2 ìg g-1). The top ten lines with high zinc concentration (31.5 to 52.05) exhibited either slightly lower or slightly higher zinc concentration than O.rufipogon (45.3 ìg g-1). These results clearly indicated the favourable effect of the introgressed genetic variability from the wild rice (O.rufipogon).

Netravati *et al.,* (2013) reported the microsatellite or simple sequence repeat (SSR) markers were used to determine the allelic diversity and relationship among 48 traditional indigenous aromatic rice germplasm grown under Eastern part of India. Out of 30 primers, 12 primers showed DNA amplification and polymorphism among 48 aromatic rice genotypes. A total of 28 bands appeared by using 12 SSR primers in 48 aromatic rice varieties/landraces. The number of alleles per locus ranged from 1 to 5 with an average 2.08. Out of 28 bands, 25 bands were polymorphic and three were monomorphic bands. The results reveal that all the tested primers showed distinct polymorphism among the landraces/varieties indicating the robust nature of SSR markers. Most of the primers, showed highest polymorphic information content (PIC). Phenotypic characteristics are significantly correlated with genotypic characters.

Gande *et al.,* (2014) reported the Grain zinc content ranged from 16.1 to 35.5 ppm with an average of 23.7 ppm. Among twenty four candidate gene markers, eight showed polymorphism and out of three simple sequence repeats (SSR) markers, three showed polymorphism. Single marker analysis revealed that four (OsNAC, OsZIP8a, OsZIP8c and OsZIP4b) candidate gene markers showed significant variation among RIL population with a phenotypic variation of 4.5, 19.0, 5.1 and 10.2% respectively. Validation with 96 rice genotypes showed three markers (OsZIP8a, OsNAC and OsZIP4b) with phenotypic variation of 11.0, 5.8 and 4.8%, respectively.

Patil *et al.,* (2014) reported the protein content analysis resulted in identification of five high protein germplasm lines namely, CGR-436 (11.2%), GP-145-48 (10.68%), CGR-446 (10.43%), CGR-52 (10.35%) and CGR-77 (9.92%). Morphological analysis for the same revealed a wide range of diversity for eight quantitative traits. Further, the genetic diversity was assessed among 58 rice

germplasm lines and varieties using 69 alleles generated by 25 SSR primers. DNA fingerprinting of identified high grain protein containing rice lines (≥ 9.0% grain protein) was carried out using 25 polymorphic SSR markers among which SSR marker RM 489 was found to be highly discriminating marker.

Indurkar *et al.*, (2016) reported the improvement of grain quality, such as Zn/Fe and grain protein content has been a major concern of rice breeders. In the present study, grain zinc (Zn), iron (Fe) and protein contents were analyzed in 60 F7 Recombinant Inbred Lines (RILs) derived from Swarna X Moroberekan cross to detect quantitative trait loci (QTLs) and their interactions. The analysisi of 20 polymorphic SSR markers showed 4 QTLs on chromosomes 1, 10, 6, significantly linked to iron and protein. Results revealed that QTL's for grain protein content (qgpc-1) on chromosome 6 and (qgpc-2) and (qgpc-3) on chromosome 10 and one QTL's for Fe content in rice grain are identified (qFe1.1) on chromosome 1. Three markers were associated to Zn content in rice grain on chromosome 2, 3 and11.

CHAPTER-III
MATERIALS AND METHODS

The present study entitled **"Identification of DNA Markers Associated with Grain Fe, Zn and Protein Contentsin Rice(*Oryza sativa* L.)"**was carried out at NutritionalGenomics Laboratory and Genomics and proteomics Laboratory of the Department of Plant Molecular Biology and Biotechnology, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur, C.G. India. The details of the experiment are explained below.

3.1 Materials

The plant material used in this study includes ninety six rice genotype selected from the C.G. collection. These ninety six genotype was selected on the basis of diverse morphological characters like panicle diversity, grain type and some trait specific genotypes includes grain protein content, grain micronutrient content and drought tolerant. The list of ninety six rice genotypes and its details are tabulated in table 3.2

3.2 Methods

3.1 Fieldobservation:-

Quantitative Character	Qualitative Character
Plant height (cm)	Basal leaf Sheath colour
Panicle: Length of main axis (cm)	Leaf Intensity of green color
Number of total tiller per plant	Leaf Distribution of anthocyanin coloration
Panicle: Number of effective tillers per plant	Leaf Pubescence on blade surface
Panicles: Colour of awns (late observation)	Leaf Anthocyanin colouration of auricles
Panicles: Attitude of branches	Leaf: Length of blade
Panicles: Exertion	Leaf: Width of blade
Grain: length	Time of heading (50% of flowering with panicles) days
Grain: width	Flag leaf: Attitude of blade(early observation)

Table 3.2 list of 96 rice genotypes from C.G. core collection used in the study

S.NO	Landraces	S.NO	Landraces	S.NO	Landraces
1	Bhathaili Gurmatia	33	Kadamphool	65	GP-145-44
2	Botki Gurmatia	34	Karigilas	66	GP-145-70
3	Chapti Gurmatia	35	Jhilli	67	RKVY-52
4	chapti Gurmatia	36	Azucena	68	GP-145-103
5	Gurmatia	37	IR-64	69	RKVY-15
6	Jhunki Gurmatia	38	Swarna	70	GP-145-78
7	Kalam Gurmatia	39	RR-100	71	GP-145-43
8	Nunki Gurmatia	40	Bhansapanchi	72	GP-145-49
9	Sultu Gurmatia	41	Banda	73	GP-145-59
10	Tulsi Gurmatia	42	Bada gada khuta	74	GP-145-119
11	Bangla Gurmatia	43	Reg-695	75	GP-145-136
12	ShriKamal	44	Dagad-Desi	76	GP-145-50
13	Elayachi	45	GP-145-40	77	GP-145-65
14	Jeeradhan	46	RKVY-104	78	GP-145-38
15	Bisni-1	47	RKVY-211	79	GP-145-130
16	RR-152	48	Dullar	80	GP-145-138
17	RR-137	49	BAM-1292	81	GP-145-5
18	RR-149	50	BAM-5446	82	GP-145-11
19	RR-8 M011	51	BAM-5926	83	GP-145-20
20	Reg-1035	52	Moroberekan	84	GP-145-34
21	Reg-1038	53	Nagina-22	85	F8 Safri-17*IR681444-41
22	CHIR-8	54	BAM-5997	86	F7 Bas-1*IR681444-4
23	CGZR-1	55	Bakal	87	F6 Kranti* Swarna
24	IET 23829	56	GP-145-37	88	F10 IR681444*Abhaya-18
25	Basmati 370	57	MTU-1010	89	F4 Moro*IR94046-31
26	R-RHZ-LI-23	58	SL-62	90	F9 IR681444*HMT-24
27	R-RHZ-IB-13	59	GP-145-41	91	F9 IR681444*IR64
28	R-RHZ-SM-14	60	GP-145-66	92	F10 Safri-17*IR681444-5
29	Basmati 1	61	CGR-1539	93	F5 MTU1010*IR94032-5
30	R-RHZ-MI-30	62	RRGM-ATN-47	94	F8 IR681444*Bas-8-3
31	Kalanamak	63	RRGM-AS-45	95	F8 IR681444*Bas-1-27
32	R-56	64	GP-145-42	96	F5 Swarna*MTU1010-2

3.2.1 Field experiment

The plant materials were planted in the field for recording the morphological observations during the wet season 2016. The seed were sown on date 23th June 2016 and transplanted on 17 July 2016. The plant to plant and row to row spacing was 10×15 cm**.** Each line was sown in two replications. The NPK fertilizer was applied @ 100-60-40 kg per hectare. All normal packages of practices were followed to raise a crop. The following observations were recorded based on the procedures described in following National guideline for the conduct of tests for distinctness, uniformity and stability of Rice (IIRR, 2006).

3.2.2 Basal leaf Sheath colour

The colour of the leaf sheath, which is wrapped around the culms above the basal node, was visually recorded at early boot stage on individual plants. The categories observed were green, light purple, purple lines and uniform purple colour at basal leaf sheath.

3.2.3 Leaf Intensity of green color

The intensity of green colour on leaf was visually recorded at early boot stage by observation of a group of plants. The major categories recorded were light, medium and dark green color.

3.2.4 Leaf Distribution of anthocyanin coloration

The distribution of anthocyanin coloration on leaf was recorded at early boot stage by visual assessment of a group of plants. The major categories are on leaf tips only, on leaf margins only, in blotches only and uniform presence of anthocyanin colour on leaf lemma.

3.2.5 Leaf Pubescence on blade surface

The intensity of leaf pubescence was recorded at early boot stage by visual assessment of individual plants of each land races. The categories observed under this character are absence, weak, medium, strong and very strong presence of pubescence on blade surface.

3.2.6 Leaf Anthocyanin colouration of auricles

The anthocyanin colouration of auricles i.e. colourless, light purple and purple colour in auricles was recorded at early boot stage with visual assessment by observation of individual plants

3.2.7 Leaf: Length of blade

The length of the leaf blade was measured in centimeter and categorized in to short, medium and long leaves.

3.2.8 Leaf: Width of blade

The width of the leaf blade was measured in centimeter and categorized in to narrow, medium and broad leaves.

3.2.9 Time of heading (50% of flowering with panicles) days

Time of heading (50% of flowering with panicles) days was recorded at ½ of inflorescence emerged through visual assessment and grouped into very early, medium, late and very late by a single observation of a group of plants or parts of plants. Number of days was recorded from date of sowing to the days when primary panicles in 50 percent plants were emerged.

3.2.10 Flag leaf: Attitude of blade (early observation)

Attitude of blade (early observation) was recorded at beginning of anthesis. It begins with the protrusion of the first dehiscing anthers in the terminal spikelets on the panicle branches through visual assessment and grouped in to erect, semi-erect, horizontal and drooping by a single observation of a group of plants or parts of plants.

3.2.11 Plant height (cm)

Pant height was measured at the time of maturity from ground level to the tip of the panicle.

3.2.12 Panicle: Length of main axis (cm)

Panicle length was measured at the time of maturity from the base of panicle to the tip of last spikelet prior to harvesting. The categories under this class

are very short (<16 cm), short (16-20 cm), medium (21-25 cm), long (26-30 cm) and very long (>30cm).

3.2.13 Number of total tiller per plant

Number of total tiller per plant was counted form randomly selected plant.

3.2.14 Panicle: Number of effective tillers per plant

Number of panicle per plant was recorded from dough development (spikelets become hard) to ripening terminal spikelets ripened through visual observation and classified into few, medium and many by observation of individual plants.

3.2.15 Panicles: Colour of awns (late observation)

The colour of awns was recorded at ripening stage through visual assessment of individual plants and grouped into classes yellowish white, yellowish brown, brown, reddish brown, light red, red, light purple, purple and black on the basis of awn colour.

3.2.16 Panicles: Attitude of branches

The attitude of panicle branches was recorded at ripening stage, and classified into erect, erect to semi-erect, semi-erect, semi- erect to spreading and spreading types. This character was observed visually on a group of plants.

3.2.17 Panicles: Exertion

The panicle exertion was recorded at ripening stage, which were classified into partly exerted, exerted and well exerted classes. The classes were recorded through visual assessment of a group of plants.

3.2.18 Grain: length

Ten grains (with husk) were taken randomly and average length was measured in centimeter. These were classified in to very short, short, medium, long and very long classes.

3.2.19 Grain: width

Ten grains (with husk) were taken randomly and average width was measured in centimeters. These were classified in to very narrow, narrow, medium, broad and very broad classes.

3.3 Estimation of total grain protein content

3.3.1 Processing of rice grains.

Before analyzing the rice samples for total grain protein, the rice grain of these lines were subjected to dehusking and polishing. Approximately 10 g seeds of each sample were hand dehusked using polyurethane coated hand dehusker unit to avoid metal contamination.The dehusked rice sample was polished using electronic polisher for 45-60 second. Whiteness range reading 35-50 of polished grains was measured using refractometer.

3.3.2 Estimation of Protein

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

3.3.2.1 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 400 $^{\circ}$C. 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.

3.3.2.2 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.

3.3.2.3 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).

3.3.3 Calculation for Nitrogen and Protein Percentage

Nitrogen and total grain protein content percentage can be calculated using the below mentioned formula

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

$$\text{Protein percentage (\%)} = \text{Nitrogen percentage (\%)} \times 5.95$$

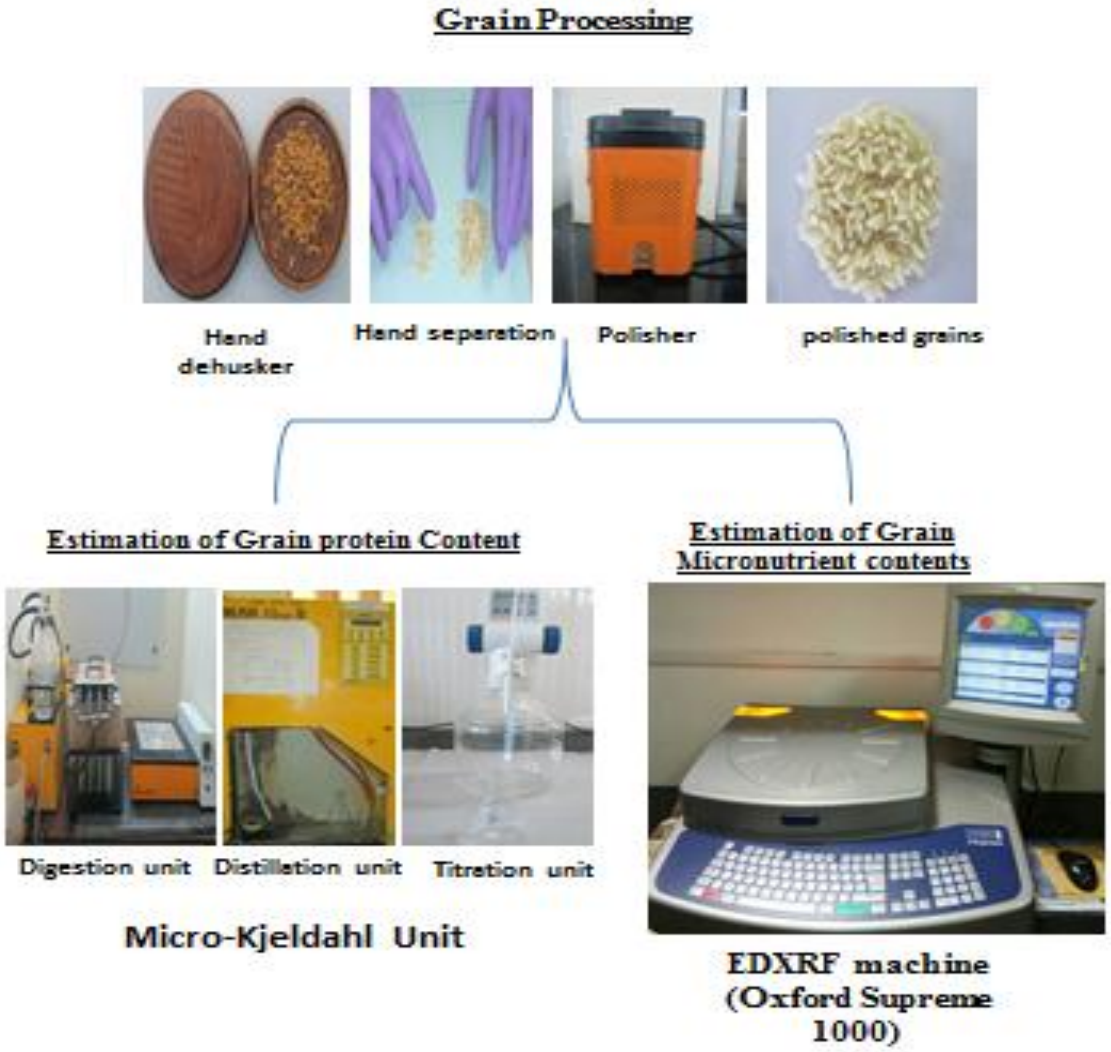


Fig: 3.1 Estimation of micronutrient and protein concentration in polished rice grain

3.3.3.1 Estimation of Fe and Zn

3.3.3.2Processing of ricegrains

Before analyzing the for Iron and Zinc concentration the rice seeds samples of all the 96 lines as well as both the parents were subjected to dehusking and seed separation

3.1.1 Dehusking

Around 200 grams of each seed sample were hand dehusked using polyurethane coated hand dehusker to avoid metal contamination.

3.1.2 Estimations of Iron andZinc

Iron and Zinc content was estimated by using standard ED-XRF Method

Described under the protocol for step-wise procedure is asfollows.

1. Rice grains of individual lines were harvested manually and hand threshed for micronutrient analysis using X-ray Florescence (XRF).
2. The grains were then manually dehusked using hand dehusker.
3. Grains were further polished using reflectance meterand recorded reflectancereadingapproxmatly 40-50and Fe, Zn content in these grains were estimated using X-ray florescence (XRF) at DRR (Hyderabad).
4. Manufacturer recommended filling rice sample in ED - XRF sample cups to a mark which is roughly 3/4th volume of the total Space.
5. 5g sample of polished rice samplesis necessary for simultaneous estimation of both iron and zinc.From each plant were separately subjected to energy dispersive X-ray fluorescent spectrophotometer (ED-XRF) for micronutrient analysis (Stanguilis*et al.,* 2014) and content in µg/gm recorded.
6. Analysis time for each sample was 186 s which included 60s acquisition time for the separate Zn and Fe conditions as well as 66 s ‘dead time’ during which the XRF establishes each measurement condition.
7. Scans were conducted in sample cups assembled from 21 mm diameter all cups combined with polypropylene inner cups sealed at one end with 4 µm Poly-4 XRF sample film.
8. Calibration of instrument was done using known ICP-OES values of high, low Zn and Fe containing genotypes.

3.4 Statistical analysis

Statistical analysis of data was done on a personal computer using software packages like, MS-EXCEL, for different analysis. All data was analyzed without any transformation

3.4.1 Analysis of variance (ANOVA)

The analysis of variance, test of significance of variance components were carried out as suggested by Panse and Sukhatme, (1961). The form of ANOVA is given below: The data obtained in present study was statistically analyzed using randomized block design (RBD) for eight agronomical traits in the field condition and completely randomized block design (CRD) for checking the grain protein content of selected rice germplasm lines and varieties in the lab condition. The skeleton of ANOVA for CRD and RBD was presented in table 3.3 and table 3.4

Table 3.3 Skeleton of ANOVA for completely randomized block design(CRD)

Source of Variation	Degree of Freedom	Sum of Square	Mean squares	Computed F	Tabular F (0.05)
Treatment	(t-1)	TrSS	TrMS= TrSS/df	TrMS/EMS	
Experimental	rt-t	ESS	EMS= ESS/df		
Error					
Total	rt-1	TSS			

Note: TrSS; treatment sum of square, ESS; error sum of square, TSS; total sum of square, TrMS;treatment mean square, EMS; error mean square, df; degree of freedom.

Table 3.4 Skeleton of ANOVA for randomized block design (RBD)

Source of Variation	Degree of Freedom	Sum of Square	Mean Squares	Computed F	Tabular F (0.05)
Replication	(r-1)	RSS	RMS= RSS/df	RMS/EMS	
Treatment	(t-1)	TrSS	TrMS= TrSS/df	TrMS/EMS	
Experimental	rt-t	ESS	EMS= ESS/df		
Error					
Total	rt-1	TSS			

Note: RSS; replication sum of square, TrSS; treatment sum of square, ESS; error sum of square,TSS; total sum of square, RMS; replication mean square, TrMS; treatment mean square, EMS; error mean square, df; degree of freedom.

3.4.2 Standard deviation (SD)

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

$$SD \sqrt{\frac{d^2}{N}}$$

Where,

$\square d^2$ = Sum of squares of deviation

n = Total number of observation

3.4.3 Coefficient of variation (CV)

Coefficient of variation in percentage was calculated by the formula; CV (%) =Standard deviation ×100

Mean

3.4.4 Standard error (SE)

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

Where,

S = Standard deviation

$\sqrt{n}$ = Total number of observation

3.5 Genotyping of rice landraces using SSR Marker

3.5.1 Genomic DNA extraction

Total rice genomic DNA was extracted from four to five week old plants of the rice genotype, by CTAB (Pervaiz *et al*., 2011) protocol.

Before starting, add β-merceptaethanol to CTAB extraction buffer @ 20 µl/20 ml. then follow the step wise protocol given below:

1. About 100 mg of young leaf was grinded in 1000 µl 2X CTAB extraction buffer with the help of tissuehomogenizer.
2. Then 700 µl of solution transferred into 1.5 ml eppendorftube.
3. Incubated at 65ºC on water bath for 15-20 min further cooled briefly and add 700 µl of Chloroform: Isoamylalcohol (24:1).
4. The content were shaken by hands intermittently and kept at room temperature for 15 min. tubes were centrifuged at 13000 rpm for 3min.
5. 600 µl of upper aqueous phase was transferred into a new 1.5 ml eppendorf

tube.

6. 900 µl of absolute ethanol was added and mixed gently and the tubes were kept for 2 hrs at-20^{o}c.
7. The samples were centrifuged for 3 min at 10,000 rpm, the supernatant was decanted
8. The pellet was washed with 70% ethanol andair-dried.
9. DNA pellet was air dried and then dissolved in 50 µl of TEbuffer.

3.5.2 Quantification and dilution of DNA:

For quantification, 4 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 50 volts for 90 minutes. The gel was stained with ethidium bromide 8 µl/100 ml and observed under Gel doc. 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. After quantification, the DNA samples were diluted in TE buffer to bring down the final concentration of DNA 50 ng/µl for PCR analysis.

3.5.3 PCR analysis to detect polymorphism among the diverse landraces

PCR analysis was done using the selected SSR markers to identify the polymorphic loci between the ninty six landraces. Forty SSR markers are selected from the Panel of 50 standard SSR markers used by the Generation Challenge Program for rice diversity analysis available in the Gramene website (McCouch *et al.,* 2002). Chosen SSR markers are well distributed among all the chromosomes. Details of SSR marker and primes sequence are described in the table 3.4

3.5.4 Gel Electrophoresis

PCR amplified SSR products were mixed with 2-4 µl of loading dye and loaded in the well of 2.5% agarose gel prepared in 1X TAE buffer, electrophoresis was carried out at 100 volt for hour. The banding patterns were observed under gel documentation system (Biorad).

3.5.5 Scoring and Data analysis

All the rice genotypes were scored for the presence and absence of the SSR bands. Moreover, the data were entered into a binary matrix as discrete variables; A for product size lower or some missing band and B for upper band of the character and this data matrix was subjected to further analysis. The resultant data file was employed to generate graphical image indicating chromosome wise contribution of A/B alleles amplified by different SSR markers.

Table 3.5 List of SSR, QTL specific and Gene specific DNA markers used in the study.

S.NO	Marker	Chrom. No	Forward primer sequence Reverse primer sequence	AT	Bp
1	RM428	1	AACAGATGGCATCGTCTTCC CGCTGCATCCACTACTGTTG	55	266
2	RM490	1	ATCTGCACACTGCAAACACC AGCAAGCAGTGCTTTCAGAG	55	101
3	RM5	1	TGCAACTTCTAGCTGCTCGA GCATCCGATCTTGATGGG	55	113
4	RM283	1	GTCTACATGTACCCTTGTTGGG CGGCATGAGAGTCTGTGATG	55	151
5	RM259	1	TGGAGTTTGAGAGGAGGG CTTGTTGCATGGTGCCATGT	55	162
6	RM1	1	GCGAAAACACAATGCAAAAA GCGTTGGTTGGACCTGAC	55	113
7	RM226	1	GAAGCTAAGGTCTGGGAGAAACC AATGGCCTTAACCAAGTAGTAGGATG	55	274
8	RM290	2	ACCCTTATTCCTGCTCTCCTC GTGCTGTAGATGGAAGGGAG	142	55
9	RM 279	2	GCGGGAGAGGGATCTCCT GGCTAGGAGTTAACCTCGCG	55	174
10	RM234	2	CTGATCGAGAGCGTTAAGGG GGGATCAAACCACGTTTCTG	55	209
11	RM154	2	GACGGTGACGCACTTTATGAACC CGATCTGCGAGAAACCCTCTCC	61	183
12	Grm33-1	2	TCGTTCTGACATGTTGAGG CCCGACAAAGTCAACGTC	56	250
13	Crm33-1	3	TCGTTCTGACATGTTGAGG CCCGACAAAGTCAACGTC	58	60.2
14	RM 231	3	CCAGATTATTTCCTGAGGTC CACTTGCATAGTTCTGCATTG	55	182
15	RM7	3	TTCGCCATGAAGTCTCTCG CCTCCCATCATTTCGTTGTT	55	180
16	RM517	3	GGCTTACTGGCTTCGATTTG CGTCTCCTTTGGTTAGTGCC	55	266
17	RM514	3	AGATTGATCTCCCATTCCCC CACGAGCATATTACTAGTGG	55	259
18	QsZip3b	4	CCTGCTGAGGCTGAGTTGAA	61.5	370

S.NO	Marker	Chrom. No	Forward primer sequence Reverse primer sequence	AT	Bp
19	RM252	4	CGAGAACAAAGTAACAGGCTGC TTCGCTGACGTGATAGGTTG ATGACTTGATCCCGAGAACG	55	216
20	RM 470	4	TCCTCATCGGCTTCTTCTTC AGAACCCGTTCTACGTCACG	55	83
21	RM592	5	TCTTTGGTATGAGGAACACC AGAGATCCGGTTTGTTGTAA	55	270
22	RM122	5	GAGTCGATGTAATGTCATCAGTGC GAAGGAGGTATCGCTTTGTTGGAC	55	227
23	RM574	5	GGCGAATTCTTTGCACTTGG ACGGTTTGGTAGGGTGTCAC	55	155
24	RM541	6	TATAACCGACCTCAGTGCCC CCTTACTCCCATGCCATGAG	55	158
25	RM475	6	GCATTGATGTGCCAATCG CATTGCAACATCTTCAACATCC	55	114
26	RM225	6	TGCCCATATGGTCTGGATG GAAAGTGGATCAGGAAGGC	55	140
27	RM501	7	GCCCAATTAATGTACAGGCG ATATCGTTTAGCCGTGCTGC	55	179
28	RM248	7	TCCTTGTGAAATCTGGTCCC GTAGCCTAGCATGGTGCATG	55	102
29	RM 8007	7	AATAGGATGGATCATGGATA CATCTCATCAGGAACCTAAC	55	178
30	RM160	7	ACAGTATCCAAGGCCCTGG CACGTGAGACAAAGACGGAG	55	156
31	RM44	8	ACGGGCAATCCGAACAACC TCGGGAAAACCTACCCTACC	55	99
32	RM137	8	GACATCGCCACCAGCCCACCAC CGGGTGGTCCCCGAGGATCTTG	55	218
33	RM271	10	TCAGATCTACAATTCCATCC TCGGTGAGACCTAGAGAGCC	55	101
34	RM484	10	TCTCCCTCCTCACCATTGTC TGCTGCCCTCTCTCTCTCTC	55	299
35	RM552	11	CGCAGTTGTGGATTTCAGTG TGCTCAACGTTTGACTGTCC	55	195
36	RM21	11	ACAGTATTCCGTAGGCACGG GCTCCATGAGGGTGGTAGAG	55	157
37	RM17	12	TGCCCTGTTATTTTCTTCTCTC TGCCCTGTTATTTTCTTCTCTC	55	184
39	RM260	12	ACTCCACTATGACCCAGAG GAACAATCCCTTCTACGATCG	55	111
40	RM452	12	TGCCCTGTTATTTTCTTCTCTC GGTGATCCTTTCCCATTTCA	55	184
41	RM19	12	CAAAAACAGAGCAGATGAC CTCAAGATGGACGCCAAGA	55	226
42	RM277	12	CGGTCAAATCATCACCTGA CAAGGCTTGCAAGGGAAG	55	124

Note:-AT; Annealing temperature, PS; Product size

3.5.3 PCR analysis to detect polymorphism among germplasm lines

PCR analysis was done using the selected SSR and designed markers to identify the polymorphic loci between the ninty six rice genotypes.

Table 3.6:- 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 Mm	1 µl
4	Primer (Reverse)	10 Mm	1 µl
5	Taq DNA Polymerase	5 U	1 µl
6	Sterile water	-	11 µl
7	Template DNA	50 ηg/µl	2.0 µl
8	Total		20.0 µl

The reaction mixture was prepared using components above mentioned in table 3.6.The mixture was overlaid with a drop of mineral oil before the amplification was carried out for 35 cycles of amplification process as mentioned in table 3.7 Amplified products were resolved by electrophoresis on agarose gel electrophoresis where bands of interest were observed.

Table 3.7:- Temperature profile used for PCR amplification

Steps	Temperature (°C)	Duration (min.)	Cycles	Activity
1	94	5 min	1	Initial denaturation
2	94	45 sec.		Denaturation
3	50-55	30-45 sec	35	Annealing
4	72	30-45sec		Extension
5	72	5 min	1	Final Extension
6	4	99 min	1	Storage

.

3.6. Association analysis

For association analysis of phenotypic measurement in the multiple replication traits were recorded for eight quantitative character and Iron, Zinc and grain protein content using National guideline for the conduct of tests for distinctness, uniformity and stability of Rice (IIRR, 2006). Genotyping of the population was carried out by 18 polymorphic markers and banding pattern was scored either A or B depending on its position. The association between trait and markers were calculated using single marker analysis (SMA) using t test in

Microsoft Excel program. The significant marker trait associations were indicated by a P-value (>0.05) with corresponding R^2 for each marker is the total phenotypic variation for a traits that is accounted by markers. It is calculated using following formula-

$$\% R^{2} = (\text{Between group SS/ Total SS}) \times 100$$

The result is statistically significant if p-value is less than 0.05.Therefore the conclusion is that, there is an association in between trait and marker.

3.7 Reagents and solutions

3.7.1 Stock solutions

A. DNA extraction buffer

Tris HCl (1M; pH-8)	5 ml
EDTA (0.5M; pH-8)	10 ml
NaCl (4M)	7.5 ml
SDS (20% W/V)	5 ml

Final volume was adjusted to 100 ml with distilled water.

B. TE buffer

1M Tris-Hcl (pH-8)	1 ml
0.25 EDTA	0.4 ml

Final volume was adjusted to 100 ml and autoclaved.

C. EDTA (0.5M; pH-8)

186.12 g of EDTA was dissolved in 700 ml of distilled water. The pH was set to 8 using NaOH. Final volume was adjusted to 1000 ml with distilled water and sterilized by autoclaving.

D. 4M NaCl

23.36 g of NaCl was dissolved in 80 ml of distilled water. Final volume was adjusted to 100 ml and sterilized by autoclaving.

E. 1M Tris HCl (pH 8.3 at 25°C)

30.28 g of Trizma base was dissolved in 200 ml of distilled water. The pH was set to 8.3 using concentrated HCl. The final volume was adjusted to 250 ml

with distilled water and sterilized by autoclaving.

F. Iso propanol (pre chilled)

G. Absolute alcohol (pre chilled)

H.70% Ethanol (pre chilled) 3.7.2

Reagents for PCR

A. Primers: Highly variable microsatellite markers from ILS, USA.

B. dNTPs: (dATP/dCTP/dGTP/dTTP) 2 mM stock of dNTP (Thermo) was used.

C. PCR buffer (10X): 10X Genaxy buffer was used

D.*Taq*polymeras: 5 unit /µl,*Taq*polymerase (Genaxy) was used for PCR.

E. Tank buffer (1X TAE): 20 ml 50X TAE + 980 ml of distilled water.

F. Orange loading dye

3.7.3 Solutions for electrophoresis

3.7.4 Instruments used in the laboratory

- Veriti 96 well thermal cycler (Applied Biosystems)
- Refrigerated centrifuge
- Microwave oven
- Transilluminator and Bio Rad Gel documentation system
- Micropipettes
- Eppendorf tubes
- Electronic balance

CHAPTER –IV
RESULTS AND DISCUSSION

The present study entitled **"Identification of DNA Markers Associated with Grain Fe, Zn and Protein Contents in Rice (*Oryza sativa* L.)"** was carried out at Nutritional Genomics Laboratory and Genomics and proteomics Laboratory in Department of Plant Molecular Biology and Biotechnology, COA, IGKV with the objectives of genotyping of selected rice genotypes basis of panicle diversity, grain type and some trait specific genotypes includes grain protein content, grain micronutrient content, drought tolerant and high and low grain protein/Fe/Zn concentration with SSR markers, QTL specific and gene specific markers followed by association of markers with the polished grain Fe, Zn and protein contents in rice. Phenotypic and morphological characterization was performed in the 96 rice genotypes under irrigated condition in each replication for morphological characters and estimation of iron, zinc and Protein content in grains. The mean data of phenotypic characters were used for association analysis. DNA was extracted from selected rice genotypes, quantified and amplified in PCR using SSR markers to generate the genotypic data. Phenotypic and genotypic data generated were further used for association analysis to identify the markers associated with the grain Fe/Zn/protein content. The results thus obtained are presented under following headings.

4.1 Mean performance and frequency distribution for quantitative traits

4.2 Frequency distribution of Morphological traits

4.3 Mean performance and frequency distribution of grain protein Contents in 96 rice genotypes

4.4 Performance of Fe and Zn content in 96 rice genotypes used in this study

4.5 Genotyping of selected rice genotypes with molecular markers

4.5.1 Polymorphism Information Content (PIC) of 18 Molecular Markers

4.5.2 Graphical genotyping of rice genotypes using SSR maker data

4.6 Association analysis between DNA markers and Iron, Zinc and grain Protein content

4.1 Mean performance and frequency distribution for quantitative traits

The observation of 96 selected rice genotypes was done to study the morphological characters and calculated the mean performance of each quantitative character (Appendix 1). The observations were recorded for each rice genotypes with five randomly selected plants. The mean performance, range (minimum and maximum), standard deviation (SD) and coeficient of variance (CV %) for all the traits was calculated in the study (Table 4.1, Figure 4.1).

Table 4.1: Trait mean, range, standard deviation (SD), Coefficient of variance (CV)

Traits	Mean	SD	Range		CV%
			Min	Max	
Plant height (cm)	135.56	2.59	69.4	203.2	3.02
Panicle length (cm)	26.79	2.19	16.4	35.4	12.97
No. of total tiller/plant	9.35	0.89	6.2	13.8	15.04
No. of effective tiller/plant	7.90	0.70	5.2	11.4	14.09
Days to 50% flowering (days)	92.7	7.87	70	109.5	8.31
Leaf length of blade (cm)	36.14	0.10	28.6	57.4	10.38
Leaf width of blade (cm)	1.56	2.10	1.32	1.94	9.19
Grain length (mm)	8.60	0.66	5.9	11.2	7.74
Grain width (mm)	2.79	0.03	2	3.9	1.06

4.1.1 Plant Height (cm)

The character plant height (cm) ranges from 69.4 cm to 203.2 cm with a mean height of 135.56 cm and having standard error mean 1.83. Highest mean performance for plant height was recorded for GP-145-20 (203.2 cm) followed by GP-145-34 (202.8cm) whereas the lowest mean performance was recorded in Safri-17/IR681444-5 (69.4 cm). Reduction in plant height may improve their resistance to lodging and reduce substantial yield losses.

4.1.2 Width of leaf blade

The mean leaf width of the 96 rice genotypes was 1.5621 cm and variability in the leaf width varies from 1.32 cm (Botki Gurmatia) to 3.9 cm (Safri-17/IR681444-41) with a mean performance of 1.56 cm. Leaf width of blade was categorized into 3 classes as narrow, medium, broad width of leaf blade, 70 rice genotypes were having narrow width of leaf blade, 17 rice genotypes were having

medium width of leaf blade and 9 of rice genotypes showed broad width of leaf blade.

4.1.3 Length of leaf blade

The mean leaf length of the population was recorded 36.14 cm. The maximum leaf length recorded for GP-145-20 (57.4cm) and minimum leaf length recorded for Basni-1/IR681444-4 (28.6 cm). Out of 96 rice genotypes, 19 rice genotypes were showing short length of leaf blade, 73 rice genotypes were showing medium length of leaf blade and remaining 4 were having long length leaf blade

4.1.4 Days to 50% flowering

The mean value of the population was 92.66 days observed for the 50% flowering. GP-145-59, Morobarakan/IR94046-31 and Swarna/MTU1010-2 (109.5 days) recorded maximum days for 50% flowering, while minimum days of 50% flowering were recorded in Morobarakan (70 days). Out of 96 rice genotypes, only 1 genotypes Morobarakan was having very early flowering duration, 43 rice genotypes were showing early flowering duration, 52 rice genotypes were showing medium flowering duration, and none of rice genotypes showed late and very late flowing duration.

4.1.5 Panicle length (cm)

The mean performance of panicle length (cm) is 26.798 cm and varied between 16.4 cm and 35.4 cm with a standard error mean 1.555. GP-145-40 (35.4 cm) having long panicle followed by Tulsi Gurmatia (33.2) and Reg-695 (16.4cm) short panicle. Panicle length was classified into 5 classes on the basis of panicle length of main axis as very short, short, medium, long, very long. Out of 96 selected rice genotypes in the study, 20 rice genotypes have short panicle length, 66 genotypes have medium panicle length, 10 genotypes were recorded long panicle length and none of genotypes were recorded very long panicle length.

4.1.6 Number of total tillers per plant

Number of tillers/plant had mean performance of 9.35 with a standard error mean 0.629. Number of tillers/plant was shown to be more in Safri-17/IR681444-41 (13.8) followed by R-RHZ IB-13 (12.8). However the lowest mean value of this

particular trait was recorded in BAM-5997 (6.2). A range of 6.2 to 13.8 tillers per plant was recorded.

4.1.7 Number of effective tillers/plant

Number of tillers/plant had mean performance of 7.90 with a standard error mean 0.498. For this character Safri-17/IR681444-5 (11.4) showed maximum number of effective tillers per plant followed by IR681444/Abhaya-18 (10.4), however less number of effective tillers/plant was recorded in BAM-5997 (5.2). Number of effective tillers per plant was classified into 3 classes as few, medium and many panicles. Out of 96 rice genotypes, 95 showed few effective tillers, only 1 genotype shown medium effective tillers/ plant.

4.1.8 Grain length (mm)

The mean performance of grain length was 8.606 mm with standard error mean 0.471. The long grain length was recorded in GP-145-38 (11.2mm) followed by GP-145-119 (10.55mm) while Srikamal (5.9mm) exhibited the lowest grain length. Grain length was classified into 5 classes on the basis of grain length as very short, short, medium, long and very long. Out of 96 rice genotypes, only one genotype Srikamal has very short grain length, 43 genotypes have short grain length, 50 genotypes were recorded medium grain length, only 2 genotypes was having long grain length.

4.1.9 Grain width (mm)

The mean performance of grain width was 2.791mm with standard error mean 0.0054. GP-145-119 (3.9mm) performed best for the character of grain width followed by chapti Gurmatia (3.7mm) and lowest grain width was observed for IR681444/IR64 (2mm). Grain width was categorized into 5 classes as very narrow, narrow, medium, broad, very broad width of grain. Out of 96 rice genotypes, none of the genotypes shown very narrow width of grain, 32 genotypes were recorded narrow width of grain, 35 genotypes were recorded medium width of grain, 27 genotypes were recorded broad width of grain and 2 genotype were recorded very broad width of grain.

4.2 Frequency distribution of Morphological traits

Morphological traits were recorded for ninety six selected rice genotypes for nine qualitative characters as per the National DUS Guidelines for characterization of rice as described in Appendix 2. Pictorial representations of distinguishable morphological features were presented in figure 4.2 and figure 4.3 Similarly frequency distribution graph of morphological traits were presented in figure 4.4 and figure 4.5

4.2.1 Basal leaf sheath color

Out of 96 rice genotypes, 78 genotypes were recorded green basal leaf sheath colour, 3 genotypes were recorded purple line, 15 genotypes were recorded light purple basal leaf sheath colour and none of rice genotypes were recorded uniform purple.

4.2.2 Intensity of green color in leaf

The colour of leaf blade was categorized into light, medium and dark green colour classes. Out of 96 rice genotypes, it was observed that 15 lines were showing light green colour, 73 lines were showing medium green colour of leaf whereas 8 under study having dark green leaf blade and none of genotypes was showing purple colour.

4.2.3 Distribution of anthocyanin coloration on leaf blade

This character is a good marker character, can distinguish a variety from others. The distribution of anthocyanin colouration was classified into tips only, on margin only, in blotch only and uniform presence of anthocyanin colouration of leaf blade. Out of 96 genotypes, 3 rice genotypes having anthocyanin colouration on leaf tip only, 25 rice genotypes having anthocyanin colouration on margin only, 8 rice genotypes having anthocyanin colouration in blotch only, 5 rice genotypes having uniform presence of anthocyanin colouration of leaf blade and remaining other 55 rice genotypes does not showing anthocyanin colouration on leaf.

4.2.4 Presence of pubescence on leaf blade surface

Out of 96, 6 rice genotypes included into medium pubescence on leaf surface, 32 included into strong pubescence on leaf surface, 58 included into very

strong pubescence on leaf surface and no one rice genotypes found in absent and weak pubescence on leaf surface.

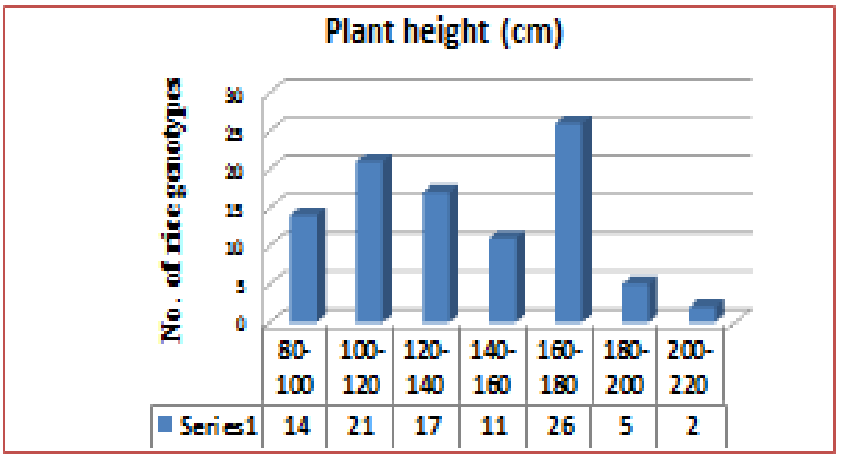
4.2.5 Anthocyanin coloration of auricles

The colouration of auricles was grouped into colorless, light purple and purple auricles. Out of 96 rice genotypes, 58 rice genotypes were recorded colourless auricles, 22 rice genotypes were recorded light purple auricle and 6 rice genotypes were recorded purple auricle.

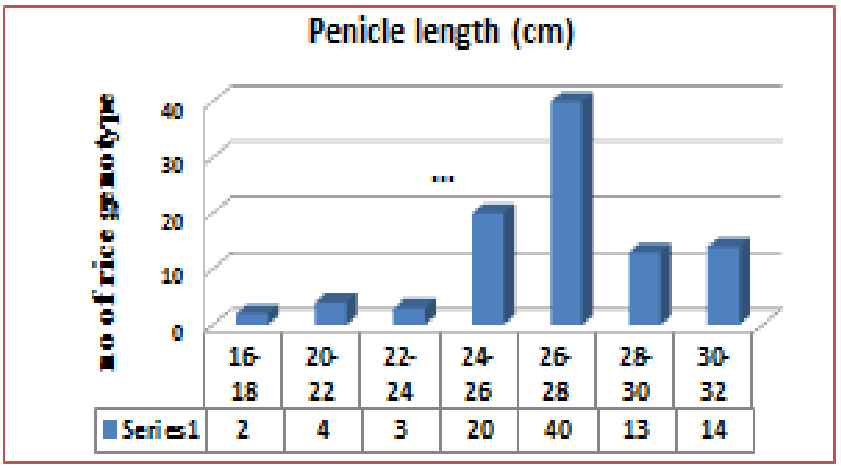
4.2.6 Flag leaf attitude

Attitude of flag leaf blade was observed for 4 class namely erect, semi-erect, horizontal and drooping attitude. Out of 96 rice genotypes, 17 rice genotypes recorded erect flag leaf attitude, 63 rice genotypes recorded semi-erect rice genotypes 7 recorded horizontal flag leaf attitude and 9 rice genotypes was found drooping flag leaf attitude flag.

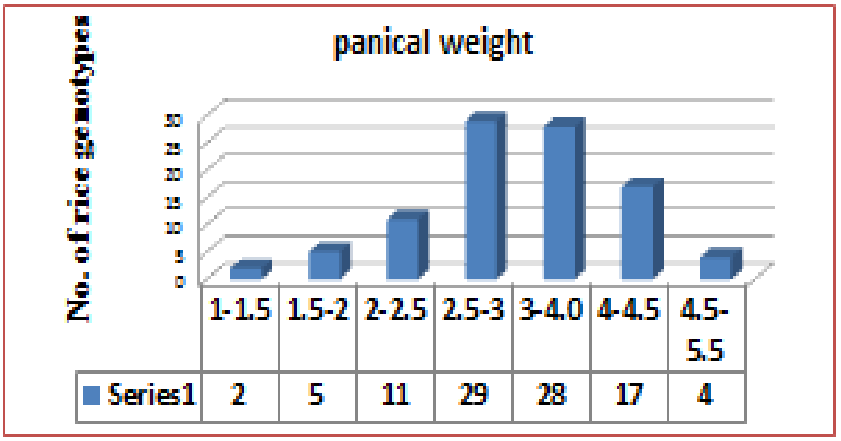
a.


Plant height (cm)
No. of rice genotypes
80-100
100-120
120-140
140-160
160-180
180-200
200-220
Series1
14
21
17
11
26
5
2

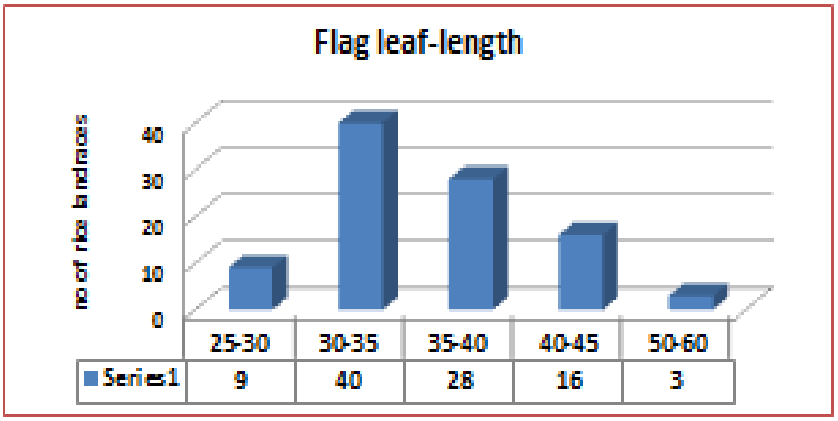
b.


Penicle length (cm)
no of rice genotype
16-18
20-22
22-24
24-26
26-28
28-30
30-32
Series1
2
4
3
20
40
13
14

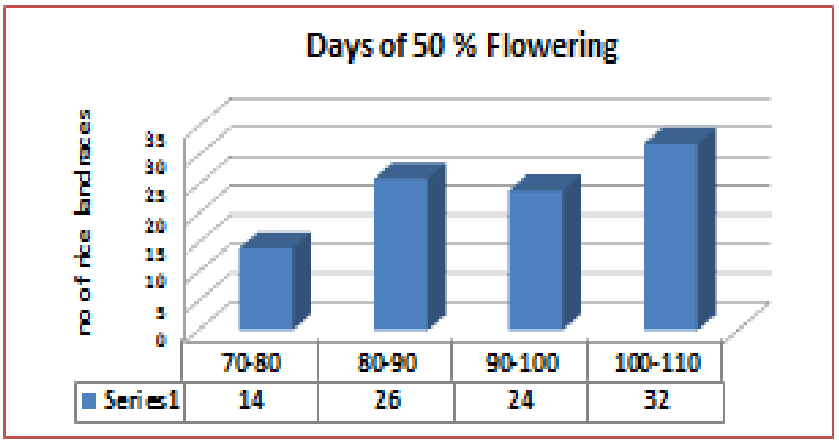
c.


panical weight
No. of rice genotypes
1-1.5
1.5-2
2-2.5
2.5-3
3-4.0
4-4.5
4.5-5.5
Series1
2
5
11
29
28
17
4

d.



e.



f.

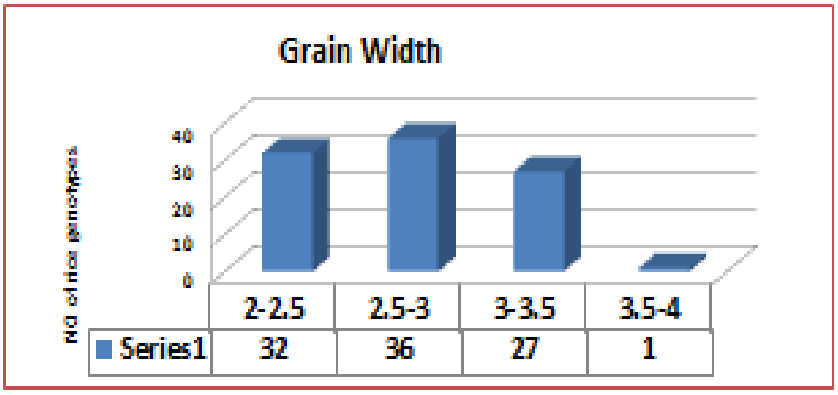


Fig4.1 Frequency distribution of genotype based on qualitative and uantitative trait in selected rice genotypes.

a. plant hight; b. Panicle Length c. Panicle weight d. flag leaf-length e. Days of 50% plants with panicles f. Grain width

Fig 4.2 Showing distinguishable morphological characters of selected rice genotypes

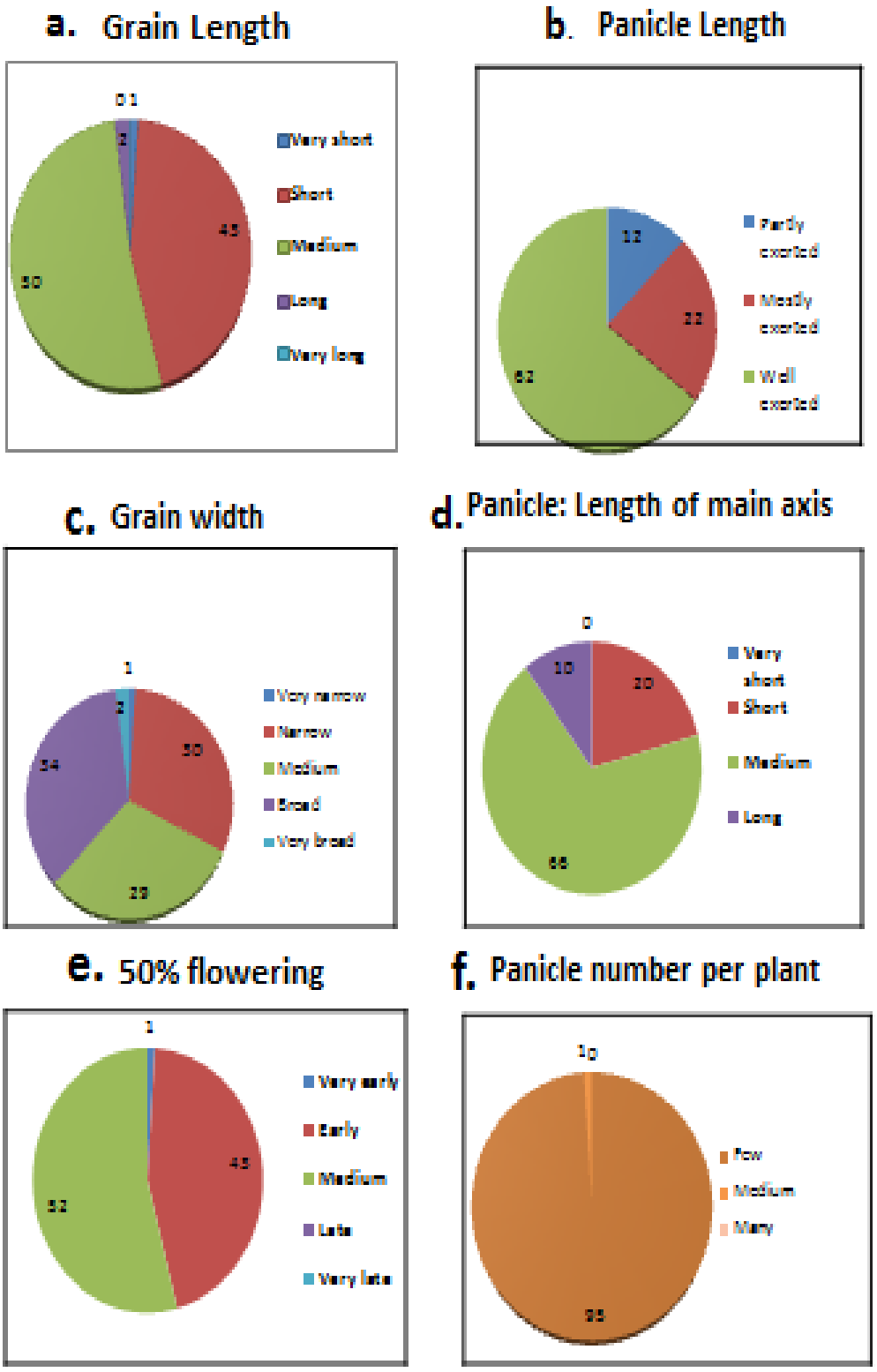


Fig.4.3 Frequency distribution of rice genotype based on quantitative trait.

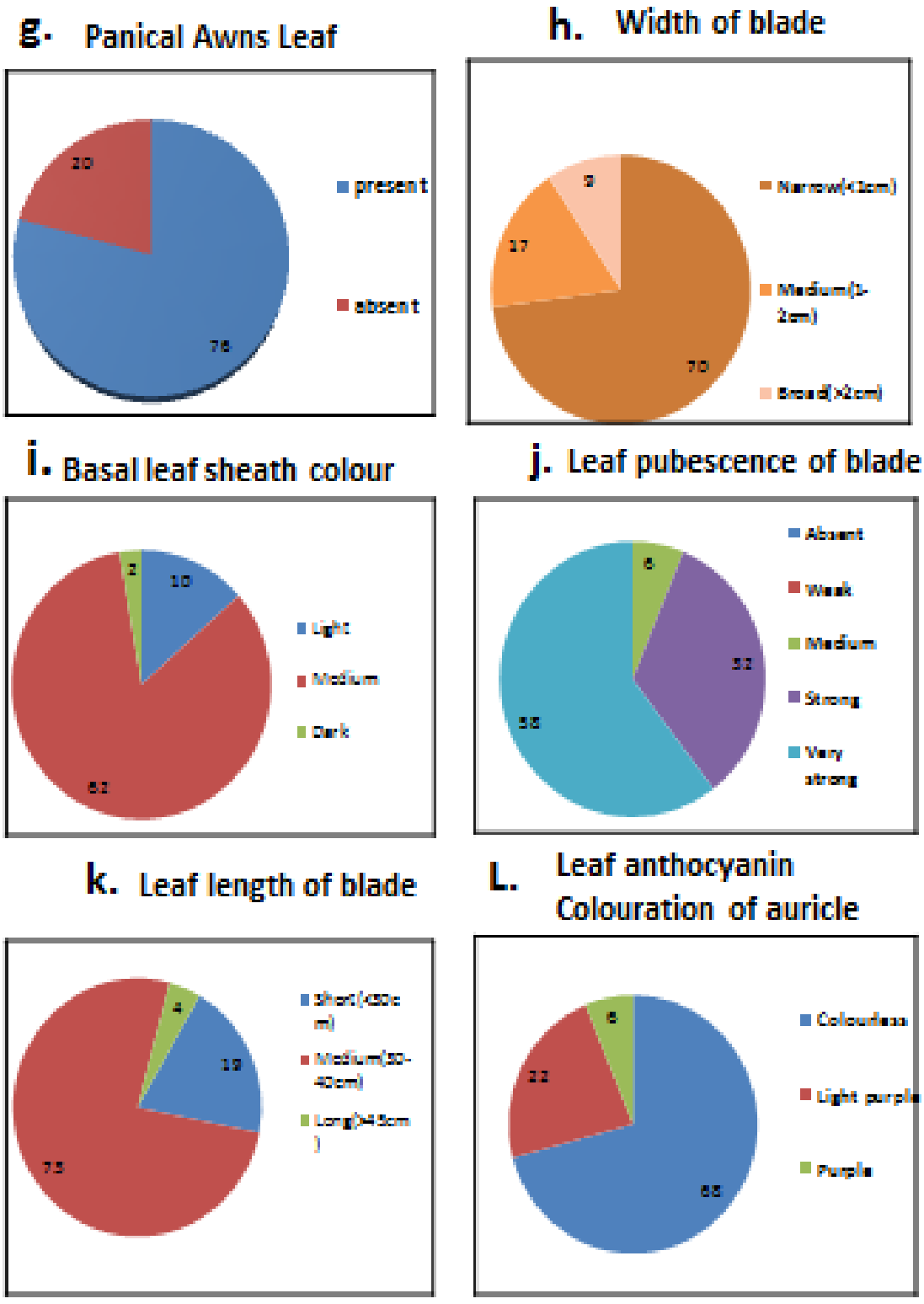


Fig.4.4 Frequency distribution of rice genotype based on qualitative trait

4.2.7 Panicle: attitude of branches

Out of 96 rice genotypes, 25 rice genotypes recorded erect type attitude of branches, 61 rice genotypes recorded semi-erect type attitude of branches, none of the rice genotypes recorded semi-erect to spreading type attitude of branches and 10 Rice genotypes recorded spreading type attitude of branches.

4.2.8 Panicle exsertion

Panicle exertions are divided into 3 class *viz*., partly exserted, mostly exserted d and well exserted panicle. Out of 96 rice genotypes, 12 rice genotypes were observed partly exserted panicle, 22 rice genotypes were observed mostly exserted panicle and remaining 62 rice genotypes were recorded well exserted panicle.

Hossain *et al.,* (2005) also studied morphological character leaf breadth, leaf orientation) and agronomic character (plant height, panicle length, total tillers per hill) of some local and modern aromatic rice varieties. All the parameter varied significantly and results showed that the maximum plant height was observed in chinigura (162.8 cm), total tillers per hill (12.5). Maximum panicle length observed in BRRI dhan 38 (24.14 cm) and minimum panicle length observed in Radhuni pagal (20.65 cm).

4.3 Mean performance and frequency distribution of Grain protein contents in rice genotypes

In this study grain protein content of 96 rice genotypes was analyzed by using micro-Kjeldahl method (Johri *et al*., 2000). The result of analysis revealed that the level of grain protein varied from to 5.78 to 10.28 % with the 0.171 % coefficient of variation (CV) and having standard error mean of 0.121. The highest grain protein content was recorded in Kalam Gurmatia (10.28%) followed by Bisni-1 (9.87) and Jeeradhan(9.87%), whereas the lowest grain protein content was observed in CGR-1539 (5.78 %) followed by Swarna (6.20%).The analysis of variance (CRD) of grain protein content revealed significant difference among rice genotypes at 5% level of significance. The protein content and its ANOVA of all the 96 rice genotypes is presented in the table 4.2 and 4.4

Riza *et al*., (2004) have also reported grain protein content range from 6.3% to 9.1% in a set of 438 rice genotype. They reported PR-27423-MS6 (6.3%) containing lowest GPC and PR-31595-PSC101 (9.1%) with the highest GPC.

Similar results were observed in a study conducted by Banerjee *et al.,* (2010) on estimation of protein content in a set of 12 diverse rice genotype including both cultivated and wild genotype of rice result showed a range of 6.19-10.75% protein in whole grains. Wide variation for protein concentration in milled grain level from 2.8% to 9.9% of rice germplasm lines of Chhattisgarh have been reported by Chandel *et al.,* (2005).

The rice genotypes identified with higher grain protein content can serve as donor lines for this trait for the improvement of elite and popular rice cultivar poor in grain protein. Efficient breeding programs planned with high grain protein content genotype identified in this study will be beneficial in improvement of local cultivars of Chhattisgarh helpful in eradicating protein malnutrition from the state.

Table 4.2 Mean Grain Protein levels among selected rice genotypes

SN	Rice genotypes	Mean Protein ± Sem	SN	Rice genotypes	Mean Protein ±Sem
1	Bhathaili Gurmatia	7.95 ± 0.04	49	BAM-1292	8.205± 0.04
2	Botki Gurmatia	8.16±0.16	50	BAM-5446	7.53±0.20
3	Chapti Gurmatia	8.74±0.25	51	BAM-5926	6.58±0.08
4	chapti Gurmatia	8.70±0.12	52	Moroberekan	7.12±0.20
5	Gurmatia	8.74±0.08	53	Nagina-22	7.87 ±0.12
6	Jhunki Gurmatia	8.41±0.16	54	BAM-5997	7.41±0.08
7	Kalam Gurmatia	10.28±0.04	55	Bakal	7.66±0.08
8	Nunki Gurmatia	8.45±0.04	56	GP-145-37	8.37±0.04
9	Sultu Gurmatia	8.45±0.12	57	MTU-1010	7.95 ±0.04
10	Tulsi Gurmatia	8.66±0.12	58	SL-62	8.20 ±0.12
11	Bangla Gurmatia	8.17±0	59	GP-145-41	8.12±0.04
12	ShriKamal	7.247±0	60	GP-145-66	7.20±0.12
13	Elayachi	6.67±0	61	CGR-1539	5.78±0.04
14	Jeeradhan	9.87±0	62	RRGM-ATN-47	8.99±0.08
15	Bisni-1	9.87±0.04	63	RRGM-AS-45	8.87±0.04
16	RR-152	9.12±0.12	64	GP-145-42	7.74 ±0.16
17	RR-137	9.08±0.08	65	GP-145-44	7.12±0.04
18	RR-149	7.70±0.04	66	GP-145-70	6.78±0.12
19	RR-8 M011	9.03±0.20	67	RKVY-52	9.70±0.04
20	Reg-1035	8.28±0.12	68	GP-145-103	7.03 ±0.12
21	Reg-1038	6.91±0.08	69	RKVY-15	8.20±0.04
22	CHIR-8	7.95±0.20	70	GP-145-78	7.12 ±0.04
23	CGZR-1	8.3±0.08	71	GP-145-43	7.66±0.08
24	IET 23829	8.24±0.08	72	GP-145-49	9.08 ±0.16
25	Basmati 370	7.91±0.08	73	GP-145-59	9.33 ±0.16
26	R-RHZ-LI-23	8.86±0	74	GP-145-119	8.87±0.62
27	R-RHZ-IB-13	9.08±0.08	75	GP-145-136	9.45±0.04
28	R-RHZ-SM-14	6.66±0	76	GP-145-50	8.45±0.12
29	Basmati 1	8.28±0.12	77	GP-145-65	6.70±0.04
30	R-RHZ-MI-30	7.41±0.08	78	GP-145-38	6.91±0.16
31	Kalanamak	7.87±0.12	79	GP-145-130	7.70±0.04
32	R-56	9.08±0.16	80	GP-145-138	6.497 ±0
33	Kadamphool	8.33±0.16	81	GP-145-5	6.45 ±0.04
34	Karigilas	7.70±0.04	82	GP-145-11	7.74±0.08
35	Jhilli	7.91±0.08	83	GP-145-20	7.37±0.12
36	Azucena	7.37±0.04	84	GP-145-34	6.83 ±0.08
37	IR-64	7.62±0.04	85	Safri-17/IR681444-41	6.95 ±0.04
38	Swarna	6.20±0.04	86	Bas-1/IR681444-4	8.45 ±0.20
39	RR-100	8.08±0.08	87	Kranti/Swarna	6.95 ±0.04
40	Bhansapanchi	6.49±0.16	88	IR681444/Abhaya-18	8.91 ±0.08
41	Banda	9.20±0.04	89	Moro/IR94046-31	9.28±0.04
42	Bada gada khuta	8.66±0.08	90	IR681444/HMT-24	8.20±0.12
43	Reg-695	7.37±0.04	91	IR681444/IR64	9.28 ±0.04

SN	Rice genotypes	Mean Protein ± Sem	SN	Rice genotypes	Mean Protein ±Sem
44	Dagad-Desi	7.53±0.12	92	Safri-17/IR681444-5	7.87 ±0.04
45	GP-145-40	7.58±0	93	MTU1010/IR94032-5	8.08 ±0.16
46	RKVY-104	7.24±0.08	94	IR681444/Bas-8-3	9.58 ±0.08
47	RKVY-211	6.66±0.08	95	IR681444/Bas-1-27	6.539±0.04
48	Dullar	9.33±0.08	96	Swarna/MTU1010-2	8.12 ±0.12

Table 4.3 Fertiliser dose/ hectare

Nitrogen	Phosporus	Potassium
100kg	60kg	40kg

Table 4.4: Analysis of variance for GPC

SV	DF	SS	MS	F-cal	F-Table		Sem	Sed	CV (%)	CD
Replication	1	0.049								
Treatment	95	170.631	1.796	61.268	0	Significant	0.121	0.171	0.171	o.34
Error	95	2.785	0.029							
Total	191	173.466								

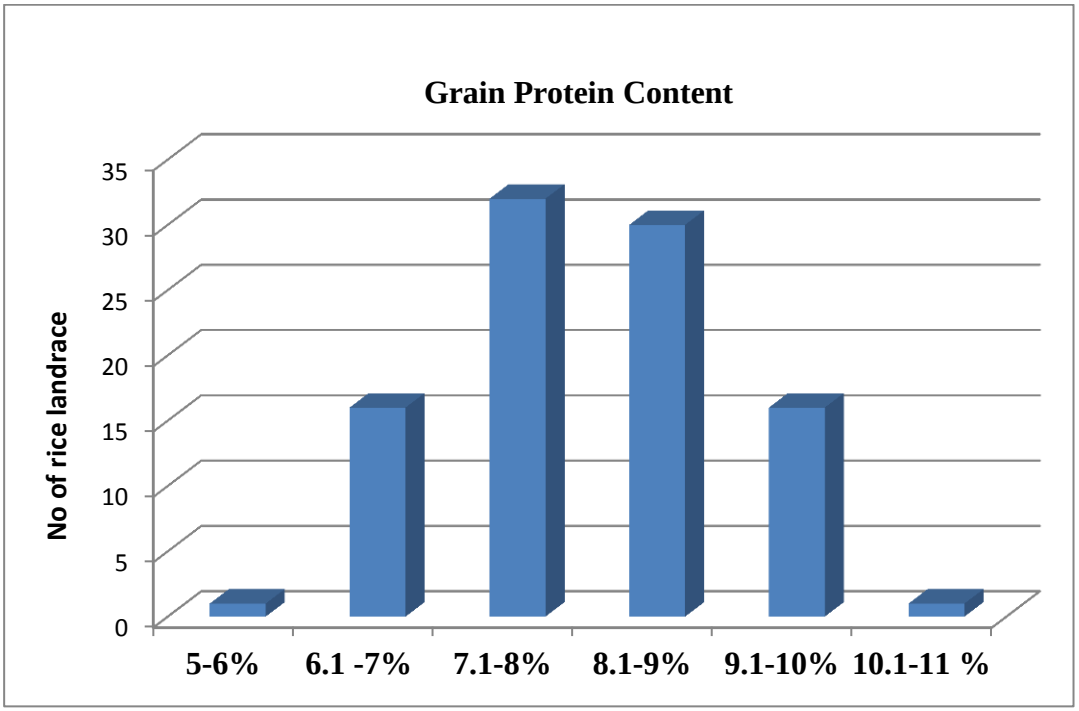


Fig 4.5 Grain Protein Content (%) of 96 rice genotypes used in this study

4.4 Performance of Fe and Zn content in 96 rice genotypes used in this study

In this study Fe and Zn content of 96 rice genotypes was analyzed by using energy dispersive X-ray fluorescent spectrophotometer (ED-XRF) (Stanguilis *et al.*,2014). Analysis revealed that the level of iron and zinc content in polished rice grain varies from 4.8 to 14.3µg/gm and 13.6 to34.3 µg/gm respectively. The high iron content was recorded in Bisni-1(14.3µg/gm) followed by Jeeradhan (13.9µg/gm) and Bangla Gurmatia (2711)13.3µg/gm Similarly, high zinc content in grains was recorded in dullar (34.3µg/gm) followed by Jeeradhan (32.4 µg/gm) and Bisni 31.49µg/gm. Whereas, the lowest iron and zinc concentration was observed in Moroberekan (4.8 µg/gm) and GP-145-138 (13.6µg/gm) respectively. The analysis of variance (CRD) for iron and zinc concentration in grains has shown significant difference among 96 rice genotypes at 5% level of significance. The Fe and Zn content of all the 96 rice genotypes is presented in the table 4.5 and its ANOVA table and figure present in table 4.5.1 and 4.6

Table 4.5 Micronutrients concentration in polished grain of selected rice genotypes (µg/gm)

S.no	Rice genotypes	Fe (µg/gm)	Zinc (µg/gm)	S.no	Rice genotypes	Fe (µg/gm)	Zn (µg/gm)
1	Bhathaili Gurmatia	8.9	19	49	BAM-1292	7	27
2	Botki Gurmatia	7.9	19.7	50	BAM-5446	6.3	22.1
3	Chapti Gurmatia	8.6	24.4	51	BAM-5926	6	17.3
4	chapti Gurmatia	8.4	18.9	52	Moroberekan	4.8	17.3
5	Gurmatia	10.9	27	53	Nagina-22	9.7	28.4
6	Jhunki Gurmatia	10.9	21.6	54	BAM-5997	10	27.1
7	Kamal Gurmatia	8.5	20.5	55	Bakal	9.6	22.7
8	Nunki Gurmatia	8.2	23.4	56	GP-145-37	6.7	26.4
9	Sultu Gurmatia	8	25.4	57	MTU-1010	7.8	17.6
10	Tulsi Gurmatia	7.1	17.8	58	SL-62	7.4	20.2
11	Bangla Gurmatia	13.3	29.7	59	GP-145-41	8.9	26.8
12	ShriKamal	7.9	16.5	60	GP-145-66	7.6	18.5
13	Elayachi	9.8	20.9	61	CGR-1539	8.4	15.5
14	Jeeradhan	13.9	32.4	62	RRGM-ATN-47	8.6	23.6
15	Bisni-1	14.3	31.4	63	RRGM-AS-45	9.5	19.2
16	RR-152	11.8	24.1	64	GP-145-42	8.1	23.4
17	RR-137	6.3	18.2	65	GP-145-44	8.8	17.5
18	RR-149	8.4	23.1	66	GP-145-70	6.7	17.5
19	RR-8 M011	6	19.7	67	RKVY-52	5.8	23.6
20	Reg-1035	8.6	26.7	68	GP-145-103	5.6	17.7
21	Reg-1038	11.3	18.5	69	RKVY-15	12.1	27.7
22	CHIR-8	9.1	27	70	GP-145-78	8	17.6
23	CGZR-1	7	21.2	71	GP-145-43	7.5	18.8
24	IET 23829	11.4	28.4	72	GP-145-49	9.4	25.3
25	Basmati 370	10	24.7	73	GP-145-59	8	18.5
26	R-RHZ-LI-23	8.6	21.9	74	GP-145-119	6.9	19.4
27	R-RHZ-IB-13	9.1	25.9	75	GP-145-136	8	18.5

S.no	Rice genotypes	Fe (µg/gm)	Zinc (µg/gm)	S.no	Rice genotypes	Fe (µg/gm)	Zn (µg/gm)
28	R-RHZ-SM-14	9.5	24.3	76	GP-145-50	5.7	15.6
29	Basmati 1	7.9	24.7	77	GP-145-65	6.4	14.2
30	R-RHZ-MI-30	8.4	24.4	78	GP-145-38	6.8	15.2
31	Kalanamak	8.8	21.7	79	GP-145-130	9.9	16.5
32	R-56	9.9	25.9	80	GP-145-138	5.9	13.6
33	Kadamphool	9.4	23.1	81	GP-145-5	7.6	15.3
34	Karigilas	5.8	22.8	82	GP-145-11	10.4	21.2
35	Jhilli	7.7	20.4	83	GP-145-20	7.9	21.2
36	Azucena	7.8	25.3	84	GP-145-34	10.1	21
37	IR-64	6.5	20.3	85	Safri-17/IR681444-41	7.9	18.1
38	Swarna	8.3	14.2	86	Bas-1/IR681444-4	9	21.8
39	RR-100	6.7	20	87	Kranti/Swarna	9	18.8
40	Bhansapanchi	7.7	18.5	88	IR681444/Abhaya-18	9	22.1
41	Banda	5.1	15.1	89	Moro/IR94046-31	6.7	16
42	Bada gada khuta	5.8	20.8	90	IR681444/HMT-24	11.3	21.4
43	Reg-695	6.4	17.6	91	IR681444/IR64	8.7	21.9
44	Dagad-Desi	10.5	28.3	92	Safri-17/IR681444-5	8	18.4
45	GP-145-40	7.5	19.3	93	TU1010/IR94032-5	9.3	20.6
46	RKVY-104	9	26.5	94	IR681444/Bas-8-3	9.7	28.5
47	RKVY-211	9.5	23.8	95	IR681444/Bas-1-27	10	28.3
48	Dullar	11.2	34.8	96	Swarna/MTU1010-2	6.8	19.2

Table 4.5.1 ANOVA for Iron content in polished rice grain

Iron	
Mean	8.407273
Standard Error	0.17368
Median	8.4
Mode	7.9
Standard Deviation	1.821568
Sample Variance	3.318112
Kurtosis	0.887218
Skewness	0.598521
Range	9.6
Minimum	4.7
Maximum	14.3
Sum	924.8
Count	110

Table 4.6 ANOVA for grain Zinc content

Zinc	
Mean	21.43636
Standard Error	0.412906
Median	20.95
Mode	18.5
Standard Deviation	4.330598
Sample Variance	18.75408
Kurtosis	-0.07741
Skewness	0.531343
Range	21.2

Minimum	13.6
Maximum	34.8
Sum	2358
Count	110

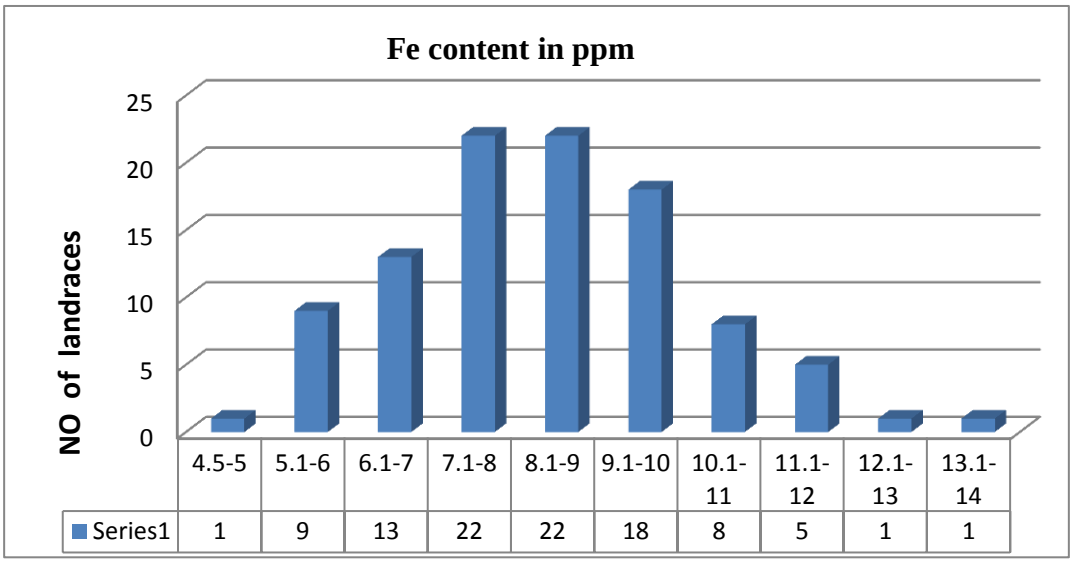


Fig:-4.6 Iron concentration of 96 rice genotypes used in this study

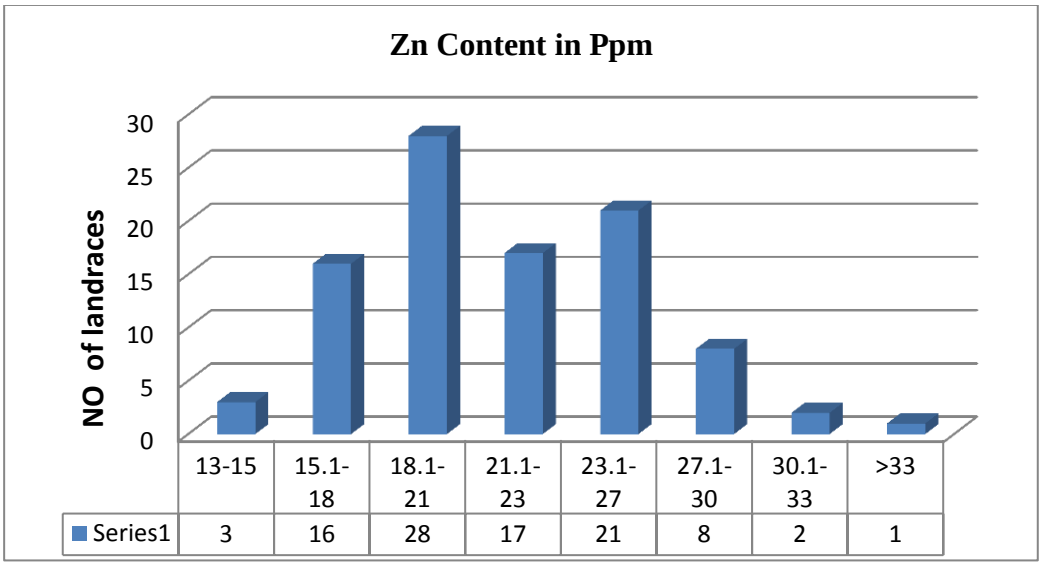
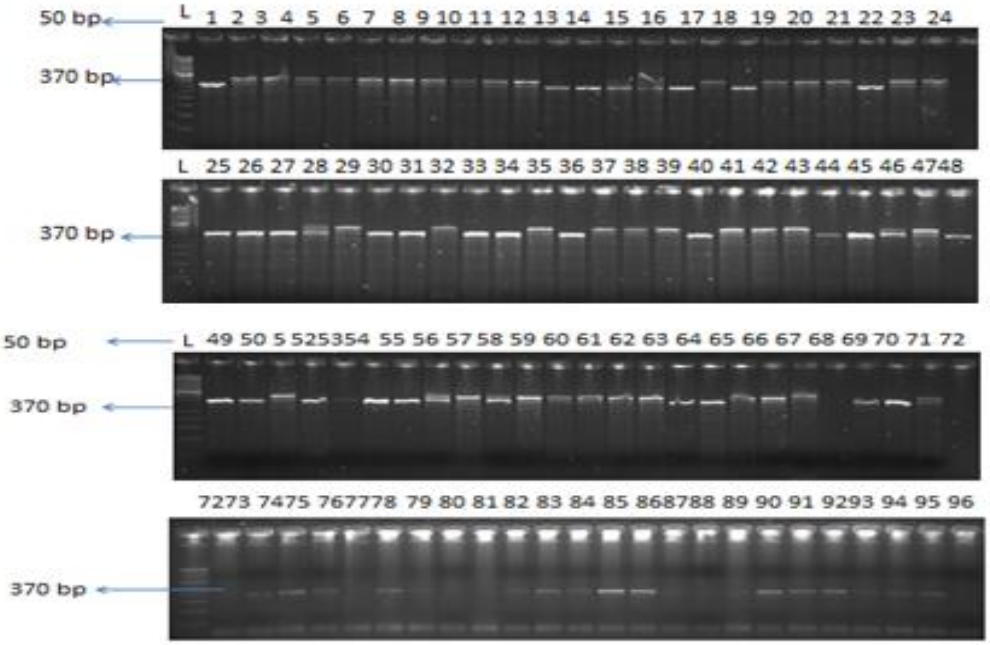


Fig:-4.7 Zinc content of 96 rice genotypes used in this study

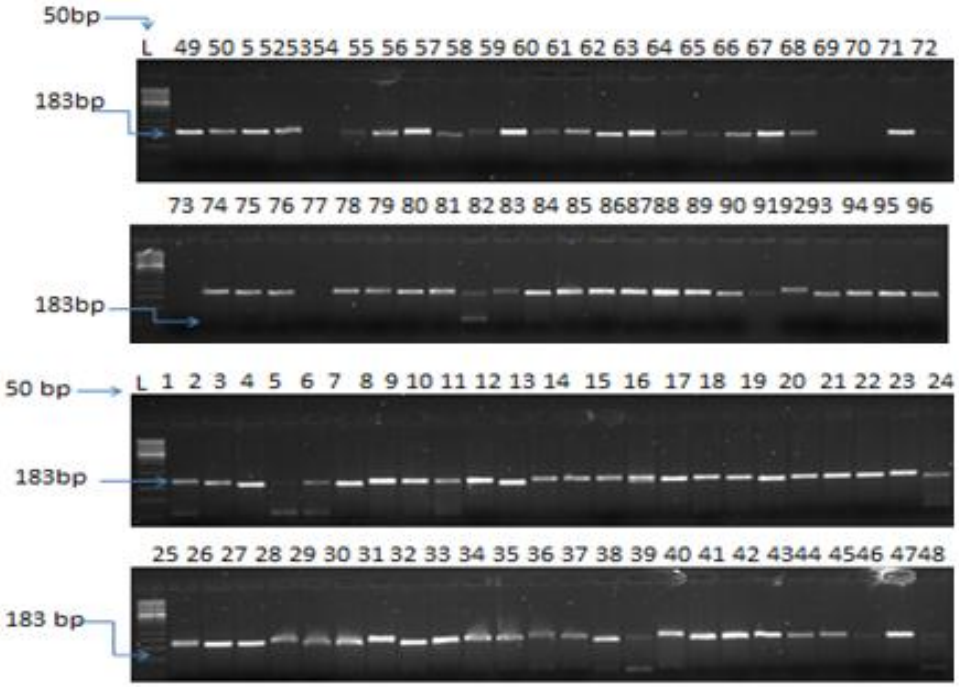
4.5 Genotyping of selected rice genotypes with molecular markers

Out of the 42 markers including 39 SSR markers, 2 QTL specific markers and 1 gene specific markers used in the study, 18 SSR markers were found to be polymorphic in the set of selected rice genotypes which were used to investigate the level of polymorphism (Table no.4.6), (Fig.4.8). The set of 42 markers used in the study included distributive randomly across the twelve chromosomes. Total 36 alleles were produced by 18 SSR primer set used in this study. Similar kind of study was performed by the Patil *et al.*, (2014), they reported 69 alleles from 25 polymorphic SSR primer set used with an average of 2.76 alleles per locus. Similarly, Jain *et al*., (2004) evaluated 24 rice genotypes using 30 SSR primers, in which high level of polymorphism was reported among the different rice genotype. They have detected 229 alleles with an average no of 4.58 per locus. Lapitan *et al* ., (2007) also carried out the DNA fingerprinting of 24 rice cultivars carrying good quality traits by using 164 SSR markers in which 890 alleles were detected by 151 polymorphic markers, with an average of 5.89 alleles per locus. Rajendran *et al.*, (2012) used hundred SSR markers for molecular fingerprinting and genetic distance analysis of twelve commercial hybrid rice parental lines, among the marker screened six ty two were polymorphic and generating 203 alleles with an average of 3.2 alleles per primers. Sajib *et al.*, (2012) studied 24 SSR markers used for the characterization and discrimination of 12 elite aromatic rice genotypes. Among these 24 markers, 9 microsatellite markers were showed polymorphism. The number of alleles per locus ranges from 2 alleles (RM-510, RM-244 and RM-277) to 6 alleles (RM-163) with an average of 3.33 alleles across 9 loci

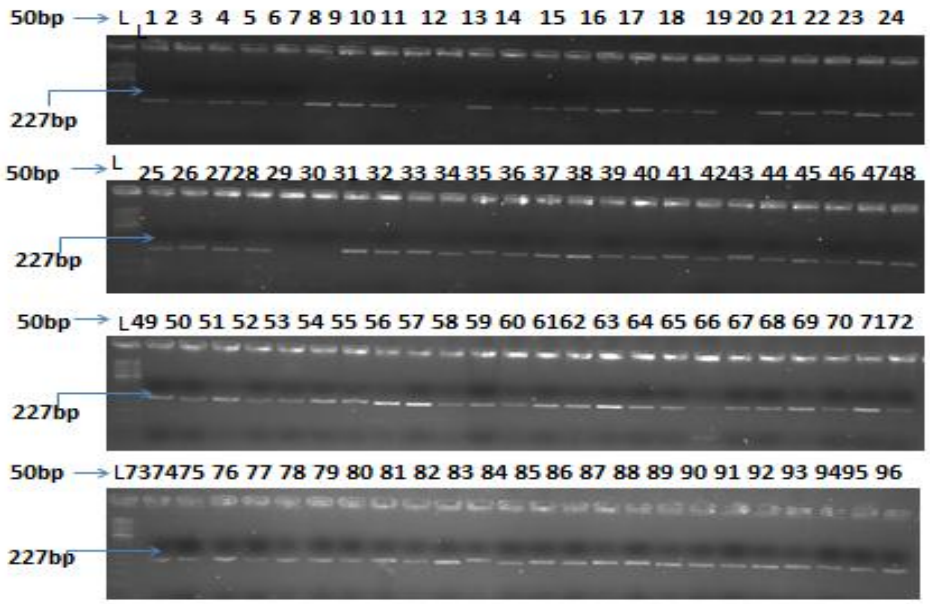
a.


50 bp
L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
370 bp
L 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 4748
370 bp
50 bp
L 49 50 5 525354 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72
370 bp
7273 7475 767778 79 80 81 82 83 84 85 868788 89 90 91 9293 94 95 96
370 bp

b.


50bp
L 49 50 5 525354 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72
183bp
73 74 75 76 77 78 79 80 81 82 83 84 85 868788 89 90 919293 94 95 96
183bp
50 bp
L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
183bp
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 4344 4546 4748
183 bp

c.



d.

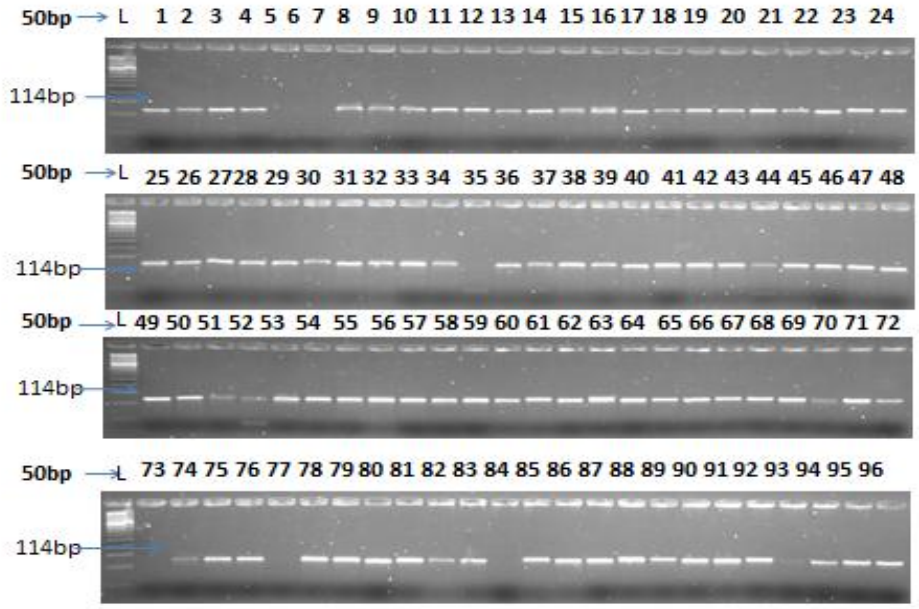


Fig.4.8 **Genotyping of 96 contrasting rice genotypes for grain Fe/Zn/Protein content. a and b polymorphism showing and c and d showing monomorphism**

a. Oszip-3b; b. RM154: c. RM122; d. RM475

4.6 Polymorphism Information Content (PIC) of 18 Molecular Markers

The PIC values are usually dependent on the genetic diversity of the landraces chosen for the specific study (Garland, 1999). Polymorphism Information Content (PIC) is a measure of diversity for SSR marker. PIC provides an estimate of discriminatory power of a locus by taking into account not only the number of alleles expressed, but also the relative frequency of those alleles. PIC value ranges from A to B (very high discriminative with many alleles in equal frequencies. The average PIC value for all 18 polymorphic marker loci in the present study was 0.47, with a range from 0.37-0.50 (Table4.7). PIC was highest for the gene specific marker OsZIP3b and SSR marker RM 234 having PIC value 0.50 followed by RM490, RM5 and was lowest for the primer RM 517 (0.37). The above markers were found to be fairly informative in revealing the genetic diversity among the varieties and will be useful in future genetic diversity analysis. The high PIC value obtained in the present investigation might be due to high genetic diversity among the rice landraces for the locus under study. Similarly, Shahriar *et al*., (2014) worked on advanced breeding lines and reported that three markers (RM147, RM167, RM215) polymorphism information content (PIC) ranged from 0.47 to 0.88 with an an average of 071. PIC was highest for RM167 (0.88) and was lowest for RM 147 (0.47), RM167 was highly informative.

Table 4.7 List of SSR markers showing polymorphism using 96 rice genotypes

Marker	Ch.No	Primers	At	Alleles	PIC	Ps (bp)	Position (cM)
RM490	1	F: ATCTGCACACTGCAAACACC R: AGCAAGCAGTGCTTTCAGAG	55	2	0.49	101	51.0
RM5	1	F: TGCAACTTCTAGCTGCTCGA R: GCATCCGATCTTGATGGG	55	2	0.49	113	94.9
RM259	1	F: TGGAGTTTGAGAGGAGGG R: CTTGTTGCATGGTGCCATGT	55	2	0.49	162	54.2
RM226	1	F:GAAGCTAAGGTCTGGGAGAAACC R:AATGGCCTTAACCAAGTAGTAGG	55	2	0.48	274	154.8
RM 279	2	F: GCGGGAGAGGGATCTCCT R: GGCTAGGAGTTAACCTCGCG	55	2	0.48	174	17.3
RM154	2	F: GACGGTGACGCACTTTATGAACC R: CGATCTGCGAGAAACCCTCTCC	61	2	0.48	183	4.8
RM231	3	F: CCAGATTATTTCCTGAGGTC R: CACTTGCATAGTTCTGCATTG	55	2	0.43	182	15.7
RM 517	3	F: GGCTTACTGGCTTCGATTTG R: CGTCTCCTTTGGTTAGTGCC	55	2	0.37	266	42.9
Crm33-1	3	F: TCGTTCTGACATGTTGAGG R: TCGTTCTGACATGTTGAGG	58	2	0.49	60.2	68.2
RM252	4	F: TTCGCTGACGTGATAGGTTG R: ATGACTTGATCCCGAGAACG	55	2	0.45	216	99.0
RM 470	4	F: TCCTCATCGGCTTCTTCTTC R: AGAACCCGTTCTACGTCACG	55	2	0.48	83.0	115.5
OsZIP3b	4	F: CCTGCTGAGGCTGAGTTGAA R: CGAGAACAAAGTAACAGGCTGC	56	2	0.50	370	124.3
RM225	6	F: TGCCCATATGGTCTGGATG R: GAAAGTGGATCAGGAAGGC	55	2	0.48	140	26.2
RM234	7	F: ACAGTATCCAAGGCCCTGG R: CACGTGAGACAAAGACGGAG	55	2	0.50	156	88.2
RM501	7	F: GCCCAATTAATGTACAGGCG R: ATATCGTTTAGCCGTGCTGC	55	2	0.49	179	30.1
RM44	8	F: ACGGGCAATCCGAACAACC R: TCGGGAAAACCTACCCTACC	55	2	0.49	99.0	60.9
RM277	12	F: CGGTCAAATCATCACCTGA R: CAAGGCTTGCAAGGGAAG	55	2	0.49	124	57.2
RM19	12	F: CAAAAACAGAGCAGATGAC R: CTCAAGATGGACGCCAAGA	55	2	0.48	226	20.9

Table 4.8 Marker wise contribution of allele A and B

Marker	Chromosome	allele A%	allele B%
RM490	1	56.25	43.75
RM5	1	55.20	44.79
RM226	1	59.37	40.62
RM259	1	48.96	51.04
RM279	2	58.33	41.66
RM154	2	58.33	41.66
RM517	3	75.00	25.00
Crm33-1	3	55.20	44.79
RM231	3	67.70	32.29
Oszip-3b	4	50.00	50.00
RM252	4	64.58	35.41
RM470	4	62.50	37.50
RM225	6	40.62	59.37
RM234	7	50.00	50.00
RM501	7	52.08	47.91
RM44	8	55.20	44.79
RM19	12	41.66	58.33
RM277	12	45.82	54.16

4.7:-Association analysis between DNA markers and grain Iron, Zinc and grain protein content in polished rice grain

The association between markers and Iron, Zinc and grain protein content were calculated using single marker analysis (SMA) in Microsoft Excel program. The significant marker Iron, Zinc and grain protein contentassociations were indicated by a P-value (<0.05) with corresponding R^2 for each marker is the total phenotypic variation for a trait that is accounted by markers. We detected a total of 8 significant marker associated with the polished grain iron, zinc and protein content ($P<0.05$) (Table 4.9). All of the 8 significant SSR loci were indentified for the iron, zinc and protein content, with the R^2, percentage of the total variation explained ranging from 4.04 to 9.1 %. The chromosomal map of each marker loci associated with the Iron, Zinc and grain protein content was prepared using software MAPCHART version 2.3 presented in figure 4.9.

Table 4.9: Association between SSR markers with Fe, Zn and grain protein content with P<0.05.

Marker	Trait	Chromosome	Position (cM)	P-value	R^2 value
RM490	Iron	1	51.0	0.0027	9.1
RM5	Iron	1	94.9	0.049	4.04
RM5	Zinc	1	94.9	0.015	6.08
RM279	Protein	2	17.3	0.041	4.34
RM154	Protein	2	4.8	0.017	5.83
Crm33-1	Protein	3	60.2	0.0035	8.66
RM225	Iron	6	26.2	0.032	4.75
RM234	Iron	7	88.2	0.024	5.27
RM19	Zinc	12	20.9	0.004	8.02

Grain protein content (GPC): We detected 3 loci with a significant association, RM279 on chromosome 2 had the less effect explaining 4.34 % of the phenotypic variation and Crm33-1 on chromosome 3 had the greatest effect explaining 8.66% of the total phenotypic variation. Crm33-1 is a microsatellite marker designed previously (data not published) from reported QTL region named ATQ033 found to be associated with the brown rice grain protein content Kaiyang et al.,(2008) and Yoshida et al.,(2002). However, in our study we have found association of this marker with protein content in polished rice grain. Similarly Patil *et al*., (2014) identified 5 significant SSR markers associated with grain protein content of the rice; RM11on chromosome 7 had the greatest effect, explaining 23.88% of the phenotypic variation. Knowledge of these loci should make a valuable contribution to rice breeding programs.

Grain iron content: We detected 4 loci with the significant association, RM5 on chromosome 1 had the less effect explaining phenotypic variation of 4.04% and RM490 on chromosome 1 had the greatest effect explaining phenotypic variation of 9.1% followed by RM234 and RM225 having phenotypic variance of 5.27% and 4.75%. Similarly, Anuradha *et al*., (2012) reported 4 markers RM535, RM137, RM152, RM260 located on chromosomes 2, 8 and 12 were linked only to Fe concentration. Out of 4, only one maker RM535 on chromosome 2 explained high (30.7) phenotypic variance (p=0.007**).

Grain zinc content: We detected 2 loci with the significant association, RM 5 and RM 19 located on chromosome 1 and 12 having phenotypic variance of 6.08% and 8.02% respectively, explaining the greatest effect. Anuradha et al., (2012) reported 14 markers located on chromosomes 1, 2, 3, 4, 5, 6, 7 and 10 were linked with Zn concentration. The range of phenotypic variance explained by these 14 loci ranged from 12 to 46%. Out of 14, 4 markers on chromosomes 3 (RM231, RM514), 6 (RM541) and 10 (RM484) explained high phenotypic variance significant at 1% (p=0.006** to 0.009**). Similarly, Atul *et al*., (2016) reported three markers RM12796 on chromosome 2, RM2489 on chromosome 3, RM287 on chromosome 11 significantly showed association with grain zinc content with a phenotypic variation of 15, 4, and 11%, respectively among the RIL population (swarna/morobarakan). Grain zinc content associated SSR markers (RM152, RM263 and RM21) with 6.1 to 11.7% phenotypic variability were reported by Berhanu *et al***.** (2013).

Further confirmation of these loci is necessary for their presence and role in grain iron, zinc and protein content in different genotypes.

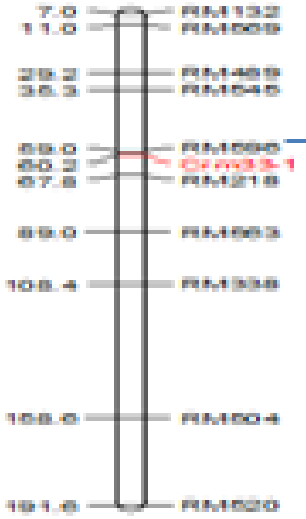
marker	cM	trait
RM490	51.0	Iron content

marker	cM	trait
RM5	94.9	Zinc content

2

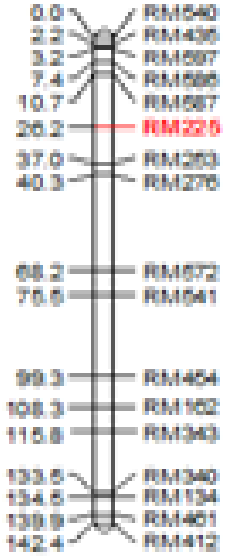
marker	cM	trait
RM154 RM279	4.8 17.3	proteinco ntent

3



marker	cM	trait
Crm33-1	60.2	protein

6



marker	cM	trait
RM225	26.2	Iron content

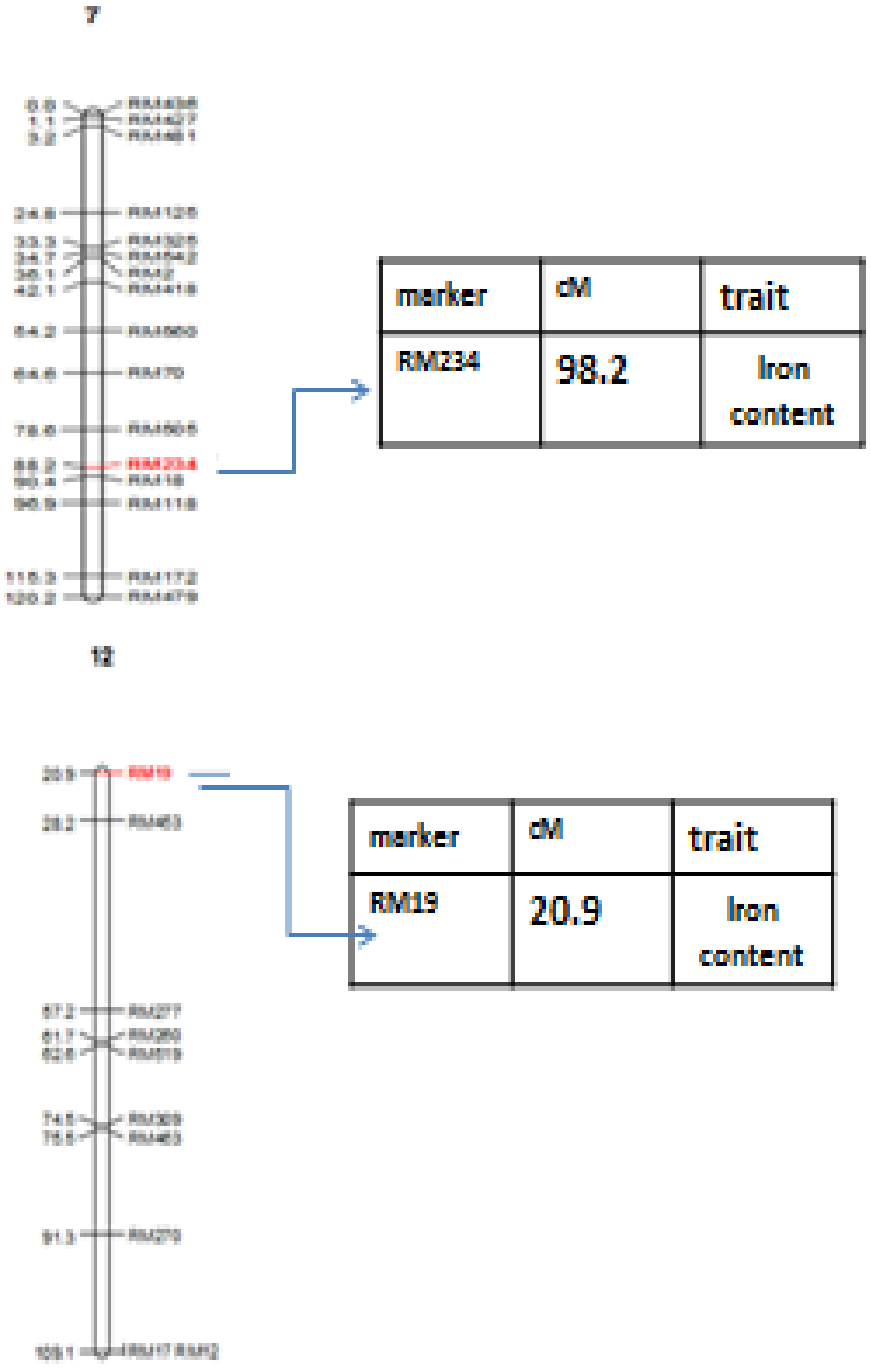


Fig4.9 The marker loci significantly associated with the traits in the study (P<0.05)

Table 4.10 List of markers used in the study

S.NO	Marker	Chrom. No	Forward primer sequence Reverse primer sequence	AT	Bp	Map Position (cM)
1	RM428	1	AACAGATGGCATCGTCTTCC CGCTGCATCCACTACTGTTG	55	266	19.3
2	RM490	1	ATCTGCACACTGCAAACACC AGCAAGCAGTGCTTTCAGAG	55	101	51
3	RM5	1	TGCAACTTCTAGCTGCTCGA GCATCCGATCTTGATGGG	55	113	94.9
4	RM283	1	GTCTACATGTACCCTTGTTGGG CGGCATGAGAGTCTGTGATG	55	151	31.4
5	RM259	1	TGGAGTTTGAGAGGAGGG CTTGTTGCATGGTGCCATGT	55	162	54.2
6	RM1	1	GCGAAAACACAATGCAAAAA GCGTTGGTTGGACCTGAC	55	113	29.7
7	RM226	1	GAAGCTAAGGTCTGGGAGAAACC AATGGCCTTAACCAAGTAGTAGGATGG	55	274	154.8
8	RM290	2	ACCCTTATTCCTGCTCTCCTC GTGCTGTAGATGGAAGGGAG	142	55	66
9	RM 279	2	GCGGGAGAGGGATCTCCT GGCTAGGAGTTAACCTCGCG	55	174	17.3
10	RM234	2	CTGATCGAGAGCGTTAAGGG GGGATCAAACCACGTTTCTG	55	209	58.4
11	RM154	2	GACGGTGACGCACTTTATGAACC CGATCTGCGAGAAACCCTCTCC	61	183	4.8
12	Grm33-1	2	TCGTTCTGACATGTTGAGG CCCGACAAAGTCAACGTC	56	250	68.2
13	Crm33-1	3	TCGTTCTGACATGTTGAGG CCCGACAAAGTCAACGTC	58	60.2	68.2
14	RM 231	3	CCAGATTATTTCCTGAGGTC CACTTGCATAGTTCTGCATTG	55	182	15.7
15	RM7	3	TTCGCCATGAAGTCTCTCG CCTCCCATCATTTCGTTGTT	55	180	64
16	RM517	3	GGCTTACTGGCTTCGATTTG CGTCTCCTTTGGTTAGTGCC	55	266	42.9
17	RM514	3	AGATTGATCTCCCATTCCCC CACGAGCATATTACTAGTGG	55	259	216.4
18	OSZip3b	4	CCTGCTGAGGCTGAGTTGAA CGAGAACAAAGTAACAGGCTGC	61.5	370	124.3
19	RM252	4	TTCGCTGACGTGATAGGTTG ATGACTTGATCCCGAGAACG	55	216	99
20	RM 470	4	TCCTCATCGGCTTCTTCTTC AGAACCCGTTCTACGTCACG	55	83	115.5
21	RM592	5	TCTTTGGTATGAGGAACACC AGAGATCCGGTTTGTTGTAA	55	270	31.4
22	RM122	5	GAGTCGATGTAATGTCATCAGTGC GAAGGAGGTATCGCTTTGTTGGAC	55	227	10.1

S.NO	Marker	Chrom. No	Forward primer sequence Reverse primer sequence	AT	Bp	Map Position (cM)
23	RM574	5	GGCGAATTCTTTGCACTTGG ACGGTTTGGTAGGGTGTCAC	55	155	41
24	RM541	6	TATAACCGACCTCAGTGCCC CCTTACTCCCATGCCATGAG	55	158	75.5
25	RM475	6	GCATTGATGTGCCAATCG CATTGCAACATCTTCAACATCC	55	114	108.3
26	RM225	6	TGCCCATATGGTCTGGATG GAAAGTGGATCAGGAAGGC	55	140	26.2
27	RM501	7	GCCCAATTAATGTACAGGCG ATATCGTTTAGCCGTGCTGC	55	179	30.1
28	RM248	7	TCCTTGTGAAATCTGGTCCC GTAGCCTAGCATGGTGCATG	55	102	99.7
29	RM 8007	7	AATAGGATGGATCATGGATA CATCTCATCAGGAACCTAAC	55	178	31
30	RM160	7	ACAGTATCCAAGGCCCTGG CACGTGAGACAAAGACGGAG	55	156	88.2
31	RM44	8	ACGGGCAATCCGAACAACC TCGGGAAAACCTACCCTACC	55	99	60.9
32	RM137	8	GACATCGCCACCAGCCCACCAC CGGGTGGTCCCCGAGGATCTTG	55	218	60.9
33	RM271	10	TCAGATCTACAATTCCATCC TCGGTGAGACCTAGAGAGCC	55	101	59.4
34	RM484	10	TCTCCCTCCTCACCATTGTC TGCTGCCCTCTCTCTCTCTC	55	299	97.3
35	RM552	11	CGCAGTTGTGGATTTCAGTG TGCTCAACGTTTGACTGTCC	55	195	40.6
36	RM21	11	ACAGTATTCCGTAGGCACGG GCTCCATGAGGGTGGTAGAG	55	157	85.7
37	RM7102	12	TTGAGAGCGTTTTTAGGATG TCGGTTTACTTGGTTACTCG	55	169	52.8
38	RM17	12	TGCCCTGTTATTTTCTTCTCTC TGCCCTGTTATTTTCTTCTCTC	55	184	109.1
39	RM260	12	ACTCCACTATGACCCAGAG GAACAATCCCTTCTACGATCG	55	111	61.7
40	RM452	12	TGCCCTGTTATTTTCTTCTCTC GGTGATCCTTTCCCATTTCA	55	184	109.1
41	RM277	12	CGGTCAAATCATCACCTGA CAAGGCTTGCAAGGGAAG	55	124	57.2
42	RM19	12	CAAAAACAGAGCAGATGAC CTCAAGATGGACGCCAAGA	55	226	20.9

CHAPTER-V
SUMMARY AND CONCLUSION

Protein, Fe and Zn content in rice grain (*Oryza sativa L.*) is an important trait for health of people whose main food in daily life is rice. The improvement in Fe, Zn and Protein content or its composition 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. Hence the present study entitled **"Identification of DNA Markers Associated with Grain Fe, Zn and Protein Contents in Rice (*Oryza sativa* L.)"** was carried out at Nutritional Genomics Laboratory and Genomics and proteomics Laboratory of the Department of Plant Molecular Biology and Biotechnology, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur (CG), with an objective of the study include morphological characterization of selected rice genotypes, Phenotyping of selected rice genotype for grain Protein, Fe and Zn content in rice, Genotyping of selected rice genotype with SSR markers and Identification of DNA Markers Associated with Grain Fe, Zn and grain Protein Contents in Rice**.**Results obtained on all these aspects in the present investigation are summarized here under:

1. A wide range of variation was observed for eight quantitative traits in 96 rice genotype studied. A significant difference was observed for the entire quantitative trait observed. On the basis of the mean performance of the quantitative trait the genotype Bisni (high Iron line) containing desirable plant type, medium plant height (162 cm), early 50 % flowering (79 days), more tiller number (8.6), long panicle length (31.6 cm), short grain length (7.1 mm) whereas Dullar(high zinc line) has been identified as a line of interest based on desirable plant type *viz*., medium plant height (142.8cm), early days to fifty percent flowering (72.5 days), more tiller number (9.2), long panicle length (26.6 cm), long grain length (8.9 mm) andKalam Gurmatia(High protein line), medium plant height(148.4cm), medium days to fifty percent flowering (89 days), more tiller number (9.2), long panicle length (28.4 cm), medium grain length (8.4 mm).

2. A wide range of morphological diversity was noticed for nine qualitative traits in 96 rice genotype studied. Out of nine qualitative characters observed, basal leaf sheath colour, leaf pubescence of blade surface, leaf anthocyanin colouration of auricle, flag leaf attitude of blade, panicle attitude of branches, panicle exsertion are recorded highest variation among accessions. After that, leaf distribution of anthocyanin colouration, Leaf intensity of green colour, colour of awn observed low variation among accessions.
3. Significant variation was found in Fe and Zn and protein content of all the 96 rice genotypeprotein varying from 5.78 % to 10.28 %, Fe varied from 4.8 to 14.3 Ppm and Zn is 13.6 to34.3 Ppm. Among these genotype the highest protein content (10.28%) was observed for Kalam Gurmatia (3053) and the lowest protein content (5.789 %) was observed for CGR-1539, highest Fe content (14.3Ppm) was observed for Bisni and the lowest Fe content for (4.7%) was observed for IR681444*Morobroken and highest Zn content (34.3 Ppm) was observed for Dullar and the lowest Zn content (13.6 Ppm) was observed for GP-145-138.Based on this analysis high Fe, Zn and protein content containing rice genotype namelyKalam Gurmatia, Bisni-1, Dullar,Jeeradhan were identified as donor lines for this trait.
4. The association between markers and Iron, Zinc and GPC were calculated using single marker analysis (SMA) in Microsoft Excel program. The results showed that, a total of 8 significant marker Iron, Zinc and GPC association ($P<0.05$) was found. All of the 8 significant SSR loci were indentified for the Iron, Zinc and GPC, with the R^2, percentage of the total variation explained ranging from 4.04 % to 9.1 %. Iron, Zinc and Grain protein content we detected eight loci with a significant association, RM5 on chromosome 1 had the less effect explaining 4.04 % of the phenotypic variation and RM490 on chromosome 1 had the greatest effect explaining 9.1% of the total phenotypic variation.

Conclusion

All the quantitative, morphological traits showed significant variability along with the Iron, Zinc and grain protein content in 96 rice genotypes. Normal distribution of quantitative traits showed that there was a sufficient amount of variation was present. Significant amount of variation also found in the grain protein content which proven by ANOVA and frequency distribution graph. These traits should be used for selection of high yielding line with high Iron, Zinc and GPC value. A total of 36 alleles generated by 18 SSR markers (out of 42 markers) were used for the genetic analysis of genotypes. Out of the 18 polymorphic SSR markers used,R^2 percentage of the total variation explained ranging from 4.04 % to 9.1 % these marker set will better to use for the diversity analysis. Association analysis by single marker analysis showed that there was 8 significant markers associated with Iron, Zinc and GPC.

Suggestion for future work

1. The genotypes used in this study should also be characterized for other remaining nutrional quality traits like micronutrients, vitamin, and amino acid contents etc.
2. Germplasm collections are sources of genes for resistance to various biotic and abiotic stresses agronomic and quality characters. Further analysis of genotypes used in this on these aspects will help in the identification of elite rice genotypes.
3. More SSR markers (including trait associated or gene specific markers) can be used for further characterization of rice genotypes. More markers should be investigated to work out discriminating markers among high and low Iron, Zinc and GPC containing genotypes.
4. Identified rice genotype with high Iron, Zinc and grain protein content can be used as donor line for further breeding program.
5. The identified genomic region contributed for the locus of micronutrinents and GPC will be further validated in high and low Iron, Zinc and grain protein content variety for study of their expression pattern.

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AppendixA:- Mean performance of 96 landraces for different quantitative trait characters

Landraces	PH (cm)	PL (cm)	NTT	NPP	DTF (days)	LLB (cm)	LWB (cm)	GL (mm)	GW (mm)
Bhathaili Gurmatia	157.20	27.60	12.00	9.60	98.00	41.20	1.78	8.50	2.20
Botki Gurmatia	134.40	27.00	11.60	10.00	90.00	34.40	1.32	7.80	2.90
CHAPTI GURMATIA	141.20	26.80	10.20	8.40	81.50	35.20	1.44	8.10	3.30
chapti Gurmatia	152.60	27.00	7.80	7.40	79.00	35.60	1.66	8.30	3.70
Gurmatia	137.40	27.00	8.00	7.00	73.00	33.60	1.34	8.40	2.80
Jhunki Gurmatia	134.40	27.00	10.00	8.20	107.0	31.00	1.46	8.80	3.00
Kamal Gurmatia	148.40	28.40	9.20	7.60	89.00	35.00	1.48	8.40	2.80
Nunki Gurmatia	107.20	24.40	11.20	9.60	96.00	32.00	1.60	8.60	2.90
Sultu Gurmatia	120.20	26.00	9.60	8.00	94.00	33.60	1.48	8.80	3.10
Tulsi Gurmatia	134.80	33.20	9.00	7.40	91.50	36.80	1.52	8.90	3.20
Bangla Gurmatia	169.60	27.60	9.00	7.00	89.50	45.40	1.70	8.90	2.90
ShriKamal	163.00	32.20	10.40	8.60	88.50	44.00	1.62	5.90	2.90
Elayachi	165.60	32.40	8.80	8.40	90.00	38.60	1.58	6.70	3.20
Jeeradhan	162.00	31.60	8.60	7.20	86.00	35.20	1.46	6.30	2.60
Bisni-1	162.00	31.60	8.60	8.00	79.00	31.60	1.48	7.10	2.50
RR-152	103.60	30.40	9.40	8.60	73.00	29.60	1.48	6.80	2.30
RR-137	133.80	30.00	8.20	7.20	107.0	36.20	1.62	8.20	2.30
RR-149	162.00	25.80	7.60	6.40	89.00	35.20	1.50	8.10	2.40
RR-8 M011	105.80	29.00	8.40	7.40	96.00	30.00	1.56	8.10	3.30
Reg-1035	105.00	30.00	10.00	8.00	94.00	29.20	1.54	8.40	2.90
Reg-1038	128.20	27.80	11.20	9.20	94.00	30.80	1.46	9.40	3.50
CHIR-8	109.60	27.60	9.60	8.00	72.50	31.80	1.58	8.90	2.70
CGZR-1	106.00	26.60	8.80	8.00	77.00	32.00	1.60	9.50	3.20
IET 23829	95.60	25.40	9.00	7.60	94.50	31.00	1.62	8.10	2.20
Basmati 370	153.80	30.80	9.40	7.60	87.00	34.40	1.66	9.70	2.40
R-RHZ-LI-23	96.40	24.80	11.60	8.60	88.00	29.60	1.36	8.90	2.40
R-RHZ-IB-13	86.80	28.40	12.80	9.40	85.50	29.60	1.34	9.80	2.20
R-RHZ-SM-14	108.40	27.00	8.40	7.00	84.00	31.20	1.68	8.40	3.10
Basmati 1	94.60	25.40	9.00	7.80	86.00	30.20	1.64	9.70	2.30
R-RHZ-MI-30	85.00	21.60	8.20	7.60	85.00	32.00	1.50	8.60	2.90
Kalanamak	174.20	32.20	10.40	8.80	102.0	43.20	1.76	9.10	2.30
R-56	107.80	26.20	9.60	8.00	84.00	30.20	1.58	9.60	2.20
Kadamphool	105.80	25.80	7.60	7.20	74.50	34.00	1.58	7.20	2.50
Karigilas	181.20	29.00	8.60	6.60	100.5	45.40	1.56	10.00	2.60
Jhilli	176.60	28.60	10.20	8.40	101.5	40.60	1.64	7.50	2.00
Azucena	134.60	26.40	7.40	6.60	85.00	31.00	1.54	10.00	2.80
IR-64	106.00	26.20	10.20	7.20	83.00	32.40	1.36	9.20	2.70
Swarna	84.40	26.40	7.80	7.20	104.0	32.40	1.68	8.00	2.50
RR-100	130.40	28.40	7.80	6.80	101.0	33.60	1.62	8.80	2.40
Bhansapanchi	161.20	32.40	9.80	8.60	91.00	39.40	1.72	6.80	3.00
Banda	162.40	28.00	10.80	8.80	101.0	36.60	1.90	8.50	2.20
Bada gada khuta	125.20	27.80	11.80	9.40	108.5	29.20	1.56	8.00	2.70
Reg-695	93.80	16.40	8.40	7.60	83.00	36.00	1.50	8.30	2.90
Dagad-Desi	161.00	26.80	9.20	8.00	109.0	42.60	1.44	9.80	3.30
GP-145-40	179.80	35.40	8.00	7.00	85.50	48.20	1.66	9.10	2.90
RKVY-104	160.20	27.00	8.00	7.20	91.50	38.60	1.42	8.60	3.10
RKVY-211	168.60	28.60	10.20	7.20	72.50	38.40	1.60	8.40	2.80
Dullar	142.80	26.60	9.80	8.40	72.50	34.60	1.44	8.90	3.20
BAM-1292	125.60	26.80	9.20	7.80	98.00	33.20	1.58	9.00	2.90
BAM-5446	153.40	22.60	9.60	7.60	100.0	38.60	1.46	9.10	3.20
BAM-5926	184.00	25.00	11.60	8.60	93.00	44.40	1.50	8.50	3.30
Moroberekan	145.00	30.00	9.40	7.60	70.00	33.60	1.68	8.30	2.80

Landraces	PH (cm)	PL (cm)	NTT	NPP	DTF (days)	LLB (cm)	LWB (cm)	GL (mm)	GW (mm)
Nagina-22	108.20	21.40	8.20	7.40	72.00	30.60	1.62	8.70	3.30
BAM-5997	106.00	18.40	6.20	5.20	96.00	30.40	1.74	9.20	2.90
Bakal	129.00	25.60	6.40	5.40	81.50	35.80	1.40	9.60	2.70
GP-145-37	171.00	24.20	7.40	6.80	100.5	38.00	1.64	9.40	3.20
MTU-1010	105.20	25.20	6.60	5.60	99.00	30.20	1.44	9.60	2.70
SL-62	100.80	24.20	8.000	7.20	82.00	32.00	1.40	9.20	2.50
GP-145-41	164.80	22.20	9.60	9.00	84.50	36.60	1.68	9.10	2.50
GP-145-66	169.60	25.40	9.20	8.20	85.00	37.40	1.72	10.00	2.80
CGR-1539	148.8	20.00	8.80	8.00	101.0	33.20	1.64	10.00	3.40
RRGM-ATN-47	103.20	22.40	9.00	8.20	101.0	29.80	1.60	9.30	3.40
RRGM-AS-45	102.00	30.20	9.80	8.00	106.5	33.60	1.64	8.10	2.90
GP-145-42	163.60	26.60	10.00	8.60	73.50	37.80	1.50	7.70	2.90
GP-145-44	166.40	27.60	8.80	7.80	103.0	38.40	1.42	8.20	3.30
GP-145-70	143.60	26.00	8.40	7.60	109.5	39.40	1.56	8.60	2.20
RKVY-52	97.20	27.00	8.80	7.60	99.00	36.60	1.56	8.30	3.30
GP-145-103	163.20	26.00	7.00	5.40	108.5	36.20	1.58	8.90	3.10
RKVY-15	131.60	26.80	8.20	7.20	105.0	33.00	1.56	7.90	2.80
GP-145-78	134.60	27.20	10.20	8.60	108.5	34.20	1.58	8.60	2.70
GP-145-43	173.20	26.60	9.60	8.20	103.5	43.40	1.60	8.10	3.40
GP-145-49	171.60	25.20	10.20	8.60	109.0	44.60	1.62	8.70	2.20
GP-145-59	172.80	27.40	8.80	8.00	109.5	45.80	1.44	8.10	2.40
GP-145-119	180.60	29.60	11.40	9.40	101.5	48.40	1.58	11.00	3.90
GP-145-136	175.20	25.20	7.40	6.80	103.5	44.00	1.60	9.20	3.10
GP-145-50	142.40	27.20	9.40	7.80	98.00	38.60	1.48	8.50	2.50
GP-145-65	173.20	28.40	7.80	7.20	94.50	46.20	1.48	8.60	3.50
GP-145-38	163.20	31.80	7.20	6.60	87.00	38.00	1.56	11.00	2.70
GP-145-130	135.60	26.40	9.00	8.20	88.00	32.80	1.54	8.10	3.20
GP-145-138	134.60	28.60	7.40	6.60	85.50	31.60	1.48	7.60	2.20
GP-145-5	182.40	26.40	9.00	7.80	84.00	50.40	1.46	9.20	2.40
GP-145-11	180.60	27.40	9.20	7.80	86.00	47.80	1.50	9.20	2.40
GP-145-20	203.20	26.60	9.60	8.20	85.00	57.40	1.54	8.20	2.20
GP-145-34	202.80	26.80	9.40	7.80	102.0	52.20	1.56	9.70	3.10
F8 Safri-17*IR681444-41	125.80	25.80	13.80	7.00	84.00	35.40	1.94	8.10	2.30
F7 Bas-1*IR681444-4	94.60	28.40	11.60	10.00	74.50	28.60	1.84	7.70	2.90
F6 Kranti* Swarna	98.20	24.60	9.40	7.20	73.50	33.00	1.68	8.30	2.30
F10 IR681444*Abhaya-18	90.00	24.60	12.00	10.40	103.0	35.20	1.80	8.60	2.20
F4 Moro*IR94046-31	102.20	24.20	11.20	10.00	109.5	29.80	1.50	7.90	2.50
F9 IR681444*HMT-24	118.00	26.80	11.00	8.60	99.00	29.40	1.52	7.90	2.60
F9 IR681444*IR64	111.00	26.60	12.40	9.60	108.5	36.20	1.64	8.30	2.00
F10 Safri-17*IR681444-5	69.40	17.80	12.20	11.40	105.0	31.40	1.38	9.00	2.80
F5 MTU1010*IR94032-5	109.00	26.40	10.40	8.80	108.5	34.40	1.58	8.60	2.90
F8 IR681444*Bas-8-3	82.40	26.00	9.60	8.20	103.5	34.80	1.44	8.80	3.10
F8 IR681444*Bas-1-27	79.80	21.60	8.20	7.40	109.0	32.80	1.50	9.00	3.20
F5 Swarna*MTU1010-2	104.80	26.80	9.20	8.00	109.5	31.60	1.58	9.20	2.90
MEAN	135.56	26.80	9.35	7.90	92.66	36.10	1.56	8.60	2.79
MINIMUM	69.40	16.40	6.20	5.20	70.00	28.60	1.32	5.90	2.00
MAXIMUM	203.20	35.40	13.80	11.40	109.5	57.40	1.94	11.0	3.70
C.D.	5.104	4.33	1.75	1.39	15.65	0.20	4.13	1.30	2.10
SE(m)	1.835	1.56	0.62	0.50	5.56	0.07	1.48	0.50	0.70
SE(d)	2.595	2.20	0.89	0.71	7.87	0.10	2.10	0.70	0.03
C.V.	3.026	13.0	15.04	14.1	8.31	10.40	9.19	7.70	6.30

Appendix B: Banding pattern of rice genotypes based on different SSR , QTL, gene specific markers

Genotype	RM231	RM225	RM252	RM226	RM501	RM490	RM279	RM5	RM277	RM19	RM44	Grm33-1	Crm34-1	Oszip-3b	RM234	RM154	RM259	RM517
Bhathaili Gurmatia	B	B	B	B	B	A	B	B	A	A	A	B	B	A	A	B	A	A
Botki Gurmatia	A	B	A	A	A	B	A	A	B	B	B	B	B	A	B	B	A	A
CHAPTI GURMATIA	A	B	A	A	A	B	A	A	B	A	B	B	B	B	B	A	B	A
chapti Gurmatia	A	B	A	A	B	B	A	A	B	B	A	A	B	A	A	A	B	B
Gurmatia	B	B	A	B	A	A	A	B	A	B	A	A	A	A	A	A	A	A
Jhunki Gurmatia	B	B	A	A	B	A	A	B	A	B	A	A	A	A	B	A	A	A
Kamal Gurmatia	B	B	A	A	A	B	B	A	B	B	A	B	A	A	B	B	A	A
Nunki Gurmatia	A	B	A	A	A	B	A	A	B	A	A	B	A	B	B	B	B	A
Sultu Gurmatia	A	B	A	B	A	A	A	A	B	B	B	A	B	B	A	A	B	A
Tulsi Gurmatia	B	B	B	A	B	B	B	B	B	B	B	A	A	B	B	B	A	A
Bangla Gurmatia	A	B	A	A	A	A	A	A	B	A	B	A	A	A	B	A	B	A
ShriKamal	A	A	A	A	B	B	A	B	A	B	B	B	A	B	A	B	A	B
Elayachi	A	A	A	A	B	B	A	A	A	A	A	B	A	B	A	B	A	B
Jeeradhan	A	A	A	B	A	B	A	A	B	A	B	A	A	B	A	B	B	A
Bisni-1	A	A	A	B	A	B	A	B	B	A	B	B	B	B	A	A	A	A
RR-152	A	A	A	A	A	B	B	A	A	B	B	A	B	A	A	A	B	A
RR-137	A	A	A	A	B	A	A	B	A	B	A	B	B	A	B	B	A	B
RR-149	B	A	A	B	A	A	B	B	A	A	A	B	B	B	B	B	B	A
RR-8 M011	A	B	A	B	B	A	A	B	A	B	A	A	B	B	B	A	A	A
Reg-1035	A	A	B	A	A	B	A	A	A	A	A	A	A	B	B	A	B	B
Reg-1038	A	B	B	B	A	B	B	B	A	B	B	A	B	A	B	B	A	B
CHIR-8	A	A	A	A	B	A	A	A	A	A	B	A	A	A	A	A	B	B
CGZR-1	B	B	A	A	A	B	B	B	B	A	A	A	A	A	A	B	A	A
IET 23829	A	A	B	B	B	A	A	A	B	B	A	A	A	B	A	A	A	A
Basmati 370	A	A	A	A	A	A	A	A	A	B	A	B	A	A	B	A	A	A
R-RHZ-LI-23	B	A	A	A	B	A	B	A	B	A	B	A	B	A	A	A	B	A
R-RHZ-IB-13	A	B	A	A	B	A	B	A	B	B	B	A	B	A	A	A	B	A
R-RHZ-SM-14	B	A	A	A	B	A	A	A	A	B	B	B	A	B	B	B	A	B
Basmati 1	A	A	B	B	B	A	B	B	A	B	B	A	B	B	B	B	A	B
R-RHZ-MI-30	A	A	A	A	B	A	B	B	B	A	A	A	A	A	B	B	B	A
Kalanamak	B	A	A	B	B	B	A	B	A	A	B	B	A	A	A	B	B	A
R-56	B	B	B	A	B	A	B	A	B	B	A	A	A	B	B	A	A	A
Kadamphool	B	A	A	B	A	B	B	A	A	B	A	B	A	A	A	A	B	B
Karigilas	B	B	B	B	B	A	A	B	A	B	B	A	A	A	B	B	B	A
Jhilli	A	B	B	B	B	A	B	A	A	B	A	A	A	B	B	A	A	A
Azucena	A	B	A	B	A	B	A	B	A	A	B	B	B	A	A	B	B	A
IR-64	B	B	A	B	A	A	A	A	A	A	A	B	A	B	B	B	A	B
Swarna	A	A	B	A	A	B	A	A	B	B	B	A	B	B	B	A	A	A
RR-100	A	B	B	A	A	A	B	B	B	B	B	A	A	B	B	A	A	A
Bhansapanchi	B	B	B	A	B	B	A	B	A	A	B	B	B	A	A	B	B	B
Banda	B	B	A	A	A	A	A	A	B	B	A	A	B	B	B	A	A	A
Bada gada khuta	A	A	A	A	A	A	A	A	B	B	B	A	A	B	B	A	B	A
Reg-695	A	A	B	A	A	A	A	B	B	B	A	A	A	B	B	A	A	A
Dagad-Desi	A	B	B	A	A	B	B	A	A	A	B	A	A	A	A	A	B	A
GP-145-40	A	B	A	A	A	A	A	B	B	B	B	A	B	A	A	B	A	A

RKVY-104	A	B	B	B	B	A	B	A	B	A	B	A	A	A	A	A	A	A
RKVY-211	A	B	B	A	B	A	A	A	B	B	A	B	A	B	A	B	B	A
Dullar	A	A	A	A	A	B	A	A	A	A	A	A	A	A	A	A	A	B
BAM-1292	B	A	B	A	B	B	B	A	B	A	B	A	A	A	A	A	B	A
BAM-5446	A	B	B	A	A	A	A	A	B	B	A	B	A	A	B	B	A	A
BAM-5926	A	B	A	B	A	A	B	B	B	A	A	B	B	B	B	B	A	A
Moroberekan	B	B	A	B	B	A	B	B	A	B	A	B	B	A	B	B	B	B
Nagina-22	A	A	B	A	B	B	B	B	A	A	A	A	A	A	A	A	B	A
BAM-5997	A	A	A	B	B	B	B	A	B	A	B	A	B	A	B	A	A	B
Bakal	A	A	A	A	B	B	B	A	B	B	B	A	B	A	B	A	A	B
GP-145-37	A	B	B	A	B	A	A	B	B	B	A	A	A	B	B	B	B	A
MTU-1010	B	B	A	B	B	B	B	A	B	B	B	A	A	B	B	A	B	A
SL-62	A	B	A	B	A	B	B	A	B	A	A	A	B	A	A	A	A	A
GP-145-41	A	B	B	A	A	A	A	B	A	B	B	A	A	B	A	B	B	B
GP-145-66	A	A	B	A	A	A	A	B	B	A	A	A	B	B	A	A	B	A
CGR-1539	B	A	A	B	B	A	A	A	B	B	A	B	A	B	A	B	A	A
RRGM-ATN-47	A	B	A	B	A	B	B	A	A	A	B	A	A	B	A	A	A	A
RRGM-AS-45	A	A	B	A	A	B	B	B	A	B	A	A	A	B	B	B	B	A
GP-145-42	A	B	A	A	B	B	A	A	B	B	A	A	B	A	A	A	B	A
GP-145-44	B	A	B	A	B	B	A	A	A	A	A	B	A	A	B	A	B	A
GP-145-70	B	B	A	B	B	A	A	B	A	B	A	A	A	B	A	A	B	A
RKVY-52	A	B	A	B	A	A	B	A	B	B	B	B	B	B	B	A	A	B
GP-145-103	B	A	A	A	A	A	A	B	B	A	A	B	A	B	B	A	B	B
RKVY-15	B	A	B	B	B	B	B	A	B	B	A	A	A	A	B	A	A	A
GP-145-78	A	A	B	A	A	A	A	B	A	B	A	A	A	A	B	A	A	A
GP-145-43	A	B	A	A	A	A	A	A	B	A	A	B	B	A	A	B	B	A
GP-145-49	A	B	A	A	B	A	A	A	A	A	B	B	B	B	A	A	B	A
GP-145-59	A	B	B	B	B	A	A	B	A	B	A	A	A	A	A	A	B	A
GP-145-119	A	B	A	A	B	A	A	A	A	B	A	A	A	A	A	B	A	A
GP-145-136	A	B	A	B	B	A	A	A	B	A	A	A	A	B	B	A	A	A
GP-145-50	A	A	B	A	B	A	B	B	B	B	B	A	B	B	B	A	B	A
GP-145-65	A	A	B	A	A	B	A	B	A	B	A	A	B	B	A	A	A	A
GP-145-38	A	B	A	B	B	B	A	B	B	B	B	B	B	A	A	B	A	B
GP-145-130	A	A	B	B	B	B	B	B	B	A	A	B	B	B	A	B	B	B
GP-145-138	A	B	B	A	A	A	A	A	A	A	B	B	A	B	B	A	B	A
GP-145-5	B	B	A	A	A	A	A	A	B	B	A	B	B	A	A	B	A	A
GP-145-11	A	B	A	A	B	A	A	B	B	B	B	A	B	A	A	A	A	A
GP-145-20	B	B	B	A	A	A	B	B	B	B	A	B	B	B	A	B	B	A
GP-145-34	A	B	A	B	B	B	A	A	A	B	A	B	B	A	A	A	B	A
F8 Safri-17*IR681444-41	B	A	B	A	A	A	B	A	B	B	B	B	B	A	B	B	A	A
F7 Bas-1*IR681444-4	A	A	A	B	A	B	B	A	A	A	A	A	B	B	B	A	A	A
F6 Kranti* Swarna	A	B	A	B	A	A	A	B	A	B	A	A	B	B	A	A	B	B
F10 IR681444*Abhaya-18	A	A	A	A	A	A	B	B	B	B	A	A	A	B	B	A	B	A
F4 Moro*IR94046-31	B	B	A	A	A	A	B	B	B	B	A	A	A	B	A	A	B	A
F9 IR681444*HMT-24	A	B	A	B	A	B	A	A	A	B	A	A	A	B	A	A	B	A
F9 IR681444*IR64	B	B	A	B	A	B	B	A	A	B	B	B	B	A	B	A	A	A
F10 Safri-17*IR681444-5	B	B	A	B	B	B	B	B	B	A	B	A	B	B	B	B	B	A
F5 MTU1010*IR94032-5	A	A	B	A	B	A	B	B	A	A	B	A	A	A	A	A	B	A
F8 IR681444*Bas-8-3	A	B	B	B	B	B	B	B	A	A	A	A	A	B	B	B	A	B
F8 IR681444*Bas-1-27	B	B	A	B	A	A	A	A	B	A	B	B	A	A	B	B	B	A
F5 Swarna*MTU1010-2	**A**	**B**	**A**	**A**	**A**	**B**	**A**	**A**	**B**	**A**	**A**	**A**	**B**	**A**	**A**	**B**	**B**	**A**

Appendix C: Description of morphological and quality charactersas per DUS test

S.No.	Characteristics	States	No. of Genotypes
1	Basal leaf sheath colour	Light	10
		Medium	82
		Dark	4
2	Leaf intensity ofgreen colour	Lightgreen colour	15
		Medium green colour	73
		Dark green leaf blade	8
		purple colour	0
3	Leaf distribution of Anthocyanincolouration	On tips only	3
		On margins only	25
		In blotches only	8
		Uniform	60
4	Leaf pubescence of blade Surface	Absent	0
		Weak	0
		Medium	6
		Strong	32
		Very strong	58
5	Leaf anthocyanin colourization of auricles	Colourless	68
		Light purple	22
		Purple	6
6	Leaf length of blade	Short(<30cm)	19
		Medium(30-40cm)	73
		Long(>45cm)	4
7	Leaf width of blade	Narrow(<1cm)	70
		Medium(1-2cm)	17
		Broad(>2cm)	9
8	Time of heading (50% plants with panicles)	Very early	1
		Early	43
		Medium	52
		Late	NIL
		Very late	NIL
9	Flag leaf attitude of blade (early observation)	Erect	17
		Semi-erect	63
		Horizontal	7
		Drooping	9
10	Panicle: length of main axis	Very short	20
		Short	66
		Medium	10
		Long	0
		Very long	NIL
11	Panicle number per plant	Few	95
		Medium	1

S.No.	Characteristics	States	No. of Genotypes
		Many	NIL
12	Panicle colour of awns (late observation)	Yellowish White	0
		Yellowish brown	NIL
		Brown	0
		Reddish brown	NIL
		Light red	NIL
		Red	NIL
		Light purple	NIL
		Purple	0
		Black	NIL
13	Panicle attitude of branches	Erect	25
		Erect to semi erect	61
		Semi-erect	0
		Semi erect to spreading	NIL
		Spreading	10
14	Panicle exertion	Partly exerted	12
		Mostly exerted	22
		Well exerted	62
15	Grain length	Very short	1
		Short	43
		Medium	50
		Long	1
		Very long	1
16	Grain width	Very narrow	0
		Narrow	35
		Medium	27
		Broad	22
		Very broad	2

RESUME

Name : Ajay Kumar

Date of birth : 21/03/1989

Present Address : Room No.105, Sundram PG, Hostel, Jora, Raipur, CG

Phones 9174845139

Fax

E. mail : binnykumari100@gmail.com

Permanent address : At- Sargati, PO- Garkha, Dist- Chapra,
Pin-841311, Bihar

Academic Qualification:

Degree	Year	University/Institute
B.Sc. (Agriculture)	*2011-15*	*DR.PDKV, AKOLA, MH*
M.Sc. (PMBB)	2015-17	I.G.K.V. Raipur, Chhatisgarh

Professional Experience (If any) : No

Membership of Professional Societies (If any) : No

Awards / Recognitions (If any) : DBT / JRF

Publications (If any) : Yes

Ajay kumar
Signature

Gmail **ajay kumar <binnykumari100@gmail.com>**

submission of manuscript

2 messages

Girish Chandel <ghchandel@gmail.com> Wed, Sep 6, 2017 at 11:18 PM
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Dear Dr Ahmad,

Please find enclosed herewith a manuscript entitled "**Identification of DNA markers associated with grain iron, zinc and protein contents in rice (*Oryza sativa* L.)"** for publication in your journal. Kindly provide the acknowledgement of the same.
with best regards
girish

Dr Girish Chandel
Professor/ NE Bolaug Fellow 2006
Department of Plant Molecular Biology and Biotechnology
Indira Gandhi Krishi Vishwavidyalaya, Raipur
Tel: 91-9406382169 (m), 91-771-2442069 (o)

7 attachments

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Fig. 1.docx
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Fig. 2.docx
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Fig.3.docx
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Table .2.docx
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Table. 1.docx
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Dr Girish Chandel
Professor/ NE Bolaug Fellow 2006
Department of Plant Molecular Biology and Biotechnology
Indira Gandhi Krishi Vishwavidyalaya, Raipur
Tel: 91-9406382169 (m), 91-771-2442069 (o)

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