Association Genetics of Grain Mineral Elements of Wheat from Pakistan

BY

MUZZAFAR SHAUKAT

Reg. No. 03041711006

Department of Plant Sciences,
Faculty of Biological Sciences,
Quaid-i-Azam University,
Islamabad
Pakistan
2021

Association Genetics of Grain Mineral Elements of Wheat from Pakistan

A thesis submitted to the Quaid-i-Azam University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

Plant Sciences

By

MUZZAFAR SHAUKAT

Reg. No. 03041711006

Department of Plant Sciences,
Faculty of Biological Sciences,
Quaid-i-Azam University,
Islamabad,
Pakistan
2021

APPROVAL CERTIFICATE

This thesis submitted by **Mr. Muzzafar Shaukat** is accepted for evaluation from foreignexperts in its present form by the Department of Plant Sciences, Faculty of Biological Sciences,Quaid-i-Azam University, Islamabad.

Supervisor: Dr. Tariq Mahmood

Professor

Department of Plant Sciences

Quaid-i-azam University, Islamabad

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and the material contained in this thesis is original. I have not previously presented any part of this work elsewhere for any other degree.

Muzzafar Shaukat

PLAGIARISM UNDERTAKING

I solemnly declare that research work presented in the thesis titled **"Association Genetics of Grain Mineral Elements of Wheat from Pakistan"** is solely my research work with no significant contribution from any other person. Small contribution/help wherever taken has been duly acknowledge and I agreed that complete thesis has been written by me.

I understand the zero-tolerance policy of the HEC and Quaid-i-Azam University, Islamabad towards plagiarism. Therefore, I as an author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis evenafter award of PhD degree, Quaid-i-Azam University, Islamabad reserves the rights to withdraw/revoke my PhD degree and that HEC and Quaid-i-Azam University, Islamabad has the right to publish my name on the HEC/University website on which names of students are placed who submitted plagiarized thesis.

Student/Author Signature:

Name: Muzzafar Shaukat

SPECIAL THANKS

I pay my special thanks to my supportive Father ***Mr. Shaukat Ali (Late)****, Mother* ***Mrs. Shaukat Ali*** *and supportive fiancé* ***Dr. Fatima Khalid*** *for their countless love, care, encouragement, guidance, and support throughout my life.*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

$	United States Dollar
%	Percentage
°C	Degree Celsius
Ae.	*Aegilops.*
Zn	Zinc
Ca	Calcium
Fe	Iron
Kg	Kilogram
Gln	Glutenin
Gld	Glidin
UN	The United Nations
WHO	World Health Organization
GWAS	Genome-wide association study
QTL	Quantitative trait locus
SNP	Single nucleotide polymorphisms
MTAs	Marker-Trait Associations
RILs	Recombinant inbred lines
MARS	Mark-Assisted Multiplication Marks
DNA	Deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
DArT	Diversity arrays technology
CIMMYT	The International Maize and Wheat Improvement Center
RNA	Ribonucleic acid
FAO	Food and Agriculture Organization of the United Nations
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
GFe	Grain iron
GZn	Grain zinc
ICP-OES	Inductively coupled plasma - optical emission spectrometry
EDXRF	Energy Dispersive X-ray Fluorescence

Al	Aluminum
NARC	National Agricultural Research Centre
RIL	Recombinant inbred lines
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeats
K	Potassium
Mn	Manganese
Na	Sodium
Se	Selenium
P	Phosphorous
RCBD	Randomized complete block design
TPP	Tillers per plant
PH	Plant height
Cm	Centimeter
SL	Spike length
GPS	Grains per spike
TKW	Thousand kernel weight
GL	Grain length
GW	Grain width
GD	Grain diameter
EDXRF	Energy-dispersive X-Ray fluorescence analysis
GBTS	Genotyping-by-targeted-sequencing
KASP	Kompetitive allele specific PCR
PCR	Polymerase chain reaction
BLUE	Best linear unbiased estimator
ANOVA	Analysis of variance
Chr	Chromosome

AKNOWLEDGMENTS

Praise is to **Almighty Allah**, the cherisher & sustainer of the worlds, the most merciful, the most beneficent, who showed his countless blessing upon us. Almighty Allah enabled me to compile my humble endeavor in the shape of this dissertation. All respect goes to the **Holy prophet Hazrat Muhammad (S.A.W)** whose teaching strengthen our faith in Allah, who clarified that pursuit of knowledge is a divine commandment.

I would like to express my gratitude to all those who gave me the courage to complete this thesis & to do the necessary research work. I have a great exhilaration for my supervisor **Dr. Tariq Mahmood**, (Professor), for his valuable guidance, constructive criticism, and encouragement throughout the course of my research work. I'm indebted to **Dr. Mushtaq Ahmed** (chairman) for his encouraging behavior & obliged to him for extending the research facilities of the department to accomplish this work.

I am also thankful to **Dr Awais Rasheed** (Assistant Professor, QAU). Thanks to all my seniors **Dr. Ilyas, Dr Waseem, Dr Ijaz, Dr. Shazia, Dr. Riffat,** and all my lab fellows **Dr. Javaid Iqbal, Dr Bainzeer Ahsan Abbasi Wasba Sarfaraz**, **Aamir Shahbaz, Syeda Anber Zahra, & Zakirullah,** for their help. I am thankful to my dearest friends **Dr. Yesrab Aman**, **Saqlain Haidar, Zia, Rahat Siddique & Saad Ehsan** for their enjoyable company, care, concern, support and encouragement.

I would like to thank my family. Words fail to express the intensity of gratitude to my parents who have devoted their lives and sacrificed their comfort for my studies. I would specially like to thank my father **Mr. Shaukat Ali (Late),** my **mother Mrs. Shaukat Ali**, my brothers **Mr. Mubashir Shaukat** & **Mr. Mudassar Shaukat**, my sisters **Dr. Fozia Shafi** & **Rabia Khalid**. I also want to thank to my respected father & mother -in-law **Mr. Raja Khalid Nazir (Late)** & **Mrs. Nazima** Almas for keeping faith on me.

Last but not the least I would like to thank my fiancé **Dr. Fatima Khalid** for her encouragement, care, support, positive attitude in all hard circumstances and trusting and believing me. Her constant love, affection and prayers helped me to pursue my goal May ALLAH ALMIGHTY infuse me with the energy to fulfill the noble inspirations and expectations and further edify my competence (Ameen).

Muzzafar Shaukat

GENERAL ABSTRACT

Wheat (*T'iticum aestivum L.*) as a staple food crop is an essential source of protein and minerals. To address the difficulties associated with malnutrition, it is critical to fortify basic grains such as wheat with vital minerals. The first part of study aimed to assess the level of 11 micronutrients, including grain iron (GFe) and zinc (GZn) in 62 wheat cultivars released in Pakistan between 1911 and 2016. In the first part of study, GFe and GZn and other mineral concentration were measured over a two-year period in wheat cultivars using inductively coupled plasma optical emission spectroscopy (ICP-OES) and an energy-dispersive X-ray fluorescence spectrophotometer (EDXRF). The GZn concentration varied from 18.4 to 40.8 mg kg^{-1}according to ED-XRF and 23.7 to 38.8 mg kg^{-1} according to ICP-OES. Similarly, ICP-OES found GFe levels ranging from 24.8 to 44.1 mg kg^{-1}. while EDEXR found levels ranging from 26.8 to 36.6 mg kg^{-1}. GZn had a greater coefficient of correlation (r = 0.90) than GFe (r = 0.68). Modern cultivars like Zincol-16 and AAS-2011 demonstrated increased GFe and GZn levels as well as enhanced yield components. Old wheat cultivars WL-711, C-518, and Pothowar-70, which were released before to 1970, also had greater GFe and GZn values; nevertheless, their agronomic performance was poor. A multivariate study of eleven micronutrients (Fe, Zn, Al, Ca, Cu, K, Mg, Mn, Na, Se, and P) as well as agronomic characteristics and genome-wide SNP markers found a promising cultivar with increased yield, biofortification, and more genetic diversity. Over the course of 100 years, genetic gain analysis revealed a substantial rise in grain yield (0.4 % year 1), but there was a negative gain for GFe (0.111 % year 1) and GZn (0.15 % year 1). Plant height and grain yield (GY) were strongly associated with the Green Revolution Rht-B1 and Rht-D1 genes, whereas semi-dwarfing alleles had a negative influence on GFe and GZn levels. The second part of the study was aimed to assess the level of nine micronutrients, including grain iron (GFe) and zinc (GZn), as well as five biochemical characteristics across 188 wheat land races. Over the course of two years, field experiments were undertaken, and GFe, GZn, and other minerals were measured using flame photometer. The coefficient of variation was highest for P contents (108.8 %), and the lowest CV % was observed for oil (17.5 %). The GZn. 2019 concentration ranges from 13.5 mg $kg^{-1}$to 54 mg $kg^{-1}$and GZn.2020 concentration ranges from 11.8 mg kg^{-1} to 54.5 mg kg^{-1}. There was no

significant increase observed in GZn concentration in wheat landraces between years 2019 to 2020. The GFe.2019 concentration ranges from 8.20 mg kg^{-1} to 56 mg $kg^{-1}$and GFe.2020 concentration ranges from 15 mg kg^{-1} to 60 mg kg^{-1}. There is prominent increase observed in GZn concentration in wheat landraces between years 2019 to 2020. The highest correlation among the morphological and mineral traits was between P and N (r=0.99). The other trait has non-significant correlation. All biochemical and mineral traits of wheat land races show higher variation across different provinces of Pakistan. The mean of fiber was highest in AJK province (1.91 mg kg^{-1}) with a range of 1.87-1.99 mg kg^{-1} and lowest in Gilgit province (1.17 mg kg^{-1}) with a range of 0.93-1.29 mg kg^{-1}. Protein has seven MTAs on chromosome 1A, 3B (2), 4A, 5A, 6B, 7D with R^2 range of 0.28-0.44. Fiber has four MTAs on chromosome 6A (2), 7A, 2B with R^2 range of 0.04-0.05. A total eighty-four MTAs were identified on different chromosomes in wheat related to mineral traits in wheat landraces. GZn. 2019 has five MTAs on chromosome 7D, 1A, 7B (3) with R^2 range of 0.84- 1.11. GZn. 2020 has four MTAs on chromosome 3D, 3B, 5D, 2A with R^2 range of 0.61-0.70. GZn average has two MTAs on chromosome 1A, 6B with R^2 range of 0.56-0.59.Fe. 2019 has large number of twenty MTAs on chromosome 1B (3), 4A, 2B (2), 2A, 3B, 2D, 2A, 7B, 5A (4),7D, 7A, 6A (2), 5B with R^2 range of 5.60-11.26. Fe 2020 has fourteen MTAs on chromosome 2B (6), 5B, 1A (3), 1B, 6B, 4B, 7A with R^2 range of 4.01-6.53.GFe average has seven MTAs on chromosome 2B (2), 3B (2), 6A (2), 4A with R^2 range of 4.51-9.17. Na has one MTA on chromosome 5A with R^2 value of 0.002. B has one MTA on chromosome 1B with R^2 value of 0.11. P has 3MTAs on chromosome 3A (2), 3B with R^2 range of 0.07-0.12. K has fifteen MTAs on chromosome 5A, 7A, 6B (4), 2B (4), 6A (2), 7B, 6D, 1A with R^2 range of 0.01-0.03. Mn has seven MTAs on chromosome 1A (2), 1B, 2B, 3A, 4A, 4B with R^2 range of 0.80-1.68. Cu has one MTA on chromosome 6D with R^2 value of 0.36. N has three MTAs on chromosome 3A (2), 3B with R^2 range of 0.42-0.69. GFe.2019 has maximum number of MTAs. B, Cu, Na has minimum number of MTAs. This study offered significant information into the biofortification status of wheat cultivars used historically in Pakistan as well as the biofortification status and GWAS of wheat landraces from Pakistan. It is a good starting point for developing a breeding strategy for simultaneous improvement in wheat phenology and biofortification.

CHAPTER 1: General Introduction and Review of Literature

Wheat (*Triticum aestivum* L.) is one of the world's most important cereal crops It is the most significant source of calories for millions of people all over the world and is consumed by more than 40% of the worldwide population as a basic food (FAO, 2020). Wheat is the most essential cultivated food crop among all species and cultivated on huge area of two hundred million hectares (FAO, 2020). However, the crop lacks several micronutrients, such as Zn, Fe, and carotenoid, which are found in extremely small amounts in wheat grain. Micronutrient deficiency affects about three billion people globally, including one-third of children in poor countries (Chattha *et al.,* 2017). Pakistan, being an agricultural country, has a wealth of natural resources, including harsh weather, irrigated land, irrigation infrastructure, and fertile soil (Khan *et al.,*2020). Agriculture employs 42.3 % of the working population and contributes for 18.9 % of the country's GDP. Agriculture employs 68 % of rural labor directly or indirectly in the production, value addition, and trade of major and minor agricultural commodities, which are the country's principal source of livelihood and supply food and raw materials to industries (Economics survey of Pakistan 2018). Being a main crop of temperate countries, it is utilized by human as a staple food and as a livestock feed (Shewry *et al.,* 2016).

Wheat can grow on a diverse range of climatic conditions ranging from 47ºS to 57ºN and it also has adaptable behavior of all crop plants that grow commercially. It is highly successful among human due to its adaptability and presence of fractions of gluten protein, which gives the elastic and viscous characteristics to wheat and very famous among peoples after processing of dough into bread, dietary products, and pasta. The whole grain of wheat contains large number of vitamins, dietary fibers, amino acids, minerals (Shewry *et al.,*2009).

1.1 Nomenclature of Wheat

The diversity of wheat has generated more confusion in the naming of it. Bread wheat belongs to Triticeae tribe; whose synonym is Hordeae. It contains over 150 different species with varying ploidy levels, among which rye (*Secale cereale* L.), barley (*Hordeum vulgare* L.), einkorn wheat (*T. monococcum* L.), durum wheat (*T. turgidum* L.), and bread wheat (*Triticum aestivum* L.) are very important and cultivated on a large scale throughout the world for food and are commercially cultivated (Ortiz *et al.,* 2008). Wheat cultivated on large scale for the very first time by human at about ten thousand years before. This was the part of the Neolithic Revolution. During this time, many changes come in existence then before time.

With the arrival of formal agriculture, the earlier method of getting food was replaced by the proper agricultural practices by man. Their genetic relationship revealed that the emmer wheat with tetraploid genome, and einkorn wheat with diploid genome were cultivated by man and these two cultivars were originated from southeastern part of Turkey (Heun *et al.,* 1997).

1.2 National and International Wheat Statistics

In 2020, China produced 134,250 tons of wheat, accounting for 21.9 % of world wheat production. India, the Russian Federation, the United States of America, and Canada are the top five countries in terms of overall production, accounting for 64.71 % of the total. In Asia, over 44 % of the area is suitable for wheat production, with India, China, and Pakistan accounting for 62 million hectares. The most recent FAO estimate for worldwide wheat output in 2018 is 736.1 million tons. Pakistan is located in an arid and semi-arid environment, with wheat accounting for 70 % of grain and 37 % of total cropland (Ahmad *et al.,* 2021).

In Pakistan, the annual production forecast for wheat production is 25.5 million tons, an increase of 1.2 million tons over the previous year (FAO, 2020). Rainfed crop farming is important both internationally and in Pakistan, since it accounts for 80 % of global cultivated land and 33 % Pakistan's cultivated land (Bradford *et al.,* 2017). Wheat production of Pakistan increased from 6.4 million tons in 1971 to 25,700 thousand tons in 2020 growing at an average annual rate of 3.18 %. Punjab has the greatest producing area (71.17 %), followed by Sindh province (13.38 %). Sindh, on the other hand, has a far higher output per hectare (2,410 kg/ha) than Punjab (2,316 kg/ha) (Ahmad *et al*., 2021). Wheat is widely produced on irrigated land in Punjab. China is the world's leading wheat producer. On global scale, Canada, European Union, Ukraine, Australia, Russia, United States, China, Pakistan, India, and Turkey are the main wheat growing countries therefore serving more than 80 % of wheat production around the world.

1.3 Nutritional Quality of Wheat

The most important useful dietary staple food of Pakistan is wheat flour, used up three times a day in the form of round flat bread (Rehman *et al.,*2007). Wheat is a main source of many essential components of food like vitamins, protein, amino acids, and dietary fibers, which used mostly in the bread and pasta making industries than any other cereal crop. Because it contains good quality and quantity of gluten protein. Wheat has many medicinal uses as it has

gluten and starch components that give us heat and energy. The outermost bran is very important in digestion process as it provides easy movement of bowels. While inner portion of wheat grain contain many minerals, phosphates, salts. Wheat protein muscular tissue of body (Kumar *et al*., 2011).

Proper growth and development require balanced and nutritional food containing calories, protein, and micronutrients with less antinutritional components. Three billion people all over the world facing malnutrition problem due to inadequate supply of micronutrient (Ehrlich *et al.,* 2015). For optimal development and energy, more starch and protein are necessary, whereas gluten, a complex protein made composed of glutenin (Gln) and gliadin (Gld), is essential for gas and water holding capabilities for creating bread and chapati. Water soluble albumin and globulin, which are also key factors in nutritional dominance, increase the biological value of protein (Dvořáček *et al.,* 2008).

Iron (Fe), zinc (Zn), and calcium (Ca) are divalent macromolecules that are essential for human health and are involved in hemoglobin production, ossification, and brain development, respectively. These elements are beginning to decline as phytic acid concentrations grow; they are inaccessible to plants owing to phytic acid's chelating activity (Ekhlom *et al.,* 2003). The most important and first amino acid that is used for the consumption of wheat protein is lysine, amino acid pattern is necessary to improve in rich wheat diets (Young and Pellett, 1990).

Nutrients are essential to human health and development. The emerging awareness of malnutrition, hidden hunger and obesity has shifted attention to the dietary safety category (Horton and Lo, 2013; Haddad *et al.,* 2015 and Willett *et al.,* 2019). The Sustainable Development Goals (SDGs) primarily aim to overcome all forms of malnutrition and reduce child mortality to less than 2.5 % by 2030 and increase the implementation of sustainable and sustainable production programs (Zimm *et al.,*2018). Similarly, the United Nations Decade of Action on Nutrition (2016-2025) was announced to overcome the global malnutrition crisis (UN, 2017). Many actions have been taken on the effects of healthy eating on the various components of the diet plan as well as the production, distribution, processing, and marketing (Popkin *et al.,* 2017; Willett *et al.,* 2019).

1.4 Micronutrient Deficiency and Biofortification

Malnutrition of critical micronutrients such as Zn and iron Fe affects more than two billion individuals throughout the world (White and Broadle, 2009 and WHO, 2013). Severe malnutrition mostly affects children under the age of five and pregnant women. Malnutrition is a more prevalent problem than low-energy diet and poor nutrition globally (Stewart *et al.,* 2010), with vitamin A, Zn, Fe, and or Mn deficiency accounting for around 20 % of mortality among children under the age of five (Prentice *et al.,* 2008). Dietary-based foods are a major source of daily diets in countries with high levels of micronutrient deficiency (Cakmak *et al.,* 2010a and Bouis *et al.,* 2011). Deficiencies of micronutrients are one of the major causes of malnutrition in humans throughout the world. Low micronutrient in the soil affects plant nutrition directly and human health indirectly (Sanchez and Swaminathan, 2005). Vegetarian diets are very low in micronutrients are commonly used in resource poor populations of developing countries (Joy *et al.,* 2014). Therefore, malnutrition has become matter of attention globally due to high rate of health issues (FAO, 2015).

The intensity of the malnutrition problem is even more prominent in low to middle income developing countries of the world like Africa and Asia where multi-nutrient deficiencies have been reported in humans (Joy *et al.,* 2014). Soils in these countries are unable to supplement optimum quantity of nutrients to the staple crops and cause decline in yield and low-quality produce (Kumssa *et al.,* 2015). Thus, the deficiency of essential micronutrient has transfer from soil to crops and then to human population. The CGIAR consortium's Harvest Plus program is continually working with people all around the globe to overcome key nutritional micronutrient shortages by biofortifying the primary with vital micronutrients and vitamins. This approach is thought to be the most effective way to address human vitamin insufficiency (Bouis, *et al*., 2007). Biofortification is very important strategy to increase nutritional quality of commercial cereals crops. It is the improvement of the micronutrient bioavailability of cereal crops achieved with genetic engineering-based techniques and conventional breeding (Kumar *et al.,* 2019).

It is better than general fortification owing to its capacity of improving the nutrient content of plants during development, therefore becoming more approachable to poor populations in the developing world. It is also accumulated more beneficial micronutrients in cereals crop grains that facilitate crop production, especially when the plant is grown in nutrient deficient soil (Kumar *et al.,* 2019). Ruel *et al*. (2013) demonstrated many direct and indirect approaches to

overcome the problem of hidden hunger. Direct approaches include nutrition specificity and are focused to changing dietary habits, industrial fortification of food products, expansion of dietary choices, medication. Indirect approaches are highly nutrient sensitive. These approaches demonstrate the primary causes of hidden hunger through biofortification of crops (Bouis *et al.,* 2011). Biofortified staple foods may not provide us with a high concentration of essential micronutrients and vitamins per day as compared to food supplements or fortified food products, but they can increase essential micronutrient intake for poor people in developing countries who consume them on a regular basis, thus complementing existing approaches (Bouis *et al.,* 2011).

1.5 Major Strategies of Biofortification

There are three major tools of biofortification which includes genetic engineering, agronomy, conventional breeding (Saltzman *et al.,* 2015). Among all these tools, agronomic biofortification is most important tool. It is less time consuming, effective, economic, and highly adaptable approaching all over the world (Cakmak *et al.,*2014 and De *et al.,* 2018). Agronomic biofortification achieve through soil and foliar applications of micronutrients to the crops (Cakmak *et al.,* 2010 and Phattarakul *et al.,* 2012). Agronomic biofortification is mainly depended on the addition of synthetic and natural fertilizers to the commercial cereals crops for the micronutrient enhancement of edible parts of crops (Velu *et al.,* 2014). It is an effective approach for handling malnutrition problem globally but genetic biofortification is a better and sustainable solution to tackle malnutrition.

Genetic biofortification including transgenic methodology and traditional breeding that leads to the introduction of nutrient enriched crop cultivars in wheat nutritional quality improvement programs (Gomez *et al.,* 2010). It is indicated in a lot of research that the content of Fe and Zn in grain varies greatly amongst wheat varieties. The most significant techniques for improving nutritional quality characteristics in wheat are genome assisted breeding and microbial assisted breeding, as well as fertilizers (Chandra *et al*., 2020). It provides a sustainable solution to the problems of malnutrition by discovering genetic diversity to increase plant-rich mineral species (Pfeiffer and McClafferty, 2007). Existing inputs have been tested by plant breeders in international banks of the germplasm to determine whether there is enough genetic diversity to breed a specific gene. At the same

time, they specifically prefer nutritious food crops, rich in Zn and Fe focus and the potential benefits of Zn and Fe (Pfeiffer and McClafferty, 2007).

1.6 Importance of Zinc

Among various essential micronutrients in plants, Zn is an important essential micronutrient that play important role in diverse metabolic processes of plant comprising respiration, photosynthesis, activation of enzymes and assimilation of some other major nutrients and thereby essential for the proper growth and development of plants (Cakmak *et al.,* 2008 and Rehman *et al*., 2012). Zn is one of the most essential micronutrients deficient among women and pre-school children, which ultimately leads to hidden hunger. Zn deficiency also exists to large extent in Pakistan. In Pakistan 37 % of population is suffering Zn deficiency (Cakmak *et al.,* 2008). Zn deficiency is considered an important health hazard problem for humans which affect about 1/3rd of the total world's population because it leads to thousands of deaths annually (White and Broadley, 2011). To resolve the emerging health problems and to reduce the rate of diseases globally, there is urgent need to increase nutrient concentration in grains of staple food crop plants along with maximum per acre yield (White and Broadley, 2011).

Zn is one of the main target micronutrients because Zn deficiency is internationally acknowledged as an essential health risk element for humans. Adequate Zn supplementation has recurrently been shown to considerably decline the occurrence of infectious diseases in humans, for example pneumonia especially among children under age of five in populations with reduced supply of Zn (Gibson *et al.,* 2012).The main causes of high levels of Zn deficiency in the world are due to low Zn food intake and low food diversity (Blacks *et al.,* 2013 and White *et al.,* 2009). Food-based foods that are widely used in developing countries are low in price and Zn availability is also a major cause of Zn deficiency. Therefore, the discovery of grain crops with higher Zn is an important global challenge and the most important study in the world (Cakmak *et al.,* 2008). Two of the most important tools in agriculture to improve the concentration of Zn grain are the use of Zn fertilizers (e.g., Agronomic biofortification), plant breeding (e.g., Biofortification). Plant breeding method however provides an effective solution for grain stabilization with Zn which is a long-term process (Cakmak *et al.,* 2008).

The genetic capacity of the newly developed Zn genotypes depends on the amount of Zn found in plants in the soil solution to obtain sufficient Zn grains. Cakmak *et al.* (2008) the use of Zn fertilizer in the crop industry provides a short-term solution to the problem and an ongoing way to address the shortage of Zn crop production strategy. The role of leaf senescence in wheat grain provides crucial molecular evidence of enhanced micronutrients. It has also been demonstrated that *NAM-B1* genetic expression is responsible for the regeneration of Zn, Fe, and N from leaf tissue to stem during leaf senescence, and that leaf delay senescence seems to lower Zn and Fe grain concentrations (Uauy *et al.,* 2006 and Delfelfeld *et al.,* 2007).

1.7 Importance of Iron

Iron (Fe) is the most important micronutrient for human health. Its deficiency can cause serious health problems. Global nutrition and food security are still a far-away goal regardless of latest growth in the fight against starvation and malnutrition in several countries around the world (Mayer *et al.,* 2008).Anemia is one of the most prevalent human disease in the world caused by Fe deficiency in the world, affecting approximately one-third of the world's population and turn out 0.8 million deaths per year globally (WHO, 2013).To overcome this problem of malnutrition, biofortification (i.e., the breeding of micronutrient-fortified crops) is highly beneficial for people who face problem in altering their dietary habits because of cultural, financial, regional, or religious constraints. In 2010, more than 46,000 persons were injured as a result of anemia induced by a Fe deficiency (Murray and Lopez, 2013). Biofortification has addressed these issues by increasing the important micronutrient density in plant edible parts and increasing their availability and absorption in the human body during digestion (Bouis *et al.,* 2011).

Organic acids are produced by organic matter and function as chelators, increasing metal (in this example, Fe) accessibility to plants by solubilizing nutrients in the soil solution in the ground (McCauley *et al.,* 2009). In poultry manure, there is a high nutritional composition and concentration of nitrogen; high nitrogen persistence increases Fe content in wheat plants by increasing Fe activity and the richness of Fe transporter proteins, such as yellow stripe 1 (YS1) in the root cell membrane (Curie *et al.,* 2009). Biochar (BC) is used to promote soil fertility by modifying the soil's physico-chemical and biological characteristics, which leads to nutrient mobilization (Xu *et al.,* 2013 and Jeffery *et al.,* 2015).

1.8 Genetic Diversity for Grain Fe and Zinc in Wheat

Most publications place the range of biofortified grains and the creation of cultivars with high Zn concentrations between 40-50 mg kg^{1} (Cakmak and Kutman, 2018). Our germplasm has phenotypic variations ranging from 25.05 to 52.65 mg g^{-1}, which corresponds to the intended range and allows us to employ the majority of genes encoding Zn genotypes in reproductive systems. In durum wheat, the variation in Zn concentrations ranges from 24.8 to 48.8 mg/kg, which is consistent with our findings (Magallanes-Lopez *et al.,* 2017). Approximately four to five times more than five grains of Fe and Zn were detected in an early analysis of several hundred wheat grains. Between hexaploid wheat, *Triticum dicoccon*, and soils cultivated under field circumstances, Fe grain concentrations varied from 25 to 56 mg kg^{1}, with a mean of 37 mg kg^{1}, and Zn grain concentrations ranged from 25 to 65 mg kg^{1}, with a mean of 35 mg kg^{1}. Genotypes with very high levels, on the other hand, were less productive, and genotypes were biased (Ortiz-Monasterio *et al.,* 2007).

The genetic variety of these micronutrients from wild wheat progenitors that have genetic micronutrients can help boost wheat feed production. *Aegilops tauschii, Triticum dicoccoides, Triticum monococcum, Aegilops speltoides, Aegilops kotschyi, Aegilops longissimi, and Aegilops speltoides, Aegilops kotschyi, Aegilops longissimi* and reported among sources and Zn concentration (Chhuneja *et al.,* 2006 and Rawat *et al.,* 2009). *Ae. tauschii* is the most important germplasm resource for expanding the genetic variety of micronutrients in a wheat crop generated by *Ae. tauschii* since it can also associate with the D-genome of hexaploid wheat (Wang *et al.,* 2016). *Triticum boeoticum, Triticum monococcum, Triticum dicoccoides, Aegilops tauschii, Aegilops speltoides,* and synthetic hexaploidy included the highest genetic diversity and Fe and Zn grain genetics.

GFe (24.50–44.30 mg kg^{1}), GZn (17.75–49 mg kg^{1}), and beta-carotene (0.5–6.5 mg kg^{1}) concentrations. Certain wheat landraces, particularly Afghan landraces (GFe: 55.14–122.2 mg kg^{1} and GZn: 15.56–87.29 mg kg^{1}), contain significant micronutrient contents (Manickavelu *et al.,* 2017). CIMMYT has 132 wheat cultivars with a wide range of micronutrient concentrations (GFe: 28.8–56.50 mg kg^{1}) in their collection (Xu *et al.,* 2011). The genotypic diversity in Fe concentrations appeared to be evenly distributed across each year. Based on BLUE readings, the average grain Fe content was around 34 dry weight (DW) in the whole panel and subpanel of genotypes, with DW values ranging from 24.42 to 52.42

mg kg^1 in the whole panel and 26.99 to 48.52 mg kg^1 in the subpanel of genotypes (Alomari *et al.,* 2019).

1.9 Function of *NAC* Gene for Fe, Zn in Wheat

The plant-precise *NAC* transcription elements (TFs) constitute a key TF circle of relatives in flora, and play widespread roles within the improvement, increase and responses to biotic and abiotic strain (Shao *et al.,* 2015). Different *NAC* family members have been shown to control developmental strategies including leaf senescence (Guo and Gan., 2006). Leaf yellowing became non-existent over time in transgenic wheat *NAM* RNA interference (RNAi) lines with decreased expression of *NAM-B1* and homoeologous genes, although Fe, Zn, and grain protein contents were dramatically reduced (Uauy *et al.,* 2006b). These observations, together with higher N, Fe, and Zn contents in mature RNAi line flag leaves, revealed a function for *NAM-B1* homoeologous in N, Fe, and Zn remobilization (Uauy *et al.,* 2006b).

These results, together with increased N, Fe, and Zn contents in mature RNAi line flag leaves, revealed a function for *NAM-B1* homoeologous in N, Fe, and Zn remobilization (Uauy *et al.,* 2006b). The *NAC* transcription component (*NAMB1*) encoded by the *GPC-B1* locus contributes to grain protein, zinc, and iron concentrations, likely by speeding up leaf senescence and hence remobilization of amino acids, zinc, and iron from flag leaves into seeds (Uauy *et al.,* 2006).

The inhibition of *NAM* gene synthesis resulted in a decrease in grain awareness of micronutrients and a corresponding delay in leaf senescence (Uauy *et al.,* 2006). As a result, it has been postulated that NAM genes from T. dicoccoides are likely to be valuable candidate genes for the creation of commercial wheat for both micronutrient and protein concentration. When comparing bread wheat to durum wheat, knowledge related with micronutrient awareness and genetic diversity has been observed. Durum wheat has a propensity to acquire more Zn and Fe, as well as Cd (Hadjivassiliou *et al.,* 2019). Its miles still unidentified that why the assembly of Fe and Zn and particularly Cd takes place to a more diploma in durum than bread wheat (Uauy *et al.,* 2006).

1.10 Identified QTLs for Fe and Zn in Wheat

Understanding the genetic basis of micronutrient accumulation in wheat grain and mapping quantitative trait loci (QTL) will lay the groundwork for creating optimal breeding techniques

for increasing grain micronutrient concentrations via (MAS) (Peleg *et al.,* 2009). *PvFER1*, genes in Wheat cv. Bobwhite are responsible for 1.6-fold increased Fe in grain tissue (Singh *et al*., 2017). *TaFER1-A* genes in Wheat cv. Bobwhite are responsible for Fe 1-1.5-fold Fe increased in grain tissue (Borg *et al*., 2012). *TaVIT2-D* genes in Wheat cv. Fielder are responsible for Fe 2-fold Fe increased in white flour (Connorton *et al*.,2019). *TaVIT-1* gene for Fe and Mn are present on chromosome 2A, 2B and 2D. Thirteen QTLs were identified for Fe, Zn, Protein in RIL, Xiaoyan 54 ×Jing 411 by using 555 SSR (Yu *et al*., 2014). Five QTLs for Fe and Zn in RIL, BSaricanak98 × MM5/4^, Adana99 × 70,711^ by using 7000DArT were identified (Crespo-Herrera *et al*., 2017). Six QTLs were found in RILS, PBW343×d Kenya Swara for Zn by using DArT, SSR (Yu *etal*., 2014). Two QTLs were identified for Zn and Fe in RILS, *T. spelta* L× synthetic hexaploid wheat by using DArT,9034 markers (Crespo-Herrera *et al*., 2017). Fifteen QTLs for Fe and Zn in RILS, SHW-L1/Chuanmai32×Chuanmai42/Chuannong wheat by using DArT markers were identified (Goudia *et al*., 2015). Two QTLs for Fe were found in wheat RIL, *T.boeoticum*×*T.monococcum* by using SSR and RFLP markers (Tiwari *et al*., 2009). One QTL for Zn found in RIL, *T.boeticum*×*T.monococcum* using SSR and RFLP markers (Tiwari *et al*, 2009). Four QTLs for Zn and, one QTL for Fe was identified in RAC875-2×Cascades using RFLP, AFLP, SSR and DArT markers (Gene *et al.*, 2009). Five QTLs for Zn and five QTLs for Fe were found in RILs, *T.spelta*× *T.aestivum* wheat by using DArT and SNP markers. Thirteen QTLs for Fe, Zn, protein in wheat RILS by using DArT and SNP markers (Xu *et al*., 2012). Eleven QTLs for Fe and six QTLs for Zn were found in Langdon×G18-16 by using DArT and SNP markers (Peleg *et al.,* 2009). Five QTLs for Zn were identified in wheat DH Clipper × Sahara by using DArT and SNP markers (Lonergan *et al.,* 2009). 90 K Infinitum SNP markers array was used to genotype 330 bread wheat lines. The study revealed two QTLs for Zn on chromosome number 2B and 7D whereas 39 marker-trait associations in three environments (Velu *et al*, 2018). DArT markers and SNP markers were used to genotype 269 Afghan wheat landraces. One QTL for Zn and Fe was found on the chromosome 6D and one marker-trait associations in two environments (Manickavelu *et al.,* 2018). SNPs were used to genotype 140 RILs, SeriM82×SHWCWI76364 CIMMYT wheat lines. One QTL was identified for Zn and Fe on chromosome number 4BS and one marker-trait associations (Crespo-Herrera *et al.,* 2016). SNPs were used to genotype 330 bread wheat lines CIMMYT biofortification program. Three QTLs were found for Zn and Fe on chromosome number 1A, 3B, 5B and 137 marker-trait associations (Cu *et al.,* 2020).

1.11 Genome-wide Association Mapping for Zinc and Iron

Genome-wide association studies (GWAS) are one of the most widely utilized methods for finding the genetic basis of complex characteristics (Lopes *et al.,*2015). To far, a number of QTL studies have been done to investigate the genetic basis of wheat grain Zn and Fe concentration (Crespo-Herrera *et al.,* 2017). The traditional QTL mapping method, on the other hand, is limited to the biparental population used and yields low-resolution QTL loci. In wheat, GWAS have been used to examine the genetic basis of complex characteristics. GWAS provides a number of advantages over traditional QTL mapping, including better QTL resolution, allele coverage, and the capacity to use large collections of natural germplasm resources including landraces, elite cultivars, and advanced breeding lines. Only a few studies have used GWAS to study the genetics of end-use quality attributes in wheat (Battenfield *et al.,* 2016).

In 246 spring wheat genotypes, Kumar *et al.* (2018) reported 33 marker-trait associations (MTAs) for GFeC and 198 MTAs for GZnC on all three A, B, and D genomes. In 330 bread wheat lines, Velu *et al.* (2018) discovered 39 significant MTA for grain Zn. There were 10 different chromosomes with corresponding SNPs: 1A, 2A, 2B, 2D, 5A, 6B, 6D, 7B, 7D, and one unknown. On chromosomes 2B and 7D, a GWAS study revealed two substantial effects QTL. On wheat chromosomes 2B and 7D, a number of QTL for high grain Fe and Zn have been discovered. SNPs were responsible for 5–10.5 percent of phenotypic variation. Seven of the 39 MTAs were discovered in three different contexts. A major SNP at 60 cM on chromosome 2A (RAC875 c34757 180) explained around 9 % of the phenotypic diversity, while another at 60 cM on chromosome 5A (IAAV1375) explained about 6 %. Another substantial SNP, wsnp Ex c5268 9320618, was identified at 120 cM on chromosome 7B and accounted for around 10.5 percent of the variance (Velu *et al.,* 2018). In a tetraploid wild emmer durum wheat recombinant inbred lines (RILs) population. Peleg *et al.* (2009) identified five QTLs for Fe concentration on chromosomes 2A, 3B, 5A, 6B, and 7A.

Another study revealed five QTLs in a *Triticum spelta* x *T. aestivum* RIL population that underlay grain Fe concentration (GFeC), three of which linked to chromosome 1A and two to chromosomes 2A and 3B. (Srinivasa *et al.,* 2014). Gorafi *et al.* (2016) looked examined grain iron levels in 47 synthetic hexaploid wheat germplasm lines. Bhatta *et al.* (2018) used GWAS to look for various grain minerals in 123 synthetic hexaploid wheat lines, including Fe. GWAS analysis identified 41 significant MTAs (log10 (p-value) 3) spread across the genome

with R2 values ranging from 2.7 to 5.22 % for the entire panel. There was a total of 41 MTAs discovered, with 17 of them located between 46.6 and 59.8 cM on chromosome 3B. The studies were based on BLUEs, with the most significant three SNPs chosen for further analysis because there were no common correlations across years. The subpanel had a higher number of significant correlations with unmapped markers, comprising 137 MTAs with R2 values ranging from 5.60 to 13.09 percent. Unmapped markers (AX-158577508 and AX-158577509) were responsible for the most phenotypic variance, accounting for 10.38 and 13.09 percent, respectively. On chromosomes 2A (763,689,738–765,710,113 bp), 3B (731,263,238–731,264,585 bp), and 5A (538,758,878–539,958,539 bp), respectively, fifteen, four, and two significant SNPs were discovered (Alomari *et al.,* 2019)

1.12 Objective

The objective of our study is to

a) Assess the temporal variation for grain mineral elements in historical wheat cultivars and rate of progress for improvement in grain mineral elements.
b) Identify the important phenological traits associated with grain mineral elements in historical wheat cultivars of Pakistan.
c) Identify the allelic effects of important Green-revolution *Rht-1* genes and other genes like *NAM-A1, TaSus2-2B, TaGW2-6B* and *TaGW2-6A* on agronomic traits and mineral contents in historical wheat cultivars from Pakistan.
d) Elucidate the grain micronutrient variations in wheat landraces from Pakistan.
e) Genome-wide association studies (GWAS) to identify potentially new loci for improving grain micronutrient concentration in a collection of wheat landraces from Pakistan.

CHAPTER 2: Genetic Gain for Grain Micronutrients and Their Association with Phenology in Historical Wheat Cultivars Released between 1911 and 2016 in Pakistan

Abstract:

Wheat (*Triticum aestivum* L.) is an important source of protein and minerals as a staple food crop. It is crucial to fortify basic grains like wheat with key minerals to address the challenges associated with malnutrition. The goal of the study was to determine the levels of 11 micronutrients in 62 wheat cultivars released in Pakistan between 1911 and 2016, including grain iron (GFe) and zinc (GZn). Inductively coupled plasma optical emission spectroscopy (ICP-OES) and an energy-dispersive X-ray fluorescence spectrophotometer were used to detect GFe and GZn during a two-year period (EDXRF). The GZn concentration varied from 18.4 to 40.8 mg kg^{-1} according to EDXRF and 23.7 to 38.8 mg kg^{-1} according to ICP-OES. Similarly, ICP-OES found GFe levels ranging from 24.8 to 44.1 mg kg^{-1} while EDEXR found levels ranging from 26.8 to 36.6 mg kg^{-1}. GZn had a greater coefficient of correlation (r = 0.90) than GFe (r = 0.68). Modern cultivars like Zincol-16 and AAS-2011 demonstrated increased GFe and GZn levels as well as enhanced yield components. Old wheat cultivars WL-711, C-518, and Pothowar-70, which were released before to 1970, also had greater GFe and GZn values; nevertheless, their agronomic performance was poor. A multivariate analysis of eleven micronutrients (Fe, Zn, Al, Ca, Cu, K, Mg, Mn, Na, Se, and P), as well as agronomic traits and genome-wide SNP markers, revealed a promising cultivar with higher yield, biofortification, and genetic diversity. Genetic gain study found a significant increase in grain yield (0.4 percent year 1) over the period of 100 years, but a negative gain for GFe (0.111 percent year 1) and GZn (0.15 percent year 1). Plant height and grain yield (GY) were closely linked to the *Rht-B1* and *Rht-D1* genes of the Green Revolution, whereas semi-dwarfing alleles had a negative impact on GFe and GZn levels. This study provided valuable insight into the biofortification status of wheat cultivars used in Pakistan in the past, and it serves as a suitable beginning point for establishing a breeding strategy to improve wheat phenology and biofortification at the same time.

2.1 Introduction

A deficiency of key micronutrients such as zinc (Zn) and iron (Fe) affects more than two billion people globally (WHO, 2021). Inadequate zinc consumption leads to stunted development and an increased risk of child mortality, and now, 17 percent of the world's population is at risk. Anemia is caused by a lack of iron, which affects 800 million women and children throughout the world. Copper (Cu), manganese (Mn), calcium (Ca), and selenium (Se) are also critical micronutrients since they participate in crucial biochemical processes for both plant development and human health (Hänschand Mendel, 2009). Although Cu and Mn shortage is uncommon in humans, these trace elements are essential for growth and development. Wheat (*Triticum aestivum L.*) is one of the most significant cereal crop plants in the world, ranking third in terms of production behind rice and maize (FAO, 2021). Wheat contributes more than 20% of the calories consumed by the world population, particularly in underdeveloped nations. Therefore, increasing the micronutrient contents, known as biofortification, in wheat cultivars is a low-cost and sustainable strategy for alleviating micronutrient malnutrition. The preliminary breeding goal for the major target nations, Pakistan, and northern India, is to boost Zn levels by 12 mg/kg, which is approximately 50 % higher than the baseline, which is the mean of popular varieties cultivated in the region (Velu *et al.,* 2014). According to Velu*et al.* (2014), dietary supplements and agronomic methods involving the use of Fe- and Zn-containing fertilizers can aid in addressing the nutritional shortage problem. Wheat cultivars often contain low levels of micronutrients such as Fe and Zn (Velu *et al.,* 2014).

It has been calculated that the GZn content in wheat grain should be greater than 50 mg kg^{1} per gram dry weight, but present wheat grains have an average of 25–30 mg kg^{1}Zn per gram dry weight (Cakmak *et al.,* 1998). Genetic biofortification, which requires the identification of cultivars with useful genetic variability for grain minerals as well as an understanding of the physiological and genetic architecture of these minerals in wheat, is a more sustainable and cost-effective approach to increasing essential mineral concentration (Ali and Borrill, 2020). There is clear evidence that modern and old wheat cultivars differs significantly for grain micronutrients, and it was discovered that with the introduction of semi-dwarf and high-yielding wheat cultivars after 1965, grain contents of 23–27 percent Fe, 33–49 mg kg^{1}, Zn, 25–39mg kg^{1}, Cu, and 29–27 mg kg^{1} (Fan *et al.,* 2008). It is most likely because *Rht-B1b* and *Rht-D1b* genotypes have decreased root system development, reducing the capacity to scavenge minerals from the soil or store minerals in the vegetative tissues prior to

redistribution to the grain (Velu *et al.,* 2017). Murphy *et al*. (2008) analyzed the mineral elements in 63 historical wheat cultivars released between 1842 and 1965 in Pacific Northwest US and concluded that all minerals except Ca significantly decreased over time. Although breeding for biofortified wheat is not a main target in Pakistan and India, high-Zn cultivars such as Zincol-2016 and Zn Shakti have been released in Pakistan and India, respectively (Singh *et al.,* 2017).

Previously, some cultivars with high grain Fe/Zn have been released, such as cv. Burnside in Canada with the *Gpc-B1* gene (Randhawa *et al.,* 2013). Therefore, it is very important to analyze the status of minerals in historical wheat germplasm for better insight when selecting germplasm resources in breeding. The present study was designed to evaluate the status of minerals contents in historical wheat cultivars released in Pakistan between 1911 to 2016. The main objectives included were: (a) to assess the temporal variation in grain mineral elements in historical wheat cultivars and rate of progress for improvement in grain mineral elements, (b) to identify the important phenological traits associated with grain mineral elements in historical wheat cultivars of Pakistan and (c) to identify the allelic effects of important Green Revolution *Rht-1* genes, and others such as *NAM-A1, TaSus2-2B, TaGW2-6B* and *TaGW2-6A*, on agronomic traits and mineral contents in historical wheat cultivars from Pakistan.

2.2 Materials and Methods

2.2.1 Plant Material and Field Trials

A set of 62 wheat cultivars released in Pakistan from 1911 to 2016 were selected for this study. The cultivar name, year of release and pedigree are given in Table 2.1. The cultivars were evaluated for two years, 2018–2019 (later as 2018) and 2019–2020 (later as 2019), in the field at the National Agriculture Research Center (NARC), Islamabad, Pakistan, using a randomized complete block design (RCBD) with two replications. The NARC site is located at 33◦43′ N 73◦04′ E. The date of sowing was 5 December in 2018 and 8 December in 2019.

Table 2.1. Pedigree of historical wheat cultivars evaluated for phenological parameters along with the grain iron and zinc content.

Cultivar	Release Year	Pedigree
T9	1911	Nil
C-518	1933	T9/8A
C-217	1944	C516/C591
C-271	1957	C230/IP165
C-273	1957	C209/C591
Dirk	1958	FORD//DUNDEE/BOBIN
Mexipak-65	1965	PJ/GB55orPJ62/GB55
Pothowar-70	1970	BURT/KENYA//QUETA(L)/3/NAD63
Pari-73	1973	CNO67//SN64/KLRE/3/8156
Parula	1973	Nil
WL-711	1978	S308/CHRIS//KAL
Pak-81	1981	KVZ/BUHO//KAL/BB
Barani-83	1983	BB/GLL/3/GTO/7C//BB/CNO67
Chakwal-86	1986	FORLANI/ACC//ANAorFln/ACS//ANA
Khyber-87	1987	KVZ/TRM//PTM/ANA
Rawal-87	1987	MAYA/MON//KVZ/TRM
Inquilab-91	1991	WL 711/CROW “S”
Pasban-90	1991	INIAF66/TH.DISTICHUM//INIAF66/3/GENAROT81
Pastor	1993	PFAU/SERI-82//BOBWHITE
Bakhtawar-94	1994	AU/UP301//GLL/SX/3/PEW/4/MAI/MAYA//PEW
Parwaz-94	1995	V.5648/PARULA
Punjab-96	1996	SA42*2/4/CC/INIA//BB/3/INIA/HD832
Suleman-96	1996	F6.74/BUN//SIS/3/VEE#7orF6-74/BUN//SIS/3/VEE#7
Tatara	1996	JUP/ALD’S’//KLT’S’
Chakwal-97	1997	BUC’S’/FCT’S’
MH-97	1997	NORD-DESPREZ(ND)/VG-9144//KALYANSONA/BLUEBIRD/3/YACO/4/VEERY-5
Margallah-99	1999	OPATA/BOW’S’
Auqab-2000	2000	CROW’S’/NAC//BOW’S’
Wafaq-2001	2001	OPATA/RAYON//KAUZ
AS-2002	2002	KHP/D31708//CM74A370/3/CNO79/4/RL6043/4*NAC
Bhakkar-2002	2002	P20102/PIMA/SKA/3/TTR’S’/BOW’S’
GA-2002	2002	DWL5023/SNB//SNB
Ufaq	2002	V.84133/V83150
Pirsabak-2004	2004	KAUZ/STAR
Pirsabak-2005	2005	MUNIA/CHTO//AMSEL
Fareed-2006	2006	PT’S’/3/TOB/LFN//BB/4/BB/HD-832-5//ON/5/G-V/ALD’S’//HPO
Seher-2006	2006	CHILL/2*STAR/4/BOW//BUC/PVN/3/2*VEE#10
Bathoor	2008	URES/JUN//KAUZ
Chakwal-50	2008	ATTILA/3/HUI/CARC//CHEN/CHTO/4/ATTILA
Faisalabad-2008	2008	PBW65/2*Pastor
Mairaj-2008	2008	SPARROW/INIA//V.7394/WL711/13/BAUS
Pirsabak-2008	2008	KAUZ/PASTOR

NARC-2009	2009	INQALAB91*2/TUKURU
Atta-Habib	2010	INQALAB91*2/TUKURU
Barsat-2009	2010	FRET2
AAS-2011	2011	PRL/PASTOR//2236(V6550/SUTLEH-86)
Dharabi-2011	2011	HXL-7573/2*BAGULA//PASTOR
Millat-2011	2011	CHENAB2000/INQ-91
NARC-2011	2011	OASIS/SKAUZ//4*BCN/3/2*PASTOR
Punjab-2011	2011	AMSEL/ATTILA//INQ-91/PEW'S'
Galaxy-2013	2013	PUNJAB-96/V-87094//MH-97
Pakistan-2013	2013	MEX94.27.1.20/3/Sokoll//Attila/3*BCN
Pirsabak-2013	2013	CS/TH.SC//3*PVN/3/MIRLO/BUC/4/MILAN/5/TII
Shahkar-2013	2013	CMH84.3379/CMH78.578//MILAN
Pakhtunkhwa-2015	2015	WBLL1*2/4/YACO/PBW65/3/KAUZ*2/TRAP//KAUZ
Ujala-2016	2015	KIRITATI/4/2*WEAVER/TSC//WEAVER/3/WEAVR
Ahsan-2016	2016	Pastor/3/Altar84/Ae.squarrosa//Opata
Borlaug-2016	2016	Sokoll/3/Pastor//HXL7573/2*BAU
Gold-2016	2016	Nil
Johar-2016	2016	KAUZ/PASTOR//V.3009

2.2.2 Phenotyping

At various growth stages, the number of tillers per plant (TPP), plant height (PH) in cm, spike length (SL) in cm, spikelet per spike (SNPS), grains per spike (GPS), thousand kernel weight (TKW) in grams, grain length (GL) in mm, grain width (GW) in mm, grain diameter (GD) in mm, and grain yield (GY) in t/ha were all measured. Plant height was measured from the ground to the peak of the spike during late grain filling (Z77). From randomly selected spikes from 15 different plants per plot, the average quantity of grains per spike was computed. At physiological maturity (Z96), the number of spikes per plot was counted, all plants in each plot were removed manually, and the above-ground total biomass weight was recorded. Grain yield was calculated using the weight of grain collected each plot. Three 200-grain samples from each plot were used to calculate the thousand kernel weight. The GL and GW of one hundred uniform seeds from each plot were recorded. Using inductively coupled plasma mass spectroscopy, eleven mineral concentrations in the grains of sixty-two wheat cultivars were determined for each year and duplicate (ICP-OES). Grain samples were digested with concentrated HNO_3, hydrogen peroxide, and hydrofluoric acid, in brief. The non-destructive high-throughput ED-XRF (energy-dispersive X-Ray fluorescence analysis) technique was used to further analyze the grain Fe and Zn. This was accomplished using an Oxford Instruments X-Supreme 8000 equipped with a ten-position auto-sampler holding 40 mm aluminum cups. Fe and Zn were measured and evaluated in 186 seconds, with an acquisition

time of 60 seconds and a dead time of 60 seconds (Paltridge *et al.,* 2012). All analyses were carried out at the Institute of Crop Science, Chinese Academy of Agricultural Sciences (CAAS), in Beijing, China.

2.2.3 Genotyping Using KASP Markers and GBTS

Total genomic DNA was extracted from each cultivar according to a previously reported methodology (Ain *et al.,* 2015). KASP markers for the genes *Rht-B1*, *Rht-D1*, *TaSus2-2B*, *TaGW2-6A*, and *TaGW2-6B* were used in a previous work (Rasheed *et al*., 2016). For NAM-A1, Cormier *et al*. (2015) used two KASP markers. 2 liters 50–100 ng/L template DNA, 2.5 liters 2X KASP master mix, 0.07 liters KASP assay mix, and 2.5 liters distilled water made up the PCR mix. The following procedure was used to perform PCR in 384-well formats (S1000, Thermal Cycler, USA): a 15-minute hot start at 95°C, followed by 10 touchdown cycles (95°C for 20 s; touchdown at 65°C initially and decreasing at 1°C per cycle for 25 s), and 30 additional annealing cycles (95°C for 10 s; 57°C for 60 s). A genotyping-by-targeted-sequencing (GBTS) technology was also used to genotype DNA samples, which included more than 100 SNPs spread across all of the wheat chromosomes. This assay was used to investigate genetic diversity among crops. The GBTS assay's design has yet to be revealed.

2.2.4 Statistical Analysis

A mixed linear model was used to estimate the best linear unbiased estimator (BLUE) for each genotype across two settings for each attribute. The entire model looked like this:

$$Y_{ijkl} = \mu + geno_i + env_j + geno \times env_{ij} + Rep(env)jk + \varepsilon_{ijkl}$$

where Y_{ijkl} is the average phenotype of an individual plot, μ is the grand mean, $geno_i$is the fixed effect of the *i*th genotype, $env_j$is the random effect of the *j*th env (year in this case), $geno \times env_{ij}$is the random effect of interaction between the *i*th genotype and the *j*th env, $Rep(env)jk$is the random effect of the *k*th Rep nested within the *j*th env and $\varepsilon_{ijkl}$is the residual effect that was assumed to be independent and identically distributed following anormal distribution with a mean of zero and variance σ^2. After removing the outliers for each phenotype, an iterative mixed linear model was fitted in *lmerTest*(R package) with the full model. The model was used to compute the BLUE values for each genotype, estimate the variance components for broad-sense heritability (H2) on a line basis, and calculate standard error using the delta approach for each trait. The pairwise Pearson's correlation coefficients were computed using R package Hmisc and plotted using R package Performance Analytics

to analyze the degree of relationship between BLUE values for each set of attributes. The usual functions in the stats and pysch packages were used to compute the mean, range, and standard deviation of BLUEs. For each trait, the genetic gain over time was estimated by a simple linear regression in R function *lm*. The model was as follows:

$y_i = \beta_0 + \beta_1 x_i + \varepsilon_i$

where $y_i$is the BLUE value of the *i*th genotype, xi is the year of release of the *i*th genotype, β_0 is the intercept of the regression line, β_1 is the regression coefficient and $\varepsilon_i$is the residual effect. The regression coefficient was used to estimate the genetic gain (Gao *et al.,* 2017). The *t*-test on the regression coefficient β_1 was carried out to examine the significance of the regression with the null hypothesis: $\beta_1 = 0$, and the significance level was 0.05. Using TASSEL version 5.0, SNP markers from the GBTS assay were utilised to perform principal component analysis (PCA) and estimations of unweighted paired group arithmetic mean (UPGMA) using the neighbor-joining (NJ) technique. SNP markers with more than 5% missing data and low allele frequency were eliminated. The allelic effects on individual attributes were assessed using the KASP markers and the Student's t-test.

2.3 Results

The historical wheat cultivars showed significant variations in morphological traits and the eleven micronutrients evaluated in this study. Moreover, the allelic effects of some of the genes were also significant on the micronutrient traits. The results are given below in each subsection.

2.3.1 Variation in Micronutrients and Morphological Traits

All sixty-two wheat cultivars were grouped into three categories according to the year of release. The first group included seven cultivars released during 1911 to 1965, the second group included 21 cultivars released during 1965–2000, and the third group included 34 cultivars released after 2000. Descriptive statistics for all morphological traits and micronutrients across three groups are described in Table 2.2. The coefficient of variation was highest for Na contents (75.4 %), and the lowest CV % was observed for GD (3.7 %). The mean and range for all traits in cultivars released in three breeding eras are also described in Table 2.2. The GY progressively increased from 1.3 to 2.09 and 2.44 t/ha in three breeding eras, respectively. Contrastingly, TKW was higher (43.4 g) in old cultivars, compared to39.1 g mid-era and 41.3 g in cultivars released after 2000 (Table2.2; Figure 2.1). Similarly, PH

was 111 cm in old cultivars, 97.6 cm in cultivars released in 1965–2000 and 93.7 cm in post-2000 cultivars. Among the micronutrients, there was no clear pattern for GFe and GZn; however, K was significantly higher and Se, Mg and Cu were significantly higher in cultivars released before 1965 (Table 2.2; Figure 2.1a, b).

Fig2.1a

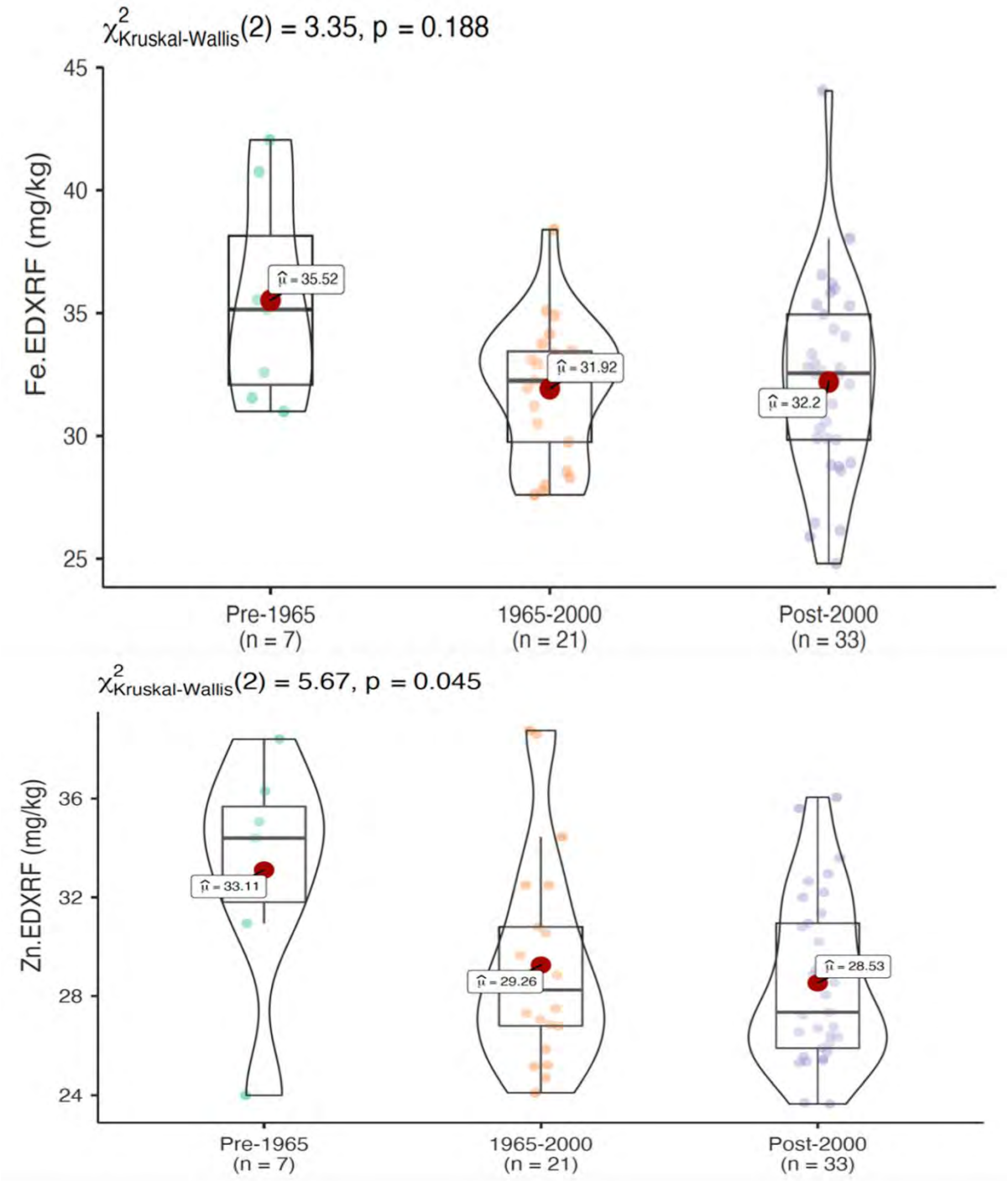

$\chi^2_{Kruskal\text{-}Wallis}(2) = 3.35, p = 0.188$
Fe.EDXRF (mg/kg)
45
40
35
30
25
$\hat{\mu} = 35.52$
$\hat{\mu} = 31.92$
$\hat{\mu} = 32.2$
Pre-1965
(n = 7)
1965-2000
(n = 21)
Post-2000
(n = 33)
$\chi^2_{Kruskal\text{-}Wallis}(2) = 5.67, p = 0.045$
Zn.EDXRF (mg/kg)
36
32
28
24
$\hat{\mu} = 33.11$
$\hat{\mu} = 29.26$
$\hat{\mu} = 28.53$
Pre-1965
(n = 7)
1965-2000
(n = 21)
Post-2000
(n = 33)

Fig2.1 b

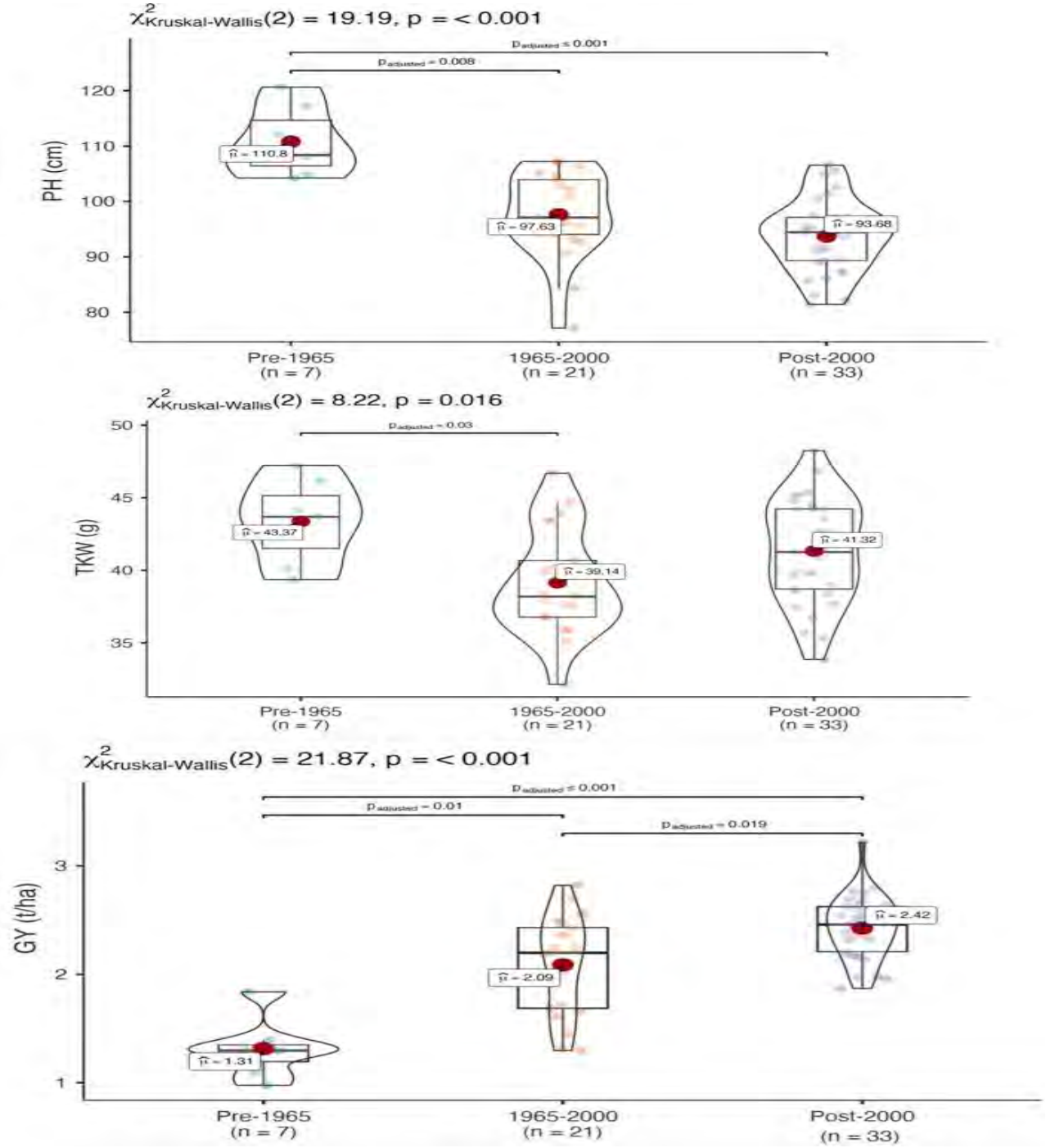


Figure 2.1. Box plots showing the variation among important micronutrients and yield-related traits distributed over three selective breeding periods.**(a)**Box plot showing the variation of Fe. EDXRF (mg/kg) and Zn. EDXRF (mg/kg). (**b**) Box plot showing the variation of PH (cm), TKW(g), GY(t/ha). The significance between mean performances of three breeding periods is shown as *p* value of Kruskal–Wallis test.

Table 2.2. Descriptive statistics of grain mineral elements and morphological traits in sixty-two wheat cultivars classified into three distinct breeding eras.

	Pre-1965 (*n* = 7)				1965–2000 (*n* = 21)				Post-2000 (*n* = 34)				Overall (*n* = 62)			
Traits	**Min**	**Mean**	**Mean**	**CV(%)**	**Min**	**Max**	**Mean**	**CV(%)**	**Min**	**Max**	**Mean**	**CV(%)**	**Min**	**Max**	**Mean**	**CV (%)**
Fe.EDXRF (mg/kg)	31	42	35.5	12.34	27.6	38.4	31.9	8.9	24.8	44	32.2	12.39	24.8	44	32.5	11.66
Zn.EDXRF (mg/kg)	24	38.4	33.1	14.14	24.1	38.8	29.3	14.2	23.6	36	28.5	11.72	23.6	38.8	29.3	13.62
Fe.ICPOES (mg/kg)	30.5	38.6	32.7	8.96	26.9	37.3	32.8	6.83	26.8	37.6	33.7	6.5	26.8	38.6	33.3	6.91
Zn.ICPOES (mg/kg)	18.8	33.1	28.8	16.63	20.4	40.8	29.8	14.7	23.9	37.2	28.5	11.82	18.8	40.8	28.9	13.43
Al (mg/kg)	2.69	4.78	3.87	21.06	2.44	7.78	4.48	23.21	2.06	4.97	3.58	18.91	2.06	7.78	3.92	23.37
Ca (mg/kg)	453	635	547	11.99	476	701	588	9.73	415	720	563	11.26	415	720	570	10.91
Cu (mg/kg)	3.81	5.28	4.68	10.51	3.25	5.69	4.16	17	3.06	5.47	4.08	14.24	3.06	5.69	4.18	15.22
K (mg/kg)	3591	4118	3892	4.83	3762	4891	4385	6.07	3732	5344	4279	8.72	3591	5344	4271	8.19
Mg (mg/kg)	984	1288	1181	8.64	923	1318	1134	8.06	970	1293	1130	7.22	923	1318	1137	7.69
Mn (mg/kg)	24.4	37.4	32.2	13.45	25.6	37.8	32	9.53	26.3	38.2	32.7	9.88	24.4	38.2	32.4	10.06
Na (mg/kg)	8.75	47.3	28.7	55.4	10	159	43.1	87.01	8.94	79.9	32.1	59.5	8.75	159	35.4	75.42
P (mg/kg)	2752	3349	3108	7.53	2862	3918	3361	7.94	2719	3657	3150	7.71	2719	3918	3217	8.33
Se (mg/kg)	0.199	0.258	0.232	8.84	0.132	0.24	0.199	18.69	0.135	0.26	0.212	14.29	0.132	0.26	0.21	15.71
TPP	3.13	4.25	3.58	12.04	2.83	4.34	3.53	12.32	2.44	4.08	3.23	11.8	2.44	4.34	3.37	12.76
PH (cm)	104	121	111	5.6	77.1	107	97.6	9.54	81.4	107	93.7	11.11	77.1	121	97	8.85
SL	12.8	18.5	15.8	12.72	15.1	22.2	17.2	9.42	14.1	19.2	16.9	6.98	12.8	22.2	16.9	8.70
SNPS	18.1	20.3	19.3	4.13	18.1	20.9	19.7	3.88	17.6	21.1	19.2	4.29	17.6	21.1	19.4	4.27
GPS	39.3	52.3	45.9	11.18	48.6	61.3	53.3	6.42	44.1	64	54.2	8.03	39.3	64	53	9.11
GY (t/ha)	0.975	1.84	1.31	20.76	1.3	2.82	2.09	21.77	1.87	3.22	2.44	12.05	0.975	3.22	2.19	22.74
TKW (g)	39.4	47.2	43.4	6.66	32.1	46.7	39.1	9.26	33.9	48.2	41.3	8.45	32.1	48.2	40.8	9.02
GL (mm)	1.11	1.36	1.25	7.01	1.16	2.04	1.46	14.11	1.16	2.23	1.38	16.09	1.11	2.23	1.39	15.32

	Pre-1965 (*n* = 7)				1965–2000 (*n* = 21)				Post-2000 (*n* = 34)				Overall (*n* = 62)			
Traits	**Min**	**Mean**	**Mean**	**CV(%)**	**Min**	**Max**	**Mean**	**CV(%)**	**Min**	**Max**	**Mean**	**CV(%)**	**Min**	**Max**	**Mean**	**CV (%)**
GW (mm)	1.97	2.44	2.11	7.91	2.15	2.73	2.35	5.62	2.02	2.52	2.27	5.55	1.97	2.73	2.27	6.56
GD (mm)	5.86	6.65	6.16	4.69	5.89	6.65	6.36	3.25	5.77	6.8	6.38	3.65	5.77	6.8	6.34	3.77

Fe calculated by EDXRF, Zn calculated by EDXRF, Fe calculated by ICPOES and Zn calculated by ICPOES. Aluminum (Al), calcium (Ca), copper (Cu), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorous (P) and selenium (Se). Plant height (PH), grains per spike (GPS), thousand kernel weight (TKW), tillers per plant (TPP), spike length (SL), spike number per spike (SNPS), grain number per spike (GpS), grain yield (GY), grain length (GL), grain width (GW), grain diameter (GD).

Analysis of variance (ANOVA) showed variation in nearly all the traits except grain Se contents (Table 2.3). All the traits showed significant variations by genotype, year and genotype x year interaction, with some exceptions. No significant variation was observed by planting year in TPP, PH, TKW, GL, GW, Al, Ca, Cu and Se content (Table 2.3). Among the micronutrients, the heritability for Fe and Zn was 0.62 and 0.68, respectively (Table 2.3). The heritability for important yield-related traits such as TKW, TPP and GY was 0.66, 0.35 and 0.68, respectively.

Table 2.3. Analysis of variance for morphological traits and micronutrient contents in historical wheat cultivars of Pakistan.

	Replication	Genotype (G)	Year (Y)	G x Y Interaction	
df	**2**	**61**	**1**	**61**	
Traits		**Means Squares**			**Heritability**
TPP	1.162 *	0.7386 ***	0.0413 ns	0.9095 ***	0.35
PH	203.1443 ns	465.0146 ***	256.8787 ns	156.766	0.67
SL	1.2842 ns	8.6695 ***	13.518 *	5.5964 **	0.49
SNPS	222.2401 ***	4.5472 ***	141.0344 ***	1.6726 ns	0.48
GNPS	675.4824 ***	93.4753 ***	598.6102 ***	51.7566 ns	0.52
GY	0.1032 ***	0.9954 ***	2.3278 ***	0.1575 **	0.68
TKW	0.3556 ns	54.2871 ***	0.0429 ns	19.7962 ns	0.66
GL	7.9117 ***	0.2239 ***	0.0032 ns	0.0451 ns	0.80
GW	8.6088 ***	0.1296 ***	0.0147 ns	0.0455 ns	0.65
GD	8.329 ***	0.2752 ***	0.0022 ns	0.0579 ns	0.78
Al	68.6896 ***	3.1559 ***	0.127 ns	0.3507 ns	0.52
Ca	35,341.2148 ***	11,898.9395 ***	81.7116 ns	75.8745 ns	0.86
Cu	8.2654 ***	1.1161 ***	0.5172 ns	0.066 ns	0.72
Fe.EDXRF	4.5506 **	40.2081 ***	0.1301 ns	37.163 ***	0.51
Fe.ICPOES	14.5627 ***	15.3527 ***	120.9392 ***	8.7979 ***	0.62
K	112,509.1797 *	455,115.7812 ***	756,099.6875 ***	113,190.0078 ***	0.79
Mg	6691.2295 *	25,110.6953 ***	52,088.6211 ***	8330.1592 ***	0.73
Mn	5.0568 *	35.1404 ***	5.8521 *	11.7127 *	0.74

	Replication	Genotype (G)	Year (Y)	G x Y Interaction	
df	**2**	**61**	**1**	**61**	
Traits		**Means Squares**			**Heritability**
Na	23.8786 ns	2031.3976 ***	6190.4634 ***	2507.9097 ***	0.43
P	49,623.0234 *	205,982.9375 ***	390,194.75 ***	120,418.9297 ***	0.61
Zn.EDXRF	11.2164 **	39.5774 ***	11.9913 *	34.2843 ***	0.52
Zn.ICPOES	8.4438 **	41.8175 ***	594.0781 ***	18.7137 ***	0.68
Se	7.3049 ***	0.045 ns	0.0319 ns	0.0518 ns	0.0057

*, significant ($p < 0.05$); **, significant ($p < 0.01$); ***, significant ($p < 0.001$); ns, non-significant ($p > 0.05$).

Among the micronutrients, two methods were used to phenotype GFe and GZn. GFe ranged between 24.8 (Auqab-2000) and 44.0 mg/kg (Zincol-16), with an average of32.5 mg/kg using EDXRF, while it ranged from 26.8 to 38.6 mg/kg with an average of33.3 mg/kg with ICP-OES (Table 2.2). The correlation coefficient between the two methods was r = 0.53 (Figure 2.2). Similarly, the GZn content ranged between 23.65 (Auqab-2000) and38.8 mg/kg (WL-711) with a mean value of 29.30 mg/kg by EDXRF and ranged from 18.4 (Rawal-87) to 40.8 mg/kg (Pothowar-70) with an average of 28.9 mg/kg by ICP-OES. The correlation coefficient between the two methods was r = 0.82 (Figure 2.2). Other important micronutrients such as Ca ranged from 415 to 720 mg/kg with an average of 570 mg/kg. Similarly, Mn ranged between 24.4 and 38.2 mg/kg with an average of 32.4 mg/kg (Table 2.2).

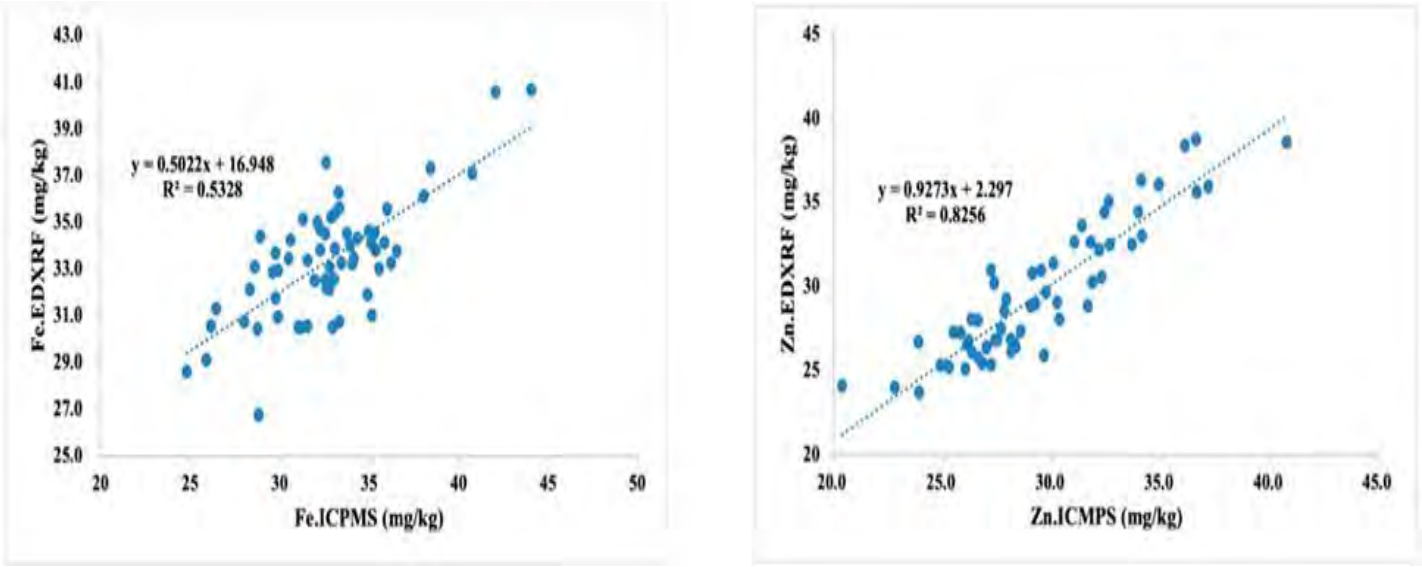


Figure 2.2. Coefficient of determination between two ICP-OES and EDXRF methods to predict grain iron (GFe) and zinc (Zn) in historical wheat cultivars released between 1911 and 2016.

2.3.2 Correlation between Traits and Multivariate Analysis

The coefficient of correlation is reported in Table2.4between all morphological traits and micronutrients. The coefficient of correlation between GFe and GZn was positive with r = 0.31. GFe had a strong positive correlation with Ca (r= 0.28), Mg (r = 0.35), Mn (r = 0.3) and Se (r = 0.26), while its correlation was non-significant with any morphological trait. GZn had a relatively higher correlation with Ca (r = 0.61), Cu (r = 0.6), K (r = 0.4), Mg (r = 0.74) and P (r = 0.63), while GZn had a strong negative correlation with GD (r = 0.48). GY had a negative correlation with TPP (r =0.3) and a positive correlation with GPS (r = 0.4). The highest correlation among the morphological traits was between SL and SNPS (r = 0.46).

The PCA biplot clearly separated the cultivars into three groups consistent with the three breeding eras defined previously (Figure 2.3a). The first two principal components explained 19 and 12.1 % of the total

variation. The pre-1965 cultivars were separated on the lower side of the PC2 in admixture, containing cultivars Chakwal-86 and Rawal-87. The dendrogram showed two major clusters, cluster I and cluster II. Cluster I consisted of 20 cultivars mostly released after 1965, except Dirk, while cluster II consisted of 42 cultivars and was further subdivided into three subclusters. The clustering was consistent with the breeding eras except that C- 273 was in admixture with some modern cultivars (Figure 2.3b). The dendrogram generated from the genome wide SNP marker also corroborated the diversity pattern showed by the phenotypic analysis (Figure 2.3 c).

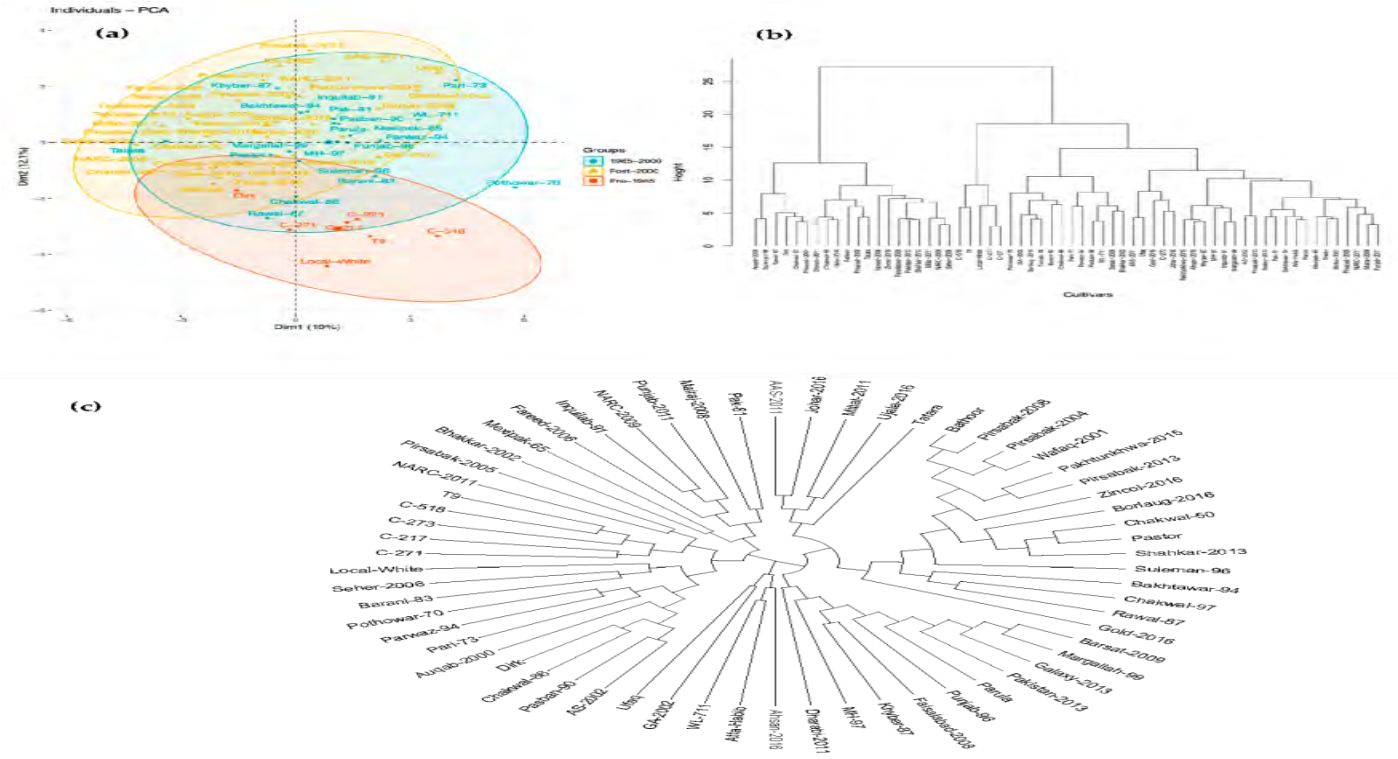


Figure2.3. Multivariate analysis of historical wheat cultivars based on morphological traits and micronutrients, and genome-wide SNP markers. (**a**) Principal components analysis-based biplot showing scattering of wheat cultivars grouped based on three breeding periods, (**b**) dendrogram showing clustering of wheat cultivars based on morphological traits and micronutrients, (**c**) dendro- gram showing clustering of wheat cultivars based on genome-wide SNP markers.

Table2.4. Coefficient of correlation between morphological traits and grain micronutrients in historical wheat cultivars of Pakistan.

Traits	Fe.ICPOES (mg/kg)	Zn.ICPOES (mg/kg)	Al (mg/kg)	Ca (mg/kg)	Cu (mg/kg)	K (mg/kg)	Mg (mg/kg)	Mn (mg/kg)	Na (mg/kg)	P (mg/kg)	Se (mg/kg)	TPP	PH (cm)	SL (cm)	SNPS	GPS	GY (t/ha)	TKW (g)	GL (cm)	GW(cm)
Zn.ICPOES (mg/kg)	0.313 *	—																		
Al (mg/kg)	−0.041	0.166	—																	
Ca (mg/kg)	0.278 *	0.613 ***	0.234	—																
Cu (mg/kg)	0.197	0.601 ***	0.359 **	0.378 **	—															
K (mg/kg)	0.149	0.399 **	0.13	0.551 ***	0.188	—														
Mg (mg/kg)	0.354 **	0.747 ***	0.058	0.521 ***	0.536 ***	0.278 *	—													
Mn (mg/kg)	0.299 *	0.24	−0.121	0.226	0.088	0.042	0.412 ***	—												
Na (mg/kg)	−0.01	0.299 *	0.286 *	0.253 *	0.331 **	0.38 **	0.321 *	−0.171	—											
P (mg/kg)	0.126	0.637 ***	0.195	0.498 ***	0.417 ***	0.555 ***	0.667 ***	0.353 **	0.279 *	—										
Se (mg/kg)	0.26 *	0.373 **	0.132	0.402 **	0.42 ***	0.174	0.536 ***	0.296 *	0.302 *	0.101	—									
TPP	−0.134	0.11	0.355 **	−0.083	0.315 *	0.027	0.043	−0.071	0.281 *	0.203	−0.024	—								
PH (cm)	−0.093	0.114	−0.004	−0.094	0.291 *	−0.317 *	0.114	0.053	0.051	0.004	0.131	0.296 *	—							
SL (cm)	0.156	0.191	−0.019	0.187	−0.018	0.118	0.09	0.084	−0.02	0.282 *	−0.127	0.181	0.252 *	—						
SNPS	−0.041	0.153	0.207	0.078	0.114	0.019	−0.07	−0.055	0.059	0.148	−0.059	0.359 **	0.344 **	0.459 ***	—					
GPS	0.113	0.012	−0.046	0.02	−0.065	0.211	−0.137	0.103	−0.069	0.123	−0.147	−0.111	−0.09	0.282 *	0.457 ***	—				
GY (t/ha)	0.2	−0.149	−0.354 **	−0.176	−0.462 ***	0.07	−0.227	0.17	−0.169	−0.108	−0.248	−0.329 **	−0.216	0.187	−0.073	0.393 **	—			

Traits	Fe.ICPOES (mg/kg)	Zn.ICPOES (mg/kg)	Al (mg/kg)	Ca (mg/kg)	Cu (mg/kg)	K (mg/kg)	Mg (mg/kg)	Mn (mg/kg)	Na (mg/kg)	P (mg/kg)	Se (mg/kg)	TPP	PH (cm)	SL (cm)	SNPS	GPS	GY (t/ha)	TKW (g)	GL (cm)	GW(cm)
TKW (g)	−0.005	−0.084	−0.152	−0.103	−0.013	−0.093	0.124	0.111	−0.042	−0.117	0.324 *	−0.157	0.175	−0.015	−0.257 *	−0.115	0.011	—		
GL (cm)	0.205	0.072	0.053	0.141	−0.166	0.108	0.113	−0.145	−0.03	0.162	−0.07	−0.108	−0.027	0.195	−0.023	0.061	0.166	−0.128	—	
GW (cm)	0.19	0.129	0.211	0.331 *	0.17	0.503 ***	0.067	−0.238	0.301 *	0.229	0.11	−0.026	−0.185	0.173	0.077	0.232	0.086	−0.124	0.37 **	—
GD (cm)	−0.059	−0.488 ***	−0.081	−0.372 ***	−0.404 **	−0.027	−0.309	−0.044	−0.004	−0.179	−0.246	0.045	0.026	0.179	−0.073	0.259 *	0.359 **	0.304 *	0.215	0.278 *

*, significant ($p < 0.05$); **, significant ($p < 0.01$); ***, significant ($p < 0.001$); ns, non-significant ($p > 0.05$).

2.3.3 Genetic Gain for Micronutrients and Morphological Traits

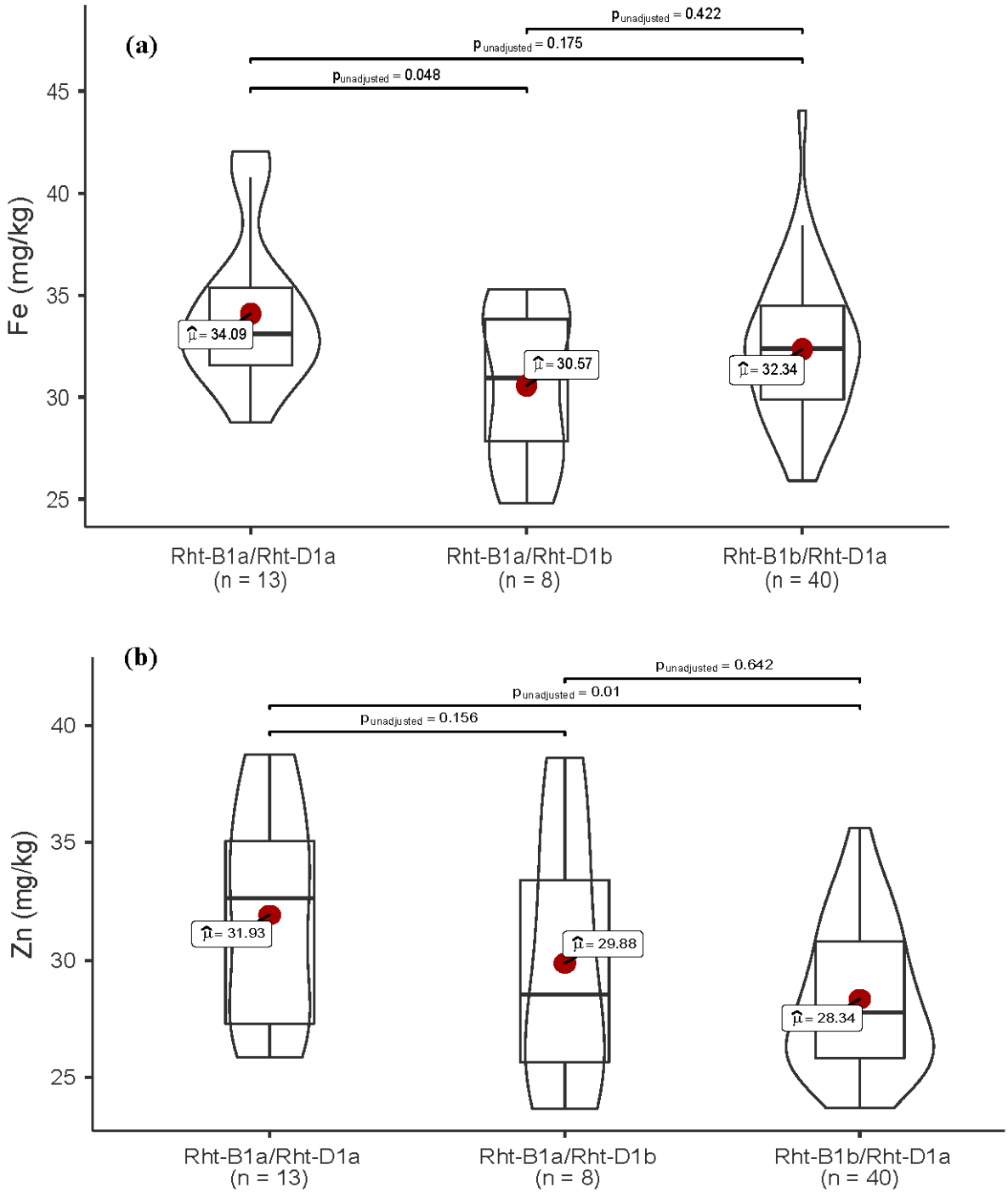
The genetic gain analysis identified the traits that significantly changed with the release year (Table 2.1). Among the morphological traits, there was significant change in TPP, PH and SNPS, which reduced significantly over time, while GY and GpS significantly increased over time. The highest yielding cultivar, Punjab-2011 (3.2 t/ha), yielded almost thrice that of the lowest yielding cultivar T9 (0.97 t/ha). The increase in genetic gain was 0.41% over the period of 105 years, while the increase was highest in the recent period after 2000. The change in TKW, GL and GW remained non-significant over the years. Among the micronutrients, GZn significantly reduced during the course of breeding to0.05 mg/kg/year (0.12 %), while GFe also reduced at the rate of 0.02 mg/kg/year, but the change was non-significant.

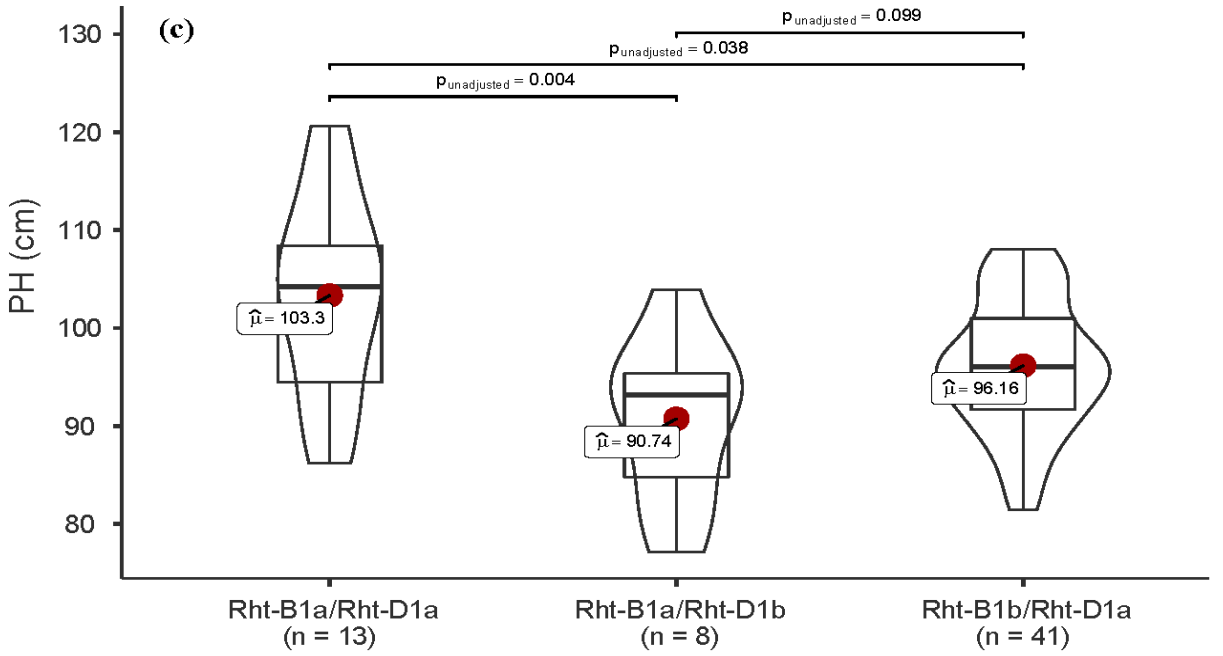
2.3.4 Allelic Variation in Functional Genes and Association with Traits

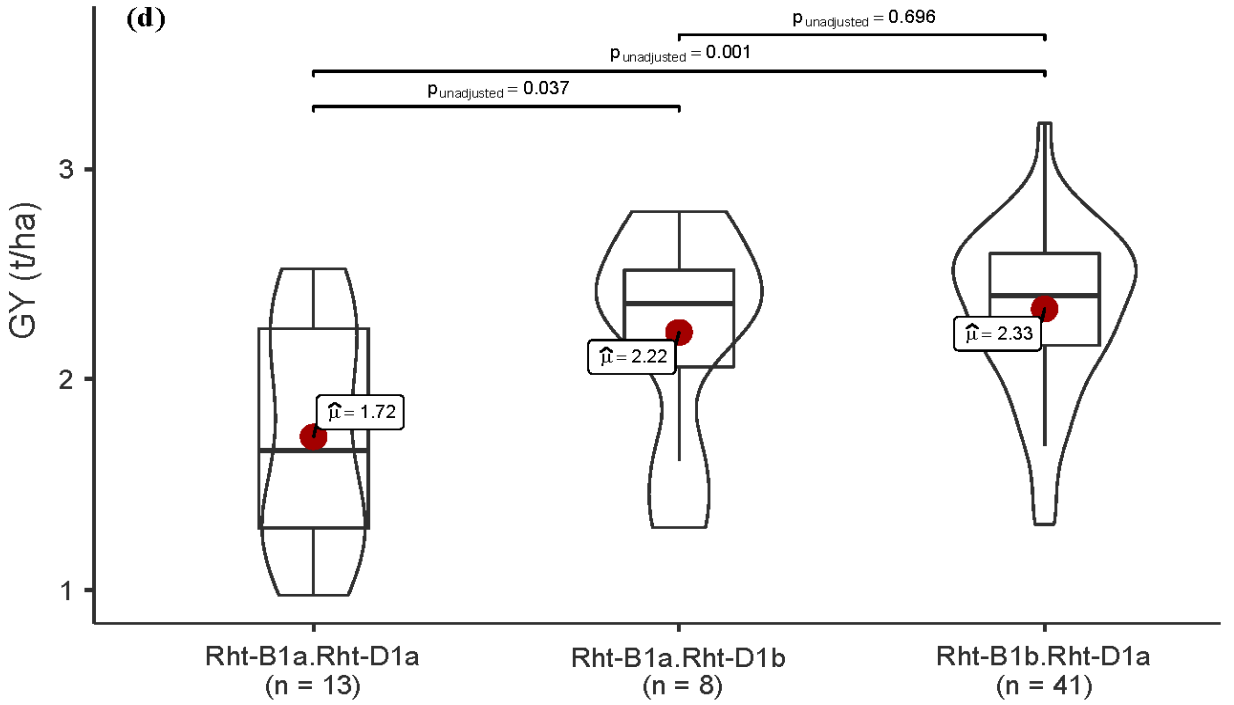
The KASP markers for six genes were used to identify the allelic variation in histor- ical wheat cultivars. The *Rht-B1* and *Rht-D1* genes were combined to identify the *Rht-1* haplotypes in the wheat cultivars. The results reveal that semi-dwarfing alleles, either *Rht-B1b* or *Rht-D1b,* were introduced after 1965 and their frequency was 79 %, compared to the 21 % frequency of the *Rht-B1a/Rht-D1a* haplotype (Table 2.5). Wheat sucrose synthase gene, *TaSus2-2B,* had two haplotypes and haplotype *Hap-L* had a very high frequency of 85.5% compared to 14.5% of the *Hap-H* frequency. Similarly, two grain-width-related genes, *TaGW2-6A* and 6B, were also surveyed. The frequency of haplotypes associated with higher TKW of *Hap-I* was 32 % at *TaGW2-6B*, while the frequency of *Hap-6A-A* was 80.6 %, which was associated with higher TKW. At the *NAM-A1* locus, two haplotypes *NAM-A1b* and *NAM-A1d* had frequencies of 40.3 and 59.7%, respectively. The association of the alleles with phenotypes revealed that *Rht-1* haplotypes had a minor but significant effect on GFe and GZn contents (Figure 2.4). The *Rht-B1a/Rht-D1a* haplotypes had slightly higher GFe and GZn contents compared to haplotypes with any semi-dwarfing allele. Similarly, the effect of *Rht-1* haplotypes was much higher on PH and GY. The presence of *Rht-B1b/Rht-D1a* and *Rht-B1a/Rht-D1b* haplotypes reduced the PH from 103.3 to 96.1 and 90.7 cm, respectively. Contrastingly, these haplotypes significantly improved GY from 1.72 to 2.33 and 2.22 t/ha, respectively. The *TaSus2-2B* haplotype Hap-H had a significant and positive effect on TKW.

Table 2.5. Allelic frequencies in percentages for important functional genes in historical wheat cultivars of Pakistan.

		Cultivar Release Era			
Genes	**Alleles**	**Pre-1965**	**1965–2000**	**Post-2000**	**Overall**
Rht-8	*Rht8*	8.1	30.6	50.0	88.7
	rht-8	3.2	3.2	4.8	11.3
Rht-1	*Rht-B1a/Rht-D1a*	9.7	6.5	4.8	21.0
	Rht-B1a/Rht-D1b	0.0	4.8	8.1	12.9
	Rht-B1b/Rht-D1a	0.0	24.2	41.9	66.1
TaSus2-2B	*Hap-H*	11.3	1.6	1.6	14.5
	Hap-L	0.0	32.3	53.2	85.5
TaGW2-6B	*Hap-I*	1.6	16.1	14.5	32.3
	Hap-II	9.7	17.7	40.3	67.7
TaGW2-6A	*Hap-6A-A*	11.3	24.2	45.2	80.6
	Hap-6A-G	0.0	9.7	9.7	19.4
NAM-A1	*NAM-A1b*	4.8	11.3	24.2	40.3
	NAM-A1d	6.5	22.6	30.6	59.7


(a)
$p_{unadjusted}$ = 0.422
$p_{unadjusted}$ = 0.175
$p_{unadjusted}$ = 0.048
Fe (mg/kg)
45
40
35
30
25
$\hat{\mu}$ = 34.09
$\hat{\mu}$ = 30.57
$\hat{\mu}$ = 32.34
Rht-B1a/Rht-D1a (n = 13)
Rht-B1a/Rht-D1b (n = 8)
Rht-B1b/Rht-D1a (n = 40)
(b)
$p_{unadjusted}$ = 0.642
$p_{unadjusted}$ = 0.01
$p_{unadjusted}$ = 0.156
Zn (mg/kg)
40
35
30
25
$\hat{\mu}$ = 31.93
$\hat{\mu}$ = 29.88
$\hat{\mu}$ = 28.34
Rht-B1a/Rht-D1a (n = 13)
Rht-B1a/Rht-D1b (n = 8)
Rht-B1b/Rht-D1a (n = 40)


(c)
PH (cm)
130
120
110
100
90
80
$p_{unadjusted}$ = 0.099
$p_{unadjusted}$ = 0.038
$p_{unadjusted}$ = 0.004
$\hat{\mu}$ = 103.3
$\hat{\mu}$ = 90.74
$\hat{\mu}$ = 96.16
Rht-B1a/Rht-D1a
(n = 13)
Rht-B1a/Rht-D1b
(n = 8)
Rht-B1b/Rht-D1a
(n = 41)


(d)
GY (t/ha)
3
2
1
$p_{unadjusted}$ = 0.696
$p_{unadjusted}$ = 0.001
$p_{unadjusted}$ = 0.037
$\hat{\mu}$ = 1.72
$\hat{\mu}$ = 2.22
$\hat{\mu}$ = 2.33
Rht-B1a.Rht-D1a
(n = 13)
Rht-B1a.Rht-D1b
(n = 8)
Rht-B1b.Rht-D1a
(n = 41)

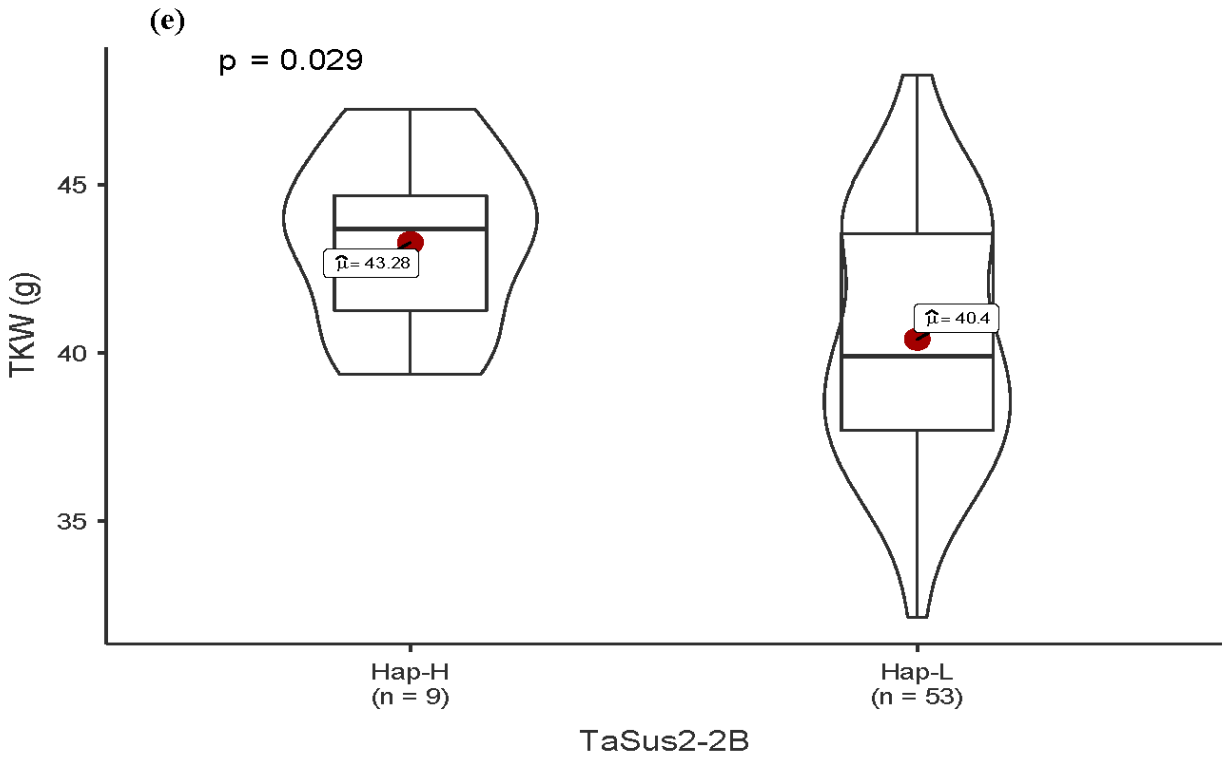


Figure 2.4. Allelic effects of *Rht-1* haplotypes on grain Fe (**a**), grain Zinc (**b**), plant height (**c**), grain yield (**d**) and *TaSus2-2B* gene on thousand kernel weight (TKW) (**e**) in historical wheat cultivars of Pakistan.

2.4 Discussion

A collection of historical wheat cultivars released over a period of 105 years were evaluated for yield-related traits and grain micronutrients. There were significant variations observed for all of the traits which indicated the progress in improving yield metrices over the course of selective breeding. The improvement in grain yield at the rate of 0.4 % per year is relatively low compared to genetic gain in yield in other parts of the world (Calderini and Slafer 1999; Morgounov *et al.,* 2013). The improvement in GY in Siberia over the period 1900 to 2010 was 0.59 % and from 0.58 to 1.25 %, in Great Plains hard winter wheat (Morgounov *et al.,* 2013). While Gao *et al.* (2017) reported a 57.5 kg $ha^{-1}yr^{-1}$gain in GY in Chinese bread wheat cultivars from 1950 to 2012 in the irrigated plain of China. The reason for the slightly slower rate of genetic gain is that the present comparison involved a relatively longer duration of 105 years, and the genetic improvement in yield in Pakistan was not temporally smooth. There were introductions of some high-yielding cultivars such as Pak-81 (1B.1R translocation) in the early 1980s, Inquilab-91 in early 1990s and their derivatives. These cultivars provided sudden increases in GY followed by stagnations in yield for many years. The current data support this hypothesis, showing that the CV (%) of the cultivars released after 2000 was half the CV (%) of the cultivars released in previous periods. This indicated the consistent progress made towards improving GY and the high stability of yield in modern cultivars. Although

significant progress has been made in improving productivity, there was limited progress in improving micronutrients in Pakistan and elsewhere. The cultivars released before the so-called Green Revolution in 1965 had relatively higher levels of GFe and GZn. It has been well established in previous studies that micronutrient concentrations decreased over time in modern wheat cultivars, and this has been validated in US hard winter wheat (Garvin *et al.,* 2006), historical and modern soft white wheat cultivars from US (Murphy *et al.,* 2006) and in a Broad Balk wheat experiment in the UK (Fan *et al.,* 2008). It was concluded that grain mineral concentration remained stable in wheat cultivars from 1845 to 1960s, while it significantly decreased in cultivars afterwards. However, some reports contradict this trend, as observed in the Siberian wheat cultivars where no significant change in mineral concentrations was observed over a period of 110 years (Morgounov *et al.,* 2013). Our results are in complete agreement with these studies, and partially supported by the allelic effect of *Rht-1* haplotypes on GFe and GZn. Previously, analysis of GFe and GZn in several bread wheat and durum wheat near-isogenic lines of *Rht-1* genes revealed that semi-dwarf lines reduced GFe by 3.2 ppm and GZn by 3.9 ppm (Velu *et al.,* 2017). In another set of near-isogenic lines of *Rht-1* genes, semi-dwarf lines showed decreased levels of GFe and GZn, while K and Ca were increased (Jobson *et al.,* 2018). However, no confounding effect of *Rht, Ppd* and *Vrn* genes was found to affect GZn and GFe concentrations in the association mapping panel of Harvest Plus (Velu *et al.,* 2016), which supports our results that such effects could not be significant in natural germplasm.

In this study, the correlation between GFe and GZn was positive, which corroborates with most of the previous findings (Morgounov *et al.,* 2007; Zhang *et al.,* 2010; Guttieri *et al.,* 2015). However, the extent of correlation is highly variable in most of the studies. The positive correlation between GFe and GZn is very useful to identify the common genetic basis to breed for both traits. It was important to observe that grain P was highly positively correlated with most of the micronutrients, including GZn, Ca, Mg, Mn and Se. In wheat grain, P is stored as phytic acid in aleurone, and significantly inhibits the bioavailability of divalent mineral cations (White and Broadley, 2005). Therefore, it is very important to devise biofortification breeding strategies to modify the distribution of P between phytic acid and inorganic P (Guttieri *et al.,* 2015). The principal component analysis suggested that most of the variation was explained by the first two principal components, and PC1 weighted towards the micronutrients with the highest loadings. Therefore, wheat cultivars with high scores for PC1 are likely to have high mineral concentrations, and most of the old cultivars are included in this category. The correlation of GZn with GY was not significant, but Cu and Al had significant negative correlations with GY. This contradicts most of the studies where a significant negative correlation was observed between GY and micronutrients (Morgounov *et al.,* 2007; Guttieri *et al.,* 2015; Zho *et al.,* 2009). However, the insignificant correlation of micronutrients with TKW has been reported elsewhere (Morgounov *et al.,* 2007; McDonald *et al.,* 2008), which corroborates our results. It is

important to carefully assemble the diversity collection for such relationships, as in our case the cultivar collection was from irrigated and rainfed areas from South and Central Pakistan, which have different yield-related attributes, such as TPP and TKW. Historically, the wheat cultivars from South Pakistan have more TKW on an average compared to cultivars from other parts of the country.

The target to increase GZn concentration by 12 mg kg^{-1}, and similarly increase GFe specifically for Pakistan, is very challenging. The approaches needed to enhance the GZn, GFe and other micronutrients will include the introduction diversity from other genetic resources or wild species of Triticeae (Peleg *et al.,* 2009; Calderini and Ortiz, 2003). In most cases, the landraces, synthetic hexaploidy wheat and wild relatives of wheat were identified with higher levels of grain micronutrients (Ortiz *et al.,* 2008; Ortiz *et al.,* 2007; Manickavelu *et al.,* 2017). The conventional breeding approaches have been successfully used to incorporate such diversity into elite germplasm. CIMMYT's biofortification breeding program has developed elite cultivars by targeting crosses between high-yielding germplasm and high-micronutrient germplasm and selecting the desired traits in large population sizes (Velu *et al.,* 2014). This strategy has resulted in the development and release of several cultivars, such as 'Zinc Shakti (Chitra)' in India, WB-02, HPBW 01 (PBW 1 Zn), Zincol-2016 in Pakistan, and BARI-Gom 33 in Bangladesh, having 33–40 % more GZn compared to Czech cultivars (Velu *et al.,* 2015).

Apart from the GFe and GZn, other micronutrients such as Ca, Se and Mn are also important for human health, and breeding for such micronutrients has been largely ignored. Our data show significant variations in other micronutrients. Selenium is an essential micronutrient with antioxidant, anti-cancer and anti-viral effects. In this study, grain Se concentration was positively correlated with GFe, GZn and TKW, and a two-fold increase was observed in some cultivars. Previously, significant variation was observed for Se concentration in bread wheat and related species, and as with other minerals, Se variation was associated with spatial variation in soil Se (Lyons *et al.,* 2005). Similarly, the decrease in Ca concentration in modern wheat cultivars might have adverse health consequences, and biofortification for grain Ca is largely ignored (AlOmari *et al.,* 2017). The correlation was positive between Ca and GFe and GZn and other micronutrients, which suggested common breeding strategies could be devised for simultaneous improvement of these micronutrients in wheat.

2.5 Conclusion

Conclusively, this study provided insight into the mineral status and yield of wheat cultivars historically deployed in Pakistan. Overall, the improvement in GY was not translated into an improvement in micro- and macronutrients. Although GFe (0.06 mg/kg/year) and GZn (0.15 % year) slightly declined in modern wheat cultivars compared to old cultivars, there are some high-yielding cultivars such as Zincol-2016 and AAS-

2011, which have high levels of micronutrients. Elucidating the genetic basis of GY and micronutrient concentrations could help to develop cultivars with both improved yield and biofortification status.

Genome-wide association studies (GWAS) for grain mineral elements in wheat landraces from Pakistan

Abstract

As a staple food crop, wheat is an important source of protein and minerals. To address the problems associated with malnutrition, fundamental cereals such as wheat must be fortified with essential minerals. The study aimed to assess the level of nine micronutrients, including grain iron (GFe) and zinc (GZn), in 188 wheat land races collected from Pakistan. Over the course of two years, field experiments were conducted, and GFe, GZn, and other minerals were measured. The coefficient of variation (CV %) was highest for P contents (58.8 %), and the lowest CV % was observed for oil (17.5 %). The GZn.2019 concentration ranges from 13.5 mg kg^{-1} to 54 mg kg^{-1} and GZn.2020 concentration ranges from 11.8mgkg^{-1} to 54.5 mg kg^{-1}. The GFe.2019 concentration ranges from 8.2 mg kg^{-1} to 56 mg kg^{-1} and GFe2020 concentration ranged from 15 mg kg^{-1} to 60 mg kg^{-1}. The highest correlation among the mineral traits was observed between P and N (r=0.99). All mineral traits in wheat landraces showed higher variation in different provinces of Pakistan. The average fiber contents were highest for landraces from AJK province (1.91 mgkg^{-1}) with a range of 1.87-1.99 mgkg^{-1} and lowest for landraces from Gilgit province (1.17 mgkg^{-1}) with a range of 0.93-1.29 mgkg^{-1}. The mean of GFe was highest in landraces from KPK province (104.16 mgkg^{-1}) with a range of 48-295 mgkg^{-1} and lowest in landraces from AJK province (29.7 mgkg^{-1}) with a range of 15.4-45.7 mgkg^{-1}. Genome-wide association studies (GWAS) using genotyping-by-sequencing (GBS) markers identified maximum number of MTAs (20) for GFe.2019. Minimum number of MTAs (1) had identified for B, Cu, and Na contents. Maximum number of MTAs were identified on chr1A (11), followed by 3B (10), chr6D and chr5B had minimum number of MTAs (2) (1) respectively. MTAs were commonly identified for ash, moisture and GZn 2019, N, P, GFe 2019, B contents. This study explored the potential of mineral elements in wheat landraces from Pakistan and identified the quantitative genetic framework underpinning biofortification in spring wheat landraces.

3.1 Introduction

Wheat provides 60 % of the world's daily caloric intake, making it the most important food crop in the world (Cakmak *et al.,* 2008). Wheat contains not only low levels of grain iron (GFe) and zinc (GZn) but is also rich in chemicals that reduce the availability of essential nutrients in the body, such as phytate and fiber (Cakmak *et al.,* 2008). Malnutrition (hidden hunger) in the world's population is increasing rapidly, with about 30 % of the world's population suffering from iron and zinc deficiency (Majumder *et al.,* 2019). Micronutrient deficiencies not only increase mortality rates but also reduce children's mental health, national growth rates, and the quality of life of developing countries (Welch and Graham, 2004). Micronutrient deficiency is expected to escalate into more severe countries in developing countries due to excessive consumption of food with a limited variety of diets, which usually have one or two basic foods (Welch and Graham, 2004). Because of poverty, the developing world relies heavily on cereals as a source of energy and less nutritious protein compared to animal Feed (Cakmak *et al.,* 2008).

Wheat micronutrient variability, such as GFe and GZn, is mostly genetic, with a complex polygenic regulatory system (Srinivasa *et al.,* 2014). This makes increasing some features through conventional breeding difficult (Velu *et al.,* 2012). MARS (marker-assisted recurrent selection) and MABC (marker-assisted backcrossing) are potentially feasible options. To discover marker-trait connections, these methodologies would involve linkage-based interval mapping and LD-based genome wide association analyses (GWAS). This will also aid in interpreting the characteristics' genetic architecture (Tiwari *et al.,* 2009). Several micronutrient QTL interval mapping experiments in wheat has previously been done (Sharma *et al.,* 2018). GWAS has been conducted on rice, pea, maize, barley, chickpea, and cassava (Rabbi *et al.,* 2017).

Understanding the genetic basis of GFe and GZn concentration in wheat grains is essential for increasing wheat's biofortification potential. The genome-wide association study (GWAS) approach, which is one of the main approaches for dissecting complex traits like nutritional quality traits that are controlled by many genes and influenced by the environment, is one of the main approaches for dissecting complex traits like nutritional quality traits that are controlled by many genes and influenced by the environment (Alomari *et al.,* 2018). GWAS provides a number of advantages over traditional QTL mapping, including better QTL resolution, allele coverage, and the capacity to use large collections of natural germplasm

resources including landraces, elite cultivars, and advanced breeding lines. Only a few studies in wheat have used GWAS to look into the genetics of end-use quality variables (Battenfield *et al.,* 2018). However, the great majority of GWAS studies to date have focused on a single gene and a single variable, offering minimal information. Despite the fact that epistasis has been investigated in only a few GWAS studies, epistasis is commonly missed in GWAS (Sehgal *et al.,* 2017). For GWAS, MLMM (multi locus mixed model) and MTMM (multi trait mixed model) have recently become accessible, which alleviate the before mentioned limits of genetic analysis (Thoen *et al.,* 2017). MVLMM has also been utilized in multi-trait analysis to evaluate more than two related attributes (Furlotte and Eskin, 2015). To date, several QTL studies have been done in an attempt to uncover the genetic basis of wheat grain GZn and GFe contents (Crespo-Herrera *et al.,* 2017). Wheat molecular breeding requires an understanding of the genetic variables that determine GFe and GZn concentrations.

The QTL analysis is a useful tool for determining which genes are responsible for natural variations in GFe and GZn concentrations (Ghandilyan *et al.,* 2006). Using recombinant inbred lines or doubled haploid populations, several QTLs influencing micronutrient content in wheat grain have been found in recent years (Peleg *et al.,* 2009). Advances in QTL mapping will help researchers better understand the genetic basis for micronutrient concentrations in wheat grains. In a tetraploid wild emmer durum wheat recombinant inbred lines (RILs) population, Peleg *et al.* (2009) revealed five QTLs for GFe concentration on chromosomes 2A, 3B, 5A, 6B, and 7A. In a *Triticum spelta* x *T. aestivum* RIL population, another study revealed five QTLs that support GFe, three of which mapped to chromosome 1A and two to chromosomes 2A and 3B. (Srinivasa *et al.,* 2014). On synthetic wheat lines, two GWAS studies have been reported. Gorafi *et al.* (2018) studied GFe content in 47 synthetic hexaploid wheat germplasm lines, whereas Bhatta *et al.* (2018) performed GWAS for multiple grain minerals, including GFe, on 123 synthetic hexaploid wheat lines.

The creation of innovative spring bread wheat genotypes with broad adaptability, high yields, high grain GZn content, better processing quality, disease resistance, and stress tolerance is a top priority for CIMMYT's biofortification breeding program. 30–40 % more GZn has been introduced into CIMMYT-derived high yielding wheat genotypes as a consequence of targeted breeding for improved nutritional quality (Velu *et al.,*2015). Kumar *et al*. (2018) identified 784 MTAs in 246 wheat genotypes using 17,937 SNP markers, yielding the following findings after Bonferroni correction using four methods: (1) A single locus single trait analysis revealed 136 marker-trait associations (MTAs); (2) multi-locus mixed model

yielded 587 MTAs; (3) multi-trait mixed model yielded 28 MTAs; and (4) matrix-variate linear mixed model yielded 33 MTAs. Using GWAS research on 330 wheat lines, Alomari *et al.* (2019) discovered 39 MTAs for grain Zn. On chromosomes 2 and 7, two greater impact QTL areas were discovered. In a panel of 369 European elite wheat types, GWAS identified 41 and 137 significant SNPs, respectively, including significant marker-trait associations (MTAs) for best linear unbiased estimates (BLUEs) of GFeC on chromosomes 2A, 3B, and 5A. (Velu *et al.,* 2018). QGZn.cimmyt-7B 2P1, QGZn.cimmyt-7B 1P1, QGZn.cimmyt-7B 1P2, QGZn.cimmyt-7B 2P2, QGZn.cimmyt-7B 2P2, and QGZn.cimmyt-7B 3P2 were discovered at physical positions of 139.4–160.6, 158.3–159.2, 485.8–506.4. On this chromosome, Crespo-Herrera *et al.* (2017) discovered two GZn QTL. QGZn.cimmyt-3B 2P2 was connected to DArT markers 4394657 at 32.6 Mb, while QGZn.cimmyt-3B 1P2 was bordered by 3533713 and 1007339 at 32.6 Mb. Wang *et al.,* (2021) also discovered a QTL for GZnin 254wheat RILs on chromosome 3BS, flanked by AX-110975262 and AX-109911679 at physical locations of 42.5 and 59.1 Mb, from a hybrid Jingdong 8/Bainong AK58. QGZn.co-3B was mapped flanked by DArT markers 1002594|F|0 and 1103633 at physical locations of 104.5 and 128.6 Mb, respectively, by Liu *et al.* (2019). Peleg *et al.* (2009) found a QTL on chromosome 3B at a physical position of 150 Mb that is closely connected to Xgwm1266.

The so-called Green Revolution has had a detrimental influence on grain quality, including micronutrients and very low GFe and GZn concentrations of 26-41 and 19-60 mg kg^{-1} respectively (Paltridge *et al.,* 2012). Wheat bread biofortification necessitates a considerable genetic variety of micronutrient particles in focused breeding systems (Ortiz-Monasterio *et al.*, 2007). Because of the positive link between the focus of GFe and GZn, both of these genes can be absorbed into new species at the same time (Monastery and Graham, 2000). The world's biofortification breeding techniques have been constrained by the modest genetic diversity in farmed wheat (Cakmak *et al.,* 2004).

Wild wheat germplasm and other discoveries, on the other hand, constitute a significant source of untapped genetic variety in terms of GFe and Zn grain content that may be exploited efficiently in biofortification for particular crops (Strobbe *et al.,* 2018). *Triticum dicoccoides, Aegilops tauschii, Triticum monococcum,* and *Triticum boeticum* are some of the prospective sources of high GFe and GZn grain concentrations ranging from 35 mg kg-1 to 85 mg kg-1 and 40 mg kg-1 to 90 mg kg^{-1}, respectively (Cakmak *et al.,* 2004 and Zhang *et al.,* 2014).

Variety with high yields from the CIMMYT-based breeding program have recently been demonstrated to exhibit more genetic diversity in GFe and GZn, as well as more popular processing features, than commercial variety (Guzmán *et al.,* 2014). However, very no information about the genetic areas that govern micronutrient in landraces is available, making molecular breeding methods difficult to implement. The GWAS investigation might be a potential technique to decode the locus traits that naturalists are interested in (Huang *et al.,* 2015). The study objective was (1) to analyze the genetic architecture of micronutrients using a GWAS technique, and (2) study the natural phenotypic variation of wheat GFeC and GZnC, as well as other minerals, in a panel of 188 wheat landraces growing for two years in the field.

3.2 Materials and Methods

3.2.1 Plant material and Field Trials

The study was conducted on a collection of 188 wheat landraces from Pakistan. The passport information of landraces is given in Annexure1.The seeds were obtained from the National Gene Bank at Plant Genetic Resources Institute (PGRI), National Agricultural Research Centre in Islamabad, Pakistan. The landraces were assessed in the field for two years, 2018–2019 (later as 2018) and 2019–2020 (later as 2019), at the National Agriculture Research Centre (NARC), Islamabad, Pakistan, using a randomised complete block design (RCBD) with two replications. The NARC location is33◦43′ N 73◦04′ E. In 2018, the sowing date was December 5th, while in 2019, it was December 8th.

3.2.2 Phenotyping

Nine mineral concentrations and five biochemical parameters in the grains of one hundred and eighty-eight wheat landraces were determined from each year and each replicate. Samples of ripe wheat seeds were coarsely crushed by hand in the lab, and grain mineral contents were measured. A 0.5 g dry matter basis subsample of crushed wheat grain was dissolved in 10 mL concentrated nitric acid and heated at reflux. After dissolving, 10 mL concentrated perchloric acid was added and heated until the nitrous gas generation stopped. The solution was placed into a 50 mL volumetric flask, which was then filled with deionized water to the top. All of the chemicals used in the sample analysis were of the greatest purity possible. The glassware was cleaned before use by soaking it overnight in a 10 % v/v HNO3 solution (Suprapur, Merck) and then washing it with water. The mineral elements (N, P, K, B, Cu, Mn, Na, GFe, and GZn) were determined using anatomic absorption (ANA 135,

Tokyo Photoelectric, Tokyo, Japan) at the University of Agriculture Faisalabad, Okara campus. Atomic absorption was performed in accordance with AOAC method 990.23 (Poitevin *et al.*, 2016). This approach has the benefit of allowing the mineral concentration to be determined directly (Chen *et al.,*2005). High-quality chemical standards were used to determine the Na and K levels. Calibrants of the highest-grade degree were employed. Calibration curves were produced with standard metal solutions in the same acid matrix for quantification. Each measurement was repeated twice, with the average taken. The sample's absorption signal was determined after removing the blank's mean value.

3.2.3 DNA extraction and GBS

DNA Using a modified cetyl trimethyl ammonium bromide method, each sample's two-week-old seedlings were extracted (Saghai-Maroof *et al.,* 1984). The DNA concentration was measured using the Quant-iT Pico Green ds DNA Assay (Life Technologies Inc.), which was then standardised to 20 ng/ul for library construction. The GBS libraries were created in the same way that Poland and colleagues did (2012). PstI and MspI (both from New England Bio Labs Inc.) were used to digest genomic DNA, and barcoded adapters were ligated to each DNA sample with T4 ligase (New England Bio Labs Inc.). All of the ligated products from each plate were pooled and cleaned using the QIA quick PCR Purification Kit. Primers that were complementary to both adaptors were used in the polymerase chain reaction (PCR). Using the Bio analyzer 7500 Agilent DNA Chip, the PCR products were cleaned and quantified (Agilent Technologies, Inc.). After size selection for 250–300 bp fragments in an E-gel system (Life Technologies Inc.), the concentration of each library was determined using the Qubit 2.0 fluoro metre and the Qubit ds DNA HS Assay Kit (Life Technologies Inc.). The size-selected library was sequenced using an Ion Proton sequencer (Life Technologies Inc.). Before being sorted into sequence tags, sequence readings were reduced to 64 bp. Internal alignment of the unique tags allowed for the detection of 3 bp mismatches in order to find single nucleotide polymorphisms (SNPs) inside the tags. Single nucleotide polymorphisms were identified using the Universal Network Enabled Analysis Kit GBS process (Lu *et al.,* 2013). TASSEL 4.0, a bioinformatics analysis software, includes it (Bradbury *et al.,* 2007). Reads with a poor-quality score (15) were discarded, and SNPs with heterozygosity 10 %, a minor allele frequency (MAF) >1 %, and missing data 20 % were utilised for further analysis. BLASTn was used to match sequence reads to the International Wheat Genome Sequencing Consortium (IWGSC) reference genome sequence (Version 1.0) (IWGSC, 2018).

3.2.4Statistical Analysis

The essential statistics for allele frequency, heterozygosity, and genetic diversity were computed using TASSEL (Version 5.0). The technique of neighbor-joining (Saitou and Nei, 1987) was used to create a diversity tree, which relaxes the premise of equal mutation rates throughout space and time, resulting in an unrooted tree. The findings were reported as percentages at the major nodes of each branch, and the confidence interval for the genetic linkages between the accessions was estimated using 1,000 bootstraps. A model-based Bayesian cluster analysis on population structure was performed using STRUCTURE (Version 2.3.4). (Pritchard *et al.,* 2000). With a burn-in period of 10,000 steps, 10,000 Monte Carlo steps, and an admixture model, the structural analysis was done ten times for each K value (probability of best fit into each number of assumed clusters) (K = 1–8). All of the settings were set to the manufacturer's recommended defaults (Pritchard *et al.,* 2010). The rate of change in the log probability of data between successive K values was used to estimate K using an ad hoc statistic K. (Evanno *et al.,* 2005). PLINK 1.9 (https://www.cog-genomics.org/plink2; Chang *et al.* (2015)) was used to look at linkage disequilibrium (LD) in different groups of landraces. Pairwise r2 values versus genetic distance were displayed in a 1-kb frame, and a locally weighted polynomial regression (LOESS) curve was fitted using R software. The pattern of LD decay was determined using the genetic distance at which the LOESS curve intersects the r2 threshold of 0.1. The GWAS was conducted using phenotypic data as response variables and a recently developed model selection technique, the fixed and random model circulating probability unification (Farm CPU) Liu *et al.* (2016)

(The FarmCPU deals for the confounding problem between factors and test markers by using a fixed effect model (FEM) and a random effect model) (REM). The first five primary components computed by TASSEL were used as covariates. The default p-value criteria in Farm CPU was a.01 Bonferroni-corrected threshold. The Bonferroni-corrected threshold was too restrictive when the LD across genotypic markers was large, thus the threshold was calculated using the formula p =.05/number of markers with 1,000 permutations. In this function, the phenotypes were permuted to break the relationship between genotypes and phenotypes. For p.threshold, the Farm CPU model recommended the 95 percent quantile value of a vector of minimum p-values from each trial. For the supplied attributes, the Farm CPU established a threshold of 4.68, which was used to build marker-trait links (MTAs). The model's fitness to population structure was assessed using the quantile–quantile (Q–Q) plot.

3.3 Results

The wheat landraces showed significant variations for biochemical and mineral traits evaluated in this study.

3.3.1 Variation in Mineral and Biochemical Traits

All one hundred and eighty-eight wheat land races show significant variation in micronutrient and biochemical traits. Descriptive statistics for all morphological and traits and micronutrients are described in Table 3.1. The coefficient of variation was highest for P contents (108.8 %), and the lowest CV % was observed for oil (17.5 %). The coefficient of variation was highest for GFe.2019 (90.5 %), and the lowest CV % was observed for GZn.2020 (22.9 %). The GZn.2019 concentration ranges from 13.5 mg $kg^{-1}$to 54 mg $kg^{-1}$and GZn.2020 concentration ranges from 11.8 mg kg^{-1} to 54.5 mg kg^{-1}. There is no significant increase observed in GZn concentration in wheat landraces between years 2019 to 2020. The GFe.2019 concentration ranges from 8.20 mg $kg^{-1}$to 56 mg $kg^{-1}$and GFe.2020 concentration ranges from 15 mg $kg^{-1}$to 60mg kg^{-1}.

3.3.2. Variation for Grain Mineral Elements in Landraces from Different Provinces

All biochemical and mineral traits of wheat land races show higher variation across different provinces of Pakistan. The mean of fiber was highest in AJK province (1.91 mg kg^{-1}) with a range of 1.87-1.99 mg kg^{-1} and lowest in Gilgit province (1.17 mg kg^{-1}) with a range of 0.93-1.29 mg kg^{-1}. The mean of oil contents was highest in Punjab province (1.93mg kg-1) with a range of 1.38-2.49 mg kg^{-1} and lowest in Gilgit province (1.62 mg kg^{-1}) with a range of 1.19-1.99 mg kg^{-1}. The mean of moisture was highest in AJK province (7.88 mg kg^{-1}) with a range of 7.65-8.1 mg kg^{-1} and lowest in Baluchistan province (7.32 mg kg^{-1}) with a range of 6-8.4 mg kg^{-1}. The mean of ash was highest in Baluchistan province (1.80 mg kg^{-1}) with a range of 0.90-6.85 mg $kg^{-1}$and lowest in AJK province (1.28 mg kg^{-1}) with a range of 1.20-1.33 mg kg^{-1}. The mean of protein was highest in Sindh province (13 mg kg^{-1}) with a range of 10.69-15.04 mg $kg^{-1}$and lowest in AJK province (11.51 mg kg^{-1}) with a range of 10.59-12.97 mg kg^{-1}. The mean of GFe was highest in KPK province (104.16 mg kg^{-1}) with a range of 48-295 mg kg^{-1} and lowest in AJK province (29.7 mg kg^{-1}) with a range of 15.4-45.7 mg kg^{-1}. The mean of Zn was highest in Gilgit province (34.85 mg kg^{-1}) with a range of 25.6-45.6 mg $kg^{-1}$and lowest in Sindh province (26.96 mg kg^{-1}) with a range of 20-33.5 mg kg^{-1}. The mean of N was highest in Sindh province (2.27 mg kg^{-1}) with a range of 1.87-2.64 mg kg^{-1} and lowest in AJK province (2.02 mg kg^{-1}) with a range of 1.85- 2.27 mg kg^{-1}. The mean of P was

highest in Gilgit province (0.31 mg kg^{-1}) with a range of 0.24-0.43 mg $kg^{-1}$and lowest in KPK province (0.21 mg kg^{-1}) with a range of 0.13-0.31 mg kg^{-1}. The mean of K was highest in Baluchistan province (0.61 mg kg^{-1}) with a range of 0.34-0.88 mg kg^{-1} and lowest in Punjab province (0.46) mg kg^{-1} with a range of 0.3-0.58 mg kg^{-1}. The mean of B was highest in Gilgit province (2.80mg kg^{-1}) with a range of 1.48-3.23 mg kg^{-1} and lowest in Sindh province (2.04mg kg^{-1}) with a range of 0.85-3.58 mg kg^{-1}. The mean of Cu was highest in Gilgit province (4.50mg kg-1) with a range of 1.2-8.5 mg kg^{-1} and lowest in KPK province (2.54mg kg^{-1}) with a range of 1.78-3.6mg kg^{-1}. The mean of Mn was highest in Punjab province (32.32mg kg^{-1}) with a range of 10.4-146.2 mg kg^{-1} and lowest in Sindh province (24.56mg kg^{-1}) with a range of 18.2-36.2mg kg^{-1}. The mean of Na was highest in Punjab province (0.04mg kg^{-1}) with a range of 0.02-0.08 mg kg^{-1} and lowest in AJK province (0.02mg kg^{-1}) with a range of 0.02-0.04mg kg^{-1}.

3.3.3 Pearson Correlation Coefficient

The coefficient of correlation is reported in Table 3.3 between all biochemical traits and micronutrients. Grain moisture has strong positive correlation with oil (r= 0.24), while its correlation was non-significant with any biochemical trait. Grain ash has negative correlation with oil (r=-0.26) and moisture (r=-0.94). Grain protein has strong positive correlation with oil(r=0.28), moisture (r=0.95) and strong negative with ash (r=-0.92). Grain N has strong positive correlation with ash (r=0.94) and has strong negative correlation with oil (r=-0.26), moisture (r=-0.97), protein (r=-0.94). Grain P has strong positive correlation with ash(r=0.94), moisture(r=0.99) and has strong negative correlation with oil (r=-0.27), moisture (r=-0.97), protein (r=-0.95). Grain K has strong positive correlation with protein (r=0.65), moisture (r=0.65) and has strong negative correlation with ash (r=-0.63), N (r=-0.66),P (r=-0.67). Grain B has strong positive correlation with protein (r=0.67), moisture(r=0.71), K (r=0.45) and has strong negative correlation with ash (r=-0.65), N (r=-0.68), P (r=-0.66). Cu has negative correlation with oil (r=-0.21). The highest correlation among the biochemical and mineral traits was between P and N (r=0.99). The other trait has Hanson-significant correlation.

Table 3.1. Descriptive statistics of biochemical and mineral parameters of wheat land races 2019-2020.

	Fiber	**Oil%**	**Moisture**	**Ash**	**Protein**	**N**	**P**	**K**	**B**	**Cu**	**Mn**	**Na**	**Zn.2019**	**Zn.2020**	**GFe.2019**	**GFe.2020**	
Mean	1.33	1.77	5.87	3.23	9.40	4.95	0.793	0.482	1.66	3.17	29.3	0.03	30.2	30	61.1	80.5	
Median	1.30	1.78	7.20	1.64	11.6	2.29	0.315	0.480	1.52	2.80	26.8	0.03	29.6	30	47.9	66.5	
S.D	0.333	0.315	2.48	2.69	5.05	4.62	0.860	0.162	1.03	1.52	22.3	0.01	7.22	6.87	55.3	103	
Minimum	0.643	0.935	1.21	0.806	0.772	1.25	0.100	0.205	0.330	1.00	7.80	0.02	13.5	11.8	8.20	15	
Maximum	1.87	2.49	8.50	8.15	16.2	16.2	2.84	0.880	3.78	9	0.08	0.08	54	54.5	56	60	
CV%	24.81	17.51	42.24	83.28	53.73	105.23	108.86	33.33	62.04	47.94	76.10	33.33	23.90	22.9	90.50	127.9	

(%): Percentage; CV: Coefficient of variation; S.D: Standard deviation

Table 3.2. Descriptive statistics of biochemical and mineral parameters of wheat land races across provinces of Pakistan.

	Punjab (n=53)		**Sindh (n=9)**		**KPK (n=6)**		**Balochistan (n=70)**		**AJK (n=3)**		**Gilgit (n=12)**	
	Mean± S.D	Range	Mean± S.D	Range	Mean± S.D	Range	Mean± S.D	Range	Mean± S.D	Range	Mean± S.D	Range
Fiber	1.56 ±0.38	0.93-2.35	1.18±0.34	0.94-1.87	1.19±0.28	0.94-1.70	1.33±0.32	0.64-2.11	1.91±0.07	1.87-1.99	1.17±0.16	0.93-1.29
Oil%	1.93± 0.23	1.38-2.49	1.62±0.26	1.19-1.99	1.71±0.24	1.36-2	1.81±0.26	1.19-2.34	1.75±0.53	1.35-2.36	1.89±0.29	1.21±2.27
Moisture	7.34± 0.43	6.1-8.5	7.46±0.26	7- 7.9	7.53±0.14	7.4-7.8	7.32±0.46	6-8.4	7.88±0.22	7.65-8.1	7.45±0.35	6.5-7.9
Ash	1.35 ± 0.14	1.14-1.92	1.39±0.31	1.20-1.94	1.48±0.27	1.231.94	1.80±1.01	0.90-6.85	1.28±0.07	1.20-1.33	1.53±0.47	0.77-2.45
protein	12.69 ± 0.85	10.6-14.03	13±1.12	10.69-15.04	12.72±2.07	11.51-16.92	12.23±1.67	7.12-16.83	11.51±1.27	10.59-12.97	12.60±1.99	8.81-16.22
GFe	32.32 ± 23.52	10.4-146.2	72.43±59.91	15.6-224.2	104.16±94.92	48-295	78.23±65.82	24.6-300	29.7±15.22	15.4-45.7	77.20±75.10	18.9-279.6
GZn	30.05 ± 5.37	20-46.4	26.96±3.73	20-33.5	33±10.44	22.6-53	30.29±8.88	13.5-54	28.7± 1.7	27-30.4	34.85±7.75	25.6-45.6
N	2.23 ± 0.15	1.86-2.6	2.27±0.19	1.87-2.64	2.23±0.36	2.02-2.97	2.15±0.30	1.25-2.95	2.02±0.22	1.85-2.27	2.20±0.35	1.54-2.84
P	0.29 ± 0.05	0.18-0.41	0.27±0.06	0.17-0.38	0.21±0.09	0.13-0.33	0.27±0.09	0.1-0.44	0.27±0.02	0.25-0.29	0.31±0.04	0.24-0.43
K	0.46 ± 0.07	0.3-0.58	0.48±0.15	0.35-0.78	0.57±0.16	0.33-0.82	0.61±0.13	0.34-0.88	0.47±0.07	0.4-0.54	0.59±0.09	0.48-0.76
B	2.23±0.75	0.91-3.59	2.04±0.98	0.85-3.58	2.16±0.56	1.64-2.95	2±0.82	0.48-3.5	2.23±0.92	1.2-3	2.80±0.49	1.48-3.28
Cu	2.87±1.23	1.2-7	3.21±1.40	1.89-6	2.54±0.65	1.78-3.6	3.51±1.69	1-9	3.03±0.66	2.6-3.8	4.50±2.17	1.2-8.5
Mn	32.32±23.52	10.4-146.2	24.56±5.21	18.2-36.2	26.16±8.83	9.2-35.4	25.53±6.4	7.8-39.6	26.43±6.28	19.2-30.5	26.69±4.01	21-34
Na	0.04±0.02	0.02-0.08	0.03±0.02	0.02-0.08	0.03±0.01	0.02-0.06	0.03±0.02	0.02-0.08	0.02±0.02	0.02-0.04	0.03±0.02	0.02-0.08

Table 3.3. Correlation between biochemical and mineral parameters of wheat landraces from Pakistan.*p<.05,**p<.01,***p<.00

	Fiber	**Oil(%)**	**Moisture**	**Ash**	**Protein**	**N**	**P**	**K**	**B**	**Cu**	**Mn**	**Na**
Fiber	—											
Oil(%)	0.105	—										
Moisture	-0.121	0.242 ***	—									
Ash	-0.006	-0.269 ***	-0.944 ***	—								
Protein	-0.086	0.283 ***	0.956 ***	-0.929 ***	—							
N	-0.095	-0.266 ***	-0.977 ***	0.949 ***	-0.943 ***	—						
P	-0.073	-0.273 ***	-0.972 ***	0.948 ***	-0.952 ***	0.996 ***	—					
K	-0.167	0.142	0.654 ***	-0.633 ***	0.653 ***	-0.661 ***	-0.670 ***	—				
B	-0.038	0.113	0.714 ***	-0.654 ***	0.675 ***	-0.681 ***	-0.666 ***	0.456 ***	—			
Cu	-0.047	-0.216 **	-0.057	0.058	-0.039	0.081	0.101	0.082	0.061	—		
Mn	-0.091	0.050	-0.093	0.079	-0.093	0.070	0.067	-0.103	-0.039	-0.046	—	
Na	-0.060	-0.005	-0.028	-0.004	-0.004	0.006	0.005	0.002	-0.006	0.004	0.077	—

3.3.4 SNP Distribution in Genome

A total of 29563 SNPs with 80 % missing data were discovered in the panel of 188 Pakistani wheat landraces, and 33,961 (77.4 %) of these were mapped to the IWGSC wheat reference genome (Version 1.0). (2018, IWGSC). SNPs are most abundant in the B genome (50), followed by the A genome (40), and the D genome (40). (11). (Fig. 3.1, Table 3.4). The B genome has the most (50) transition-type (Ts) SNPs, whereas the D genome had the fewest (10) (Table 3.5). Tv SNPs exhibited a similar chromosomal distribution pattern as Ts SNPs, however Tv SNPs were much more common (64.3%) than Ts SNPs (35.7%), with a Ts/Tv SNP ratio of 1.80. The Ts/Tv ratios in the A and B genomes were substantially higher. As expected, more A/G and C/T transitions were discovered than G/A and T/C transitions. Transversions such as C/G, A/C, G/T, and A/T, on the other hand, had higher frequency than G/C, C/A, T/G, and T/A. In Pakistani landraces, there were 997 monomorphic SNPs; these monomorphic SNPs were removed from subsequent SNP analyses, with the exception of Eigen GWAS for selective sweeps. Average gene diversity and polymorphism information content (PIC) values are higher among Pakistani landraces (Table 3.5). In Pakistani landraces, the proportion of heterozygous SNPs was also higher (8.41 percent). In general, Pakistani wheat landraces had somewhat higher mean MAF and heterozygosity (H) values (MAF = 0.19, H = 0.08) (Table 3.5).

3.3.5 MTAs identified in Wheat Landraces for Biochemical Traits (GWAS)

A total twenty-nine MTAs were identified on different chromosomes in wheat land races related to biochemical traits as shown in table 3.4. The Ash content has five MTAs on chromosome 1A, 3B, 6B, 7B, 4D with R square range of 0.15-0.26. Moisture content has six MTAs on chromosome 1A, 3B, 4D, 5B, 7B (2) with R square range of 0.17-0.24. Oil content has seven MTAs on chromosome 2A (3), 3D, 6A, 7B, 4A R square range of 0.02-0.06.Protein has seven MTAs on chromosome 1A, 3B (2), 4A, 5A, 6B, 7D R square range of 0.28-0.44. Fiber has four MTAs on chromosome 6A (2), 7A, 2Bwith R square range of 0.04-0.05. The QQ plot and Rectangular Manhattan plot of biochemical traits of wheat landraces from Pakistan are shown in figure 3.2.

3.3.6 MTAs Identified in Wheat Landraces for Mineral Traits (GWAS)

A total eighty-four MTAs were identified on different chromosomes in wheat related to mineral traits as shown in table 3.4. GZn 2019 has five MTAs on chromosome 7D, 1A, 7B

(3) R^2 range of 0.84- 1.11. GZn 2020 has four MTAs on chromosome 3D, 3B, 5D, 2AR square range of 0.61-0.70. Zn average has two MTAs on chromosome 1A, 6B R square range of 0.56-0.59. GFe 2019 has large number of twenty MTAs on chromosome 1B (3), 4A, 2B (2), 2A, 3B, 2D, 2A, 7B, 5A (4), 7D, 7A, 6A (2), 5B with square range of 5.60-11.26. GFe 2020 has fourteen MTAs on chromosome 2B (6), 5B, 1A (3), 1B, 6B, 4B, 7A with R^2 range of 4.01-6.53. GFe average has seven MTAs on chromosome 2B (2), 3B (2), 6A (2), 4Awith R^2 range of 4.51-9.17. Na has one MTA on chromosome 5A with R square value of 0.002. B has one MTA on chromosome 1Bwith R^2 value of 0.11. P has 3MTAs on chromosome 3A (2), 3B R^2 range of 0.07-0.12. K has fifteen MTAs on chromosome 5A, 7A, 6B (4), 2B (4), 6A (2), 7B, 6D, 1A with R square range of 0.01-0.03. Mn has seven MTAs on chromosome 1A (2), 1B, 2B, 3A, 4A, 4B with R^2 of 0.80-1.68. Cu has one MTA on chromosome 6D with R^2 value of 0.36. N has three MTAs on chromosome 3A (2), 3B with R square range of 0.42-0.69. The QQ plot and Rectangular Manhattan plot of mineral traits of wheat landraces from Pakistan are shown in figure 3.2 to 3.4.

3.3.7 Common MTAs and Frequency in Wheat Landraces for Mineral and Biochemical Traits

Ash content, Moisture content, Protein, GZn 2019, GZn average, GFe 2020, K, Mn has common MTAs on chromosome 1A.Ash content, Moisture content, Protein, GZn 2020, GFe average, P, N has common MTAs on chromosome 3B. Ash content, Protein, K has common MTAs on chromosome 6B.Ash content, Moisture content, Oil content, GZn 2019, GFe 2019, K has common MTAs on chromosome 7B.Ash content, Moisture content has common MTAs on chromosome 4D.Moisture content; GFe 2020 has common MTAs on chromosome 5B. Oil content, GZn 2020,GFe 2020has common MTAs on chromosome 2A. Oil content, GZn 2020 also has common MTAs on chromosome 3D.Oil content, Fiber, GFe 2019,GFeaverage, K has common MTAs on chromosome 6A.Protein,GFe 2019, Oil content, GFe average has common MTAs on chromosome 4A. Na, Protein, GFe 2019 has common MTAs on chromosome 5A. Protein, GFe 2019, GZn 2019 has common MTAs on chromosome 7D. Fiber, GFe2020 has common MTAs on chromosome 7A. Fiber, GFe 2020,GFe 2019,GFeaveragehas common MTAs on chromosome 2B. B, GFe 2020,GFe 2019 has common MTAs on chromosome 1B. Total number of MTAs on individual chromosomes in wheat landraces are as follows Chromosome 1A has 11MTAs, 2A (2), 4A (3), 3A (5), 5A (7), 3B (10), 6B (8), 7B (9), 5B (1), 7D (2), 3D (2), 6D (2), 7D (3), 4D (2). GFe 2019 has maximum number of MTAs. B, Cu, Na has minimum number of MTAs. Chromosome 1A

(11),3B (10) has maximum number of MTAs. Chromosome 6D (2), 5B (1) has minimum number of MTAs. A and B genome has high frequency of MTAs and D genome has low frequency of MTAs. That's mean A and B genome can be utilized in further breeding for high micronutrient in wheat grain.

Table 3.4. MTAs identified in wheat landraces for biochemical and mineral parameters.

Trait	MTAs	Chromosome
Ash	5	1A, 3B, 6B, 7B, 4D.
Moisture	6	1A, 3B, 4D, 5B, 7B (2).
Oil	7	2A (3), 3D, 6A, 7B, 4A.
Protein	7	1A, 3B (2), 4A, 5A, 6B,7D.
Fiber	4	6A (2), 7A, 2B.
GZn 2019	5	7D, 1A, 7B (3)
GZn 2020	4	3D, 3B, 5D, 2A
GZn average	2	1A, 6B
GFe 2019	20	1B(3),4A,2B(2),2A,3B,2D,2A,7B,5A(4),7D,7A,6A (2),5B.
GFe 2020	14	2B (6), 5B, 1A (3), 1B, 6B, 4B, 7A.
Na	1	5A
B	1	1B
P	3	3A(2), 3B.
K	15	5A, 7A, 6B(4), 2B(4), 6A(2), 7B, 6D, 1A.
Mn	7	1A(2), 1B, 2B, 3A, 4A, 4B
Cu	1	6D
N	3	3A (2), 3B.

Table 3.5. MTAs for mineral element and biochemical parameters in wheat land races.

Trait	SNP	Chr	Position	Effect	R.square	P.value
Ash	M57275	1A	2.67E+08	0.64	0.15	1.63E-06
Ash	M35674	3B	7.8E+08	1.48	0.25	3.92E-10
Ash	M19709	4D	4.66E+08	1.05	0.19	3.04E-09
Ash	M61777	6B	6.26E+08	1.02	0.26	1.61E-06
Ash	M3333	7B	7.02E+08	-1.08	0.24	7.45E-07
B	M56605	1B	6.62E+08	-0.57	0.11	9.63E-07
Cu	M33253	6D	1.12E+08	1.87	0.36	8.49E-07
GFe 2019	M29304	1B	6.12E+08	30.47	6.24	2.56E-06
GFe 2019	M22662	4A	4.63E+08	48.49	10.17	4.18E-06
GFe 2019	M39354	2B	79313046	35.13	7.43	4.97E-06
GFe 2019	M29746	1B	5.17E+08	50.85	10.96	7.29E-06
GFe 2019	M61706	2A	46332659	51.10	11.26	1.11E-05
GFe 2019	M28078	3B	1.13E+08	29.83	6.64	1.36E-05
GFe 2019	M4542	1B	41526729	24.99	5.60	1.55E-05
GFe 2019	M56300	2D	27652650	45.94	10.56	2.43E-05
GFe 2019	M55920	2A	32913984	28.79	6.63	2.55E-05
GFe 2019	M56772	7B	5.93E+08	34.24	7.96	3.00E-05
GFe 2019	M5464	5A	7.08E+08	26.16	6.19	4.00E-05
GFe 2019	M5465	5A	7.08E+08	27.78	6.59	4.26E-05
GFe 2019	M13227	7D	13851002	27.74	6.59	4.30E-05
GFe 2019	M33744	7A	6.83E+08	40.60	9.69	4.62E-05
GFe 2019	M39355	2B	79313046	31.99	7.70	5.38E-05
GFe 2019	M29630	5A	5.45E+08	-30.83	7.42	5.41E-05
GFe 2019	M56694	5A	4.72E+08	33.98	8.30	6.74E-05
GFe 2019	M38320	6A	1.05E+08	43.56	10.75	7.91E-05
GFe 2019	M38319	6A	1.05E+08	44.31	10.95	8.12E-05
GFe 2019	M9918	5B	7.03E+08	26.15	6.51	9.22E-05
GFe2020	M42834	2B	7.84E+08	26.21	5.28	1.76E-06
GFe2020	M43263	2B	6.96E+08	21.30	4.60	7.83E-06
GFe2020	M36689	5B	54966647	29.16	6.53	1.50E-05
GFe2020	M55829	1A	20420222	-18.17	4.15	2.20E-05
GFe2020	M60685	1B	46376309	17.75	4.14	3.21E-05
GFe2020	M10784	2B	7.42E+08	20.76	4.88	3.58E-05
GFe2020	M8863	6B	1.83E+08	-16.89	4.01	4.34E-05

GFe2020	M11271	2B	7.52E+08	24.90	6.05	6.27E-05
GFe2020	M43339	1A	3.63E+08	20.83	5.08	6.57E-05
GFe2020	M11270	2B	7.52E+08	24.77	6.07	7.11E-05
GFe2020	M37336	2B	7.65E+08	26.17	6.46	8.04E-05
GFe2020	M4100	4B	4.97E+08	21.67	5.35	8.08E-05
GFe2020	M37353	1A	3.18E+08	16.69	4.12	8.22E-05
GFe2020	M38269	7A	91661541	18.42	4.56	8.39E-05
GFeAv	M43263	2B	6.96E+08	24.51	5.31	8.28E-06
GFeAv	M42834	2B	7.84E+08	26.54	6.19	3.19E-05
GFeAv	M21723	3B	6.25E+08	38.38	9.17	4.75E-05
GFeAv	M38319	6A	1.05E+08	30.50	7.34	5.38E-05
GFeAv	M38320	6A	1.05E+08	29.63	7.22	6.52E-05
GFeAv	M5755	4A	5.27E+08	32.77	8.03	7.13E-05
GFeAv	M28078	3B	1.13E+08	18.35	4.51	7.49E-05
Fiber	M57111	6A	6.12E+08	0.25	0.05	2.84E-06
Fiber	M57110	6A	6.12E+08	0.23	0.05	1.65E-05
Fiber	M2181	7A	5.34E+08	0.19	0.04	1.76E-05
Fiber	M56884	2B	7.6E+08	0.20	0.04	3.41E-05
K	M33023	5A	6.74E+08	-0.10	0.02	8.83E-06
K	M6016	7A	1.43E+08	-0.07	0.01	1.37E-05
K	M47559	6B	44032373	0.11	0.02	1.94E-05
K	M47560	6B	44032373	0.11	0.02	1.94E-05
K	M7492	2B	6.83E+08	-0.07	0.01	2.05E-05
K	M56659	6A	6.07E+08	-0.07	0.01	2.84E-05
K	M27635	7B	1.12E+08	-0.08	0.01	2.84E-05
K	M4582	6D	5594221	-0.08	0.02	3.44E-05
K	M54993	6B	6.79E+08	-0.07	0.01	3.53E-05
K	M54994	6B	6.79E+08	-0.07	0.01	3.53E-05
K	M26136	1A	5.65E+08	-0.06	0.01	4.77E-05
K	M26039	2B	1.96E+08	0.12	0.03	5.01E-05
K	M21215	2B	3.88E+08	0.07	0.01	6.66E-05
K	M21216	2B	3.88E+08	0.07	0.01	6.66E-05
K	M29864	6A	6.05E+08	-0.06	0.01	8.53E-05
Mn	M26223	1A	22581576	5.45	1.11	7.49E-08
Mn	M9525	1A	5.55E+08	9.61	1.68	6.38E-10
Mn	M27227	1B	19234426	8.17	1.30	9.81E-11

Mn	M15133	2B	6.49E+08	-3.44	0.80	4.94E-07
Mn	M63751	3A	25183387	-5.34	1.52	7.78E-07
Mn	M56632	4A	6.09E+08	7.73	1.42	3.35E-09
Mn	M21582	4B	4.98E+08	7.91	1.45	1.90E-10
moisture	M2600	1A	8633434	-1.18	0.24	1.44E-07
moisture	M35674	3B	7.8E+08	-1.04	0.23	1.06E-07
moisture	M19709	4D	4.66E+08	-1.00	0.17	8.77E-10
moisture	M64029	5B	2.24E+08	0.72	0.17	1.14E-06
moisture	M55557	7B	5.53E+08	0.83	0.19	1.02E-06
moisture	M40818	7B	7.36E+08	-1.00	0.23	7.95E-07
N	M54326	3A	13151104	2.19	0.42	8.36E-07
N	M54327	3A	13151104	2.27	0.43	4.20E-07
N	M35674	3B	7.8E+08	3.60	0.69	6.26E-07
Na	M7746	5A	6.73E+08	-0.01	0.00	5.40E-05
Oil	M56665	2A	7.59E+08	-0.28	0.06	7.87E-06
Oil	M16043	3D	2.92E+08	-0.14	0.03	3.36E-05
Oil	M2218	6A	5.94E+08	-0.19	0.04	4.22E-05
Oil	M63198	7B	7.04E+08	0.11	0.02	5.17E-05
Oil	M40639	2A	4.82E+08	-0.20	0.04	5.55E-05
Oil	M5005	2A	5.05E+08	0.18	0.04	6.95E-05
Oil	M37905	4A	3.83E+08	-0.17	0.04	8.27E-05
P	M54326	3A	13151104	0.39	0.07	1.34E-06
P	M54327	3A	13151104	0.41	0.08	6.25E-07
P	M35674	3B	7.8E+08	0.67	0.12	5.31E-07
Protein	M60894	1B	23978419	-1.38	0.29	2.92E-07
Protein	M33108	3B	5.21E+08	-1.18	0.28	1.04E-06
Protein	M35674	3B	7.8E+08	-1.86	0.44	1.61E-06
Protein	M13046	4A	6.12E+08	-1.73	0.38	3.31E-07
Protein	M32506	5A	67475242	-1.35	0.31	6.49E-07
Protein	M16451	6B	80681748	1.84	0.44	1.33E-06
Protein	M29610	7D	19106313	-1.82	0.42	6.72E-07
GZn,2019	M13227	7D	13851002	3.81	0.84	1.27E-05
GZn,2019	M10577	1A	5.49E+08	3.72	0.88	4.07E-05
GZn,2019	M2858	7B	2.71E+08	-4.28	1.05	7.49E-05
GZn,2019	M24440	7B	3.74E+08	4.50	1.11	8.27E-05
GZn,2019	M45667	7B	5.67E+08	3.81	0.95	9.23E-05

GZn,2020	M30729	3D	5.15E+08	-2.69	0.61	1.82E-05
GZn,2020	M55362	3B	7.22E+08	3.03	0.70	2.91E-05
GZn,2020	M8047	5D	2.74E+08	2.68	0.65	6.41E-05
GZn,2020	M55358	2A	6.78E+08	2.61	0.65	9.05E-05
GZnAv	M10577	1A	5.49E+08	2.66	0.59	1.31E-05
GZnAv	M48989	6A	28013599	2.29	0.56	7.35E-05

Chr: Chromosome; Position: position along chromosome; SNP: Single nucleotide polymorphism

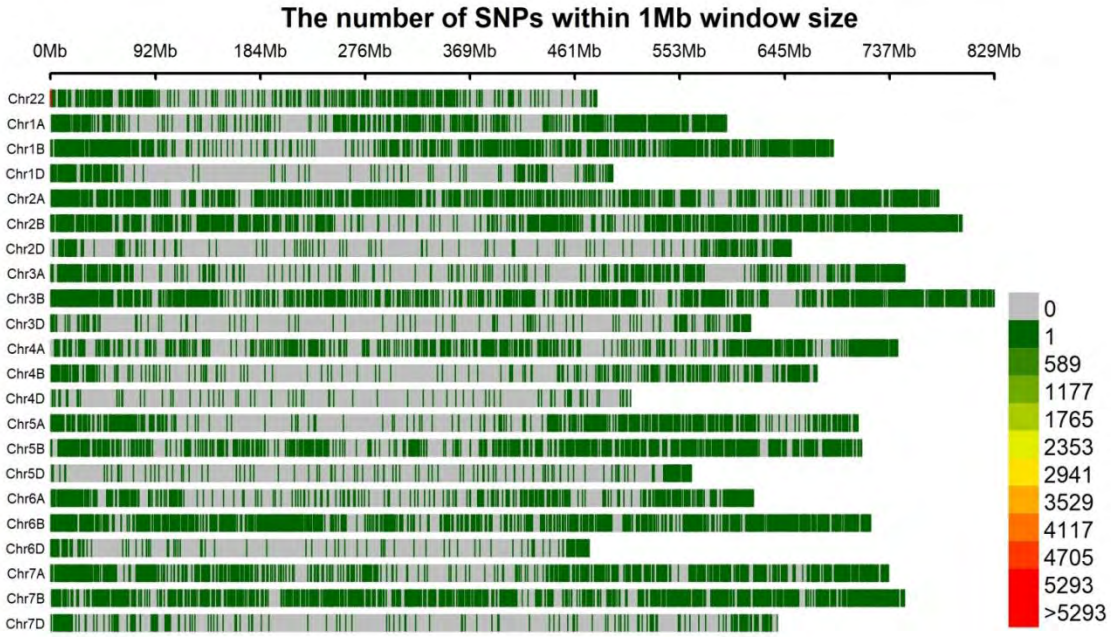


Figure 3.1. SNP density plots of29563 SNPs within 1-Mb wheat landraces from Pakistan. The different colors represent different density levels and "Chr" refers to wheat chromosomes.

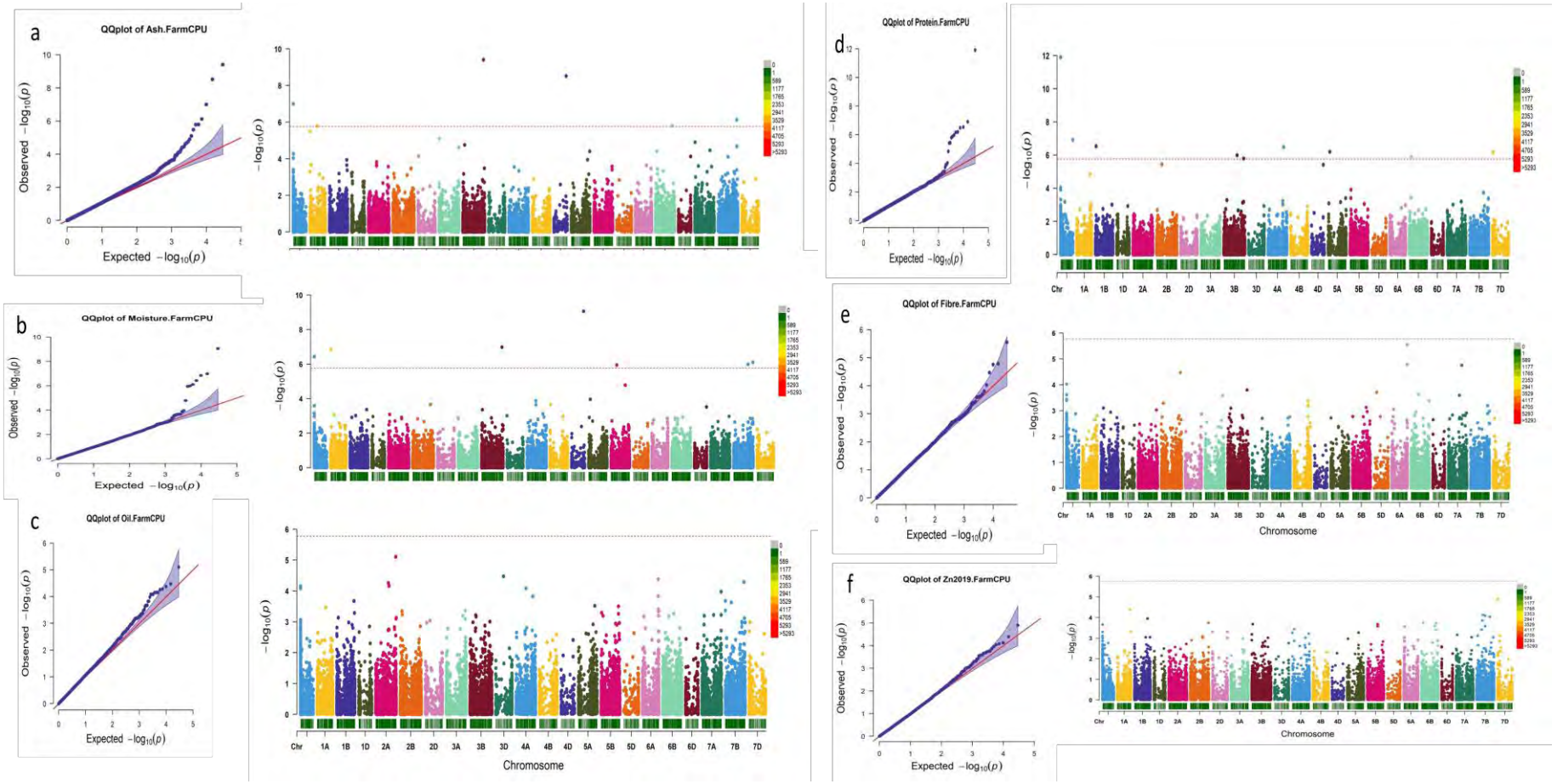


Figure 3.2. QQ plot and Rectangular Manhattan plot of (a) Ash,(b) moisture, (c)oil%, (d) protein, (e) fiber, (f) GZn 2019 Fram.cpu of wheat landraces from Pakistan.

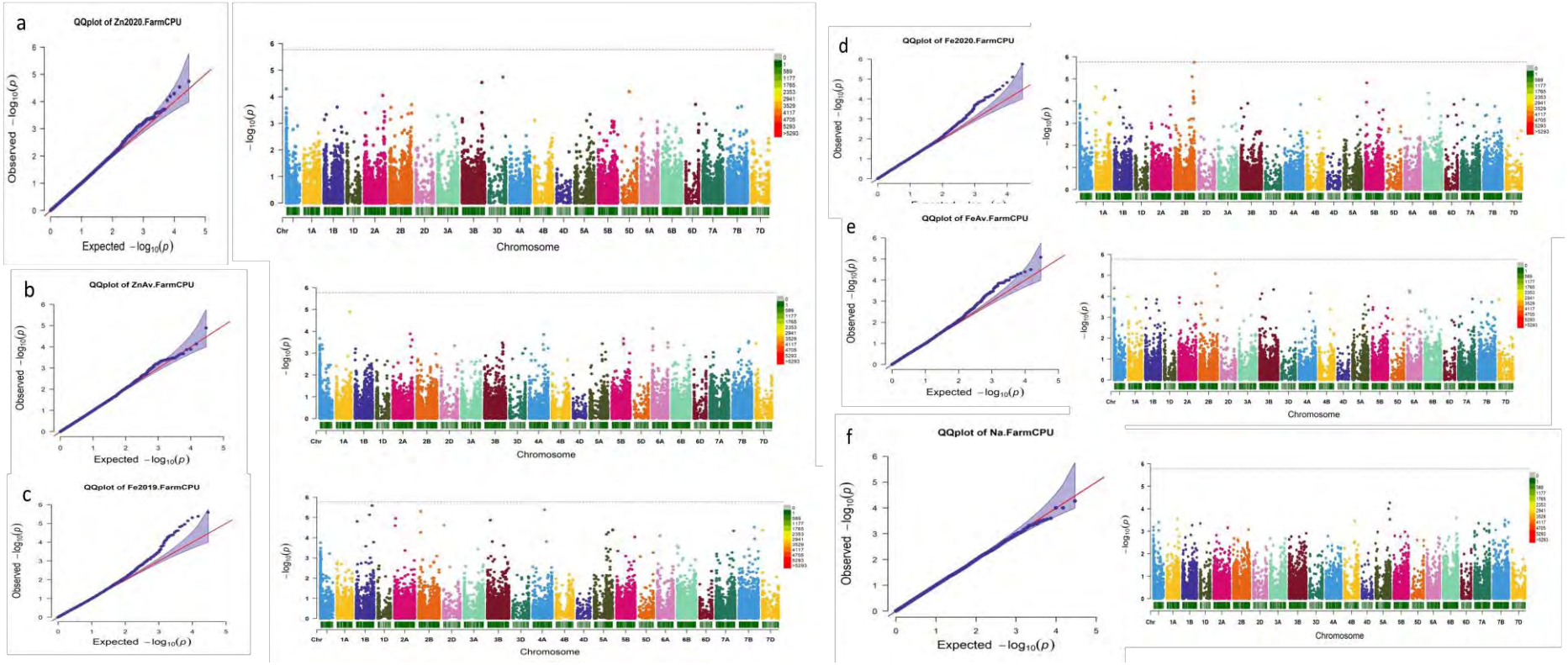


Figure 3.3. QQ plot and Rectangular Manhattan plot of (a) GZn 2020, (b) GZn. Av,(c) GFe 2019, (d) GFe 2020, (e) GFe.Av, (f) Na,Fram.cpu of wheat landraces from Pakistan

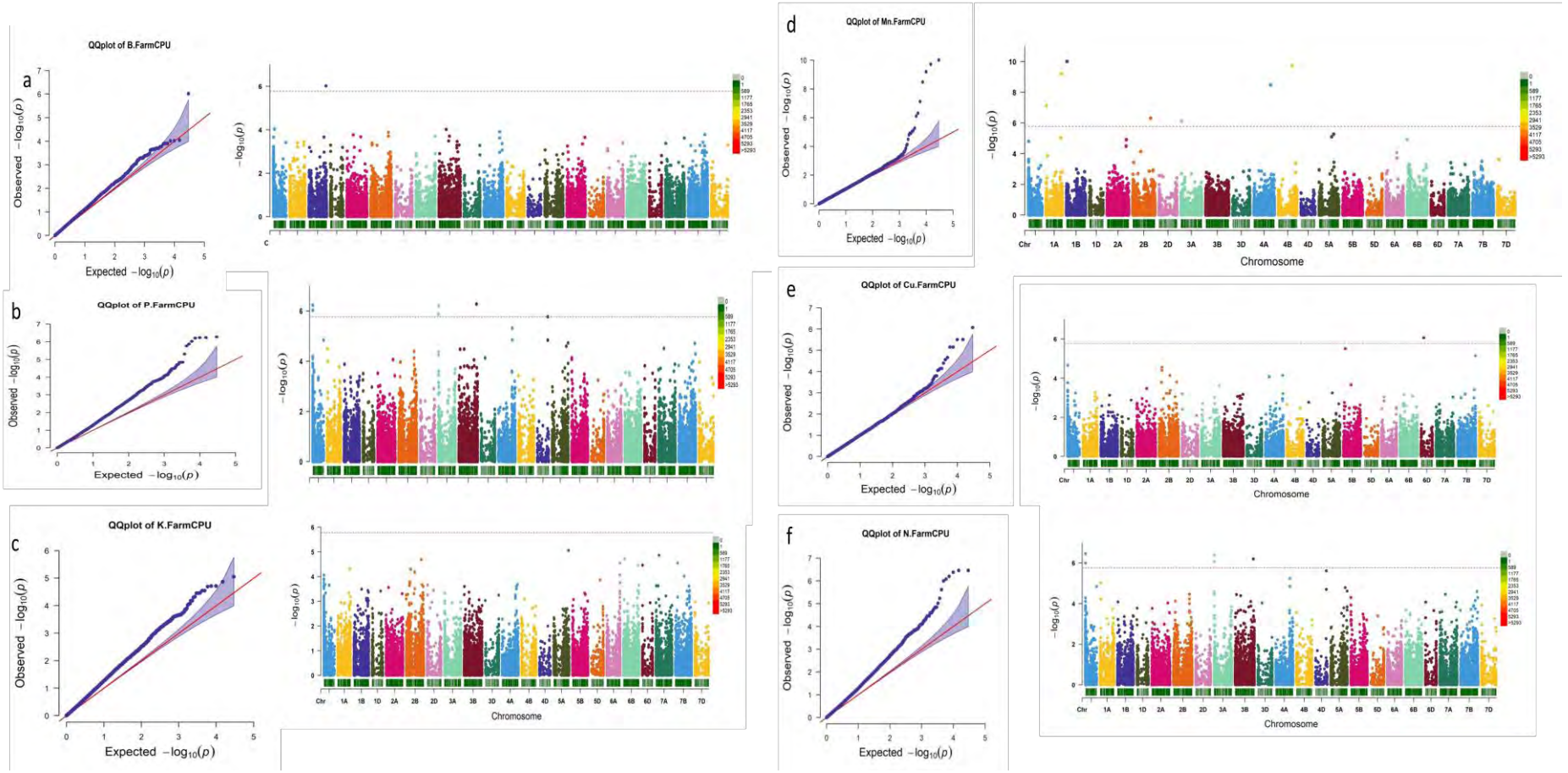


Figure 3.4. QQ plot and Rectangular Manhattan plot of (a) B, (b) P, (c) K,(d) Mn,(e) Cu,(f) N, Fram.cpu of wheat landraces from Pakistan.

3.4 Discussion

A collection of 188 wheat landraces were evaluated for grain mineral element and biochemical traits. There was a remarkable variation observed in all aspects that have demonstrated their potential of high levels of grain minerals in wheat. Biofortification, which enhances the concentration of major micronutrients in cereal grains, is an important field of research to solve the issue of healthy food safety (Neeraja *et al.,* 2017). Information regarding the genetic nature of a characteristic is necessary for effective genetic modification of crops. The majority of previous studies on wheat grain elemental composition focused on GFe and GZn (Velu *et al.,* 2016). Because these two nutrients are typically inadequate in the human diet, they are important biofortification goals (Bouis *et al.,* 2010).

Elements are commonly linked, showing that elemental interactions take place throughout plant metabolic processes (Baxter *et al.,* 2010). In order to optimize the concentration of micronutrients like GFe and GZn, it may be beneficial to investigate the mobility of other major and minor elements. In addition to GFe and GZn deficiencies, many industrialized and developing countries suffer from deficiencies in other micronutrients such as Ca, Mg, and Cu (White *et al.,* 2009). We found minimal to strong correlations between elements in our study, indicating the value of integrated analysis of several elements. For example, the GFe and GZn concentrations in this research were 15-60mg kg^{-1} and 11.8-54.5 mg kg^{-1}, respectively, which were lower than the amounts reported for 269 Afghan wheat landraces evaluated for grain micronutrient contents (GFe: 55.14–122.2mg kg^{-1}; GZn: 15.56–87.29mg kg^{-1}) (Manickavelu *et al.,*2017). The mineral element amounts were also higher than those reported for 132 cultivars evaluated by the International Maize and Wheat Improvement Center (CIMMYT) (GFe: 28.8–56.5mg kg^{-1}; GZn: 25.2–53.3 mg kg^{-1}) (Roohani *et al.,* 2013). Our contents were greater than those of 150 bread wheat lines from various sources (GFe: 28.8–50.8 mg kg^{-1}; GZn: 13.5–34.5mg kg^{-1}) (Cakmak *et al.,* 2018).

In our study all biochemical and mineral traits of wheat land races shows higher variation across different provinces of Pakistan. The mean of GFe was highest in KPK province (104.16mg kg^{-1}) with a range of 48-295mg kg^{-1}.The mean of GZn was highest in Gilgit province (34.85mg kg^{-1}) with a range of 25.6-45.6mg kg^{-1}which were higher than the amounts reported for 269 Afghan wheat landraces evaluated for grain micronutrient contents (GFe: 55.14–122.2 mg kg-1; GZn: 15.56–87.29mg kg^{-1}) (Manickavelu *et al.,* 2017).

According to these findings, several Pakistani landraces might be helpful genetic resources for raising grain micronutrient concentrations. When comparing results from different studies, it's important to keep in mind that environmental factors like soil nutrient composition can have a big influence on plant element content. The most significant minerals have been identified as GZn and GFe; unfortunately, both of these elements are lacking in many plants across the world. Significant genetic diversity in the focus of GFe and GZn was discovered in big grain grains and wild cousins (Myers *et al.,* 2014). CIMMYT looked at around 3000 GZn and GFe accessions, including hexaploid, tetraploid, and diploid wild relatives (Monasterio and Graham, 2000). High GFe and GZn levels were discovered in a variety of einkorn wheat and wild emmer wheat accessions, as well as landraces (Cakmak *et al.,* 2008).

Better wheat cultivars, on the other hand, have less genetic diversity. As a result, the current focus of study should shift to a complete analysis of all grain landraces. Wheat GZn levels have been reported to range between 30 and 98 mg kg (Cakmak *et al*., 2009). Wheat had GFe contents ranging from 20 to 60 mg kg^{-1} in another research (Welch and Graham, 1999). Landrace wheat has the greatest amounts of GZn and GFe, according to germplasm screening. The GFe and GZn concentrations detected in the wheat land races in our investigation were likewise quite high. That is, the concentration of GZn. 2019 varies from 13.5mg $kg^{-1}$to 54mg kg^{-1}, whereas the concentration of GZn. 2020 ranges from 11.8mg $kg^{-1}$to 54.5mg kg^{-1}. The concentration of GFe.2019 varies from 8.20mg $kg^{-1}$to 56 mg kg^{-1}whereas the concentration of GFe.2020 ranges from 15mg $kg^{-1}$to 60mg kg^{-1}. Between 2019 and 2020, there is a significant increase in GZn content in wheat landraces. This necessitates an understanding of the genetic composition of each micronutrient and biochemical component. Furthermore, because assessing the content of many distinct micronutrients in a breeding program can be time-consuming and wasteful, selective-assisted selection (MAS) is an effective technique when information about markers (MTAs) is provided (Kumar *et al.,* 2018).

Understanding the genetic basis of GFe concentration in wheat grains is crucial for developing new varieties with higher GFe values. One of the major ways for dissecting complex variables like nutritional quality traits that are regulated by multiple genes and impacted by the environment is the genome-wide association study (GWAS) methodology (Alomari *et al.,* 2018). In wheat, GWAS has been used to examine the genetic control of complex traits. For wheat breeding using MAS, understanding the genetic variables that

determine GFe and GZn concentrations is crucial. The QTL analysis is a valuable technique for identifying the genes that cause natural variation in GFe and GZn concentrations (Ghandilyan *et al.,* 2006). Wheat also has several reports involving GWAS for GZn, GFe, and carotenoid content (Colasuonno *et al.,* 2017). In our research, one hundred and thirteen significant MTAs for biochemical and mineral traits were found on various chromosomes in wheat landraces. The number of MTAs in GFe, 2019 is at its peak. MTAs in the elements B, Cu, and Na are at a bare minimum. Chromosome 1A (11), 3B (10) has maximum number of MTAs. Chromosome 6D (2), 5B (1) has minimum number of MTAs. A, B genome has high frequency of MTAs and D genome has low frequency of MTAs. That's mean A and B genome can be utilized in further breeding for high micronutrient in wheat grain. Other micronutrients and biochemical parameter like as N, P, K, B, Cu, Mn, Na, protein, oil, ash in addition to GFe and GZn, are vital for human health, and breeding for these micronutrients has been largely overlooked. The highest correlation among the biochemical and mineral traits was between P and N (r=0.99). Understanding the genetic factors that influence GFe and GZn concentrations is critical for wheat breeding with MAS. The QTL analysis is a valuable technique for identifying the genes that cause natural variation in GFe and GZn concentrations (Ghandilyan *et al.,* 2006). Many QTLs controlling micronutrient content in wheat grain have been discovered in recent years using recombinant inbred lines or doubled haploid populations (Peleg *et al.,* 2009). QTL mapping advancements will improve understanding of the genetic basis determining micronutrient concentrations in wheat grains. Furthermore, identifying, and labeling key QTLs for micronutrient-related characteristics with large impacts would aid in QTL selection in early generations using the MAS approach and would significantly expedite wheat cultivar development for increasing mineral concentration in grain (Ortiz-Monasterio *et al*., 2007).

3.4.1 Comparison with previous reports

3.4.1.1 MTAs for GZn

A total of eleven MTAs were identified for GZn in current study on chromosome 7D, 1A, 7B (3),3D, 3B, 5D, 2A,1B, 6B in wheat landraces. The MTAs detected for GFe were forty-one. Which are present on chromosomes 1B (3), 4A, 2B (2), 2A, 3B, 2D, 2A,7B, 5A (4), 7D, 7A, 6A (2), 5B, 2B (6), 5B, 1A (3), 1B, 6B, 4B, 7A, 2B (2), 3B (2), 6A (2), 4A in wheat landraces. Five MTAs were identified for Zn. 2019 flanked by SNPs on chromosome 7D, 1A,7B (2) in wheat landraces. Crespo-Herrera *et al.* (2017) found five QTLs, including QGZn.cimmyt-7B 2P1, QGZn.cimmyt-7B 1P1, QGZn.cimmyt-7B 1P2, QGZn.cimmyt-7B

2P2, and QGZn.cimmyt-7B 3P2 at physical locations of 139.4–160.6, 158.3–159.2,485.8–506.4, 590.1, and 633.6–637.3 Mb, respectively. The current MTAs found on chromosomes 1A and 7B appear to be novel. Four MTAs were identified for GZn. 2020 flanked by SNPs *M30729, M55362, M8047, M55358,* at position 5.15, 7.22, 2.47, 6.78 on chromosome 3D, 3B, 5D, 2A in wheat landraces. Wang *et al.* (2021) also identified one QTL for GZnin 254wheat (RILs) from a cross Jingdong 8/Bainong AK58 on chromosome *3BS*, flanked by *AX-110975262* and *AX-109911679* at physical positions of 42.5 and 59.1 Mb.Crespo-Herrera *et al.* (2017) identified two QTL for GZn on this chromosome. *QGZn.cimmyt-3B_2P2* was at the physical position 32.6 Mb linked with DArT markers *4394657*, and *QGZn.cimmyt-3B_1P2* flanked by 3533713 and 1007339. Liu *et al.* (2019) mapped *QGZn.co-3B* flanked by DArT markers *1002594|F|0* and *1103633* at physical positions of 104.5 and 128.6 Mb, respectively. The remaining three MTAs on chromosome were novel. Alomari *et al.* (2019) identified 39 marker-trait associations for grain GZn by using GWAS analysis in 330 wheat lines. On chromosomes 2 and 7, two greater impact QTL areas were discovered. In wheat landraces, two MTAs for GZn average were discovered, bordered by SNPs on chromosome 1A and 6B. These MTAs are new and have never been seen before in wheat landrace study.

3.4.1.2 MTAs for GFe

Twenty MTAs were identified for GFe 2019 flanked by SNPs on chromosome1B (3), 4A, 2B (2), 2A, 3B, 2D, 2A, 7B, 5A (4), 7D, 7A, 6A (2), 5B in wheat landraces. One MTA on chromosome 3B in current study is a very important MTA identified in wheat landraces. This MTA is reported in many studies. In a panel of 369 European elite wheat types, GWAS identified 41 and 137 significant SNPs, respectively, including significant marker-trait associations (MTAs) for best linear unbiased estimates (BLUEs) of GFeC on chromosomes 2A, 3B, and 5A. (Velu *et al.,* 2018). Wang *et al.* (2021) found one QTL for GFe in 254 wheat (RILs) from a Jingdong 8/Bainong AK58 cross on chromosome 3BS, bordered by AX-111016352 and AX-94835626 at physical locations 764.7 and 822.9 Mb. Crespo-Herrera *et al*. (2017) discovered two QTL for GFe in the same location as the previously described QTL for GZn, both on the short arm of chromosome 3B. Peleg *et al.* (2009) discovered a QTL on chromosome 3B that is tightly related to Xgwm1266 at a physical location of 150 Mb. Liu *et al.,* 2019 discovered QGFe.co-3B.1, and QGFe.co-3B.2 flanked by DArT markers 1089107 and 1127875|F|0, 1233878-4262223|F|0 at 37.2–754.8 and 12.3–536.6 Mb, respectively. Fourteen MTAs were identified for GFe 2020 flanked by SNPs on chromosome 2B(6), 5B,

1A(3), 1B, 6B, 4B, 7A in wheat landraces. Liu *et al.*(2019) identified nine QTLs for GFe on chromosome 2B, 3B, 4A, 6B, 6D, 7B and 7D in wheat RILs. These QTL are similar with most of the MTAs identified in current wheat landraces.

3.4.1.3 MTAs for Cu

In our research, we found one MTA for Cu on chromosome 6D in wheat land races. On chromosomes 1B, 2A, 3A, 3B, 4B, 5A, 5B, 5D, 6A, and 6B, Bhatta *et al.* (2018) discovered 13 MTAs for Cu. In diploid wheat, Ozkan *et al*. (2007) discovered one QTL for Cu concentration on chromosome 5A. (*T. monococum*). In tetraploid wheat, there are 10 QTLs on chromosomes 1A, 2A, 3B, 4A, 4B, 5A, 6A, 6B, 7A, and 7B, and six QTLs on chromosomes 2A, 4A, 4D, 5A, 6A, and 7B (Peleg *et al.,* 2009). (Shi *et al.,* 2013). The one MTA found on chromosome 6D in this study has never been reported before, and it might represent a novel MTA that regulates grain Cu content.

3.4.1.4 MTAs for Mn

In wheat land races, seven MTAs for Cu were discovered in our research on chromosomes 1A (2), 1B, 2B, 3A, 4A, 4B. On chromosomes 2D, 3A, 4B, 5D, and 6B, Bhatta *et al.* (2018) discovered six MTAs for Mn concentration. In *T. monoccocum*, one QTL was previously found on chromosome 5A (Ozkan *et al.,* 2007). In tetraploid wheat, two QTLs for Mn concentration were discovered on chromosomes 2B and 7B (Peleg *et al.* 2009). In hexaploid wheat, four QTLs on chromosomes 1A, 2B, and 3B were discovered (Shi *et al.,* 2013). The two MTAs found on chromosomes 1B and 4A in this study have never been described before, and they might be novel MTAs that regulate grain Mn content.

3.4.1.5 MTAs for P

Three MTAs were identified in our study for P on chromosome 3A (2), 3B in wheat land races. Cu *et al.* (2020) also identified 5 MTAs on chromosome 1D, 2A (2), 3B, 5B. Earlier studies have identified 8 MTAs on chromosome 2B, 4A, 4B, 6B, 7A, 1A, 2B,5B tetraploid wheat.

3.4.1.6 MTAs for Biochemical Traits

In all, twenty-nine MTAs were found on distinct chromosomes in wheat land races that were linked to biochemical features in our study. Grain protein content (GPC) has garnered specific attention among biochemical properties as a traditional indication for determining the nutritional value of food (Zhao et al., 2010). Grain protein content includes seven MTAs on chromosomes 1A, 3B (2), 4A, 5A, 6B, and 7D with R squares ranging from 0.28 to 0.4 in our research. Kumar *et al.* (2018) also identified 214 MTAs for grain protein contents on chromosome 3B, 2B, 6B, 6A, 6B, 5A, 5B, 4A, 4B in 246 genotypes of spring wheat. The

mean of protein in wheat landraces was 13 mg kg^{-1} with a range of 10.69-15.04 mg kg^{-1}. This was lower than the mean 12.72 mg kg-1 with a range of 9.99-17.87 mg kg^{-1}reported by Kumar et al., (2018). Bhatta *et al.* (2018) also identified GPC ranged from 130 to 168 mg $kg^{-1}$with an average of 151 mg kg^{-1} in 123 SHWs.

3.4.2 Conclusion

Conclusively, it is paramount to recognize grain mineral and biochemical traits of wheat land races. The wheat land races could be very important genetic resources for breeding high grain mineral element in wheat. This study provided insight into the mineral status and biochemical traits in wheat land races. Overall, the improvement in GY was not translated into an improvement in micro- and macronutrients. The observed GFe and GZn concentration in the wheat land races in our study was also very high. That is GZn. 2019 concentration ranges from 13.5 mg kg^{-1} to 54 mg kg^{-1} and GZn 2020 concentration ranges from 11.8mg kg^{-1} to 54.5 mg kg^{-1}. The GFe.2019 concentration ranges from 8.20 mg $kg^{-1}$to 56 mg kg^{-1} and GFe.2020 concentration ranges from 15 mg kg^{-1} to 60 mg kg^{-1}. The GFe contents were highest in KPK province with a range of 48-295 mg kg^{-1} and lowest in AJK province with a range of 15.4-45.7 mg kg^{-1}. The GZn contents were highest in Gilgit province with a range of 25.6-45.6 mg kg^{-1} and lowest in Sindh province (with a range of 20-33.5 mg kg^{-1}. A total of 10 MTAs for GZn and 34 for GFe are identified in this study. In our knowledge, this is the first study on wheat landraces of Pakistan from all provinces. This highlights the status of mineral elements and biochemical parameters in wheat landraces from Pakistan. The data can be serves as the good source of wheat breeding for higher mineral and biochemical traits.

CHAPTER 4: References

Abis, S. (2012) Geopolitics of wheat in the Mediterranean. *Futuribles*, (387), 65-82.

Adsule, R. N., Kadam, S. S., Salunkhe, D. K., &Luh, B. S. (1986) Chemistry and technology of green gram (*Vigna radiata* [L.] Wilczek). *Critical Reviews in Food Science & Nutrition*, *25*(1), 73-105.

Agrawal, P. K., & Gupta, H. S. (2006) Enhancement of nutritional quality of cereals using biotechnological options. *Proceeding of ICPHT*, 48-58.

Ahmad, S., Zhang, C., & Ekanayake, E. M. B. P. (2021) Smallholder Farmers' Perception on Ecological Impacts of Agroforestry: Evidence from Northern Irrigated Plain, Pakistan. *Polish Journal of Environmental Studies*, *30*(4).

Ain, Q. U., Rasheed, A., Anwar, A., Mahmood, T., Imtiaz, M., He, Z., ... & Quraishi, U. M. (2015) Genome-wide association for grain yield under rainfed conditions in historical wheat cultivars from Pakistan. *Frontiers in Plant Science*, *6*, 743.

Ali, M. W., &Borrill, P. (2020) Applying genomic resources to accelerate wheat biofortification. *Heredity*, *125*(6), 386-395.

Ali, S., Liu, Y., Ishaq, M., Shah, T., Ilyas, A., & Din, I. U. (2017) Climate change and its impact on the yield of major food crops: Evidence from Pakistan. *Foods*, *6*(6), 39.

Alomari, D. Z., Eggert, K., Von Wirén, N., Alqudah, A. M., Polley, A., Plieske, J., ... &Röder, M. S. (2018) Identifying candidate genes for enhancing grain Zn concentration in wheat. *Frontiers in Plant Science*, *9*, 1313.

Alomari, D. Z., Eggert, K., Von Wirén, N., Pillen, K., &Röder, M. S. (2017) Genome-wide association study of calcium accumulation in grains of European wheat cultivars. *Frontiers in Plant Science*, *8*, 1797.

Alomari, D. Z., Eggert, K., Von Wirén, N., Polley, A., Plieske, J., Ganal, M. W., ... &Röder, M. S. (2019) Whole-genome association mapping and genomic prediction for iron concentration in wheat grains. *International Journal of Molecular Sciences*, *20*(1), 76.

Amarakoon, D., McPhee, K., &Thavarajah, P. (2012) Iron-, zinc-, and magnesium-rich field peas (*Pisum sativum L.)* with naturally low phytic acid: A potential food-based solution to global micronutrient malnutrition. *Journal of Food Composition and Analysis*, *27*(1), 8-13.

Anonymous. (2000) Report, Planning Division Islamabad, Government of Pakistan.

Anuradha, N., Satyavathi, C. T., Bharadwaj, C., Nepolean, T., Sankar, S. M., Singh, S. P., ... & Srivastava, R. K. (2017) Deciphering genomic regions for high grain iron and zinc content using association mapping in pearl millet. *Frontiers in Plant Science*, *8*, 412.

Appels, R., Eversole, K., Feuille, C., Keller, B., Rogers, J., & Stein, N. (2018) The International Wheat Genome Sequencing Consortium (IWGSC). Shifting the limits in wheat research and breeding using a fully annotated reference genome. *Science*, *361*(6403), 10-1126.

Badakhshan, H., Moradi, N., Mohammadzadeh, H., &Zakeri, M. R. (2013) Genetic variability analysis of grains Fe, Zn and beta-carotene concentration of prevalent wheat varieties in Iran. *International Journal of Agriculture and Crop Sciences*, *6*(2), 57.

Battenfield, S. D., Guzmán, C., Gaynor, R. C., Singh, R. P., Peña, R. J., Dreisigacker, S.,Fritz, A. K., & Poland, J. A. (2016) Genomic selection for processing and end-use quality traits in the CIMMYT spring bread wheat breeding program. *The Plant Genome*, *9*(2), plantgenome2016-01.

Baxter, I. (2010) Ionomics: The functional genomics of elements. *Briefings in Functional Genomics*, *9*(2), 149-156.

Belderok, B., Mesdag, H., & Donner, D. A. (2013) *Bread-making quality of wheat: A Century of Breeding in Europe*. Springer Science & Business Media.

Bhatta, M., Baenziger, P. S., Waters, B. M., Poudel, R., Belamkar, V., Poland, J., &Morgounov, A. (2018) Genome-wide association study reveals novel genomic regions associated with 10 grain minerals in synthetic hexaploid wheat. *International Journal of Molecular Sciences*, *19*(10), 3237.

Bhatta, M., Baenziger, P. S., Waters, B. M., Poudel, R., Belamkar, V., Poland, J., &Morgounov, A. (2018) Genome-wide association study reveals novel genomic regions associated with 10 grain minerals in synthetic hexaploid wheat. *International Journal of Molecular Sciences*, *19*(10), 3237.

Black, R. E., Victora, C. G., Walker, S. P., Bhutta, Z. A., Christian, P., De Onis, M., Ezzati, M., Grantham-McGregor, S., Katz, J., Martorell, R., Uauy, R., & Maternal and Child Nutrition Study Group. (2013) Maternal and child undernutrition and overweight in low-income and middle-income countries. *The lancet*, *382*(9890), 427-451.

Borg, S., Brinch-Pedersen, H., Tauris, B., Madsen, L. H., Darbani, B., Noeparvar, S., & Holm, P. B. (2012) Wheat ferritins: improving the iron content of the wheat grain. *Journal of Cereal Science*, *56*(2), 204-213.

Bouis, H. E. (2007) The potential of genetically modified food crops to improve human nutrition in developing countries. *The Journal of Development Studies*, *43*(1), 79-96.

Bouis, H. E., & Welch, R. M. (2010) Biofortification a sustainable agricultural strategy for reducing micronutrient malnutrition in the global south. *Crop Science*, *50*, S-20.

Bouis, H. E., Hotz, C., McClafferty, B., Meenakshi, J. V., & Pfeiffer, W. H. (2011) Biofortification: a new tool to reduce micronutrient malnutrition. *Food and Nutrition Bulletin*, *32*(1_suppl1), S31-S40.

Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y., & Buckler, E. S. (2007) TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics*, *23*(19), 2633-2635.

Bradford, J. B., Schlaepfer, D. R., Lauenroth, W. K., Yackulic, C. B., Duniway, M., Hall, S., ... & Tietjen, B. (2017) Future soil moisture and temperature extremes imply expanding suitability for rainfed agriculture in temperate drylands. *Scientific Reports*, *7*(1), 1-11.

Breseghello, F., & Sorrells, M. E. (2006) Association mapping of kernel size and milling quality in wheat (*Triticum aestivum L.*) cultivars. *Genetics*, *172*(2), 1165-1177.

Bujoczek, G., Oleszkiewicz, J., Sparling, R., & Cenkowski, S. (2000) High solid anaerobic digestion of chicken manure. *Journal of Agricultural Engineering Research*, *76*(1), 51-60.

Cakmak, I. (2008) Enrichment of cereal grains with zinc: agronomic or genetic biofortification *Plant and Soil*, *302*(1), 1-17.

Cakmak, I. (2014) Agronomic biofortification. Conference brief# 8. In *Proceedings of the 2nd Global Conference on Biofortification: Getting Nutritious Foods to People, Rwanda*.

Cakmak, I., &Kutman, U. Á. (2018) Agronomic biofortification of cereals with zinc: a review. *European Journal of Soil Science*, *69*(1), 172-180.

Cakmak, I., Kalayci, M., Kaya, Y., Torun, A. A., Aydin, N., Wang, Y., Arisoy,Z., Erdem, H., Yazici, A., Gokmen, O., Ozturk, L., & Horst, W. J. (2010) Biofortification and localization of zinc in wheat grain. *Journal of Agricultural and Food Chemistry*, *58*(16), 9092-9102.

Cakmak, I., Ozkan, H., Braun, H. J., Welch, R. M., &Romheld, V. (2000) Zinc and iron concentrations in seeds of wild, primitive, and modern wheats. *Food and Nutrition Bulletin*, *21*(4), 401-403.

Cakmak, I., Pfeiffer, W. H., & McClafferty, B. (2010) Biofortification of durum wheat with zinc and iron. *Cereal Chemistry*, *87*(1), 10-20.

Cakmak, I., Torun, B., Erenoğlu, B., Öztürk, L., Marschner, H., Kalayci, M., ... & Yilmaz, A. (1998) Morphological and physiological differences in the response of cereals to zinc deficiency. *Euphytica*, *100*(1), 349-357.

Calderini, D. F., & Ortiz-Monasterio, I. (2003) Are synthetic hexaploids a means of increasing grain element concentrations in wheat. *Euphytica*, *134*(2), 169-178.

Calderini, D. F., & Slafer, G. A. (1999) Has yield stability changed with genetic improvement of wheat yield. *Euphytica*, *107*(1), 51-59.

Chandra, A. K., Kumar, A., Bharati, A., Joshi, R., Agrawal, A., & Kumar, S. (2020) Microbial-assisted and genomic-assisted breeding: a two-way approach for the improvement of nutritional quality traits in agricultural crops. *3 Biotech*, *10*(1), 1-15.

Chang, C. C., Chow, C. C., Tellier, L. C., Vattikuti, S., Purcell, S. M., & Lee, J. J. (2015) Second-generation PLINK: rising to the challenge of larger and richer datasets. *Giga Science*, *4*(1), s13742-015.

Chattha, M. U., Hassan, M. U., Khan, I., Chattha, M. B., Mahmood, A., Nawaz, M., ... & Khan, S. (2017) Biofortification of wheat cultivars to combat zinc deficiency. *Frontiers in Plant Science*, *8*, 281.

Chen, M. J., Hsieh, Y. T., Weng, Y. M., &Chiou, R. Y. Y. (2005) Flame photometric determination of salinity in processed foods. *Food Chemistry*, *91*(4), 765-770.

Chhuneja, P., Dhaliwal, H. S., Bains, N. S., & Singh, K. (2006) *Aegilops kotschyi* and *Aegilops tauschii*as sources for higher levels of grain iron and zinc. *Plant Breeding*, *125*(5), 529-531.

Colasuonno, P., Lozito, M. L., Marcotuli, I., Nigro, D., Giancaspro, A., Mangini, G., ... & Blanco, A. (2017) The carotenoid biosynthetic and catabolic genes in wheat and their association with yellow pigments. *BMC Genomics*, *18*(1), 1-18.

Connorton, J. M., & Balk, J. (2019) Iron biofortification of staple crops: lessons and challenges in plant genetics. *Plant and Cell Physiology*, *60*(7), 1447-1456.

Cormier, F., Throude, M., Ravel, C., Gouis, J. L., Leveugle, M., Lafarge, S., ... &Praud, S. (2015) Detection of NAM-A1 natural variants in bread wheat reveals differences in haplotype distribution between a worldwide core collection and European elite germplasm. *Agronomy*, *5*(2), 143-151.

Crespo-Herrera, L. A., Govindan, V., Stangoulis, J., Hao, Y., & Singh, R. P. (2017) QTL mapping of grain Zn and Fe concentrations in two hexaploid wheat RIL populations with ample transgressive segregation. *Frontiers in Plant Science*, *8*, 1800.

Crespo-Herrera, L. A., Velu, G., & Singh, R. P. (2016) Quantitative trait loci mapping reveals pleiotropic effect for grain iron and zinc concentrations in wheat. *Annals of Applied Biology*, *169*(1), 27-35.

Cu, S. T., Guild, G., Nicolson, A., Velu, G., Singh, R., &Stangoulis, J. (2020) Genetic dissection of zinc, iron, copper, manganese and phosphorus in wheat (*Triticum aestivum L.)* grain and rachis at two developmental stages. *Plant Science*, *291*, 110338.

Cu, S. T., Guild, G., Nicolson, A., Velu, G., Singh, R., &Stangoulis, J. (2020). Genetic dissection of zinc, iron, copper, manganese and phosphorus in wheat (*Triticum aestivum L.*) grain and rachis at two developmental stages. *Plant Science*, *291*, 110338.

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Le Jean, M., Misson, J., Schikora, J., Czernic, P., & Mari, S. (2009) Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Annals of Botany*, *103*(1), 1-11.

De Santis, M. A., Kosik, O., Passmore, D., Flagella, Z., Shewry, P. R., & Lovegrove, A. (2018) Comparison of the dietary fibre composition of old and modern durum wheat (*Triticum turgidum spp. durum*) genotypes. *Food Chemistry*, *244*, 304-310.

Distelfeld, A., Cakmak, I., Peleg, Z., Ozturk, L., Yazici, A. M., Budak, H., Saranga, Y., & Fahima, T. (2007) Multiple QTL-effects of wheat Gpc-B1 locus on grain protein and micronutrient concentrations. *Physiologia Plantarum*, *129*(3), 635-643.

Du Laing, G., Van de Moortel, A. M. K., Moors, W., De Grauwe, P., Meers, E., Tack, F. M. G., &Verloo, M. G. (2009) Factors affecting metal concentrations in reed plants (Phragmites australis) of intertidal marshes in the Scheldt estuary. *Ecological Engineering*, *35*(2), 310-318.

Dvořáček, V., Kodeš, A., Stehno, Z., Hučko, B., &Mudřík, Z. (2008) Nutritive effect of protein composition and other grain properties of doubled haploid wheat lines with/without translocation 1B/1R in a model feeding test. *Czech Journal of Animal Sciences*, *53*, 487-498.

Economics survey of Pakistan (2018). GOP. 2018 Economics survey of Pakistan, 2017–2018. *Economic Advisory Wing, Finance Division, Islamabad, Pakistan.*

Ehrlich, P. R., & Harte, J. (2015) Food security requires a new revolution. *International Journal of Environmental Studies*, *72*(6), 908-920.

Ekholm, P., Virkki, L., Ylinen, M., & Johansson, L. (2003) The effect of phytic acid and some natural chelating agents on the solubility of mineral elements in oat bran. *Food Chemistry*, *80*(2), 165-170.

Evanno, G., Regnaut, S., &Goudet, J. (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, *14*(8), 2611-2620.

Fan, M. S., Zhao, F. J., Fairweather-Tait, S. J., Poulton, P. R., Dunham, S. J., & McGrath, S. P. (2008) Evidence of decreasing mineral density in wheat grain over the last 160 years. *Journal of Trace Elements in Medicine and Biology*, *22*(4), 315-324.

Fan, X., Bi, Y., Zhang, Y., Jeffers, D., Yin, X., & Kang, M. (2018) Improving breeding efficiency of a hybrid maize breeding program using a three heterotic-group classification. *Agronomy Journal*, *110*(4), 1209-1216.

FAO (2015) Food and Agriculture Organization: statistics. www.fao.or/stats.

FAO (2020) Food and Agriculture Organization: statistics. www.fao.or/stats.

FAO (2021) Food and Agriculture Organization: statistics. www.fao.or/stats.

Faostat, F. A. O. (2017) Available online: http://www. fao. org/faostat/en/# data. *QC (accessed on January 2018)*.

Finkelstein, J. L., Mehta, S., Udipi, S. A., Ghugre, P. S., Luna, S. V., Wenger, M. J., Murray-Kolb, L.E., Przybyszewski., & Haas, J. D. (2015) A randomized trial of iron-biofortified pearl millet in school children in India. *The Journal of Nutrition*, *145*(7), 1576-1581.

Furlotte, N. A., &Eskin, E. (2015) Efficient multiple-trait association and estimation of genetic correlation using the matrix-variate linear mixed model. *Genetics*, *200*(1), 59-68.

Gao, F., Ma, D., Yin, G., Rasheed, A., Dong, Y., Xiao, Y., ... & He, Z. (2017) Genetic progress in grain yield and physiological traits in Chinese wheat cultivars of southern yellow and Huai Valley since 1950. *Crop Science*, *57*(2), 760-773.

Garvin, D. F., Welch, R. M., & Finley, J. W. (2006) Historical shifts in the seed mineral micronutrient concentration of US hard red winter wheat germplasm. *Journal of the Science of Food and Agriculture*, *86*(13), 2213-2220.

Genc, Y., Verbyla, A. P., Torun, A. A., Cakmak, I., Willsmore, K., Wallwork, H., & McDonald, G. K. (2009) Quantitative trait loci analysis of zinc efficiency and grain zinc concentration in wheat using whole genome average interval mapping. *Plant and Soil*, *314*(1), 49-66.

Ghandilyan, A., Vreugdenhil, D., &Aarts, M. G. (2006) Progress in the genetic understanding of plant iron and zinc nutrition. *Physiologia Plantarum*, *126*(3), 407-417.

Gibson, R. S. (2012) A historical review of progress in the assessment of dietary zinc intake as an indicator of population zinc status. *Advances in Nutrition*, *3*(6), 772-782.

Gomez-Becerra, H. F., Yazici, A., Ozturk, L., Budak, H., Peleg, Z., Morgounov, A., Fahima, T., Saranga, Y., &Cakmak, I. (2010) Genetic variation and environmental stability of grain mineral nutrient concentrations in *Triticum dicoccoides* under five environments. *Euphytica*, *171*(1), 39-52.f

Gorafi, Y. S., Ishii, T., Kim, J. S., Elbashir, A. A. E., &Tsujimoto, H (2018) Genetic variation and association mapping of grain iron and zinc contents in synthetic hexaploid wheat germplasm. *Plant Genetic Resources*, *16*(1), 9-17.

Gorafi, Y. S., Ishii, T., Kim, J. S., Elbashir, A. A. E., &Tsujimoto, H. (2018) Genetic variation and association mapping of grain iron and zinc contents in synthetic hexaploid wheat germplasm. *Plant Genetic Resources*, *16*(1), 9-17.

Goudia, B. D., & Hash, C. T. (2015) Breeding for high grain Fe and Zn levels in cereals. *International Journal of Innovation and Applied Studies*, *12*(2), 342-354.

Graham, R. D., Welch, R. M., Saunders, D. A., Ortiz-Monasterio, I., Bouis, H. E., Bonierbale, M.,Haan, Thiele, G., Liara, R., &Twomlow, S. (2007) Nutritious subsistence food systems. *Advances in Agronomy*, *92*, 1-74.

Graham, R., Senadhira, D., Beebe, S., Iglesias, C., &Monasterio, I. (1999) Breeding for micronutrient density in edible portions of staple food crops: conventional approaches. *Field Crops Research*, *60*(1-2), 57-80.

Gregorio, G. B., Senadhira, D., Htut, H., & Graham, R. D. (2000) Breeding for trace mineral density in rice. *Food and Nutrition Bulletin*, *21*(4), 382-386.

Grusak, M. A. (2002) Enhancing mineral content in plant food products. *Journal of the American College of Nutrition*, *21*(sup3), 178S-183S.

Grusak, M. A., Pearson, J. N., & Marentes, E. (1999) The physiology of micronutrient homeostasis in field crops. *Field Crops Research*, *60*(1-2), 41-56.

Guttieri, M. J., Baenziger, P. S., Frels, K., Carver, B., Arnall, B., & Waters, B. M. (2015) Variation for grain mineral concentration in a diversity panel of current and historical Great Plains hard winter wheat germplasm. *Crop Science*, *55*(3), 1035-1052.

Guzmán, C., Medina-Larqué, A. S., Velu, G., González-Santoyo, H., Singh, R. P., Huerta-Espino, J., ... & Peña, R. J. (2014) Use of wheat genetic resources to develop biofortified wheat with enhanced grain zinc and iron concentrations and desirable processing quality. *Journal of Cereal Science*, *60*(3), 617-622.

Haddad, N. M., Brudvig, L. A., Clobert, J., Davies, K. F., Gonzalez, A., Holt, R. D., Lovejoy, T., Sexton, J.O., Austin, M.P., Collins, C.D., Cook, W. M., Damschen, E.I., Ewers, R.M., Foster, B., Jenkins, C.N., King, A.J., Laurance, W.F., & Townshend, J. R. (2015) Habitat fragmentation and its lasting impact on Earth's ecosystems. *Science Advances*, *1*(2), e1500052.

Hadjivassiliou, M., & Sanders, D. (Eds.). (2019) *Extraintestinal Manifestations of Coeliac Disease*. MDPI.

Hänsch, R., & Mendel, R. R. (2009) Physiological functions of mineral micronutrients (cu, Zn, Mn, Fe, Ni, Mo, B, cl). *Current Opinion in Plant Biology*, *12*(3), 259-266.

Heun, M., Schäfer-Pregl, R., Klawan, D., Castagna, R., Accerbi, M., Borghi, B., &Salamini, F. (1997) Site of einkorn wheat domestication identified by DNA fingerprinting. *Science*, *278*(5341), 1312-1314.

Horton, R., Lo, S., Lemma, F., Matji, J., Pinstrup-Andersen, P., & Nabarro, D. (2013) Nutrition: a quintessential sustainable development goal. *Lancet Series Maternal Child Nutr*.

Hotz, V. J. IV. Designing Experimental Evaluations of Social Programs: The Case of the National JTPA Study.

Huang, Y., Wang, C., Yao, Y., Zuo, X., Chen, S., Xu, C., ... & Wang, Q. K. (2015) Molecular basis of gene-gene interaction: cyclic cross-regulation of gene expression and post-GWAS gene-gene interaction involved in atrial fibrillation. *PLoS Genetics*, *11*(8), e1005393.

Hussain, Z., Tyagi, R. K., Sharma, R., & Agrawal, A. (2008) Genetic diversity in in vitro-conserved germplasm of Curcuma L. as revealed by RAPD markers. *Biologia Plantarum*, *52*(4), 627-633.

International Wheat Genome Sequencing Consortium (IWGSC). Shifting the limits in wheat research and breeding using a fully annotated reference genome. Science 2018, 361, eaar7191.

Jeffery, S., Meinders, M. B., Stoof, C. R., Bezemer, T. M., van de Voorde, T. F., Mommer, L., & van Groenigen, J. W. (2015) Biochar application does not improve the soil hydrological function of a sandy soil. *Geoderma*, *251*, 47-54.

Jia, C., Zhao, F., Wang, X., Han, J., Zhao, H., Liu, G., & Wang, Z. (2018) Genomic prediction for 25 agronomic and quality traits in alfalfa (Medicago sativa). *Frontiers in Plant Science*, *9*, 1220.

Jobson, E. M., Martin, J. M., Schneider, T. M., & Giroux, M. J. (2018) The impact of the Rht-B1b, Rht-D1b, and Rht-8 wheat semi-dwarfing genes on flour milling, baking, and micronutrients. *Cereal Chemistry*, *95*(6), 770-778.

Joshi, A. K., Crossa, J., Arun, B., Chand, R., Trethowan, R., Vargas, M., & Ortiz-Monasterio, I. (2010) Genotype× environment interaction for zinc and iron concentration of wheat grain in eastern Gangetic plains of India. *Field Crops Research*, *116*(3), 268-277.

Joy, E. J., Ander, E. L., Young, S. D., Black, C. R., Watts, M. J., Chilimba, A. D., Chilima, B., Siyame, E.W.P., Kalimbira, A.A., Hurst, R., Fairweather-Trait, S.J., Stein, A.J., Gibson,R.S., White, P.J., & Broadley, M. R. (2014) Dietary mineral supplies in Africa. *Physiologia Plantarum*, *151*(3), 208-229.

Khan, M. S., Rizvi, A., Saif, S., & Zaidi, A. (2017) Phosphate-solubilizing microorganisms in sustainable production of wheat: current perspective. In *Probiotics in Agroecosystem* (pp. 51-81). Springer, Singapore.

Khan, N., Fahad, S., Naushad, M., & Faisal, S. (2020) Grape Production Critical Review in the World. *Available at SSRN 3595842*.

Khush, G. S., Lee, S., Cho, J. I., & Jeon, J. S. (2012) Biofortification of crops for reducing malnutrition. *Plant Biotechnology Reports*, *6*(3), 195-202.

Kumar, J., Jaiswal, V., Kumar, A., Kumar, N., Mir, R. R., Kumar, S., Dhariwal, R., Tyagi, S., Khandelwal, M., Prabhu, K.V., Prasad, R., Balyan, H.S., & Gupta, P. K. (2011) Introgression of a major gene for high grain protein content in some Indian bread wheat cultivars. *Field Crops Research*, *123*(3), 226-233.

Kumar, J., Saripalli, G., Gahlaut, V., Goel, N., Meher, P. K., Mishra, K. K., ... & Gupta, P. K. (2018) Genetics of Fe, Zn, β-carotene, GPC and yield traits in bread wheat (*Triticum aestivum L.*) using multi-locus and multi-traits GWAS. *Euphytica*, *214*(11), 1-17.

Kumar, S., Palve, A., Joshi, C., & Srivastava, R. K. (2019) Crop biofortification for iron (Fe), zinc (Zn) and vitamin A with transgenic approaches. *Heliyon*, *5*(6), e01914.

Kumssa, D. B., Joy, E. J., Ander, E. L., Watts, M. J., Young, S. D., Walker, S., & Broadley, M. R. (2015) Dietary calcium and zinc deficiency risks are decreasing but remain prevalent. *Scientific Reports*, *5*(1), 1-11.

Kutman, U. B., Yildiz, B., Ozturk, L., &Cakmak, I. (2010) Biofortification of durum wheat with zinc through soil and foliar applications of nitrogen. *Cereal Chemistry*, *87*(1), 1-9.

Labarrere, C. A., Woods, J. R., Hardin, J. W., Campana, G. L., Ortiz, M. A., Jaeger, B. R., ... & Wozniak, T. C. (2011) Early prediction of cardiac allograft vasculopathy and heart transplant failure. *American Journal of Transplantation*, *11*(3), 528-535.

Ladha, J. K., Tirol-Padre, A., Reddy, C. K., Cassman, K. G., Verma, S., Powlson, D. S., ... & Pathak, H. (2016) Global nitrogen budgets in cereals: A 50-year assessment for maize, rice and wheat production systems. *Scientific Reports*, *6*(1), 1-9.

Lipka, A. E., Tian, F., Wang, Q., Peiffer, J., Li, M., Bradbury, P. J., ... & Zhang, Z. (2012) GAPIT: genome association and prediction integrated tool. *Bioinformatics*, *28*(18), 2397-2399.

Liu, J., Huang, L., Wang, C., Liu, Y., Yan, Z., Wang, Z., ... & Wu, B. (2019) Genome-wide association study reveals novel genomic regions associated with high grain protein content in wheat lines derived from wild emmer wheat. *Frontiers in Plant Science*, *10*, 464.

Liu, J., Wu, B., Singh, R. P., &Velu, G. (2019) QTL mapping for micronutrients concentration and yield component traits in a hexaploid wheat mapping population. *Journal of Cereal Science*, *88*, 57-64.

Liu, X., Huang, M., Fan, B., Buckler, E. S., & Zhang, Z. (2016) Iterative usage of fixed and random effect models for powerful and efficient genome-wide association studies. *PLoS Genetics*, *12*(2), e1005767.

Lonergan, P. F., Pallotta, M. A., Lorimer, M., Paull, J. G., Barker, S. J., & Graham, R. D. (2009) Multiple genetic loci for zinc uptake and distribution in barley (Hordeum vulgare). *New Phytologist*, *184*(1), 168-179.

Lopes, M. S., Dreisigacker, S., Peña, R. J., Sukumaran, S., & Reynolds, M. P. (2015) Genetic characterization of the wheat association mapping initiative (WAMI) panel for dissection of complex traits in spring wheat. *Theoretical and Applied Genetics*, *128*(3), 453-464.

Lu, F., Lipka, A. E., Glaubitz, J., Elshire, R., Cherney, J. H., Casler, M. D., ... &Costich, D. E. (2013) Switchgrass genomic diversity, ploidy, and evolution: novel insights from a network-based SNP discovery protocol. *PLoS Genetics*, *9*(1), e1003215.

Lyons, G., Ortiz-Monasterio, I., Stangoulis, J., & Graham, R. (2005) Selenium concentration in wheat grain: is there sufficient genotypic variation to use in breeding?. *Plant and Soil*, *269*(1), 369-380.

Magallanes-López, A. M., Hernandez-Espinosa, N., Velu, G., Posadas-Romano, G., Ordoñez-Villegas, V. M. G., Crossa, J., ... & Guzmán, C. (2017) Variability in iron, zinc and phytic acid content in a worldwide collection of commercial durum wheat cultivars and the effect of reduced irrigation on these traits. *Food Chemistry*, *237*, 499-505.

Majumder, S., Datta, K., & Datta, S. K. (2019) Rice biofortification: high Iron, Zinc, and vitamin-A to fight against "hidden hunger". *Agronomy*, *9*(12), 803.

Manickavelu, A., Hattori, T., Yamaoka, S., Yoshimura, K., Kondou, Y., Onogi, A., ... & Ban, T. (2017) Genetic nature of elemental contents in wheat grains and its genomic prediction: toward the effective use of wheat landraces from Afghanistan. *PloS One*, *12*(1), e0169416.

Masson, P., Dalix, T., &Bussiere, S. (2010) Determination of major and trace elements in plant samples by inductively coupled plasma–mass spectrometry. *Communications in Soil Science and Plant Analysis*, *41*(3), 231-243.

Mayer, J. E., Pfeiffer, W. H., & Beyer, P. (2008) Biofortified crops to alleviate micronutrient malnutrition. *Current Opinion in Plant Biology*, *11*(2), 166-170.

McCauley, A., Jones, C., & Jacobsen, J. (2009) Soil pH and organic matter, nutrient management's module. *Bozeman, USA: Montana State University*.

McDonald, G. K., Genc, Y., & Graham, R. D. (2008) A simple method to evaluate genetic variation in grain zinc concentration by correcting for differences in grain yield. *Plant and Soil*, *306*(1), 49-55.

Mitchell, D. O., & Mielke, M. (2005) Wheat: The global market, policies, and priorities. *Global Agricultural Trade and Developing Countries*, 195-214.

Monasterio, I., & Graham, R. D. (2000) Breeding for trace minerals in wheat. *Food and Nutrition Bulletin*, *21*(4), 392-396.

Morgounov, A. I., Belan, I., Zelenskiy, Y., Roseeva, L., Tömösközi, S., Bekes, F., ... &Crossa, J. (2013) Historical changes in grain yield and quality of spring wheat varieties cultivated in Siberia from 1900 to 2010. *Canadian Journal of Plant Science*, *93*(3), 425-433.

Morgounov, A., Gómez-Becerra, H. F., Abugalieva, A., Dzhunusova, M., Yessimbekova, M., Muminjanov, H., Zelenskiy, Y., Ozturk, L., &Cakmak, I. (2007) Iron and zinc grain density in common wheat grown in Central Asia. *Euphytica*, *155*(1), 193-203.
Morgounov, A., Gómez-Becerra, H. F., Abugalieva, A., Dzhunusova, M., Yessimbekova, M., Muminjanov, H., ... &Cakmak, I. (2007) Iron and zinc grain density in common wheat grown in Central Asia. *Euphytica*, *155*(1), 193-203.

Murphy, K. M., Reeves, P. G., & Jones, S. S. (2008) Relationship between yield and mineral nutrient concentrations in historical and modern spring wheat cultivars. *Euphytica*, *163*(3), 381-

Murray, C. J., & Lopez, A. D. (2013) Measuring the global burden of disease. *New England Journal of Medicine*, *369*(5), 448-457.

Myers, S. S., Zanobetti, A., Kloog, I., Huybers, P., Leakey, A. D., Bloom, A. J., ... & Usui, Y. (2014) Increasing CO 2 threatens human nutrition. *Nature*, *510*(7503), 139-142.

Naylor, C., Lu, M., Ma, J. A., Prentice, A. M., & Petri, W. A. (2016) The impact of environmental enteropathy and systemic inflammation on infant growth failure. *The FASEB Journal*, *30*, 296-4.

Neeraja, C. N., Babu, V. R., Ram, S., Hossain, F., Hariprasanna, K., Rajpurohit, B. S., ... & Datta, S. K. (2017) Biofortification in cereals: progress and prospects. *Current Science*, 1050-1057.

Ortiz, R., Braun, H. J., Crossa, J., Crouch, J. H., Davenport, G., Dixon, J., ... & Iwanaga, M. (2008) Wheat genetic resources enhancement by the International Maize and Wheat Improvement Center (CIMMYT). *Genetic Resources and Crop Evolution*, *55*(7), 1095-1140.

Ortiz, R., Sayre, K. D., Govaerts, B., Gupta, R., Subbarao, G. V., Ban, T., Hodson, D., Dixon, J.M., Ortiz-Monasterio, J.I., & Reynolds, M. (2008) Climate change: can wheat beat the heat. *Agriculture, Ecosystems & Environment*, *126*(1-2), 46-58.

Ortiz-Monasterio, J. I., Palacios-Rojas, N., Meng, E., Pixley, K., Trethowan, R., & Pena, R. J. (2007) Enhancing the mineral and vitamin content of wheat and maize through plant breeding. *Journal of Cereal Science*, *46*(3), 293-307.

Oury, F. X., Leenhardt, F., Remesy, C., Chanliaud, E., Duperrier, B., Balfourier, F., &Charmet, G. (2006) Genetic variability and stability of grain magnesium, zinc and iron concentrations in bread wheat. *European Journal of Agronomy*, *25*(2), 177-185.

Ozkan, H., Brandolini, A., Torun, A., Altintas, S., Eker, S. E. L. İ. M., Kilian, B., ... &Cakmak, I. (2007) Natural variation and identification of microelements content in seeds of einkorn wheat (*Triticum monococcum*). In *Wheat Production in Stressed Environments* (pp. 455-462). Springer, Dordrecht.

Paltridge, N. G., Milham, P. J., Ortiz-Monasterio, J. I., Velu, G., Yasmin, Z., Palmer, L. J., Guild, G.E.,&Stangoulis, J. C. (2012) Energy-dispersive X-ray fluorescence spectrometry as a tool for zinc, iron and selenium analysis in whole grain wheat. *Plant and Soil*, *361*(1), 261-269.

Pearson, J. N., &Rengel, Z. (1994) Distribution and remobilization of Zn and Mn during grain development in wheat. *Journal of Experimental Botany*, *45*(12), 1829-1835.

Peck, A. W., McDonald, G. K., & Graham, R. D. (2008) Zinc nutrition influences the protein composition of flour in bread wheat (*Triticum aestivum L.*). *Journal of Cereal Science*, *47*(2), 266-274.

Peleg, Z., Cakmak, I., Ozturk, L., Yazici, A., Jun, Y., Budak, H., Korol, A.B., Fahima, T., &Saranga, Y. (2009) Quantitative trait loci conferring grain mineral nutrient concentrations in durum wheat× wild emmer wheat RIL population. *Theoretical and Applied Genetics*, *119*(2), 353-369.

Pfeiffer, W. H., &McClafferty, B. (2007) HarvestPlus: breeding crops for better nutrition. *Crop Science*, *47*, S-88.

Phattarakul, N., Rerkasem, B., Li, L. J., Wu, L. H., Zou, C. Q., Ram, H., Sohu, V.S., Kang, B.S., Surek, H., Kalayci, M., Yazici, A., Zhang, F.S., &Cakmak, I. (2012) Biofortification of rice grain with zinc through zinc fertilization in different countries. *Plant and Soil*, *361*(1), 131-141.

Poitevin, E. (2016) Official methods for the determination of minerals and trace elements in infant formula and milk products: a review. *Journal of AOAC International*, *99*(1), 42-52.

Poland, J. A., Brown, P. J., Sorrells, M. E., &Jannink, J. L. (2012) Development of high-density genetic maps for barley and wheat using a novel two-enzyme genotyping-by-sequencing approach. *PloS One*, *7*(2), e32253.

Popkin, B. M. (2017) Relationship between shifts in food system dynamics and acceleration of the global nutrition transition. *Nutrition Reviews*, *75*(2), 73-82.

Pozniak, C. J., Knox, R. E., Clarke, F. R., & Clarke, J. M. (2007) Identification of QTL and association of a phytoene synthase gene with endosperm colour in durum wheat. *Theoretical and Applied Genetics*, *114*(3), 525-537.

Prentice, A. (2008) Vitamin D deficiency: a global perspective. *Nutrition Reviews*, *66*(suppl_2), S153-S164.

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000) Inference of population structure using multilocus genotype data. *Genetics*, *155*(2), 945-959.

Pritchard, J. K., Wen, W., &Falush, D. (2010) Documentation for STRUCTURE software: Version 2. *University of Chicago, Chicago, IL*.

Rabbi, I. Y., Udoh, L. I., Wolfe, M., Parkes, E. Y., Gedil, M. A., Dixon, A., ... &Kulakow, P. (2017). Genome-wide association mapping of correlated traits in cassava: dry matter and total carotenoid content. *The Plant Genome*, *10*(3).

Randhawa, H. S., Asif, M., Pozniak, C., Clarke, J. M., Graf, R. J., Fox, S. L., ... &Spaner, D. (2013) Application of molecular markers to wheat breeding in C anada. *Plant Breeding*, *132*(5), 458-471.

Rasheed, A., Hao, Y., Xia, X., Khan, A., Xu, Y., Varshney, R. K., & He, Z. (2017) Crop breeding chips and genotyping platforms: progress, challenges, and perspectives. *Molecular Plant*, *10*(8), 1047-1064.

Rasheed, A., Wen, W., Gao, F., Zhai, S., Jin, H., Liu, J., ... & He, Z. (2016) Development and validation of KASP assays for genes underpinning key economic traits in bread wheat. *Theoretical and Applied Genetics*, *129*(10), 1843-1860.

Rawat, N., Tiwari, V. K., Neelam, K., Randhawa, G. S., Chhuneja, P., Singh, K., & Dhaliwal, H. S. (2009) Development and characterization of *Triticum aestivum–Aegilops kotschyi amphiploids* with high grain iron and zinc contents. *Plant Genetic Resources*, *7*(3), 271-280.

Rehman, H. U., Aziz, T., Farooq, M., Wakeel, A., &Rengel, Z. (2012) Zinc nutrition in rice production systems: a review. *Plant and Soil*, *361*(1), 203-226.

Rehman, S. U., Paterson, A., & Piggott, J. R. (2007) Chapatti quality from British wheat cultivar flours. *LWT-Food Science and Technology*, *40*(5), 775-784.

Roohani, N., Hurrell, R., Kelishadi, R., &Schulin, R. (2013).Zinc and its importance for human health: An integrative review. *Journal of Research in Medical Sciences: The Official Journal of Isfahan University of Medical Sciences*, *18*(2), 144.

Roshanzamir, H., Kordenaeej, A., &Bostani, A. (2013) Mapping QTLs related to Zn and Fe concentrations in bread wheat (*Triticum aestivum*) grain using microsatellite markers. *Iranian Journal of Genetics and Plant Breeding*, *2*(1), 10-17.

Ruel, M. T., Alderman, H., & Maternal and Child Nutrition Study Group. (2013). Nutrition-sensitive interventions and programmes: how can they help to accelerate progress in improving maternal and child nutrition.*The lancet*, *382*(9891), 536-551.

Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A., & Allard, R. W. L. (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Sciences*, *81*(24), 8014-8018.

Saltzman, A., Andersson, M. S., Asare-Marfo, D., Lividini, K., De Moura, F. F., Moursi, M., Oparinda, A., &Taleon, V. (2015) Biofortification Techniques to Improve Food Security. Reference *Module in Food Sciences.*

Sanchez, P. A., & Swaminathan, M. S. (2005) Cutting world hunger in half. *Science*, *307*(5708), 357-359.

Sehgal, D., Autrique, E., Singh, R., Ellis, M., Singh, S., &Dreisigacker, S. (2017) Identification of genomic regions for grain yield and yield stability and their epistatic interactions. *Scientific Reports*, *7*(1), 1-12.

Shao, H., Wang, H., & Tang, X. (2015) NAC transcription factors in plant multiple abiotic stress responses: progress and prospects. *Frontiers in Plant Science*, *6*, 902.

Sharma, R., Draicchio, F., Bull, H., Herzig, P., Maurer, A., Pillen, K., Thomas, W.T.B., & Flavell, A. J. (2018) Genome-wide association of yield traits in a nested association mapping population of barley reveals new gene diversity for future breeding. *Journal of Experimental Botany*, *69*(16), 3811-3822.

Shewry, P. (2016) Cultivation and impact of wheat. In *Oxford Research Encyclopedia of Environmental Science*.

Shewry, P. R. (2007) Improving the protein content and composition of cereal grain. *Journal of Cereal Science*, *46*(3), 239-250.

Shewry, P. R. (2009) Wheat. *Journal of Experimental Botany*, *60*(6), 1537-1553.

SHI, R. L., TONG, Y. P., JING, R. L., ZHANG, F. S., & ZOU, C. Q. (2013) Characterization of quantitative trait loci for grain minerals in hexaploid wheat (*Triticum aestivum L.*). *Journal of Integrative Agriculture*, *12*(9), 1512-1521.

Shi, R., Li, H., Tong, Y., Jing, R., Zhang, F., & Zou, C. (2008) Identification of quantitative trait locus of zinc and phosphorus density in wheat (*Triticum aestivum L.)* grain. *Plant and Soil*, *306*(1), 95-104.

Singh, R., Govindan, V., & Andersson, M. S. (2017) *Zinc-biofortified wheat: Harnessing Genetic Diversity for Improved Ntritional Quality* (No. 2187-2019-666).

Singh, R., Govindan, V., & Andersson, M. S. (2017) *Zinc-biofortified wheat: harnessing genetic diversity for improved nutritional quality* (No. 2187-2019-666).

Srinivasa, J., Arun, B., Mishra, V. K., Singh, G. P., Velu, G., Babu, R., Vasistha, N.K., & Joshi, A. K. (2014) Zinc and iron concentration QTL mapped in a *Triticum spelta*× *T. aestivum cross. Theoretical and Applied Genetics*, *127*(7), 1643-1651.

Stehno, Z., Konvalina, P., &Dotlačil, L. (2008) Emmer wheat growing. *Praha, VúRV*, *22*.

Stein, A. J., Nestel, P., Meenakshi, J. V., Qaim, M., Sachdev, H. P. S., &Bhutta, Z. A. (2007) Plant breeding to control zinc deficiency in India: how cost-effective is biofortification. *Public health nutrition*, *10*(5), 492-501.

Stewart, R. C., Bunn, J., Vokhiwa, M., Umar, E., Kauye, F., Fitzgerald, M., & Creed, F. (2010) Common mental disorder and associated factors amongst women with young infants in rural Malawi. *Social Psychiatry and Psychiatric Epidemiology*, *45*(5), 551-559.

Strobbe, S., De Lepeleire, J., & Van Der Straeten, D. (2018) From in planta function to vitamin-rich food crops: the ACE of biofortification. *Frontiers in Plant Science*, *9*, 1862.

Thoen, M. P., Davila Olivas, N. H., Kloth, K. J., Coolen, S., Huang, P. P., Aarts, M. G., ... &Dicke, M. (2017) Genetic architecture of plant stress resistance: multi-trait genome-wide association mapping. *New Phytologist*, *213*(3), 1346-1362.

Tiwari, S., Sl, K., Kumar, V., Singh, B., Rao, A. R., Mithra SV, A., Rai, V., Singh, A.K., & Singh, N. K. (2016) Mapping QTLs for salt tolerance in rice (*Oryza sativa L.*) by bulked segregant analysis of recombinant inbred lines using 50K SNP chip. *PloS One*, *11*(4), e0153610.

Tiwari, V. K., Rawat, N., Chhuneja, P., Neelam, K., Aggarwal, R., Randhawa, G. S., ... & Singh, K. (2009) Mapping of quantitative trait loci for grain iron and zinc concentration in diploid A genome wheat. *Journal of Heredity*, *100*(6), 771-776.

Uauy, C., Brevis, J. C., & Dubcovsky, J. (2006) The high grain protein content gene Gpc-B1 accelerates senescence and has pleiotropic effects on protein content in wheat. *Journal of Experimental Botany*, *57*(11), 2785-2794.

Velu, G., Crossa, J., Singh, R. P., Hao, Y., Dreisigacker, S., Perez-Rodriguez, P., ... & Mavi, G. S. (2016) Genomic prediction for grain zinc and iron concentrations in spring wheat. *Theoretical and Applied Genetics*, *129*(8), 1595-1605.

Velu, G., Ortiz-Monasterio, I., Cakmak, I., Hao, Y., & Singh, R. Á. (2014) Biofortification strategies to increase grain zinc and iron concentrations in wheat. *Journal of Cereal Science*, *59*(3), 365-372.

Velu, G., Singh, R. G., Balasubramaniam, A., Mishra, V. K., Chand, R., Tiwari, C., ... & Pfeiffer, W. H. (2015) Reaching out to farmers with high zinc wheat varieties through public-private partnerships: an experience from eastern-gangetic plains of India.

Velu, G., Singh, R. P., Crespo-Herrera, L., Juliana, P., Dreisigacker, S., Valluru, R., ... & Joshi, A. K. (2018) Genetic dissection of grain zinc concentration in spring wheat for mainstreaming biofortification in CIMMYT wheat breeding. *Scientific Reports*, *8*(1), 1-10.

Velu, G., Singh, R. P., Huerta, J., & Guzmán, C. (2017) Genetic impact of Rht dwarfing genes on grain micronutrients concentration in wheat. *Field Crops Research*, *214*, 373-377.

Velu, G., Singh, R. P., Huerta-Espino, J., Peña, R. J., Arun, B., Mahendru-Singh, A., ... & Pfeiffer, W. H. (2012) Performance of biofortified spring wheat genotypes in target environments for grain zinc and iron concentrations. *Field Crops Research*, *137*, 261-267.

Velu, G., Singh, R. P., Huerta-Espino, J., Peña-Bautista, R. J., & Ortíz-Monasterios, I. (2011) Breeding for enhanced zinc and iron concentration in CIMMYT spring wheat germplasm.

Velu, G., Tutus, Y., Gomez-Becerra, H. F., Hao, Y., Demir, L., Kara, R., Crespo-Herrera, L.A., Orhan, S., Yazici,A., Singh, R.P., &Cakmak, I. (2017) QTL mapping for grain zinc and iron concentrations and zinc efficiency in a tetraploid and hexaploid wheat mapping populations. *Plant and Soil*, *411*(1-2), 81-99.

Wang, Y., Wang, C., Quan, W., Jia, X., Fu, Y., Zhang, H., & Ji, W. (2016) Identification and mapping of PmSE5785, a new recessive powdery mildew resistance locus, in synthetic hexaploid wheat. *Euphytica*, *207*(3), 619-626.

Wang, Y., Xu, X., Hao, Y., Zhang, Y., Liu, Y., Pu, Z., Tian, Y., Xu, D., Xia, X., He, Z., & Zhang, Y. (2021) QTL mapping for grain zinc and iron concentrations in bread wheat.

Welch, R. M., & Graham, R. D. (2004) Breeding for micronutrients in staple food crops from a human nutrition perspective. *Journal of Experimental Botany*, *55*(396), 353-364.

White, P. J., & Broadley, M. R. (2005) Biofortifying crops with essential mineral elements. *Trends in Plant Science*, *10*(12), 586-593.

White, P. J., & Broadley, M. R. (2009) Biofortification of crops with seven mineral elements often lacking in human diets–iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytologist*, *182*(1), 49-84.

White, P. J., & Broadley, M. R. (2011) Physiological limits to zinc biofortification of edible crops. *Frontiers in Plant Science*, *2*, 80.

Willett, W., Rockström, J., Loken, B., Springmann, M., Lang, T., Vermeulen, S., ... & Murray, C. J. (2019) Food in the Anthropocene: the EAT–Lancet Commission on healthy diets from sustainable food systems. *The Lancet*, *393*(10170), 447-492.

World Health Organization. (2013) *Global tuberculosis report 2013*. World Health Organization.

World Health Organization. (2018) *The state of food security and nutrition in the world 2018: building climate resilience for food security and nutrition*. Food & Agriculture Org.

Xu, X., Cao, X., & Zhao, L. (2013) Comparison of rice husk-and dairy manure-derived biochars for simultaneously removing heavy metals from aqueous solutions: role of mineral components in biochars. *Chemosphere*, *92*(8), 955-961.

Xu, Y., An, D., Li, H., & Xu, H. (2011) Breeding wheat for enhanced micronutrients. *Canadian Journal of Plant Science*, *91*(2), 231-237.

Xu, Y., An, D., Liu, D., Zhang, A., Xu, H., & Li, B. (2012) Molecular mapping of QTLs for grain zinc, iron and protein concentration of wheat across two environments. *Field Crops Research*, *138*, 57-62.

Xu, Y., Wang, R., Tong, Y., Zhao, H., Xie, Q., Liu, D., ... & An, D. (2014) Mapping QTLs for yield and nitrogen-related traits in wheat: influence of nitrogen and phosphorus fertilization on QTL expression. *Theoretical and Applied Genetics*, *127*(1), 59-72.

Yilmaz, A., Ekiz, H., Torun, B., Gultekin, I., Karanlik, S., Bagci, S. A., &Cakmak, I. (1997) Effect of different zinc application methods on grain yield and zinc concentration in wheat cultivars grown on zinc-deficient calcareous soils. *Journal of Plant Nutrition*, *20*(4-5), 461-471.

Young, V. R., &Pellett, P. L. (1990) Current concepts concerning indispensable amino acid needs in adults and their implications for international nutrition planning. *Food and Nutrition Bulletin*, *12*(4), 1-13.

Yu, J., & Buckler, E. S. (2006) Genetic association mapping and genome organization of maize. *Current Opinion in Biotechnology*, *17*(2), 155-160.

Zhang, Y., Song, Q., Yan, J., Tang, J., Zhao, R., Zhang, Y., ... & Ortiz-Monasterio, I. (2010) Mineral element concentrations in grains of Chinese wheat cultivars. *Euphytica*, *174*(3), 303-313.

Zhao, F. J., Su, Y. H., Dunham, S. J., Rakszegi, M., Bedo, Z., McGrath, S. P., &Shewry, P. R. (2009) Variation in mineral micronutrient concentrations in grain of wheat lines of diverse origin. *Journal of Cereal science*, *49*(2), 290-295.

Zhao, F. J., Su, Y. H., Dunham, S. J., Rakszegi, M., Bedo, Z., McGrath, S. P., &Shewry, P. R. (2009) Variation in mineral micronutrient concentrations in grain of wheat lines of diverse origin. *Journal of Cereal Science*, *49*(2), 290-295.

Zhao, N., Yu, X., Jie, Q., Li, H., Li, H., Hu, J.,. Zhai, H., & Liu, Q. (2013) A genetic linkage map based on AFLP and SSR markers and mapping of QTL for dry-matter content in sweet potato. *Molecular Breeding*, *32*(4), 807-820.

Zhu, C., Gore, M., Buckler, E. S., & Yu, J. (2008) Status and prospects of association mapping in plants. *The Plant Genome*, *1*(1).

Zimm, C., Sperling, F., & Busch, S. (2018) Identifying sustainability and knowledge gaps in socio-economic pathways vis-à-vis the Sustainable Development Goals. *Economies*, *6*(2), 20.

QUAID-I-AZAM UNIVERSITY, ISLAMABAD

DEPARTMENT OF PLANT SCIENCES

Subject: **Publication of W-category Mr. Muzzafar Shaukat (PhD Scholar)**

This is in reference to circular regarding the publication requirement for PhD in Department of Plant Sciences, Faculty of Biological Sciences. It is certified that **Mr. Muzzafar Shaukat** has published research paper in W-category as given below.

Sr. No	Paper title	Year	Impact factor
1	Genetic Gain for Grain Micronutrients and Their Association with Phenology in Historical Wheat Cultivars Released between 1911 and 2016 in Pakistan	2021	3.41

Supervisor: _____

agronomy

MDPI

Article

Genetic Gain for Grain Micronutrients and Their Association with Phenology in Historical Wheat Cultivars Released between 1911 and 2016 in Pakistan

Muzzafar Shaukat [1], Mengjing Sun [2], Mohsin Ali [2], Tariq Mahmood [1], Samar Naseer [1], Saman Maqbool [1], Shoaib Rehman [3], Zahid Mahmood [4], Yuanfeng Hao [2], Xianchun Xia [2], Awais Rasheed [1,2,5,*] and Zhonghu He [2,5,*]

1 Department of Plant Sciences, Quaid-i-Azam University, Islamabad 45320, Pakistan; m.muzaffar.shoukat@gmail.com (M.S.); tmahmood@qau.edu.pk (T.M.); samar_naseer@yahoo.com (S.N.); samanmaqbool@bs.qau.edu.pk (S.M.)
2 Institute of Crop Science, Chinese Academy of Agricultural Sciences (CAAS), 12 Zhongguanccun South Street, Beijing 100081, China; smj970626@163.com (M.S.); mali1990@yahoo.com (M.A.); haoyuanfeng@caas.cn (Y.H.); xiaxianchun@caas.cn (X.X.)
3 Institute of Biotechnology, Muhammad Nawaz Shareef University of Agriculture, Multan 66000, Pakistan; shoaib.rehman10@hotmail.com
4 Wheat Program, Institute of Crop Sciences, National Agriculture Research Center (NARC), Park Road, Islamabad 45320, Pakistan; zeearid@gmail.com
5 CIMMYT-China c/o CAAS, 12 Zhongguanccun South Street, Beijing 100081, China
* Correspondence: arasheed@qau.edu.pk (A.R.); z.he@cgiar.org (Z.H.)

Citation: Shaukat, M.; Sun, M.; Ali, M.; Mahmood, T.; Naseer, S.; Maqbool, S.; Rehman, S.; Mahmood, Z.; Hao, Y.; Xia, X.; et al. Genetic Gain for Grain Micronutrients and Their Association with Phenology in Historical Wheat Cultivars Released between 1911 and 2016 in Pakistan. *Agronomy* **2021**, *11*, 1247. https://doi.org/10.3390/agronomy11061247

Academic Editors: Ahmad M. Alqudah and Andreas Börner

Received: 8 May 2021
Accepted: 10 June 2021
Published: 19 June 2021

Abstract: Wheat (*Triticum aestivum* L.), being a staple food crop, is an important nutritional source providing protein and minerals. It is important to fortify staple cereals such as wheat with essential minerals to overcome the problems associated with malnutrition. The experiment was designed to evaluate the status of 11 micronutrients including grain iron (GFe) and zinc (GZn) in 62 wheat cultivars released between 1911 and 2016 in Pakistan. Field trials were conducted over two years and GFe and GZn were quantified by both inductively coupled plasma optical emission spectroscopy (ICP-OES) and energy-dispersive X-ray fluorescence spectrophotometer (EDXRF). The GZn ranged from 18.4 to 40.8 mg/kg by ED-XRF and 23.7 to 38.8 mg/kg by ICP-OES. Similarly, GFe ranged from 24.8 to 44.1 mg/kg by ICP-OES and 26.8 to 36.6 mg/kg by EDEXR. The coefficient of correlation was higher for GZn (r = 0.90), compared to GFe (r = 0.68). Modern cultivars such as Zincol-16 and AAS-2011 showed higher GFe and GZn along with improved yield components. Old wheat cultivars WL-711, C-518 and Pothowar-70, released before 1970, also exhibited higher values of GFe and GZn; however, their agronomic performance was poor. Multivariate analysis using eleven micronutrients (Fe, Zn, Al, Ca, Cu, K, Mg, Mn, Na, Se and P) along with agronomic traits, and genome-wide SNP markers identified the potential cultivar with improved yield, biofortification and wider genetic diversity. Genetic gain analysis identified a significant increase in grain yield (0.4% year^{-1}), while there was negative gain for GFe (−0.11% year^{-1}) and GZn (−0.15% year^{-1}) over the span of 100 years. The Green Revolution *Rht-B1* and *Rht-D1* genes had a strong association with plant height and grain yield (GY), while semi-dwarfing alleles had a negative effect on GFe and GZn contents. This study provided a valuable insight into the biofortification status of wheat cultivars deployed historically in Pakistan and is a valuable source to initiate a breeding strategy for simultaneous improvement in wheat phenology and biofortification.

Keywords: wheat; biofortification; iron; zinc; rht genes

1. Introduction

More than two billion people in the world are severely affected by the dietary deficiency of essential micronutrients such as zinc (Zn) and iron (Fe) [1]. Zinc deficiency

Title: Association Genetics of Grain Mineral Elements in wheat from Pakistan

ORIGINALITY REPORT

18%	9%	16%	4%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.frontiersin.org Internet Source	2%
2	Submitted to Higher Education Commission Pakistan Student Paper	1%
3	link.springer.com Internet Source	1%
4	www.bioone.org Internet Source	1%
5	Sanu Arora, Jitender Cheema, Jesse Poland, Cristobal Uauy, Parveen Chhuneja. "Genome-Wide Association Mapping of Grain Micronutrients Concentration in Aegilops tauschii", Frontiers in Plant Science, 2019 Publication	1%
6	Cakmak, Ismail, Wolfgang H. Pfeiffer, and Bonnie McClafferty. "REVIEW: Biofortification of Durum Wheat with Zinc and Iron", Cereal Chemistry, 2010. Publication	1%