Mapping genetic determinants for grain physicochemical and nutritional traits in unpolished rice using genome-wide association analysis

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Abstract

Public awareness is gradually growing in favour of consuming nutritionally superior brown and pigmented rice. But unpolished rice is relatively poor in eating and cooking quality. Understanding inheritance pattern of nutritional and quality traits is prerequisite for further improvement exercising molecular tools. A holistic approach was adopted to identify unique and common genomic regions regulating 17 grain nutritional as well as physiochemical traits using a diverse panel of 96 rice genotypes. Seventy eight significant marker-trait association distributed in all chromosomes with PVE ranging from 4–27% were detected. Marker RM 467 was co-localized with previously identified QTL *qPC10.1* and gene *OsGluA2.* Grain protein and metal content were associated with cooking quality which was also supported by the marker-trait association. Two QTLs for each of grain protein and amylose content with associated markers RM 17600 and RM 1272 were found co-localized with additive effect in opposite direction. Similarly, RM 162 was associated with both zinc content and gelatinization temperature (alkali spreading value). Iron content was also associated with grain size which was supported by the association of RM8050 with the both Fe content and kernel length/breadth ratio. Phenotypically pigmented rice was detected with low amylose content and one genetic loci for anthocyanin (*qANTH5*.1) was also found to be co-localized with a QTL for AC (*qAC5.1*) with additive effect in opposite direction. Co-localized associated loci for nutritional and cooking and eating quality can guide strategic biofotification programs for improving rice for nutritional traits without distracting consumer preference.

Introduction

More than half of the world's population considers rice to be "food of life", as it is their primary source of calories. Milled rice is commonly consumed, which has a lesser nutritional value than brown or pigmented rice (Kushwaha 2016). Milling removes most of the minerals, protein, vitamins, and other antioxidants found in rice bran. In general, colored or pigmented rice contains higher amounts of proteins, vitamins, minerals, fibre, and phytochemicals such as tocopherols, tocotrienols, γ-oryzanols, phenolic compounds, and other phytochemicals, all of which provide numerous health benefits by supplying required nutrients. Phenolics and anthocyanins in the aleurone layer offers pigmentation on rice grains; hence, pigmented rice is consumed as brown rice (Descalsota-Empleo et al. 2019). Iron and Zinc, as well as anthocyanin-related antioxidants, are required for normal growth and development as well as living a healthy life (Goufo and Trindade 2014; Oki et al. 2002). According to Saleh et al. (2019), Fe and Zn deficiencies, malnutrition, and oxidative stress-related health problems impact more than one-third of the world's population. The deficiencies of micronutrients like Fe and Zn lead health complications like anaemia, poor cognitive development, poor immunity, low fertility, etc (Cheng et al, 2005; Bouis, 2007; Singh et al. 2007). Oxidative stress, on the other hand, is linked to a number of health issues, including stroke, psoriasis, and rheumatoid arthritis (Galli et al. 2005). Consumption of pigmented rice appears to protect against certain malignancies, according to a large body of evidences. The extracts of black and red rice demonstrates to have significant impact on inhibiting breast cancer (Ghasemzadeh et al. 2018) and anthocyanin pigments known to reduce blood cholesterol in human body (Sompong et al. 2011).

Significant raise in awareness on nutrition and health among public, the nutritional benefits of pigmented or brown rice draws public attention. Thus, the field of breeding science prioritized Fe, Zn and other anti-oxidant compounds for bio-fortification staple food sources like rice (Garg et al. 2018). Apart from micro-nutrients, protein in rice has significant role as calorie source and also for improving cooking quality of rice. Hence, improving rice varieties with significant amounts of micro-nutrients along with protein is need of the hour in the

present era of nutritional security. As a prelude to bio-fortification, it is important to identify genomic regions controlling nutritional quality traits through advanced mapping approaches (Patra et al. 2020; Chattopadhyay et al. 2019a). However, few QTL controlling Fe, Zn and protein content in brown and milled rice have been reported on all 12 rice chromosomes (Sharma et al, 2020). Among these, a putative amino acid polymerase gene *OsAAP6* inside QTL in chromosome 1 *qPC1.1* (Peng et al. 2014) and putative gene for glutelin *OsGluA2* inside QTL on chromosome 10 (*qGPC10*) (Yang et al, 2019) were cloned. Similarly, anthocyanin content in the pericarp of black rice was reported to regulated by *Ra*, *Rc*, *Rd*, *Kala1*, *Kala3*, and *Kala4* genes (Winkel-Shirley 2001), of which *Kala4* produces purple or black pericarp in the complementation of *Kala1* and *Kala3* (Maeda et al. 2014). Although brown rice is nutritionally more superior to milled rice, storability in brown rice is a serious concern due to rancidity. Moreover, brown rice has comparatively poor eating quality due to firm, chewy, hard and loose texture in cooked rice, and resulting lower acceptability by the consumers. The eating quality of red rice also makes it difficult and challenging for consumers to eat red rice on regular basis. Even biofortified rice cannot be acceptable to consumers without acceptable cooking and eating quality. Among cooking quality, amylose (AC) and gel consistency (GC) along with alkali spresding value (ASV) are considered most important. AC has been reported to be controlled mainly by *waxy* gene locus (*Wx*) which affect the cooking quality was reported on chromosome 6 and few other minor QTL for cooking quality were mapped on chromosomes 1, 3, 4, 7, 8 and 11 (Zheng et al. 2008; Tian et al. 2009). Many of these QTL were identified from bi-parental population, which restricts there transferability to other genetic backgrounds; hence, limits the practical application of these findings. Further, the variation for quantitative traits like nutritional quality should be captured by following a holistic approach instead of bi-parental mapping approach (Mather et al. 2004).

The limitations of bi-parental mapping to capture wide variation of quantitative traits may be resolved by adopting modified forms of linkage disequilibrium mapping, popularly known as genome-wide association analysis (GWAS). GWAS captures historic recombination frequency instead of parental recombination frequency for identification of genomic determinants of quantitative traits (Yu et al. 2017). Considering the existence of huge allelic diversity for nutritional traits in rice, GWAS could be the most promising strategy for simultaneous mapping of QTL for several nutritional traits with high precision (Huang and Han 2014). Hence, GWAS can be deployed for effective identification of causative alleles for rice grain nutritional traits with modest number of markers distributed over all the chromosomes.

In this milieu, a genome-wide association analysis was performed with statistically strong and diverse association panel evaluated for 17 physicochemical and nutritional traits in rice. Selected traits are considered most important in bio-fortification programs to develop mineral dense rice varieties to combat mineral malnutrition. The main objective of the study was to identify unique QTL/associated markers with traits of importance for bio-fortification and grain quality. We also aimed to identify pleiotropic QTL/marker trait association (MTA) for physicochemical and nutritional quality traits to deploy them in bio-fortification breeding programs. The results of this study have significant scope in developing strategic marker aided breeding program for bio-fortification of nutritional and grain quality traits in rice.

Materials And Methods

Plant materials

A set of 96 top performing germplasm accessions in terms of yield and quality were selected from a pool of 300 rice accessions. This panel of 96 genotypes comprised of 36 rice landraces, 39 released varieties and 21 breeding lines from indigenous and exotic germplasm collection maintained at ICAR-NRRI, Cuttack (Supplementary Table 1). These were either originated or released for cultivation in Indian states such as Odisha, Chhattisgarh, West Bengal, Andhra Pradesh, Tamil Nadu, Kerala, Karnataka, Goa, Maharastra, Gujarat, Madhya Pradesh, Uttar Pradesh, Jharkhand, Punjab, Assam, Tripura, Manipur, Nagaland and Meghalaya. Many of the landraces considered are well known for their nutritional quality among rice farmers of these states. Six of these germplasm were exotic collections from International Rice Research Institute, Philippines. Thirty genotypes of this set were either identified donors or advanced breeding lines in breeding programme for improvement of grain and nutritional quality as part of bio-fortification programs. This germplasm set also comprised of landraces, ARC 10075 and ARC10063 and released varieties CR Dhan 310 and Mukul (CR Dhan 311) with high grain protein content (Chattopadhyay et al. 2019b), indigenous cultivars such as *Bindli* and *Chittimitiyalu* and high zinc content bio-fortified variety CR Dhan 315 (Sanghamitra et al. 2022) and landraces *Kalobhat*, *Manipuri Black rice* and *Mamihanger* (Sanghamitra et al. 2018; Bagchi et al. 2021) reported as rich sources of anti-oxidative compounds (anthocyanin, flavonoids, etc.). Glutinous rice such as *Kala Bora*, *Chakhao*, *Kalobhat*, etc. with very low amylose content (AC) and variety such as Mahsuri with high amylose contain were part of this panel; it also included ten aromatic rice germplasm accessions. The genotypes of this panel were highly diverse in terms of their nutritional quality and geographic distribution, hence, the panel designed for this study was most ideal for association analysis of nutritional and grain quality traits. The genotypes of association panel were planted in randomized complete block design in three rows with spacing of 20 × 15 cm in two replications at the farm of ICAR-National Rice Research Institute (ICAR-NRRI) in *kharif* 2018. The recommended packages of practices were followed to raise a healthy crop for better experimental results. Upon harvesting, the seeds of each genotype were dried separately to reduce the moisture content to 11–13% first in the sun, then in a hot air oven to homogenize the grain samples. The five random samples were drawn from each replication and subjected to physicochemical and quality analysis in order to minimize the error and ensure promising results.

Estimation of Physicochemical parameters

Physicochemical parameters and grain parameters of association panel were measured as phenotypes for performing GWAS. The digital grain vernier (Indosaw Pvt. Ltd., India) was used for measurement of kernel length (KL: mm), breadth (KB: mm) and L/B ratio (LB). Alkali spreading value (ASV) was measured using 1.7% KOH solution on milled rice grain. For measurement of kernel length after cooking (KLAC: mm), 5g of milled rice was cooked with 15 ml of distilled water in a glass cooking tube, subsequently the length was measured by a scale and expressed in mm. For measuring VER- the ratio of volume after cooking to volume before cooking, the cooked rice was added in to a 100 ml measuring cylinder containing 50 ml of water (Pal et al. 2019). Amylose content (AC) of the grain was measured following the standard method (Juliano 2003) using 1N NaOH, 1N acetic acid, ethanol and iodine solution and expressed on % basis. Gel consistency (GC) of the rice flour was measured by ethanol containing 0.03% thymol blue and 2 ml 0.2 N KOH and expressed as mm of gel length (Juliano 1980). The hardness (HD) of the sample was measured through texture analyzer model TAXT plus (Stable Micro Systems Ltd, Surrey, UK) as per modified method of Jaiboon et al. (2016) and hardness was expressed as Newton.

Estimation of grain protein, Fe and Zn content

The total grain protein content (GPC) was estimated by standard *Kjeldhal* method. GPC (%) was calculated by multiplication of N_2 content by 5.95 (factor for rice) (FAO, 1970). Iron and Zinc content was estimated by high resolution atomic absorption spectrophotometer (AAS) (Analytic Jena, Germany) using flame ionization method. The brown rice sample (1 g) was digested with conc. HNO_3, ultrapure distilled water and H_2O_2 by a micro wave digester before subjecting to estimation (Tyagi et al. 2020).

Estimation of anti-oxidant properties

Anthocyanin (ANTH) was extracted from the sample by using methanol HCL solvent as described by Fuleki and Francis (1968) with minor modifications. Total anthocyanin content (TAC) of the sample was calculated by using a multiplication factor of 16.73 × absorbance at 535 nm and expressed as 'mg' total anthocyanin per 100 g of sample. Standard procedure (Chen and Bergman 2005) with some simplification was used for γ-oryzanol (GORY) extraction (2–3 times) from sample by using HPLC grade isopropanol. Shimadzu High Performance Liquid Chromatography (RP-HPLC) system equipped with an LC-20AT pump and PDA detector (Shimadzu, Kyoto, Japan) was used to separate the filtered extract. Mobile phase was operated in low pressure gradient mode with 35% acetonitrile, 55% methanol and 10% isopropanol with run time of 35 min including column equilibration. The quantification of total γ-oryzanol was based on the sum of the peak area of four main peaks obtained between 13 and 18 minutes by using PDA detector at 325nm.

The total phenolics (TPC) was estimated according to Zilic et al. (2011) by using Folineciocaltue reagent at 725 nm using catechol (CE) as a standard and expressed as mg catechol (CE)100 g^{-1}. Total flavonoid content (TFC) was determined according to Eberhardt et al. (2000) at 510 nm using catechine (CE) as a standard and expressed as mg catechine (CEt) 100 g^{-1}. Procedure of Zhu et al. (2006) with minor modification was used for diphenylpicrylhydrazyl (DPPH) radical scavenging assay at 517nm and expressed as % inhibition.

DNA extraction and fingerprinting with SSR markers

The genomic DNA (gDNA) of individuals of the association panel was extracted from younger leaves of seedlings. Around 1 g leaf sample of each of 96 genotypes were used for DNA extraction and purification following CTAB method (Murray and Thompson 1980). The genomic DNA of each sample was diluted accordingly to 20ng/µl to use in PCR amplification of SSR markers. Initially, 250 Type I and II SSR markers were tested using 5 randomly selected genotypes. Finally, 122 markers with good amplification and even distribution over all 12 rice chromosomes were selected for genotyping the association panel (Supplementary Fig. 1). Even though the marker number was smaller, 100 well distributed SSR markers can provide the information similar to 1000 SNP markers and markers utilized in the study were properly distributed on all 12 rice chromosomes (Yesmin et al. 2014). Hence, the results obtained in the study can be used to draw constructive interpretation followed by utilization in MAS breeding programs.

The polymerase chain reaction was done in a solution (25 µl) containing 10 mM Tris-HCl buffer (pH 8.2), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatine, 200 µM dNTPs, 0.2µM primers, 1 unit *Taq* DNA Polymerase and 20ng/µl of the template DNA. The amplification reaction consisted of pre-heating for 5 min at 94°C followed by 36 cycles of denaturation at 94°C for 1 minute, annealing at 53–61°C for 1 minute and elongation at 72°C for 1 minute, followed by extension at 72°C for 5 minutes in a PCR system (Ependorf make). The amplified products were separated in 2% agarose gel containing 0.5 ng mL^{-1} of EtBr (ethidium bromide). The separated PCR products were visualized and photographed in Systronics Gel documentation System.

Statistical analysis

Phenotypic data analysis

Observations recorded on 17 physicochemical and grain quality recorded from five random samples on each genotype over two replications was subjected to estimation of best linear unbiased predictor values (BLUP). BLUP estimation on number of samples over replication shrinks the phenotype values into a single value by reducing the mean squared error (Piepho et al. 2008). BLUP values of phenotypes were estimated using CIMMYT developed META-R software (Alvarado et al. 2020). The BLUP values for phenotypes on association panel were considered for preliminary variation analysis before subjecting to GWAS. To ensure the best fit of panel for association analysis, the trait variation, pattern of phenotypic distribution and descriptive statistics of all phenotypes were analyzed using RStudio version 1.4.17 (R Core Team 2021). To understand the inter-relationship among traits, correlation coefficients were estimated following Pearson's correlation approach using "PerformanceAnalytics" package in R software (Peterson et al. 2018) and a correlation plot was developed using 'corrplot' package in R software (Wei and Simko 2021).

Population stratification and GWAS analysis

The detailed understanding of population structure and relatedness among individuals of association panel is a prelude for performing GWAS. An integrated package called GAPIT (Genomic Association and Prediction Integrated Tool) was employed on R software background to perform the analysis. GAPIT assess the population structure by estimating kinship coefficients between individuals of association panel based on VanRanden algorithm. The results of population structure were made available to visualize through kinship heat map and also with principle component plots. The genotypic information of 133 microsatellite markers scanned on all the individuals of the association panel and phenotype information of 17 physicochemical traits were provided as input files for association analysis. Compressed MLM (CMLM) approach was used for GWAS analysis in GAPIT package in R software (Lipka et al. 2012). The CMLM is compressed and optimized model of MLM approach. Efficient mixed-model association (EMMA) (Kang et al, 2008) was adopted for simultaneous identification of marker trait associations and correction for population structure. Significant associations were identified based on significant marker *p* values.

Results

Phenotype variation analysis

Significant variation for 17 physicochemical and nutritional traits studied on 96 genotypes of the association panel was observed (Table 1). Significantly high range for values of all nutritional traits was detected in GPC (3.81–13.13%), Zn content (14.18–69.20 ppm), Fe content (10.46–73.42 ppm). These germplasm set contained a wide range of coloration in kernel starting from white, brown, red, purple and black. Therefore, as expected a wide range of variation in anti-oxidant traits such as ANTH (2.50-96.14 mg), GORY (27.69–82.25), TPC (15.63–664.20), TFC (65.78- 428.56) and DPPH (2.16–99.87%) from unpolished brown rice samples were recorded. This germplasm panel included low amylose glutinous rice and also high amylose rice; hence, amylose content varied from 5.4% (Kala Birohin) to 27.4% (Sahabhagidhan). As normal distribution of trait variation is preliminary requirement for GWAS analysis, phenotypic distribution of variation of all the traits were tested and presented in

Fig. 1. Most of the traits exhibited normal distribution except ASV, Fe, ANTH, TPC and TFC which showed skewness in the distribution either slightly towards higher or lower extremes. However, the bell shaped distribution phenotype from association panel was considered an indication that the traits are controlled by polygenes following quantitative inheritance pattern and more suitable for association analysis. For better understanding the relationship between variables considered for the study, Pearson correlation analysis was performed (Fig. 2). Significant positive and high correlation coefficients were obtained between TFC with TPC (r = 0.94), TPC with DPPH (r = 0.80), DPPH with TFC (r = 0.77) and KL with KLAC (r = 0.63). Apart from that many of the relationships were found positive with medium level of associations and some were with very modest level of relations among the traits considered for the study. Positive correlations were also found between ANTH and TPC (r = 0.47), TFC (r = 0.55) and DPPH (r = 0.42). However, LB was found significantly negatively related with KB and GC was significant but negatively related with AC. Fe content was found positively associated with KL and KB while it had negative association with AC which eventually was negatively associated with most of the anti-oxidative nutritional compounds.

Table 1
Phenotypic variations of nutritional and quality traits assessed on association panel

Trait	Range		Mean	SD	SE
	Min	Max			
KL	3.64	7.03	5.37	0.67	0.07
KB	1.23	2.58	1.91	0.30	0.03
LB	1.81	4.33	2.85	0.46	2.85
ASV	3.00	7.00	4.10	1.08	0.11
VER	3.25	4.25	3.82	0.16	0.016
KLAC	2.96	11.60	9.30	1.42	0.14
AC	5.40	27.49	18.62	4.49	0.45
GC	31.00	77.33	51.98	10.23	1.04
GPC	3.81	14.88	8.88	1.83	0.18
Zn	14.18	129.09	39.21	12.87	1.31
Fe	10.46	73.42	21.43	10.79	1.10
HD	26336	45215	36806.41	3842.53	392.17
ANTH	2.49	96.14	5.64	14.07	1.43
GORY	27.68	82.25	44.28	12.17	1.24
TPC	15.62	664.20	109.27	119.51	12.19
TFC	65.77	428.55	104.01	58.19	6.01
DPPH	2.15	99.87	38.91	28.27	2.88

(Note: KL: kernel length, KB: kernel breadth, LB: L/B ratio, ASV: Alkali spreading value, LKAC: kernel length after cooking, ASV: alkali spreading value, VER: Volume expansion ratio, HD: kernel hardness, AC: amylose content, GC: gel consistency, GPC: grain protein content, Zn: zinc content, Fe: iron content, ANTH: Anthocyanin content, TAC: Total anthocyanin content, GORY: γ-oryzanol, TPC: total phenolics content, TFC: total flavonoid content., DPPH: diphenylpicrylhydrazyl radical scavenging assay)

Population structure

Among 122 SSR markers which were distributed on all 12 rice chromosomes, 18 were monomorphic and the rest were all polymorphic (Supplementary Table 2). These markers generated 249 unique loci upon genotyping 96 germplasm accessions of association panel. The genotypic data was subjected to population structure analysis. It is most important to control the population structure before performing GWAS in plant species. The results of population structure with VanRaden kinship matrix derived heatmap stratified the association panel into three subpopulations (Fig. 3A). The results of PCA confirmed the presence of three subpopulations within the association panel (Fig. 3B). Appropriate K value determined by scree plot re-iterated the results of heatmap and PCA (Fig. 3C). Two larger subpopulations comprised 40 (Cluster-III) and 37 (Cluster-I) genotypes and the smaller

subpopulation (Cluster-II) contained 19 genotypes. Subpopulation-I contained many genotypes with high antioxidant activities as well as low amylose content. Subpopulation-III had genotypes with high protein donors such as ARC 10075 and ARC10063. Subpopulation-II contained genotypes such as Pusa- 1176 with high Fe and Zn content.

Marker-trait association by GWAS

Upon ensuring population structure through principle component analysis and VanRanden kinship analysis, the association panel was subjected to marker trait association. The mean phenotypic values on 17 quantitative traits recorded from 96 individuals of the association panel which was genotyped with 122 SSR markers was fitted with CMLM model to find marker trait associations. A total of 78 significant marker trait associations for all 17 quantitative traits were recorded at $p < 0.05$ which were distributed over almost all the rice chromosomes (Table 2 and Fig. 4). For all the traits studied, at least two markers were linked significantly. Eight markers were significantly linked with the trait AC with explained phenotypic value (PVE) ranging from 25 to 27%, most of them were having positive allelic effect on trait expression except only two markers with negative effects. The trait ANTH was significantly associated with three markers present on chromosome 9, 12 and 5 with allelic effect − 9.12, 5.47 and − 7.64, respectively. For the trait ASV, eight markers were significantly associated with a phenotypic variation explained that ranged between 18 and 20%, while, four markers were associated with the trait DPPH with explained phenotypic variation that ranged from 7 to 8%. Six markers were found to be associated significantly with Fe, three markers on chromosome 1, two on chromosome 2 and one marker on chromosome 3 with phenotypic variation explained ranged between 16 and 18%, whereas, three markers were associated with trait GC, each one on chromosome 5, 10 and 2, and explaining phenotypic variation of 24%. Traits GORY and HD both were found associated by four markers and their phenotypic variation explained was also minor. Six markers were found to be associated with trait GPC with minor phenotypic variation explained, whereas, traits KB and KL each were associated by seven markers and their explained phenotypic variation ranged from 10 to 14%. Traits KLAC, TFC, TPC and VER were associated with two markers each and, for KLAC both markers explained phenotypic variation of 7% each, for TFC markers explained 14 and 15% of phenotypic variation respectively, for TPC 16 and 17% and for VER it was 10 and 11% respectively. The trait LB was significantly associated with four markers with minor phenotypic variation explained, while, six markers were significantly associated to Zn with explained phenotypic variation of 7 to 8%. The Manhattan and Q-Q plots developed based on CMLM model representing same results is also presented as Fig. 4. The Q-Q plot represents the deviation of observed *p* values from the expected indicating significant marker trait associations.

Table 2
Significant marker trait association identified for 17 physicochemical and nutritional traits in rice

Trait	*QTLs*	Marker	Chromosome number	Position (bp)	p-value	R^2	Allele effect	*Co-localized QTL*
AC	*qAC2.1*	RM1075	2	3833177	0.024	0.26	1.15	*qFe2.1*
	qAC2.2	RM324	2	11389704	0.017	0.26	1.94	*qKB2.1*
	qAC4.1	RM17600	4	33596398	0.037	0.25	4.43	*qGPC4.1*
	qAC4.2	RM1272	4	35110870	0.037	0.25	4.43	*qGPC4.2*
	qAC5.1	RM18136	5	7764190	0.034	0.25	2.3	*qKB3.1*
	qAC5.2	RM 87	5	129200000	0.012	0.27	2.01	
	qAC7.1	RM 21521	7	15985258	0.048	0.25	-1.15	*qKL7.1*
	qAC10.1	RM25022	10	3589942	0.046	0.25	-1.55	*qGC10.1*
ASV	*qASV2.1*	RM181	2	201993	0.033	0.18	-0.33	
	qASV3.1	RM14761	3	9782360	0.009	0.2	-0.41	*qKL3.1*
	qASV3.2	RM135	3	27411671	0.022	0.18	0.4	
	qASV6.1	RM197	6	3085639	0.029	0.18	0.3	*qGORY6.1*
	qASV6.2	RM276	6	6230045	0.035	0.18	0.3	
	qASV6.3	RM162	6	24035491	0.04	0.18	0.47	*qZn6.2*
	qASV8.1	RM210	8	22471837	0.035	0.18	-0.55	
	qASV8.2	RM149	8	24721365	0.03	0.18	-0.43	
GC	*qGC2.1*	RM13928	2	30409991	0.029	0.24	-2.99	
	qGC5.1	RM32	5	4200000	0.021	0.24	3.72	
	qGC10.1	RM25022	10	3589942	0.027	0.24	4.02	*qAC10.1*
HD	*qHD2.1*	RM13604	2	24549546	0.017	0.06	1376.6	*qVER2.1*
	qHD3.1	RM175	3	3865706	0.036	0.04	-998.4	
	qHD8.1	RM281	8	27895505	0.004	0.09	2702.8	*qZn8.1*
	qHD12.1	RM28828	12	27478976	0.017	0.06	-1764.2	*qGPC12.1*
KB	*qKB2.1*	RM324	2	11389704	0.01	0.13	0.16	*qLB2.1*

(Note: KL: kernel length, KB: kernel breadth, LB: L/B ratio, ASV: Alkali spreading value, LKAC: kernel length after cooking, ASV: alkali spreading value, VER: Volume expansion ratio, HD: kernel hardness, AC: amylose content, GC: gel consistency, GPC: grain protein content, Zn: zinc content, Fe: iron content, ANTH: Anthocyanin content, TAC: Total anthocyanin content, GORY: γ-oryzanol, TPC: total phenolics content, TFC: total flavonoid content., DPPH: diphenylpicrylhydrazyl radical scavenging assay)

Trait	*QTLs*	Marker	Chromosome number	Position (bp)	p-value	R^2	Allele effect	*Co-localized QTL*
	qKB3.1	RM16138	3	34611863	0.036	0.11	-0.11	*qVER3.1*
	qKB4.1	RM142	4	20518899	0.037	0.11	-0.16	
	qKB5.1	RM3663	5	21363398	0.037	0.11	-0.09	*qLB5.1*
	qKB7.1	RM182	7	77700000	0.048	0.1	0.15	
	qKB9.1	RM257	9	17719660	0.046	0.1	0.07	
	qKB9.2	RM285	9	66000000	0.009	0.13	0.2	*qLB9.1*
KL	*qKL2.1*	RM12678	2	5764761	0.039	0.1	0.19	
	qKL2.2	RM71	2	8760433	0.045	0.1	0.17	*qFe2.2*
	qKL3.1	RM14761	3	9782360	0.02	0.12	0.26	*qASV3.1*
	qKL4.1	RM142	4	20518899	0.009	0.13	-0.45	
	qKL7.1	RM 21521	7	15985258	0.004	0.14	0.31	*qZn7.1*
	qKL11.1	RM286	11	383711	0.021	0.11	0.23	
	qKL11.2	RM4862	11	9980302	0.038	0.1	0.4	*qTPC11.1*
LB	*qLB1.1*	RM8050	1	42072506	0.027	0.06	0.19	*qFe1.3*
	qLB2.1	RM324	2	11389704	0.027	0.06	-0.21	*qKB2.1*
	qLB5.1	RM3663	5	21363398	0.015	0.07	0.16	
	qLB9.1	RM285	9	66000000	0.006	0.09	-0.32	*qANTH9.1*
KLAC	*qKLAC10.1*	RM25754	10	20198093	0.026	0.07	-0.65	
	qKLAC11.1	RM27177	11	25297111	0.021	0.07	0.56	*qDPPH11.1*
VER	*qVER2.1*	RM13604	2	24549546	0.015	0.11	0.06	*qHD2.1*
	qVER3.1	RM16138	3	34611863	0.023	0.1	-0.06	*qKB3.1*
TFC	*qTFC4.1*	RM17115	4	23207668	0.022	0.15	26.18	*qDPPH4.1*
	qTFC9.1	RM24616	9	19580580	0.037	0.14	-19.22	*qTPC9.1*
TPC	*qTPC9.1*	RM24616	9	19580580	0.022	0.17	-44.81	*qTFC9.1*
	qTPC11.1	RM4862	11	9980302	0.03	0.16	73.08	*qKL11.2*
ANTH	*qANTH5.1*	RM18136	5	7764190	0.037	0.13	-7.64	*qAC5.1*

(Note: KL: kernel length, KB: kernel breadth, LB: L/B ratio, ASV: Alkali spreading value, LKAC: kernel length after cooking, ASV: alkali spreading value, VER: Volume expansion ratio, HD: kernel hardness, AC: amylose content, GC: gel consistency, GPC: grain protein content, Zn: zinc content, Fe: iron content, ANTH: Anthocyanin content, TAC: Total anthocyanin content, GORY: γ-oryzanol, TPC: total phenolics content, TFC: total flavonoid content., DPPH: diphenylpicrylhydrazyl radical scavenging assay)

Trait	*QTLs*	Marker	Chromosome number	Position (bp)	p-value	R^2	Allele effect	*Co-localized QTL*
	qANTH9.1	RM285	9	66000000	0.006	0.16	-9.12	*qDPPH9.1*
	qANTH12.1	RM28828	12	27478976	0.027	0.13	5.47	*qGPC12.1*
DPPH	*qDPPH4.1*	RM17115	4	23207668	0.022	0.08	12.94	*qTFC4.1*
	qDPPH8.1	RM502	8	26492117	0.038	0.07	43.64	
	qDPPH9.1	RM285	9	66000000	0.042	0.07	14.65	*qANTH9.1*
	qDPPH11.1	RM27177	11	25297111	0.031	0.07	-10.04	*qKLAC11.1*
GORY	*qGORY2.1*	RM12678	2	5764761	0.044	0.08	-3.53	
	qGORY3.1	RM14761	3	9782360	0.002	0.14	-6.63	
	qGORY6.1	RM197	6	3085639	0.038	0.08	3.86	*qASV6.1*
	qGORY9.1	RM24217	9	13094087	0.043	0.08	6.05	
GPC	*qGPC4.1*	RM17600	4	33596398	0.017	0.09	-2.31	*qAC4.1*
	qGPC4.2	RM1272	4	35110870	0.017	0.09	-2.31	*qAC4.2*
	qGPC9.1	RM105	9	55000000	0.037	0.07	-0.91	
	qGPC10.1	RM4455	10	11665805	0.016	0.09	-0.63	
	qGPC10.2	RM467	10	13488471	0.042	0.07	-0.51	
	qGPC12.1	RM28828	12	27478976	0.033	0.07	0.72	*qANTH12.1, qHD12.1*
Fe	*qFe1.1*	RM10725	1	11456035	0.049	0.16	-3.19	
	qFe1.2	RM11292	1	23439423	0.047	0.16	-3.45	
	qFe1.3	RM8050	1	42072506	0.028	0.17	-4.1	*qLB1.1*
	qFe2.1	RM1075	2	3833177	0.012	0.18	-3.42	*qAC2.1*
	qFe2.2	RM71	2	8760433	0.01	0.18	3.06	*qKL2.2*
	qFe3.1	RM489	3	4333680	0.035	0.16	-3.76	
Zn	*qZn5.1*	RM18600	5	19018217	0.043	0.07	6.18	
	qZn6.1	RM510	6	2831443	0.031	0.08	-3.8	
	qZn6.2	RM162	6	24035491	0.042	0.07	-5.78	*qASV6.3*

(Note: KL: kernel length, KB: kernel breadth, LB: L/B ratio, ASV: Alkali spreading value, LKAC: kernel length after cooking, ASV: alkali spreading value, VER: Volume expansion ratio, HD: kernel hardness, AC: amylose content, GC: gel consistency, GPC: grain protein content, Zn: zinc content, Fe: iron content, ANTH: Anthocyanin content, TAC: Total anthocyanin content, GORY: γ-oryzanol, TPC: total phenolics content, TFC: total flavonoid content., DPPH: diphenylpicrylhydrazyl radical scavenging assay)

Trait	*QTLs*	Marker	Chromosome number	Position (bp)	p-value	R^2	Allele effect	*Co-localized QTL*
	qZn7.1	RM 21521	7	15985258	0.042	0.07	3.77	*qKL7.1*
	qZn8.1	RM281	8	27895505	0.029	0.08	-6.47	*qHD8.1*
	qZn9.1	RM24448	9	16699860	0.028	0.08	-3.86	

(Note: KL: kernel length, KB: kernel breadth, LB: L/B ratio, ASV: Alkali spreading value, LKAC: kernel length after cooking, ASV: alkali spreading value, VER: Volume expansion ratio, HD: kernel hardness, AC: amylose content, GC: gel consistency, GPC: grain protein content, Zn: zinc content, Fe: iron content, ANTH: Anthocyanin content, TAC: Total anthocyanin content, GORY: γ-oryzanol, TPC: total phenolics content, TFC: total flavonoid content., DPPH: diphenylpicrylhydrazyl radical scavenging assay)

Pleiotropic QTL/ multiple trait associations

Among these QTL with significant marker trait association, 48 were found pleiotropic in nature. Three QTL for AC-*qAC4.1*, *qAC4.2*, *qAC 10.1* were co-localized with two QTL for GPC-*qGPC4.1*, *qGPC4.2* and one QTL for GC, *qGC10.1*, respectively. Similarly two QTL for flavonoid content, *qTFC4.1* and *qTFC9.1* were co-localized with *qDPPH4.1* and *qTPC9.1*, respectively. Another QTL for DPPH, *qDPPH9.1* was found pleiotropic to *qANTH9.1*. Two QTLs for Fe, *qFe1.3* and *qFe2.2* were also found pleiotropic to QTL for grain size, *qLB1.1* and *qKL2.2*, respectively. One QTL for Zn content *qZn7.1* shared the same physical position with the QTL for kernel length *qKL7.1* (Table 2).

Discussion

The brown rice contained higher amount of protein, fat, vitamins, and minerals, and also bioactive compounds such as phenolic acids, falvonoids, *γ* - oryzanol, anthocyanin, aminobutyric acid etc. as compared to milled rice. Therefore, unpolished brown rice or its derived products has significant potential of health benefits such as antioxidant, antidiabetic, anticancer, neuroprotective, and cholesterol lowering effects (Pang et al. 2018; Saleh et al. 2019). In addition, increasing demand for dehuled pigmented rice and its by-products from food, health, and cosmetic industries, which has created market and export opportunities for rice-producing countries in Asia (Issara and Rawdkuen 2017). Although brown, red or black rice is rich in nutritional sources but it was reported inferior in cooking and eating quality leading to lower consumer acceptability (Mohan et al. 2017; Saleh et al. 2019). Therefore, understanding genetics of nutritional traits and bio-fortification of those traits cannot be accomplished in isolation without simultaneously considering cooking and eating parameters. Hence, understanding the pattern of inheritance and identification of genomic regions regulating these traits is perhaps prerequisite for bio-fortification. We made an attempt to determine the genomic regions for nutritional and quality traits in rice through GWAS analysis. A diverse panel of 96 rice genotypes in respect of grain physicochemical properties and nutritional traits was considered for the present study. The diversity in association panel was prerequisite to identify associated markers for the targeted traits. In this present study, we found significant variability in all 17 traits. The results of descriptive statistics and trait variation indicated that these traits are controlled by many genes with minor effects. These results provided strong statistical support to the choice of genotypes for hypothesized study.

Associated loci for cooking and other grain quality

The properties of amylose and amylopectin starch polymers are generally associated with the cooking and eating quality of rice, usually expressed by amylose content (AC), gelatinization temperature (GT), and gel consistency (GC). GT is measured through ASV, where high GT value indicates low ASV. Different combinations of these traits classify rice genotypes in various quality groups. GC generally shows negative correlation with AC and GT (Zhang et al. 2020) supporting the fact that indeed GC is the important factor affecting cooking quality traits (Bao et al. 2006). Similarly, in our study also GC was negatively correlated with AC and positively correlated with ASV (Fig. 2). Several QTL were identified for cooking quality and grain quality traits in the association panel. Among them, QTL *qGC10.1* was co-localized with the QTL *qAC10.1* with allelic effect in opposite direction (Table 2). The physical position of these QTL was adjacent to the previously reported QTL (Zhang et al. 2020). Similar kind of reports for AC, GT and GC were available in public domain (Ponce et al. 2018; Yang et al. 2018). However, only few of them have been fine-mapped or cloned. Zhang et al (2020) detected a major QTL over environments for GC (*qGC10.1*) using a RIL population derived from a cross between PA64s and 93-11suitable for fine mapping further.

Associated loci for GPC and related grain quality

In general, the anti-waxy gene increases protein content and decreases the amylose content of rice grains (Li et al. 2009). A negative association of grain protein and amylose content has been observed in previous studies (Yang et al. 2004; Chattopadhyay et al. 2018). In the present association analysis, we could not detect markers in association with *Wx* locus. However, two QTL for GPC-*qGPC 4.1* and *qGPC 4.2* associated with markers RM 17600 and RM 1272, respectively were found co-localized with other two QTL for AC, *qAC 4.1* and *qAC 4.2*, respectively with additive effect in opposite direction (Table 2). Bruno et al. (2017) reported a QTL for protein content (*qPC7*) identified at RM8261 co-localized with QTL for amylose content (*qAC7*). One QTL for GPC-*qGPC10.2* was associated with the marker RM 467, this marker was also detected by Leng et al. (2014) as flanking marker of the QTL *qGPC10*. Using *indica* x *japonica* population Yang et al. (2019) also identified *qGPC10* in the same position as Wang et al. (2017) identified *qPC10.1* through association mapping. The *OsGluA2* gene was found within the *qGPC10* QTL which regulates glutelin synthesis.

Associated markers for Fe and Zn content and quality traits

In many earlier experiments, grain Fe content had significant positive correlation with Zn content, both Fe and Zn content was found positively associated with grain size (Bollinedi et al. 2020; Maganti et al. 2020; Sanghamitra et al. 2022). Positive association of Zn and Fe content was evidenced by the co-location of QTL for Fe and Zn in brown rice (Swamy et al. 2016). In the present study, although we did not find any association between Zn and Fe content, but significant positive association between Fe content and grain size was detected (Fig. 2). One QTL, *qFe1.3* and associated marker RM8050 was co-localized with a QTL for length/breadth ratio, *qLB1.1*. In the similar position *qLBR1.1* (for length/ breadth ratio) was reported earlier (Swamy et al. 2018; Suman et al. 2021). Another QTL *qFe3.1* and associated marker RM 489 was found in nearby location with metal assimilation genes *OsNAS1* and *OsNAS2*. On the other hand, one QTL for zinc content *qZn6.2* and associated marker RM 162 was pleiotropic to *qASV6.3*. In the similar position, QTL for Zn and Fe content (*qZn6.2* and *qFe6.2*) was reported by Dixit et al. (2019).

Associated loci for antioxidant activities and other quality traits

A wide range of variability in total phenolics, total flavonoids, total anthocyanin, *γ* - oryganol and resultant antioxidant activity was found. In the present study, a linear relationship among anti-oxidant traits was found using Pearson's correlation approach and results indicated the strong significant positive correlation between TFC and TPC, TPC with DPPH, DPPH with TFC. Most of the anti-oxidants of brown rice were contributed by phenolic compounds (Ye et al. 2015). Positive correlations were also found between ANTH and TPC, TFC and DPPH. A strong positive correlation between the TAC and TPC was also observed in Attakkari, Bg2907, and Bg406 by Priyanthi and Sivakanesan (2021). Moreover, a strong positive correlation between the TPC and TFC indicates that the flavonoids are the major polyphenols. One QTL for flavonoid content (*qTFC9.1)* was co-localized with another QTL of TPC (*TPC9.1*) with additive effect in the same direction, signified their positive association. This is also applicable for pleiotropic QTL pair (*qTFC4.1* and *qDPPH4.1*) for TFC and DPPH with positive additive effect. Another QTL for DPPH (*qDPPH9.1)* was co-localized with QTL for TAC content (*qANTH9.1)* but with additive effect in opposite direction. *qANTH12.1*, *qGPC12.1* and *qHD12.1* were co-localized with associated marker RM 28828. We know that high protein content in brown rice causes hardness in cooked rice. Additive effect in opposite direction for *qGPC12.1* and *qHD12.1* also indicated the negative association of these two traits especially for those genetic loci. In nearby position Shao et al. (2014) detected QTL (*qPAC12.2*) for proanthocyanic content, also co-localized with QTL for different antioxidant traits such as TPC (*qPC12)*, TFC (*qFC12)* and DPPH (*qACD12)*. QTLs for TPC and TFC were previously detected in similar position by linkage mapping (Jin et al. 2009). Significant negative association (r=-0.34) between AC and total anthocyanin content (TAC) also indicated that coloured rice had low amylose content and are sticky in nature (Fig. 2). One important QTL for ANTH (*qANTH5*.1) was found to be co-localized with a QTL for AC (*qAC5*.1) with additive effect in opposite direction supporting their nature of association particularly for this locus. Positive association of ASV and GORY signified a pleiotropic locus and associated marker RM 197 with additive effect in the same direction (Table 2).

The QTL with significant phenotypic effect on trait variation have great potential to be utilized in bio-fortification and genetic improvement of quality traits in rice. Further, QTL with minor effect can be accumulated through marker assisted breeding for a significant positive effect on trait phenotype following a strategy based on the principles of population improvement. Since, these traits were found to inherit quantitatively, it is important to accumulate all the causative alleles for each trait in a single elite genetic background to realize their actual potential of improvement for the respective traits. Further, pleiotropic or association of markers with more than one trait have significant scope for simultaneous improvement of associated traits for better rewards in breeding programs. Deployment of markers associated with more than one trait in MAS programs helps to faster accumulation of alleles for related traits in one go and reduces resources and time requirement of breeding program. The phynotypic traits considered in this study have significant scope in bio-fortification for development of mineral dense rice varieties. Hence, results discussed in this study have significant application fighting mineral malnutrition. The information generated from this study has potential scope in planning strategic breeding programs for improving nutritional security through marker assisted rice breeding and bio-fortification programs.

Declarations

Conflict of interest

The authors declare no conflict of interest

Author Contribution

KC made design of the experiment, coordinated the study, analyzed data and made initial draft. AC analyzed for association mapping and contributed in drafting the manuscript. TBB, PS, SS, AK, NM, SSM and SKS generated biochemical and molecular data and did initial statistical analysis. BCM and SS provided geramplsm for the study.

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Figures

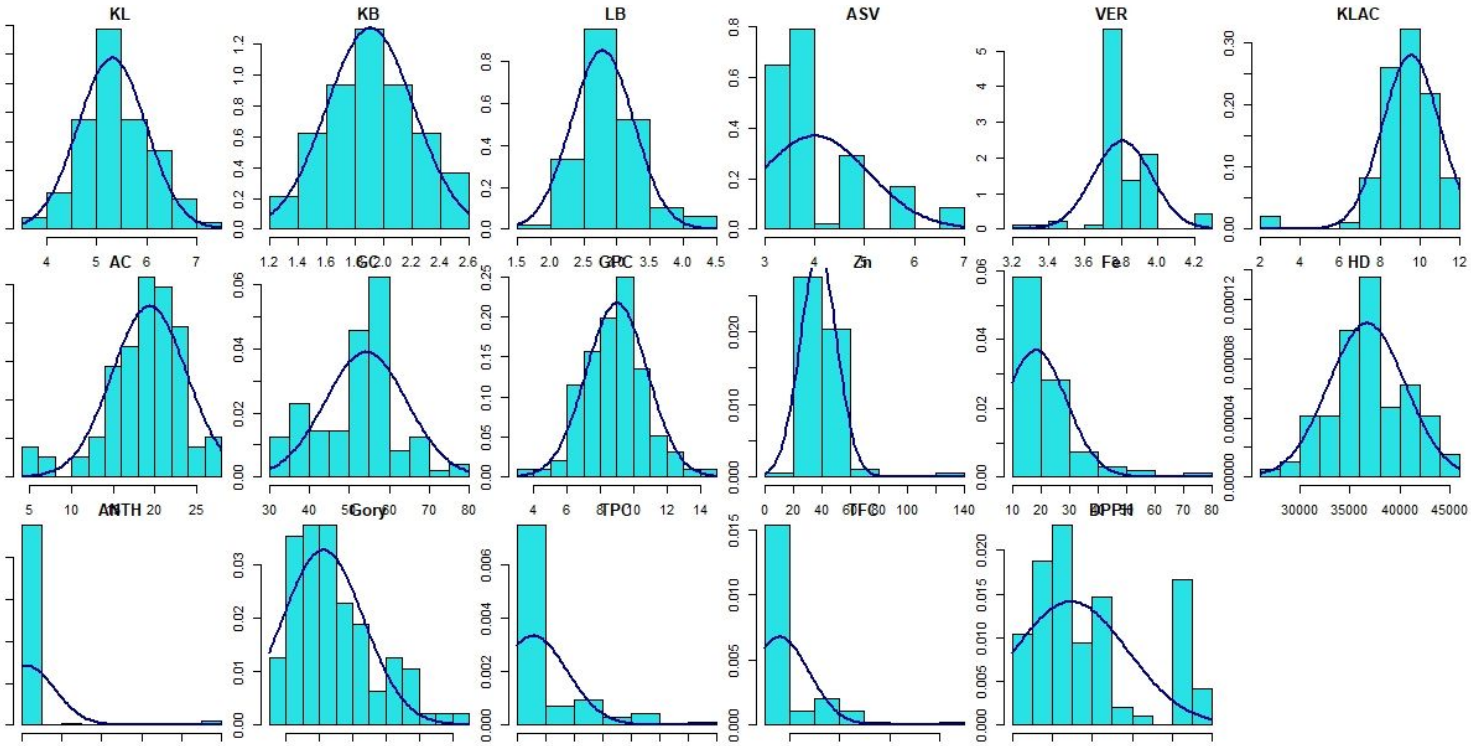

Figure 1

Variation and distribution pattern of 17 nutritional and quality traits assessed on association panel

(Note: KL: kernel length, KB: kernel breadth, LB: L/B ratio, ASV: Alkali spreading value, LKAC: kernel length after cooking, ASV: alkali spreading value, VER: Volume expansion ratio, HD: kernel hardness, AC: amylose content, GC: gel consistency, GPC: grain protein content, Zn: zinc content, Fe: iron content, ANTH: Anthocyanin content, TAC: Total anthocyanin content, GORY: g-oryzanol, TPC: total phenolics content, TFC: total flavonoid content., DPPH: diphenylpicrylhydrazyl radical scavenging assay)

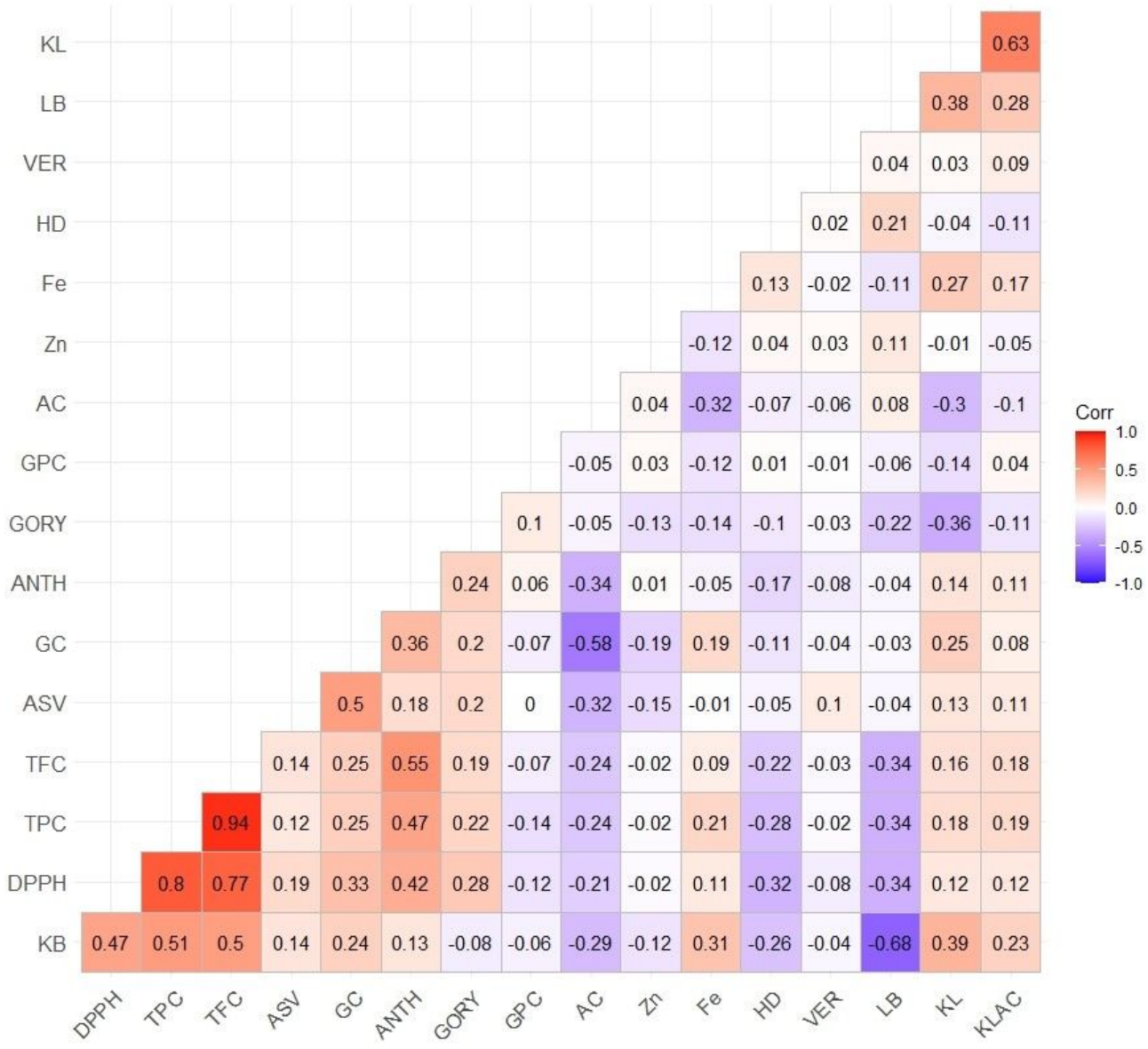

Figure 2

Correlation coefficients among nutritional and quality parameters estimated on association panel. (Note: KL: kernel length, KB: kernel breadth, LB: L/B ratio, ASV: Alkali spreading value, LKAC: kernel length after cooking, ASV: alkali spreading value, VER: Volume expansion ratio, HD: kernel hardness, AC: amylose content, GC: gel consistency, GPC: grain protein content, Zn: zinc content, Fe: iron content, ANTH: Anthocyanin content, TAC: Total anthocyanin content, GORY: γ-oryzanol, TPC: total phenolics content, TFC: total flavonoid content., DPPH: diphenylpicrylhydrazyl radical scavenging assay)

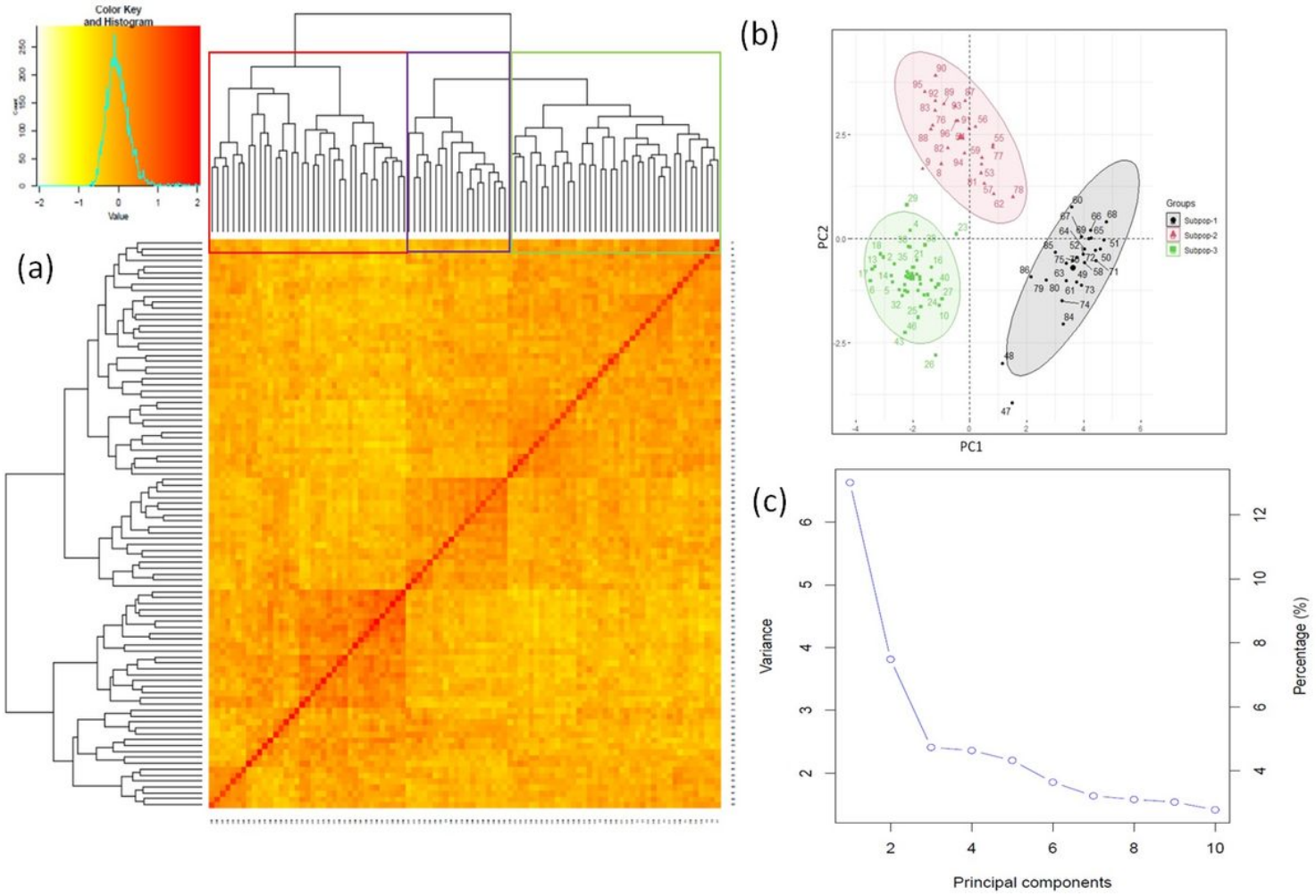

Figure 3

Population structure analysis: (A) heat map of kinship matrix, the heat map shows the level of relatedness among the population, (B) principle component analysis showing three subpopulations and (C) Scree Plot explaining the Variance of principal component indicating significance of first three components. The darker areas show the level of relatedness between varieties and colored line boxes at the top depicts clustering of subpopulations.

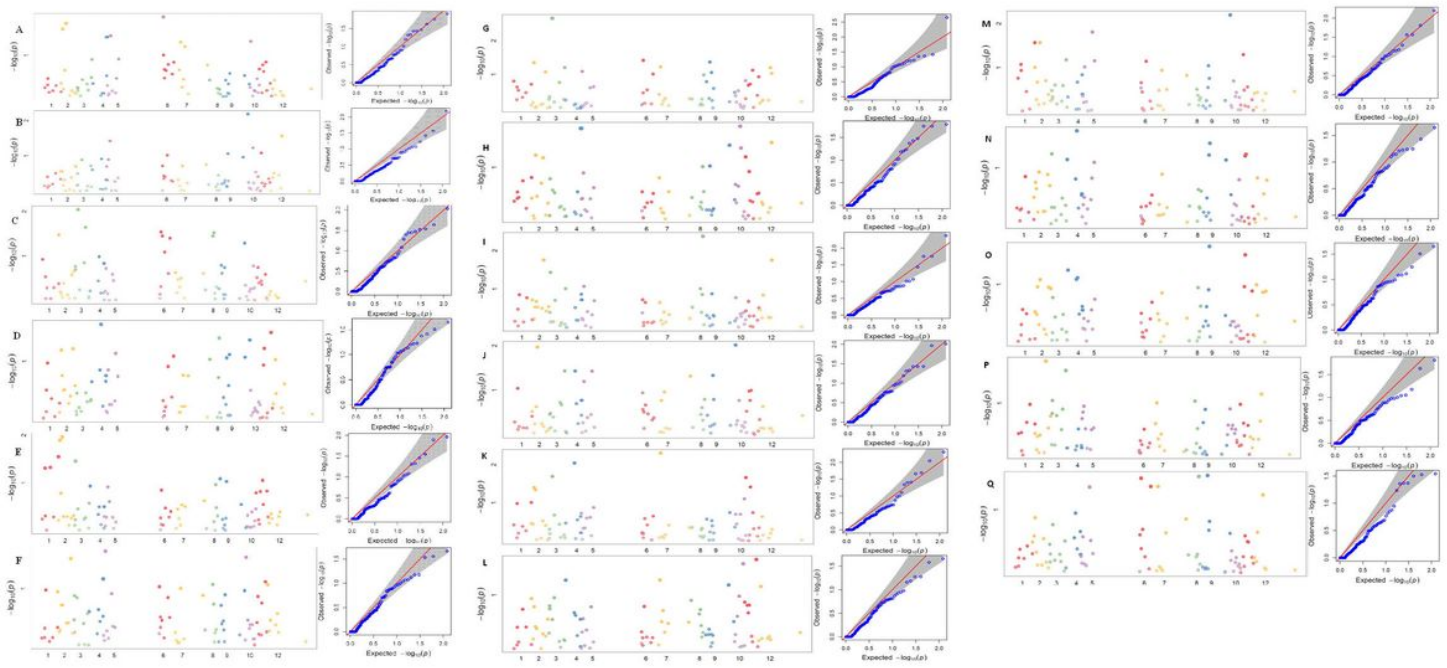

Figure 4

Genome-wide association analysis results obtained by compressed MLM approach represented by Manhattan Plots and Q-Q plots for A:AC, B:ANTH, C:ASV, D:DPPH, E:Fe, F:GC, G:GORY, H:GPC, I:HD, J:KB, K:KL, L:KLAC, M:LB, N:TFC, O:TPC, P:VER, Q:Zn traits. QQ plots illustrate the relationship between observed and expected p-values of each marker considered for association. The grey area of the plots shows confidence interval up to 95% for p-values for their accuracy. Most of the data points will lie on the diagonal line since they are not associated with trait. Any deviation in trends from the expected is due to unaddressed population structure in the association panel. Significant markers lie above the expected line.

Supplementary Files

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