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ORIGINAL ARTICLE

Assessment of Marker-trait Associations for Amylose Content, Gelatinization Temperature and Yield in Sri Lankan Rice Varieties

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Abstract

Rice provides a balanced diet, and regular rice consumption helps maintain a healthy life and prevents many common illnesses. Amylose content and gelatinization temperature are considered major physicochemical factors which significantly affect the nutrition and cooking quality of rice. The *Waxy* (Wx) gene controls the synthesis of amylose in the endosperm and the *ALK* gene controls the gelatinization temperature of rice. Both genes are present on chromosome 6. Improving the rice traits is crucially essential to the cultivators and the consumers to overcome the increasing demand for high-quality and nutritionally rich rice. Many studies have been successfully conducted to improve the agronomical traits of rice. However, the concern for improving nutrition and cooking quality-related traits of rice is less common among the breeders. Therefore, considering the current trends, introducing consumer-friendly novel varieties that can fulfill the nutritional requirements is essential to face the increasing domestic demand for high-quality rice. In the present study, we used frequently grown 54 local rice varieties and carried out a marker-based genetic analysis for three traits: amylose content, gelatinization temperature, and yield. Then statistical analysis was carried out using quantitative data of different agronomical and physicochemical traits to identify the correlation and potential to improve such essential traits. The level of amylose content is significantly associated with the gelatinization temperature indicating that AC and GT have a positive correlation. The DNA markers *SSIIa* and *Seq1-2* markers showed monomorphic patterns indicating the narrow genetic basis of the rice varieties tested. This study revealed that existing high-yielding rice varieties could be successfully employed to increase the amylose content while decreasing the level of gelatinization temperature through breeding strategies to make nutritionally rich consumer-friendly novel varieties.

***Keywords**: ALK gene, Marker-assisted selection, Plant breeding, Rice yield, Waxy gene*

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1. Introduction

Rice (*Oryza sativa* L.) is the staple food for half of the world's population, including 90% of Asians (Chaudhari et al. 2018). Around 114 countries worldwide are employed in the rice industry by cultivating over 40,000 rice varieties (The Rice Association 2020), producing about 491.4 million metric tons of rough rice from 160.6 million hectares (Datta et al. 2017). In Sri Lanka, around 55 varieties have been recommended for cultivation (RRDI 2020). Nearly 1.9 million families cultivate rice about 870,000 ha, yielding 2.7 million metric tons (RRDI 2020) annually. This yield can fulfil 45% calorie and 40% protein requirement of an average Sri Lankan individual (Fari et al. 2011). Rice is a highly nutritious cereal crop that provide 80% of energy, 80% carbohydrate, 7–8% protein, 3% fat, and 3% fibre (Chaudhari et al. 2018) with some vitamins and minerals (RRDI 2020) for Asians. Therefore, rice is mostly used in food-based industries such as breakfast cereals, biscuits, baby food, crackers, candies (Bao et al. 2006a), and noodles (Fari et al. 2011) productions. Furthermore, rice flour is the primary ingredient of local traditional sweets for many localities.

The protein in rice contains eight essential amino acids with a high proportion of lysine. These amino acids help nourishing the heart, lungs, tendons, and ligaments and help maintain healthy skin and hair, while offering good health and muscle activity. The B complex vitamins like riboflavin, thiamin, and niacin present in brown rice promote nourishment in the skin and blood vessels (Chaudhari et al. 2018). Rice grains are rich in iron (Fe) and zinc (Zn) like minerals, which are essential for the enzymatic process and haemoglobin production in the body (Ahuja et al. 2008). Many Asian countries utilize the medicinal value of the rice in the traditional, Ayurveda and Unani medicinal methods (Chaudhari et al. 2018). Rice can be added to a well-balanced diet since it is free from cholesterol, fat, and salt. Rice bran contains fatty acids that are removed in the milling process helps fulfilling up to 80% of the requirement of unsaturated fatty acid (Frei & Becker 2003), which is also necessary to maintain the proper function the cell membrane and the nervous system.

Different genes present in the rice govern the production of these critical nutritional and medicinal constituents. The level of amylose content (AC), gelatinization temperature (GT), and gel consistency (GC) are the key determinants of rice processing, cooking, and eating quality. The synthesis of amylose requires a granule bound starch synthase (GBSS) enzyme, which is the product of the Waxy (*Wx*) gene (Rohilla et al. 2000), located on chromosome six (6) (Harushima et al. 1998). Amylose content of rice can be classified as low (<20%), intermediate (20–25%), and high (>25%) (Kongseree & Juliano 1972). Amylose and Amylopectin are the two major glucose polymers found in rice starch. Amylose is relatively unbranched and presents in 20–30% of storage starch, while highly branched Amylopectin presents in 70-80% (Robyt 2008). Amylose content positively correlates to the expansion volume and the water absorption rice in the rice cooking process (Juliano 1985), while negatively correlates to the qualities like cohesiveness, tenderness, and glossiness in the rice boiling process. Amylose content also influences the texture and retrogradation (Yu 2009) characteristics of cooked rice. The structure and the characteristics of Amylose and Amylopectin also contribute to the texture of cooked rice (Yang et al. 2016). Generally, high amylose content is associated with the hard texture of cooked rice (Juliano 1972).

The gelatinization temperature is another critical parameter determines the quality of cooked rice. Gelatinization temperature is defined as the temperature at which rice starch granules start to irreversibly lose the crystal and birefringence nature. This particular trait is governed by *ALK* (alkali degeneration) gene (Gao et al. 2003), also known as *SSIIa* (Starch synthase IIa), located in chromosome 6 (Wang et al. 2019). The range of gelatinization temperature varies from variety to variety of rice. It is classified according to the temperature as low (<74 ºC), intermediate (70–74 ºC), and high (>74 ºC) (Bhattacharya 1979). Rice with low gelatinization temperature needs a low amount of water, a shorter period to cook (Bhattacharya 1979), and exhibits a soft texture. Rice with high gelatinization temperature requires a high amount of water, a more extended period, and exhibits a hard texture (Bao et al. 2006b). Also, low gelatinization temperature rice varieties require less energy than high gelatinization temperature varieties during the cooking process (Umemoto et al. 2004). Therefore, the consumers prefer rice varieties with low gelatinization temperature since it causes time and energy saving (Waters et al. 2006).

Currently, many rice breeders aim to increase rice yield due to the rapidly rising population, while paying less consideration to the quality of rice. The quality of rice can be improved by enhancing the grain qualities and the nutritional value of the rice. The outcomes of many successful findings in rice improvement research have been employed in increasing rice grain qualities. However, the concern of elevating the nutritional value of high-yielding rice varieties is not very common. Sri Lanka needs better varieties, offering high-level nutrition while becoming consumer-friendly, which suits the current economy.

Analysing the genetic variation of the amylose content and gelatinization temperature and predicting the possibility of such improvement using statistical analysis of frequently grown high-yielding rice varieties might provide an excellent base to introduce novel rice varieties to overcome the increasing domestic demand for high-quality rice in the market and help alleviate the nutrition deficiencies of the local people. Therefore, the present study focused on finding the variation and correlation of critical parameters that affect the nutrition and cooking qualities of rice using molecular biological and statistical approaches as the time-saving and non-laborious methods. Based on the outcomes, this study further identified the potential to make predictions for creating new consumer-friendly rice cultivars that possess high nutritional quality by combining valuable agronomical parameters to fulfil the preferences of both consumers and rice cultivators.

2. Materials and Methods

Genotyping and screening the population

The breeder seeds of landraces and newly improved 54 ice varieties (6 landraces and 48 newly improved rice varieties) were obtained from Rice Research and Development Institute (RRDI) Bathalagoda, Sri Lanka (Table 1). The seeds were germinated under greenhouse conditions for two weeks at the Department of Molecular Biology and Biotechnology, Faculty of Science, University of Peradeniya. Then the young leaf samples were collected to perform DNA extraction.

Table 1: Rice varieties considered for the study and their specific characteristics.

#	Variety name	Amylose content (AC)	Gelatinization temperature (GT)	Pericarp colour	Grain shape	Average yield (MT ha^{-1})
1	H4	high	high	red	long-medium	3.5
2	H7	intermediate	intermediate	red	intermediate-bold	3.6
3	At303	high	high	red	long-medium	6.2
4	At306	high	intermediate	white	long-slender	4.7
5	At307	high	high intermediate	white	intermediate-bold	7.0
6	At308	high	high	white	short-round	6.5
7	At309	low	low	white	long-slender	6.0
8	At311	high	high	red	long-slender	5.2
9	At353	high	low	red	long-medium	5.2
10	At354	high	low-intermediate	white	long-medium	6.5
11	At401	intermediate	intermediate	red	long-medium	5.0
12	At402	high	intermediate	red	long-medium	6.7
13	At405	low	low	white	long-slender	4.7
14	At362	intermediate	low	red	long-medium	7.9
15	At373	high	high intermediate	white	short-round	4.9
16	Bg34_6	low	intermediate	red	intermediate-short	5.5
17	Bg38	intermediate	intermediate	white	short-round	6.0
18	Bg94_1	high	high intermediate	white	long-medium	4.1
19	Bg250	high	intermediate	white	intermediate-bold	4.5
20	Bg251	high	intermediate	white	long-medium	5.5
21	Bg252	intermediate	intermediate	red	short-round	4.2
22	Bg300	high	intermediate	white	intermediate-bold	5.0
23	Bg304	high intermediate	intermediate	white	long-medium	4.7

#	Variety name	Amylose content (AC)	Gelatinization temperature (GT)	Pericarp colour	Grain shape	Average yield (MT ha^{-1})
24	Bg305	high	intermediate	white	intermediate-bold	6.9
25	Bg310	high	intermediate	white	intermediate-bold	5.6
26	Bg352	intermediate	low	white	intermediate-bold	4.9
27	Bg357	high	high intermediate	white	long-medium	5.8
28	Bg358	high-intermediate	intermediate	white	short-round	4.8
29	Bg359	high	high	white	intermediate-bold	5.9
30	Bg360	intermediate	high intermediate	white	short-round	4.3
31	Bg366	intermediate	intermediate	white	intermediate-bold	6.0
32	Bg369	high	intermediate	white	long-medium	7.0
33	Bg370	high	intermediate	white	short-round	5.4
34	Bg374	high-intermediate	high	white	intermediate-bold	5.9
35	Bg379_2	high	high intermediate	white	intermediate-bold	6.1
36	Bg406	intermediate	low	red	intermediate-bold	5.0
37	Bg407H	high	low	white	long-slender	8.0
38	Bg407	intermediate	intermediate	white	intermediate-bold	5.5
39	Bg450	high	intermediate	white	short-round	3.0
40	Bg454	high	high intermediate	white	intermediate-bold	4.2
41	Bg750	high	intermediate	Red	intermediate-bold	3.0
42	Bw351	intermediate	intermediate	red	intermediate-bold	4.5
43	Bw361	high	low	red	intermediate-bold	4.5
44	Bw367	high	intermediate	white	short-round	5.2
45	Bw372	high	high	red	intermediate-bold	4.2
46	Ld356	high	intermediate	red	long-slender	4.5
47	Ld365	high-intermediate	intermediate	red	short-round	4.5
48	Ld368	high-intermediate	intermediate	Red	short-round	4.6
49	*Herath banda*	high	intermediate	red	intermediate-bold	5.1
50	*Kalu heenati*	high	high intermediate	red	intermediate-bold	4.4

#	Variety name	Amylose content (AC)	Gelatinization temperature (GT)	Pericarp colour	Grain shape	Average yield (MT ha^{-1})
51	*Pachchape rumal*	high	intermediate	red	intermediate-bold	4.9
52	*Sudu heenati*	high	intermediate	red	intermediate-bold	3.1
53	*Suduru samba*	intermediate	intermediate	white	short-round	3.4
54	*Sulai*	high	intermediate	red	intermediate-bold	5.2

(RRDI, Sri Lanka)

DNA extraction, Agarose gel electrophoresis, and PCR

The genomic DNA was extracted from two-week-old young rice leaves using the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle 1991) and stored at -20 ºC. Then the PCR was performed as 15 µL reaction mixtures, containing the 0.3 µL of DNA template, 7.5 µL GoTaq® Green Master mix, nuclease-free water, 0.6 µL of each forward and reverse primers for the genes *Wx*, *ALK,* and *Ghd7* (Table 2) using a Takara thermal cycler (Otsu Shiga, Japan).

The PCR conditions were as follows: initial denaturation for 5 min at 94 °C; 35 cycles of 30 sec at 94 °C for denaturation; 90 sec at 55 °C for primer annealing; 2 min at 72 °C for extension; and final extension of 10 min at 72 °C (Jin et al. 2010). Then the amplified DNA was subjected to size separation using ethidium bromide-stained 2.5% agarose gel electrophoresis.

Statistical analyses: The important characteristics of the improved local rice varieties were collected under eight different parameters from information sources available at RRDI (RRDI, 2018) (Table 1 and Table 3). The data obtained for the AC and GT were converted into categorical values as low = 1, low-intermediate = 2, intermediate = 3, high-intermediate = 4, and high = 5. All the eight parameters were categorized into three main criteria, according to Table 3. Then the statistical analysis was carried out using Statistical Package Minitab 18 (Minitab Inc.). The frequency distribution of AC and GT in rice varieties was analysed with with Kolmogorov-Smirnov test for normality, and the most common level of each parameter was identified.

Table 2: Details of the genetic markers used in analysis.

Marker	Forward and reverse primer sequence (5'→3')	Reference
Wx	484: CTTTGTCTATCTCAAGACAC	Jin *et al*. (2010)
	485: TTGCAGATGTTCTTCCTGATG	
SSIIa	NF1: CGAGGCGCAGCACAACAG	Jin *et al*. (2010)
	NR1: GGCCGTGCAGATCTTAACCAT	
Seq1-2	GCAAGGGGATGTCTAAACGA	Lu *et al*. (2012)
	AATTTTTGACCGTCGGATTC	

Then the principal component (PC) variance was analysed separately for the yield-related parameters and the rice processing-related and cooking quality and nutritional-related parameters to identify the specific trends. The correlation between yield-related and cooking quality and nutrition-related parameters was analysed to identify the strength and direction of the association between variables. The correlation coefficient values of significance level $P<0.05$ were determined. Finally, the distribution of the three parameters under the yield-related criteria was interpreted according to the XYZ plot.

Table 3: The recorded quantitative and qualitative data categorization parameters

Parameter	Sub-parameters
Yield-related parameters	Average yield (metric tonnes/ha)
	1000 grain weight (g)
	Bushel weight (kg)
Rice processing-related parameters	Brown rice recovery (%)
	Milled rice recovery (%)
	Head rice recovery (%)
Cooking quality and nutrition-related parameters	Amylose content
	Gelatinization temperature (°C)

3. Results and Discussion

Genetic analysis

DNA marker *Waxy* showed a significant band polymorphism in the 2.5% agarose gel electrophoresis, while *SSIIa* and *Seq1-2 s*how monomorphic band patterns for all 54 rice varieties (Fig. 1). Therefore, the outcomes revealed that the selected 54 frequently grown improved and traditional rice varieties have the genes responsible for the AC and GT. Concerning the marker used for detecting the gene responsible for the yield was present only in some varieties. Furthermore, the band pattern obtained for *SSIIa* and *Seq1-2* markers showed monomorphic patterns in the gel. When considering the distribution of each parameter, all three parameters under each criterion have shown continuous distributions when tested with Kolmogorov-Smirnov test. The obtained data under the AC and GT levels were converted to numerical values as; low-1, low-intermediate-2, intermediate-3, high-intermediate-4, and high-5 for the statistical analysis. When focusing on the distribution of the considered local 54 varieties, according to the results obtained, most Sri Lankan varieties contain a high or high-intermediate amount of GT (75.6%) and an intermediate level of GT (53.7%) (Fig. 2). However, varieties with low or low-intermediate GT were rarely observed (16.6%), indicating that most local varieties are rich in amylose while expressing an intermediate level of GT. In the variance analysis between the yield-related parameters, the first principal component (PC) accounts for 39.5% of the total variance (Table 4). The results shown in Figure 3a revealed that the bushel weight and 1000 grain weight are negatively correlated, and both parameters showed no correlation with the average yield. Therefore, it was predicted that even though the average yield increased, the 1000 grain weight or bushel weight of rice might not increase. The multivariate clustering based on PCs of the yield traits is given in Figure 3b, indicating the presence of two major clusters. Most varieties present in Cluster A contained high or high-intermediate AC and intermediate or low-intermediate GT. The cultivars are shown in Cluster B express intermediate or high-intermediate AC with intermediate GT. The varieties in Cluster A have a considerably high average yield compared to the varieties in Cluster B. In this clustering, some inter-cluster varieties/landraces were also observed.

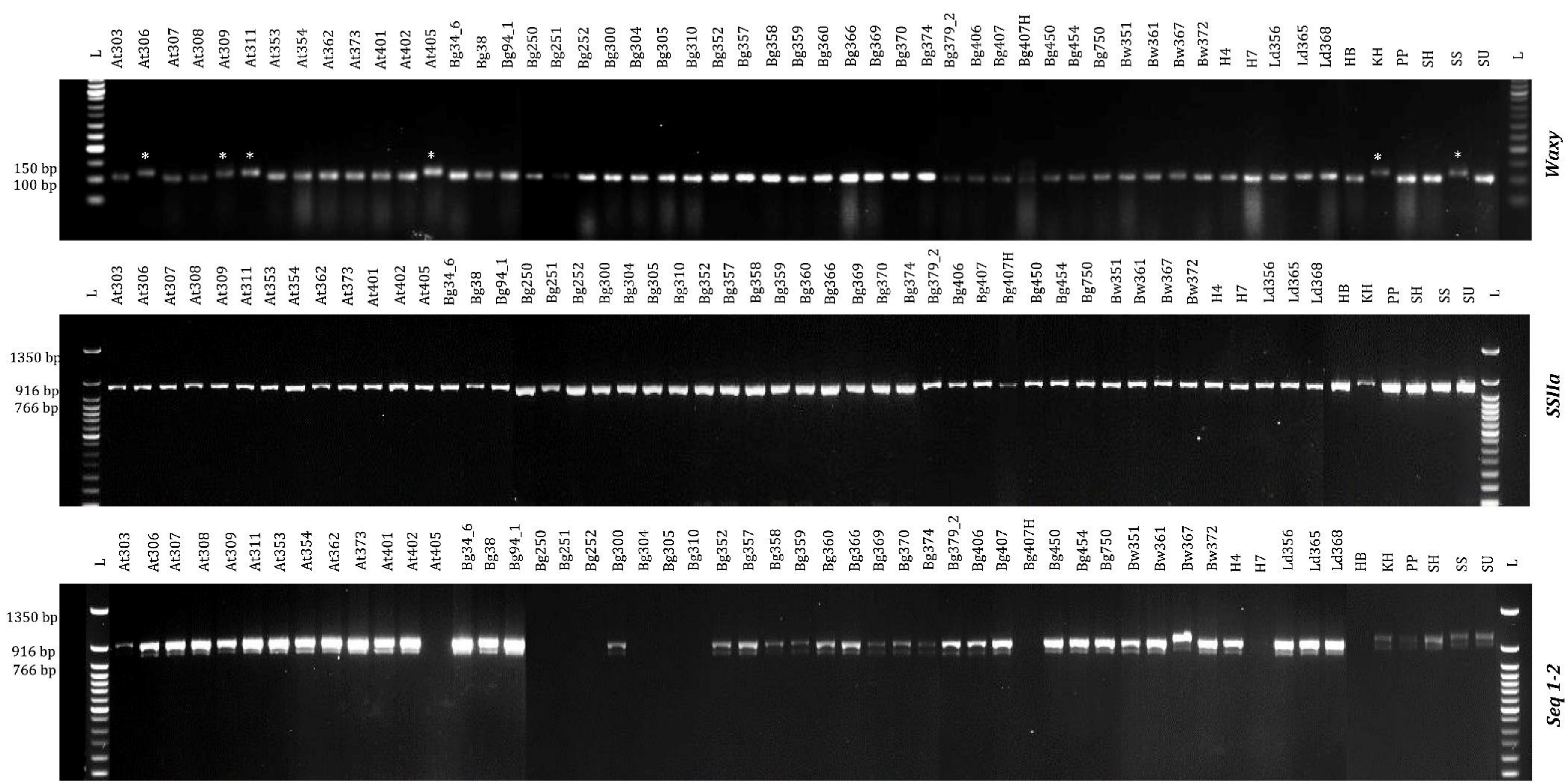

Figure 1: The polymorphism of three DNA markers linked to *Waxy*, *ALK* and *Ghd7* in 54 rice varieties. The band sizes are indicated at the left side of the figure and DNA marker names are indicated at the right side. The cultivar names are given at the top. "*" indicates the band size 110 bp (HB: *Herath banda*, KH: *Kalu heenati*, PP: *Pachchaperumal*, SH: *Sudu heenati*, SS: *Suduru samba*, SU: *Sulai*, L: Ladder)

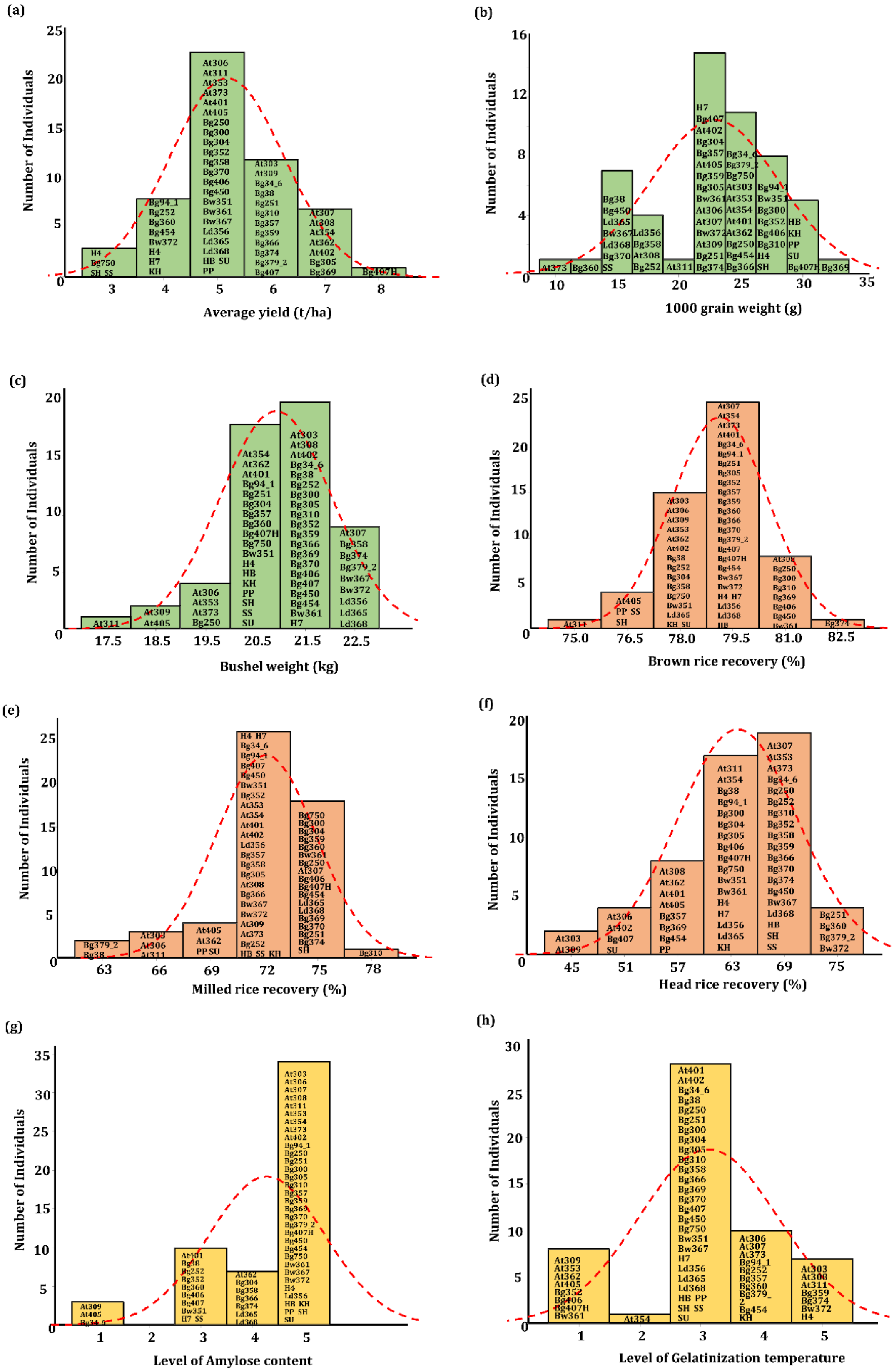

Figure 2: The classification of different improved and local rice varieties based on major eight quality parameters/trait distribution. (a) Average yield, (b) 1000 grain weight, (c) Bushel weight, (d) Brown rice recovery, (e) Milled rice recovery, (f) Head rice recovery, (g) Amylose content, and (h) Gelatinization temperature. (HB: *Herath banda*, KH: *Kalu heenati*, PP: *Pachchaperumal*, SH: *Sudu heenati*, SS: *Suduru samba*, SU: *Sulai*).

Table 4: Eigen analysis of the correlation matrix for the yield-related parameters.

	PC1	PC2	PC3
Eigenvalue	1.186	1.094	0.721
Proportion	0.395	0.365	0.240
Cumulative variance	0.395	0.760	1.000

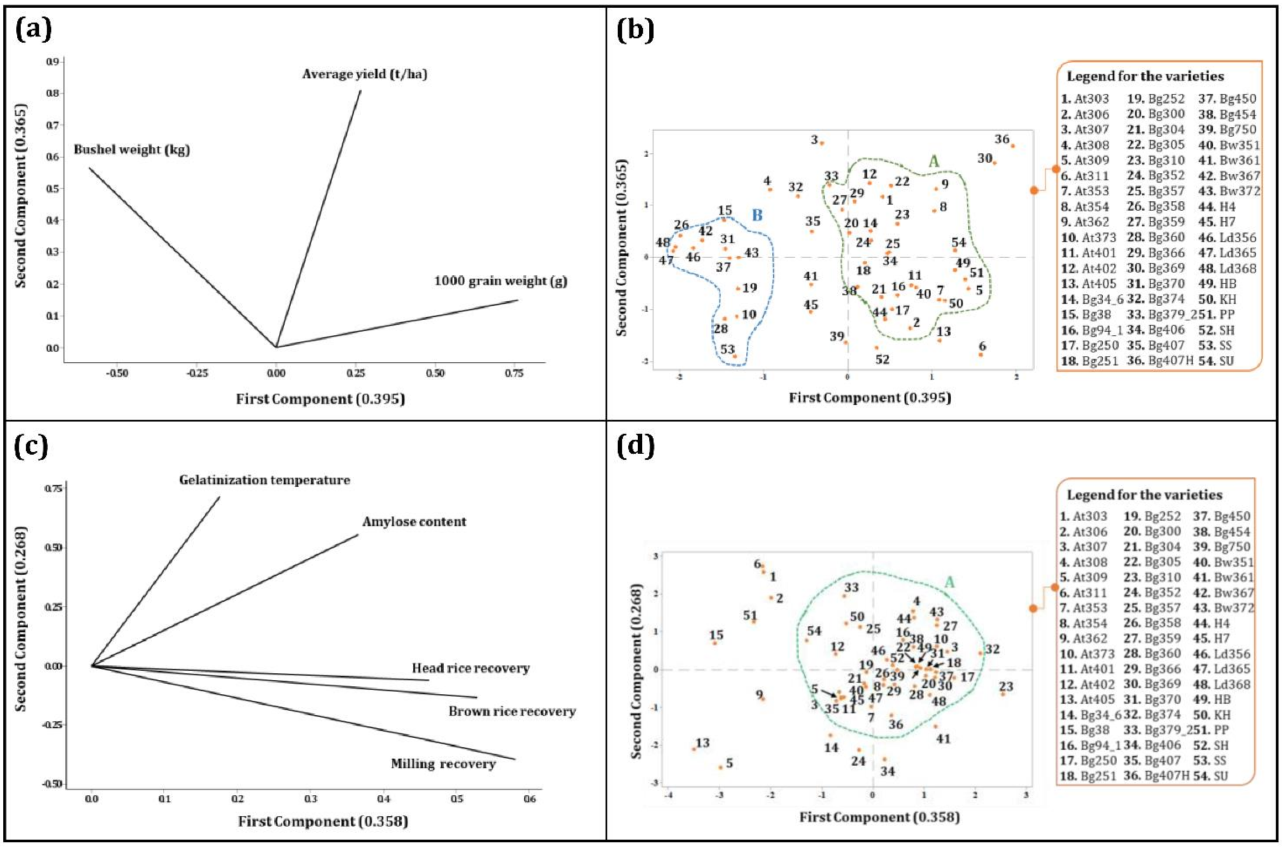

Figure 3: Variance analysis of yield-related parameters (a and b) and nutrition and cooking quality-related parameters with rice processing-related parameters (c and d). (a) and (c) are the PC loading plots, while (b) and (d) are the Score plots. The less than 90° angles between the parameters indicate a positive correlation. In (a), the bushel and 1000 grain weight are negatively correlated. Plot (c) indicates positive correlations among all five parameters.

Therefore, using molecular breeding approaches, these high-intermediate AC expressing varieties can be improved into high AC level producing varieties while decreasing the intermediate or low-intermediate level of GT into low GT. Since the clustered varieties are frequently grown in *Yala* and *Maha* seasons, the improvement of those cultivars might add high nutrition value to diets, ultimately bringing a solution for the nutrient deficiencies. Since the low GT level varieties require less time and energy for the cooking process, those improved novel varieties will also become more consumer-friendly than the current varieties.

According to the outcomes shown in Table 5, the variation analysis of rice processing-related parameters and cooking quality and nutrition-related parameters, the first PC accounted for

35.8% of the total variance. The first three PC values explained 80.4% of the variance. The variables that correlate most with the PC1 were brown rice recovery, milled rice recovery, head rice recovery, and amylose content. Therefore, PC1 was positively correlated with all these four parameters. The PC loading plot (Fig. 3c) showed a more positive correlation between rice processing-related parameters (brown rice recovery, milled rice recovery, and head rice recovery) with AC showed no correlation with GT. Considering the correlation between the rice processing-related parameters with AC, it clearly showed that high AC producing varieties give a high yield. Furthermore, this outcome is further verified by the score plot in Figure 3d. A clear cluster can be seen in the score plot with the high or intermediate-high AC expressing varieties (Cluster A).

Table 5: Eigen analysis of the correlation matrix for rice processing-related parameters and cooking quality and nutrition-related parameters.

	PC1	**PC2**	**PC3**	**PC4**	**PC5**
Eigenvalue	1.790	1.338	0.893	0.615	0.363
Proportion	0.358	0.268	0.179	0.123	0.073
Cumulative variance	0.358	0.626	0.804	0.927	1.000

In correlation analysis of yield-related parameters and cooking quality and nutrition-related parameters, a positive correlation was observed only within the two cooking quality and nutrition-related parameters (Table 6). The significance level *P*<0.05 value obtained only for AC with GT indicated that the level of AC is significantly associated with the GT (0.366, *P*<0.05), indicating that AC and GT have a positive correlation. However, Wang et al. (2007) mentioned that any correlation between AC and GT was not identified yet. Nevertheless, Sri Lankan rice germplasm might positively correlate between the AC and GT at a minor level which could be used for future predictive purposes in breeding strategies.

Table 6: Correlation values obtained for the yield-related parameters and cooking quality and nutrition-related parameters.

	Average yield (MT ha^{-1})	**1000-grain weight (g)**	**Bushel weight (kg)**	**Amylose content**
1000 grain weight (g)	0.141			
Bushel weight (kg)	0.098	-0.177		
Amylose content	0.114	0.130	0.186	
Gelatinization temperature (°C)	-0.139	-0.165	0.206	0.366*

* indicates the significance level P<0.05.

When considering the distribution of the yield-related parameters of these local varieties, an apparent clustering was observed with 40 (74.0%) rice cultivars out of 54 rice cultivars (Fig. 4). Moreover, the majority of the members (55.0%) in this group recommended for general cultivation. When considering the other phenotypes which belong to those cultivars, such as grain shape and pericarp colour, cooking quality and nutrition-related parameters, any clear-cut correlation with this clustering was not observed.

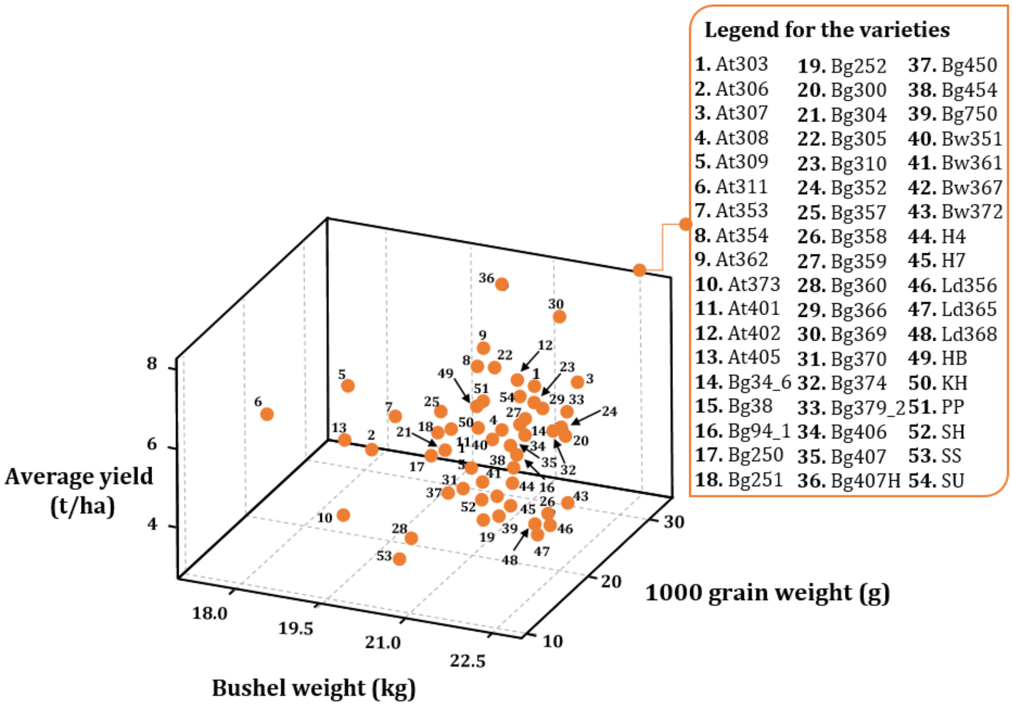

Figure 4: The distribution of the yield-related parameters. This 3D scatterplot indicates the distribution of the selected varieties between the three parameters. A precise cluster formation can be obtained, including 39 varieties out of 54 (HB: *Herath banda*, KH: *Kalu heenati*, PP: *Pachchaperumal*, SH: *Sudu heenati*, SS: *Suduru samba*, SU: *Sulai*).

The overall results of this study provide clues of the potential to improve these local varieties to obtain nutritionally rich and consumer-preferred rice, while providing high yield to the growers. Through the breeding approaches, novel varieties can be produced, or the existing varieties can be improved to fulfil the aforesaid requirements. As the cooking quality and nutrition-related parameters, AC and GT of rice are crucially essential to overcome the problems associated with the nutritional aspects of rice and economic gains in rice farming. Therefore, using the outcomes of this study, high-yielding rice varieties with high or intermediate-high AC can be selected and using the molecular breeding approaches, novel varieties with a reduced level of GT are expected to be introduced.

In addition, since many of these varieties are recommended for general cultivation and popular among the growers newly improved varieties will also be accepted by growers in many of the regions in the country and be well adapted to current agro-ecological context and agronomic approaches. Moreover, due to the strong positive correlation among the rice processing-related parameters with the amylose content, breeding of new varieties through the MAS approaches could be further expanded, which will positively affect the economic returns of rice farming by implementing improved varieties. Therefore, local rice varieties in the Sri Lankan germplasm have the potential to improve further to maximize their nutritional and economic value.

4. Conclusions

The *Wx* and *ALK* genes responsible for the AC and GT were present in all 54 rice varieties. The level of AC in the Sri Lankan rice germplasm has a significant association with the level of GT. There was a positive correlation between the rice processing-related parameters (brown rice recovery, milled rice recovery, and head rice recovery) with the AC. Therefore, there is a potential in improving the nutrition and cooking quality of rice with the existing high-yielding varieties of Sri Lankan germplasm of rice to obtain nutritionally rich, consumer-preferred varieties.

Conflicts of Interest: The authors have no conflicts of interest regarding this publication.

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