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Validation of Molecular Markers for the Analysis of Genetic Diversity of Amylase Content and Gel Consistency among Representative Rice Varieties in Sri Lanka

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ABSTRACT: *Starch is the major storage compound in almost all cereals including rice (Oryza sativa L.) and is used as a primary source of energy for humans. Furthermore, it is the main determinant of the eating and cooking quality (ECQ) of rice as amylose content (AC) and gel consistency (GC) are two parameters that significantly contribute to the ECQ. These parameters are mainly controlled by a major gene designated as Waxy or Wx, other minor genes such as ADP-glucose pyrophosphorylase isomerase (AGPiso) and environmental factors. The Waxy gene that encodes for the granule bound starch synthase (GBSS) enzyme which synthesizes amylase (meaning is not clear). The AGPiso (AGPase) gene, codes the protein glucose-1-phosphate adenylytransferase which plays a role in starch synthesis by catalyzing the synthesizing of activated glycosyl donor, ADP-glucose. In this study, an attempt was taken to demonstrate the correlation of AC and GC to three simple sequence repeat/ short tandem repeat (SSR/STS) molecular markers, AGPiso (linked to AGPiso gene), GBSS1 and WX (linked to Waxy gene) using 31 representative Sri Lankan rice varieties. Among the tested rice varieties, the AC and GC varied from 14.8 to 27.7%; and 3.4 to 9.0 cm, respectively. All three markers gave multiple alleles (2-5 alleles), however, these markers did not show a significant association to the AC and GC of the tested rice varieties. Further analysis of the genetic variation of Suduru Samba and Kalu Heenati will be useful as they clustered separately from the rest of the tested varieties based on the polymorphism for markers AGPiso, GBSS1 and WX.*

Keywords: *Amylose content, gel consistency, molecular markers, rice eating and cooking quality*

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important and widely consumed food crops in the world. The rapid digestion of rice starch than any other high starchy food makes it distinctive among other cereals (Kent, 1982). Rice supplies nutrition for more than half of the world's population and it accounts for 21% of energy, 14% of protein and 21% of fat intake (Kennedy and Burlingam, 2003). The genus *Oryza* comprises of 25 species with only two cultivated species: *O. sativa* L. and *O. glaberrima* Steud. (Grist, 1986). There are three sub-

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species of *O. sativa* in Asia; *indicia*, *japonica* and *javanica* and cultivated varieties in Sri Lanka are of the *indica* sub-species. Based on the production statistics of World Food and Agriculture Organization, Sri Lanka is ranked 18th among the rice producing countries in 2012 (FAO, 2013). There are more than 100 varieties currently cultivated in Sri Lanka, including both traditional and elite varieties (Jayawardena *et al.*, 2010) grown in two seasons per year: *Maha* and *Yala* (Department of Census and statistics, 2012).

The grain quality of rice is conditioned by its physiochemical properties which are influenced greatly by genotype and environmental factors such as location, cultural practices and post harvest management (Hsieh *et al.*, 1982). Rice quality basically depends on the satisfaction of the consumers. In general, the grain quality is derived into four categories; physical quality, milling quality, eating and cooking quality (ECQ) and nutritional quality.

ECQ is one of the key aspects that influence consumer's acceptability and their buying decisions. Consumers tend to buy quality rice despite the price (Cramer *et al.*, 1993). ECQ is generally measured by tenderness, cohesiveness and glossiness of boiled rice and is controlled by both intrinsic and extrinsic parameters. There are mainly three intrinsic grain quality parameters that influence the ECQ of rice. They are amylase content (AC), gel consistency (GC) and gelatinization temperature (GT) (Khush *et al.*, 1979). However, it is generally accepted that AC is the key determinant of the ECQ of rice (Juliano, 1971). Amylase content is directly correlated to volume expansion and water absorption during cooking (Cheng *et al.*, 2012) and negetively correlated to cohessiveness, tenderness, colour and glossiness of boiled rice (Cruz and Khush, 2000). It is also a determinant of the market value (Larkin *et al.*, 2003) and an indicator for classsifying rice varieties (Juliano, 1985). Amylose content influences texture and retrogradation potential of cooked grains (Champagne *et al.*, 2004). In addition to AC, rice texture is also depends on structure and characteristic of amylopectin (Takeda and Hizukuri, 1987).

Gel consistency is a measure of firmness of cooked rice. It is used to classify rice varieties by measuring the length of a cooled gel made from flour previously cooked in 0.2M KOH (Cagampange *et al.,* 1973). Gel consistency used in rice improvement programs focusing on rice varieties with intermediate and lower AC classes. Rate of hardnening and hardness differences in cooked rice correlate with GC (Rohilla *et al.*, 2000). If the GC is hard, then the cooked rice tends to be less sticky and if the GC is soft, then the cooked rice is more tender (Juliano, 1985). Gelatinization temperature is used in varietal development as an indicator of the cooking time (Cuevas *et al.*, 2010). It is an economically important indicator of quality, because selecting for varieties with shorter cooking time can lead to savings in fuel costs (Fitzgerald *et al.*, 2009).

Over the past several decades, various methods have been reported for the determination of ECQ parameters using phenotypic markers. Some of the limitations associated with phenotypic markers are the need for large quantity of sample for analysis, time and labour consuming analysis and high experimental errors involved (Takeda *et al.*, 1986). Molecular markers are the most promising alternative to overcome these limitations and to evaluate the eating quality accurately for selections in rice breeding programs (Boulaphanh *et al.,* 2011).

It is well documented that starch plays a key role in determining ECQ of rice (Kumar and Khush, 1987; Lee *et al.*, 2007; Tian *et al.*, 2009; Sun *et al.*, 2011). Starch in rice endosperm contains two types of polysaccharides: amylose and amylopectin. Amylose accounts to approximately 0–30% of total starch in the endosperm and amylopectin to approximately 70–100% (Martin and Smith, 1997). The ratio of these two polysaccharides is heritable and

varies from cultivar to cultivar (Cai *et al*., 1998). Four groups of enzymes, such as ADP-glucose pyrophosphorylase *(*AGP), starch synthase (SS), starch branching enzyme (SBE) and debranching enzyme (DBE) (James *et al.,* 2003; Hannah and James, 2008) play a distinct role in starch biosynthesis (Ball, 1996; Myers *et al*., 2000), but presumably function as a part of a network (Sun *et al.,* 2011). Some with various isoforms lead to a specific starch end product (Pandey *et al.,* 2012).

ADP glucose pyrophosphylase catalyzes the first reaction in starch synthesis, producing the activated glucosyl donor ADP-glucose (ADPG). *AGPiso* (*AGP* large subunit isoform) codes for the protien glucose-1-phosphate adenylyltransferase which is composed of 518 amino acids. Previous research revealed that *AGPiso* gene is responsible for GC of *japonica* rice (Sun *et al*., 2011) and acts as a minor gene affecting GC in all rice cultivars (Tian *et al*., 2009). *AGPiso* is a PCR based SSR marker linked to the *AGPiso* gene and the expected product size is approximately 98 bp (Hsu *et al*., 2014).

Starch synthases (SS) utilize ADPG to elongate linear chains (James *et al*., 2003). A granule-bound isoform, GBSSI, which is encoded by the *Waxy* (*Wx*) locus in cereals, functions specifically to elongate amylose (Shure *et al*., 1983). A major quantitative trait loci (QTL) contributing largely to the ECQ has been mapped to chromosome 6 corresponding to the *Waxy* locus. It encodes GBSS1*,* a key gene determining the percentage of amylose and ratio between amylose to amylopectin, the two critical factors affecting the ECQ. Six alleles of *Wx* have been found in natural germplasm (Hsu *et al*., 2014). *GBSS1* marker is a SSR type PCR based marker derived from *OSR19* to *RM587* of QTL region (Kwon *et al*., 2008). The expected PCR product is 170 bp in size. The *WX* marker is a STS type PCR based marker with an expected band size of 100 bp (Han *et al*., 2004). There are two classes of brancing enzymes (BE);BEI and BEII*;* that differ in terms of the length of the chains they transfer (Guan and Preiss, 1993). The amylopecten structures (Umemoto *et al*., 2002) and the percentage amylose content (Takeda *et al.,* 1986) vary between *japonica* and *indica* rice.

Previous studies (Sun *et al.,* 2011) have been carried out to show the genetic basis of ECQ with respect to DNA markers (13 markers including *GBSS1* and *AGPiso*) and some of these markers were found to be useful in marker-assisted selection (MAS) of *japonica* rice (Hsu *et al*., 2014). However, no previous research has been carried out test them on *indica* background and therefore, the present study was carried out to validate the three molecular markers (*AGPiso*, *GBSS*1 and *WX*) in analyzing the genetic diversity of AC and GC in selected rice varieties representing the Sri Lankan rice germplasm, aiming to evaluate the usefulness of these molecular markers MAS in rice breeding programs.

METHODOLOGY

Plant materials and preparation of seedlings

Thirty one varieties of *O. sativa* (Table 3) were selected to represent the Sri Lankan rice germplasm. Rice seeds were obtained from an ongoing research at the Rice Research and Development Institute (RRDI), Bathalagoda and the seedlings were maintained at plant house of the Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya for DNA extraction. The *japonica* rice variety Nipponbare was used as the reference variety. A total of 10 seeds from each variety were germinated and seedlings were planted separately in labelled pots and maintained in the plant house. After three weeks, the leaves were harvested from each variety for DNA extraction.

Genomic DNA extraction and quantification

Genomic DNA was extracted from three weeks old immature tender leaves using a modified CTAB method described by Murray and Thompson (1980). The extracted DNA was stored at -20 °C. Confirmation and quantification of the extracted genomic DNA was performed using a spectrophotometric method and agarose gel electrophoresis.

Polymerase chain reaction

Polymerase chain reaction (PCR) amplification was performed using a PCR reaction mixture of 20 µl containing 1 unit of Taq DNA, 200 µM of dNTPs, 0.5 pM each of forward and reverse primers (Table 1) and 100 ng of template genomic DNA. The thermal cycling conditions given are as follows: an initial denaturation for 5 min at 94 °C; 35 cycles of 1 min at 94 °C; 1 min at optimized annealing temperature of 57 °C (*AGPiso*) /56 °C (*WX*) /58 °C (*GBSS1*); 2 min at 72 °C; and final extension of 10 min at 72 °C. The PCR was performed on a thermal cycler (Thermal cycler 2720, Applied Bio systems USA).

Table 1. Primer sequences of the molecular markers tested in analyzing genetic diversity of *Waxy* and *AGPiso genes*

Gene	Marker name	DNA sequence (5' to 3')	Reference	Melting temperature (°C)	Product size (bp)
AGPiso	*AGPiso*	F-CAA TCG CTG CCA TCG GTT G	Hsu *et al*., 2014	60	90-120
		R- TTC CAC ATC GTT AGG TAC ACG		54	
Waxy	*GBSS1*	F-CAA ATA GCC ACC CAC ACC AC	Kwon *et al*., 2008	56	170-250
		R-CTT GCA GAT GTT CTT CCT GAT G		54	
	WX	F-CTC TCT CAC CAT TCC TTC AG	Han *et al.,* 2004	52	90-120
		R-CAC AAG CAG AGA AGT GAA GC		53	

The PCR products were first visualized using 2% agarose gel. Two-µl of loading buffer (98% formaldehyde, 10 mM *EDTA*, 0.025% bromophenol blue, 0.025% xylene cyanol) was mixed with 5 µl of PCR product and was loaded to the agarose gel. Gel electrophoresis was carried out using a Mupid-2Plus gel electrophoresis apparatus (Advance, Japan) at 50 V for 1 hr. After staining with ethidium bromide (0.05 µl/ml) it was visualized using the UV light documentation system (BIO RAD, USA).

Polyacrylamide gel electrophoresis (PAGE) was carried out to detect the polymorphism among the thirty two varieties using amplified PCR products on an 8% acrylamide gel. The 100-bp ladder or the PCR products (1.5 µl) were mixed with 1.5 µl of 2× loading dye (98% formaldehyde, 10 mM *EDTA*, 0.025% bromophenolblue, 0.025% xylene cyanol) and was loaded to the gel. PAGE was done at 20 mA/gel for 1.5 hr using a vertical gel electrophoresis

apparatus (Atto, Japan) and the gel was visualized using silver staining method described by Chevallet *et al.* (2006).

Determination of amylase content and gel consistency

Seeds from each variety cultivated in *Maha* (2012/2013) and *Yala* (2013) seasons at RRDI, Bathalagoda were harvested at maturity and shade dried to reduce the moisture content to 12-14 %. Seed samples of 150 g were dehulled using a Sakate dehusking machine (THU 358) and polished to a level of ± 8% of bran removal using a Satake dehusking machine (THO 5.0). Amylose content of each rice variety was estimated using the method described by Juliano (1971) and GC was determined by the method described by Cruz and Khush (2000) at the Grain Quality laboratory, RRDI, Bathalagoda.

Data analysis

The analysis of variance for data on AC and GC was performed using SAS 9.0 program and mean separation was performed using duncan multiple range test. Based on the marker polymorphism, a dendrogram was developed for 30 rice varieties (excluding two rice hybrids, Bg 407H and Bg 94-1) by performing a hierarchical cluster analysis (with 'complete linkage' clustering method and 'euclidean' distance measure) using the 'Rcmdr' package v2.0-4 in RStudio v0.98.978 (R Development Core Team, 2008).

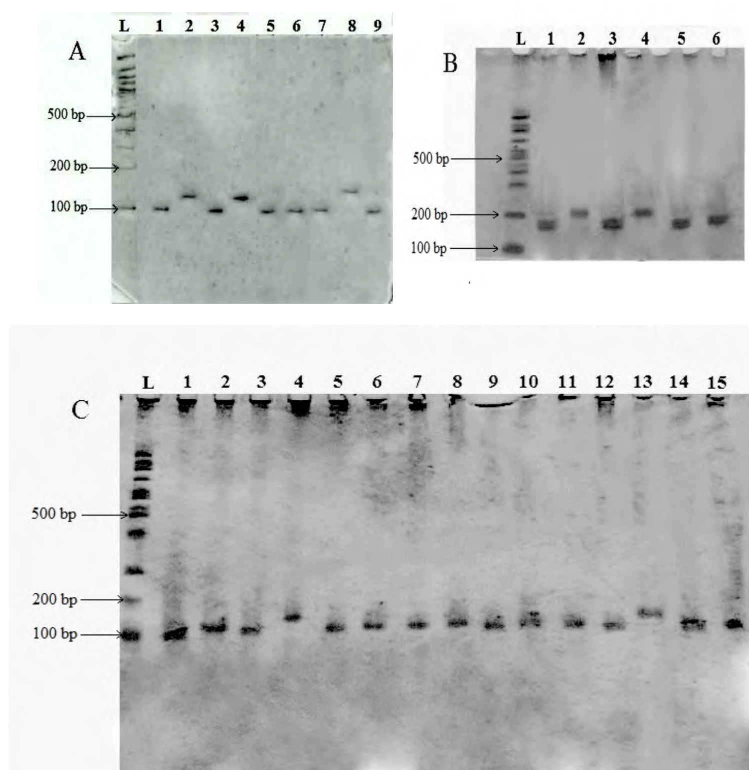
RESULTS AND DISCUSSION

Analysis of the genetic diversity at *Wxay* and *AGPiso* genes using molecular markers

Five alleles were detected (designated as 1,2,3,4 and 5) for the marker *AGPiso* in the selected rice germplasm and twenty eight varieties out of the thirty two showed no polymorphism for the target SSR site in *AGPiso* gene. The varieties/hybrids that showed polymorphisms at the locus are Kalu Heenati (allele 2), Suduru Samba (allele 3), Bg 94-1(alleles 1 and 4) and Nippon bare (allele 5). Among tested varieties, three alleles (designated as 1, 2 and 3) were detected for markers *WX* and *GBSS1*. For those two markers, Kalu Heenati and Suduru Samba (both carrying the allele 2) showed polymorphism from all other Sri Lankan rice varieties (allele 1), while Nippon bare carried a different allele (allele 3) (Fig. 1 and Table 2). Therefore, two traditional rice varieties Kalu Heenati and Suduru Samba, and the reference variety Nippon bare showed polymorphism for all tested markers. For the marker *AGPiso*, Suduru Samba gave an amplified product which is slightly longer than 98 bp. Kalu Heenati amplified a longer product than Suduru Samba, which implies that it has a longer SSR sequence. Kalu Heenati and Suduru Samba (Fig. 1B, C) showed a different allele for both the target sites with slightly larger amplified product than all other varieties with approximate sizes of 200 bp and 100 bp for markers *GBSS1* and *WX*, respectively.The *japonica* rice variety Nipponbare showed a different allele for all tested markers with a size in between 100 and 200 bp for marker *AGPiso* (Fig. 1A).

Table 2. Allele types generated by each molecular marker for selected rice varieties

Rice Variety/ Hybrid	Molecular marker		
	AGPiso	***WX***	***GBSS1***
Kalu Heenati	2	2	2
Suduru Samba	3	2	2
Bg 94-1	1,4	1	1
Bg 407H	1	1,2	1,2
Nippon Bare	5	3	3
Total no of alleles	5	3	3



A: Polymorphism with marker *AGPiso* (L-100 bp ladder, 1-Bg 360, 2-Kalu Heenati, 3-Rathal, 4-Suduru Samba, 5-At 306, 6-Bg 407-H, 7-Pachchaperumal, 8-Nipponbare, 9-Kahatawee) B: Polymorphism with marker*GBSS1* (L- 100 bp Ladder, 1-Dular, 2-Suduru Samba, 3-Bg 58, 4-Kalu Heenati, 5-Masuran, 6-At 306) C: Polymorphism with marker *WX* (L-100 bp Ladder, 1-Wanni Dahanala, 2-Pachchaperumal, 3-Kahata wee, 4-Suduru Samba, 5-Devaradiri, 6-Kuruluthuda, 7- Masuran, 8-Sudu Heenati, 9-Gonabaru, 10-Hondarawalu, 11-Sulai, 12-Madathawalu, 13-Kalu Heenati, 14-Maawee, 15-Inginimitiya)

Fig. 1. Polymorphism showed by the selected rice varieties for molecular marker *AGPiso, GBSS1* and *WX*, on 8% polyacrylamide gels.

In the dendrogram resulted from the hiarachical cluster analysis, Kalu Heenati and Suduru Samba could be clustered separatly from the other 27 inbred varieties (Fig. 2). This indicates

a close relationship among Sri Lankan rice for the tested markers. Suduru Samba and Kalu Heenati have shown different alleles with respect to all three markers tested. However, the marker vizualyzation technique used in the study was insufficient to clearly resolve the PCR products of the *GBSS1* and *Wx* markers for above two varieties. Therefore, these two traditional rice vareities would be ideal for further studies in understanding their genetic background in relation to starch synthesis. The *japonica* reference variety Nipponbare clustered separately showing the clear genetic distance between *japonica* and *indica* type rice including genes responsible for starch sysntheis.

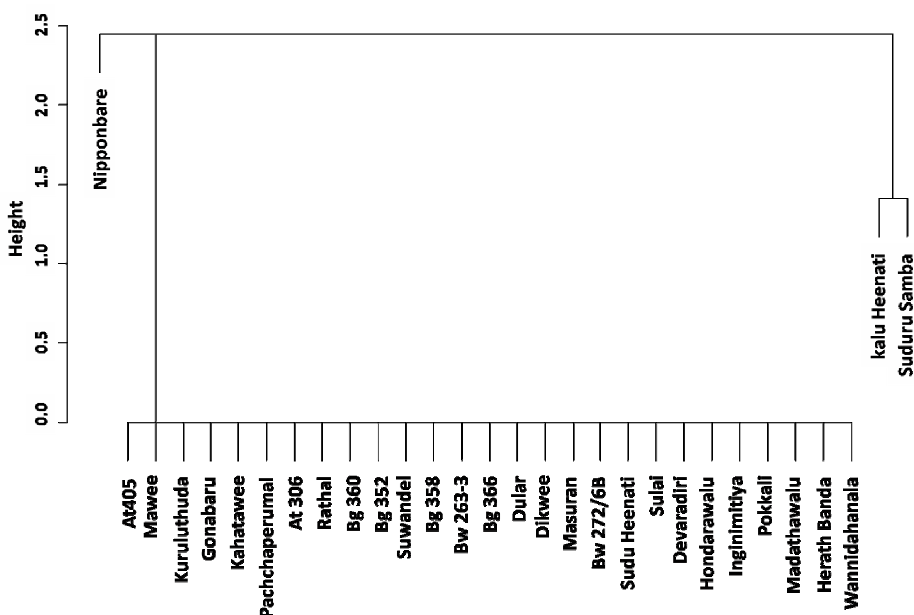


Fig. 2. Genetic relationship of Sri Lankan rice varieties based on molecular markers *AGPiso, GBSS1, WX.*

Amylose content and gel consistency of selected rice varieties

Amylose content of the evaluated varieties varied from the lowest of 14.8% (At 405) to the highest of 27.7% (Kahata Wee) with a majority (19 varieties out of 31) grouped in to the intermediate AC class (Table 3) according to the five amylose classes given by Kumar and Khush (1987) (high (>25%); intermediate (20-25 %); low (10-19 %); very low (3-9 %) and waxy (0-2 %)). Only At 405 could be catogorized under low AC group and there were 11 varieties that could be classified as high AC. The AC values determined by previous studies for some rice varieties showed relatively high for all the sampled varieties (Wickramasinghe and Noda, 2008; Rajapaksha *et al*., 2011). This could be due to differences in analysis procedures and influence of environmental factors. Amylose content is significantly affected by storage intervals, treatments (Khush *et al.,* 1979) and method of measurement. A prevalent high ambient temperature during the ripening stage of the crop may increase the AC of cereals (Sun *et al*., 2011).

Traditional vareity Kuruluthuda has the highest GC value (9.00 cm) among the tested varieties, therefore, it may have the softest texture in cooked rice as research evidences

proved that GC has a strong negative relationship to the texture of the cooked rice . Bg 360 has the lowest GC with a value of 3.40 cm. According to the classification; soft (>6), medium (4-6) and hard (<4), Bg 360 is catogorized as having a "hard" texture. Nine varieites had low GC, but they all have a GC value very close to 4.0, therefore, eventhough they are catogorized under "hard" class, their texture falls barely in to the hard textured category when cooked. The majority of *indica* varieties have high AC, and low GC and GT values, while *japonica* varieties show low AC and high GC and a GT values (Tian *et al*., 2009). Varieties such as Herath Banda, Pokkali, Hondarawalu, Kuruluthuda, Sulai and At 405 are catogorized under soft GC class and are prefered over other varieties (Rohilla *et al.,* 2000) by the consumers. Eleven varieties were classified to the medium GC class category.

Table 3. Variation of the average amylose content and the gel consistency of the tested rice varieties of Si Lanka

Rice Variety	Accession No	Average AC (%)	AC Class	GC/ cm	GC Class
Herath Banda	280	26.7	High	7.67	Soft
Kalu Heenati	33	27.1	High	3.95	Hard
Wanni Dahanala	591	24.2	Intermediate	5.83	Medium
Pachchaperumal	799	23.5	Intermediate	3.70	Hard
Suwandel	579	23.6	Intermediate	3.52	Hard
Madathawalu	-	24.2	Intermediate	4.10	Medium
Pokkali	809	23.3	Intermediate	7.30	Soft
Mawee	87	23.2	Intermediate	4.52	Medium
Gonabaru	28	21.9	Intermediate	3.50	Hard
Rathal	133	21.7	Intermediate	3.65	Hard
Hondarawaalu	285	25.6	High	7.15	Soft
Kahata Wee	324	27.7	High	4.27	Medium
Kuruluthuda	69	22.9	Intermediate	9.00	Soft
Devaradiri	243	25.9	High	5.85	Medium
Suduru Samba	903	20.6	Intermediate	3.61	Hard
Sulai	502	25.2	High	6.42	Soft
Dik Wee	246	24.4	Intermediate	3.75	Hard
Sudu Heenati	491	23.8	Intermediate	3.75	Hard
Masuran	86	23.1	Intermediate	3.65	Hard
Inginimitiya	-	25.1	High	4.30	Medium
Dular	-	22.4	Intermediate	5.30	Medium
At 405	-	14.7	Low	6.62	Soft
Bg 366	-	23.4	Intermediate	3.65	Hard
Bg 360	-	22.9	Intermediate	3.40	Hard
At 306	-	24.4	Intermediate	4.27	Medium
Bg 407H	-	25.2	High	3.80	Hard
Bg 358	-	26.9	High	3.60	Hard
Bg 352	-	27.6	High	4.70	Medium
Bg 94-1	-	25.8	High	3.97	Hard
Bw 267-3	-	22.2	Intermediate	4.35	Medium
Bw 272/6B	-	26.1	Intermediate	5.45	Medium

AC – amylose content; GC- gel consistency

Correlation analysis

Eventhough the previous studies have reported a strong correlation between AC and GC (Cruz and Khush, 2000; Rohilla *et al.,* 2000), in this study the correlation between AC and GC was not significant (p = 0.07; R^2 = 0.11). Cruz and Khush, 2000 have also reported that differences in GC exists among varieties of similar amylose content. In cotrast, Singh *et al.* (2000) showed that AC have significant negative correlation with GC. Therefore, the correlation between AC and GC might depend on the varieties used and the diversity of the characters.

Furthermore it was not possible to detect an allelic association of any tested molecular marker with AC or GC, though some previous studies have shown such relationships with these markers to the ECQ in *japonica* rice (Sun *et al*., 2011; Hsu *et al.,* 2014). The major reason for not detecting significant alleleic association in the current study could be due to the *indica* genetic background of the varieties tested. Therefore, more molecular markers should be examined and further linkage studies should be carried out with Sri Lankan rice varieties.

CONCLUSION

Amylose content of the evaluated varieties varied from 14.8 (At 405) to 27.7% (Kahata Wee) and the GC variation was from 3.4 cm (Bg 360) to 9.0 cm (Kurulu thuda). No significant correlation was shown between the above two characters. The three molecular markers *AGPsio*, *WX* and *GBSS1* gave multiple alleles, however, none of the markers clearly correlated to either AC or GC of the tested varieties. Therefore, they could not be used for MAS for the selected rice varieties. Since Suduru Samba and Kalu Heenati have different alleles in all three molecular marker target regions, they would appeared to have a deviated genetic background with respect to the target regions of both *Waxy* and *AGPiso* genes. Therefore these two traditional rice vareities would be ideal for further studies in understanding their genetic background in relation to starch synthesis.

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