Original Article **Asian J Agri Biol, 2014, 2(1):44-50.**

APPLICATION OF DNA MARKERS FOR THE DETECTION OF AMYLOSE CONTENT IN SRI LANKAN RICE (*ORYZA SATIVA* L.) VARIETIES

N.S. Kottearachchi[1*], R.K. Peiris[1] and S. Rebeira[2]

[1]Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP), Sri Lanka. [2]Department of Grain Quality, Rice Research and Development Institute, Bathalagoda, Sri Lanka

ABSTRACT

Grain quality improvement of rice is considered as prioritized research area in rice breeding programs in Sri Lanka. Amylose Content (AC) is a key determinant of end quality of rice. Amylose Content is controlled by granule bound starch synthase (GBSS) enzyme which is encoded by *waxy* gene, located on chromosome 6. Similarly another character associated with grain quality, Gelatinization Temperature (GT), is controlled by the alkali degeneration locus (*alk*), which encodes for a soluble enzyme called starch synthase IIa (SSIIa). Two SSR markers RM 190 and RM 314 were selected from the previously developed QTL maps that showed association with AC and GT. This study demonstrates the feasibility of RM 190 and RM 314 for the detection of polymorphism associated with AC and GT. In total, 18 different rice varieties were analysed. The relationship between marker genotype and amylose content was detected by comparing the pooled AC under 2 different allele categories using Mann-Whitney Test. The results indicated that RM 190 was able to detect genetic variation among the rice varieties quite efficiently, on the basis of which, the rice varieties could be identified with two categories; with low AC and with intermediate and high AC. Therefore, the SSR marker, RM 190, could be efficiently employed in large-scale screening programmes to predict AC through Marker Assisted Selection (MAS).

Keywords: Amylose content, Gelatinization temperature, Microsatellite Markers, *Oryza Sativa* L.

INTRODUCTION

Rice is one of the most important staple food crops of more than one-half of population with approximately 95% of production in Asia (Bhattacharjee *et al.*, 2002). Starch is the major component of the rice endosperm, which consists of amylose and amylopectin. The amylose is made up of linear molecule composed of α (1,4)- linked glycosidic chains, whereas, the amylopectin exhibits highly branched glucan with α (1,6) glycosidic bonds that connect linear chains. Amylose content (AC) (Juliano, 1985), gel consistency (GC) (Cagampang *et al.*, 1973) and Gelatinization Temperature (GT) (Little *et al* ., 1958) are the three physiochemical characteristics of starch, involve in determination of eating, cooking and processing quality of rice.

Amylose content is considered as one of the most important indicators of rice grain quality. The AC is determined as the ratio of amylose amount present in endosperm to total starch content. The high AC leads to a dry, fluffy and separated cooked rice which becomes hard upon cooling (Juliano, 1985), whereas, the low AC gives a moist, sticky and glossy rice grain after cooking. Rice cultivars are commonly categorized as glutinous/ waxy (0-5% of amylose), low AC (<20% of amylose), intermediate AC (21-25% of amylose) and high AC (>25% amylose) (Kongseree and Juliano, 1972).

Earlier work on rice genetics, has identified major genes as well as QTLs (Quantitative trait loci), which are considered responsible for AC in rice endosperm. The QTLs are present on chromosome 5 and 6 and, the major gene, *waxy*, located in chromosome 6, accounts for 91.9% of the total variation of AC (He *et al.*, 1999). The *waxy* gene plays a key role in amylose synthesis by encoding the enzyme, granule bound starch synthase (GBSS) (Tan *et al.*, 1999 and Fan *et al.*, 2005). Amylose content polymorphism in rice has been explained by the *waxy* alleles: wx^a and wx^b (Sano, 1984). The wx^a allele is predominantly involved in the synthesis of GBSS. According to the study performed by Wang and co-workers (1995), the variation in AC accumulation in rice is primarily governed at post transcriptional levels. Gelatinization

**Corresponding author:* e-mail: kottearachchins@yahoo.com

temperature (GT) of rice starch is defined as the range of temperature at which nearly all the starch granules start to swell irreversibly in hot water with simultaneous loss of birefringence and crystallinity (Virmani, 1994). GT of rice is controlled by the alkali degeneration locus (*alk*) on chromosome 6, which encodes soluble starch synthase IIa (SSIIa) isoform (Fan *et al*., 2005). Low GT rice needs lesser energy input during cooking than high GT rice (Bao *et al.*, 2004). Based on GT values, rice cultivars can be broadly categorized into three classes: low (<70 ^{o}C), intermediate (70-74 ^{o}C) and high (>74 ^{o}C) (Kongseree and Juliano, 1972). Development of cultivars with improved agronomic traits while maintaining the desired AC and GT is of major concerns of rice breeders. Conventional methods for measuring of these quality traits are time consuming and expensive. DNA markers provide a useful, easy and cost-effective way to identify plants with desired level of AC and GT values (DeOliveira *et al.*, 2010 and Tabkhkar *et al.*, 2012). Theretofore, this study was carried out to detect a suitable DNA marker, which is closely linked with AC and GT in order to facilitate rice breeding programs.

MATERIALS AND METHODS

Plant Materials

Both mature seeds and husked and polished grains of 18 different rice varieties were collected from Rice Research and Development Institute, Bathalagoda, Sri Lanka (see Table 2 for details).

Analysis of Amylose Content

Initially rice samples were husked and polished prior to milling. Twenty whole-milled rice kernels of 18 rice samples were ground separately in a Udy cyclone mill (sieve mesh size 60). Amylose content per 100 mg was determined through measuring blue value of rice varieties as described by Juliano (1971). A 100-mg of rice sample was put into a 100 ml volumetric flask and 1 ml of 95% (v/v) ethanol was added. Then 1 ml of 1 N (w/v) NaOH was added. The content was then boiled for 20 min to gelatinize starch. After cooling the content, the volume was made up to 100 ml. A 5-ml of starch solution was pipetted out into a 100-ml volumetric flask. 1 ml of 1 N acetic acid and 2 ml of iodine solution (0.2 g iodine and 2.0 g potassium iodide in 100 ml aqueous solution) were added. Volume was made up to 100 ml with distilled water and solution was allowed to stay for 20 min after shaking. The absorbance of the solution was measured at 620 nm with spectrophotometer (JENWAY 6305, UK).

The standard curve was prepared using 40 mg of potato amylose to calculate amylose content of rice varieties. Fourty (40) mg of potato amylose was put into a 100 ml of volumetric flask and 1 ml of 95% (v/v) ethanol and 9 ml of 1N NaOH were added and content was heated for 20 min at boiling temperature. After cooling, the content volume of the solution was made up to 100 ml using distilled water. Then 1, 2, 3, 4, and 5 ml of amylose solution were pipetted out into 100 ml flasks. Then 0.2, 0.4, 0.6, 0.8 and 1 ml of 1 N acetic acid were added to the flasks respectively. Finally, 2 ml of iodine solution was added to each flask and volumarized up to 100 ml with distilled water. Solutions were stood up for 20 min after shaking. Then absorbance values were measured at 620 nm. Measured absorbance values were plotted at 620 nm against the concentration of anhydrous amylose (mg).

Analysis of Gelatinization Temperature

The GT was indirectly measured on rice by the alkali spreading value (Little *et al* ., 1958). Husked and polished rice samples were used for the analysis. Selected duplicate sets of six milled grains without cracks of each sample were put into petridishes. 10 ml of 1.7% (w/v) KOH was added and grains were spread in the petridish to provide enough space. Constant temperature of 30 ^{o}C was maintained to ensure better reproducibility. After 24 hrs, GT was identified based on the score given from 1 to 7, by visual appearance of the gelatinization. Then the gelatinization temperature was identified based on the score value of the samples as reported by Cruz and Khush (2000).

Genomic DNA Extraction and Quantification

Genomic DNA was extracted from 2 to 3-week-old leaves. Approximately, a 3-cm tender leaf piece was ground with 300 µl of DNA extraction buffer (1 M KCl, 1 M Tris HCl, 0.5 M EDTA) in a micro centrifuge tube separately for each variety. Then homogenate was incubated at 70 ^{o}C for 15 min at room temperature and centrifuged at 15000 rpm for 15 min immediately after the incubation. 100 µl of isopropanol was added into a new eppendorf

tube. Then supernatant was transferred into the tube containing isoproponol and mixed gently. Tubes was kept at 4 ^{o}C overnight and centrifuged at 15000 rpm for 15 min at 4 ^{o}C. The supernatant was removed and DNA pellet was washed with 150 µl of 70% (v/v) ice cold ethanol by centrifuging for 10 min at 15000 rpm at 4 ^{o}C. Finally, the supernatant was removed and pellet was air dried. After the removal of all the ethanol traces, the DNA pellet was dissolved in 300 µl of 1/10th TE buffer (10 mM Tris, 1 mM EDTA) and used for PCR.

Selection of SSR Markers

The SSR markers linked with AC and GT were selected by QTL maps developed by Fan *et al.* (2005) and linkage association studies as reported previously (Septiningsih *et al.*, 2003; Shu *et al.*, 2006; Tabkhkar *et al.*, 2012). Accordingly two SSR primers; RM 190, RM 314 (Table 1) were selected for PCR amplification

.

Table 1: DNA sequence information of SSR markers used in the study

Marker	Motif	Primer Sequence	AC*	GT**	Reference
RM190	(CT)11	F: 5' TTTGTCTATCTCAAGACAC 3' R: 5' TTGCAGATGTTCTTCCTGATG 3	*ac6a*	*asv6a*	Fan *et al.* (2005), Tabkhkar *et al.* (2012)
RM314	(GT)8(CG)3 (GT)5	F: 5' CTAGCAGGAACTCCTTTCAGG 3' R: 5' AACATTCCACACACACACGC 3'	*ac6b*	*alk2(t)*	Septiningsih *et al*. (2003), Fan *et al.* (2005), Shu *et al.* (2006), Tabkhkar *et al* . (2012)

*QTL reported for Amylose content, **QTL reported for Gelatinization temperature

No.	Variety	AC (%)	Allele type produced by RM 190	GT
1	At 303	28.72	A	High
2	At 306	23.93	B	High
3	At 308	28.03	A	In
4	At 402	21.54	A	High
5	At 405	13.29	B	Low
6	Bg 250	26.15	A	In
7	Bg 300	26.41	A	In
8	Bg 305	27.35	A	High
9	Bg 358	25.21	A	H/I
10	Bg 379-2	31.88	A	High
11	Bg 403	31.88	A	Low
12	Bg 406	21.41	A	High
13	Bg 407-H	26.84	A & B	Low
14	Basmati 370	16.24	B	In
15	Ld 356	25.3	A	In
16	Ld 365	21.67	A	High
17	Ld 408	21.79	A	Low
18	Pusa basmati	22.09	A	Low

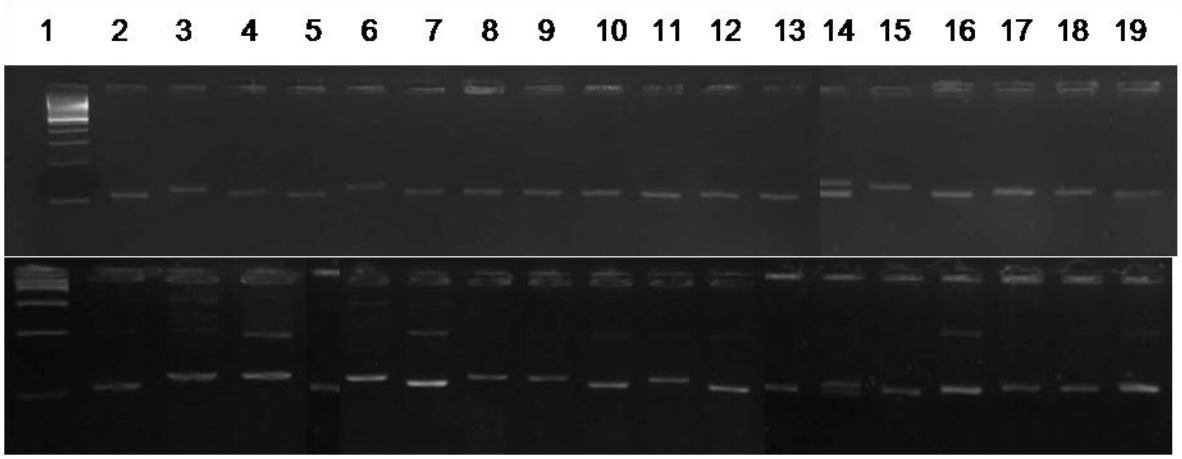
Table 2. Characteristics of rice varieties used in the study

GT; In: Intermediate, H/I: High/Intermediate
Allele type produced by RM 190; A allele-amplified short DNA fragment, B allele-amplified long DNA fragment

Table 3: Analysis of genotypes by Mann-Whitney test

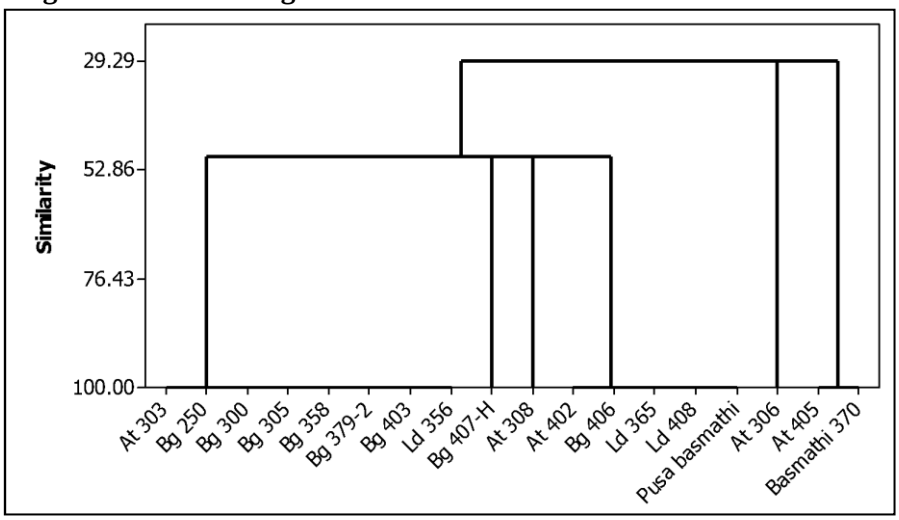
Primer	Median AC(%) for allele types		p value
	A	B	
RM 190	16.239	25.470	0.0041

Figure 1. PCR profile generated by markers, RM 190 (upper gel) and RM314 (lower gel) for the tested rice varieties



Lane 1- 100 bp ladder, 2-19: At 303, At 306, At 308, At 402, At 405, Bg 250, Bg 300, Bg 305, Bg 358, Bg 379-2, Bg 403, Bg 406, Bg 407-H, Basmati 370, Ld 356, Ld 365, Ld 408, Pusa basmati respectively

Figure 2. Dendrogram constructed based on genotypes of RM190 and groups of Amylose Content showing similarities among rice varieties



PCR Amplification

The PCR amplification was performed in a 12-µl reaction volume, which consisted of 5 µl of diluted DNA (10ng/µl), 1.5 µl of 10X PCR buffer (provided with Dream Taq polymerase, Fermentas, USA), 1.2 µl of dNTPs (2.5 mM), 0.9 µl of primers (20 pmol/µl), 0.12 µl of Taq DNA polymerase (Dream Taq, Fermentas, USA) and 3.28 µl distilled water. Amplification was carried out in a BioRad (My Cycler^{TM}) thermal cycler with following parameters: initial denaturation at 95 ^{o}C for 5 min followed by 35 cycles of 95 ^{o}C for 1 min, 52 ^{o}C (for RM 190) or 57 ^{o}C (for RM 314) for 30 sec, 72 ^{o}C

for 1 min and final extension at 72 ^{o}C for 5 min. The amplified PCR products were detected under agarose gel electrophoresis using 3.8 % gel by precast staining with ethidium bromide. After the electrophoresis, the gels were visualized by using a gel documentation system.

Statistical Analysis

The banding patterns of amplified alleles from SSR markers were observed and based on the allele size, plants showing the presence of similar alleles were categorized viz. A (short allele), B (long allele) etc. The Mann-Whitney test (Fay *et al.*, 2010) was performed to detect whether RM190 marker shows strong association with AC and allele type using Minitab 15. 1. A dendrogram was constructed using the hierarchical clustering procedure based on RM190 genotype and AC values and 'euclidean' distance was calculated by single linkage method using Minitab version 15.1.

RESULTS

Analysis of Amylose Content and Gelatinization Temperature

The AC and GT were measured in 18 locally prevalent rice varieties by Blue value method described by Juliano (1971). According to the analysis At 405 and Basmati 370 were categorized as low AC rice varieties as they were in the range of 10-19%, while At 306, At 402, Bg 406, Ld 365, Ld 408 and Pusa basmati were categorized as intermediate AC rice varieties as they exhibited AC values in the range of 20-25%. Other rice varieties, which showed more than 25% of AC were categorized as high AC containing rice varieties.

Similarly, based on GT values the varieties were grouped into three categories: high, intermediate and low GT varieties (Table 2).

Genotyping by RM 190

With the RM 190 primer pair the PCR amplification reaction produced two distinguishable alleles. Both alleles were between 100-150 base pairs (bp). Except in Bg 407-H, all the other rice varieties had one allele, either A or B (A allele- amplified a shorter DNA fragment, B allele- amplified longer DNA fragment) (Figure 1.). All the low AC rice varieties tested here showed the presence of allele 'B' while At 306, whose AC values were in intermediate range also amplified allele 'B' exceptionally. All the other intermediate and high AC rice varieties showed the presence of allele 'A'.

In order to detect whether there is significant relationship between genotype produced by marker RM190 and the AC values, the pooled amylose categories were compared by Mann-Whitney test. As a result it appeared that RM 190 could significantly ($p<0.01$) distinguish between low AC rice varieties from intermediate and those with high AC (Table 3).

All varieties that possessed 'A' allele of RM190 marker were separately clustered with nearly 50% similarity index giving four subclusters further. All intermediate AC varieties, At402, Bg406, Ld365, Ld408 and Pusa basmati except Bg306 were separately subclustered with 100% similarity index. All varieties that contained high amylose content were separately subclustered except Bg308 and Bg407-H. The dendrogram showed that three varieties that contained allele 'B' of RM190 marker were separately clustered and of those, At 405 and Basmati 370, which had low amylose, were separately sub-clustered with 100% similarity index (Figure 2).

Genotyping by RM 314

The marker RM314 was found polymorphic among all the varieties used in the study and produced three distinguishable alleles ranging from bp 111-128. The marker RM 314 locates close to the QTL of AC, *ac6b* (Fan *et al.*, 2005) and QTL of GT *alk2 (t)* (Shu *et al.*, 2006). However, phenotypic variation of AC and GT in rice varieties tested in this study has not linked with any of the RM 314 alleles. Bg 407-H, the hybrid rice variety, also expressed heterozygosis nature through producing two alleles by RM 314.

DISCUSSION

In this study, we have tested the power of two rice markers associated with rice grain quality. Our results show that at least one of the two markers, RM 190, could be efficiently used for the identification of rice population with a desired amylose content (AC). Hence, it could be a useful component of the future breeding programmes aimed at rice crop improvement against various biotic and abiotic stresses in a background of desired AC.

Ayres *et al.* (1997), Bergman *et al.* (2001), Lang and Buu (2004) and Chen *et al.* (2008)

suggested that RM 190 primer was suitable to use as a marker for the detection of AC polymorphism during breeding process. It flanks major QTL, *ac6a*, linked with AC (Fan *et al.*, 2005; Tabkhkar *et al.*, 2012). Therefore, RM 190 primer was mainly used in this study for the detection of AC polymorphism in Sri Lankan rice varieties. The AC values were determined using blue value method. The RM 190 was able to distinguish two types of alleles among tested varieties and showed a strong association with AC values.

Fan *et al.*, 2005 have reported that marker RM190 is one of the closer markers to the main QTL (*asv6a*) responsible for GT. However, our study could not find any relationship between RM 190 and GT. Another marker, RM 314, one of the closest markers to the another AC QTL, *ac6b* and another GT QTL, *alk2 (t)*, located in the same chromosome 6, showed a good level of polymorphism in the rice varieties used. However, once again this marker did not show any linkage with AC and GT values. According to Fan *et al.*, 2005 the effect of *ac6b* was relatively small, giving contributions of about 0.85% to the phenotypic variation of AC in their tested population. This fact indicates that the effect of such minor QTLs are not likely to be distinguished among diverse population.

CONCLUSION

The RM 190 was able to distinguish two types of alleles among tested varieties and showed a strong association with AC values of tested rice. On the basis of our results it can be concluded that RM 190 has the potential to be used in distinguishing low AC rice varieties over the intermediate and high AC. Local rice variety At 405 may have the potential to be used as parental line in developing low amylose content varieties in addition to the donor parent Basmati 370 following RM190 marker based selection. Analysis of segregation population using RM190 would be useful for further validation.

ACKNOWLEDGEMENTS

The authors express their gratitude to the research grant (RG/2011/BT/02) of National Science Foundation, Sri Lanka for donating primers for this study.

REFERENCES

Ayres NM, McClung AM, Larkin PD, Bligh HFJ, Jones CA and Park WD, 1997. Microsatellites and a single-nucleotide polymorphism differentiate apparent amylose classes in an extended pedigree of US rice germplasm. Theor. Appl. Genet. 94: 773-781.

Bao JS, Sun M, Zhu LH and Corke H, 2004. Analysis of quantitative trait loci for some starch properties in rice (*Oryza sativa* L.), thermal properties, gel texture, swelling volume. J. Cereal Sci. 39: 379-385.

Bergman CJ, Delgado JT, McClung AM and Fjellstrom RG, 2001. An improved method for using a microsatellite in the rice *waxy* gene to determine amylose class. Cereal Chem. 78: 257-260.

Bhattacharjee P, Singhal RS and Kulkarni PR, 2002. Basmati rice: A review. International J. Food Sci. Tech. 37: 1-12.

Cagampang GB, Perez CM and Juliano BO, 1973. A gel consistency test for eating quality in rice. J. Sci. Food Agric. 24: 1589-1594.

Chen MH, Bergman C, Pinson S and Fjellstrom R, 2008. *Waxy* gene haplotypes: Association with apparent amylose content and the effect by the environment in an international germplasm collection. J. Cereal Sci. 47: 536-545.

Cruz N D and Khush GS, 2000. Rice grain quality evaluation procedures, In: Aromatic rices. Eds: Singh RK. Singh US and Khush GS. New Dhelhi, Oxford & IBH Publishing Co. Pvt. Ltd, New Delhi. pp. 15-28.

DeOliveira TC, Brondani RPV, Breseghello F, Coelho ASG, Mendonca JA, Rangel PHN, Brondan IC, 2010. Association mapping for yield and grain quality traits in rice (*Oryza sativa* L.). Genet. Mo. Biol. 33(3): 515–524

Fan CC, Yu XQ, Xing YZ, Xu CG, Luo LG and Zhang QF, 2005. The main effects, epistatic effects and environmental interactions of QTLs on the cooking and eating quality of rice in a doubled-haploid line population. Theor. Appl. Genet. 110: 1445-1452.

Fay MP, and Proschan MA, 2010. Wilcoxon-Mann-Whitney or t-test? On assumptions for hypothesis tests and multiple interpretations of decision rules. Statistics Survey. 4: 1–39

He P, Li SG, Qian Q, Ma YQ, Li JZ, Wang WL, Chen Y and Zhu LH, 1999. Genetic analysis of rice grain quality. Theor. Appl. Genet. 98: 502-508.

Juliano BO, 1971. A simplified assay for milled- rice amylose. Cereal Sci. Today. 16 (4): 334-338.

Juliano BO, 1985. Criteria and test for rice grain quality. Rice chemistry and technology. American Association of Cereal Chemists, 2nd edition. Incorporated Saint Paul, Minnesota, USA. American Association of Cereal Chemists, 443-513.

Kongseree N. and Juliano BO, 1972. Physiochemical Properties of Rice Grain and Starch from Lines Differing in Amylose content and Gelatinization Temperature. J. Sci. Food Agric. 20 (3): 714-718.

Lang NT and Buu BC, 2004. Quantitative analysis of Amylose content by DNA Markers through backcross population of Rice (*Oryza sativa* L.). Omonrice. 12: 13-18.

Little RR, Hilder GB and Dawson EH, 1958. Differential effect of Dilute alkali on 25 varieties of milled white rice. Cereal Chem. 35: 11-126.

Sano Y, 1984. Differential regulation of waxy gene expression in rice endosperm. Theor. Appl. Genet. 68: 467-473.

Septiningsih EM, Trijatmiko KR, Moeljopawiro S, McCouch SR, 2003. Identification of quantitative trait loci for grain quality in an advanced backcross population derived from the *Oryza sativa* variety IR64 and the wild relative *O. rufipogon*. Theor. Appl. Genet. 107: 1441-1433

Shu XL, Shen SQ, Bao JS, Wu DX, Nakamura Y, Shu QY, 2006. Molecular and biochemical analysis of the gelatinization temperature characteristics of rice (*Oryza sativa* L.) starch granules. J. Cereal Sci. 44: 40-48.

Tabkhkar N, Rabiei B and Sabouri A, 2012. Genetic diversity of rice cultivars by microsatellite markers tightly linked to cooking and eating quality. Australian J. Crop Sci. 6 (6): 980-985.

Tan YF, Li JX, Yu SB, Xing YZ, Xu CG and Zhang Q, 1999. The three important traits for cooking and eating quality of rice grains are controlled by a single locus in an elite rice hybrid, Shanyou 63. Theor. Appl. Genet. 99: 642-648.

Virmani SS, 1994. Heterosis and hybrid rice breeding. In; Monographs on Theoretical and Applied Genetics 22, Eds: Frankel R, Grossman M and Maliga P, Springer-Verlag Berlin Heidelberg. Germany. p. 129

Wang ZY, Zheng FQ, Shen GZ, Gao JP, Snustad DP, Li MG, Zhang JL and Hong MM, 1995. The amylose content in rice endosperm is related to the post transcriptional regulation of the *waxy* gene. The Plant J. **7**: 613-622.