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An Assessment of Wx Microsatellite Allele, Alkali Degradation and Differentiation of Chloroplast DNA in Traditional Black Rice (*Oryza sativa* L.) From Thailand and Lao PDR

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Abstract: Thailand and Lao PDR are the country's rich rice diversity. To contribute a significant knowledge for development new rice varieties, a collection of 142 black rice (*Oryza sativa*) accessions were determined for variation of physico-chemical properties, *Wx* microsatellite allele, *Wx* allele and chloroplast DNA type. The results showed that amylose content of black rice accessions were ranged from 1.9 to 6.8%. All of the alkali disintegration types (high, intermediate and low) was observed in these rice with average of 1.75 on the 1-3 digestibility scale. The unique *Wx* microsatellite allele $(CT)_{17}$ was found in these samples and all black rice strains carried Wx^b allele. In addition, all black rice accessions were found the duplication of the 23 bp sequence motif in the exon 2 of the *wx* gene. This evidence is a common phenomenon in glutinous rice. Based on two growing condition for black rice, rainfed lowland and rainfed upland, chloroplast DNA type was distinct from each other. All rice strains from rainfed lowland was deletion plastotype, but all other rainfed upland strains were non-deletion types.

Key words: Rice, microsatellite, amylose, Waxy gene

INTRODUCTION

Colored rice, most of which is black or red, is not the most commonly consumed rice product; that distinction belongs to white rice. Nonetheless, black and red rice are widely planted throughout South Asia, Italy, Greece and the United States. Black rice is a very popular food with Europeans, who consume more of it than South Asians (Simmons and Williams, 1997). In China colored rice has long been considered a health food. The potential health benefits to be gained from the consumption of black or red rice have been proven by Ling *et al.* (2001) in his work on atherosclerotic plaque formation and antioxidant status in rabbits.

Historically, black rice has been reserved for use in festival foods and desserts in East Asia. Typically, black rice grains are aromatic and, because grain fragrance is an important feature of premium-value rice, it commands higher prices in domestic and international markets. In Southeast Asia, especially in Lao PDR and the north and northeastern regions of Thailand, black rice serves as the staple food. *Khao Kam* is the traditional name of black rice in these regions. In Vietnam the local inhabitants probably planted black rice (*nêp câm*) as long as 4,000 year ago in the time of the Hung dynasty (Nguyen, 2001).

Black rice varieties are represented in two categories: grain with purple pigmentation on glumes and various color shades on the pericarp and grain with yellow glumes and purple pericarp. In most cases the purple pericarp color is associated with purple glumes. The rice cultivars show great diversity in genetic background, composition, granule size and thermal and rheological properties of starches (Sodhi and Singh, 2003). The starch separated from the rice varies significantly in its composition during rice grain development (Asaoka *et al.*, 1985). For example, composition and cooking quality can vary significantly between rice planted in different areas of India (Sodhi and Singh, 2003). Generally, starch is composed of two types of glucan polymer, amylose and amylopectin (Smith *et al.*, 1997). It has been established that the amylose in the endosperm is the most important characteristic for predicting the cooking quality of rice. The *waxy* gene encoding granule-bound starch synthase (GBSS) is responsible for the synthesis of amylose in developing grain. Predicated on the amylose content in the endosperm, rice is commonly categorized into several amylose classes: waxy (0-2%), very low amylose (3-9%), low amylose (10-19%), intermediate amylose (20-24%), or high amylose (>24%) (Juliano, 1971).

Due to the complexity of black rice morphological characteristics resulting from the influence of

environmental conditions and since information about the genetics of black rice germplasm is very limited, especially the diversity of the *waxy* gene of black rice varieties, genetic evidence from DNA analysis data was a good candidate to reflect the diversity of rice strains. Therefore, for the purpose of conservation and utilization, black rice samples were collected from Lao PDR and the north and northeastern regions of Thailand. The objectives of this study were to conduct an extensive survey of domesticated black rice germplasm for assessment of the diversity of the *waxy* gene; to examine chloroplast DNA variation; and to examine disintegration of starch granules in alkali solution.

MATERIALS AND METHODS

Materials: Seed of black rice accessions were collected from farmers' fields in all regions of Thailand and the country's Sakon Nakhon Rice Research Center. Twenty-three additional accessions were obtained from the Lao PDR. A total of 142 accessions were used in the investigation of the diversity of the *waxy* microsatellite allele and the variation of the chloroplast DNA using the PCR and DNA sequencing techniques. Table 1 contains a summary of black rice accessions used in this study. For DNA isolation, the rice accessions were grown under field conditions in the wet season in Thailand (May-October). The disintegration of the starch granules of the rice accession in alkali (1.7% KOH) solution was determined.

Determination of amylose content: Amylose content was determined by using the Juliano (1971) method. One hundred milligrams of rice starch was homogenized with 1 mL of 95% ethanol and 9 mL of 1N NaOH. The sample was heated for 10 minutes in a boiling water bath to gelatinize the starch. Then it was cooled and transferred into a 100 mL volumetric flask and filled to full volume with water. Two milliliters of iodine solution (0.2% I_2, 2% KI) was added to 5 mL of the starch solution. The solution was made up to 100 mL with water and shaken well. After standing the solution for 20 min, the solution absorbance at 620 nm was measured using a spectrophotometer. The amylose content was determined based on a standard curve.

Disintegration of starch granules in KOH solution: The degrees of disintegration of the starch granules of each accession were determined using a procedure described in a publication by Umemoto *et al.* (2002). Three seeds of each accession were hulled and halved vertically with a razor. The disintegration of starch granules was detected by standing three-halved seeds of each accession in 1 mL of 1.7 % (w/v) KOH solution for 23 h at 30°C. The degree of disintegration was determined by comparing with Japonica rice cv. Todorokiwase, which has a high level of disintegration and indica rice cv. KDML 105, which shows a low level, as positive and negative standards, respectively. The alkali digestibility in each accession was quantified with alkali spreading a score of 1, 2 and 3 for low, intermediate and high digestibility. (Prathepha *et al.*, 2005).

DNA isolation: Fresh leaves of a 4-week-old plant were harvested for isolation of genomic DNA using a CTAB extraction procedure. (Roger and Bendich, 1994).

***Wx* microsatellite assay:** To amplify the beginning of exon 1 and the beginning of the first intron the following primers were used: Waxy-F: 5′-ACCATTCCTTCAGTTCTTTGTCT-3′(Waxy-F, forward primer complementary to pos. 1-23 in exon 1); Waxy-R: 5′-TAGCATGTATGA GACTACTTGTAA-3′ (Waxy-R, reverse primer complementary to pos. 80-103 in intron 1). The PCR was carried out using 0.5 units of *Taq* DNA polymerase, 0.5 μg total DNA, 10 pmol of each primer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs in a final volume of 40 μL. The amplification conditions were 94°C for 1 min and 60°C for 1 min followed by an extension step of 1.5 min at 72°C for 35 cycles. PCR products were separated on 2% agarose gel electrophoresis in a 0.5 × TBE buffer, stained with ethidium bromide.

DNA sequence analysis of wx microsatellite: PCR products were purified from agarose gels. The fragments were cloned into pGEM-T vector and sequenced using the M13 forward primer and cycle sequencing on an ABI 377 automated sequencer, according to the manufacturer's instructions (Applied Biosystems, Richmond, CA).

Determination of the molecular marker for glutinous rice: The 23 bp insertion in the exon 2 was uniquely present among all glutinous rice varieties (Wanchana *et al.*, 2003). In this study a pair of primers (Glu-23F: 5′-TGCAGAGATCTTCCACAGCA-3′ and Glu-23R: 5′-GCTGGTCGTCA CGCTGAG-3′) flanking the duplication was used to generate amplicons.The polymerase chain reaction (PCR) analysis was done as described by Wanchana *et al.*, 2003. DNA sequence of

the amplicons was determined according to the manufacturer's instructions (ABI Co.) in an automated fluorescent sequencer (model ABI 373).

Chloroplast -DNA type analysis: According to Kanno *et al.* (1993), the ORF100 region of chloroplast DNA was amplified by PCR using primer ORF100-F, 5′-CCTGCAGTAAGTGGACCTGACTCC-3′;ORF100-R,5′-GCGGATCCGAGGTCGTGGTAAATCC-3′. The PCR reaction mixture was done under the same condition as described by Prathepha and Baimai (2004). The PCR reactions were performed using the following profile: 94°C, 1 min; 50°C, 1 min; and 72°C, 1.5 min for 35 cycles and a final extension of 5 min at 72°C. After PCR, the amplified products were electrophoresed for 45 min at 75 V. Bands were detected by ethidium bromide staining to determine which accessions were *Indica* or *Japonica* when compared to positive control using. cv. KDML 105 and Jaw Haw served respectively as the reference of Indica and Japonica for chloroplast DNA.

RESULTS

Amylose content and physico-chemical properties: The amylose content of starches separated from the 142 black rice accessions ranged from 1.9 to 6.8% (Table 1). All of the alkali disintegration types, i.e., high, intermediate and low, were observed in the black rice accessions tested (Fig. 1). Based on the results, black rice strains showed intermediate degrees of disintegration of starch granules in alkali solution with the average value of 1.75 on the 1-3 digestibility scale. Black rice strains were grown under two growing conditions, rainfed lowland and rainfed upland. The strains showed a variation in alkali disintegration of the starch granules. Some accessions of the rainfed upland rice showed a low level of disintegration, whereas starch granules of the rainfed upland rice showed high degrees of disintegration. Figure 2 shows the grain of black rice strains collected from Luang Prabang in Lao PDR that were completely degraded in alkali solution.

***Wx* microsatellite allele in black rice:** Black rice strains used in this study showed monomorphism in *Wx* microsatellite allele, revealed by the length of a repeated sequence, i.e., CT microsatellite in the truncated exon 1 of rice *waxy* gene. The *wx* microsatellite allele $(CT)_{17}$ was found in these samples and all black rice strains carried Wx^b allele. The first intron 5′ splice acceptor site AGTTATA, which exists downstream from the $(CT)_{17}$ repeats, was found in all black rice strains. The G-to-T base substitution is one of the characteristics of the Wx^b allele commonly found among glutinous and non-glutinous rice srains with low amylose content.

Duplication of the 23 bp sequence in a coding region of *wx* gene of black rice: Since duplication of the 23 bp insertion in the exon 2 uniquely exists in all glutinous rice strains (Wanchana *et al*., 2003), as expected, all black rice strains used in this study were found to have duplicates of the 23 bp sequence motif, ACGGGTTCCAGGGCCTCAAGCCC, in the exon 2 of the *wx* gene. Therefore, the duplication sequence in glutinous groups was also a common phenomenon in black rice. Thus, the duplication of the 23 bp sequence in exon 2 of the rice *waxy* gene is a characteristic of glutinous rice strains.

Chloroplast DNA type of black rice: It has been reported that indica rice has chloroplast DNAs with the deletion at the *Pst* I-12 fragment, while japonica rice has cpDNAs without the deletion (Kanno *et al*., 1993). Results from this study showed that upland rice strains of black rice from northern Thailand and Lao PDR were classified as japonica. While, all lowland rice strains of black rice from other regions of Thailand and Lao PDR were indica type (Fig. 3).

Table 1: Summary of characteristics of the 142 black rice accessions used in this study

Code	Province/Country	Cultural type	Amylose content (%)	*Wx* microsatellite allele	*Wx* allele	Chloroplast DNA type
K01-K42	Mahasarakham, Thailand	Rainfed lowland	4.0-6.8	CT = 17	Wx^b	Indica
A01-A20	Vientiane,Champassak, Lao PDR	Rainfed lowland	1.9-5.4	CT = 17	Wx^b	Indica
LBP1-LBP3	Luang Prabang, Lao PDR	Rainfed upland	2.4-5.2	CT = 17	Wx^b	Japonica
KN1-KN13	Chiangrai, Thailand	Rainfed upland	3.5-5.4	CT = 17	Wx^b	Japonica
K-VCR-1-KVCR-4	Chiangrai, Thailand	Rainfed lowland	3.8-5.6	CT = 17	Wx^b	Indica
SKN1-SKN53	Sakon Nakhon Rice Research Center Thailand	Rainfed lowland	5.4-6.2	CT = 17	Wx^b	Indica
TR1-TR2	Trat, Thailand	Rainfed lowland	4.5-6.5	CT = 17	Wx^b	Indica
MDH1-MDH5	Mukdahan, Thailand	Rainfed lowland	6.2-6.5	CT = 17	Wx^b	Indica

Fig. 1: Degrees of alkali disintegration of starch granules in black rice. Left to right; low, intermediate and high, respectively

Fig. 2: Kernel of three strains of upland black rice from Luang Prabang, Lao PDR

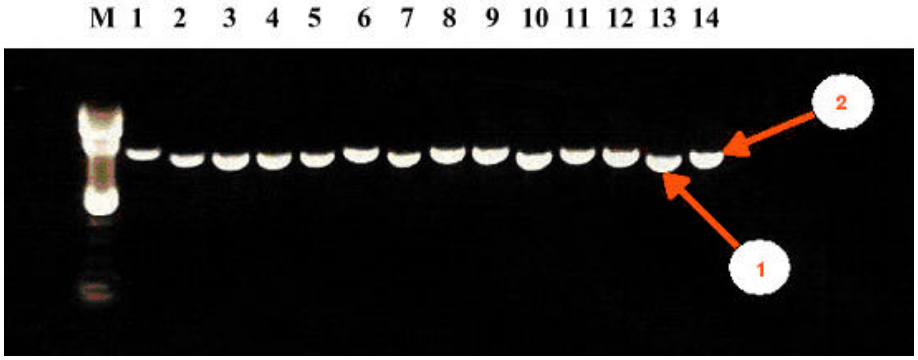

Fig. 3: Chloroplast DNA type of black rice accessions analyzed by PCR. 1= indica type, 2 = japonica type

DISCUSSION

Microsatellite *Wx* allele and amylose content of black rice strains: Amylose content of black rice strains obtained in this study ranged from 1.9 to 6.8 %. Variation on amylose content among them may be caused by genotypic differences. Amylose content was affected by heredity as well as the environment. In general, a kernel of glutinous rice is round in shape and opaque white in color. For example, as shown in Figure 2, a kernel of black rice showed opaque white in color. The glutinous phenotype of black rice strains corresponded to their characteristics obtained from this study, i.e., the duplication of the 23 bp sequence in exon 2 of the rice *waxy* gene, amylose content and they carried Wx^b allele (AAGTTATA) which found in glutinous and non-glutinous rice with low amylose content (<19%). The molecular evidences indicated that the glutinous phenotype arose through the disrupted expression of the *waxy* gene which encoded a key enzyme, granule-bound starch synthase (GBSS) that controls amylose biosynthesis in rice endosperms. Glutinous phenotype contained a G to T mutation at the 5′ splice site of wx intron 1 that leads to incomplete post-transcriptional processing of *Wx* pre-mRNA (Wang *et al*., 1995; Bligh *et al*., 1998; Cai *et al*., 1998; Hirano *et al*., 1998; Isshiki *et al*., 1998). In addition, Isshiki *et al*.(2001) reported that a point mutation created internal stop codon within the coding sequences and could reduce the abundance of the GBSS mRNA transcribed from the gene in a process termed nonsense-mediated mRNA decay (NMD). One possible explanation for the glutinous

phenotype is the presence of the 23 bp duplicated sequence in the exon 2 resulting in a chain termination of waxy protein (or GBSS) synthesis (Isshiki *et al.* , 2001; Wanchana *et al.*, 2003)

Further, results from this study showed that unique microsatellite alleles were detected among these black rice strains. They carried the $(CT)_{17}$ allele, which is the highest frequency allele found in rice cultivars from Thailand (Prathepha and Baimai, 2004). Thus, this marker would not be suitable for a marker assisted breeding program. However, research on the chemical constituents of black rice and their grain quality need to be evaluated. These black glutinous rice strains are crucial not only for food and nutrition security, but also offer opportunities for export.

The wide distribution of black glutinous-rice eaters and their large number ensures a lot of differences in ethnic, religious and cultural, conditions. For example, Thais and Laotians have two ways of preparing cooked rice for eating based on this rice. The two main ways of preparation are *Khao lam* (cook in a bamboo tube) and stream using a rice streamer on a fire. *Khao lam* and cooked black rice were found everyday at the morning markets in Lao PDR and in the northern and northeastern regions of Thailand.

Rainfed lowland and rainfed upland black rice showed variation of cpDNAs and variation in degrees of alkali digestion: A deletion or non-deletion of nucleotides (69 bp) in the chloroplast of rice was detected at the ORF 100 region within a *Pst* I-12 fragment (Kanno *et al*., 1993). In this study, lowland and upland glutinous black rice have different chloroplast DNA type. Upland black rice had chloroplast DNAs without a deletion at the *Pst*I-12 fragment (japonica type), while lowland black rice had chloroplast DNAs with the deletion (indica type). In Thailand and Lao PDR most upland black rice cultivation was done under a slash-and-burn system concentrating on slopes ranging from 300 to 600 m and only traditional varieties were grown in the uplands with mixtures of different phenotypes being grown in the same field. These mixtures are of the same height and maturity but differ for panicle and/or grain characters, such as in shape, size and pigmentation of panicle and grains. In general, most upland varieties are early-maturing (October to December).

All of the alkali digestion types, i.e. high, intermediate and low, were observed in the glutinous black rice strains used in this study. All three accessions (or strains), which are upland rice from Luang Prabang, Lao PDR showed a high degree of alkali digestibility of starch granules. While, the 16 upland black rice accessions from northern Thailand showed a low degree of disintegration. For lowland black rice, approximately 50% of the samples showed intermediate and high levels of disintegration. From previous study only intermediate and high alkali digestibility were observed in glutinous Thai rice varieties (Prathepha *et al.*, 2005), while glutinous black rice showed all of the alkali digestion. As mentioned above, the black rice samples used in this study were traditional black rice. The difference in degrees for alkali digestion found in these samples may result from varietal differences in amylopectin fine structure that were reported by Nakamura *et al.* (2002). In addition, Satoh *et al.* (2003) suggested that mutation in gene encoding of an amylopectin-synthesizing enzyme (starch-branching enzyme I) in the rice varieties is responsible for the difference in physico-chemical properties of starch granules through structural alteration of their amylopectin molecules.

This research demonstrates that black rice is classified as glutinous rice with different amylose content. This rice is grown in rainfed lowland and rainfed upland ecosystem in Thailand and Lao PDR. The chloroplast DNA type of upland rice differ from that of the lowland rice. The unique *Wx* microsatellite allele $(CT)_{17}$ and Wx^b allele were observed in black rice. In addition, variations in the alkali digestion of starch granules were also observed among them. Thailand and Lao PDR are countries rich in rice diversity. The richness of rice diversity in terms of genetic resource should be considered as part of the country's heritage developed by farmers. Through the collection, characterization and conservation of black rice resources in a genebank, significant knowledge will be contributed to the development of new black rice varieties.

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