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Characterization of *Waxy* microsatellite classes that are closely linked to the rice *Waxy* gene and amylose content in Thai rice germplasm

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Abstract

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Sixty-eight strains belonging to two species of *Oryza* were characterized by using *Wx* microsatellite, $(CT)_n$ repeats that are closely linked to the rice *Waxy* gene. These strains included high-, intermediate- and low-amylose strains as well as glutinous rice. The polymerase chain reaction (PCR) was used to amplify a DNA fragment including the beginning of exon 1 and the beginning of the intron 1 of the *Waxy* gene. The amplified DNAs were sequenced an ABI Model 377 automatic sequencer.

Ten *Wx* microsatellite classes were identified. The popular Thai jasmine rice, KDML105, contained the $(CT)_{17}$ repeats in the *wx* locus. Moreover, the $(CT)_{17}$ repeat is the predominant class of the strains tested, whereas the $(CT)_{11}$ class associated with high-amylose strains. In addition, a unique microsatellite class, $(CT)_{18}$, was detected in some traditional glutinous strains and glutinous wild rice collected from the north-eastern region.

The restriction pattern of polymerase chain reaction-amplified *Waxy* DNA digested with *AccI* was also characterized. Polymorphism was observed between rice strains with low amylose and intermediate and high amylose content.

Key words : *Oryza sativa*, *Waxy* microsatellite, *Waxy* gene, amylose

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บทคัดย่อ

ปรีชา ประเทพา

การตรวจสอบรูปแบบของไมโครแซทเทลไลท์ที่อยู่บนยีนแวคซี และความสัมพันธ์กับปริมาณอมิโลสในเชื้อพันธุ์ข้าวไทย

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สายพันธุ์ข้าวปลูก (*Oryza sativa* L.) และข้าวป่า (*Oryza nivara*) จำนวน 68 สายพันธุ์ ได้ถูกนำมาตรวจสอบรูปแบบของไมโครแซทเทลไลท์แบบ CT ที่มีตำแหน่งอยู่ในยีนแวคซีของข้าว

สายพันธุ์ข้าวเจ้าแบ่งออกเป็นสามกลุ่มตามปริมาณอมิโลสคือ กลุ่มอมิโลสต่ำ (<19%) กลุ่มอมิโลสปานกลาง (20-25%) และกลุ่มอมิโลสสูง (>25%) และสายพันธุ์ข้าวเหนียว เทคนิคที่ใช้สำหรับตรวจสอบคือเทคนิค PCR และ direct sequencing ที่วิเคราะห์ผลโดยเครื่องวิเคราะห์ลำดับนิวคลีโอไทด์แบบอัตโนมัติ (ABI Model 377 automatic sequencer) ผลจากการวิเคราะห์พบว่าข้าวสายพันธุ์เหล่านี้ประกอบด้วยไมโครแซทเทลไลท์ CT จำนวน 10 แบบ โดยใช้เกณฑ์คือจำนวนซ้ำของ CT (หรือ $(CT)_n$) โดยมีจำนวนซ้ำดังนี้ n = 4, 8, 9, 10, 11, 12, 14, 17, 18 และ 19 ในข้าวขาวดอกมะลิ 105 มีจำนวนซ้ำเท่ากับ 17 ซึ่งจำนวนซ้ำ n=17 พบมากที่สุดในข้าวที่ใช้ในการตรวจสอบครั้งนี้ ในขณะที่จำนวนซ้ำ n=11 พบเฉพาะในข้าวกลุ่มอมิโลสสูงเท่านั้น และข้อค้นพบที่น่าสนใจคือการที่พบว่า $(CT)_{18}$ ปรากฏในข้าวเหนียวพื้นบ้านและข้าวเหนียวที่เป็นข้าวป่าจากภาคอีสานเท่านั้น โดยไม่ปรากฏในข้าวเหนียวพื้นบ้านจากชนกลุ่มน้อยทางภาคเหนือและข้าวเหนียวพื้นบ้านจากสาธารณรัฐประชาธิปไตยประชาชนลาว นอกจากนี้ข้าวในกลุ่มอมิโลสปานกลางและสูง สามารถวินิจฉัยได้จากการใช้บริเวณจดจำของเอนไซม์ตัดจำเพาะชนิด *AccI* ซึ่งสามารถตัดชิ้นดีเอ็นเอที่เป็นผลลัพธ์ของปฏิกิริยาลูกโซ่ได้ ในขณะที่ชิ้นดีเอ็นเอที่เป็นผลลัพธ์ของปฏิกิริยาลูกโซ่ของข้าวเหนียวและข้าวเจ้าในกลุ่มอมิโลสต่ำเอนไซม์ไม่สามารถตัดดีเอ็นเอชิ้นนี้ได้

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The rice *waxy* locus encodes granule-bound starch synthase (GBSS), a key enzyme in amylose synthesis of plants (Nelson and Pan, 1995; Preiss, 1991). In cereals GBSS is expressed in endosperm, pollen and embryosac. Previously, the use of microsatellite DNA sequences in rice has been reported by Wu and Tanksley (1993). In rice breeding program, polymorphisms of microsatellite markers associated with genes affecting desired phenotypic traits have a practical value for rice breeders (Tanksley *et al*., 1989). Previously, a polymorphic microsatellite was identified in the rice *wx* locus (Bligh *et al*., 1995), located 55 bp upstream of the 5' splice site of the first intron. In a screen of 13 commercial rice strains, four different classes designated as CT repeats, $(CT)_n$: n=10, 13, 18 and 20 were identified. They suggested that this marker has potential for use in selective rice breeding for starch quality. Moreover, Ayres *et al*. (1997) determined the relation between the *Wx* microsatellite polymorphism and the amylose content in the 92 US rice strains. They demonstrated that the *Wx* microsatellite is polymorphic enough to distinguish most rice strains in different amylose classes, and the inheritance of the CT repeats can be traced through a cross-section of the US rice pedigree. In almost all cases, the *Wx* microsatellite show the expected simple pattern of inheritance, which can be traced back to the original introductions of foreign germplasm. Recently, Bergman *et al*. (2001) have demonstrated that the CT repeats classes with n=10 or 11, 14 or 20 and 17 or 18 were categorized as high-amylose, intermediate-amylose, and low-amylose types, respectively.

The rice *Wx* locus is characterized by two functional alleles, which are defined on the basis of the amount of *Wx* protein that accumulates in

the mature seeds (Sano, 1984). The allele Wx^a that controls the production of higher amounts of Wx protein is widely distributed in cultivated rice (*O. sativa* Indica) and their wild progenitors. The other allele, Wx^b, produces levels of Wx protein about 10-fold lower than that of Wx^a, and is mainly distributed in *O. sativa* Japonica (Sano, 1984). The evolutionary relationships of the species in the genus *Oryza* indicated that Wx^b in Japonica type has evolved from Wx^a of *O. rufipogon* (Sano, 1984; Sano *et al*., 1991). Moreover, Wx^a alleles from both wild rice and cultivated species had the sequence of AGGTATA at the 5' splice junction of the first intron. On the other hand, Wx^b alleles had the sequence AGTTATA at the 5' splice junction. This single nucleotide substitution could be assayed by *AccI* digestion of the amplified fragments (Ayres *et al.,* 1997). Interestingly, Ayres *et al*. (1997) demonstrated that the polymorphism in this sequence correlated with variation in apparent amylose content. All of the strains with 18% or less amylose had the sequence AGTTATA at the putative leader intron 5' splice site, while all strains with a higher proportion of amylose had AGGTATA.

Genetic analysis has enabled the rice *Waxy* gene in Thai rice strains to be characterized (Prathepha and Muosri, 2002). This report aimed to shed some light on gene pool of Thai rice germ plasm by investigating and accumulating data on the *Waxy* microsatellite, $(CT)_n$, located in the rice *Waxy* gene, as well as determining the relationships between this feature and amylose content in their seeds.

Materials and methods

Plant materials: Rice strains (*Oryza sativa* and *O. nivara*) used in this study are listed in Table 1. These strains represent low-amylose, intermediate-amylose, and high-amylose strains. Seed samples for each strain were cut to divide into half-seed with- or without-embryo part. The latter part of each strain was used to determine amylose content using the method of Juliano (1971). In this study, type of endosperm of each strain was examined. The waxy endosperm (glutinous rice strain) with amylose starch (1-9%) stains red or brown with iodine, whereas nonwaxy endosperm (nonglutinous) with amylose starch (>10%) stains purple-blue to blue. According to Juliano (1971), the amylose content for nonglutinous rice strains obtained from this investigation were classified as low (<19%); intermediate (20-25%); and high (>25%).

Total DNA isolation

The half-seeds with embryo were placed in petri dishes containing filter paper moistened with distilled water. For DNA isolation, rice strains were grown in a greenhouse and leaves of 4-week-old seedlings were harvested. Rice genomic DNA was extracted from fresh leaves using the CTAB method of Doyle and Doyle (1987). The synthetic primers used for both amplification and sequencing were designed from the published nucleotide sequence of the *Waxy* gene that is available from GenBank under accession number AF031162 (Figure 1). The primer pair (F-primer: 5'ACCATT

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1    ACCATTGCTTCAGTTTTTTG TCTATCTCAA GACACAAATA ACTGCAGTCT CTCTCTCTCT
61   CTCTCTCTCT CTCTCTCTCT CTCTCTCTGC TTCACTTCTC TGCTTGTGTT GTTCTGTTGT
121  TCATCAGGAA GAACATCTGC AAGGTATACA TATATGTTTA TAATTCTTTG TTTCCCCTCT
181  TATTCAGATC GATCACATGC ATCTTTCATT GCTCGTTTTT CCTTTACAAGTGAGTCTCATAG
241  ATGGTAATTT CTGTAAGGTG TTGGGCTGGA AATTAATTAA TTAATTAATT GACTTGCCAA
301  GATCCATATA TATGTCCTGA TATTAAATCT TCGTTCGTTA TGTTTGGTTA GGCTGATCAA
361  TGTTATTCTA GAGTCTAGAG AAACACACCC AGGGGTTTTC CAACTAGCTC CACAAGATGG
421  TGGGCTAGCT GACCTAGATT TGAAGTCTCA CTCCTTATAA TTATTTTATA TTAGATCATT
481  TTCTAATATT CGTGTCTTTT TTTATTCTAG AGTCTAGATC TTGTGTTCAA CTCTCGTTAA
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Figure 1. Nucleotide sequence of the rice Waxy gene (5' to 3'). It starts at base 1 and runs through base 540. The shaded sequence segments in the sequence are the position of primer pairs. The microsatellite DNA, $(CT)_n$, is underlined.

Table 1. *Waxy* microsatellite classes and apparent amylose content of 68 rice strains. Also shown is whether amylose content classes were detected by *AccI* digestion.

Variety/strain	$(CT)_n$	Amylose (%)	*AccI**	Locality
KDML105 (1) (ขาวดอกมะลิ 105)	17	13.72	-	Nakhon Phanom
KDML105 (2)	17	14.11	-	Roi Et
KDML105 (3)	17	14.11	-	Yasothon
KDML105 (4)	17	12.98	-	Maha Sarakham
RD15 (กข 15)	17	16.48	-	Pathum Thani
Chainaht 60 (ชัยนาท 60)	8	17.21	-	Pathum Thani
Mali (breeding line 10) (มะลิ)	17	15.63	-	Suphan Buri
Mali Hawm (มะลิหอม)	17	17.04	-	Suphan Buri
Mali Daeng (มะลิแดง)	17	15.35	-	Kalasin
Jow Dam1/2 (เจ้าดำ 50%)	17	16.19	-	Suphan Buri
Jow Dam1/4 (เจ้าดำ 25%)	17	14.45	-	Suphan Buri
Jaw Haw (เจ้าฮ่อ)	17	17.5	-	Chiang Rai
Khao Pong Krai (1) (ขาวโป่งไคร้)	17	14.67	-	Chiang Mai
Khao Pong Krai (2) (ขาวโป่งไคร้)	17	4.31*	-	Chiang Mai
PSL60-1 (พิษณุโลก 60-1)	17	17.8	-	Phitsanulok
PSL60-2 (พิษณุโลก 60-2)	11	30.78	+	Phitsanulok
Ang Jerng Jahn (อั้งเจิงจาน)	17	15.57	-	Phetchaburi
Khao Hawm (ขาวหอม)	17	13.38	-	Suphan Buri
Khao' Dam 1/2 (ข้าวดำ 50%)	17	14.17	-	Suphan Buri
Khao' Dam 90% (ข้าวดำ 90%)	17	17.21	-	Suphan Buri
Khao' Rai Kaset (ข้าวไร่เกษตร)	17	9.77*	-	Mukdahan
Hao' Kaen Du (ฮ้าวแก่นดู่)	17	8.65*	-	Mukdahan
U Kam (อุคำ)	18	6.51*	-	Mukdahan
Pong' Aew (ป้องแอ้ว)	18	8.7*	-	Mukdahan
RD6 (กข 6)	11	5.95*	-	Maha Sarakham
Kam Poon (กำปุน)	17	9.44*	-	Mukdahan
Kam Nai (กำใน)	17	8.94*	-	Kalasin
Kai Ngaw (ไก่งอ้อ)	17	8.67*	-	Sakon Nakhon
Khao' Kam no.1 (ข้าวกำ)	17	9.21*	-	Mukdahan
Khao' Kam no.2 (ข้าวกำ)	17	9.44*	-	Roi Et
E-Pon (อีโพน)	17	7.6*	-	Mukdahan
Ma Yom (มะยม)	18	7.8*	-	Mukdahan
Khi Tom (ขี้ตม)	18	8.48*	-	Maha Sarakham
Jow Daeng (เจ้าแดง)	11	28.19	+	Roi Et
Mali Tawng (มะลิทอง)	11	28.75	+	Pathum Thani
Bawng Kasat (บองกษัตริย์)	17	26.16	+	Surin
Niang Mow (เนียงเมา)	17	25.93	+	Surin
Khao' Nak (ข้าวหนัก)	19	22.05	+	Buri Ram
Leuang Pratew (เหลืองประทิว)	11	30.33	+	Phetchburi
Jek Chuey (เจ๊กเชย)	11	27.51	+	Phetchburi
Khaw Kaw (ขาวกอ)	19	26.22	+	Saraburi
Leuang Lao Khan (เหลืองเลาขวัญ)	19	28.36	+	Kanchanaburi
Bal Khao Seu (แบลเกาซือ)	14	13.99	-	Chiang Rai

* + : Restriction enzyne *AccI* can cleave the PCR products
* : Waxy endosperm

(to be continued)

Table 1. (continued)

Variety/strain	$(CT)_n$	Amylose (%)	*AccI**	Locality
Bal La Mi (แบลลามี้)	17	15.13	-	"Hilltribe, Chiang Rai"
Bal Cha Plau (แบลชาเปลา)	17	3.39*	-	"Hilltribe, Chiang Rai"
Biaw Ku (เบี้ยวกู่)	9	3.62*	-	"Hilltribe, Chiang Rai"
Biaw Mai Yan Rai (เบี้ยวไมยั่นไร)	17	16.51	-	"Hilltribe, Chiang Rai"
Yim (ยิม)	11	3.74*	-	"Hilltribe, Chiang Rai"
Jow Ne (เจ้าหนี้)	9	16.49	-	"Hilltribe, Chiang Rai"
Hua Nah (หัวนา)	17	24.92	+	Songkhla
Tam Me Rai (ตำเมไหร)	4	27.65	+	Songkhla
Leb Nog Pattani (เล็บนกปัตตานี)	17	23.7	+	Pattani
Chaw Lung (ช่อลุง)	12	23.76	+	Phatthalung
Dawk Kam (ดอกคำ)	12	4.42*	-	Laos
Traditional waxy rice (ข้าวเหนียวพื้นบ้านลาว)	14	4.19*	-	Laos
O. nivara no.1 (ข้าวป่า)	17	12.98	-	Nakhon Phanom
O. nivara no.2	17	13.72	-	Nakhon Phanom
O. nivara no.3	17	10.45	-	Mukdahan
O. nivara no.4	12	25.03	+	Mukdahan
O. nivara no.5	12	22.27	+	Mukdahan
O. nivara no.6	10	22.11	+	Kalasin
O. nivara no.7	10	24.84	+	Kalasin
O. nivara no.8	9	21.94	+	Sakon Nakhon
O. nivara no.9	18	3.62*	-	Maha Sarakham
O. nivara no.10	10	22.5	+	Kalasin
O. nivara no.11	9	22.45	+	Sakon Nakhon
O. nivara no.12	11	23.45	+	Mukdahan
O. nivara no.13	18	4.56*	-	Kalasin

* + : Restriction enzyne *AccI* can cleave the PCR products
* : Waxy endosperm

CCTTCAGTTCTTTGTCT 3'; R-primer: 5'TAG CATGTATGAGACTACTTGTAA 3') used for PCR flanks the beginning of exon 1 and the beginning of the intron 1.

PCR amplification

For the microsatellite assay, PCR reactions consisted of 40 µl containing 1 microgram of total rice DNA was used as a template, 10 pmol each of primer pairs, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, and 0.5 units *Taq* polymerase (Promega). Thirty-five cycles of amplification were carried out under the following conditions: denature 1 min at 94ºC, 1 min 60ºC, and 1.5 min at 72ºC; and a final extension of 5 min at 72ºC was done following the final cycle. PCR products were loaded onto 2% agarose gels using 0.5x TBE buffer and separated for 45 min at 75 volts, stained with ethidium bromide, and photographed under UV light using GDS 8000 Gel Documentation System (UVP Inc., California, USA). Molecular weights were estimated using a 100 bp ladder (FMC). This PCR reaction yielded a single fragment of about 250 bp in length for all examined strains as could be detected by ethidium bromide staining.

Sequence analysis

For sequence analysis, the PCR products were purified by gel electrophoresis (2% Seakem agarose) and purified PCR product was sequenced directly using the Taq Dye Terminator Cycle Sequencing kit (Applied Biosystem). The PCR

Figure 2. Example of agarose gel electrophoresis of amplified band by PCR using a primer pairs (F-primer: 5'ACCATTCCTTCAGTTCTTTGTCT 3'; R-primer: 5' TAGCATGTATGAGA-CTACTTGTAA 3'. M=100 bp ladder used as MW marker.

products were analyzed on an ABI 737A auto-sequencer.

To determine if intermediate- and high-amylose strains could be differentiated from low-amylose strains by AFLP analysis, PCR products were digested by *AccI* as described by Ayres *et al*. (1997).

Results and Discussion

Sixty-eight strains of rice were tested using primers flanking the first intron of the *Waxy* gene. The amplified products were approximately 250 bp in length (Figure 2). According to the Rice Research Institute, Thailand, the non-glutinous rice represents 'low'(<19%), 'intermediate' (20-25%) and 'high' (>25%) amylose strains. The strains were classified into four groups, waxy endosperm (or glutinous rice), low-, intermediate- and high-amylose strains with frequency distribution of 20, 25, 12 and 10, respectively (Table 1). Ten classes of *Wx* microsatellites, containing $(CT)_n$ repeats were detected, ranging from n=4 to 19 (Table 2). An example of the CT repeats in some strains is shown in Figure 3.

The $(CT)_{13}$ and $(CT)_{20}$ repeats were not found in the Thai rice germplasm, while these classes have been reported in US rice (Bligh *et al*., 1995; Ayres *et al*., 1997). The $(CT)_{17}$ class is prominent in the Thai gene pool, 36 of the strains tested contained this class. Also, the popular Thai jasmine rice (KDML105) contained this microsatellite class.

The $(CT)_{18}$ class was found only in traditional glutinous rice strains both cultivated rice (*O. sativa* Indica) and wild rice (*O. nivara*). In contrast, all strains of non-glutinous rice with high amylose content (>25%) contained the $(CT)_{11}$ class. Similar results have also been reported in US rice samples (Aryes *et al*., 1997).

Restriction patterns of polymerase chain reaction-amplified Waxy DNA digested with *AccI* were characterized. Polymorphism was observed between rice strains with low amylose (<19%) and intermediate and high amylose content (Figure 4). Thus, rice strains with low amylose content could be differentiated from rice strains with intermediate and high amylose content by using this rapid procedure.

Table 2. Summary of microsatellite classes of the *Wx* locus among 68 rice strains used in this study.

Microsatellite class	Number of rice strains
$(CT)_4$	1
$(CT)_8$	1
$(CT)_9$	4
$(CT)_{10}$	3
$(CT)_{11}$	8
$(CT)_{12}$	4
$(CT)_{14}$	2
$(CT)_{17}$	36
$(CT)_{18}$	6
$(CT)_{19}$	3

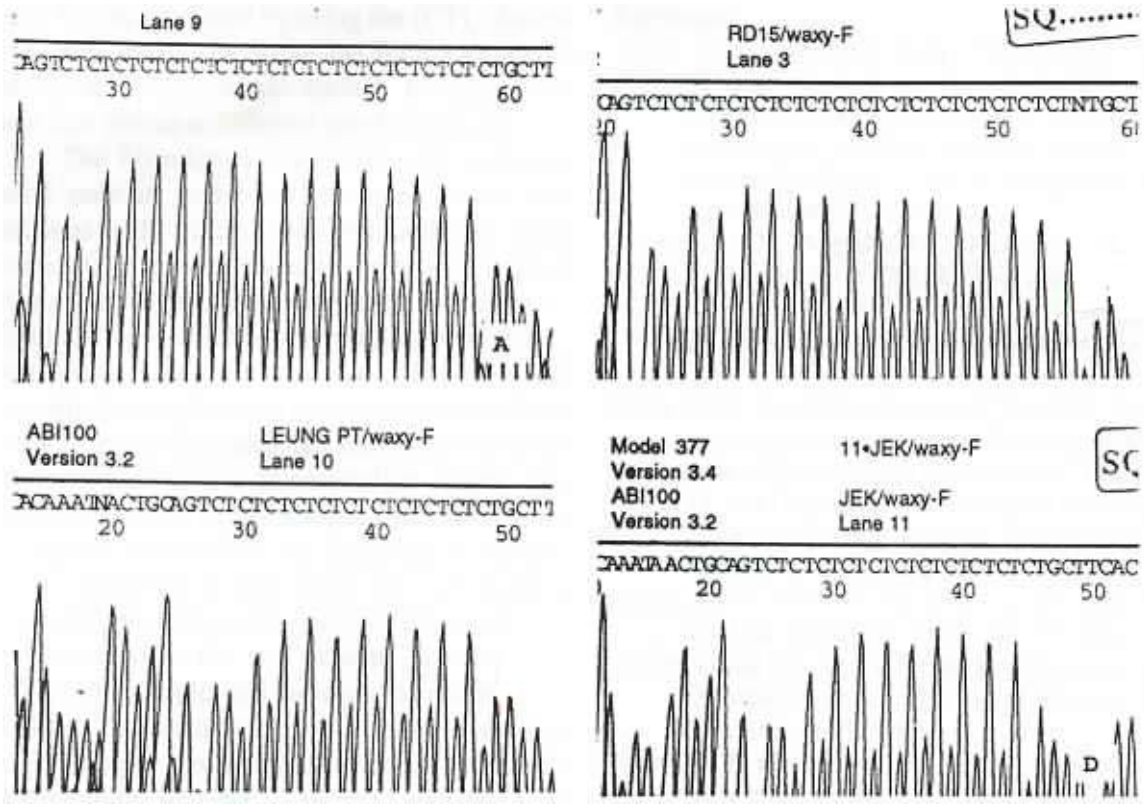

Figure 3. An example of *Waxy* microsatellite,CT repeats, in some strains obtained from ABI 373A autosequencer.
A: KDML105,$(CT)_{17}$; B: Leuang Pratew, $(CT)_{11}$; C: RD15,$(CT)_{17}$; Jek Chuey, $(CT)_{11}$.

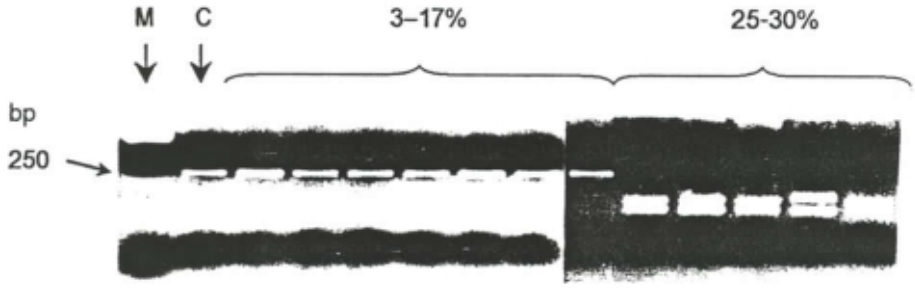

Figure 4. An example of PCR products (250 bp) digested with *AccI* with representatives of glutinous rice and low-amylose strains (lane 3-9) and Intermediate- and high-amylose strains (lane 10-14). M=DNA marker. C=negative control.

The traditional Thai rice strains Leuang Pratew (LPT), Mali Tawng and Jek Chuey have Thai genetic background and represent strains with high-amylose content, while Chainaht 60 and RD15 varieties have Thai-based genetic background with low-amylose content. These strains

could be differentiated by using the $(CT)_n$ classes. In the Thai strains, therefore, the *Wx* microsatellite classes are polymorphic enough to distinguish most rice strains in different amylose classes.

The *Wx* microsatellite class with n=18 was found only in traditional glutinous strains and glutinous wild rice (*O. nivara*) collected from northeastern region, but not in rice strains from other regions, such as some strains of traditional glutinous rice obtained from hilltribe people in mountainous regions in northern Thailand and Laos which use glutinous rice strains in their local diet. However, a possible reason for the observed differences in the *Wx* microsatellite among glutinous rice strains is that sampling may have been inadequate. In addition, the pedigrees of the rice strains analyzed in this study are not known. However, the differences may reflect a real distinction between the rice genome domesticated by hilltribe people and farmers in northeastern Thailand. The strains used in this study also showed a difference in frequency distribution of the *Wx* microsatellite between different regions. Rice domestication and evolution studies can provide valuable information about genetic diversity. The domestication pattern of rice in Thailand should be further investigated using molecular evidence. Therefore an extensive survey of domesticated rice germ plasm for the diversity of the *Wx* microsatellite in Thailand will be useful. Also, the answers may explain the history of rice domestication and the evolutionary context of rice strains in Thailand and neighboring countries in the mainland of Southeast Asia, which has been recognized as a center of rice diversity.

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