Diversity of Starch Physico-Chemical Properties and Haplotypes of Starch-Synthesizing Genes in Thai Rice

Siriphorn Jangsutthivorawat[1,2*] and Hugo Volkaert[2,3]

[1]*Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand*

[2]*Center for Agricultural Biotechnology, Kasetsart University, Kamphaengsaen, Nakhorn Pathom 73140, Thailand*

[3]*BIOTEC, Thailand Science Park, Phaholyothin Road, Khlong Luang, Pathumthani 10120, Thailand*

**Corresponding author. E-mail: s.jang@chiangmai.ac.th*

ABSTRACT

The physico-chemical properties of starch in the rice grain which include the apparent amylose content (AAC), the gelatinization temperature (GT) and the gel consistency (GC) are important characters of interest to breeders. We studied starch physico-chemical properties and the genotype of starch-synthesizing genes in Thai rice varieties. One hundred and sixty accessions of Oryza sativa from Thailand were evaluated for AAC, GT and GC, and were assayed using PCR-SSCP at seven loci coding for starch-synthesizing genes. The AAC varied from 1.11% to 30.07%. The GT alkali score varied from score 2 to score 7 and the GC varied from 1 to 15 cm The statistical analyses suggested an inverse correlation between AAC and GT and GC, and a correlation between GT and GC. The numbers of alleles at the GBSSI, SSSIIIa, SSSIIIb, SSSIVa, SSSIVb, RBE1 and RBE3 loci were 13, 14, 8, 8, 7, 5 and 5, respectively. The ANOVA analysis comparing the mean values of starch AAC and GC for each of the 7 genes showed significant differences ($P < 0.05$) at each of the loci except at the SSSIIIb and RBE1 loci for AAC, and at the SSSIIIa, SSSIIIb and RBE1 loci for GC. For the GT, significant differences were indicated only at the GBSSI locus.

Key words: Starch, Apparent amylose content, Gelatinization temperature, Gel consistency, Rice

INTRODUCTION

The quality of the rice grain is a major character that determines prices and demand in markets. However, different products require different types of rice with distinctive starch properties, e.g., high amylose starches are used in fried snack products to create crispness and are widely used as thickeners or strong gelling agents in the production of jellies. Sticky rice containing starches with

low percentage of amylose are widely used in the food industry. They improve uniformity, stability and texture, and impart better freezeñthaw stability in frozen foods (Slattery et al., 2000). Starch is composed of two different types of glucan chains, linear amylose and branched amylopectin. The relative proportions of the two components and their molecular structure (degree of branching and length of the glucan chains) specify the physical and chemical properties of the starch in the endosperm such as the apparent amylose content (AAC), the gel consistency (GC) and the gelatinization temperature (GT) (Zhou et al., 2003). The apparent amylose content indicates the proportion of amylose in the total starch.

The starch synthase isoform responsible for the amylose synthesis is the granule-bound starch synthase (GBSS) (James et al., 2003). For amylopectin synthesis, soluble starch synthases (SSSs) are involved in chain extension while starch-branching enzymes (SBEs) and starch-debranching enzymes (DBEs) determine the degree of branching of the chains (Nakamura, 2002). Multi-sequence alignment analysis of the starch synthase proteins from rice and other plant species suggested that they can be grouped into five classes: soluble starch synthase I (SSSI), SSSII, SSSIII, SSSIV and granule-bound starch synthase (GBSS). The rice genome contains 10 loci coding for starch synthases. One locus codes for SSSI, three for SSSII (SSSIIa, b and c), two for SSSIII (SSSIIIa and b), two for SSSIV (SSSIVa and b) and two for GBSS (GBSSI and II) (Hirose and Terao, 2004). Plant starch-branching enzymes (SBEs) consist of two groups, families A and B, that differ in terms of the length of chain transferred *in vitro*, with family A transferring shorter chains than family B (James et al., 2003). In rice, family A consists of two isoforms: RBE3 and RBE4. The family B consists of only a single isoform (RBE1) (Mizuno et al., 2001).

Thailand has a great diversity of rice germplasm. Rice has been cultivated for a very long time. The earliest and most convincing archeological evidence for domestication of rice in Thailand was discovered by Welhelm G. Solheim II in 1966. Pottery shreds bearing the imprints of grain and husk of *O. sativa* were discovered at Non Nok Tha in Nakhon Ratchasima Province (Khush, 1997).

In 1989, Orita et al., developed a mobility shift assay of single-stranded DNAs, using non-denaturing polyacrylamide gel electrophoresis to detect DNA polymorphism. The mobility shift is thought to be caused by nucleotide substitutions that impart a conformational change of the single-stranded DNAs. SSCPs were found to be allelic variants of true Mendelian traits, and therefore they should be useful genetic markers. Sequencing is the best method to study the diversity of alleles. Sequencing technology has developed rapidly over the past two decades. The power of the technique has ensured that DNA sequencing has become one of the most-utilized molecular approaches for inferring phylogenetic history (Hillis et al., 1996).

The objectives of this study are to compare starch physico-chemical properties with the genotypes as determined by SSCP analysis of starch-synthesizing genes in Thai rice varieties. Specifically, we aim to clarify whether SSCP haplotypes of seven loci coding for starch-synthesizing genes could differentiate the indigenous rice varieties according to starch physico-chemical quality groups.

MATERIALS AND METHODS

Plant materials

One hundred and sixty accessions of *O. sativa* were sampled from the Biotechnology Research and Development Office, Pathum Thani. These accessions were selected from all parts of Thailand, representing different cultivation methods and a wide range of percent amylose content.

Starch physico-chemical properties

Determination of amylose content

The apparent amylose content (AAC) in starch granules was determined using a near-infrared reflectance spectroscopy method, based on an iodine colorimetric assay (Juliano, 1998). Serial dilutions of purified amylose from potato were used as standards. Twenty mg of starch was gelatinized by treating with 0.5 ml of 95% ethanol and 4.5 ml of 1 M NaOH and stood for 24 h at room temperature. After addition of distilled water up to 50 ml, the solution was homogenized. An aliquot (2.5 ml) of the solution was taken and added by 35 ml of distilled water, 0.5 ml of 1 M CH_3COOH, 0.2 ml of 0.2% (w/v) I_2 and 2% (w/v) KI solution. After adjusting the volume to 50 ml with distilled water, the solution was homogenized and stood for 20 min. at room temperature. The absorbance at 680 nm (the blue value) and at the wavelength of maximal absorbance (λ-max) was measured. The apparent amylose content was calculated using a calibration line obtained from the blue value at 680 nm by serial dilutions of purified amylose from potato in the iodine solution.

Determination of gel consistency

The GC was measured in duplicates according to the method of Cagampang et al., (1973). Briefly, 100 mg of rice flour was weighed in a 13 mm x 150 mm culture tube, to which 0.2 ml of 95% ethanol containing 0.025% bromthymol blue was added to prevent clumping of the powder during gelatinization. Two ml of 0.2 N KOH was added and the solution was vortexed thoroughly. The tubes were covered with parafilm and boiled vigorously in a water bath for 8 min. After standing at room temperature for 5 min, the tubes were put on ice for 20 min, and then laid down horizontally on a table surface. The gel spreading length was measured 1 h later as the distance from the bottom of the tube to the front of the gel migration. The gel length thus obtained provided a measurement of the gel consistency: the longer the distance, the softer the gel.

Determination of gelatinization temperature

The GT was measured on the basis of individual grains expressed as the alkali spread value (ASV), using the method of Little et al., (1958) with minor modifications. Ten intact milled grains from each variety were put in a weighing boat, to which 15 ml of 1.7% KOH was added. The grains were carefully separated from each other, using a forceps and incubated at room temperature for 23 h to allow spreading of the grains. The spreading value of the grains was scored by visual assessment. Rice kernels with low GT disintegrate completely

whereas rice kernels with intermediate GT show partial disintegration and rice kernels with high GT remain nearly intact. ASVs corresponding to GT are as follows: 1-2, high (GT: 74-80°C); 3-5, intermediate (GT: 70-74°C) and 6-7 low (GT < 70°C).

PCR-SSCP

DNA extraction

Genomic DNA was extracted from 5-day-old seedlings, using a MATAB method which was similar to the CTAB method by Agrawal et al., (1992) but CTAB was substituted by MATAB in the extraction buffer. The quality of the extracted DNA was assessed by electrophoresis on 0.8% agarose gel. A set of λ DNA standards of known amount (Fermentas) was used for comparison to estimate the concentration.

PCR-SSCP

DNA fragments were PCR-amplified from genomic DNA using specific primers. Details of all primers are presented in Table 1. Each PCR amplification reaction (15 µl) contained 20 ng of template DNA, 10 pmole of each of the primers, 200 µM of each dNTP (Fermentas), 1X PCR buffer with $(NH4)_2SO_4$, 2 mM $MgCl_2$ and 0.5 unit of *Taq* DNA polymerase (Fermentas). Amplification was performed on a T1 Thermocycler (Biometra™). Cycling started with an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 50°C for 45 sec, 72°C for 1.30 min and a final extension at 72°C for 5 min. The quality and size of the amplifications were checked by agarose gel electrophoresis.

The PCR fragments were mixed with 2 volumes of loading dye (95% (v/v) formamide, 0.025% bromphenol blue, 0.025% xylene cyanol and 10 mM NaOH), then denatured at 95°C for 10 min and immediately placed on ice-cold water to stabilize single strands. The electrophoresis was performed on non-denaturing polyacrylamide gels (Sequagel MD, National Diagnostics, U.S.A.), using the Single-Strand DNA Polymorphism approach (Orita et al., 1989), 3.5 µl of the aliquots were loaded and run in 0.6X TBE buffer at constant 10 watt for 14-20 hr in a 4°C refrigerator. After the electrophoresis, silver staining was used to reveal the DNA fragments following the procedure of Bassam and Caetano-Anollés (1993).

DNA sequencing

The banding patterns that appeared in the silver-stained SSCP gels were scored. Accessions representing each of the SSCP patterns were selected for a new PCR amplification, using the same conditions except that primer and dNTP concentrations were reduced by 15% and the volume was increased to 50 µl. PCR products were sent for direct sequencing, using specific internal sequencing primers.

Table 1. Gene-specific PCR primers used for SSCP technique.

Locus	Accession (Position*)	Primers
GBSSI	AB002542 (2919-3480)	F-ACCATTCCTTCAgTTCTTTgTC R-gTTTCTCCAgTggCgAgAg
SSSIIIa	AF432915 (219-828)	F-TCCTAAAAgCTgggCCAATg R-CggTggATCggCATCTCTC
SSSIIIb	AP005441 (1900-2516)	F-AAAATAACCTACATATTTCAAAACAgC R-TAgCTTAgCTTCATCCgTCgCATC
SSSIVa	AP003292 (812-1414)	F-TTggTTgTgAAACCgTgAAAgC R-CggCCCACTCTgACTTTgg
SSSIVb	AC12135 (1371-1865)	F-TCTCAgTAgTCTgCTCCTgC R-TCACTggAAACAgATgCTTC
RBE1	D10838 (3004-3586)	F-AgTgTCAgCATAgAAATCTC R-gAAACCACgCCCAggCgAAC
RBE3	AP004879 (607-1169)	F-CCCTCCgCTCCTCCTAgCTTC R-TCgCCCTCggggATCATCAC

*numbering following the indicated GenBank accession

Statistical analysis

The results were analyzed using the SPSS 15.0 (Computer Pro System Corp, USA). Correlation analysis was conducted to characterize the relationships between AAC, GT and GC and expressed by the Pearson's correlation coefficient (r). The analysis of variance (ANOVA) of the starch physico-chemical properties and haplotypes at different gene loci was performed. Tukey's multiple range tests were conducted for comparison between means of each of the starch physico-chemical properties and each gene locus with $P < 0.05$.

RESULTS AND DISCUSSION

PCR-SSCP and allele identification

One hundred and sixty accessions of *Oryza sativa* were assayed using PCR-SSCP and alleles at seven starch-synthesizing gene loci (GBSSI, SSSIIIa, SSSIIIb, SSSIVa, SSSIVb, RBE1 and RBE3) were identified. Each of the primer sets (Table 1) successfully amplified a single fragment in all samples. The fragment sizes ranged from 420 to 620 bp. Differences among alleles were not observed on agarose gel. All amplified DNA fragments were denatured and electrophoresed on non-denaturing polyacrylamide gels. Accessions representing each of the SSCP patterns were selected for large-scale PCR amplification and sent for direct sequencing. The sequence data for all accessions were obtained by matching the observed SSCP pattern with the corresponding sequences of those samples that had an identical SSCP pattern. A complete congruence between SSCP pattern and DNA sequence haplotype was obtained. The number of haplotypes observed for the seven loci ranged from 5 (RBE1, RBE3), 7 (SSSIVb), 8 (SSSIIIb, SSSIVa) to 13 (GBSSI) and 14 (SSSIIIa). The sequences corresponding to each of the alleles were submitted to GenBank (accession numbers EF990806-EF990818 (GBSSI),

EF990822-EF990835 (SSSIIIa), EF990843-EF990850 (SSSIIIb), EF990853-EF990859 and EF990864 (SSSIVa), EF990883-EF990888 and EF990891 (SSSIVb), EF990867-EF990871 (RBE1) and EF990877-EF990881 (RBE3).

Diversity of starch properties in Thai rice

In 160 accessions of *O. sativa* from Thailand, the apparent amylose content (AAC) varied from 1.11% to 30.07%, including 59 accessions of waxy rice (36.88% of total) and 101 accessions of non-waxy rice, i.e., 31 accessions of intermediate amylose content (19.37 % of total) and 70 accessions of high amylose content (43.75 % of total). The gelatinization temperature (GT) alkali score varied from score 2 (GT: 74-80°C) to score 7 (GT < 70°C), including one accession of high GT (74-80°C, score 2), 52 accessions of intermediate GT (70-74°C, score 3-5) and 107 accessions of low GT (< 70°C, score 6-7). The gel consistency (GC) varied from 1 to 15 cm, including 33 accessions of high GC values (>6 cm), 34 accessions of medium GC values (4.1 - 6 cm) and 93 accessions of low GC values (<4 cm).

Comparison between starch physico-chemical properties and SSCP haplotypes

Statistical analyses were performed to determine whether there was a correlation between AAC, GC and GT. Plot of AAC levels against GC score are shown in a scattergram (Figure 1) in which the Pearson correlation coefficient (*r*) relating the two variables was -0.772. Likewise, a correlation between AAC and GT (Figure 2) was -0.467 and the statistical analyses suggested an inverse correlation between AAC with GC and AAC with GT. Plot of GC levels against GT score are shown in a scattergram (Figure 3) in which the *r* relating the two variables was 0.325. The statistical analyses suggested a correlation between GC against GT (Table 2).

Table 2. The correlation coefficients among AAC, GC and GT for Thai rice varieties. Data used for the calculation are from Figures 1 to 3.

		AAC	GC	GT
AAC	Pearson Correlation	1	-0.467**	-0.772**
	Sig. (2-tailed)		0.000	0.000
	N	160	160	160
GC	Pearson Correlation	-0.467**	1	0.325**
	Sig. (2-tailed)	0.000		0.000
	N	160	160	160
GT	Pearson Correlation	-0.772**	0.325**	1
	Sig. (2-tailed)	0.000	0.000	
	N	160	160	160

**The indicate correlation significance at the $P = 0.01$ of probability.

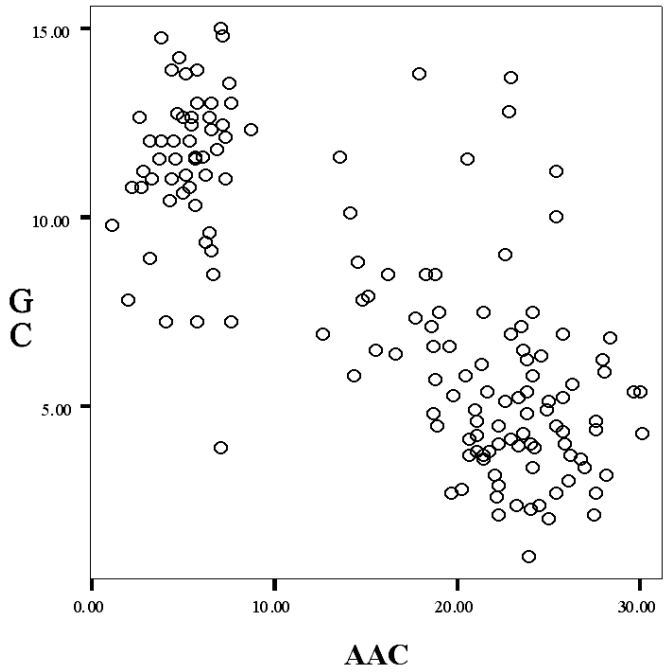

Figure 1. Scattergram showing an inverse correlation between AAC and GC.

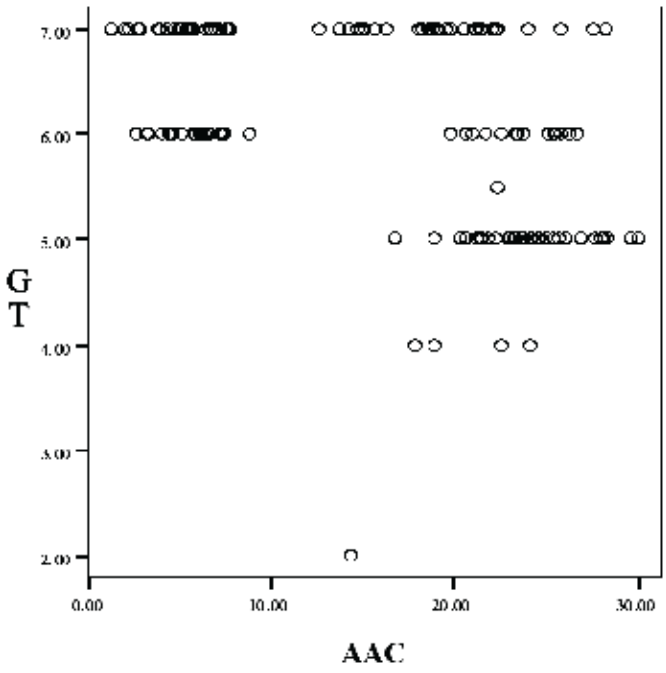

Figure 2. Scattergram showin inverse correlation between AAC and GT.

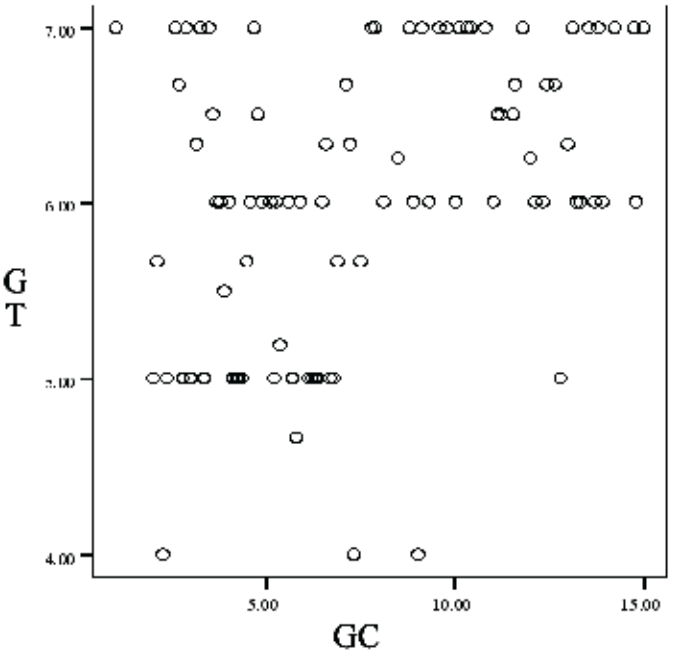

Figure 3. Scattergram showin inverse correlation between GC and GT.

The accessions were grouped according to the alleles observed at each of the loci and the values of the starch properties were compared across alleles. Some alleles were very rare, represented by only a single or couple of accessions. In such cases, those accessions were grouped with another accession that was nearly identical in DNA sequence with just one mutation different. If no nearly-identical allele could be identified, the accessions were deleted from the analysis.

The ANOVA indicated that highly significant differences ($P < 0.01$) for each of the starch properties were present for the alleles of the GBSSI locus. For the other loci, the ANOVA indicated some significant differences in AAC and GC across alleles ($0.01 < P < 0.05$). For AAC, the ANOVA results were significant ($P < 0.05$) at all genes except for SSSIIIb and RBE1, implying that there was at least one allele which showed a means different from other alleles. The ANOVA results of GT were significant ($P < 0.05$) for GBSSI only. Furthermore for GC, the ANOVA results were significant at the GBSSI, SSSIVa, SSSIVb and RBE3 loci (Table 3). The ANOVA results of AAC and GC were significant ($P < 0.05$) in many starch-synthesizing gene loci because the ranges of AAC and GC in Thai rice vary widely. Tukey's multiple range tests were conducted for comparison between means of each of the cooking quality characters and alleles at each locus. The results are shown in Table 4. Although some significant differences between haplotypes could be detected for several genes, almost all groups that showed significantly different means contained a small number of accessions.

Table 3. The ANOVA results of comparison of the mean values of starch physio-chemical properties of the haplotypes at each locus.

Genes	AAC[a]		GT[b]		GC[c]	
	***F*-statistic**	***P*[d]**	***F*-statistic**	***P*[d]**	***F*-statistic**	**P[d]**
GBSSI	8.96	0.00*	4.00	0.00*	6.76	0.00*
SSSIIIa	2.36	0.01*	1.00	0.45	1.59	0.12
SSSIIIb	2.32	0.08	1.18	0.32	2.45	0.07
SSSIVa	2.77	0.01*	1.88	0.09	2.19	0.05*
SSSIVb	2.90	0.02*	0.42	0.80	3.00	0.02*
RBE1	1.98	0.14	1.18	0.31	2.89	0.06
RBE3	4.20	0.02*	0.65	0.52	3.12	0.05*

[a]*AAC* apparent amylose content, %
[b]*GT* gelatinization temperature, Alkali score 1 to 7
[c]*GC* gel consistency, cm
[d]*P*-value of normality test
*Significant at $P < 0.05$

The $(CT)_n$ microsatellite in the upstream region of GBSSI gene has been widely studied. More alleles of the microsatellites in GBSSI gene were identified in Thai rice in the present study than in any previous studies (e.g., Ayres et al., 1997; Bao et al., 2002, 2006). The alleles $(CT)_7$ and $(CT)_9$ are reported here for the first time. The analysis of $(CT)_n$ in Chinese and US rice germplasm indicated that the alleles with fewer repeats ($n \leq 12$) had a significantly higher AAC, and those with more repeats had a significantly lower AAC (Ayres et al., 1997; Bao et al., 2006). However, in the present study we do not find a good correlation between repeat number and AAC. Thus, the length of the $(CT)_n$ repeat in the GBSSI locus is not useful to determine the amylose class in Thai rice germplasm.

SSSIIIa, SSSIIIb and RBE3 had the association of haplotypes which apparently corresponded more to the AAC than other starch physico-chemical properties whereas the means of GT and GC between haplotypes did not differ significantly. For the SSSIIIa gene, the haplotypes B, E and M presented high AAC same as the haplotype K whereas the haplotype L showed low AAC. For SSSIIIb locus, the haplotype E showed significantly low AAC. For RBE3 locus, the allele C showed a significantly lower AAC compared to accessions with other alleles. The SSSIVa, SSSIVb and RBE1 loci did not show differences between means of each of the starch physico-chemical properties.

All the genes examined in this study encoded key enzymes in the synthesis of amylose and amylopectin (Nakamura, 2002). However, other genes such as SSSIIa may play additional roles in influencing the physico-chemical behavior of rice starch. Different alleles of SSSIIa in rice contribute to different fine structures of amylopectin, leading to different gelatinization temperature of starch, as indicated by Umemoto et al., (2002). It is not well understood whether different alleles of these genes exist in natural populations and whether these

Table 4. Comparison of the mean values of starch physiochemical properties with the haplotypes at each locus.

Genes	Haplotypesa	No. of Accessions	AAC[b]		GT[c]		GC[d]	
			Mean	σ[e]	Mean	σ[e]	Mean	σ[e]
GBSSI	A $(CT)_{17}$	18	8.6099a	5.0478	6.3889b	1.1950	10.1556b	2.9404
	B $(CT)_{11}$	35	15.0575abc	8.4655	6.3429ab	0.7648	7.9571ab	4.0808
	C $(CT)_{18}$	16	18.3557abc	8.2778	5.8125ab	1.0468	7.5313ab	3.5118
	D $(CT)_{8}$	5	7.6281a	9.2345	6.2000ab	0.8367	10.0200b	1.7427
	E $(CT)_{18}$	5	18.9341abc	7.5132	6.8000b	0.4472	5.2400ab	2.3776
	F $(CT)_{9}$+I $(CT)_{7}$	22	11.2275ab	8.9630	6.3182ab	0.7162	9.6227ab	3.4112
	G $(CT)_{17}$+J $(CT)_{19}$	35	22.9791bc	6.1844	5.5714ab	0.9167	4.6771a	2.4860
	H $(CT)_{12}$	4	10.2507a	8.1117	6.7500b	0.5000	9.9250b	3.9953
	K $(CT)_{10}$+L $(CT)_{10}$	4	23.5926c	0.9432	5.0000a	0.0000	7.7750ab	3.4596
	Total	144						
SSSIIIa	A	48	15.7010ab	9.2888	6.1042	0.9048	7.1900	4.0653
	B+E+M	6	23.8014b	3.8476	5.8333	0.9832	5.5800	3.1403
	C	26	12.8286ab	8.9060	6.0385	0.9584	8.7200	3.7445
	D	15	19.9303ab	7.9292	5.8667	0.9904	6.3700	2.9531
	F	3	12.7784ab	8.2152	7.0000	0	7.7000	4.3555
	G	23	13.7196ab	9.6324	6.2609	0.7518	9.5300	3.5898
	H	7	11.9085ab	9.2720	5.7143	1.8898	9.3300	4.5305
	I+N	6	13.5622ab	6.9769	6.3333	1.2111	7.5300	2.6326
	J	4	20.0878ab	4.7349	6.5000	0.5774	7.5300	2.8906
	K	4	25.5750b	1.9186	5.2500	0.5000	6.2500	3.1300
	L	2	5.9473a	1.9551	6.0000	0	11.3000	0.3536
	Total	144						
SSSIIIb	A	81	16.9830b	8.6450	5.9506	1.0356	7.3300	3.8077
	B	54	15.8143b	9.5442	6.1852	0.8027	7.7400	3.6501
	C	7	13.3564ab	7.9924	5.8571	1.0690	9.8900	3.8247
	E	3	4.0784a	1.7709	6.6667	0.5774	12.1000	1.6258
	Total	145						
SSSIVa	A	6	20.7450	4.5323	6.5000	0.8367	5.0000	3.1887
	C	3	17.9350	9.8699	7.0000	0	4.9300	4.0550
	D	8	17.4830	7.8823	5.7500	1.1650	7.8800	3.6714
	E	7	19.8980	10.4097	5.5714	1.1339	6.4600	4.7689
	F	104	16.5410	8.8408	6.0192	0.9752	7.4600	3.5982
	G	7	19.1530	9.42934	5.7143	0.7559	8.0600	4.4211
	L	14	8.0722	6.8828	6.5000	0.5189	10.3000	3.0417
	Total	149						
SSSIVb	A	121	17.0500	8.8064	6.0413	0.9866	7.3300	3.7073
	B+I	11	14.6800	9.2416	6.0000	1.0000	8.1800	3.4388
	C+F	4	10.5100	7.0485	6.2500	0.5000	8.1300	4.5573
	D	5	5.4420	0.7489	6.4000	0.5477	12.9000	1.3008
	E	4	12.3000	10.6725	6.5000	1.0000	8.9300	2.9477
	Total	145						
RBE1	A	120	15.3800	9.1298	6.0250	0.9912	8.0400	3.8758
	B	19	17.3770	8.4622	6.3684	0.8307	6.3100	3.3454
	C+E	10	20.8970	8.6240	5.9000	0.8756	5.9500	2.5692
	Total	149						
RBE3	A+E	129	16.7502b	8.9646	6.0388	0.9715	7.3900	3.7785
	B	21	13.9409b	8.6790	6.0952	0.8891	8.7400	3.4150
	C	3	3.0919a	1.1731	6.6667	0.5774	11.8000	1.7898
	Total	153						

Means having a different letter are significantly different ($P < 0.05$).

[a] Some haplotypes that were represented by 1-3 accessions were combined with a closely related one or were excluded from the analysis if no close relative could be identified. A few accessions for which no SSCP pattern could be obtained were not included in the analysis.

[b] AAC apparent amylose content, %.

[c] GT gelatinization temperature, Alkali score 1 to 7.

[d] GC gel consistency, cm.

[e] standard deviation

alleles are correlated with starch physico-chemical properties (Nakamura et al., 2005). Therefore, further studies are needed to investigate the roles these genes play in relation to naturally-occurring variation in starch properties.

CONCLUSION

This is the first report of comparison between starch physico-chemical properties and haplotypes of starch-synthesizing genes in Thai rice. The sensitivity of the SSCP method to detect single-nucleotide substitution mutations or insertion/deletion mutations is demonstrated here by the large number of alleles that could be detected at each locus. We found that the ANOVA results were significant ($P < 0.05$) at all loci for at least one of the physico-chemical properties, implying that there was at least one haplotype which showed a means different from other haplotypes. The present information can be used to develop molecular marker-assisted selection in breeding program.

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