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Genetic Diversity of Japonica Rice (Oryza sativa L.) Based on Markers Corresponding to Starch Synthesizing Genes

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Cover Page Footnote

We would like to express our thanks to Hee-Jong Koh, Seoul National University (SNU), Korea for facilitating this work. We are also grateful for the friendly cooperation of the members of Lab. Crops Molecular Breeding, SNU, Korea.

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Genetic Diversity of *Japonica* Rice (*Oryza sativa* L.) Based on Markers Corresponding to Starch Synthesizing Genes

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Abstract

Genes related to starch synthesis and the metabolism contribute to a variety of physicochemical properties that determine the eating/cooking qualities of rice. Our previous study suggested that a set of molecular markers was able to estimate the eating quality of *japonica* rice. The present study reports the genetic diversity of 22 *japonica* rice varieties based on markers corresponding to starch synthesizing genes. The mean of the polymorphic information content (PIC: 0.135) value and the diversity index (0.171) indicated a low genetic diversity in these varieties. The phylogenetic tree clearly demonstrated three main clusters: 1) cluster I contained seven varieties with similar physicochemical properties; 2) cluster II only showed a Japanese variety, Koshihikari, and 3) cluster III included the most Korean varieties (14 varieties). This phylogenetic analysis did not completely represent the physicochemical properties differentiation of the *japonica* varieties, although it did reveal an initial clue to the close relationship between Korean rice and the Japanese and Chinese varieties. Notably, these markers were also able to identify a premium *japonica* rice. The molecular markers and information concerning the genetic relationship would be useful in improving the *japonica* rice along with its starch quality of in breeding program.

Abstrak

Keragaman Genetik Padi Japonica berdasarkan Marka terkait Gen Sintesis Pati. Gen terkait sintesis dan metabolism pati berkontribusi pada berbagai sifat fisiko-kimia yang menentukan mutu rasa dan hasil masak (*cooking*) beras. Penelitian sebelumnya menunjukkan bahwa satu set marka molekuler mampu memprediksi mutu rasa beras japonica. Pada studi ini dilaporka keragaman genetik 22 varietas padi japonica berdasarkan marka untuk gen-gen pensintesis pati. Rata-rata nilai *polymorphic information content* (PIC:0,135) dan indeks keragaman (0,171) menunjukkan keragaman genetik yang rendah dalam varietas padi ini. Pohon filogenetik menunjukkan tiga kelompok utama yang dibentuk: 1) klaster I terdiri dari tujuh varietas dengan sifat fisikokimia yang mirip; 2) klaster II hanya terdiri dari verietas premium Jepang, Koshihikari, dan 3) klaster III mengelompokkan sebagian besar varietas Korea (14 varietas). Analisis filogenetik ini belum sepenuhnya menggambarkan diferensiasi varietas japonica berdasarkan sifat fisiko-kimia, namun hasil ini mengungkapkan petunjuk awal korelasi yang erat antara padi Korea dengan varietas Jepang dan Cina. Marka-marka tersebut juga mampu mengidentifikasi beras premium japonica. Marka molekuler dan informasi kekerabatan genetik ini akan berguna dalam membantu mengembangkan padi japonica terkait dengan mutu pati dalam program pemuliaan

Keywords: genetic diversity, japonica rice, molecular markers, starch-synthesizing gene

Introduction

Rice (*Oryza sativa* L.) is a major food crop consumed daily by most people in temperate Asian countries, including Japan and South Korea. The climate in these countries allows for only one crop season due to changes in the temperature and length of day in summer. *Japonica* rice, which is characterized as sticky, moist, and soft when cooked, is preferred in South Korea; therefore, the price of the rice depends on the eating quality. In the past, rice breeders generally focused on improving the rice yield and the resistance to biotic and abiotic stresses. However, at present, one of the major goals of rice breeding programs in the northeastern countries is to improve the eating and cooking qualities by altering the physicochemical properties of rice to meet consumers' demands [1,2].

The physicochemical properties of rice starch, which accounts for about 90% of milled rice, significantly affect its eating and cooking quality. Starch comprises linier amylose and branching amylopectin, which affect rice's grain texture and quality. The amylose content (AC) and the protein content (PC) are considered to be important components that determine the quality of rice products. Other determinants such as pasting viscosities can explain the different qualities among rice varieties with similar AC [3]. Thus, the AC, PC, and pasting properties are key determinants that need to be investigated in rice germplasm in order to differentiate their genetics with respect to eating quality [4,5].

Many genes are known to be involved in starch synthesis and metabolism. The waxy gene (*Wx*) encoding granule-bound starch synthase contributes to amylase synthesis. Starch branching enzymes, starch debranching enzymes, and starch synthases play major roles in amylopectin synthesis [6,7]. The *Wx* locus harbors two alleles, namely *Wx*a and *Wx*b, in non-waxy rice cultivars. The *Wx*a allele prefers to contribute to greater waxy protein than the *Wx*b allele, resulting in a high amylose content in rice grain. The *Wx*a allele predominantly exists in *indica* rice, while the *Wx*b allele preferentially dominates in the *japonica* subspecies [8-10]. Isoamylase and pullulanase, which are two types of starch debranching enzyme, are both involved in amylopectin biosynthesis in rice endosperm [11]. These starch-synthesizing genes may contribute to the variation in starch physicochemical properties because they affect the amount and structure of amylose and amylopectin in rice grain.

Molecular marker technology has been progressively developed, including markers related to starch synthesizing genes in rice. Several types of pronounced markers have been established, including simple sequence repeat (SSR), sequence tagged site (STS), single nucleotide polymorphism (SNP) for starch synthase, starch debranching enzyme, starch branching enzyme, isoamylase, and pullulanase. The benefits of these markers have been reported for genetic studies and marker-assisted selection in breeding programs aiming at improved eating and cooking qualities [6,7,12-16]. To assist in the evaluation of *japonica* rice germplasm for improving rice grain quality in the future, this study was conducted to investigate the genetic diversity of *japonica* varieties mostly bred in Korea using markers associated with starch synthesizing genes.

Materials and Methods

Rice materials and DNA isolation. A total of 22 *japonica* rice varieties mostly bred in Korea were used in this study. For the physicochemical properties analysis, the rice varieties were grown until harvest in an experimental field following the standard recommendations for cultivation [14]. For the DNA extraction, all of the rice materials were grown in a greenhouse until the tillering stage. Young and healthy leaf tissues were collected and then stored at -80 ^{o}C until used for DNA extraction. The leaf tissues were ground into a fine powder in liquid nitrogen using a pestle and mortar, and the DNA was extracted from the leaf powder using the CTAB method [17].

Primers and PCR amplification. A total of nine primers related to starch synthesizing genes that had been previously developed [6,7,12,14,16] were used in this study (Table 1). PCR amplification of the markers

Table 1. List of Primers Corresponding to Starch Synthesizing Genes and their Sequences used in this Study

Primer name	Chromosome	Type	Description	Sequences (5'---3')	Sources
S3cI	7	Indel	Sucrose synthase 3	F: CCACTCTCATGTCCTTGAAC R: GCCATGACATTTGGACAT	[14]
S3cII	7	dCAPS/TaqI	Sucrose synthase 3	F: TTCCATGATGTGCCACTCTC R: GGACAAATGTTTTCAGTGAATAAAT	[14]
GBSS1	6	SSR	Granule-bound starch synthase1	F: CAAATAGCCACCCACACCAC R: CTTGCAGATGTTCTTCCTGATG	[14]
BE2	2	CAPS/SpeI	1.4 alpha-glucan branching enzyme IIB	F: GCCCCGAACATGATTCTA R: GGCTTTACCGACCTTACTGT	[16]
SSIIa	6	SNP	Soluble starch synthase 2-3	F7:CTGGATCACTTCAAGCTGTACGAC R1:GCCGGCCGTGCAGATCTTAAC F22:CAAGGAGAGCTGGAGGGGGC R21:ACATGCCGCGCACCTGGAAA	[6,7]
SSI	6	SSR	Starch synthase 1	F: GATCCGTTTTTGCTGTGCCC R: CCTCCTCTCCGCCGATCCTG	[6,7]
SBE2	2	dCAPS/SpeI	1,4-alpha-glucan-branching enzyme	F: GTCTTGGACTCAGATGCTGGACTC R: ATGTATAACTGGCAGTTCGAACGG	[6,7]
Isa	8	STS	Isoamylase	F: CCTGTCTTGCACGTGCGGTA R: GCACGGTTCTGATGTACGAGAG	[12]
Pul3	4	STS	Pullulanase	F: GGGTTCGCTTTCACAACACAG R: GTCACGACATAAGAGAAGCTGC	[12]

was carried out using a PTC-200 Peltier Thermal Cycler (MJ Research, Inc.) in a total volume of 20 µL with the following genotyping PCR reagents: 1 µL of DNA at 20 ng/µL, 2 µL of 10 x buffer containing 25 mM $MgCl_2$, 1 µL of 2.5 mM dNTPs, 1 unit of ExTaq Polymerase (Intron Biotechnology, Korea), and 1 µL each of forward and reverse primers (10 µM). The PCR reaction was performed under the following conditions: 5 min at 94 °C followed by 35 cycles of 45 s at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, with a final extension of 7 min at 72 °C. The PCR products were then visualized using stained ethidium bromide electrophoresis on 3% agarose gels and/or 8% polyacrylamide gels.

Data analysis. The molecular data gathered from all the primers were prepared as a binary matrix, with 1 (one) and 0 (zero) representing the reference *japonica* variety (Nipponbare) and the alternate alleles, respectively. The polymorphic information content (PIC) values were calculated for the total accessions and for the markers. The genetic diversity index and the phylogenetic tree were generated based on Nei's method, and the support for the clusters was evaluated using a bootstrap analysis of 1000 permutations conducted with PowerMarker V3.25 [18,19].

Results and Discussion

The advancing progress of genetic studies and the completion of the whole genome of *japonica* rice cultivars allows us to generate a large number of molecular markers based on sequence diversities [1]. A number of markers corresponding to starch synthesizing genes have been developed and applied with respect to rice's eating and cooking qualities [6,7,12-16]. Such markers can be used for a genetic relationship study using the reference cultivar Nipponbare for the reference alleles, as demonstrated in this study.

In this study, diverse alleles produced by a number of loci for starch synthesizing genes were identified on rice varieties through comparison with the alleles found in the reference cultivar, Nipponbare. The results revealed that a total of 19 *japonica* rice varieties from Korean, two Japanese varieties, and one China variety showed a variation of alleles on loci of SSIIa, S3cI, S3cII, GBSSI, and SSI, but not on Isa, BE2, SBE2, and Pul3. It is likely that the use of relatively few varieties in this study led to the monomorphism on loci of the starch branching enzymes and debranching enzymes, which significantly influences amylopectin synthesis [11].

Interestingly, various alleles found in the *japonica* varieties were produced from loci corresponding to starch synthase (SSIIa: soluble starch synthase 2-3, SSI: starch synthase 1), sucrose synthase 3 (S3cI, S3cII), and granule-bound starch synthase (GBSSI) encoded by Waxy gene or *Wx*, which were located in different chromosomes of the rice genome (Table 1). Considering the high contribution of this *Wx* gene to amylose content, the *Wx* allele along with the ADP-glucose pyrophosphorylase isomerase were validated for the genetic diversity analysis among Sri Lankan rice varieties [20]. In contrast, the alleles of loci corresponding to starch branching enzymes seem to have the same profile among the *japonica* varieties studied. In particular, loci of Isa and Pul3, which correspond to isoamylase and pullulanase, respectively, are kinds of starch debranching enzymes. BE2 and SBE2 denote starch branching enzymes, namely 1.4 alpha-glucan branching enzyme IIB and 1.4 alpha-glucan branching enzyme, respectively. [21,22] also indicated their more limited effect on the genetic variation of *japonica* rice. Moreover, since the *japonica* varieties used in this study showed a wide range of amylose content (AC) and pasting properties [14], allele variation was found in GBSSI, SSI, S3cI, and S3cII given their influence on starch properties. Relevant to the use of markers corresponding to starch synthesizing genes in previous genetic diversity studies and genetic maps [6,7,12], the selected polymorphic markers could, as anticipated, distinguish *japonica* rice varieties in our study. Additionally, those markers related to sucrose synthase 3 (S3cI and S3cII) worked well in *indica* rice. Sucrose synthase 3 is known to be involved in grain filling in rice, and it plays a role in the defense response. These markers could also be applied in the evaluation of rice accessions/lines with respect to tolerance to abiotic stresses, in addition to eating/cooking quality.

Alleles at a certain starch synthesis locus could be easily identified as originating from *japonica* based on marker profiling. Based on (CT)n repeat numbers, Wx (CT)n microsatellites were identified in 22 *japonica* rice varieties, while (CT)17 and (CT)18 were also detected in a previous report [6]. The (CT)18 allele was the most frequent of the (CT) microsatellites in *japonica* rice, indicating that (CT)18 was the major allele of the *Wx* gene in *japonica* varieties with a frequency of 0.955. The deletion of CTC on locus S3cI and the G allele on S3cII were found more frequently than their alternatives. The GC alleles were comparable with TT, since 12 varieties contained the GC allele (a major allele frequency of 0.545). Of all the varieties, only one variety had a 220 length amplicon of Isa, whereas the rest were 230 bp in length. The genotypic data produced by all the markers on *japonica* rice varieties are shown in Table 2. The alleles of all loci contributing to the genetic relationship of the *japonica* varieties were observed. Furthermore, these alleles are thought to be very valuable as genetic sources to support breeding programs related to rice grain quality, especially eating and cooking qualities.

Table 2. Variation of Alleles Observed on 22 *japonica* Rice Varieties based on Genotypic Evaluation using Nine Markers Corresponding to Starch Synthesizing Genes

Variety	Origin	SSIIa	S3cI[a]	S3CII	GBSSI	BE2	Isa	SSI[b]	SBE2	Pul3
Koshihikari	Japan	GC	CTC	G	(CT)17	C	230 bp	NI	G	281 bp
Gopum	Korea	GC	ND	T	(CT)18	C	230 bp	NI	G	281 bp
Samgwang	Korea	GC	CTC	G	(CT)18	C	230 bp	NI	G	281 bp
Ilpum	Korea	TT	CTC	G	(CT)18	C	230 bp	NI	G	281 bp
Chucheong	Korea	TT	ND	T	(CT)18	C	230 bp	NI	G	281 bp
Dongjin	Korea	GC	ND	T	(CT)18	C	230 bp	NI	G	281 bp
Sinkeumo	Korea	TT	CTC	G	(CT)18	C	230 bp	NI	G	281 bp
Hwaseong	Korea	GC	ND	T	(CT)18	C	220 bp	NI	G	281 bp
Hwacheong	Korea	TT	ND	T	(CT)18	C	230 bp	NI	G	281 bp
Dobong	Korea	GC	CTC	G	(CT)18	C	230 bp	NI	G	281 bp
Samnam	Korea	TT	CTC	G	(CT)18	C	230 bp	NI	G	281 bp
Palkong	Korea	GC	CTC	G	(CT)18	C	230 bp	NI	G	281 bp
Hitomebore	Japan	TT	CTC	G	(CT)18	C	230 bp	NI	G	281 bp
Baekjinju1	Korea	TT	CTC	G	(CT)18	C	230 bp	NI	G	281 bp
Seonong4	Korea	TT	CTC	G	(CT)18	C	230 bp	NI	G	281 bp
Onnuri	Korea	GC	CTC	G	(CT)18	C	230 bp	NI	G	281 bp
Manmi	Korea	TT	CTC	G	(CT)18	C	230 bp	NI	G	281 bp
Giho	Korea	TT	CTC	G	(CT)18	C	230 bp	NI	G	281 bp
Geuman	Korea	TT	CTC	G	(CT)18	C	230 bp	NI	G	281 bp
Nakdong	Korea	GC	CTC	G	(CT)18	C	230 bp	NI	G	281 bp
Hexi41	China	TT	ND	T	(CT)18	C	230 bp	NI	G	281 bp
Samdeok	Korea	GC	ND	T	(CT)18	C	230 bp	NI	G	281 bp

[a] ND: no deletion, [b] NI: no insertion

The *japonica* varieties exhibited higher polymorphism at the SSIIa (PIC: 0.373), S3cI (PIC: 0.340), and S3cII (PIC: 0.340) loci than those at other loci, which was demonstrated by the polymorphic information content of the corresponding markers. Since only five markers showed polymorphism in the observed varieties, the mean of the PIC value is somewhat low (0.135) (Table 3). The low mean of the PIC value is relevant to the low value of the genetic diversity index of these varieties, which indicates their low genetic diversity.

Phylogenetic analysis was performed using only those polymorphic markers observed among the *japonica* rice varieties. Thus, the phylogenetic tree was generated based on five markers corresponding to starch synthesizing genes on 22 rice varieties (Figure 1). Three main clusters were successfully produced and they demonstrated clear separation. Cluster I grouped the seven varieties with similar physicochemical properties, in which six were Korean rice and one was from China (Hexi41), as reported previously [14]. Hwacheong with Hwaseong, Dongjin with Gopum, and Chucheong with Samdeok had similar palatability values of around 77, 78, and 75-76, respectively. However, it was not Hexi41 that possessed the lowest value (66.7). All of the seven varieties showed PC and AC in the range of 6-7% and of 17.6-19.9%, respectively. Koshihikari, which is known as a premium Japanese variety with a good eating quality [14], preferentially stayed by itself in cluster II, and it remained a far distance from the Korean varieties. Cluster III included the most varieties (14 varieties) from Korea and one from Japan. Interestingly, Hitomebore, which is bred in Japan, was genetically closer to the Korean varieties than to Koshihikari. Based on these results, information can be gleaned relating to the distribution of rice accessions among countries in northeast Asia. Some of the *japonica* rice varieties from Korea had a close relationship with those from Japan and China, suggesting that introducing rice accessions from Japan and China contributes to the breeding and selection of germplasm for improved variety in Korea.

Although these markers were not able to clearly differentiate *japonica* varieties according to their starch physicochemical properties, some varieties with distinctive

Table 3. Statistical Summary of Alleles from Nine Markers Observed in *Japonica* Varieties in this Study

Marker	Major allele frequency	Genetic diversity index	PIC
SSIIa	0.546	0.496	0.373
S3cI	0.682	0.434	0.340
S3cII	0.682	0.434	0.340
GBSSI	0.955	0.087	0.083
BE2	1.	0	0
Isa	0.955	0.087	0.083
SSI	1	0	0
SBE2	1	0	0
Pul3	1	0	0
Mean	0.868	0.171	0.135

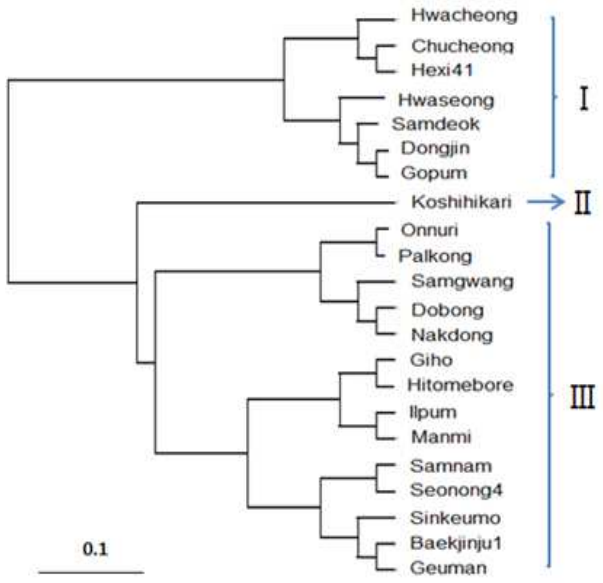

Figure 1. UPGMA Tree based on Nei's Dissimilarity Coefficients of 22 *japonica* rice Varieties using Molecular Markers Corresponding to Starch Synthesizing Genes

rice eating quality could be distinguished. These markers may be used in more diverse rice accessions, not only varieties, as well as breeding lines and local cultivar/landraces from widespread geographical regions. Notably, some of these markers were successfully included in the formulated marker set to evaluate the eating quality of *japonica* and *indica* rice [14,16]. Some markers corresponding to starch synthesizing genes have shown correlation with the eating quality of *japonica* rice [23,24]. Overall, the markers corresponding to starch synthesizing genes could prove useful in the selection of appropriate parents in breeding programs intended to improve rice eating and cooking qualities.

Conclusions

Based on these results, it can be concluded that five out of nine primers corresponding to starch synthesizing genes showed polymorphism in the *japonica* rice varieties. Some alleles commonly existed in the *japonica* varieties, especially the (CT)18 of GBSSI, fragment of 230 bp of Isa, deletion of CTC allele of S3cI, and G allele of S3cII. Three markers, namely SSIIa, S3cI, and S3cII, had greater potency in differentiating rice accessions than GBSSI and Isa, which was demonstrated by their high polymorphic information content. Five polymorphic markers were able to generate three cluster groupings of rice varieties according to their genes. Even though these markers were not able to clearly differentiate *japonica* varieties according to their starch physicochemical properties, some varieties with distinctive rice eating quality could be distinguished.

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