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

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Cover Page Footnote
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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.

**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 linear 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 Wxa and Wxb, in non-waxy rice cultivars. The Wxa allele prefers to contribute to greater waxy protein than the Wxb allele, resulting in a high amylose content in rice grain. The Wxa allele predominantly exists in indica rice, while the Wxb 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 °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

| Primer name | Chromosome | Type | Description | Sequences (5′---3′) | Sources |
|-------------|------------|------|-------------|---------------------|---------|
| S3cI        | 7          | Indel| Sucrose synthase 3 | F: CCACCTCCTGTTCTTGGAAC<br>R: GCCATGACATTTGAGCAT | [14]    |
| S3cII       | 7          | dCAPS/TaqI | Sucrose synthase 3 | F: TTGCATGATGCTTGACACTC<br>R: GGACAAATGTCTTCTATGAAAAAT | [14]    |
| GBSS1       | 6          | SSR  | Granule-bound starch synthase 1 | F: CAAATACCCACACACACAC<br>R: CGAGGAATGTCTTCTATGAAAAAT | [14]    |
| BE2         | 2          | CAPS/SpeI | 1.4 alpha-glucan branching enzyme IIB | F: GCATATATGCTGAGAGGAAGG<br>R: ATGTATAACTGGCAGTTCGAACGG | [16]    |
| SSIa        | 6          | SNP  | Soluble starch synthase 2-3 | F: F7:CCTGAGTTCCCTTACCCGCTGAC<br>R1:GCCGCCGCTGACATCTTAC<br>F2:CAAGGAGAGCTGAGAGGAAGG<br>R2:ACATCCCGGTGAGACCTGGAAA | [6,7]    |
| SSI         | 6          | SSR  | Starch synthase 1 | F: GATCCGTTCTTCTGCTTGCC<br>R: CCTCCTCCTCGCCGATCTTCTG | [6,7]    |
| SBE2        | 2          | dCAPS/SpeI | 1.4-alpha-glucan-branching enzyme | F: GTCTTGAGCTCAGATGCTTGAC<br>R: ATGTATAACTGGCAGTGGGAAGG<br>R2:ACATCCCGGTGAGACCTGGAAA | [6,7]    |
| IsA         | 8          | STS  | Isoamylase | F: CCTCGCTTGACGGAGGGCTG<br>R: GACCGGTTCCTGTAGACAGAGAG<br>R: GTACGACATACGAGAGAGGAGG | [12]    |
| Pul3        | 4          | STS  | Pullulanase | F: GGGTGTCCTTCTCCTGACAC<br>R: GTACGACATACGAGAGAGGAGG | [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 MgCl₂, 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 sucrase 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 | S3CII | GBSSI  | BE2  | Isa | SSIb | 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 |
| Manni     | 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 |

* 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 mean of the PIC value is somewhat low (0.135) (Table 3). The low mean of the PIC value is relevant to the low mean of the PIC value is somewhat low (0.135) (Table 3).

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 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|

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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