Quantitative Identification of Rice Cultivars by Real-Time PCR

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Application of a real-time PCR system for the detection of blending and the quantification of the blending ratio of rice was investigated. Quantitative measurement by real-time PCR was performed using two types of primer sets; one was an improved rice cultivar identification kit, while the other was newly developed for the determination of total amount of rice DNA. Calculation formulae for blending ratios were developed based on standard curves of both primer sets. These formulae were verified using DNA solutions extracted from the Koshihikari containing 5% or 25% of another rice sample. Calibration errors were less than 30% of the expected values.

Keywords: rice, Oryza sativa, real-time PCR, quantification of ratio, Koshihikari

Introduction

Revision of the Japanese Agricultural Standards (JAS) Act now stipulates that cultivars, location and year of production should be labeled on bags of rice as this is indispensable for identifying them objectively. High quality rice is expensive in Japan, but Japanese consumers tend to purchase high quality rice nonetheless. As specific rice cultivars, such as Koshihikari, become increasingly popular, so too does the risk of intentional mislabeling of rice to increase the price of the cultivar on display. Ohtsubo and co-workers have researched methods for denitrifying rice cultivars using DNA (Ohtsubo et al., 1997; 1999; 2001; Nakamura et al., 2004). Several rice identification kits have been developed and commercialized (Ohtsubo et al., 2002; 2003) The JAS Act also prescribes that producers indicate the ratio of the cultivars on the labels of the bags of rice when two or more kinds of rice cultivar are packaged together.

Real-time PCR is well suited to the quantification of DNA (Rho et al., 2004). In traditional PCR, detection was only possible at the end of the reaction. However, using real-time PCR it is now possible to obtain results while the reaction is in progress. The present authors attempted to apply real-time PCR to the detection of blended rice cultivars and to quantify the blending ratio.

Materials and Methods

DNA Extraction All brown rice samples were polished to 90% of yields using a friction-type rice-polishing machine followed by pulverizing with a coffee mill. From these rice powders, DNA was extracted based on methods described previously (Roger and Bendich, 1988; Ohtsubo et al., 1997). To 6 g of rice powder was added 8 ml of 2% cetyl trimethyl ammonium bromide (CTAB) solution (solid of CTAB solution; 0.1 M Tris-HCl buffer solution (pH 8.0), 2 mM EDTA, 1.4 M NaCl), before another 8 ml of 1% CTAB solution was added. The mixture was incubated at 65°C for 30 min in a water bath. To remove fat and sugar, 12 ml of chloroform/isoamylalcohol (24/1, v/v) was added and the supernatant was recovered after centrifugation (5,000 xg, 20 min). This purification step was repeated twice. RNase A (10 mg/ml, 5 μl) was added to the extracted DNA sample before being removed using phenol/chloroform/isoamylalcohol (25/24/1, v/v). To 15 ml of the DNA solution obtained, 3 M NaOAc (1.5 ml) and ethanol (30 ml) were added and the solution was centrifuged after standing for 20 min. After washing with 70% ethanol, 1 mM Tris-0.1 mM EDTA solution was added to the DNA solution.

Primers for real-time PCR For the detection of specific rice cultivars in a mixed rice sample, several primer sets were prepared: WKAq, S1q, E30q, and F6q (Table 1). Each of the forward primers employed were those for which patents were pending (Ohtsubo et al., 2003), while each reverse primer was newly designed.

Calculation of total rice DNA was determined using the waxy gene (Okagaki and Wessler, 1988) WXq (Table 1).

Real-time PCR Real-time Polymerase Chain Reaction (PCR) was carried out using LightCycler ver. 5.32 (Roche Diagnostics, Mannheim, Germany) and the software associated therewith. Reaction solutions were assembled in glass capillary tubes. Each 20 μL reaction mix contained 2 μL of sample DNA (various concentrations), 4.0 mM MgCl2 (final concentration), 0.5 mM concentrations of each primer, 2 μL of 10x FastSTART DNA SYBR Green I reagent (Roche) containing FastSTART Taq DNA polymerase, reaction buffer, and deoxynucleoside triphosphates, and PCR-grade water to achieve the final volume in each capillary. PCR was performed using a LightCycler (Roche Diagnostics) using the cycling program described in the
user manual. The PCR conditions consisted of an initial denaturation at 94°C for 10 min, followed by amplification for 45 cycles of 15 sec at 94°C, 10 sec at 68°C, and 20 sec at 72°C, with fluorescence data acquisition at the end of each PCR extension phase (72°C), except when the WKA9q primer set was used (79°C). Amplification was followed immediately using a melt program consisting of 10 sec at 73°C following moment at 95°C, and a stepwise temperature increase of 0.1°C/s until 95°C with fluorescence acquisition at each temperature transition. Melt curve analysis was carried out to check the specificity of the assay.

Results and Discussion

For quantitative identification of specific rice cultivars in mixed rice samples, two types of primer sets were designed. One is for the identification of contaminated rice cultivar, and another is for determining the total amount of rice DNA.

First, primer sets for cultivar identification were designed based on those of the commercially available identification kit. These primer sets were improved to generate amplicons that were shorter than those generated using conventional kits, to increase the quantitative detection by real-time PCR equipment. The pattern generated by amplicons did not change after the improvement of primers. For example, no amplification was achieved using the improved primer set WKA9q for Koshihikari DNA (Fig. 1a), which was the same as the result obtained using the primer set WKA9 contained in the kit (Ohtsubo et al., 2002). Conversely, when Hoshinoyume DNA was reacted with primer set WKA9q, amplification products were generated (Fig. 1a).

The PCR products were detected using SYBR Green I fluorescent reagent. Amplification per cycle was monitored with the LightCycler (Roche Diagnostics) and amplification curves were plotted. Real time PCR enables the user to analyze data during the log-linear phase rather than the end point of a reaction. A plot of the fluorescence vs. cycle number produced a sigmoid shaped curve. To analyze a real time PCR assay, the log-linear phase of the reaction is used to determine the cycle threshold for each sample. To avoid the likelihood user-

<table>
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<tr>
<th>Primer set</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Sequence</th>
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<tr>
<td>W99F</td>
<td>W99F</td>
<td>5'-CCC GCA GTT AGA TGC ACC ATT-3'</td>
<td></td>
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<tr>
<td>W99F176</td>
<td>W99F176</td>
<td>5'-TAG GTT TAT CTC TCA CTA CTA CC-3'</td>
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<tr>
<td>S13F</td>
<td>S13F</td>
<td>5'-GTC GTT CCT GTG GTT ATT ACA GGG T-3'</td>
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<tr>
<td>S13F174</td>
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<td>WX9</td>
<td>5'-CTC AAA GCT CTG TGC ATC TC-3'</td>
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<tr>
<td>WXr</td>
<td>WXr</td>
<td>5'-ACT TAA GGA GCA GGT TCT TG-3'</td>
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Table 1. Sequences of primer sets for quantitative distinction.

Fig. 1. Amplification curves for various concentrations of different DNAs with corresponding primer sets.

Note: a, Hoshinoyume and WKA9q; b, Mutsuhomare and F6q; c, Hitomebore and E30q; d, Sasanishiki and E30q; e, Kirara397 and S13q.

Amount of DNA in capillary tube: ■, 100 ng; ▲, 40 ng; ●, 16 ng; +, 6.4 ng; ×, 100 ng of Koshihikari DNA.
induced discrepancies, cycle threshold values were calculated using the second derivative maximum method with an arithmetic baseline adjustment. The highest points of the second derivatives represented the point of maximum curvature, that is, the maximum rate of change in the slope, for the growth curves.

Standard curves were generated by plotting the maximum second derivative cycle number versus the logarithm of the DNA concentration for each sample (Fig. 3a). To create a standard curve, Hoshinoyume DNA was extracted and prepared using several concentration steps. The PCR reaction for every sample was carried out in duplicate and in parallel with four different reagent conditions. A range of dilutions per PCR reaction (6.4 to 100 ng) was used to construct standard curves of the crossing point (cycle number) against log concentration of the rice DNA to quantify cultivar-specific DNA sequences. The amplification curve for Hoshinoyume DNA at various concentrations and the primer set for WKAq is shown in Fig. 1a. Standard rice DNA samples obtained from the rice strains described above were diluted to 3.2, 8, 20 and 50 ng/µl were correctly identified in the melting curve analysis. Confirmation of the specificity of the amplification reaction and the homogeneity of the products was shown by the formation of a melting curve with a single peak, indicating the formation of a single PCR product and absence of nonspecific PCR artifacts and primer-dimers (data not shown). Melting curve analysis permitted the clear identification of each of the amplified products. The Tm values for the standard DNAs were highly reproducible on every melt curve runs.

Another primer set was designed to detect the total amount of DNA using real-time PCR in the LightCycler (Roche Diagnostics). The waxy gene was chosen as the target since it is contained in all nonglutinous rice cultivars with single copy of the gene in Oryza sativa (Hirano and Sano, 1991). The consensus primer set, WXq, was designed after comparing the partial waxy gene sequences for several cultivars. DNA solutions extracted from other rice cultivars were expected to mimic same rice cultivar. Another standard curve (Fig. 2a) was also obtained when the same DNA solutions and primer set WXq reacted.

Following equation was developed from both standard curves. The calculation formula for the ratio of the specific rice that was mixed with Koshihikari was created from these two standard curves:

\[
R_{Hoshinoyume} = \frac{\text{Exp}(C_{WKAq} - 31.881)/(−1.6856))}{\text{Exp}(C_{WXq} - 35.625)/(−1.6144))} \quad \text{Eq. (1)}
\]

where \(C_{WKAq}\) and \(C_{WXq}\) represent cycle numbers of the maximum second derivative acquired when the DNA of unknown rice sample were amplified with the WKAq and WXq primer set, respectively. Koshihikari DNA and WKAq generated no amplification product, indicating that the formula derived from both standard curves corresponds with the rate of mixing between Hoshinoyume to Koshihikari.

To verify this calibration formula, DNA extracted from a rice sample of Koshihikari containing 5% or 25% Hoshinoyume was used to test the prediction. The maximum mixing ratio was expected to be 5% in cases where mixed cultivars are labeled as consisting only of one specific cultivar. This is because mixing ratios of genetically modified of up to 5%, including imported grains, are permitted. However, 25% was thus taken to be a representative value, because the maximum ratio for a mixed cultivar is 50%. When the mixing ratio exceeds 50%, it becomes the main cultivar. The quantitative identification at mixing ratios of 25% as the half-maximum, median, was therefore used. These DNA solutions of unknown samples were found to react with primer sets WKAq and WXq, individually. The number of cycles for maximum second derivatives was determined by their

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**Fig. 2.** Amplification curves of various concentration of several DNA with WXq.  
Note: a, Hoshinoyume; b, Mutsuhomare; c, Hitomebore; d, Sasanishiki; e, Kirara397.  
Amount of DNA in capillary tube: □, 100 ng; ▲, 40 ng; ○, 16 ng; +, 6.4 ng.
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Table 2. Results of prediction tests.

<table>
<thead>
<tr>
<th>Blended cultivars</th>
<th>Hoshinoyume</th>
<th>Mutsuhomare</th>
<th>Hitomebore</th>
<th>Sasanihiki</th>
<th>Kirara397</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio of blending</td>
<td>5%</td>
<td>25%</td>
<td>5%</td>
<td>25%</td>
<td>5%</td>
</tr>
<tr>
<td>Calculated mixed ratio (%)</td>
<td>4.4</td>
<td>24.1</td>
<td>3.8</td>
<td>29.4</td>
<td>3.6</td>
</tr>
<tr>
<td>Average (S.D.)</td>
<td>5.1</td>
<td>25.4</td>
<td>3.8</td>
<td>30.3</td>
<td>5.1</td>
</tr>
</tbody>
</table>

Fig. 3. Standard curves for various DNAs with primer sets for identification and WXq.

Note: a, Hoshinoyume and WKA9q; b, Mutsuhomare and F6q; c, Hitomebore and E30q; d, Sasanihiki and E30q; e, Kirara397 and S13q.

○, with identification primer sets; ●, with WXq.

respective amplification curves. Consequently, the ratio of Hoshinoyume mixing in an unknown sample was calculated using equation (1) described above. (Table 2)

There are four primer sets, WKA9, F6, S13 and E30, in the identification kit, all of which did not generate amplification products with Koshihikari DNA. In addition to WKA9q from WKA9, other primer sets for identification, F6, E30 and S13 from the rice cultivar identification kit, were improved upon to produce F6q, E30q and S13q. The points acquired when corresponding DNA solutions of various concentrations and several corresponding primer sets were made to react resulted in the generation of amplification curves (Fig. 1b-e). Standard curves were also generated by the maximum second derivative cycle number (Fig. 3b-e). The same DNA solutions with WXq drew other amplification curves, (Fig. 2b-e) and created another standard curves. (Fig. 3b-e) The ratios of mixing other cultivars to Koshihikari were derived from the reaction of these primer sets and DNA solutions from several corresponding rice DNA solutions under following primer-cultivar combinations: F6q-Mutsuhomare, E30q-Hitomebore, E30q-Sasanishiki and S13q-Kirara397.

\[
R_{\text{Mutsuhomare}} = \frac{\exp \left(C_{\text{F6q}} - 32.096 \right)}{\exp \left(C_{\text{WXq}} - 37.441 \right)}^{\left(-1.5623\right)} \quad \cdots\text{Eq. (2)}
\]

\[
R_{\text{Hitomebore}} = \frac{\exp \left(C_{\text{E30q}} - 36.752 \right)}{\exp \left(C_{\text{WXq}} - 35.572 \right)}^{\left(-1.7309\right)} \quad \cdots\text{Eq. (3)}
\]

\[
R_{\text{Sasanishiki}} = \frac{\exp \left(C_{\text{E30q}} - 31.529 \right)}{\exp \left(C_{\text{WXq}} - 37.227 \right)}^{\left(-1.4728\right)} \quad \cdots\text{Eq. (4)}
\]

\[
R_{\text{Kirara397}} = \frac{\exp \left(C_{\text{S13q}} - 30.925 \right)}{\exp \left(C_{\text{WXq}} - 37.381 \right)}^{\left(-1.8726\right)} \quad \cdots\text{Eq. (5)}
\]

In equations (2)–(5), \(C_{\text{F6q}}, C_{\text{E30q}}, C_{\text{S13q}}, \) and \(C_{\text{WXq}}\) represent cycle numbers for maximum point of second derive acquired when DNA of unknown rice sample reacts with the primer set F6q, E30q, S13q and WXq, respectively. These calibration formulas were verified using DNA solutions from Koshihikari sample containing 5% or 25% rice cultivars (Table 2). Calibration errors accounted for less than 30% from the expected values.

Conclusion

The ability to measure the quantity of a specific rice cultivar in a mixed rice sample is an important factor for enforcing the JAS as well as improving the traceability of rice shipments that become contaminated with other cultivars during rice polishing or storage. To date, the
conventional PCR was demonstrated to be capable of identifying rice cultivars, but it was not able to quantify the proportion of rice cultivars, because amplified products could only be analyzed upon termination of the PCR reaction. We have successfully developed a technique to resolve this problem.

This technology can be used to estimate the mixed ratio of blended rice and could be applied to the measurement of ratio of different rice cultivars in processed rice products such as rice crackers.

Confirmation of specificity by other (probe-based type) means would have required construction of individual probes for each of the rice cultivars likely to be encountered. In future, it may be possible to design a multiplex PCR using hybridization probes labeled with different fluorophores. The quantitative determination of the ratio for specific rice cultivars in mixed samples will thus open the way for extensive analysis of other food materials.

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References


