JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Comparative Evaluation of Three Different Extraction Methods for Rice (*Oryza sativa* L.) Genomic DNA

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Recently, more immediate and precise cultivar-identifying methods targeting the specific and/or introduced gene(s) have been put into practical use for various rice cultivars. However trustworthy and innovative the biotechnological analyses may be, DNA purity and quality do have unpredictable influences upon the identification. Extraction methods of rice DNA have hardly ever been compared in a comprehensive manner. In this study, we investigated extraction characteristics of three methods by using 10 rice cultivars and then examined template availability of rice DNAs thereby extracted. An UV spectrophotometric study with a view toward methods revealed three different facts: The Isoplant II kit method with inhibitor absorption yielded the most DNAs, the Takara kit method with magnetic trapping produced the best DNAs free from contaminative proteins, and the enzymatic digestion method exclusively with enzymatic digestions prepared the best DNAs free from contaminative polysaccharides. Moreover, with a view toward cultivars, an insignificant difference in yield was not entirely bore out, and some difference in cultivar might cause significant difference in yield; however, no significant difference in purity was found among the cultivars used. On the other hand, electrophoretic images of the DNAs from the same cultivars showed considerable differences in quality among the methods. Furthermore, the DNA extracts from certain brands of rice proved really available for cultivar identification by using polymerase chain reaction (PCR) related to sequence-tagged sites. Therefore, this study suggested that these extraction methods may be used as the situation demands and that the DNAs thereby extracted might work successfully even in cultivar-identifying PCRs.

KEYWORDS: ***Oryza sativa*** **L.; cultivar; DNA; extraction; discrimination; PCR; evaluation**

INTRODUCTION

One of the fundamental grains, especially in many Asian countries, rice (*Oryza sativa* L.), has been bred over hundreds of years. Many attractive cultivars with desirable traits, such as palatability, drought resistance, or high-yield ability, have been diligently developed in both *indica* and *japonica* subspecies through selection, crossing, or backcrossing with wild or modern cultivars. In Japan, as well as in other countries, however, brand-camouflage scandals have often happened with brand rices. To regulate the worsening imitative scams, laws related to agricultural policy, such as the Japan Agricultural Standard Act, which had been established to improve agricultural and forestry products and maintain fair trade, were partially amended and executed. According to these decrees, wholesalers and retailers are required to clearly show the production district and year, the name of the cultivar, the mixture ratio of material rough rice and so forth on the package of dehulled and polished rice.

To identify their genetic differences, methods such as morphological (*1, 2*) and isozyme (*3, 4*) analyses have already been reported and put into practical use. Advanced molecular marker technology, the restriction fragment length polymorphisms (RFLPs) technique, contributed to the initial construction of the molecular map in rice (*5*) and successfully distinguished *japonica* from *indica* cultivars (*6*). These beneficial techniques, however, have been considered unsuitable for field operations since reliability constraints seemed due to cultivation conditions (location of the cultivation, year of the production, etc.), particularly in related-cultivar identification. Therefore, technological development and practical application of precise genotype-identifying methods are required not only in breeding strategy but also in regulatory administration. Since the random amplified polymorphic DNA (RAPD) technique based on polymerase chain reaction (PCR) was introduced in 1990 (*7*), it has been widely applied to rice-cultivar classification (*8*−*14*). More importantly, simple qualitative PCR amplification was also used to identify genetically modified organisms (GMOs), for example, long-lasting Flavr Savr tomato (Calgene, United States) and herbicide-resistant Roundup Ready soybean (Monsanto, United States), which hold GMO-specific transgenes as PCR targets (*15, 16*).

In contrast, extraction methods of genomic DNA should be chosen carefully according to DNA amount, purity, and size necessary for investigative purposes. Additionally, in plant DNA

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10.1021/jf803473q CCC: $40.75 © 2009 American Chemical Society
Published on Web 03/02/2009

extraction, drastic elimination of troublesome PCR inhibitors (polysaccharides and polyphenols) is also very important when PCR amplification and/or restriction digestion are scheduled afterward. The extraction methods may have unpredictable influences upon discriminative PCRs to risk misidentification, and so, DNA purity (how much are template DNAs contaminated with proteins, polysaccharides, and RNAs?) and quality (how much do template DNAs remain intact?) should be strictly evaluated. Various extraction methods to isolate genomic DNA from cereal seeds have been used, but they have hardly ever been compared regarding the yield and purity (*17, 18*). The evaluation study using high molecular weight soy DNA revealed that the cationic surfactant or cetyltrimethylammonium bromide (CTAB) method and three commercial methods (Wizard, DNeasy, and Nucleon Phytopure) gave a relatively low recovery rate of below 20%, although they yielded the best DNA quality (*17*). On the basis of nucleic acid solubility in CTAB/NaCl solution, the CTAB method has demonstrated extraordinary benefit for plant DNA purification (*19, 20*). The multistage extractions with chloroform, however, cause substantive loss of intrinsic DNA despite thousands of man hours related to the manipulations. Hence, the CTAB method seems inconvenient or even unsuitable for such a practical case as hundreds of sample DNAs are supposed to individually be extracted from grain seeds or from DNA-degraded cereal products.

Therefore, to reveal the choice criteria for single-seed extraction methods, we comprehensively compared and evaluated extraction characteristics of three methods by using 15 kinds of brand rices. These brand rices are greatly popular among Japanese consumers since they have a pleasant taste and delightful scent, and so, they have a strong tendency to illegally be traded. Moreover, to clarify template availability of the DNA extracts thereby, we checked the validity of the tip-top brand of rice, Koshihikari, by using a pair of Koshihikari-identifying PCR kits in the marketplace.

MATERIALS AND METHODS

Rice Samples. Fifteen kinds of authoritative rice samples were obtained as dehulled seeds from the Japan Agricultural Cooperatives and rice growers around Japan. They were composed of 10 *japonica* cultivars (Japanese short-grain types) harvested in 13 local districts. The cultivar names (production prefectures) were as follows: Hoshinoyume (Hokkaido), Kirara397 (Hokkaido), Hitomebore (Iwate and Miyagi), Akitakomachi (Akita), Haenuki (Yamagata), Koshihikari (Tochigi, Ibaraki, and Niigata), Dontokoi (Niigata and Hyogo), Kinuhikari (Shiga), Hanaechizen (Fukui), and Hinohikari (Fukuoka and Kagoshima).

Extraction and Purification of Genomic DNA. Three types of extraction methods were examined for performance evaluation by using different varieties of rices since their action principles seemed distinctly notable and different from one another as described in the next section. Genomic DNAs of the dehulled rices were extracted and purified grain by grain for all of the 15 sample groups under repeatable conditions (within a few months, by the same operator, and with the same equipments). To pulverize the rice seeds, the use of liquid nitrogen was left out of consideration mainly because they were too dried even to be frozen. Alternatively, a concise and inexpensive procedure, buffer or water immersion, was adopted to soften them as described in the next section.

Isoplant II Kit Method. The Isoplant II kit (Nippon Gene, Japan), a DNA extraction kit suitable for plants, yeast, and bacteria, was purchased and tried. Taken into a 1.5 mL Eppendorf tube, a single grain was vortexed in 1 mL of appurtenant wash buffer containing 2-mercaptoethanol (final concentration, 0.5%) for 2 s and then soaked in 50 μL of TE buffer [composition: 10 mM Tris-HCl (pH 8.0) and 1 mM ethylendiaminetetraacetic acid (EDTA)] containing 2-mercaptoethanol (final concentration, 1.0%) overnight at room temperature beforehand. The considerably swollen rice grain was squished carefully with a pestle in 300 μL of solution I containing 2-mercaptoethanol (final concentration, 1.0%) and protein denaturant. Next, the sample was mixed with 150 μL of solution II, chiefly including benzyl chloride and vortexed for 10 s. The mixture was incubated for 10 min at 50 °C in a thermoshaker. Then, 100 μL of solution III-A and 120 μL of solution III-B were added for PCR-inhibitor absorption. The lysate was vortexed for 2 s and put on ice for 10 min. After centrifugation for 10 min at 14000*g* at 4 °C, the supernatant was transferred to a new 1.5 mL Eppendorf tube. Two volumes of ethanol was added, and the mixture was centrifuged for 5 min at 6000*g* at room temperature. After the supernatant was discarded, the resultant pellet was washed once with 1 mL of 70% ethanol and centrifuged for 1 min at 6000*g* at room temperature. The crude DNA pellet was air-dried and dissolved in 100 μL of TE buffer. Furthermore, the DNA extract was submitted to RNase-A digestion according to the Japanese administrative Notification No. 158 (*21*). That is, RNase A (10 mg/mL) was added to the extract (final concentration, 20 μg/mL), and the mixture was incubated for 30 min at 37 °C. Next, 200 μL of CTAB buffer [composition: 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, and 55 mM hexadecyltrimethylammonium bromide] and 250 μL of chloroform/isoamyl alcohol (24/1, v/v) were added, and the mixture was vortexed for 1 min. The mixture was centrifuged for 15 min at 7500*g* at room temperature. Then, 200 μL of the upper aqueous phase was transferred to a new 1.5 mL Eppendorf tube and mixed with 1 volume of 2-propanol. The mixture was centrifuged for 10 min at 7500*g* at room temperature. The supernatant was discarded, and the resultant pellet was washed once with 200 μL of 70% ethanol. Finally, after centrifugation for 1 min at 7500*g* at room temperature, the supernatant was discarded, and the pellet was adequately vacuumized. The purified DNA pellet was dissolved in 50 μL of 1/10 × TE buffer. Thereafter, it was adjusted to 10 ng/μL with 1/10 × TE buffer. The DNA sample was then stored at −20 °C until cultivar-discriminative PCR analysis.

Takara Kit Method. A commercial DNA extraction kit designed for uncooked dehulled and polished rice (Takara Bio, Japan), abbreviated here as the Takara kit, was also purchased and tried. For pretreatment, a single grain in a 1.5 mL Eppendorf tube was soaked in 36 μL of sterile MilliQ water overnight at room temperature. The considerably swollen rice was squished carefully with a pestle in 180 μL of appurtenant lysis buffer. Next, 10 μL of proteinase K (20 mg/ml) was added, and the sample was vortexed and immediately incubated for 60 min at 55 °C. In the interim, the sample was mixed three times by inversion. After the lysate was centrifuged for 5 min at 13500 rpm at 4 °C, 110 μL of the supernatant was transferred to a new 1.5 mL Eppendorf tube, in which 83 μL of DNA-binding buffer was predistributed. Immediately after mixing by inversion, the mixture was put on ice for 10 min, with intermittent mixing by inversion twice. Then, the mixture was centrifuged for 5 min at 13500 rpm at 4 °C, and 125 μL of the supernatant and 60 μL of magnetic particles dilution were mixed well in a new 1.5 mL Eppendorf tube and left for 5 min at room temperature. In the meantime, the mixture was stirred once for suspension. Thereafter, by using the Magnetight Separation Stand (Merck, United States), magnetic particle trapping DNAs were separated from the liquid phase. After the liquid phase was discarded, the magnetic particles were mixed with 150 μL of 1× wash buffer and vortexed well. Next, the magnetic particles were separated from the wash buffer in the same way. After the resultant buffer was discarded, the magnetic particles were mixed with 100 μL of fresh 1× wash buffer and vortexed well. Then, the magnetic particles were separated from the wash buffer in the same way. After the resultant buffer was discarded, the magnetic particles were air-dried on the Magnetight Separation Stand for 20 min, mixed well with 50 μL of elution buffer, and left for 5 min at room temperature. Finally, the magnetic particles were separated from DNA eluate in the same way. Transferred to a new 1.5 mL Eppendorf tube, the DNA eluate was made homogeneous with pipetting and centrifuged for 5 min at 13500 rpm at 4 °C. The DNA eluate was adjusted and stored as in the Isoplant II kit method.

Enzymatic Digestion Method. This DNA extraction method (Takara Bio, Japan) was also tried, and results were compared with the earlier two methods. In advance, a single seed in a 1.5 mL Eppendorf tube

was soaked in 50 μL of autoclaved MilliQ water overnight at room temperature. With a pinhole opened on the tube cap, the considerably swollen rice grain was cooked with a microwave intermittently for 12 min and squished carefully with a pestle in 300 μL of autoclaved extraction buffer [composition: 100 mM Tris-HCl (pH 8.0) and 100 mM NaCl]. Next, 10 μL of *Bacillus licheniformis*-derived thermostable α-amylase (Sigma, United States), dissolved in sterile water (15 mg/mL), was added, and the gruel was mixed well and left at 80 °C for 1 h. Then, 33 μL of filtration-sterilized 2% SDS and 20 μL of proteinase K (20 mg/mL; Takara Bio, Japan) were also added and left at 55 °C for 1 h. After the mixture was centrifuged for 1 min at 15000 rpm, the supernatant was transferred to a 2.0 mL Eppendorf tube, mixed with 2 volumes of cold ethanol (−20 °C) by inversion, and put on ice for 15 min. After centrifugation for 15 min at 15000 rpm at 4 °C, the crude DNA pellet was dissolved in 300 μL of autoclaved TE buffer [composition: 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA] and mixed with 1 μL of RNase A (10 mg/mL; Qiagen, Germany). The mixture was left at 55 °C for 1 h. Moreover, 300 μL of phenol/chloroform/isoamyl alcohol (PCI, 25/24/1, v/v/v) was added, and the mixture was slowly rotated for 15 min. After centrifugation for 15 min at 15000 rpm at 4 °C, the upper phase was transferred to a new 2 mL Eppendorf tube. Likewise, 1 volume of PCI was added, and the mixture was rotated for 15 min as well. After centrifugation for 15 min at 15000 rpm at 4 °C, the upper phase was transferred to a new 2 mL Eppendorf tube and quickly put on ice. Then, 2 volumes of cold ethanol (−20 °C) was added and mixed by inversion, and the mixture was left on ice for 10 min. After centrifugation for 15 min at 15000 rpm at 4 °C, the resultant pellet was washed with 50 μL of cold 70% ethanol (−20 °C). Lastly, after centrifugation for 15 min at 15000 rpm at 4 °C, the pellet was air-dried as adequately as possible. The purified DNA pellet was dissolved in 30 μL of 1/10× TE buffer. The DNA solution was adjusted and stored as in the Isoplant II kit method.

Examination of DNA Yield, Purity, and Quality. The DNA concentration was determined by using UV/Visible spectrometer Ultraspec 3300 Pro (Amersham, United States) with 5 μL of the stock solution diluted to 1/10 in TE buffer. The absorbance was measured for both blank and dilutions at four wavelengths, that is, 230, 260, 280, and 320 nm. The absorbance at 320 nm (OD 320) was used for background compensation, and then, the DNA concentration was calculated upon the assumption that an OD 260 of 1 corresponds to nearest 50 ng/μL for double-stranded DNA (*22*).

The equation that describes DNA quantity or yield was

$$\text{DNA yield(ng/grain)} = (\text{OD } 260 - \text{OD } 320) \times 500 \times \text{DNA solution volume}$$

The equations that describe OD ratios indicative of DNA purity (contamination with protein, polysaccharide, and/or RNA) were

$$\text{OD } 260/280 \text{ ratio} = (\text{OD } 260 - \text{OD } 320)/(\text{OD } 280 - \text{OD } 320)$$

$$\text{OD } 260/230 \text{ ratio} = (\text{OD } 260 - \text{OD } 320)/(\text{OD } 230 - \text{OD } 320)$$

The sample DNAs were electrophoresed on 0.8% agarose gels (100 ng/lane) in 1× TAE buffer [40 mM Tris-acetate and 1 mM EDTA (pH 8.0)], stained with ethidium bromide and photographed under UV light (*22*). The electrophoretic image, indicating DNA quality (fragmentation, denaturation, and so on), was optically observed.

Koshihikari-Identifying PCRs. Three authoritative rice samples, Koshihikari harvested in Tochigi, Ibaraki, and Niigata, were used for Kosihikari-identifying PCRs, where a pair of commercial PCR kits was utilized in tandem approach. The identifying PCRs were done in a final volume of 20 μL on a Gene Amp PCR System 9700 (Applied Biosystems, United States). All PCRs were tentatively carried out in triplicate since there were no manufacturer's instructions on how many reactions should be designed per sample DNA.

In the first stage, the Koshihikari identifying PCR kit I (Takara Bio, Japan), called the positive kit, was utilized. The reaction composition and step-cycle program were as follows: 2 μL of 10× PCR buffer, 1.3 μL of primer mixture, 2.5 mM $MgCl_2$, 250 μM dNTPs, 0.75 U Takara Taq polymerase, and 10 or 40 ng of Koshihikari DNA; preincubation at 96 °C for 2 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min, and extension at 72 °C for 2 min; and terminal elongation at 72 °C for 7 min. Aliquots of 8 μL of PCR products were resolved by electrophoresis through 2% agarose gel and photographed similarly as mentioned in the previous section. On the basis of the manufacturer's indications, the authenticity was proved upon positive standard for cultivar Koshihikari, which should be practically necessary and sufficient for single-grain analysis: Three bands responsible for the positive standard ought to appear nearly at the positions of 650, 770, and 870 bp.

In the second stage, the Koshihikari-identifying PCR kit II (Takara Bio, Japan), called the negative kit, was utilized. The reaction composition was as follows: 2 μL of 10× PCR buffer, 2.38 μL of primer mixture, 2.5 mM $MgCl_2$, 250 μM dNTPs, 0.75 U Takara Taq polymerase, and 40 ng of Koshihikari DNA. For PCR amplification of positive control, Hitomebore DNA (from Iwate) was extracted in the same way and added at the same dose. The step-cycle program and electrophoretic procedure were the same as described in the first stage. The authenticity was further confirmed upon negative standard against the other cultivars, where scam brands should be evinced reversely: No DNA band ought to appear at any position of 800, 1200, 1600, or 1800 bp.

If the electrophoretic pattern due to the sample, authoritative Koshihikari, was consistent with both of the standards and if enough amplification efficiency was found for the positive control, the sample was verified as genuine Koshihikari. The template availability related to the extraction methods was evaluated on the basis of a triplex- and a simplex-reaction format, respectively. That is, at least one of the triplicate PCR tubes is responsible for the identification in the former format, while such identification depends on only a simple tube in the latter.

Statistics. Data obtained from the DNA extraction experiments were classified on two factors of rice cultivar and extraction methods and then analyzed using two-way factorial ANOVA (Excel 2003, Microsoft, United States) followed by Steel−Dwass multiple comparison test (Statlight 2000; Yukmus, Japan) as posthoc comparisons. Meanwhile, data from the Koshihikari-discriminative PCRs were assessed using a χ^2 test. The differences were deemed significant at $p < 0.05$.

RESULTS

Spectrophotometric Evaluation of DNA Yield and Purity. The two-way factorial ANOVA made clear the significant differences in genomic DNA yield and in purity, OD 260/280 and OD 260/230 ratio, among the three methods. Moreover, the statistical test elucidated some cultivar−method interaction in DNA yield but not in DNA purity ($p = 0.23$ and 0.28 for OD 260/280 and OD 260/230 ratios, respectively). Noteworthy results of on-target multiple comparisons are shown below, while pointless results of cross-coupled multiple comparisons are excluded.

From a DNA yield aspect (**Figure 1A**), the rank order of the three methods used to extract DNA from the same sample was Isoplant II kit > Takara kit and enzymatic digestion method. Additionally, significant differences, enzymatic digestion > Takara kit method, were shown in Hoshinoyume and Kirara397 from Hokkaido, Hitomebore from Miyagi, and Akitakomachi from Akita. In these four samples, therefore, the rank order was Isoplant II kit > enzymatic digestion > Takara kit method. The DNA yields were in the range of 2.06−2.89 μg/grain for the Isoplant II kit method, 0.76−1.53 μg/grain for the enzymatic digestion method, and 0.68−0.97 μg/grain for the Takara kit method.

With regard to OD 260/280 ratio (**Figure 1B**), an index for contaminative proteins, the rank order was Isoplant II kit and/or Takara kit > enzymatic digestion method in Hitomebore from Iwate and Miyagi, Koshihikari from Tochigi and Ibaraki and Niigata, Donntokoi from Hyogo, and Hinohikari from Fukuoka and Kagoshima; Takara kit > Isoplant II kit and/or enzymatic digestion method in Kirara 397 from Hokkaido, Akitakomachi

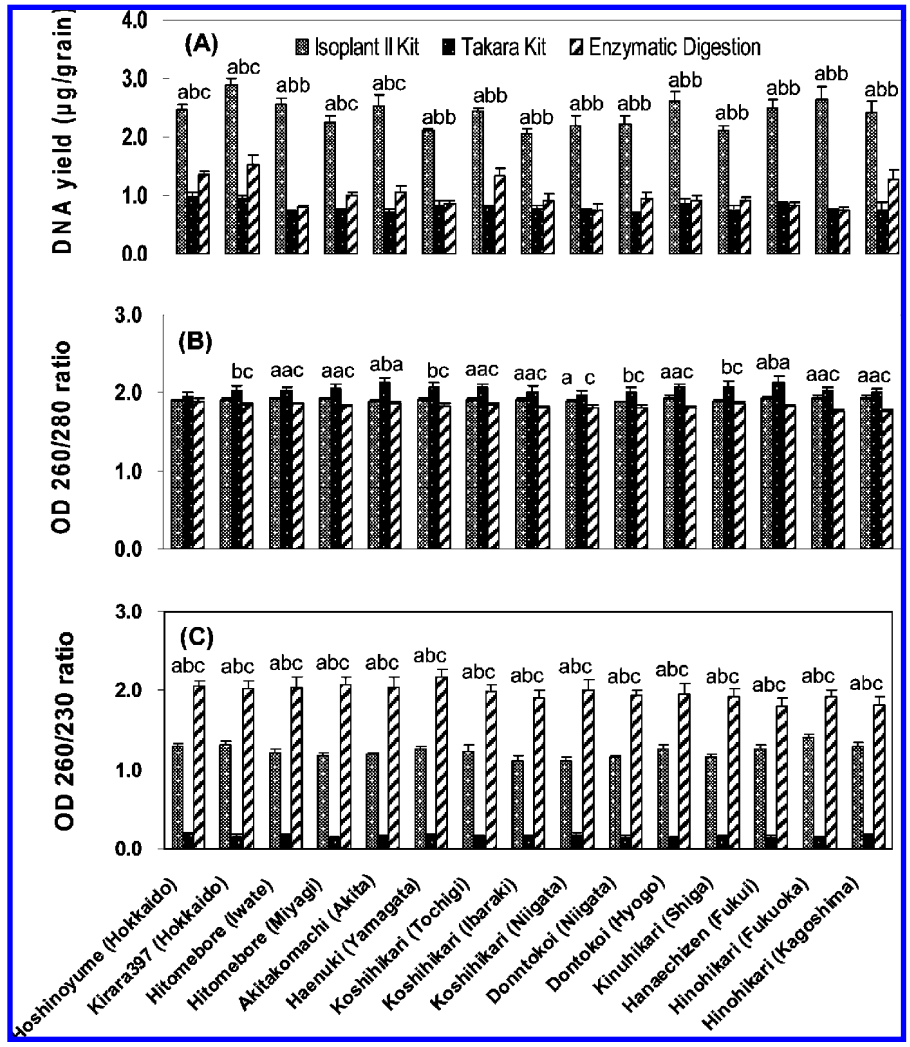

Figure 1. Intermethod comparison of DNA yields (**A**), OD 260/280 ratios (**B**), and OD 260/230 ratios (**C**) using 10 japonica cultivars (13 productive centers). Values are means of six independent extractions, and vertical bars represent standard errors. Different letters on the top of columns indicate significant differences among the three methods at $p < 0.05$.

from Akita, Haenuki from Yamagata, Donntokoi from Niigata, Kinuhikari from Shiga, and Hanaechizen from Fukui. No significant differences were shown in Hoshinoyume.

As for OD 260/230 ratio (**Figure 1C**), an index for contaminative polysaccharides and phenolics, the rank order was enzymatic digestion > Isoplant II kit > Takara kit method in all samples. No other significant differences were shown among the three methods.

Gel Electrophoresis Analysis for DNA Quality. Every sample DNA extracted with the Isoplant II (**Figure 2A**) and the Takara kit (**Figure 2B**) appeared as a single distinct band greater than 10 kb, which was strongly indicative of high molecular weight DNA. Almost no smears appeared in the case of the Isoplant II kit, whereas lurking images were recognized as background in the case of the Takara kit. In contrast, every DNA extracted by the enzymatic digestion method (**Figure 2C**) showed almost nothing but faint smearing, which spread widely in the range less than 10 kb. The faint smearing was clearly indicative of severe degradation or fragmentation of sample DNAs. Needless to say, there were obvious differences in electrophoretic images between the enzymatic digestion and the other methods.

Cultivar Influences on DNA Yield, Purity, and Quality. The two-way factorial ANOVA indicated the significant difference in DNA yield among the rice cultivars but not in purity. The Steel−Dwass test, however, failed to make clear the significance among the rice cultivars. All of the data shown in **Figure 1** were rearranged in a cultivar-oriented manner as in **Figure 3**. In terms of DNA yield, 15 samples or 10 cultivars showed no significant differences among them if the same methods were applied (**Figure 3A**). So did the same brand rices, Hitomebore, Koshihikari, and Dontokoi, harvested in the different districts. With respect to DNA purity, neither the OD 260/280 (**Figure 3B**) nor the OD 260/230 (**Figure 3C**) ratio differed significantly among 10 cultivars when their DNAs were extracted in the same way. Naturally, none of the same brand rices from different districts showed contradictory findings. Consequently, some of the cultivars used might have an influence on DNA yield, but none of the cultivars influenced on DNA purity together with DNA quality as shown above (**Figure 2A−C**).

Template Availability in Koshihikari Identification. Independent of the production districts, the results of Koshihikari identification were grouped together in **Table 1**. When the sample DNA due to authoritative Koshihikari was added at the 10 ng/reaction dose on the triplex-reaction format in the case of the positive kit, eight of the 18 triplexes with the Isoplant II kit resulted in misidentification of the cultivar, Kosihikari (**Figure 4A**), which should have generated the three specific DNA bands. However, the other triplexes with the Takara kit

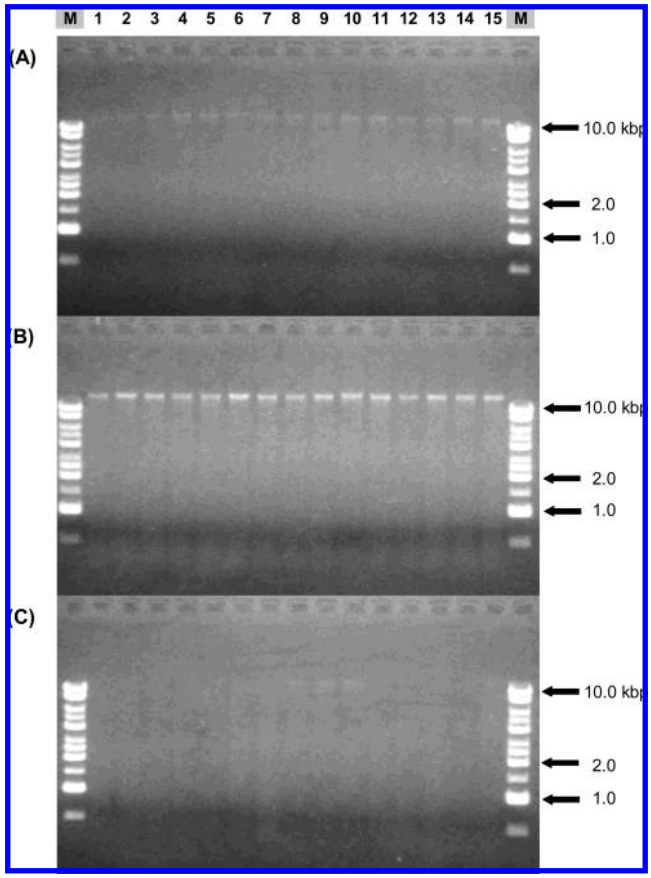

Figure 2. Agarose gel electrophoresis of rice DNAs from 15 grain samples or 10 japonica cultivars (13 productive centers), which were extracted by the Isoplant II kit (**A**), Takara kit (**B**), and enzymatic digestion method (**C**): M, kilobase DNA marker; 1, Hoshinoyume (Hokkaido); 2, Kirara397 (Hokkaido); 3, Hitomebore (Iwate); 4, Hitomebore (Miyagi); 5, Akitakomachi (Akita); 6, Haenuki (Yamagata); 7, Koshihikari (Tochigi); 8, Koshihikari (Ibaraki); 9, Koshihikari (Niigata); 10, Dontokoi (Niigata); 11, Dontokoi (Hyogo); 12, Kinuhikari (Shiga); 13, Hanaechizen (Fukui); 14, Hinohikari (Fukuoka); and 15, Hinohikari (Kagoshima). The DNAs were applied at a dose of 100 ng/lane.

(**Figure 4B**) and the enzymatic digestion method (**Figure 4C**) resulted in the positive identification that the sample was authentic Koshihikari rice. When the template dose was increased to 40 ng/reaction, no more significant differences in the yes−no decision were shown among the three methods (**Figure 5A−C**), where all of the triplexes contributed to the positive identification. On the simplex-reaction format, however, the significant differences were found between the Isoplant II kit and the other two, no matter which dose was tried out in the case of the positive kit. Of the 54 reactions concerning the Isoplant II kit, 37 reactions at a dose of 10 ng and 12 reactions at a dose of 40 ng led to the misidentification.

On the other hand, a preliminary study using the negative kit revealed that the amplification of positive control, Hitomebore DNA extracted with the Isoplant II kit, was hardly observed at the 10 ng/reaction dose (data not shown). Therefore, every template DNA was used at the 40 ng/reaction dose where the positive control showed the PCR amplification. Accordingly, no bands due to the other cultivars were observed (**Figure 6A−C**), and then, all of the triplexes or the 54 reactions contributed to the negative identification that the sample corresponded to no other cultivars than Kosihikari. Unlike in the case of the positive kit, even on a simplex-reaction format, there were no significant differences in the yes−no decision among the three methods. Both identification approaches taken together, the Koshihikari rice tested here proved to be truly authentic.

For extensive practical use of the three methods, we normalized and summarized all of the data presented above, taking into account cost advantage and user friendliness (**Figure 7**). The cost advantage was approximately calculated on the basis of the total price of materials, while user friendliness was roughly determined on the actual times necessary for each step except the overnight preimmersion. We found that the enzymatic digestion method cost the least and the Takara kit method handled most deftly.

DISCUSSION

Extraction Characteristics of the Isoplant II Kit. As a whole (overall mean ± standard deviation without distinction of cultivars used), the Isoplant II kit method gave more than twice the DNA yield (2.40 ± 0.13 μg/grain) as compared with the Takara kit (0.79 ± 0.08 μg/grain) and the enzymatic digestion method (1.02 ± 0.11 μg/grain). Although the Isoplant II kit adequately deproteinized genomic DNAs (OD 260/280 ratio = 1.91 ± 0.02), the purified DNAs poorly worked as PCR templates at the10 ng/reaction dose. Neither PCR inhibitor nor DNA fragmentation was likely to cause this low PCR efficiency since we could find neither dose-dependent inhibition nor smearing image that should be evident.

However, some double-stranded DNAs were likely denatured to single-stranded ones, which lead to spectrophotometric overestimation because of the hyperchromic effect, as was reported with Chelex 100, Alkali, or AlkaliX methods (*17*). This explanation is supported by the finding that the single band indicative of genomic DNA appeared more weakly with the Isoplant II kit than with the Takara kit since poorly stained single-stranded DNAs were considerably formed during the extraction with the Isoplant II kit.

It is known that in food products, some genetic engineering steps would damage intact DNA chemically (divalent cations, pH), physically (heating, shearing), and/or enzymatically (various nucleases) and cause unfavorable fragmentation and a decrease in high molecular weight DNA through hydrolytic cleavage and/or depurination (*23*, *24*). Whether samples may be processed or not, the extraction characteristics of the Isoplant II kit method suggest that this kit should be used for rice foods that may contain more protein by nature or for ones that may be DNA-poor due to excessive manufactured conditions.

Extraction Characteristics of the Takara Kit and the Enzymatic Digestion Method. On the contrary, we also found wholly that the Takara kit and the enzymatic digestion method, respectively, showed the highest OD 260/280 (2.04 ± 0.03) and OD 260/230 (1.98 ± 0.05) ratio, while neither method gave higher yield than the Isoplant II kit did.

As the OD 260/280 ratio was found nearest 2.0 in the Takara kit, there arises some concern that even purified DNAs might remarkably be contaminated with coextracted RNAs. However, the product developer Takara Bio commented that there was a least-likely or a slight possibility of residual RNAs since no evidence of contamination or smearing was found around the low molecular weight field in electrophoretic image. Extracted by using the Isoplant II kit with RNA digestion, genomic DNAs free from RNAs showed the single band at the same position (over 10 kb) as genomic DNAs with the Takara kit did. Both of them enabled the positive identification of Koshihikari. Therefore, the single band over 10 kb was strongly indicative of genomic DNAs. Moreover, it should be noted that OD 260/

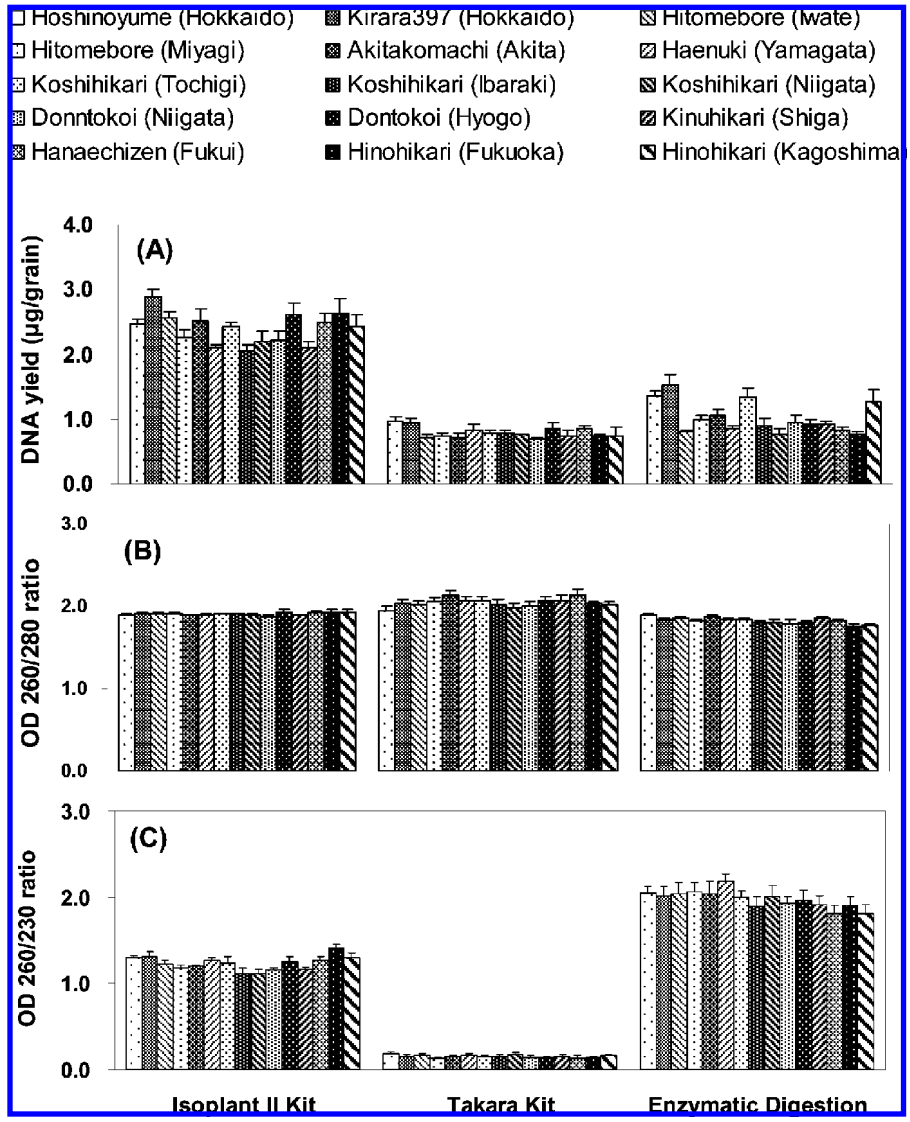

Figure 3. Intercultivar comparison of DNA yields (**A**), OD 260/280 ratios (**B**), and OD 260/230 ratios (**C**) with the three extraction methods. Values are means of six independent extractions, and vertical bars represent standard errors. No significant differences are seen among the rice cultivars at $p < 0.05$.

Table 1. Comparison of Yes−No Decision Based upon Koshihikari-Identifying PCRs[a]

	DNA dose (ng/reaction)	triplex-reaction basis			simplex-reaction basis		
		positive	negative	total	positive	negative	total
		positive kit					
Isoplant II Kit	10	10*	8*	18	17*	37*	54
Takara Kit		18	0	18	52	2	54
enzymatic digestion		18	0	18	48	6	54
Isoplant II Kit	40	18	0	18	42*	12*	54
Takara Kit		18	0	18	54	0	54
enzymatic digestion		18	0	18	54	0	54
		negative kit					
Isoplant II Kit	40	0	18	18	0	54	54
Takara Kit		0	18	18	0	54	54
enzymatic digestion		0	18	18	0	54	54

[a] Data represent frequencies in the yes−no decision. Differences were assessed by using a χ^2 test. *$p < 0.05$ was regarded as significant.

280 as well as OD 260/230 ratios are expedient indices for the contaminants, although both ratios are deemed helpful and popular. Considering all of these points together, this OD 260/280 ratio, even if indicative of pure RNAs, may not reflect the predominant contamination any more.

Referring also to the enzymatic digestion method, it seems strange enough that there was less misidentification of Koshihikari rice than we apprehended despite inexpedience that remarkable DNA fragmentation may have happened. The microwave cooking in the earlier step appears to have caused such adverse fragmentation in physical manners. However, it is probable that the majority of template DNAs was spared the worst damage that they were sheared to the size of 870 bp or below since the three-band pattern responsible for the identification still appeared at the positions of 650, 770, and 870 bp with the positive kit. Additionally, even with the negative kit, the single band due to the positive control of Hitomebore DNA also appeared at the larger size of 1600 bp.

Therefore, the extracting characteristics of the Takara kit and the enzymatic digestion method suggest that both methods should be applied to such rice foods rich in protein and polysaccharide, respectively, as are produced at low or moderate degree of processing.

Faint Likelihood of Some Effect Due to Rice Cultivars. The two-way factorial ANOVA (parametric analysis with *F* value) clarified the significant difference in DNA yield not only

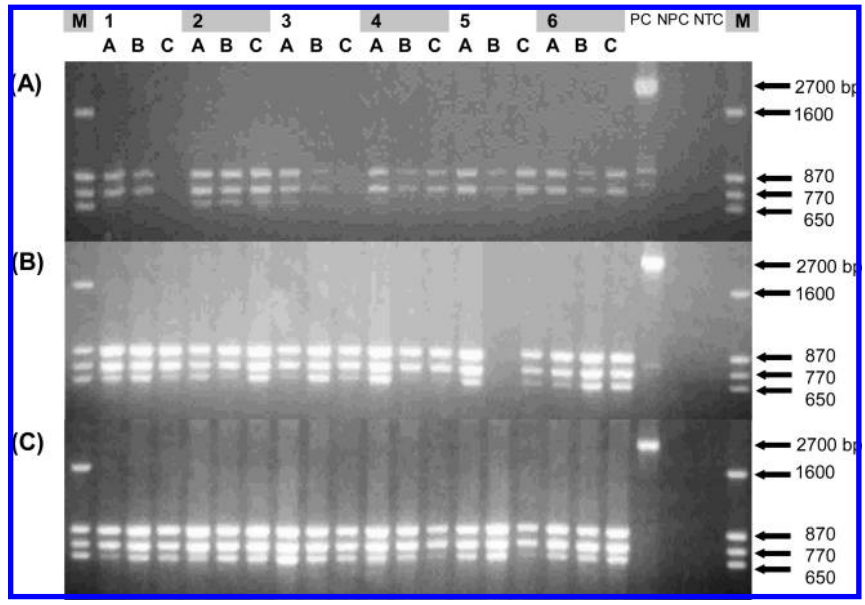

Figure 4. Representative results of Koshihikari-identifying PCRs with the positive kit by using authoritative Koshihikari grains. The template DNAs were extracted by the Isoplant II kit (**A**), Takara kit (**B**), and enzymatic digestion method (**C**). Six Koshihikari grains harvested in Niigata were subjected to the DNA extractions (upper row, 1−6) and to the discriminative PCRs in triplicate (lower row, A−C): M, indicated DNA marker; PC, positive control; NPC, no primer control; and NTC, no template control. The template DNA was added at the dose of 10 ng/reaction. The positive control presented a corresponding band nearly at 2700 bp occasionally with a weak extra.

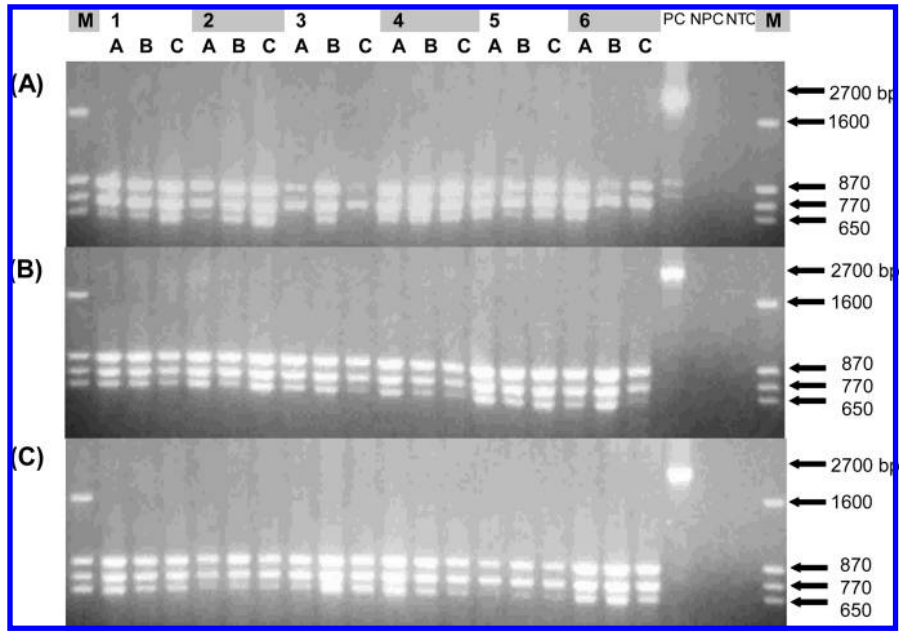

Figure 5. Representative results of Koshihikari-identifying PCRs with the positive kit by using authoritative Koshihikari grains. The template DNAs were extracted by the Isoplant II kit (**A**), Takara kit (**B**), or enzymatic digestion method (**C**). Six Koshihikari grains harvested in Niigata were subjected to the DNA extractions (upper row, 1−6) and to the discriminative PCRs in triplicate (lower row, A−C): M, indicated DNA marker; PC, positive control; NPC, no primer control; and NTC, no template control. The template DNA was added at the dose of 40 ng/reaction. The positive control presented a responsible band nearly at 2700 bp occasionally with a weak extra.

among the methods but also among the cultivars, although the ANOVA itself could not specify which pair should be accountable for the significance. However, neither the Steel−Dwass test (nonparametric analysis without *F* value) nor the Scheffe test (nonparametric analysis without *F* value) failed to bear out the significant difference in DNA yield among the cultivars (Scheffe test's data not shown). Furthermore, the two-way factorial ANOVA made clear the cultivar−method interaction that the difference in cultivar would cause difference in DNA yield with the methods.

Because the cultivar valiability and the cultivar−method interaction have never been reported in conjunction with DNA extraction, it seems highly difficult and speculative to explain what would cause the variability and the interaction. Perhaps cultivar difference in ingredient composition and/or intranuclear protein might be responsible for the unexpected results. Alternatively, the difference in mathematical assumptions of the statistical analyses might explain the subsidiary findings. Even if further closely focused studies should be performed, however, these extraordinary findings may prove slight or insignificant.

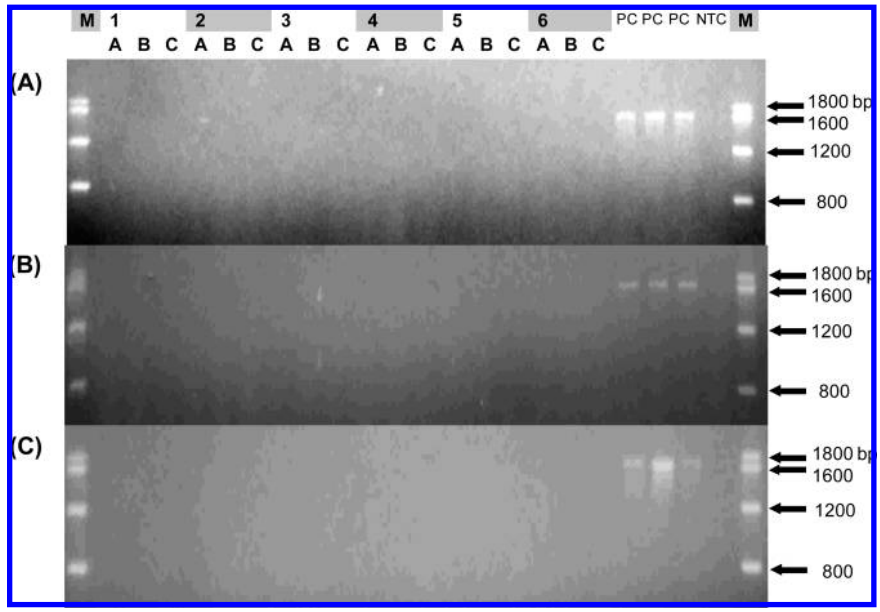

Figure 6. Representative results of Koshihikari-identifying PCRs with the negative kit by using authoritative Koshihikari grains. The template DNAs were extracted by the Isoplant II kit (**A**), Takara kit (**B**), or enzymatic digestion method (**C**). Six Koshihikari grains harvested in Niigata were subjected to the DNA extractions (upper row, 1−6) and to the discriminative PCRs in triplicate (lower row, A−C): M, indicated DNA marker; PC, positive control; and NTC, no template control. The template DNAs were added at the dose of 40 ng/reaction. The positive control or Hitomebore DNA presented a responsible band nearly at 1600 bp.

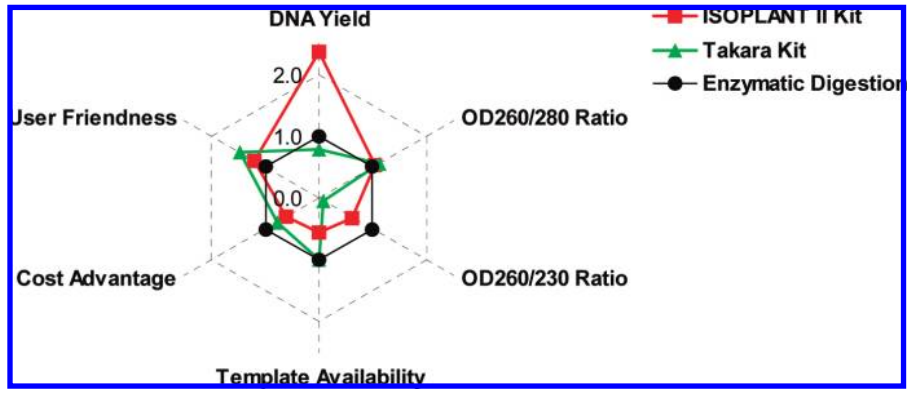

Figure 7. Characteristics profile of the three extraction methods for rice DNA. The enzymatic digestion method is regarded here as a benchmark for drawing practice; the performance index of cost advantage is expressed in reciprocal ratios. The template availability shows the aspect in the case of the 10 ng/reaction but not the 40 ng/reaction DNA dose. These results are based on their respective overall means from the three methods.

The statistical significance, the questionable one, is not always sufficient grounds for something of value.

On the other hand, various crops (rice, wheat, maize, and so on), whether conventional or genetically modified, are under development to enhance the nutritional value partly from a nutritional standpoint. Sooner or later, the cultivar−method interaction may attract some attention in plant thremmatology or so. In any way, we hazard the description that some cultivars utilized might have a significant impact on DNA yield with the three methods since we have no more data to flatly throw out their likelihood.

Background of the False Negative in Koshihikari Identification. The 10 ng/reaction dose did not present the same accuracy in the identification as shown in **Table 1** when the three methods were compared. As is generally noticed (*22*), spectrophotometric quantitation at 260 nm has various measuring nucleic acids: double-stranded DNA, single-stranded DNA and RNA, and single-stranded oligonucleotides. Although the concentration of each template DNA was adjusted in a spectrophotometric manner, they may vary in composition of nucleic acids due to the extraction methods. Therefore, this composition heterogeneity is likely to explain such discrepancy as was found according to the dose and the method.

As far as the Isoplant II kit method is concerned, however, it seems fairly difficult to specify what factor made the identification accuracy much improved at the 40 ng/reaction dose. The electrophoretic image in **Figure 2A** is unlikely to explain poor availability of the DNA extracts since they seemed rather intact even if fragmentation and/or degeneration might happen to some extent. At the 10 ng/reaction dose, the DNA extracts with the Isoplant II kit method apparently consisted of less available template DNA than those with the other methods did. Whatever the case may be, the higher DNA dose might make up for the lower availability of the template DNA itself.

Improvement Strategy for Koshihikari Identification. Both the positive and the negative kits, the multiplex primer set for Koshihikari identification, were developed on RAPD analyses for sequence-tagged sites (STSs), wherein a total of eight STS primers comprehensively proved available to discriminate the top 50 rice cultivars with a nearly 95% share of the Japanese market (*25, 26*). Our faithful compliance to the manufacturer's indications was

ensured especially by preparing template DNAs with the Takara kit and by adding them at the 10 ng/reaction dose.

As shown in **Table 1**, however, two false negatives of the 54 reactions were observed with the positive kit using the simplex-reaction format. The false negative cases further happened more frequently in the case of the Isoplant II kit whichever dose (10 or 40 ng/reaction) was tried in Koshihikari identification. Now that there is no manufacturer's indication of how many reactions should be planned for sample DNA, we suggest that the triplex-reaction format should be adopted for Koshihikari identification and that at least two doses of template DNA that may be free from PCR inhibition should be tried for the confirmation if the results are nonetheless doubtful. In fact, we found no discrepancies in the identification based on the triplex-reaction format.

Taken all together, it is most likely that choice criteria or rate scale for extraction and purification method of rice DNA might be defined with no less than three practical parameters, that is, template availability, cost advantage, and user friendliness. However, template availability itself should be valued more than anything. In view of the present situation, a generally useful mathematical presentation for the choice adequacy remains unclear since no standard methods are discussed or determined worldwide. This proposal is a highly abstract topic, and more studies are required to establish the choice criteria in an evenhanded fashion. In conclusion, the present study showed that the Isoplant II kit method yielded the most rice DNAs and that the Takara kit and the enzymatic digestion method best purified rice DNAs, which were practically free from protein and polysaccharide contamination, respectively. All of the DNAs extracted from the cultivar Koshihikari proved available for the cultivar-discriminative PCRs. Therefore, these three methods may be used for rice-cultivar identification as food ingredients and manufactured conditions demand.

ABBREVIATIONS USED

PCR, polymerase chain reaction; STS, sequence-tagged site; GMO, genetically modified organism; RFLP, restriction fragment length polymorphism; RAPD, random amplified polymorphic DNA; CTAB, cetyltrimethylammonium bromide; PCI, phenol/chloroform/iso-amyl alcohol.

ACKNOWLEDGMENT

The excellent statistical assistance of Dr. Kuniaki Tayama is gratefully acknowledged.

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Received for review November 6, 2008. Revised manuscript received February 2, 2009. Accepted February 2, 2009.

JF803473Q