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Cultivar Identification of Rice (*Oryza sativa* L.) by Polymerase Chain Reaction Method and Its Application to Processed Rice Products

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As the cultivars of rice markedly affect eating quality, processing suitability, and price, identification or differentiation of rice cultivar is very important. We developed suitable 14 STS (sequence-tagged site) primers for PCR (polymerase chain reaction), and it became possible to differentiate 60 Japanese dominant rice cultivars from each other using template DNA extracted and purified from rice grains. A multiplex primer set was shown to be useful to effectively differentiate rice cultivars produced in various countries by PCR. A novel multiplex primer set for PCR has been developed to differentiate KoshihikariBL, which is closely related with the premium cultivar, Koshihikari, in Japan. The application of the cultivar identification method by PCR method to commercially processed rice products was investigated. We developed an enzyme treatment method, in which the gelatinized starch is decomposed by the heat-stable α-amylase at 80 °C, followed by the hydrolysis of proteins by proteinase K with sodium dodecyl sulfate and purification of extracted DNAs by phenol/chloroform/*iso*-amyl alcohol. It became possible to identify the material rice cultivars of the commercially processed rice products, such as cooked rice, rice cake, or rice cracker, by a PCR method using template DNA prepared by the enzyme treatment method and novel multiplex primer sets.

KEYWORDS: Rice; DNA; cultivar identification; PCR; STS primer; SCAR marker; multiplex PCR; processed rice product; boiled rice; rice cake

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the leading food crops in the world and the staple food for more than half the world's population (*1*). Nearly 420 500 samples of rice and its related species are maintained in germplasm collections across the world (*2*). Although rice is markedly diversified from the viewpoint of genetics, morphology, and properties, high-quality rice is closely related by inbred breeding to attain high palatability, high processing suitability, or characteristic aroma, etc.

Rice grains of famous cultivars are traded or distributed at higher prices as "premium rice" than the ordinary rice because of their high palatability, processing suitability, or special aroma, etc. Because those premium rice grains sell at high prices, some dishonest rice wholesalers or retailers blend low-quality cheap rice with high-quality premium rice and mislabel it as "high-quality premium rice" (*2*). Recently, rice consumers have demanded more information, such as cultivar name, location of production, year of production, etc., about the rice that they purchase. For example, wholesalers and retailers are obliged to display the name of the rice cultivar on its package under the JAS LAW (law concerning standardization and proper labeling of agricultural and forestry products in Japan). Under such circumstances, the technology to identify rice cultivar is very important for breeders, farmers, inspectors, wholesalers, retailers, food industries, and consumers (*2*, *3*). Therefore, it is necessary to develop a time-saving technology to clearly and precisely differentiate rice cultivars.

DNA fingerprinting was developed in 1985 (*4*) and is used for criminal investigation and trial at court. Recently, a novel cultivar identification method based on DNA polymorphism has been developed that accompanies the progress in molecular biology. DNA-based markers have the obvious advantage of sampling the genome directly, and restriction fragment length polymorphism (RFLP) analysis has been widely used for assessing the variation of plants (*5*−*7*). RFLP analysis has been used to distinguish between species of *Oryza* (*8*) and particularly between Indica and Japonica types of *O. sativa* (*9*).

Recently, a polymerase chain reaction (PCR)-based marker system has been developed by Williams et al. (*10*). In this RAPD (random amplified polymorphic DNA) method, short oligonucleotides of arbitrary sequences are used to support the amplification of regions of the test plant genome and amplified DNAs are separated by gel electrophoresis.

There are some reports on RAPD analysis of rice germplasm including Indica and Japonica types to identify suitable parents for linkage map construction and for gene tagging for drought resistance (*11*, *12*). RAPD analysis was revealed to be reproducible and amenable for identification of each single plant line of F1 hybrid rice in China (*13*) and Australian rice cultivars (*14*).

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Diversified rice cultivars were classified into separate groups by PCR using RAPD markers, but many primers were needed to resolve closely related Japonica cultivars (*15*−*17*).

In the case of RAPD markers, many other DNA bands than the target DNA for cultivar differentiation appear in the electrophoregram after PCR. Therefore, it is recommended to develop the STS (sequence-tagged site) primers or SCAR (sequence characterized amplified region) markers based on RAPD markers (*18*, *19*). By the development of forward and reverse primers based on the sequence of the target DNA region, which had been proliferated using RAPD primers, it became possible to amplify only the target DNA fragment by PCR. For example, Talbert et al. sequenced and designed STS primers from 22 wheat RFLP clones and indicated that most of the primers amplified sequences that mapped to the expected chromosomes in wheat, which suggested that the STS-based PCR analysis would be useful for generation of informative molecular markers in hexaploid wheat (*18*). Jain et al. developed SCAR markers from the results of AFLP and RAPD to select the pathogen resistance gene of rice and localized those markers on the genetic map to identify the chromosomal location of the resistance gene (*19*).

In addition to RAPD markers, microsatellites or simple sequence repeats (SSRs), which are DNA sequences with repeat lengths of a few base pairs and variation in the number of nucleotide repeats, can be detected with PCR by selecting the conserved DNA sequences flanking the SSR as primers. In 1993, SSR markers were developed for rice (*20*, *21*). SSR markers are useful not only to characterize the relationship between heterosis and marker genotype heterozygosity but also to identify chromosome segments that may have significant effects on yield and its component traits in rice (*22*).

Genetic diversity among Indian Elite rice varieties was evaluated using three different types of DNA markers and parentage analysis (*23*). SSLP was reported to be more reliable than AFLP for identifying rice cultivars (*24*). RAPD, RFLP, nuclear SSLP, and chloroplast SSLP analyses were carried out to clarify the phylogenetic relationships among A-genome species of rice (*25*). SSR was reported to provide useful genetic information on weedy rice (*26*).

The development of a genome-wide DNA polymorphism database for map-based cloning of rice genes was investigated using SNP markers (*27*), and SNPs were used for the discussion on the sequence variations between the rice cultivars (*28*). Although SSR and SNPs are very useful DNA markers, it is impossible to use several PCR primers simultaneously, which is "multiplex PCR", to simplify the PCR and electrophoresis and save time.

We have investigated the cultivar identification of rice by a PCR method using RAPD markers and STS primers developed from RAPD markers (*3*, *29*−*32*). We also developed STS primers to identify a Japanese premium rice cultivar, Koshihikari (*3*), to report on the identification of Japanese rice cultivars by the RAPD method using a single grain of cooked rice as a sample (*29*), to identify glutinous rice cultivars using rice cakes as samples by the PCR method (*30*), to differentiate and search for palatability factors of worldwide rice grains by the PCR method (*31*), and to develop a PCR-based marker set for identifying Koshihikari Niigata BLs, near-isogenic lines harboring rice blast resistance genes (*32*).

In the present study, the application of PCR technology to cultivar identification of rice grains was investigated. Novel SCAR markers and multiplex primer sets were developed based on RAPD marker analyses to clearly and efficiently differentiate closely related rice cultivars, such as KoshihikariBL, by PCR.

An efficient method to prepare template DNA for PCR from processed rice products, such as cooked rice, rice cakes, or rice crackers, was investigated. In our former report, a comparison of template DNAs prepared by the three methods, commercial kit method, cetyl tri-methyl ammonium bromide (CTAB) method, and enzyme treatment method, was carried out. In the case of the enzyme treatment method, the amount of extracted and purified DNA from a single grain of boiled rice was 11.30 μg and the ratio of absorbance at 260 nm to that at 280 nm was 1.87. On the contrary, the amount of DNA by the CTAB method was 3.81 μg and that by the commercial kit was 9.21 μg. Furthermore, the amplified DNA did not appear after PCR in the case of the template DNA prepared from the boiled rice grain by the commercial kit (*29*). The enzyme treatment method was shown to be profitable from the viewpoint of quality and quantity of the template DNA prepared from processed rice products.

Furthermore, it was necessary to expand the differentiation of the material rice cultivars by the PCR method using the commercially processed rice products by the development of novel primer sets. The novel SCAR markers and primer sets were developed to differentiate the closely related BL cultivars, and the differentiation of material rice cultivars became possible using the commercially processed rice products as samples, by the enzyme treatment method and novel multiplex primer sets for PCR.

MATERIALS AND METHODS

Materials. Fifteen various kinds of rices were collected or purchased. The sample rice grains used were as follows: Koganemochi (Japan), Hinohikari (Japan), Akitakomachi (Japan), Kirara397 (Japan), Ilpum (Korea), Calmochi101 (United States), medium grain (United States), Fobidden rice (United States), Pelde (Australia), Kyeema (Australia), Paellea (United States), long grain (United States), Doongara (Australia), Nanjing 11 (China), and IR2061 (Philippines).

Thirty-three original Koshihikari rice seeds, produced in 1999, 2000, and 2001, were kindly provided by the National Food Agency, Japan, and used for DNA extraction and purification. All of the rice samples including another 49 different cultivar rice samples other than Koshihikari were kindly provided by the National Center for Seeds and Seedlings, Japan.

Paddy rice samples were hulled by an experimental huller (Ketto Science Laboratory, Tokyo) and milled to the yield of 90% by an experimental rice polisher (Pearlest, Ketto Science Laboratory, Tokyo). Milled rice flours were prepared by a coffee mill (Millser IFM-100, Iwatani, Japan). Commercial rice products were purchased at the supermarket in Tsukuba city.

Physical and Chemical Properties of the 20 Rice Cultivars. Amylose contents were measured by the iodine−colorimetric method using the milled rice flours by Juliano (*33*). Protein contents were calculated by the multiplication of nitrogen−protein conversion coefficient (5.95 for rice) to the nitrogen contents measured by the Kjeldahl method (*34*). Physical properties of the boiled rice grains were measured by the method of Okadome (*35*) using a Tensipresser.

Preparation of Boiled Rice and Rice Cake. White rice prepared by the experimental rice polisher (Ketto Science Laboratory) was boiled by an electric rice cooker (RC183, Toshiba, Tokyo) after soaking for 1 h, and each single boiled rice grain was subjected to the extraction of template DNA for PCR.

Milled waxy rice was pulverized using an experimental impact mill (Udy cyclone Mill, Udy Corp., Fort Collins, United States). Thirty-five grams of water was added to 50 g of the rice powder and kneaded manually. The rice cake was prepared using an electric rice cake maker for home use (SMK-1800, Tiger Co. Ltd., Tokyo). The rice cake was

Table 1. Sequence of DNA Amplified by the Use of Primer B43[a]

1	**TGGCCGGCATGACTCAC**ATACCCAACATATAGCATATCTGGATGTCTGTT	50
51	TGCAATCATGTCGGGTGATCGAGCCAACAACGTTTGCGAGGAATTTATCC	100
101	GTTAACGACATATTCTCCAATAGTTCGGACATGGCCGGCGCTATACATGAG	150
151	ATAAGTCGTCAGTCTTCATTGGTAGGTTGGGTGCGACAACTCAGCCTTTAT	200
201	CGAGAATGTTCTTGACAATGGGGTGTACTCGACCACAACCTACTCGAGTG	250
251	CTCAATTGTTCCTGAAAAATATTTTCTAGAAGACAAGATATATAGCAG	300
301	ATAGTATCGAGCATATACTCAAAAGACTGCATGAATCTTCTATTATTATC	350
351	ATGATATAACGAACAGATTAAATATCATACATTATGTAAACCATAAGTAA	400
401	GCATGATTTATACATAAGAAAATAAAGCGAGCCCCCAGTATTAAACCGTTG	450
451	TTGACCTAATATAGGGAATGCTCACACAATAGATATTGTATAGAAAACA	500
501	TGCTCTAGGTAAACATAATAAATTTGAAATCTGACAATATCGTATTTATC	550
551	TGAAGAATAGGTACGATAAATCACATATAAAATATTGCGTAGCTTTGTAG	600
601	ATACATGTAGGATGTATCTACGAACGAAATTAGTAGGTCAACTTAAAAAA	650
651	TTAATCTACTAATTACCTTTCTGACATGTGTATTCGGAGTAGATTAGATC	700
701	GATGCAATCATACTTCTCCAAGTGGACAGCCGCATGTATGCAGTAAACAC	750
751	ATGCACTTGCTGTTAATTGATCTCATGCATGCACATATTGACGTCATCCAA	800
801	ATTGGTTG**GTCTTGATGCCGGCCAGT**	837

[a] Bold type letter sequences were used as primers for PCR.

lyophilized and pulverized using a coffee-mill (IFM-100 Co. Ltd.) and subjected to DNA extraction.

Extraction and Purification of Template DNA. According to the CTAB method (*29*, *30*, *36*, *37*), DNAs of the milled rice flours were extracted. Milled rice flours (0.4 g) were placed in the microcentrifugal tube (2 mL), and DNAs were extracted into 0.6 mL of 2 × CTAB [2% CTAB, 20 mM ethylene diamine tetra actate (EDTA), 1.4 M NaCl, and 0.1 M tris-hydroxyl aminomethane/HCl buffer, pH 8.0] solution and 0.2 mL of distilled water for 30 min at 65 °C. The solution of chloroform and *iso*-amyl alcohol (24:1, v/v) (0.8 mL) was added and stirred gently for 15 min using a rotater. Thereafter, the solution was centrifuged (8000*g*, 15 min) by a refrigerated centrifuge (hi-mac CR21F, Hitachi, Hitachi) and the upper layer was transferred to another microtube. CTAB solution (10%, 0.08 mL) and chloroform/*iso*-amyl alcohol (24:1, v/v) were added to the solution, and it was stirred gently for 15 min followed by centrifugation (8000*g*, 15 min). The upper layer was transferred to another tube and stood for 5 min in the freezer (−80 °C) after the addition of 2.5× volumes of the precipitation buffer (50 mM tris-buffer, pH 8.0, 10 mM EDTA, and 1% CTAB). The precipitate was collected by the centrifugation (6000*g*, 15 min) and dissolved in 0.5 mL of Tris-EDTA buffer (TE) and was added with the same volume of iso-propyl alcohol. After gentle stirring by a rotater for 15 min, the precipitate was collected by the centrifugation (6000*g*, 15 min). The precipitate was dissolved in 0.2 mL of TE followed by the decomposition of RNAs by the addition of 1 μL of RNase (RNase A, bovine pancreas, 10 mg/mL, Nippon-gene Inc. Tokyo) and incubation for 1 h at 55 °C. Thereafter, neutral phenol solution was added and the upper layer was transferred to another tube after the centrifugation (8000*g*, 15 min). The same volume of the solution of phenol/chloroform (1:1, v/v) was added to the solution followed by centrifugation (8000*g*, 15 min), and the upper layer was transferred to another tube. The solution was added with 0.2 M NaCl and two times the volume of cold ethanol to generate the precipitate of DNAs. The DNAs were washed with 50 μL of 70% ethanol and dissolved in 30 μL of 0.1 TE and were subjected to PCR.

Preparation of DNA in Case of Processed Rice Products. In the case of processed rice products, such as grains of boiled rice or rice cake, a different DNA extraction/purification method was developed based on the enzyme utilization method as described in our former reports (*29*, *30*). The processed rice product was subjected to the decomposition of starch by heat-stable α-amylase (790 unit/mg solid, 30 mg/mL, from *Bacillus Richenoformis*, Sigma, United States) for 60 min at 80 °C. For the starch digestion, 20 μL of the above-mentioned α-amylase (1 mg/1 mL) was added to 1.5 mL of the sample solution (single grain of boiled rice in the solution of 300 μL of Tris-HCL buffer, 50 mM, pH 8.0) and digestion was performed for 1 h at 80 °C. Thereafter, protein hydrolysis was carried out by proteinase K (Takara-bio Inc., Japan). For the protein digestion, 10 μL of proteinase K solution (27.7 u/mg, 20 mg/mL) was added to the starch digested solution to 1% of sodium dodecyl sulfate (SDS). The protein digestion was carried out for 1 h at 55 °C. Thereafter, DNA was extracted by the same amount of Tris-EDTA-saturated phenol solution and purified by the phenol/chloroform/*iso*-amyl alcohol (PCI, 25/24/1, v/v/v) and 70% ethyl alcohol.

PCR. DNAs were proliferated by the PCR method using 600 commercial random primers (10-mers or 12-mers) as primers. Taq-DNA polymerase (Takara-bio Inc.) was used for amplification of DNAs. Three microliters of template DNA (400 ng/μL by absorbance at 260 nm) was used for each PCR with RAPD primers. Each DNA was denatured for 1 min at 94 °C, annealed for 1 min at 38 °C, and elongated for 2 min at 72 °C. This procedure was repeated 40 times. As a PCR apparatus, Thermal Cycler MP (Takara-bio Inc.) was adopted. When the STS primers were used for PCR, the annealing temperature was 62 °C. The reproducibility of these PCR was ascertained three times for every primer and reaction.

Electrophoreses of the Amplified DNAs. Proliferated DNAs were subjected to the electrophoresis for 30 min through the agarose gel (2%) using a Mupid-2 electrophoresis system (Cosmo-bio, Japan) at the charge of a direct current of 100 V. After the electrophoresis, the DNA was stained by ethydium bromide and detected by irradiation of UV light.

Development of STS Primers for SCAR Markers and Multiplex Primer Sets. In the present study, STS primers for PCR were developed based on RAPD analysis to differentiate rice cultivars by PCR as described in our former report (*3*). DNAs were extracted from the agarose gel after the electrophoresis of the PCR products using Easy Trap (Takara-bio Inc.). DNA cloning was carried out using a TOPO XL PCR Cloning Kit (Invitrogen Corp., Carlsbad, CA). The DNA sequence was determined using a commercial DNA preparation kit (QIAprep Spin Miniprep kit, Qiagen, K.K., Tokyo, Japan) and DNA proliferation kit (Big Dye Terminator Cycle sequencing kit, V1-1, Applied Biosystems Japan, Tokyo, Japan) and automatic DNA sequencing system (DNA sequencer ABI PRISM Genetic Analyzer 310, Applied Biosystems Japan). SCAR markers were designed based on the DNA sequences from 20-mers to 29-mers so that the transition temperatures were around 62 °C. The combinations of these STS primers for PCR, multiplex primer set, were developed to identify Koshihikari, the most dominant rice cultivar in Japan.

RESULTS

Development of STS Primers for PCR. An example for the development of STS primers for PCR to identify or differentiate rice cultivars is shown in **Table 1**. The sequence of the differential DNA, which was extracted from the electrophoresis agarose gel after PCR of the RAPD method, was determined, and the suitable forward and reverse primers, 20-mers, were designated as shown in **Table 1**.

The electrophoregrams after PCR using the RAPD marker, B43, and the SCAR marker, B43, are shown in **Figure 1**. As compared with the RAPD marker, the SCAR marker amplified

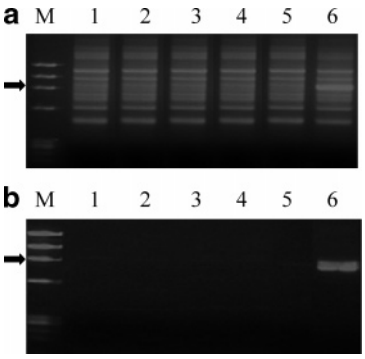

Figure 1. Comparison of RAPD and SCAR markers in PCR and electrophoresis. M, DNA molecular weight markers (marker 4, Wako Pure Chemical Co. Ltd., Japan); 1, Hinohikari; 2, Haenuki; 3, Kinuhikari; 4, Akitakomachi; 5, Kirara397; and 6, Koshihikari. Arrow: differential DNA for (**a**) RAPD marker (B43, Operon Co. Ltd.) and (**b**) SCAR marker (B43 in **Table 1**).

Table 2. Various STS Primers for PCR[a]

PCR primer		sequence
WKA9L	F	CCCGCAGTTAGATGCACCATTAGAATTGCTTCATTGCCTGTGGA
	R	CCGCAGTTAGATCAAGTGGCAAGGTTCCATGTTTGGACTCAA
WKA9	F	CCCGCAGTTAGATGCACCATT
	R	CCGCAGTTAGATCAAGTGGC
B43	F	TGGCCGGCATGACTCAC
	R	ACTGGCCGGCATCAAGAC
B43a	F	TGGCCGGCATGACTCACA
	R	ACTGGCCGGCATCAAGA
M11	F	GTCCACTGTGACCACAACAT
	R	GTCCACTGTGGGGATTGTTC
G22	F	CTCACTCAAATTTACAGTGCATTTTCTTG
	R	AGGGCCATGATACAAGACTCTGT
S13	F	GTCGTTCCTGTGGTTAGGACAGGGT
	R	GTCGTTCCTGCTGGTGTCTCAGAT
F6	F	ACCACTCCATATATATCATCCAAAG
	R	ACCACTCCATATCACCACAAGG
E30	F	TACCTGGTTGATGTATACAGATCTGGTT
	R	ATCCCTCGATCCCTCTAGCATTAT
G49A	F	AATCCAGACATGAAATTTATATGCAGATA
	R	AATCCAGACATGTTGTCCTCAATTTTTG
G49AL	F	AATCCAGACATGAAATTTATATGCAGATATTAATTTTGAATGCT
	R	AATCCAGACATGTTGTCCTCAATTTTTGAATCAAACTACCTTA
G81	F	TACCTGAACCAGCAAGCATGCGCG
	R	TACCTGAACCAGTATAATCTTTG
M2CG	F	ACAACGCCTCCGATGA
	R	ACAACGCCTCCGACAACAAGAT
A6	F	CCAGCTGTACGCCTGTACTAC
	R	CCAGCTGTACGTCTTCCCCAGC
B1	F	GTTTCGCTCCTACAGTAATTAAGGG
	R	GTTTCGCTCCCATGCAATCT
B7	F	CAGGTGTGGGTTACAAGGATGA
	R	CAGGTGGTTCACGGCCTTT
T16	F	GGTGAACGCTGTAGTTGGAATATA
	R	GGTGAACGCTCAGATTTAATAT
*P*3	F	AACGGGCCAAAAACGGAGGT
	R	AACGGGCCAACGCAG
G28	F	GGCGGTCGTTCTGCGAT
	R	GGAGAATCCCACAGTAAGTTTTTCTTTG

[a] F, forward; R, reverse.

by the use of STS primer is more useful because only the differential DNA is amplified, which makes it easy and accurate to differentiate rice cultivars.

Developed STS primers for PCR to identify or differentiate rice cultivars are shown in **Table 2**.

Physicochemical Properties of Rice Samples. Various rice samples were collected from different districts in the world, and their chemical and physical properties were measured. For example, Indica subspecies, such as IR2061 and Nanjing 11, showed high amylose contents (27.8−32.0%) and less adhesiveness. On the contrary, Japonica subspecies, such as Hinohikari and Akitakomachi, revealed low amylose contents (16.3−18.0%) and more adhesiveness as reported in our former report (*31*).

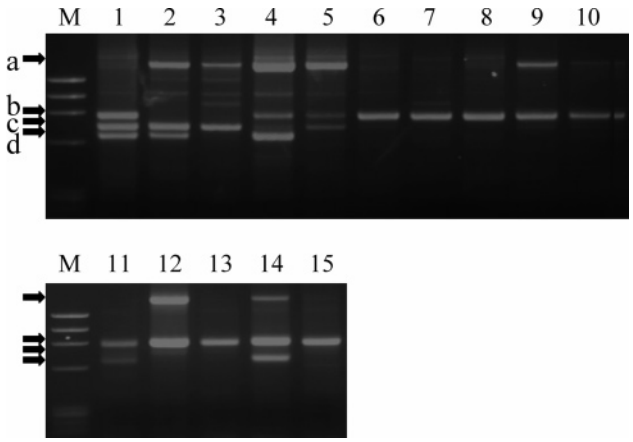

Figure 2. Example of the PCR using the primer set for the differentiation of various kinds of rice cultivars: 1, Koganemochi; 2, Hinohikari; 3, Akitakomachi; 4, Kirara397; 5, Ilpum; 6, Calmochi101; 7, medium grain; 8, forbidden rice; 9, Pelde; 10, Kyeema; 11, Paellea; 12, long grain; 13, Doongara; 14, Nanjin11; and 15, IR2061. Markers: a, Wka9; b, B43; c, M11; and d, G22.

Application of PCR to Various Rice Samples. The CTAB method can be applied to milled rice flour for extraction and purification of template DNA for PCR (*29*, *30*, *36*). The result of PCR using 15 rice cultivars from various countries, such as the United States, Korea, Australia, China, the Philippines, and Japan, as samples, is shown in **Figure 2**. The primer set for PCR was the Koshihikari Positive Kit. Three DNA bands appeared clearly in the case of Koganemochi and three other DNA bands appeared clearly in the case of Kirara 397, Ilpum, or Nanjin No. 11, which were revealed to be useful to differentiate various rice cultivars by a single time PCR and electrophoresis.

Differentiation of Japanese Japonica Rice Cultivars by PCR. We developed 19 different STS primers as shown in **Table 2**. By the use of 14 STS primers, it became possible to differentiate 60 Japanese dominant Japonica cultivars as shown in **Table 3**.

STS Primer Set for Differentiation of Koshihikari. As shown in **Figure 3**, Koshihikari (lane 41) can be discriminated from any other Japanese Japonica cultivar using this STS primer set for differentiation, which generated three specific DNA bands for Koshihikari. There is no other cultivar rice that shows the same three-band pattern than Koshihikari except KoshihikariniigataBLs (near-isogenic blast resistant cultivars of Koshihikari) among 50 dominant nonglutinous rice cultivars in Japan.

Novel PCR Primers for the Differentiation of KoshihikariBL from Koshihikari. New primers, G49A, G81, and WKA9L, were developed in our previous study as shown in **Table 2** (*32*). In the present investigation, it was shown that these three STS primers are useful to differentiate KoshihikariBL from the original Koshihikari as shown in **Figure 4**. Primer WKA9L is useful to differentiate KoshihikariniigataBL1, primer G49A is useful to differentiate KoshihikariniigataBL2, and primer G81 is useful to differentiate KoshihikariniigataBL3 from the original Koshihikari.

Differentiation of Material Rice Cultivars of Rice Products by PCR. In the case of commercially boiled white rice grain,

Table 3. Result of PCR Using 12 Kinds of PCR Primers and 60 Japanese Rice Cultivars

	PCR primer														
rice cultivar	Wka9	B43	M11	G22	M2CG	F6	S13	E30	B1	P3	G28	B7	T16	A6	digitized value
Hitomebore	+	+	+	+	+	−	−	−	−	−	+	−	+	−	11,111,000,001,010
Manamusume	+	+	+	+	+	−	−	−	−	−	+	−	−	−	11,111,000,001,000
Karinomai	+	+	+	+	−	−	−	−	−	+	+	−	−	−	11,110,000,011,000
Hohohonoho	+	+	+	+	−	−	−	−	−	+	−	−	−	−	11,110,000,010,000
Domannaka	+	+	+	−	+	−	−	−	+	−	+	−	+	+	11,101,000,101,011
Naganohomare	+	+	+	−	+	−	−	−	+	−	+	−	+	−	11,101,000,101,010
Hanaechizen	+	+	+	−	+	−	−	−	−	−	+	−	+	−	11,101,000,001,010
Yumeakari	+	+	+	−	+	−	−	−	−	−	+	−	−	−	11,101,000,001,000
Yumetsukushi	+	+	+	−	−	−	−	−	+	+	+	−	+	−	11,100,000,111,010
Akiho	+	+	−	+	+	−	−	−	−	+	−	−	+	−	11,011,000,010,010
Yukimaru	+	+	−	+	+	−	−	−	−	−	+	−	+	−	11,011,000,001,010
Hatsuboshi	+	+	−	+	+	−	−	−	−	−	+	−	−	−	11,011,000,001,000
Tsukinohikari	+	+	−	+	+	+	−	−	+	+	−	−	+	−	11,001,100,110,010
Kakehashi	+	+	−	+	+	−	−	+	−	+	+	−	+	−	11,001,001,011,010
Yumehikari	+	−	+	+	+	−	−	−	+	+	+	−	+	+	10,111,000,111,011
Morinokumasan	+	−	+	+	+	−	−	−	+	+	−	+	−	−	10,111,000,110,100
Hinohikari	+	−	+	+	+	−	−	−	+	+	−	−	−	−	10,111,000,110,000
Fusaotome	+	−	+	+	+	−	−	−	−	−	+	−	−	−	10,111,000,001,000
Aichinokaori	+	−	+	−	+	−	−	+	−	+	−	−	+	+	10,101,001,010,011
Dontokoi	+	−	+	−	+	−	−	−	+	+	+	−	+	+	10,101,000,111,011
Haenuki	+	−	+	−	+	−	−	−	−	−	+	−	−	+	10,101,000,001,001
Kinuhikari	+	−	+	−	−	−	−	−	+	+	+	+	+	+	10,100,000,111,111
Matsuribare	+	−	+	−	−	−	−	−	+	+	−	−	+	−	10,100,000,110,010
Tsugaruroman	+	−	+	−	−	−	−	−	−	−	+	+	+	−	10,100,000,001,110
Akitakomachi	+	−	+	−	−	−	−	−	−	−	+	−	−	−	10,100,000,001,000
Kirara397	+	−	−	+	+	−	+	−	−	+	+	−	+	−	10,011,010,011,010
Hoshinoyume	+	−	−	+	+	−	+	−	−	+	+	−	−	−	10,011,010,011,000
Goropikari	+	−	−	+	+	−	−	−	+	+	−	−	−	−	10,011,000,110,000
Kirarimiyazai	+	−	−	+	+	−	−	−	−	+	+	−	+	−	10,010,000,011,010
Natsuhiari	+	−	−	+	+	−	−	−	−	−	−	−	+	−	10,010,000,000,010
Takaneminori	+	−	−	−	+	+	−	−	−	−	+	−	−	−	10,001,100,001,000
Yumesansa	+	−	−	−	+	−	−	−	−	−	+	−	+	−	10,001,000,001,010
Asanohikari	+	−	−	−	−	+	−	−	+	+	−	−	+	−	10,000,100,110,010
Asahinoyume	+	−	−	−	−	−	−	−	+	+	−	−	+	−	10,000,000,110,010
Akanezora	−	+	+	+	−	−	−	−	+	+	−	+	+	−	1,110,000,110,110
Koshihikari	−	+	+	+	−	−	−	−	−	+	+	−	−	−	1,110,000,011,000
Koganenishiki	−	+	+	−	+	+	+	+	+	−	+	−	+	+	1,101,111,101,011
Kiyonishiki	−	+	+	−	+	+	+	+	−	−	+	+	+	−	1,101,111,001,110
Koganemasari	−	+	+	−	+	+	−	−	+	+	−	−	+	+	1,101,100,110,011
Yukinosei	−	+	+	−	+	+	−	−	+	−	+	−	+	+	1,101,100,101,011
Mineasahi	−	+	+	−	+	−	−	+	+	−	+	−	+	−	1,101,001,101,010
Yueminori	−	+	+	−	+	−	−	−	+	+	+	+	+	−	1,101,000,111,110
Hatsushimo	−	+	+	−	+	−	−	−	+	+	−	−	+	+	1,101,000,110,011
Notohikari	−	+	+	−	−	−	−	+	+	+	−	−	−	+	1,100,001,110,001
Koshijiwase	−	+	+	−	−	−	−	+	+	−	+	−	−	+	1,100,001,101,001
Fukuhikari	−	+	+	−	−	−	−	+	+	−	−	−	+	+	1,100,001,100,011
Yamahikari	−	+	−	+	−	+	−	+	−	+	−	−	+	+	1,010,101,010,011
Reiho	−	+	−	−	+	+	+	+	−	−	+	−	+	+	1,001,111,001,011
Asahi	−	+	−	−	+	+	+	−	+	+	−	−	+	−	1,001,110,110,010
Nakateshinsenbon	−	+	−	−	+	+	+	−	+	−	−	−	+	−	1,001,110,100,010
Nipponbare	−	+	−	−	+	+	−	−	+	+	−	−	+	+	1,001,100,110,011
Mutsuhomare	−	+	−	−	+	+	−	−	+	−	+	+	+	−	1,001,100,101,110
Akebono	−	+	−	−	+	+	−	−	+	−	−	−	+	−	1,001,100,100,010
Nishihomare	−	+	−	−	+	−	−	+	+	−	+	+	+	+	1,001,001,101,111
Mutsukaori	−	+	−	−	+	−	−	+	−	−	+	+	+	+	1,001,001,001,111
Akitsuho	−	+	−	−	+	−	−	−	+	−	−	−	+	−	1,001,000,100,010
Akinishiki	−	+	−	−	−	+	−	−	−	+	+	−	+	−	1,000,100,011,010
Yamahoushi	−	+	−	−	−	−	−	+	+	+	+	−	−	+	1,000,001,111,001
Chiyonishiki	−	−	+	−	+	−	−	+	+	−	+	−	+	+	101,001,101,011
Sasanishiki	−	−	+	−	−	−	−	+	−	−	−	+	−	−	100,001,000,100

it was necessary to search for new primers, such as A6, and we developed the novel primer set as shown in **Table 2**.

As shown in **Figure 5**, it became possible to differentiate Haenuki, Kirara397, Yukinosei, and Tsugaruroman from Koshihikari, their parental cultivar, by PCR using the template DNA prepared from each single grain of boiled rice by the enzyme treatment method, and the novel primer set, which was described in **Table 2** and the Materials and Methods.

Differentiation of Material Rice Cultivars of Rice Cake by PCR. The results of PCR using template DNAs prepared from each rice cake or rice cracker by the enzyme treatment method were the same as with those using template DNAs from material rice flour directly by CTAB method, and it became possible to differentiate Himenomochi, Hiyokumochi, Mangetsumochi, Minenoyukimochi, and Miyakoganemochi, Japanese principal waxy rices, as shown in **Figure 6**. It also became possible to differentiate Wataboshi, Mochihikari, and Mangetsumochi from each other using the template DNA for PCR prepared by the enzyme treatment method from the commercial rice crackers as shown in **Figure 7**.

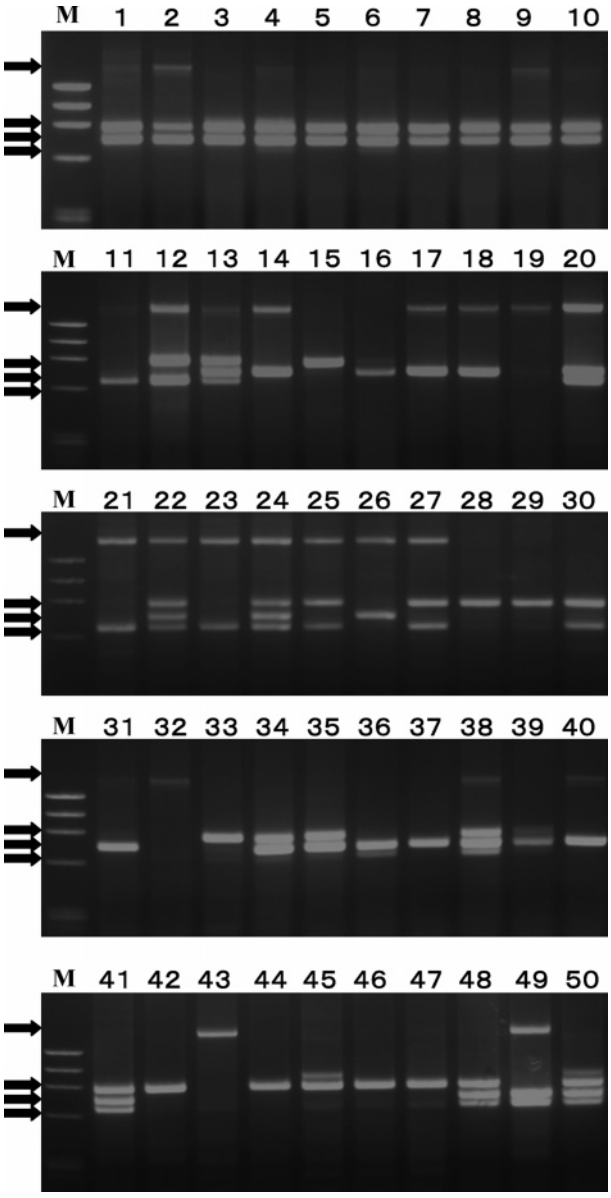

Figure 3. Results of PCR using the primer set for identification or detection of the cultivar Koshihikari using various Japanese-registered rice cultivars: 1, Koganebare; 2, Naganohomare; 3, Mutsukaroi; 4, Shimahikari; 5, Koganehikari; 6, Michikogane; 7, Tomohikari; 8, Kitaake; 9, Natsuhikari; 10, Wakamizu; 11, Hoshinoyume; 12, Akiho; 13, Karinomai; 14, Tsugaruroman; 15, Yumetoiro; 16, Yumehitachi; 17, Okiniiri; 18, Yumemusubi; 19, Asahinoyume; 20, Morinokumasan; 21, Hoshitaro; 22, Ishikawa 43; 23, Hanasatsuma; 24, Fukumirai; 25, Nanatsuboshi; 26, Takitate; 27, Mienoyume; 28, Asatsuyu; 29, Syun-you; 30, Ayahime; 31, Kinuhikari; 32, Tsugaruotome; 33, Hatsuyume; 34, Kibinohana; 35, Yukinosei; 36, Hinohikari; 37, Aoinokaze; 38, Hitomebore; 39, Yumehikari; 40, Tokimeki 35; 41, Koshihikari; 42, NipponbarekantoBL1; 43, NipponbarekantoBL2; 44, NipponbarekantoBL3; 45, NipponbarekantoBL4; 46, NipponbarekantoBL5; 47, NipponbarekantoBL6; 48, KoshihikariniigataBL1; 49, KoshihikariniigataBL2; and 50, KoshihikariniigataBL3.

DISCUSSION

Properties of Rice Grains of Various Rice Cultivars. In the case of trade contracts, inspection or grading, or the survey of traceability of rice grains, the PCR method must be applied using rice grains as samples. Shoot or leaves are not available as sample specimens. Therefore, milled rice flour, a single kernel of polished rice, or even boiled rice is used as the material for PCR in the inspection carried out postharvest.

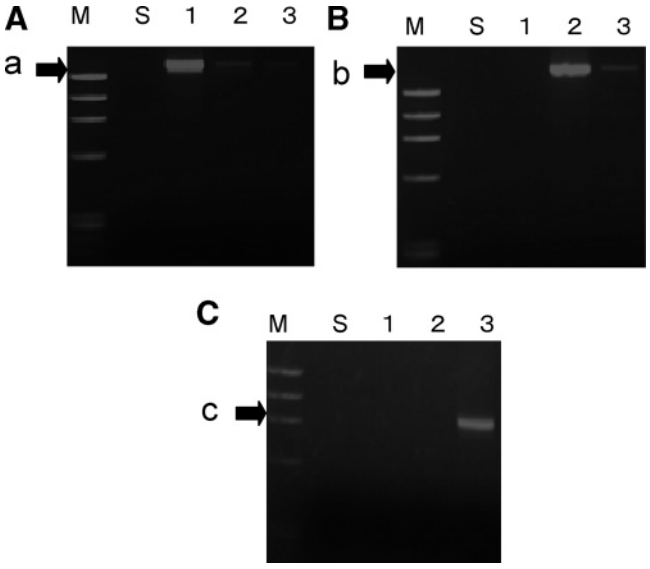

Figure 4. Results of PCR for the differentiation of KoshihikariBLs from Koshihikari. M, DNA molecular weight marker; S, original Koshihikari; 1, KoshihikariniigataBL1; 2, KoshihikariniigatBL2; and 3, KoshihikariniigataBL3. (**A**) PCR using the primer a, (**B**) PCR using the primer b, and (**C**) PCR using the primer c. Markers: a, WKA9L; b, G49A; and c, G81.

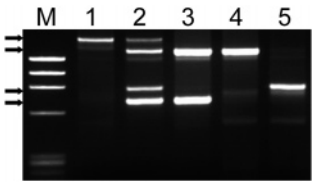

Figure 5. Differentiation of Japanese rice cultivars by PCR using template DNA extracted from a single boiled rice grain by the enzyme treatment method. Arrows, DNAs proliferated by PCR; 1, Kirara397; 2, Yukinosei; 3, Haenuki; 4, Tsugaruroman; and 5, Koshihikari. The primers used were WKA9, S13, G22, and A6.

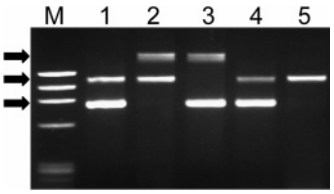

Figure 6. Differentiation of Japanese rice cultivars by PCR using template DNA extracted from the rice cake by the use of enzyme treatment method. M, DNA molecular weight marker; 1, Himenomochi; 2, Hiyokumochi; 3, Mangetsumochi; 4, Minenoyukimochi; and 5, Miyakoganemochi. Template DNAs were prepared from rice cake by the enzyme treatment method. The primers used were WKA9, E30, and F6.

Rice is markedly diversified from the viewpoint of genetics, morphology, and properties. On the contrary, high-quality rice is closely related by inbred breeding to attain high palatability, high processing suitability, or characteristic aroma, etc. (*38*). Rice grains of famous cultivars are traded or distributed at higher prices as premium rice because of their high palatability, processing suitability, or special aromas, etc. Premium rice grains sell at high prices (*3*, *31*).

In the present study, various rice samples were collected from different districts in the world and their chemical and physical properties were measured. As described in Results, the grain properties between Indica subspecies, such as IR2061, and

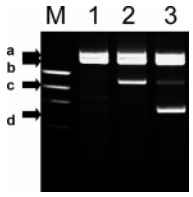

Figure 7. Differentiation of Japanese rice cultivars by PCR using template DNA extracted from the rice cracker by the use of enzyme treatment method. M, DNA molecular weight marker; 1, Wataboshi; 2, Mochihikari; and 3, Mangetsumochi. Template DNAs were prepared from rice cracker by the enzyme treatment method. The primers used were a, S13; b, WKA9; c, F6; and d, E30.

Japonica subspecies, such as Hinohikari, were revealed to be very different, but those among the same subspecies are not so different.

Development of SCAR Markers. As shown in **Table 1** and **Figure 1**, SCAR markers are more useful than RAPD markers (*3*, *37*) because they are clearer in electrophoregrams (*18*, *19*). Furthermore, it is possible to use several SCAR markers simultaneously in PCR, which leads to time and labor savings. As shown in **Table 2**, several new kinds of SCAR markers were developed. The length of markers was adjusted so that the transition temperatures of the markers were around same temperature, 62 °C. Therefore, several SCAR markers can be used simultaneously in multiplex PCR.

Multiplex Primer Set, Koshihikari Positive Kit. As shown in **Figure 2**, the multiplex primer set for PCR, Koshihikari Positive Kit, is very useful to differentiate various rice cultivars from different districts in the world. It was shown that RAPD analysis is a useful tool in determining the genetic relationships among rice cultivars (*12*−*17*). Nevertheless, it is necessary to perform many PCRs and electrophoreses to distinguish rice cultivars by RAPD or SSR markers because those markers are difficult to use simultaneously in the same experiment. It became possible to save experimental time and costs by the use of multiplex primer set based on SCAR markers.

Figure 2 is an example that the multiplex SCAR marker set is useful to differentiate various rice cultivars by a single-time PCR and electrophoresis. Three DNA bands appeared clearly in the case of Koganemochi, a premium Japonica waxy rice, and three other ones appeared clearly in the case of Hinohikari, a premium nonglutinous Japonica rice. As the three DNA band patterns of Hinohikari are different with those of Ilpum, a high-quality Japonica rice in Korea, these three SCAR markers amplified by the STS primer set are very useful for the differentiation among the high-quality Japonica rice cultivars in Asian countries.

Furthermore, Nanjing 11, a typical Indica nonglutinous rice, showed the three different DNA bands, which are useful to differentiate it from the other Indica rice, IR2061. This STS primer set would be useful for differentiation among the Indica rice cultivars.

Differentiation of Japanese Domestic Japonica Rice Cultivars and Digitizing of the Results of PCR. If we digitize the results of PCR to 1 or 0 in the case of appearance or disappearance of the amplified DNA band after PCR, it becomes simple to judge the cultivar name and easy to sort and compare the data of PCR in a DATA base for germplasm.

STS Primer Set for Differentiation of Koshihikari. In Japan, the leading variety Koshihikari shares more than one-third of the total cultivation area of rice because it is palatable and traded at a higher price than other rice cultivars. As the premium rice Koshihikari sells at a high price, mislabeling "Koshihikari 100%" occurs by dishonest rice retailers in Japan (*3*).

Wholesalers and retailers are obliged, in Japan, to state the name of the rice cultivar, location of the cultivation, and year of rice production on the package of rice by the Japan Agricultural Standard (JAS) Act. Therefore, it was necessary to develop the technology to identify the Koshihikari cultivar by DNA analysis.

As shown in **Figure 3**, Koshihikari (lane 41) can be differentiated from any other Japanese Japonica cultivars using this STS primer set for differentiation, which generated three specific DNA bands for Koshihikari. There is no other cultivar rice that shows the same three-band pattern other than Koshihikari except for KoshihikariniigataBLs (near-isogenic blast resistant cultivars of Koshihikari) among 50 dominant nonglutinous rice cultivars in Japan.

In our former report, all of the Koshihikari from different districts showed the same pattern of three DNA bands, which corresponded to the same grouping under the Japanese Seeds and Seedling Law based on representative characteristics of rice plant (*3*). On the contrary, no cultivars other than Koshihikari showed the same DNA patterns with Koshihikari or even Hitomebore or Hinohiari, which are descendant cultivars of Koshihikari (*3*).

SSR markers are useful not only to characterize the relationship between heterosis and marker genotype heterozygosity but also to identify chromosome segments that may have significant effects on yield and its component traits in rice (*20*−*26*). SNPs were also used for the discussion on the sequence variations between the rice cultivars (*27*, *28*). Nevertheless, multiplex primer sets using SCAR markers were revealed to be more useful and practical for efficient and precise cultivar differentiation.

Novel PCR Primers for the Differentiation of KoshihikariBL from Koshihikari. In the present study, the Koshihikari differentiation primer set was shown to be useful to differentiate Koshihikari from almost all of the other Japanese newly bred Japonica rice cultivars. However, there were some problems with this primer set because KoshihikariniigataBLs have been bred and these BL cultivars are very similar in their DNA sequences because they were bred through the 5−10× back-crossings with the Koshihikari in order to resemble Koshihikari in their qualities except for the blast resistance (*32*).

For the purpose of differentiation of newly bred KoshihikariniigataBLs from Koshihikari, we developed three new PCR primers, such as G49A, G81, and WKA9L, as shown in **Table 2**. These three primers are very useful to differentiate the closely related three KoshihikariBL cultivars from the original Koshihikari as shown in **Figure 4**. Although it is impossible to differentiate them by the Koshihikari identification kit as shown in **Figure 3**, it became possible to discriminate these three near-isogenic cultivars by the development of the new PCR primers as shown in **Figure 4**.

Enzyme Treatment Method for Preparation of Template DNA from Processed Rice Products. In the case of boiled white rice grain, gelatinized rice starch and heat-denatured proteins inhibit the extraction of DNA. Therefore, it is necessary to remove these starch and proteins without the damage or decomposition of DNAs themselves. The high temperature of 80 °C during the starch decomposition by the heat-stable α-amylase and coexistence of SDS during the protein digestion are meaningful to inhibit the activity of endogenous DNase.

Although the commercial DNA extraction kit is very useful to prepare the template DNAs from rice leaves, it is not suitable for boiled rice as described in our former report (*29*). In the case of the CTAB method, it is possible to prepare purified DNAs, but it was inferior to the enzyme treatment method in terms of yield of DNAs as described in our former study (*29*).

Lysozyme is sometimes used for DNA preparation (*39*). However, lysozyme mainly decomposes the cell wall, and it is difficult to digest the gelatinized starch and denatured proteins.

We developed the new primer set for the differentiation of the material rice cultivars used for commercially boiled rice products as shown in **Figure 5**. It became possible to apply the PCR method to the commercial rice products.

It became possible to proliferate specific DNAs by PCR using template DNA prepared by the enzyme treatment method, and the multiplex primer set is very useful to differentiate five kinds of rice cultivars by a single PCR and electrophoresis as shown in **Figure 5**.

Multiplex PCR for Identification of Material Rice of Rice Cake. The enzyme treatment method is also useful for the preparation of template DNAs from rice cake (*30*). The solubility of rice cake in boiling water and expansion on heating varies depending on cultivar and the producing area of the material glutinous rice (*40*). Therefore, identification of cultivars of the material glutinous rice using rice cake as a sample is very important.

As shown in **Figure 6**, it became possible to differentiate five kinds of material waxy rice cultivars by the single PCR and electrophoresis by the development of a novel primer set for multiplex PCR to identify waxy rice cultivars.

Multiplex PCR for Identification of Material Rice of Rice Cracker. The enzyme treatment method is also useful for the preparation of template DNAs from rice crackers. Rice crackers are made from rice cakes by roasting the rice cake dough at about 230 °C. Therefore, identification of cultivars of the material glutinous rice using rice crackers as samples was very difficult.

As shown in **Figure 7**, it became possible to differentiate three kinds of material waxy rice cultivars by the single PCR and electrophoresis by the development of an enzyme treatment method for the preparation of template DNA and the design of the novel primer set for multiplex PCR to identify waxy rice cultivars.

Meaning of the Development of SCAR Markers for Multiplex Primer Sets and Enzyme Treatment Method for DNA Preparation from the Processed Rice Products. In conclusion, it became possible to identify or differentiate rice cultivars by PCR. A practical primer set for Koshihikari, the dominant cultivar in Japan, was developed. The merit of this method is to identify or differentiate the dominant cultivar by a single PCR without time-consuming RAPD analysis or elaborate electrophoresis for SSR analysis. The results were the same with ones based on the plant morphological characteristics, such as grain shape, plant height, color of leaves, etc.

Furthermore, it became possible to differentiate the blast resistant near-isogenic KoshihikariBL from the original Koshihikari by a single multiplex PCR. It became possible to use not only raw rice grains but also processed rice products, such as boiled rice or rice cakes, as materials for cultivar identification by the PCR method by the development of an enzyme treatment method. The interference of DNA extraction by the gelatinized starch and denatured proteins of the processed rice products is removed by the decomposition of starch and proteins. Heat-stable α-amylase was very useful because the high temperature of 80 °C inhibits the DNase activities during the starch decomposition and SDS is useful to prohibit the DNase during the protein digestion. Single boiled rice grains of commercial rice products or rice cakes can be used as samples for the identification or differentiation of material rice cultivars by the PCR method.

ABBREVIATIONS USED

STS, sequence-tagged site; PCR, polymerase chain reaction; CTAB, cetyl trimethyl ammonium bromide; PCI, phenol/chloroform/*iso*-amyl alcohol; JAS LAW, law concerning standardization and proper labeling of agricultural and forestry products in Japan; SCAR, sequence-characterized amplified region; RAPD, random amplified polymorphic DNA.

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LITERATURE CITED

(1) Childs, N. W. Production and utilization of rice. In *Rice*, 3rd ed.; Champagne, E. T., Ed.; American Association of Cereal Chemists: St. Paul, MN, 2004; pp 1−23.

(2) Bergman, C. J.; Bhattacharya, K. R.; Ohtsubo, K. Rice end-use quality analysis. In *Rice*, 3rd ed.; Champagne, E. T., Ed.; American Association of Cereal Chemists: St. Paul, MN, 2004; pp 415−472.

(3) Ohtsubo, K.; Nakamura, S.; Imamura, T. Development of the primer sets for identification of the cultivar, Koshihikari, by PCR. *Nippon Nogeikagaku Kaishi* **2002**, *76* (4), 388−397 (in Japanese).

(4) Jeffreys, A. J.; MacLeod, A.; Tamaki, K.; Neil, D. L.; Monckton, D. G. Minisatellite repeat coding as a digital approach to DNA typing. *Nature* **1985**, *316*, 76−79.

(5) Virk, P. S.; Ford-Lloyd, B. V.; Jackson, M. T.; Newbury, H. J. Use of RAPD for the study of diversity within plant germplasm collections. *Heredity* **1995**, *74*, 170−179.

(6) Helentjaris, T.; King, G.; Slocum, M.; Siedenstrang, C.; Wegman, S. Restriction fragment polymorphism as probes for plant diversity and their development as tools for applied plant breeding. *Plant Mol. Biol.* **1985**, *5*, 109−118.

(7) Tanksley, S. D.; Young, N. D.; Paterson, A. H.; Bonierbale, M. W. RFLP mapping in plant breeding: New tools for an old science. *Bio/Technology* **1989**, *7*, 258−264.

(8) Wang, Z. Y.; Tanksley, S. D. Polymorphism and phylogenetic relationships among species in the genus Oryza as determined by analysis of nuclear RFLPs. *Theor. Appl. Genet.* **1992**, *83*, 565−581.

(9) Zang, Q.; Saghai, M. M. A.; Lu, T. Y.; Shen, B. Z. Genetic diversity and differentiation of Indica and Japonica rice detected by RFLP analysis. *Theor. Appl. Genet.* **1992**, *83*, 495−459.

(10) Williams, J. G.; Kubelik, A. R.; Livak, K. J.; Rafalski, J. A.; Tingey, S. V. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **1990**, *18*, 6531−6535.

(11) Fukuoka, S.; Hosaka, K.; Kamijima, O. Use of random amplified polymorphic DNAs for identification of rice accessions. *Jpn. J. Genet.* **1992**, *67*, 243−252.

(12) Yu, L.-X.; Nguyen, H. T. Genetic variation detected with RAPD markers among upland and lowland rice cultivars. *Theor. Appl. Genet.* **1994**, *87*, 668−672.

(13) Wang, G.; Castiglione, S.; Zhang, J.; Fu, R.; Ma, J.; Li, W.; Sun, Y.; Sala, F. Hybrid rice (*Oryza sativa* L.) identification and parentage determination by RAPD fingerprinting. *Plant Cell Rep.* **1994**, *14*, 112−115.

(14) Ko, H. L.; Cowan, D. C.; Henry, R. J.; Graham, G. C.; Blakeney, A. B.; Lewin, L. G. Random amplified polymorphic DNA analysis of Australian rice (*Oryza sativa* L.) varieties. *Euphytica* **1994**, *80*, 179−189.
(15) Mackill, D. J. Classifying Japonica rice cultivars with RAPD markers. *Crop Sci.* **1995**, *35*, 889−894.
(16) Ishii, T.; Nakano, T.; Maeda, H.; Kamijima, O. Phylogenetic relationships in A-genom species of rice as revealed by RAPD analysis. *Genes Genet. Syst.* **1996**, *71*, 195−201.
(17) Cao, D.; Oard, J. H. Pedigree and RAPD-based DNA analysis of commercial U.S. Rice cultivars. *Crop Sci.* **1997**, *37*, 1630−1635.
(18) Talbert, L. E.; Blake, N. K.; Chee, P. W.; Blake, T. K.; Magyar, G. M. Evaluation of "sequence-tagged-site" PCR products as molecular markers in wheat. *Theor. Appl. Genet.* **1994**, *87*, 789−794.
(19) Jain, A.; Ariyadasa, R.; Kumar, A.; Srivastava, M. N.; Mohan, M.; Nair, S. Tagging and mapping of a rice gall midge resistance gene, Gm8, and development of SCARs for use in marker-aided selection and gene pyramiding. *Theor. Appl. Genet.* **2004**, *109*, 1377−1384.
(20) Wu, K. S.; Tanksley, S. D. Abundance, polymorphism and genetic mapping of microsatellites in rice. *Mol. Gen. Genet.* **1993**, *241*, 225−235.
(21) Zhao, X.; Kochert, G. Phylogenetic distribution and genetic mapping of a $(GGC)_n$ microsatellites from rice (*Oryza sativa L.*). *Plant Mol. Biol.* **1993**, *21*, 607−614.
(22) Yang, G. P.; Maroof, M. A.; Xu, C. G.; Zhang, Q.; Biyashev, R. M. Comparative analysis of microsattelite DNA polymorphism in landraces and cultivars of rice. *Mol. Gen. Genet.* **1994**, *245*, 187−194.
(23) Davierwala, A. P.; Chowdari, K. V.; Kumar, S.; Reddy, A. P. K.; Ranjekar, P. K.; Gupta, V. S. Use of three different marker systems to estimate genetic diversity if Indian elite rice varieties. *Genetica* **2000**, *108*, 269−284.
(24) Bligh, H. F. J.; Blackhall, N. W.; Edwards, K. J.; McClung, A. M. Using amplified fragment length polymorphisms and simple sequence length polymorphisms to identify cultivars of brown and white milled rice. *Crop Sci.* **1999**, *39*, 1715−1721.
(25) Bautista, N. S.; Solis, R.; Kamijima, O.; Ishii, T. RAPD, RFLP and SSLP analyses of phylogenetic relationships between cultivated and wild species of rice. *Genes Genet. Syst.* **2001**, *76*, 71−79.
(26) Yu, G. Q.; Bao, Y.; Shi., C. H.; Dong, C. Q.; Ge, S. Genetic diversity and population differentiation of Liaoning weedy rice detected by RAPD and SSR markers. *Biochem. Genet.* **2005**, *43*, 261−270.
(27) Shen, Y. J.; Jiang, H.; Jin, J. P.; Zhang, Z. B; Xi, B.; He, Y. Y.; Wang, G; Wang, C.; Qian, L.; Li, X.; Yu, Q. B.; Liu, H. J.; Chen, D. H.; Gao, J. H.; Huang, H.; Shi, T. L.; Yang, Z. N. Development of genome-wide DNA polymorphism database for map-based cloning of rice genes. *Plant Physiol.* **2004**, *135*, 1198−1205.
(28) Shirasawa, K.; Monna, L.; Kishitani, S.; Nishio, T. Single nucleotide polymorphisms in randomly selected genes among japonica rice (*Oryza sativa* L.) varieties identified by PCR-RF-SSCP. *DNA Res.* **2004**, *11*, 275−283.
(29) Ohtsubo, K.; Nakamura, S.; Morooka, H.; Fujii, T.; Fuse, T.; Kawasaki, S. Identification of domestic rice cultivars by RAPD method using a single grain of cooked rice as a sample. *Nippon Shokuhin Kagaku Kogaku Kaishi* **1999**, *46*, 262−267 (in Japanese).
(30) Ohtsubo, K.; Nakamura, S.; Yoza, K.; Shishido, K. Identification of glutinous rice cultivars using rice cake as samples by the PCR method. *Nippon Shokuhin Kagaku Kogaku Kaishi* **2001**, *48* (4), 306−310 (in Japanese).
(31) Nakamura, S.; Okadome, H.; Yoza, K.; Haraguchi, K.; Okunishi, T.; Suzuki, K.; Satoh, H.; Ohtsubo, K. Differentiation and search for palatability-factors of world-wide rice grains by PCR method. *Nippon Nogeikagaku Kaishi* **2004**, *78* (8), 764−779 (in Japanese).
(32) Nakamura, S.; Suzuki, K.; Ban Y.; Nishikawa, T.; Tokunaga, K. Development of a PCR-based marker set for identifying Koshihikari Niigata BLs, Near-isogenic lines harboring rice blast resistance genes. *Ikusyugaku Kenkyu* **2006**, *8* (3), 79−87 (in Japanese).
(33) Juliano, B. O. A simplified assay for milled−rice amylose. *Cereal Sci. Today* **1971**, *16*, 334−340, 360.
(34) AOAC. *Protein (Total) in Flour, Final Action*; AOAC Official Method 920.87; AOAC: Gaithersburg, MD, 1995.
(35) Okadome, H.; Toyoshima, H.; Ohtsubo, K. Multiple measurements of physical properties of individual cooked rice grains with a single apparatus. *Cereal Chem.* **1999**, *76*, 855−860.
(36) Rogers, S. O.; Bendich, A. J. Extraction of DNA from plant tissues. *Plant Molecular Biology Manual A6*; Kluwer Academic Publishers: Dordrecht, Belgium, 1988; pp 1−10.
(37) Stewart, C. N., Jr.; Via, L. E. A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR application. *Biotechniques* **1993**, *14*, 748−750.
(38) Ohtsubo, K.; Toyoshima, H.; Okadome, H. Quality assay of rice using traditional and novel tools. *Cereal Foods World* **1998**, *43*, 203−206.
(39) Summerton, J. T.; Bestwick, A. R. A rapid method for preparation of bacterial plasmids. *Anal. Biochem.* **1983**, *133*, 79−84.
(40) Yanase, H.; Ohtsubo, K.; Hashimoto, K. Studies on quality and processing suitability of glutinous rice. *Rep. Natl. Food Res. Inst.* **1984**, *45*, 1−8 (in Japanese with an English summary).

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