Review

Cultivar Identification of Rice (*Oryza sativa* L.) by PCR Method and its Application to Processed Rice Products

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Abstract: As the cultivars of rice affect markedly eating quality, processing suitability and price, identification or differentiation of rice cultivars is very important. The present authors developed suitable STS (sequence-tagged-site) primers for PCR (Polymerase Chain Reaction) and it became possible to identify rice cultivars using template DNA extracted and purified from rice grains. A multiplex primer set was shown to be useful to differentiate effectively rice cultivars produced in various countries by PCR. Two kinds of multiplex kit for identification of Koshihikari, the dominant cultivar in Japan, have been developed. The application of the cultivar identification method by PCR to processed rice products was investigated. The present authors developed an “enzyme treatment method,” in which the gelatinized starch is decomposed by the heat-stable α -amylase at 80°C, followed by hydrolysis of proteins by proteinase K with SDS and purification of extracted DNAs by PCI (Phenol/Chloroform/iso-amyl alcohol). It became possible to identify the material rice cultivars of the processed rice products, such as cooked rice and rice cake, by the PCR method using template DNA prepared by the “enzyme treatment method.”

Key words: rice, DNA, cultivar identification, PCR, processed rice product

Rice is one of the most important edible crops in the world, along with wheat and corn. There are more than several tens of thousands of varieties or cultivars of rice in the world. Rice is markedly diversified from the viewpoint of genetics, morphology and properties. On the other hand, high-quality rice is closely related by inbreeding to attain high-palatability, high processing suitability, characteristic aroma, *etc.*

Rice grains of the famous cultivars are traded or distributed at higher prices as “premium rice” than the ordinary rice because of their high palatability, processing suitability, special aroma, *etc.* As those premium rice grains sell at high price, some dishonest rice wholesalers or retailers blend low quality cheap rice with high quality premium rice and mislabel it as “high-quality premium rice.” Recently, rice consumers have tended to demand more information, such as cultivar name, location of production, year of production, *etc.*, about rice which they purchase. For example, it is required under the JAS LAW (Law Concerning Standardization and Proper Labeling of Agricultural and Forestry Products in Japan) to display the name of rice cultivar on the package. Therefore, technology to identify rice cultivars is very important for breeders, farmers, inspectors, wholesalers, retailers, food industries and consumers.

Rice cultivars have been identified on the basis of morphological characteristics of rice plants or grains, ripening ratio after crossing, isozyme patterns, *etc.* Cultivated rice (*Oryza sativa* L.) is one of the most polymorphic crop species in the world. In contrast, many cultivars of high-quality rice or premium rice are closely related because inbred lines are often used for their development. Therefore, it is necessary to develop a time-saving technology to differentiate rice cultivars clearly and precisely.

DNA fingerprinting was developed in 1985⁽¹⁾ and is used for criminal investigation and trial at court. Recently, a novel cultivar identification method based on DNA polymorphism has been developed accompanied with the progress in molecular biology. DNA-based markers have the obvious advantage of sampling the genome directly and RFLP analysis has been widely used for assessing variation of plants.⁽²⁻⁴⁾ RFLP analysis has been used to distinguish between species of *Oryza*⁽⁵⁾ and particularly between *indica* and *japonica* types of *Oryza sativa*.⁽⁶⁾

Recently, a PCR-based marker system has been developed by Williams *et al.*⁽⁷⁾ In this RAPD (Random Amplified Polymorphic DNA) method, short oligo-nucleotides 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.^(8,9) RAPD analysis was revealed to be reproducible and amenable for identification of each single plant line of F1 hybrid rice in China⁽¹⁰⁾ and Australian rice cultivars.⁽¹¹⁾ Diversified rice cultivars were classified into separate groups by PCR using RAPD markers, but many primers were needed to resolve closely

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Abbreviations: STS, sequence-tagged-site; PCR, polymerase chain reaction; CTAB, cetyl tri-methyl 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.

related *japonica* cultivars.^{12–14)}

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 STS (sequence tagged site) markers or SCAR (sequence characterized amplified region) markers based on RAPD markers.

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.^{15,16)} 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.¹⁷⁾

Genetic diversity among Indian elite rice varieties was evaluated using three different types of DNA markers and parentage analysis.¹⁸⁾ SSLP was reported to be more reliable than AFLP for identifying rice cultivars.¹⁹⁾ RAPD, RFLP, nuclear SSLP and chloroplast SSLP analyses were carried out to clarify the phylogenetic relationships among A-genome species of rice.²⁰⁾ SSR was reported to provide useful genetic information on weedy rice.²¹⁾

Development of a genome-wide DNA polymorphism database for map-based cloning of rice genes was investigated using SNP markers.²²⁾ SNPs were also used for the discussion on the sequence variations between the rice cultivars.²³⁾

Although SSR and SNPs are very useful DNA markers, it is impossible to use several PCR primers, or “multiplex PCR,” simultaneously to simplify the PCR and electrophoresis and save time. Furthermore, no report has claimed to identify or differentiate the material rice cultivars by PCR method using the processed rice products, such as boiled rice or rice cakes.

The present authors have investigated cultivar identification of rice by the PCR method.^{24–28)}

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

An efficient method to prepare the template DNA for PCR from the processed rice products, such as cooked rice or rice cakes, was investigated. The identification or the differentiation of material rice cultivars by multiplex PCR was carried out using processed rice products as samples.

MATERIALS AND METHODS

Materials. Fifteen various kinds of rices were collected or purchased. Sample rice grains used are as follows; Koganemochi (Japan), Hinohikari (Japan), Akitakomachi (Japan), Kirara397 (Japan), Ilpum (Korea), Calmochi101 (USA), Medium grain (USA), Forbidden rice (USA), Pelde (Australia), Kyeema (Australia), Paellea (USA), Long grain (USA), Doongara (Australia), Nanjing

11 (China), IR2061 (Philippines).

Thirty three original Koshihikari rice seeds, produced in 1999, 2000 and 2001, were used for DNA extraction and purification. All the rice samples including 49 other cultivar rice samples besides Koshihikari were kindly provided by National Food Agency, Japan. The 49 cultivars are Hitomebore, Hinohikari, Akitakomachi, Kirara397, Kinuhikari, Hoshinoyume, Haenuki, Mutsuhomare, Niopponbare, Sasanishiki, Tsugaruroman, Hanaechizen, Yumetsukushi, Hatsushimo, Asanohikari, Tsukinohikari, Aichinokaori, Maturibare, Akiho, Yukimaru, Mutsukaori, Manamusume, Kakehashi, Kiyonishiki, Domannaka, Koshijiwase, Yukinosei, Hohohonoho, Yumeakari, Notohikari, Akitsuho, Akebono, Asahi, Yamahoushi, Yamahikari, Koganenishiki, Koganemasari, Reiho, Mineasahi, Fusao-tome, Karinomai, Dontokoi, Akinishiki, Naganohomare, Fukuhikari, Goropikari, Hatsuboshi, Nakateshinsenbon and Morinokumasan.

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, Tokyo).

Physical and chemical properties of the 20 rice cultivars. Amylose contents were measured by the iodine-colorimetric method using the milled rice flours by the method of Juliano.²⁹⁾ 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.³⁰⁾ Physical properties of the boiled rice grains were measured by the method of Okadome³¹⁾ using a Tensipresser.

Preparation of boiled rice and rice cake. White rice prepared by the experimental rice polisher (Ketto Science Laboratory) was boiled in 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 Corporation, Fort Collins, USA). Thirty-five grams of water was added to 50 g of the rice powder and kneaded manually. A rice cake was prepared using an electric rice cake maker for home use (SMK-1800, Tiger Co., Ltd., Tokyo). The rice cake was lyophilized and pulverized using a coffee-mill (IFM-100) and subjected to DNA extraction.

Extraction and purification of template DNA from milled rice flours. According to the CTAB method,^{32,33)} DNAs of the milled rice flours were extracted. Milled rice flours (0.4 g) were placed in the micro-centrifugal tube (2 mL) and DNAs were extracted into the 0.6 mL of 2 × CTAB (Cetyl trimethylammonium bromide, 2% CTAB, 20 mM EDTA (ethylenediamine-*N,N,N',N'*-tetraacetic acid), 1.4 M NaCl, 0.1 M tris-hydroxyl aminomethane/HCl buffer, pH 8.0) solution and 0.2 mL distilled water for 30 min at 65°C. The solution of chloroform and isoamyl alcohol (24:1, v/v) (0.8 mL) was added and stirred gently for 15 min using a rotator. Thereafter, the solution was centrifuged (8000× *g*, 15 min) in a refrigerated centrifuge (hi-mac CR21F, Hitachi, Hitachi) and the upper

layer was transferred to another micro-tube. CTAB solution (10%, 0.08 mL) and chloroform/isoamyl alcohol (24:1, v/v) were added to the solution and it was stirred gently for 15 min followed by centrifugation ($8000\times g$, 15 min). The upper layer was transferred to another tube and was stood for 5 min in the freezer (-80°C) after the addition of 2.5 volumes of the precipitation buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% CTAB). The precipitate was collected by the centrifugation ($6000\times g$, 15 min) and dissolved in the 0.5 mL of Tris-EDTA buffer (TE) and was added with the same volume of isopropyl alcohol. After the gentle stirring by a rotator for 15 min, the precipitate was collected by the centrifugation ($6000\times g$, 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 ($8000\times g$, 15 min). The same volume of the solution of phenol/chloroform (1:1, v/v) was added to the solution followed by the centrifugation ($8000\times g$, 15 min) and the upper layer was transferred to another tube. The solution was supplemented with 0.2 M NaCl and two volumes of cold ethanol to generate the precipitate of DNAs. The DNAs were washed by the 50 μL of 70% ethyl alcohol 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 boiled rice grains or rice cakes, a different DNA extraction/purification method was developed.^{25,26)} The processed rice product, such as a cooked rice grain, was subjected to the decomposition of starch by heat-stable α -amylase (790 U/mg solid, 30 mg/mL, from *Bacillus licheniformis*, Sigma, USA) for 60 min at 80°C . For the starch digestion, 20 μL of above-mentioned α -amylase (mg/mL) was added to the 1.5 mL of the sample solution (0.1 g of single boiled rice grain in the solution of 50 mM Tris-HCL buffer, pH 8.0) and digestion was performed for 1 h at 80°C . Thereafter, the protein hydrolysis was carried out by proteinase K (Takara-bio Inc., Otsu, 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 with 1% of sodium-dodecylsulphate (SDS). The protein digestion was carried out for 1 h at 55°C . Thereafter, DNAs were 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. For the control, a commercial DNA extraction kit (Isoplant, Nippongene, Tokyo) was used.

Polymerase chain reaction (PCR). DNAs were proliferated by the PCR method using 600 commercial random primers (10 mers or 12 mers) as primers.²⁴⁾ Taq-DNA polymerase (Takara-bio Inc., Otsu, Japan) was used for amplification of DNAs. Each DNA was denatured for 1 min at 94°C , annealed for 1 min at 62°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., Otsu, Japan) was adopted.

Electrophoreses of the amplified DNAs. Proliferated DNAs were subjected to electrophoresis for 30 min through agarose gel (2%) using a Mupid-2 electrophoresis system (Advance, Tokyo, Japan) at the charge of 100 V of direct current. After the electrophoresis, the DNAs were stained with ethidium bromide and detected by the irradiation of UV light.

Development of SCAR markers and multiplex primer sets. In the present study, SCAR markers were developed based on RAPD analysis to differentiate rice cultivars by PCR.²⁵⁻²⁸⁾ DNAs were extracted from the agarose gel after the electrophoresis of the PCR products using Easy trapTM (Takara-bio Inc., Otsu, Japan). DNA cloning was carried out using a TOPO XL PCR Cloning KitTM (Invitrogen Corporation, Carlsbad, USA). DNA sequence was determined using a commercial DNA preparation kit (QIAprep Spin Miniprep kit, Qiagen, K.K., Tokyo) and DNA proliferation kit (Big Dye Terminator Cycle sequencing kit, V1-1, Applied Biosystems Japan, Tokyo) and automatic DNA sequencing system (DNA sequencer ABI PRISM Genetic Analyzer 310, Applied Biosystems Japan, Tokyo). SCAR markers were designed based on the DNA sequences with from 20 mers to 29 mers so that the transition temperatures are around 62°C . The combinations of these SCAR markers, the "multiplex primer sets," were developed to identify Koshihikari, the most dominant rice cultivar in Japan.

RESULTS

Development of SCAR markers.

An example of the development of SCAR markers for identification or differentiation of 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, about 20 mers, were designated as shown in Table 1.^{27,28)}

The electrophoregrams after PCR using RAPD marker, B43, and SCAR marker, B43, are shown in Fig. 1. Compared with the RAPD marker, the SCAR marker is more useful because only the differential DNA is amplified, which makes it easy and accurate to differentiate the rice cultivar.

Developed SCAR markers for identification or differentiation of 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.²⁸⁾ The results are shown in Table 3. As shown in Table 3, Indica subspecies, such as IR2061 and Nanjing 11, showed high amylose content and less adhesiveness; in contrast, Japonica sub-species, such as Hinohikari and Akitakomachi, revealed low amylose content and more adhesiveness.

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.³²⁻³⁴⁾ The result of PCR using 15 rice cultivars from

Table 1. Sequence of DNA amplified by the use of primer B43.

1	TGGCCGGCATGACTCACATACCCAACATATAGCATATCTGGATGTCTGTT	50
51	TGCAATCATGTCGGGTGATCGAGCCAACAACGTTTTCGAGGAATTTATCC	100
101	GTTAACGACATATTCTCCAATAGTTCCGACATGGCCGGCGCTATACATGA	150
151	GATAAGTCGTCAGTCTTCATTGGTAGGTTGGGTGCGACAACCTCAGCCTTT	200
201	ATCGAGAATGTTCTTGACAATGGGGTGTACTCGACCACAACCTACTCGAG	250
251	TGCTCAATTGTTCTGAAAAATATTTTCTAGAAGACAAGATATATAGCAG	300
301	ATAGTATCGAGCATATACTCAAAAAGACTGCATGAATCTTCTATTATTATC	350
351	ATGATATAACGAACAGATTAAATATCATACATTATGTAAACCATAAGTAA	400
401	GCATGATTATACATAAAGAAAATAAAGCGAGCCCCCAGTATTAACCGTT	450
451	GTTGACCTAATATAGGGAATGCTCACACAATAGATATTGTATAGAAAACA	500
501	TGCTCTAGGTAAACATAATAAATTTGAAATCTGACAATATCGTATTTATC	550
551	TGAAGAATAGGTACGATAAATCACATATAAAATATTGCGTAGCTTTGTAG	600
601	ATACATGTAGGATGTATCTACGAACGAAATTAGTAGGTCAACTTAAAAAA	650
651	TTAATCTACTAATTACCTTTCTGACATGTGTATTCCGAGTAGATTAGATC	700
701	GATGCAATCATACTTCTCCAAGTGACAGCCGCATGTATGCAGTAAACAC	750
751	ATGCACTTGCTGTTAATTGATCTCATGCATGCACATATTGACGTCATCCA	800
801	AATTGGTTGGTCTTGATGCCGGCCAGT	837

Underlined sequences were used as primers for PCR.

Fig. 1. Comparison of RAPD and SCAR markers in PCR and electrophoresis.

M, DNA molecular weight markers (Marker 4, Wako Pure-chemical Co., Ltd.); 1, Hinohikari; 2, Haenuki; 3, Kinuhikari; 4, Akitakomachi; 5, Kirara397; 6, Koshihikari. Arrow, differential DNA. (a) RAPD marker (B43, Operon Co., Ltd.), (b) SCAR marker (B43 in Table 1).

various countries, such as the USA, India, Thailand or Japan, as samples is shown in Fig. 2. The primer set used for PCR was our commercial Koshihikari Positive Kit (Takara-bio Inc.). Four DNA bands appeared clearly in the case of Hitomebore and three appeared clearly in the case of Koshihikari, which were revealed to be useful to differentiate various rice cultivars by a single time PCR and electrophoresis.

Primer set for Koshihikari identification.

As shown in Fig. 3, Koshihikari can be discriminated from any other cultivars using this “primer set for identification” making three specific DNA bands.²⁷⁾ There is no other cultivar rice which shows the same three-band pat-

Table 2. Sequences of SCAR markers.

Primer	F/R	Sequences
B1	F	GTTTCGCTCCTACAGTAATTAAGGG
	R	GTTTCGCTCCCATGCAATCT
B7	F	CAGGTGTGGGTTACAAGGATGA
	R	CAGGTGTGGGTTCACGGCCTTT
B43	F	TGGCCGGCATGACTCAC
	R	ACTGGCCGGCATCAAGAC
B43a	F	TGGCCGGCATGACTCACA
	R	ACTGGCCGGCATCAAGA
E30	F	TACCTGGTTGATGTATACAGATCTGGTT
	R	ATCCCTCGATCCCTCTAGCATTAT
F6	F	ACCACTCCATATATATCATCCAAAG
	R	ACCACTCCATATCACCACAAGG
G22	F	CTCACTCAAATTTACAGTGCATTTTCTTG
	R	AGGGCCATGATACAAGACTCTGT
M2CG	F	ACAACGCCTCCGATGA
	R	ACAACGCCTCCGACAACAAGAT
M11	F	GTCCACTGTGACCACAACAT
	R	GTCCACTGTGGGGATTGTTC
S13	F	GTCGTTCTGTGGTTAGGACAGGGT
	R	GTCGTTCTGTGGTGTCTCAGAT
Wka9	F	CCCGCAGTTAGATGCACCATT
	R	CCGCAGTTAGATCAAGTGGC

F, forward; R, reverse.

Table 3. Proximate components and physical properties of various cultivar rice.

Cultivar	Amylose (%)	Protein (%)	Physical properties	
			Adhesion	Consistency
Koganemochi	0.0	6.1	2.12	15
Hinohikari	16.3	5.6	1.65	92
Akitakomachi	18.0	5.3	1.32	116
Kirara397	19.9	6.5	1.54	106
Ilpum	16.9	5.9	1.07	104
Calmochi101	0.9	5.6	2.00	44
Medium grain	17.6	6.1	0.68	125
Forbidden rice	17.1	7.4	0.58	127
Pelde	18.6	7.2	0.39	110
Kyeema	18.9	7.8	0.59	143
Paellea	20.4	6.9	0.51	139
Long grain	15.4	7.0	0.31	207
Doongara	24.7	8.8	0.32	185
Nanjing11	27.8	11.0	0.19	190
IR2061-214	32.0	9.0	0.11	180

Adhesion was measured by a Tensipresser and the unit is mm. Consistency was measured by a Rapid Visco Analyser and the unit is RVU.

tern as Koshihikari among 50 dominant non-glutinous rice cultivars in Japan (Fig. 3A). On the other hand, all Koshihikaris of the different prefectures showed the same DNA patterns (Fig. 3B).

Primer set for differentiation of other rice with Koshihikari.

As shown in Fig. 4, no DNA is proliferated in the case

Fig. 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, Paellaa; 12, Long grain; 13, Doongara; 14, Nanjin11; 15, IR2061. Marker a, Wka9; b, B43; c, M11; d, G22.

A

of all Koshihikari rice grains of various prefectures (Fig. 4B), and multiple DNA bands never fail to increase by PCR in the case of rice grains of other cultivars (Fig. 4A).²⁷⁾

Novel DNA extraction/purification method from processed rice products.

Comparison of template DNAs prepared by the three methods, the commercial kit method, the CTAB method and the “enzyme treatment method,” was carried out.²⁵⁾ The quantity and quality of the template DNAs are shown in Table 4 and Fig. 5. The “enzyme treatment method” was shown to be profitable from the viewpoint of quality and quantity.

Differentiation of material rice cultivars of boiled rice by PCR.

In the case of boiled white rice grains, as shown in Fig. 6, it became possible to differentiate Hitomebore or Hinohikari from Koshihikari, their parental cultivar by PCR using the template DNA prepared from each single grain of boiled rice by the “enzyme treatment method,” which was described in MATERIALS AND METHODS.²⁵⁾

Differentiation of material rice cultivars of rice cake by PCR.

The results of PCR using template DNAs prepared from each rice cake by the “enzyme treatment method”

B

Fig. 3. Primer set for identification of Koshihikari.

(A) Comparison of DNA patterns between Koshihikari and other rice cultivars. No. 1, Koshihikari; Nos. 2–50, Other cultivars. (B) Results of Koshihikaris of different prefectures. A, Hitomebore; B, Hinohikari; C, Akitakomachi. Used primers are Wka9, B43, M11 and G22.

Fig. 4. Primer set for detection of other cultivar rice grains from Koshihikari.

(A) Comparison of DNA patterns between Koshihikari and other rice cultivars. No.1, Koshihikari (No DNA is proliferated by PCR.); Nos. 2–50, Other cultivars (DNA band appears with PCR). (B) Results of Koshihikaris of different prefectures. A, Hitomebore; B, Hinohikari; C, Kirara397; D, Mutsuhomare; Used primers are Wka9, S13, F6 and E30.

Table 4. DNA extraction by the different methods.

Cultivar	Enzyme method		CTAB method		Commercial kit (Isoplant)	
	Extracted DNA (μg)	OD260/OD280	Extracted DNA (μg)	OD260/OD280	Extracted DNA (μg)	OD260/OD280
Koshihikari	9.89	1.84	3.10	1.95	7.08	2.06
Hitomebore	8.67	1.80	1.97	1.97	11.88	2.01
Akitakomachi	11.63	1.94	3.79	2.17	10.31	2.10
Sasanishiki	10.70	1.84	3.69	2.03	9.18	2.10
Nipponbare	14.04	1.88	4.96	1.89	8.03	1.99
Hinohikari	11.50	1.76	3.68	2.17	8.57	2.06
Yukihikari	9.80	2.02	4.25	2.12	9.65	1.74
Kirara397	16.70	1.88	5.27	2.09	10.31	2.10
Mutsuhomare	11.57	1.97	4.20	2.17	7.94	2.20
Kinuhikari	8.46	1.73	2.96	2.05	9.15	2.10
Average	11.30	1.87	3.81	2.06	9.21	2.06
SD	2.38		0.95		1.33	

were same as those using template DNAs from material rice flour directly by the CTAB method, as shown in Fig. 7.²⁶⁾

DISCUSSION

Properties of rice grains of various rice cultivars.

In the case of trade contract, inspection or grading or the survey of traceability of rice grains, the PCR method must be applied using rice grains as samples. Shoots 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 materials for PCR in the post-harvest inspection.

Rice is markedly diversified from the viewpoint of genetics, morphology and properties. On the other hand, high-quality rice is closely related by inbreeding to attain high-palatability, high processing suitability, characteristic aroma, *etc.*

Rice grains of the famous cultivars are traded or distributed at higher prices as “Premium rice” because of their high palatability, processing suitability, special aroma, *etc.*^{34,35)}

In the present study, various rice samples were col-

Fig. 5. Ultra-violet absorbance spectra of DNAs prepared by the different methods.

—, Enzyme method; ----, CTAB method; kit method.

Fig. 6. 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, Koshihikari; 2, Hitomebore; 3, Hinohikari; 4, Mutsuhomare; 5, Kinuhikari. Used primers are M2CG, M11 and B7.

Fig. 7. Differentiation of Japanese rice cultivars by PCR using template DNA extracted from the raw rice flours or the rice cake by the use of "enzyme treatment method."

M, DNA molecular weight marker; 1, Hiyokumochi; 2, Koganemochi; 3, Hakuchomochi; 4, Wataboushi. (A) Template DNAs were prepared from milled rice flours, (B) template DNAs were prepared from rice cake. Used primers are Wka9, E30, G22 and B1.

lected from different district around the world and their chemical and physical properties were measured. As shown in Table 3, the grain properties between Indica subspecies, such as IR2061, and Japonica sub-species, such as Hinohikari, were revealed to be very different, but those among the same sub-species are not different.

Development of SCAR markers.

As shown in Table 1 and Fig. 1, SCAR markers are more useful than RAPD markers^{27,28,34,35} because they are

clearer in electrophoregrams. Furthermore, it is possible to use several SCAR markers simultaneously in PCR, which leads to saving time and labor. As shown in Table 2, several kinds of SCAR markers were developed. The length of markers was adjusted so that the transition temperatures of the markers are around the same temperature, 62°C. Therefore, several SCAR markers can be used simultaneously in "multiplex PCR."

Multiplex primer set, Koshihikari positive kit.

As shown in Fig. 2, the multiplex primer set for PCR, Koshihikari Positive Kit, is very useful to differentiate various rice cultivars from the different districts in the world. It was shown that RAPD analysis is a useful tool in determining the genetic relationships among rice cultivars.⁹⁻¹⁴ 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 experiment time and cost by the use of multiplex primer sets based on SCAR markers.

Figure 2 is an example of how the multiplex SCAR marker set is useful to differentiate various rice cultivars by a single PCR and electrophoresis. Three DNA bands appeared clearly in the case of Koganemochi, a premium Japonica waxy rice, and three others appeared clearly in the case of Hinohikari, a premium non-glutinous Japonica rice. In the case of Nanjing 11, a typical Indica non-glutinous rice, three different bands appeared, which are useful to differentiate it from the other Indica rice, IR2061.

Multiplex primer set, "Detection kit of mislabeling."

In Japan, 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. It is required, in Japan, that the name of the rice cultivar, location of cultivation, and year of rice production are labeled 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 Fig. 3A, Koshihikari can be discriminated from any other cultivars using this "primer set for identification" showing three specific DNA bands.²⁷ There is no other cultivar rice which shows the same three-band pattern as Koshihikari among 50 dominant non-glutinous rice cultivars in Japan. All the Koshihikari from the different districts showed the same pattern of three DNA bands, which correspond to the same grouping under Japanese Seeds and Seedling Law based on representative characteristics of rice plants (Fig. 3B). On the other hand, no cultivars other than Koshihikari showed the same DNA patterns as Koshihikari, not even Hitomebore or Hinohikari, which are descendant cultivars of Koshihikari, as shown in Fig. 3.²⁷

As the premium rice Koshihikari sells at a high price, mislabeling "Koshihikari 100%" occurs with dishonest rice retailers in Japan. For the purpose of detection of rice grains of other cultivars which were dishonestly blended into Koshihikari, a detection-kit was developed. In the

case of the detection-kit, no DNA is proliferated in the case of any Koshihikari rice grains of any prefecture (Fig. 4B), and multiple DNA bands never fail to increase by PCR in the case of rice grains of other cultivars (Fig. 4A). This “primer set for detection” can be used to detect the illegal blending of other rice with Koshihikari labeled as “Koshihikari 100% premium rice.”²⁷⁾

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.^{15–20)} SNPs were used for the discussion on the sequence variations between the rice cultivars.^{22,23)} Nevertheless, “multiplex primer sets” using SCAR markers were revealed to be more useful and practical for efficient and precise cultivar differentiation.

“Enzyme treatment method” for preparation of template DNA from processed rice products.

In the case of boiled white rice grains, gelatinized rice starch and heat-denatured proteins inhibit the extraction of DNA. Therefore, it is necessary to remove these starches and proteins without the damage or decomposition of the 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 a commercial DNA extraction kit is very useful to prepare the template DNAs from rice leaves, it is not suitable for boiled rice as shown in Fig. 5. No amplified DNA bands appeared after PCR in the case of template DNA prepared by the commercial kit.²⁵⁾ 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 the quantity of DNAs, as shown in Table 4.²⁵⁾

Lysozyme is sometimes used for DNA preparation.³⁶⁾ But lysozyme decomposes cell walls mainly, and it is difficult to digest the gelatinized starch and denatured proteins with it.

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 various rice cultivars by a single PCR and electrophoresis, as shown in Fig. 6.²⁵⁾

Multiplex PCR for identification of material rice of rice cakes.

The “enzyme treatment method” is also useful for preparation of template DNAs from rice cakes. The solubility of rice cakes in boiling water and expansion on heating varies depending on the cultivar and production area of the material glutinous rice.³⁷⁾ Therefore, identification of cultivars of the material glutinous rice using rice cakes as samples is very important. The results of PCR using template DNAs prepared from each rice cake by the “enzyme treatment method” were same as those using template DNAs from material raw rice flour directly by the CTAB method, as shown in Fig. 7. It became possible to differentiate each material waxy rice cultivar by a single PCR and electrophoresis by the development of a

primer set for “multiplex PCR” to identify waxy rice cultivars.²⁶⁾

Meaning of the development of scar markers for multiplex primer sets and the “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. It became possible to identify or differentiate the dominant cultivar by a single PCR without time-consuming RAPD analysis or elaborate electrophoresis for SSR analysis. Furthermore, the results were same as those based on the plant characteristics.^{27,35)}

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 the “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.²⁵⁾ A single boiled rice grain or rice cake can be used as a sample for identification or differentiation of material rice cultivars by the PCR method.^{25,26)}

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PCR 法による米 (*Oryza sativa* L.) の品種判別およびその米加工品への応用

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米 (*Oryza sativa* L.) の品種は、食味、加工適性、価格等に影響するので、その判別技術の開発が必要とされている。筆者らは、米粒から鋳型 DNA を抽出・精製する方法および適正な STS プライマーを開発することにより、PCR 法による米の DNA 品種判別を可能にした。マルチプレックスプライマーセットは各国の広範な米の効率的な判別に有用であることを示し、2 種類のコシヒカリ判別用セットを開発した。また、米飯等の米加工品からの PCR 用鋳型 DNA の抽出・精製用に、耐熱性 α -アミラーゼによる 80°C 処理、次に SDS 共存下でのプロテイナーゼ K 処理と PCI 精製処理を行う「酵素法」を開発した。これにより、米飯 1 粒や餅などを試料とする原料米の DNA 品種判別が可能になった。