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Rapid Species Identification of Cooked Poisonous Mushrooms by Using Real-Time PCR▽

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Species-specific identification of the major cooked and fresh poisonous mushrooms in Japan was performed using a real-time PCR system. Specific fluorescence signals were detected, and no nonspecific signals were detected. Therefore, we succeeded in developing a species-specific test for the identification of poisonous mushrooms within 1.5 h.

Mushrooms are identified by morphological characteristics; however, the morphological characteristics are inconsistent and unstable criteria because they are strongly influenced by the environmental conditions (2, 9). For this reason, poisonous wild mushrooms can be misidentified as edible mushrooms, and they can be eaten by mushroom hunters.

In general, rapid identification of poisonous mushroom species eaten by patients is required for proper medical treatment. However, cooked and eaten mushrooms do not retain their original shape. Therefore, techniques such as DNA-based identification, which do not depend on morphology, are required. Identification of wild and cultivated mushrooms and

TABLE 1. Poisonous mushroom strains used in this study

Species	Strain	Source[a]	Accession no.	Remarks
Omphalotus japonicus	NBRC 4931	NBRC	AB301601	Stock culture (mycelium)
	NBRC 6992	NBRC		Stock culture (mycelium)
	NBRC 8341	NBRC		Stock culture (mycelium)
	NBRC 8342	NBRC		Stock culture (mycelium)
	NBRC 8616	NBRC		Stock culture (mycelium)
	NBRC 8765	NBRC		Stock culture (mycelium); cultivated fruiting bodies were used for cooking and DNA extraction
	NBRC 8917	NBRC		Stock culture (mycelium)
	NBRC 8918	NBRC		Stock culture (mycelium)
	NBRC 30243	NBRC		Stock culture (mycelium)
	Tottori Oj-1	Tottori, Japan		Fruiting body isolated from the field
	Tottori Oj-2	Tottori, Japan		Fruiting body isolated from the field
Entoloma rhodopolius	Tottori Er-1	Tottori, Japan	AB301602	Fruiting body isolated from the field
	Tottori Er-2	Tottori, Japan		Fruiting body isolated from the field
	Tottori Er-3	Tottori, Japan		Fruiting body isolated from the field
	Tottori Er-4	Tottori, Japan		Fruiting body isolated from the field
	Tottori Er-5	Tottori, Japan		Fruiting body isolated from the field
Tricholoma ustale	NBRC32808	NBRC		Stock culture (mycelium)
	NBRC32825	NBRC		Stock culture (mycelium)
	Tottori Tu-1	Tottori Japan		Fruiting body isolated from the field
	Tottori Tu-2	Tottori Japan		Fruiting body isolated from the field
	611	DDBJ	AB036894	Only nucleotide sequence used
Clitocybe acromelalga	NBRC30567	NBRC	AB301606	Stock culture (mycelium)

[a] NBRC, National Institute of Technology and Evaluation (NITE) Biological Resource Center, Japan; DDBJ, DNA Data Bank of Japan.

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TABLE 2. Edible mushroom strains used in this study

Species	Strain	Source[a]	Accession no.	Remarks
Lentinula edodes	#5-8	Tottori University		Stock culture (mycelium)
	271	DDBJ	DQ467886	Only nucleotide sequence used
Pleurotus ostreatus	Nichinou-Chusei	Tottori University		Stock culture (mycelium)
	S474	DDBJ	AY540332	Only nucleotide sequence used
Panellus serotinus	NBRC 30264	NBRC		Stock culture (mycelium)
	CBS 722.83	DDBJ	AY265847	Only nucleotide sequence used
Entoloma sarcopus	Tottori Es-3	Tottori, Japan	AB301603	Fruiting body isolated from the field
Lyophyllum shimeji	OK1L	Kinki University	AB301604	Stock culture (mycelium)
Lyophyllum decastes	NBRC33134	NBRC	AB301605	Stock culture (mycelium)
Armillaria mellea	NBRC31621	NBRC		Stock culture (mycelium)
	M1	DDBJ	AJ250051	Only nucleotide sequence used
Clitocybe clavipes	NBRC30524	NBRC	AB301607	Stock culture (mycelium)
Clitocybe gibba	NBRC100092	NBRC	AB301608	Stock culture (mycelium)
Lactarius akahatsu	NBRC33157	NBRC	AB301609	Stock culture (mycelium)
Lactarius chrysorrheus	NBRC32791	NBRC	AB301610	Stock culture (mycelium)
Lactarius hatsudake	NBRC32778	NBRC	AB301611	Stock culture (mycelium)
Tricholoma matsutake	JK	Kyoto, Japan		Fruiting body isolated from the field
	MC1	DDBJ	AB036892	Fruiting body isolated from the field
Pholiota lubrica	NBRC32453	NBRC	AB301612	Stock culture (mycelium)
Cortinarius tenuipes	Nara Ct-1	Nara, Japan	AB301613	Only nucleotide sequence used
Auricularia auricula	TU-AA1	Tottori University		Stock culture (mycelium)
Agaricus bisporus	B100	HFPRI		Stock culture (mycelium)
Flammulina velutipes	HATUYUKI	Tottori university		Stock culture (mycelium)
Grifola frondosa	MS1	HFPRI		Stock culture (mycelium)
Hypsizigus marmoreus	TU-HM1	Tottori University		Stock culture (mycelium)
Pholiota nameko	3024	Tottori University		Stock culture (mycelium)
Pleurotus eryngii	MH006062	Tottori University		Stock culture (mycelium)

[a] DDBJ, DNA Data Bank of Japan; NBRC, National Institute of Technology and Evaluation (NITE) Biological Resource Center, Japan; HFPRI, Hokkaido Forest Products Research Institute.

determination of genetic differences among basidiomycetes by using DNA techniques are usually performed by comparing the nucleotide sequences of amplified DNA fragments from nuclear and mitochondrial ribosomal DNAs (rDNAs) (1, 4, 6, 9, 10, 12, 13).

In this study, we tried to develop a rapid system of poisonous mushroom identification using a real-time PCR system. We herein propose a new strategy for mushroom identification in medical facilities.

Strains and culture conditions. In this study, 4 poisonous mushroom species that are frequently eaten by mistake in Japan and 27 popular edible mushroom species (Tables 1 and 2) were used. The mycelia of most strains were grown on GP medium (7); the mycelia of *Clitocybe* and *Lactarius* strains were

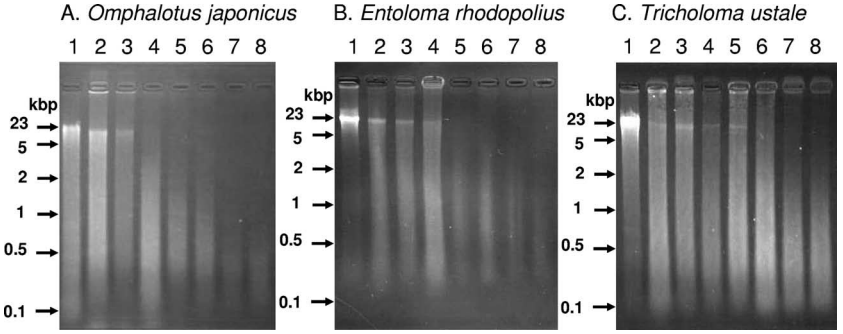

FIG. 1. Electrophoretic analysis of extracted DNA. Samples were electrophoresed on 2.0% agarose gels and stained with ethidium bromide. Panels A, B, and C correspond to DNA purified from the fruiting bodies of *O. japonicus*, *E. rhodopolius*, and *T. ustale*, respectively. In each panel, lanes and samples are lane 1, fresh; lane 2, baked for 4 min; lane 3, stir-fried for 2 min; lane 4, tempura-style (deep-fried in tempura batter for 2 min); lanes 5 to 8, boiled for 30, 60, 120, or 180 min, respectively. The size markers are EcoRI/HindIII double digests of lambda DNA and 100-bp ladders.

TABLE 3. Species-specific primers for each poisonous mushroom species

Targeted species	Primer					Product length (bp)
	Name	Length (bp)	Sequence (5′–3′)	Position[b]	T_m (°C)[c]	
O. japonicus	OJSP-F	19	GTGCACGTTTCCTTTCAAT	59–77	60.12	107
	OJSP-R	20	AGAATCATCAACAGAGCTGC	165–146	59.55	
E. rhodopolius	ERSP-F	22	TTTGAGAACTGCTGTGAAAATC	126–148	60.81	110
	ERSP-R	23	GGCACAAAGTCCCTATATGTTTA	235–213	60.61	
C. acromelalga	CASP-F	19	GGTGCACACCTGATAACCA	53–71	62.09	108
	CASP-R	20	AGCTTAAGCTTTCGCACCAG	160–141	63.39	
T. ustale	TUSP-F	22	TAGTAGGGACCTCTGTTGCCTT	501–522	62.77	80
	TUSP-R	25	AACCTCCAATTTAAAGCTGCTTCAC	580–556	66.06	
Universal[a]	ITS1	19	TCCGTAGGTGAACCTGCGG	Variable	68.4	Variable
	ITS2	20	GCTGCGTTCTTCATCGATGC		68.13	
	ITS3	20	GCATCGATGAAGAACGCAGC	Variable	68.13	Variable
	ITS4	20	TCCTCCGCTTATTGATATGC		61.49	

[a] White et al. (12).
[b] Positions of nucleotides correspond with sequence of ITS region in each species. The nucleotide sequences were published in the DDBJ nucleotide sequence database under accession numbers AB301601, AB301602, AB301606, and AB036894 respectively.
[c] T_m, melting temperature. Values were calculated with oligonucleotide calculator software (Sigma-Aldrich, Tokyo, Japan) on the web site (http://www.genosys.jp/adt/SGJ_NN_3rd.html).

grown on modified Hamada's medium (14); and the mycelia of the *Armillaria* strains were grown on malt extract medium (5) for later DNA extraction.

The fruiting body of *Omphalotus japonicus* was cultivated on sawdust medium (2.5 kg wet weight, 80% sawdust, 20% wheat bran, 65% final moisture content) in polypropylene bags. *O. japonicus* mycelia were inoculated to the medium and grown at room temperature for 2 months. At the end of this period, the bags were removed and the colonized substrates were irradiated with visible light (12 h dark and 12 h light) at room temperature for 2 months.

Cooking of fruiting bodies. Fruiting bodies were cooked as follows. For baking, the pilei of fruiting bodies were cut into 3-cm square pieces and baked on both sides in an indoor

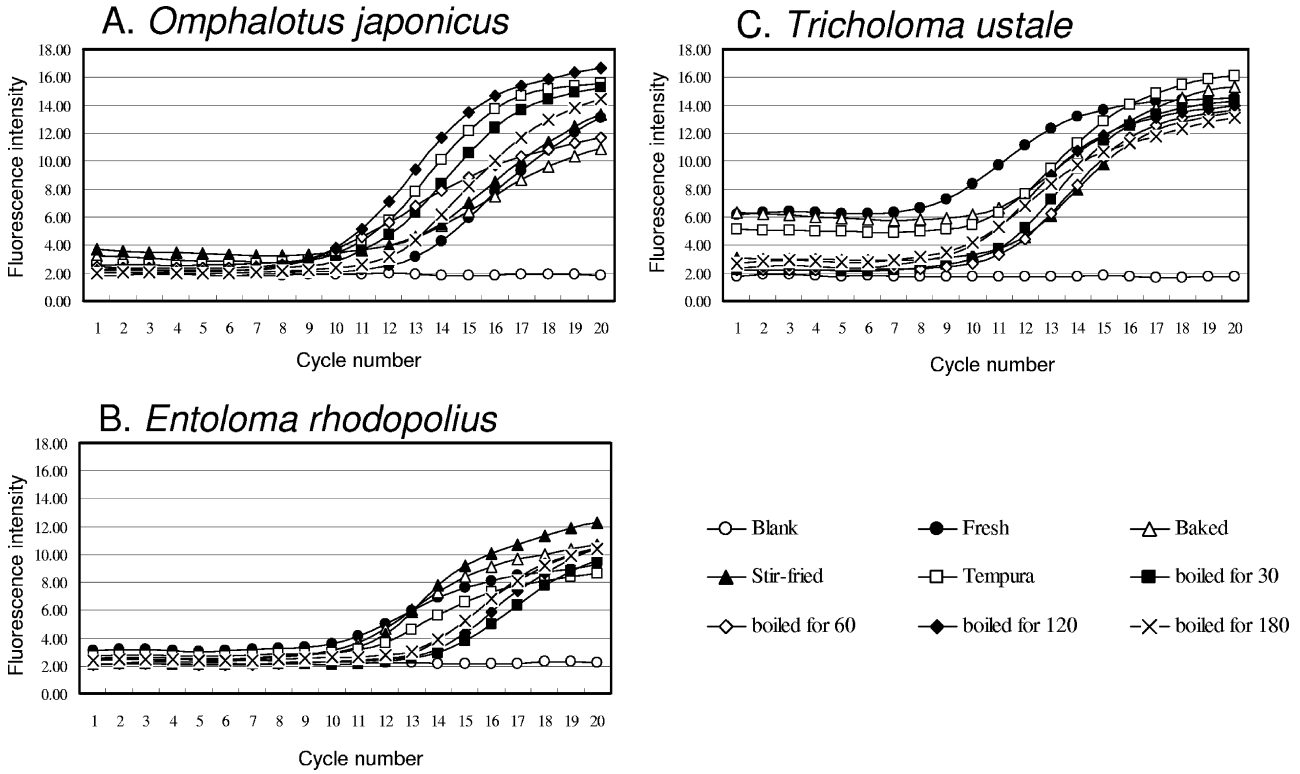

FIG. 2. Fluorescence intensity profile during real-time PCR with species-specific primer pairs using genomic DNAs from cooked and uncooked mushrooms as templates. (A) Amplified with OJSP-F/OJSP-R using genomic DNA of *O. japonicus*. (B) Amplified with ERSP-F/ERSP-R using genomic DNA of *E. rhodopolius*. (C) Amplified with CASP-F/CASP-R using genomic DNA of *T. ustale*. Blank, no genomic DNA; Fresh, genomic DNA from fresh fruiting body; Baked, baked for 4 min; Stir-fried, stir-fried for 2 min; Tempura, deep-fried in tempura batter for 2 min.

electric grill (HPS-39G-T; Sanyo Electric Co., Osaka, Japan) without oil at 240°C for 2 min each. For stir-frying, the pilei of fruiting bodies were cut into 3-mm-thick slices and stir-fried with mixing on the oiled electric grill at 240°C for 2 min. For tempura-style cooking (deep-frying in tempura batter), the pilei of fruiting bodies were cut into 3-cm square pieces and battered. The battered mushroom pieces were deep-fried in oil at 180°C for 2 min. For boiling, the pilei of fruiting bodies were cut into 3-cm square pieces and boiled in water for 30, 60, 120, or 180 min.

DNA extraction from fruiting bodies. Fruiting body tissues and mycelia were frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. DNAs were extracted from 300 mg of frozen powder (3). Figure 1 compares the size distributions of DNA isolated from fresh and cooked mushrooms. A broad range of DNA sizes up to 23 kbp was consistently present in the fresh, baked, stir-fried, and tempura-style samples, and the sizes of DNAs extracted from boiled mushroom samples were smaller than those in the other samples. For DNA isolated from *O. japonicus* boiled for 120 and 180 min, only DNA of less than 500 bp in size was observed.

Primer design for species-specific primers. DNA sequencing of the rDNA ITS region (12) was carried out as previously described (8). The nucleotide sequences of the rDNA ITS region of four poisonous mushrooms and of edible mushrooms that are morphologically similar were aligned, and four oligonucleotide primer pairs were designed for the specific amplification of *O. japonicus*, *Entoloma rhodopolius*, *Clitocybe acromelalga*, and *Tricholoma ustale* rDNA (Table 3), because these poisonous mushrooms have been responsible for approximately 85% of the mushroom poisoning events in Japan over the last five years.

Species-specific detection. PCR amplifications were performed using four species-specific primer pairs, using genomic DNAs from twenty-seven mushroom species as templates. DNA was also extracted from 300 mg of frozen powder using a GeneAll plant SV mini kit (GeneAll Biotechnology, Seoul, Korea) in accordance with the manufacturer's recommendations. Real-time PCR was carried out using Sybr green real-time PCR master mix (Toyobo Co., Osaka, Japan) with 2 μM forward and reverse primers for detection of *O. japonicus* and *T. ustale* and 0.4 μM forward and reverse primers for detection of *E. rhodopolius* and *C. acromelalga*. Real-time PCR was carried out in a 10-μl reaction mixture volume containing 5 μl of the master mix, about 2.5 ng of extracted genomic DNA, and appropriate primers. Thermocycling was performed using a LineGene real-time thermal cycler (BioFlux, Tokyo, Japan). The reaction was performed for 20 cycles, and the following cycling profile was used. The first denaturing step was at 95°C for 1 min, and then the PCR cycles were 5 s of denaturation at 95°C, 5 s of annealing at 65°C, and then 15 s of extension at 72°C. Fluorescence data were acquired during the elongation step in every cycle.

When the specific primer pairs were used, increases in fluorescence intensity were detected only for the targeted species (data not shown). Thus, we concluded that the designated primers were species specific for each poisonous mushroom. In order to confirm the universality of the four species-specific primer pairs for each species, PCR amplification was performed using genomic DNAs from 11 strains of *O. japonicus*, 5 strains of *E. rhodopolius*, and 4 strains of *T. ustale* (data not shown).

To confirm the amplification of specific DNA fragments of cooked mushrooms, real-time PCR was carried out. The results are shown in Fig. 2. For each primer set used for amplifying DNA from the poisonous mushroom species, species-specific detection was successful. In our proposed system, less than 1 h is required for detection via real-time PCR. However, in order to perfect the system we developed in this study, more specific primers should be designed in further studies.

Nucleotide sequence accession numbers. The nucleotide sequence data obtained in this study have been deposited in the DDBJ nucleotide sequence database under accession numbers AB301601 to AB301613.

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