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# Identification and Detection Method for Genetically Modified Papaya Resistant to Papaya Ringspot Virus YK Strain

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**Unauthorized genetically modified (GM) papaya (*Carica papaya* LINNAEUS) was detected in a commercially processed product, which included papaya as a major ingredient, in Japan. We identified the transgenic vector construct generated based on resistance to infection with the papaya ringspot virus (PRSV) YK strain. A specific detection method to qualitatively monitor papaya products for contamination with the GM papaya was developed using the real-time polymerase chain reaction.**

**Key words** genetically modified organism; papaya; polymerase chain reaction; genomic DNA

Papaya (*Carica papaya* LINNAEUS) is an important fruit crop in tropical and subtropical areas.[1] Infection with the papaya ringspot virus (PRSV) causes disastrous damage to papaya harvests.[2] In response to this problem, genetically modified (GM) papayas have been developed in various places, such as Hawaii, Florida, China, Jamaica, Taiwan, Thailand, Australia, Malaysia, Philippines and Vietnam.[2]

Japan has announced a mandatory safety assessment of GM foods and processed foods containing GM ingredients, and the importation of any unauthorized GM foods to Japan has been prohibited since April 1, 2001. Therefore, the establishment of qualitative detection methods for unauthorized GM foods was required for monitoring purposes. We previously developed and reported qualitative detection methods for various GM crops, such as potato,[3,4] maize,[5—8] rice,[9] and flax,[10] using polymerase chain reaction (PCR) methods. In the case of papaya, we established a qualitative detection method for GM papaya (Line 55-1), which was the first commercialized PRSV-resistant GM papaya developed in Hawaii, using a PCR test and a histochemical assay.[11—13] A safety assessment for Line 55-1 by the Food Safety Commission of Japan was finished in 2009.[14] Since Japan imports many papayas from Southeast Asia, we are required, in Japan, to monitor commercially processed products that include papaya as a major ingredient for contamination with other unauthorized GM papayas generated in the region.

GM papayas carry the transgenic vector construct generated based on resistance to PRSV infection by expressing the PRSV's coat protein (CP) gene. Since the other unauthorized GM papayas developed may differ in the transgenic vector construct of the authorized GM papaya (Line 55-1), we developed a method for detecting contamination with unauthorized GM papaya. In the present study, we found the unauthorized GM papaya, PRSV-YK, in processed products containing papaya as a major ingredient, papaya-leaf-tea, pickles and jam, and developed a method for the detection of PRSV-YK using the real-time PCR.

## MATERIALS AND METHODS

**Papaya Samples** Papaya products were purchased through the internet in Japan. Hawaiian non-GM papaya (Sunset) fruit was purchased from a Japanese trade agency *via* the Hawaii Papaya Industry Association through the Consumer Affairs Agency, Government of Japan.

**Purification of DNA** Dried papaya leaves in papaya-leaf-tea, papayas in pickles and Sunset sarcocarp were ground using a mixing mill. Papaya jam was used for purification of DNA without grinding. DNA was extracted and purified from 2 g of the samples using an ion-exchange resin-type DNA extraction and purification kit (Genomic-tip; QIAGEN, Hilden, Germany) as follows: 30 ml Buffer G2 (QIAGEN), 20 $\mu$l 100 mg/ml RNase (QIAGEN) and 500 $\mu$l cellulase (Sigma-Aldrich, St. Louis, MO, U.S.A.) were added to the sample and vortexed thoroughly, then incubated at 50 °C for 1 h. The mixture was incubated at 50 °C for another 1 h after the addition of 200 $\mu$l Proteinase K (QIAGEN). During the incubation, the samples were mixed several times by inverting the tubes. The samples were then centrifuged at $3000\times g$ at 4 °C for 20 min. The supernatant was applied to a Genomic-tip 100/G column (QIAGEN), which was pre-equilibrated with 4 ml Buffer QBT (QIAGEN). The tip was washed three times with 7.5 ml Buffer QC (QIAGEN) and transferred to a fresh centrifuge tube, and 3 ml pre-warmed Buffer QF (QIAGEN) (50 °C) was added to elute the DNA. The DNA sample was transferred to a centrifuge tube, an equal volume of isopropyl alcohol was added, and the sample was mixed thoroughly. DNA was collected by centrifugation at $12000\times g$ for 15 min. The pellet was rinsed with 1 ml 70% (v/v) ethanol and centrifuged at $12000\times g$ for 3 min. The supernatant was discarded and the precipitate was dried. The DNA was dissolved in 20 $\mu$l water for use in analyses. The DNA was quantified by measuring UV absorption at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, U.S.A.).

**PCR** Each PCR reaction mixture (25 $\mu$l) contained 2.5 $\mu$l 10×PCR buffer II (Life Technologies, Carlsbad, CA, U.S.A.), 0.16 mM of each deoxyribonucleotide triphosphate (dNTP) (Life Technologies), 1.5 mM $MgCl_2$, 1.2 $\mu$M forward and reverse primers, 0.8 U AmpliTaq Gold (Life Technologies) and 25 ng template DNA. The PCR conditions were as follows: 95 °C for 10 min, followed by 50 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 30 s, extension at 72 °C for 60 s and terminal elongation at 72 °C for 7 min. PCRs were carried out using the GeneAmp PCR System 9700 (Life Technologies). To determine the nucleotide sequence of the transgenic vector construct harbored in GM

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papaya, DNA fragments were amplified by PCR using the following primer set. Forward primer: 5′-GACATCTCCA-CTGACGTAAGGG-3′ (p324). Reverse primer: 5′-CTATCR-CTCTCTCCAGTTTTTG-3′ (p323).

**DNA Sequencing** The PCR-amplified DNA fragments were extracted from the agarose-gel and purified using a QIAquick PCR purification kit (QIAGEN). The DNA fragments were directly sequenced from both strands using forward and reverse primers with an ABI PRISM 3700 DNA analyzer and Terminator v3.1 Cycle Sequencing Kit (Life Technologies), according to the manufacturer's instructions. Nucleotide sequences were analyzed using Lasergene version 7.2 software (DNASTAR Inc., Madison, WI, U.S.A.).

**Real-Time PCR** Real-time PCR assays were performed using the ABI PRISM™ 7900 Sequence Detection System (Life Technologies). The 25 $\mu$l reaction mixture consisted of 2.5 $\mu$l sample DNA solution (25 ng), 12.5 $\mu$l Universal Master Mix® (Life Technologies), 0.8 $\mu$M forward and reverse primers, and 0.1 $\mu$M probe. The PCR conditions were as follows: 2 min at 50 °C, 95 °C for 10 min followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. GM papaya was detected using the following primers and probe. Forward primer: 5′-GATCCCCGGGTGGTCAGT-3′ (YK-1F). Reverse primer: 5′-CCGGTATCCACAGCTTCATTTT-3′ (YK-1R). Probe: 5′-FAM-AGACGCCATGGAAGG-MGB-3′ (YK-P).

For detecting the papaya endogenous internal control gene, *chymopapain* (*Chy*; GenBank accession No.: AY803756), we designed the following primers and probe referring to published report.[15] Forward primer: 5′-CCATGCGATCCTC-CCA-3′ (Q-Chy-1F2). Reverse primer: 5′-CATCGTAGCCA-TTGTAACACTAGCTAA-3′ (Q-Chy-2R). Probe: 5′-FAM-TTCCCTTCAT(BHQ1)CCATTCCCACTCTTGAGA-3′ (Q-Chy-P). Black-hole quencher 1 (BHQ1) was labeled for Q-Chy-P at the underlined thymidine in the nucleotide sequence.

All primers and probes were diluted with an appropriate volume of distilled water, and stored at −20 °C until use. Results were analyzed using SDS 2.1 sequence detection software (Life Technologies) for ABI PRISM™ 7900 Sequence Detection System.

**Real-Time PCR Data Analysis** Typically, the baseline was set to cycles 3 through 15. The ΔRn threshold for plotting the cycle threshold (Ct) values was set to 0.2 during exponential amplification. Reactions with Ct values of less than 48 and exponential amplification plots were scored as positive. If the Ct value could not be obtained, the reaction was

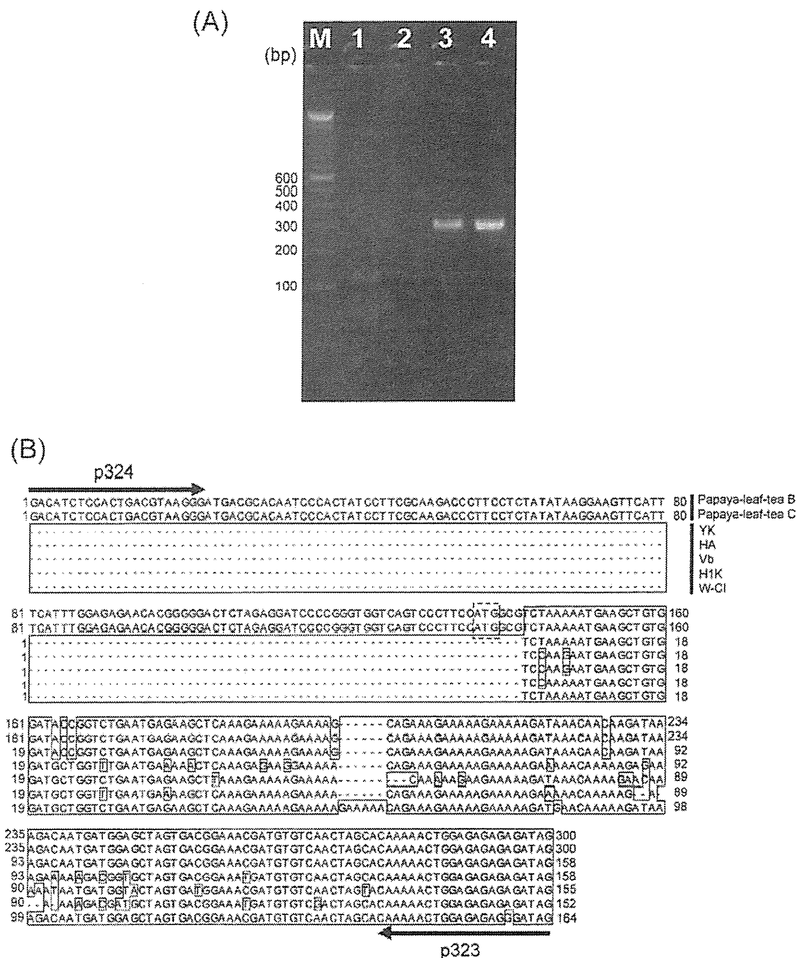


Fig. 1. PCR Targeting Construct Specific Sequence of PRSV-Resistant GM Papaya

(A) DNA templates (lane 1, non-GM papaya (Sunset); lane 2, papaya-leaf-tea A; lane 3, papaya-leaf-tea B; lane 4, papaya-leaf-tea C) were used for the PCR test using the p324 and p323 primer set. The PCR-amplified products were run on a 3% (w/v) agarose-gel. Lane M, 100-bp DNA ladder marker (B) Nucleotide sequence alignment of PRSV CP cloned from YK, HA, Vb, H1K, W-CI strains and the PCR products obtained using papaya-leaf-tea B and C. Homologous sequences were boxed. The initiation codon for the GM papaya was boxed with a dashed line. Numerals beside the sequence indicate the numbers of nucleotides from the 5′ terminus.

scored as negative. Reactions with Ct values of less than 48, but without exponential amplification as judged by visual inspection of the respective ΔRn plots and multi-component plots were scored as negative.

## RESULTS AND DISCUSSION

**Detection of Unauthorized GM Papaya** To investigate the contamination with unauthorized GM papaya in commercially processed products, containing papaya as a major ingredient, in Japan, we used genomic DNA purified from the papaya-leaf-tea products as a template for the PCR test. The forward primer (p324) was designed to hybridize in the cauliflower mosaic virus (CaMV) 35S promoter sequence, which is the most common promoter used in the transformation of papaya for various GM papaya traits,[2] and the reverse primer (p323) was designed in the highly conserved sequence of the CP gene, which is cloned from various strains of PRSV (GenBank accession no.: YK, X97251; HA, S46722; Vb, AF243496.1; H1K, AF196839.1; W-CI, AY027810.2). Electrophoresis of the PCR products using p324 and p323 primers showed a single band of about 300 bp in length using DNA purified from two of the three papaya-leaf-tea products (papaya-leaf-tea B and C) (Fig. 1A). The DNA purified from non-GM papaya (Sunset) as a control and papaya-leaf-tea A generated no PCR products with the identical length. Direct sequence analysis of the PCR product and BLASTn analysis indicated that the 3′ end sequence was identical to the CP gene in a Taiwan isolate of PRSV (PRSV YK strain)[16] (Fig. 1B). Furthermore, the multiple cloning site (containing restriction sites for *Bam*HI and *Nco*I) and two amino acid mutations (methionine and alanine) between the CaMV 35S promoter and the N-terminus of CP gene were detected (Fig. 2A). According to the literature,[17] the design of this transgenic vector construct was identical to that of the GM papaya, which was generated to resist infection of the PRSV YK strain. These results suggest that the papaya-leaf-tea products were contaminated with the unauthorized GM papaya (PRSV-YK).

**Development of a Construct-Specific Detection Method for PRSV-YK** In order to qualitatively detect PRSV-YK in processed products, containing papaya as a major ingredient, with high specificity and sensitivity, we designed specific primers and a probe for a real-time PCR assay producing a short amplicon (57 bp), based on the detected transgenic construct sequence. The forward (YK-1F) and the reverse (YK-1R) primers were designed in the region between the transgenic vector backbone and the CP gene sequence. The probe

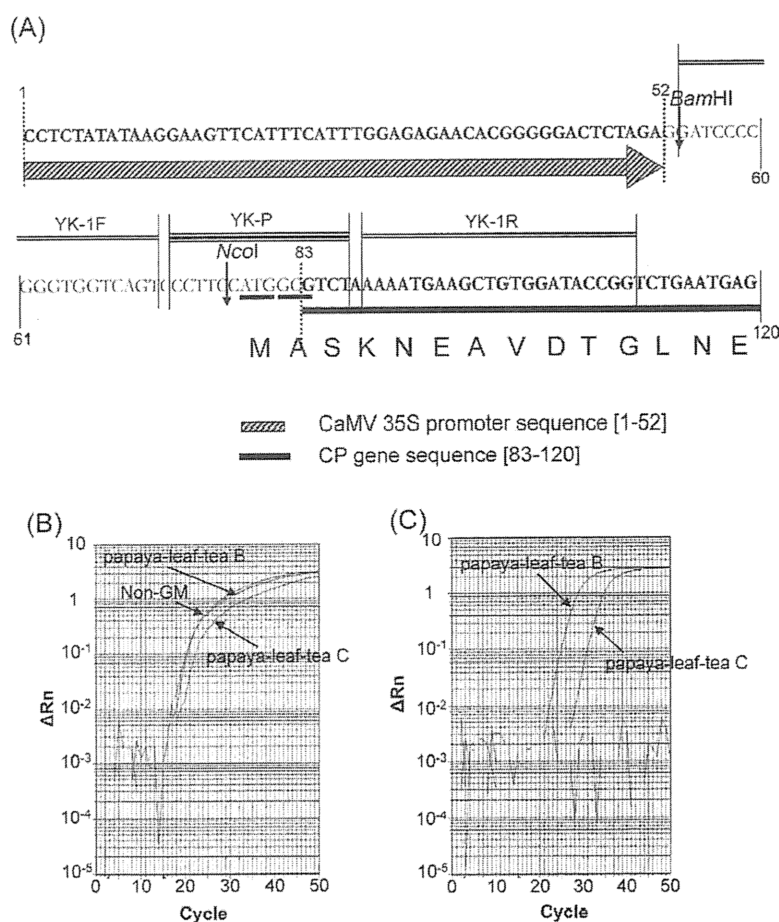


Fig. 2. Detection of PRSV-YK Using Real-Time PCR

(A) A fragment of the transgenic vector construct sequence was obtained and restriction sites were marked by vertical arrows. Design of the primers (YK-1F and YK-1R) and the probe (YK-P) for detecting construct-specific sequence of PRSV-YK is indicated by lines above the sequence. Numerals indicate the numbers of nucleotides from the 5′ terminus. (B) Endogenous *Chy* detection using a primer set (Q-Chy-1F2 and Q-Chy-2R) and probe (Q-Chy-P) (C) PRSV-YK detection using a primer set (YK-1F and YK-1R) and probe (YK-P). The threshold value was set at 0.2. Positive amplification curves are designated by arrows.

(YK-P) was designed on the site of the initiation codon of the CP gene (Fig. 2A).

Since the forward primer sequence for detecting the papaya endogenous internal control gene, *Chy*, had an unintentional error of a single nucleotide sequence in the previous report[15] (according to personal communication), we used the right sequence for the forward primer (Q-Chy-1F2), the reverse primer (Q-Chy-2R) and the probe (Q-Chy-P). The real-time PCR assay for PRSV-YK detection confirmed that the papaya-leaf-tea products B and C were positive for PRSV-YK, producing Ct values of 25.93 and 31.88 with a threshold value of 0.2, respectively. Endogenous *Chy* detection was positive for all samples, with the papaya leaf-tea product B, C and the non-GM papaya (Sunset) producing Ct values of 21.55, 23.82 and 21.45, respectively, with a threshold value of 0.2 (Figs. 2B, C). The copy numbers of PRSV-YK construct and *Chy* sequence were calculated from Ct values using standard curves which were generated using the positive control plasmid. Papaya-leaf-tea products B and C contained 1 copy of PRSV-YK construct sequence in 27 copies and 167 copies of *Chy* sequence, respectively (data not shown). Because the genetic background of PRSV-YK used in the processed papaya products was unknown, estimation of the content of PRSV-YK in a papaya product was not possible. The non-template control and the genomic DNA derived from other crops, such as maize, rice, soybean, flax and canola, gave no amplification signals in the PRSV-YK and the endogenous *Chy* detection systems (data not shown). These results indicated that the developed method is specific for detecting PRSV-YK.

In the present study, as a result of monitoring processed products, which included papaya as a major ingredient, for contamination with unauthorized GM papaya, we found a transgenic vector construct for expression of the CP gene, which was cloned from the YK strain, in papaya-leaf-tea products. The design of a part of the transgenic vector construct was identical to the one reported in 1996.[17] We also detected PRSV-YK contamination in 1 out of 7 products of papaya jam and 2 out of 3 products of papaya pickles in real-time PCR test for PRSV-YK detection (data not shown). The origin of the GM papaya contamination in the papaya products in Japan remains to be clarified. Furthermore, we successfully developed a construct-specific real-time PCR detection method for PRSV-YK. Further studies are required to determine the detection limits, and whether the method can be used for detection in other commercially processed products containing papaya as a major ingredient.

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## FOOD COMPOSITION AND ADDITIVES

# Interlaboratory Study of DNA Extraction from Multiple Ground Samples, Multiplex Real-Time PCR, and Multiplex Qualitative PCR for Individual Kernel Detection System of Genetically Modified Maize

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**In many countries, the labeling of grains, feed, and foodstuff is mandatory if the genetically modified (GM) organism content exceeds a certain level of approved GM varieties. We previously developed an individual kernel detection system consisting of grinding individual kernels, DNA extraction from the individually ground kernels, GM detection using multiplex real-time PCR, and GM event detection using multiplex qualitative PCR to analyze the precise commingling level and varieties of GM maize in real sample grains. We performed the interlaboratory study of the DNA extraction with multiple ground samples, multiplex real-time PCR detection, and multiplex qualitative PCR detection to evaluate its applicability, practicality, and ruggedness for the individual kernel detection system of GM maize. DNA extraction with multiple ground samples, multiplex real-time PCR, and multiplex qualitative PCR were evaluated by five laboratories in Japan, and all results from these laboratories were consistent with the expected results in terms of the commingling level and event analysis. Thus, the DNA extraction with multiple ground samples, multiplex real-time PCR, and multiplex qualitative PCR for the individual kernel detection system is applicable and practicable in a laboratory to regulate the commingling level of GM maize grain for GM samples, including stacked GM maize.**

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Genetically modified (GM) crops have been developed and are widespread as food and feed in many countries (1). These GM crops have been assessed and authorized for food use by administrative authorities over the past two decades. Under the current regulatory conditions, the labeling of grains, feed, and foodstuff is mandatory if the GM crop content exceeds a certain level of the approved GM varieties. For instance, the European Union, Japan, and Korea have set threshold values of 0.9, 5, and 3%, respectively, of GM organism material in a non-GM background as the basis for labeling (2–8). In the United States, GM crops are not regarded as a food safety issue, and labeling is voluntary. Depending on national philosophy, governmental regulation differs on the use and application of the technology for GM crops. In Japan, non-GM crops are segregated as non-GM material and imported from the United States using an identity preserved handling system that requires document certification from U.S. farms to Japanese processing

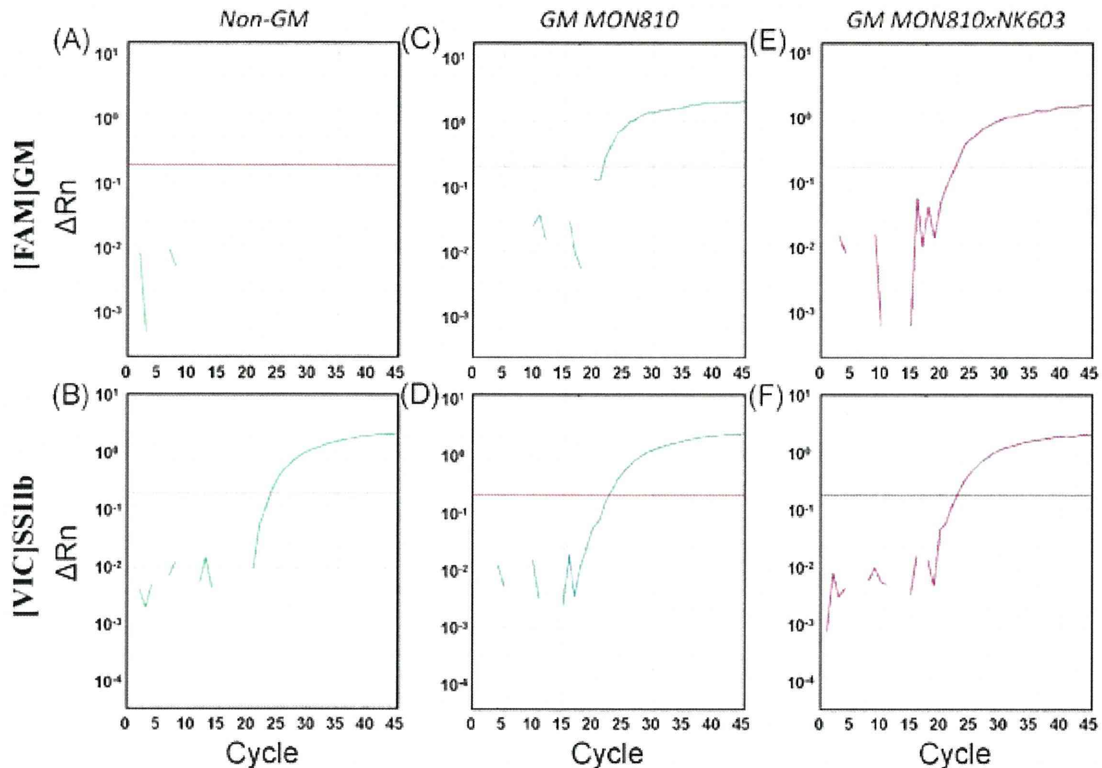


**Figure 1. Typical multiplex real-time PCR amplification plots. The amplification plot was established using genomic DNA derived from (A, B) non-GM maize, (C, D) MON810 maize, or (E, F) MON810xNK603 stacked maize as the template. The probes used to specifically detect GM maize-specific gene (A, C, E) and endogenous SSIIb gene (B, D, F) in multiplex real-time PCR were labeled with VIC- and FAM-fluorescent dyes, respectively. The horizontal axis indicates the number of PCR cycles, and the vertical axis indicates Δnormalized reporter signal (ΔRn) values, which are the relative values automatically calculated by the analysis software based on the signal intensities of FAM dye, dependent on the target amplification and ROX passive reference.**

traders. Recently, the production of stacked GM maize events, in which two or more different characteristic traits are inserted, has increased in the United States due to enhanced production efficiency (1). Although the levels of adventitious commingling of GM maize into non-GM maize in the labeling system refer to GM maize on a weight per weight (w/w) percentage basis, the conventional applicable detection methods, such as quantitative real-time PCR, do not directly measure the w/w percentage of GM maize. The GM maize percentages calculated using current quantitative real-time PCR methods are calculated by converting relative copy numbers between a specific rDNA sequence and a taxon-specific DNA sequence into a w/w percentage using appropriate reference materials. The GM maize content in a maize sample containing stacked GM maize events, as determined by current quantitative real-time PCR methods, is likely to be overestimated compared to the actual w/w percentage of GM maize in the sample because the relative copy numbers are calculated on a haploid basis.

We have developed an individual kernel detection system that consists of grinding individual maize kernels, DNA extraction from multiple ground maize kernels, multiplex real-time PCR using the extracted DNAs from individual ground maize kernels for GM detection, and multiplex qualitative PCR using the extracted DNAs for GM event detection to analyze the exact commingling level and varieties of GM maize (9–11). The detection system has already been implemented in Japan as an official GM organism detection method (12).

However, as a routine test in the laboratory, the single-kernel detection system appears to be time-consuming. As multitube samples are tested and the DNA of each kernel is extracted from each well in the 96-well plate in the single-kernel detection system, it is likely that the analyst will make some errors.

Therefore, in the single-kernel detection system it was necessary to demonstrate that an analyst is able to decontaminate and quantify each kernel individually, then determine the GM maize level (the percentage of kernel/kernel) on a multiple laboratory basis.

In the present study, the steps in the individual kernel

**Table 1. In-house validation of the individual maize kernel test**

| | SSIIb[b] | | | | | | GM[b] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Non-GM | | MON810 | | MON810xNK603 | | Non-GM | | MON810 | | MON810xNK603 | |
| Day[a] | (+)[c] | (–)[c] | (+) | (–) | (+) | (–) | (+) | (–) | (+) | (–) | (+) | (–) |
| 1 | 12 | 0 | 6 | 0 | 6 | 0 | 0 | 12 | 6 | 0 | 6 | 0 |
| 2 | 12 | 0 | 6 | 0 | 6 | 0 | 0 | 12 | 6 | 0 | 6 | 0 |
| 3 | 12 | 0 | 6 | 0 | 6 | 0 | 0 | 12 | 6 | 0 | 6 | 0 |
| 4 | 12 | 0 | 6 | 0 | 6 | 0 | 0 | 12 | 6 | 0 | 6 | 0 |
| 5 | 12 | 0 | 6 | 0 | 6 | 0 | 0 | 12 | 6 | 0 | 6 | 0 |
| Total | 60 | 0 | 30 | 0 | 30 | 0 | 0 | 60 | 30 | 0 | 30 | 0 |

[a] Real-time PCR was run independently on 5 different days.

[b] The probes used to specifically detect endogenous SSIIb and GM maize-specific genes were labeled with 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein (VIC) and 6-carboxyfluorescein (FAM)-fluorescent dyes, respectively.

[c] (+) = Number of positive samples, (–) = number of negative samples.

detection system requiring the most attention, i.e., DNA extraction from multiple ground samples, multiplex real-time PCR, and multiplex qualitative PCR, were evaluated to clarify their applicability, practicality, and ruggedness for use in the determination of GM maize kernel samples, including stacked GM maize events, in an interlaboratory study.

## Experimental

### *Maize (Zea mays) Materials*

Non-GM maize grain, MON810 seeds, and stacked maize seeds (MON810xNK603) were kindly provided by Monsanto Co. (St. Louis, MO) as positive controls for GM maize.

### *Oligonucleotide Primers and Probes for Multiplex Real-Time PCR*

Sets of primer pairs and probes for the construct-specific and universal GM quantification were the same as those described in our previous papers (9, 10). The SSIIb-3 system (SSIIb 3-5′ and SSIIb 3-3′ with SSIIb-TaqV) was used as the primers and probe for the detection of the taxon-specific gene encoding the maize starch synthase IIb gene sequence (SSIIb) by multiplex real-time PCR. The p35S-1 (P35S 1-5′ and P35S 1-3′ with P35S-Taq) and GA21-3 systems (GA21 3-5′ and GA21 3-3′ with GA21-Taq) were used for multiplex real-time PCR. All sets of primer pairs and probes (p35S-Taq and GA21-Taq) for detection of the cauliflower mosaic virus 35s promoter sequence (p35S) and GA21-specifc sequence, respectively, were purchased from Fasmac Co., Ltd (Kanagawa, Japan). SSIIb-TaqV, which is labeled with VIC® and 6-carboxytetramethyl-rhodamine (TAMRA) at the 5′ and 3′ ends, was synthesized by Life Technologies (St. Louis, MO) and used as a probe for detection of SSIIb. The target sequence of the p35S-1 system to detect the 35S promoter region derived from CaMV is widely found in recombinant DNA of almost all GM events, with the exception of GA21. The GA21-3 system was designed to detect the construct-specific sequence GM maize event GA21 (9, 10).

### *Grinding of Maize Materials*

For the in-house validation study of DNA extraction from multiple ground samples and multiplex real-time PCR detection, individual maize kernels were placed in a sample tube that contained the pulverizing medium (MC0316MZ; Yasui Kikai Co., Osaka, Japan), and the tube was closed with an attached cap (ST-0350MZ; Yasui Kikai Co.). The 24 sample tubes were arrayed in the tube holder (Type SH-123; Yasui Kikai Co.). Two tube holders can be accommodated in a multibeads shocker (Model MB601NIHS; Yasui Kikai Co.) at a time. Next, the maize kernels were ground by heavily shaking the tubes using a multibeads shocker at 2500 rpm for 1 min and repeated for 1 min after the tube holder was reversed (9).

In the interlaboratory validation of DNA extraction from multiple ground samples and multiplex real-time PCR, the non-GM maize grains, MON810 seeds, and stacked maize seeds (MON810xNK603) were ground in an ultra-centrifugal mill (ZM100; Retsch GmbH, Haan, Germany) using a 0.5 mm sieve ring. Samples were stored at –20°C until further use.

Table 2. Ruggedness of DNA extraction from multiple ground samples and multiplex real-time PCR using different real-time PCR instruments

| | SSIIb[a] | | | | | | | | GM[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Non-GM (86)[b] | | MON810xNK603 (2) | | MON810 (2) | | Total (90) | | Non-GM (86) | | MON810xNK603 (2) | | MON810 (2) | | Total (90) | |
| Instrument | (+)[c] | (−)[c] | (+) | (−) | (+) | (−) | (+) | (−) | (+) | (−) | (+) | (−) | (+) | (−) | (+) | (−) |
| AB7900 | 86 | 0 | 2 | 0 | 2 | 0 | 90 | 0 | 0 | 86 | 2 | 0 | 2 | 0 | 4 | 86 |
| AB7500 | 86 | 0 | 2 | 0 | 2 | 0 | 90 | 0 | 0 | 86 | 2 | 0 | 2 | 0 | 4 | 86 |
| Expected | 86 | 0 | 2 | 0 | 2 | 0 | 90 | 0 | 0 | 86 | 2 | 0 | 2 | 0 | 4 | 86 |

[a] Probes used to specifically detect endogenous SSIIb and GM maize-specific genes were labeled with VIC- and FAM-fluorescent dyes, respectively.
[b] Number in parentheses indicates the number of samples analyzed.
[c] (+) = Number of positive samples, (−) = number of negative samples.

### *DNA Extraction from Each Maize Kernel or Ground Maize Samples Using DNeasy® 96 Plant Kit*

Genomic DNA extraction from finely ground maize powder was performed using the DNeasy 96 plant kit (QIAGEN, Hilden, Germany) according to previous reports (9, 10). Buffer AP1 (preheated to 65°C) and RNase A (QIAGEN; final concentration, 100 μg/mL) were combined to make the working solution. One milliliter of working solution was added to each sample tube containing the ground maize powder. The tubes were capped and incubated for 30 min at 65°C (inverted 10 times at intervals of 10 min). A 170 μL aliquot of Buffer AP2 solution was then added to each solution. After sealing to avoid leakage, the tubes were vigorously shaken for 15 s, incubated for 10 min at −20°C, and centrifuged for 20 min at 3000 rpm using a metalfuge (MBG100; Yasui Kikai Co.). A 400 μL aliquot of each supernatant was carefully transferred to a new microtube and centrifuged again for 5 min at 12 000 rpm. Each supernatant was carefully transferred to a new microtube, and 1.5 volumes (typically 600 μL) Buffer AP3/E were added to each sample. After carefully transferring 1 mL of each sample to the DNeasy 96 plates, the plates were sealed with tape and then aspirated until each DNeasy membrane was dry. After removing the tape, 800 μL Buffer AW was carefully added to each sample. The plate was again sealed with tape and aspirated until each DNeasy membrane was dry. The washing was repeated three times. An 800 μL aliquot of 100% ethanol was then added to each sample. The plate was aspirated for 15 min to dry each DNeasy membrane. After removing the tape to elute the DNA, the plate was placed in the correct orientation on a rack of elution microtubes, and then 75 μL distilled water (preheated to 65°C) was added to each sample. The plate was resealed and incubated for 5 min at room temperature, followed by aspiration until each DNeasy membrane was dry.

### *Multiplex Real-Time PCR Conditions*

To simultaneously detect genomic DNA from ground maize samples and confirm the validity of the extracted genomic DNA, multiplex real-time PCR analyses were performed according to previous reports (9, 10). The amplification curves of the target sequence were monitored with a fluorescent dye that labeled the designed oligonucleotide probes using the ABI PRISM® 7900HT sequence detection system (Life Technologies Corp., Carlsbad, CA). The reaction volume (25 μL) contained 2.5 μL sample genomic DNA solution, 12.5 μL Universal Master Mix® (Life Technologies Corp.), 0.5 μM primer pair, and 0.2 μM probe (for p35S: 0.1 μM probe). The PCR step-cycle program was as follows: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 30 s at 95°C and 90 s at 59°C.

**Table 3. Interlaboratory validation of DNA extraction from multiple ground samples and multiplex real-time PCR**

| Laboratory | Instrument | SSIIb[a] | | | | | | | | GM[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Non-GM (86)[b] | | MON810xNK603 (2) | | MON810 (2) | | Total (90) | | Non-GM (86) | | MON810xNK603 (2) | | MON810 (2) | | Total (90) | |
| | | (+)[c] | (−) | (+) | (−) | (+) | (−) | (+) | (−) | (+) | (−) | (+) | (−) | (+) | (−) | (+) | (−) |
| A | AB7500 | 86 | 0 | 2 | 0 | 2 | 0 | 90 | 0 | 0 | 86 | 2 | 0 | 2 | 0 | 4 | 86 |
| B | AB7900 | 86 | 0 | 2 | 0 | 2 | 0 | 90 | 0 | 0 | 86 | 2 | 0 | 2 | 0 | 4 | 86 |
| C | AB7900 | 86 | 0 | 2 | 0 | 2 | 0 | 90 | 0 | 0 | 86 | 2 | 0 | 2 | 0 | 4 | 86 |
| D | AB7500 | 86 | 0 | 2 | 0 | 2 | 0 | 90 | 0 | 0 | 86 | 2 | 0 | 2 | 0 | 4 | 86 |
| E | AB7900 | 86 | 0 | 2 | 0 | 2 | 0 | 90 | 0 | 0 | 86 | 2 | 0 | 2 | 0 | 4 | 86 |
| Expected | | 86 | 0 | 2 | 0 | 2 | 0 | 90 | 0 | 0 | 86 | 2 | 0 | 2 | 0 | 4 | 86 |
| Agreement, %[d] | | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

[a] Probes used to specifically detect endogenous SSIIb and GM maize-specific genes were labeled with VIC and FAM-fluorescent dyes, respectively.
[b] Numbers in parentheses indicate the number of samples analyzed.
[c] (+) = Number of positive samples, (−) = number of negative samples.
[d] Value was calculated from comparison with the expected results and results obtained from five different laboratories.

Generally, the baseline was set to cycles 3 through 15. The Δnormalized reporter signal (ΔRn) threshold cycle (Ct) for plotting values was set to 0.1–0.5 during exponential amplification. The nonexponential curves, such as the rise of the baseline or the phenomenon known as 6-carboxy-X-rhodamine (ROX) dye dropping, could be observed, although it occurred with low frequency. In our previous studies, we concluded that the clearness of the exponential amplification curves after 15 cycles (last cycle of the baseline) of real-time PCR enabled the adoption of this as the threshold for the discrimination of GM from non-GM maize (9, 10). If the exponential amplification curves for GMO detection could be clearly observed after 15 cycles, the samples were judged to be positive for GM maize; otherwise, they were judged to be negative. In this study, the GM maize detection plasmid set, ColE1/TE (Nippon Gene Co., Tokyo, Japan), was used as the positive control. This plasmid set contained six concentrations of the reference plasmid pMul5, into which was inserted the amplification products of p35S, GA21, and SSIIb diluted with Tris-EDTA buffer (pH 8.0) containing 5 ng/μL ColE1 plasmid (9, 10). The ColE1 plasmid contained none of the amplification GM products and was used as the negative control. The positive controls were prepared using the two concentrations of the plasmid set with 250 000 and 1500 copies/plate. In the negative control, the ColE1 plasmid was also used as the nontemplate control for the analysis. In the reaction plate, real-time PCR was performed in duplicate using two reaction vessels for the nontemplate control as the negative control and positive control (two concentrations of the plasmid set). The other 90 reaction vessels were used for the genomic DNA samples extracted from the ground maize samples.

### *Multiple Qualitative PCR Conditions*

To identify which GM traits are contained in genomic DNA extracted from ground maize samples, multiple qualitative PCR detection was performed according to our previously reported method with some modifications (11). The reaction mixture for PCR was prepared in a 96-well plate. The reaction volume (25 μL) contained 25 ng genomic DNA, 0.2 mmol/L deoxynucleotide triphosphate (dNTP), 1.5 mmol/L $MgCl_2$, 0.2 μmol/L each of 5′ and 3′ primers, 1.25 units AmpliTaq Gold DNA polymerase (Life Technologies Corp.), and 15 primers at the following concentrations: 0.2 μmol/L for M810 1-5′, NK603 1-3′ M863 1-5′, M863 1-3′, Bt11 1-5′, and CryIA 1-3′; 0.1 μmol/L for T25 2-5′, T25 2-3′, GA21 1-5′, GA21 1-3′, TC1507 1-5′, and TC1507 1-3′; 0.05 μmol/L for Event 176 1-5′; and 0.045 μmol/L for SSIIb 1-5′ and SSIIb 1-3′. For the combination of primers for the detection of each event, M810 1-5′ and NK603 1-3′ for NK603 detection, Event 176 1-5′ and CryIA 1-3′ for Event 176 detection, T25 2-5′ and T25 2-3′ for T25 detection, GA21 1-5′ and GA21 1-3′ for GA21 detection,

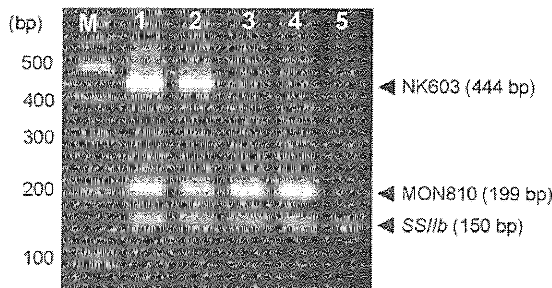


**Figure 2. Representative results of multiplex qualitative PCR. PCR amplification products using genomic DNA templates purified from No. 10 (Lane 1), No. 45 (Lane 2), No. 59 (Lane 3), No. 79 (Lane 4), and non-GM (Lane 6) samples were analyzed by agarose gel [3% (w/v)] electrophoresis. Arrowheads indicate the PCR amplification products of the taxon-specific gene MON810, NK603, and SSIIb at 199, 444, and 155 bp, respectively. Lane M: DNA size marker.**

M863 1-5′ and M863 1-3′ for MON863 detection, M810 1-5′ and CryIA 1-3′ for MON810 detection, SSIIb 1-5′ and SSIIb 1-3′ for SSIIb detection, TC1507 1-5′ and TC1507 1-3′ for TC1507 detection, and Bt11 1-5′ and CryIA 1-3′ for Bt11 detection were used.

The reactions were buffered with PCR buffer II (Life Technologies Corp.) and amplified in a thermal cycler (Silver 96-well GeneAmp PCR System 9700; Life Technologies Corp.) in max mode, according to the following PCR step-cycle program: preincubation at 95°C for 10 min; 10 cycles of denaturation at 95°C for 0.5 min; annealing at 65°C for 1 min; extension at 72°C for 1 min; 27 cycles of denaturation at 95°C for 0.5 min; annealing at 65°C for 1 min; extension at 72°C for 1 min; and final extension at 72°C for 7 min. The cycle was repeated 40 times followed by a final extension at 72°C for 7 min.

### *Interlaboratory Study*

The interlaboratory study was organized by the National Institute of Health Sciences (NIHS) to evaluate the validity of DNA extraction from multiple ground samples, multiplex real-time PCR detection, and multiplex qualitative PCR detection in the individual kernel detection system in terms of its applicability, practicality, and ruggedness. The study was conducted with the participation of five laboratories. The validation test consisted of DNA extraction from individual ground maize samples, and multiplex real-time and multiplex qualitative PCR detection for the purpose of partial evaluation of the individual kernel detection system. For complete validation of the individual kernel detection system, it was necessary to distribute the maize kernel samples of GM seeds to the five laboratories. In distributing the maize kernel samples to the five laboratories, it was essential to know whether each maize kernel in the samples is GM or non-GM, as well as the events of each GM kernel prior to sample distribution. However, we could not confirm whether the maize kernel samples were GM or non-GM without grinding the seeds and subjecting the samples to detection, even though we could obtain some pure GM seeds as the positive sample. Of the several steps in the individual detection system, we consider that the steps requiring the most attention are the DNA extraction from individual ground kernels and the subsequent multiplex real-time PCR and multiplex PCR detection in terms of applicability, practicality, and ruggedness, since the steps involving the grinding of individual maize kernels appear to be uncontaminated due to the individual closed tubes.

Therefore, we weighed out the average weight of a normal maize kernel (0.37 g) from each ground sample as reference GM and non-GM samples and distributed it to individual tubes. These blind tube samples were thus sent to the laboratories. In addition to the 90 blind tube samples, solutions of the three primer pairs (each 6 μM), all reagents for PCR, a vacuum pump (DA-60D; ULVAC, Kanagawa, Japan), and the experimental protocol were provided to the five participating laboratories from the NIHS. Thus, a total of 450 blind tube samples were analyzed by the real-time PCR systems in the interlaboratory study. The guidelines for a collaborative study were referenced to determine the general procedure of this interlaboratory study (13).

## Results and Discussion

### *In-House Validation Study of DNA Extraction from Multiple Ground Samples and Multiplex Real-Time PCR*

To assess the applicability and reproducibility of DNA extraction from multiple ground samples and multiplex real-time PCR, these were assessed in an in-house study. Genomic DNA was individually extracted from 12 GM kernels (six MON810 kernels and six MON810xNK603 stacked kernels) and 12 non-GM kernels/day. Genomic DNA was amplified using multiplex real-time PCR. The tests were repeated for a total of 120 kernels (60 GM maize kernels and 60 non-GM maize kernels) on 5 different days. Figure 1 shows typical amplified products of genomic DNA from non-GM maize, MON810 maize, and MON810xNK603 stacked maize. Table 1 shows the results of in-house validation of multiplex real-time PCR for the same day and 5 different days. Neither false-positive nor false-negative results were observed (0%), verifying that both GM kernels and non-GM kernels can be clearly detected using the detection assay with good accuracy and precision at a confidence level of 95%, since the 95%

**Table 4. Interlaboratory validation of multiplex qualitative PCR**

| Laboratory/sample | No. 10 | No. 45 | No. 59 | No. 79 |
|---|---|---|---|---|
| A | MON810xNK603 | MON810xNK603 | MON810 | MON810 |
| B | MON810xNK603 | MON810xNK603 | MON810 | MON810 |
| C | MON810xNK603 | MON810xNK603 | MON810 | MON810 |
| D | MON810xNK603 | MON810xNK603 | MON810 | MON810 |
| E | MON810xNK603 | MON810xNK603 | MON810 | MON810 |
| Expected result | MON810xNK603 | MON810xNK603 | MON810 | MON810 |
| Accuracy, %[a] | 100 | 100 | 100 | 100 |

[a] Value was calculated from comparison with the expected result and results obtained in 5 different laboratories.

confidence interval of $P = 0.95$, calculated based on the Clopper-Pearson method, is 0.861–0.990 ($n = 60$; 14).

### *Interlaboratory Validation of DNA Extraction from Multiple Ground Samples, Multiplex Real-Time PCR, and Multiplex Qualitative PCR*

To intentionally assess the applicability, practicability, and ruggedness of the individual maize kernel detection system in multilaboratory use, we conducted an interlaboratory validation of DNA extraction from multiple ground samples, multiplex real-time PCR, and multiplex qualitative PCR for the individual maize kernel detection system. The study consisted of three steps: DNA extraction from multiple ground samples; multiplex real-time PCR for detection of GM maize samples, including stacked GM maize; and multiplex qualitative PCR for GM event detection.

We first examined the ruggedness of the multiplex real-time PCR using an AB7900 and AB7500 at NIHS. As shown in Table 2, we confirmed that the DNA extracted from test samples (non-GM samples, 86 tubes and GM samples, two tubes of MON810, and two tubes of MON810xNK603) were detected using both real-time PCR instruments. Five laboratories were then invited to take part in the interlaboratory study. Each laboratory was equipped with either an AB7900 or AB7500 real-time PCR instrument; three laboratories had an AB7900 and two laboratories had an AB7500. Table 3 summarizes the detection of the *SSIIb* gene, and GM detection of MON810 maize and MON810xNK603 maize for all samples in the interlaboratory study.

For the detection of the *SSIIb* gene, the positive rates of non-GM samples and GM samples were both 100% (Table 3). For GM detection of MON810 maize and MON810xNK603 maize, the positive rates of both MON810 event and MON810xNK603 were 100%, although only 10 tubes of each (two tubes × five laboratories) were tested. The positive rate of non-GM samples was 0% (Table 3).

Next, it was attempted to validate the multiplex qualitative PCR method for GM event detection. Before validation, we confirmed four GM samples using multiplex qualitative PCR. As shown in Figure 2, electrophoresis of samples from tube Nos. 10 and 45 showed the three amplified products of MON810, NK603, and SSIIb, which were judged to be MON810xNK603, while tube Nos. 59 and 79 showed the two amplified products of MON810 and SSIIb, which were judged to be MON810. All five laboratories judged the four tubes, Nos. 10, 45, 59, and 79, to be GM tube samples (Table 4), corresponding to MON810xNK603, MON810xNK603, MON810, and MON810, respectively, as confirmed by electrophoresis. These results suggest that multiplex qualitative PCR for events detection would be valid and suitable for reproducibility and ruggedness.

## Conclusions

This study suggested that the DNA extraction from multiple ground samples, multiplex real-time PCR, and multiplex qualitative PCR for the individual kernel detection system is suitable for enforcement purposes with respect to its interlaboratory reproducibility and transferability, applicability, and ruggedness. The interlaboratory study, including the DNA extraction step, was conducted in close adherence to internationally accepted guidelines for collaborative trials (15). In practical terms, the present study suggests these methods can be readily adopted by any laboratory. These methods would provide a practical tool for the detection of GM maize containing stacked maize. However, for complete validation of the individual kernel detection system, it is necessary to conduct the validation on a multiple laboratory basis with samples of seeds mixed to various GM levels and with various numbers of staked events such as MON810xNK603xMON863 or MON863xNK603. The present study suggests that the quantification as kernel-based measurement appears to be accurate in the case of testing pure GM seeds or non-GM bulk lot. When this proposed system can accurately monitor GM commingling level in maize samples, it will be an

appropriate tool for the implementation of respective Japanese regulatory requirements concerning authorized GM maize products.

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# Remarkable growth variation in a natural Japanese population of *Pleurocybella porrigens*

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## Abstract

In 2004, an outbreak of serious acute encephalopathy exclusively occurred in patients with chronic kidney diseases after the intake of basidiomycetous wood rotting fungus *Pleurocybella porrigens*. The exact factors that induced encephalopathy by this mushroom remain unknown partly due to its extreme slow growth. We attempted to develop media suitable for vegetative growth of *P. porrigens* for application in various fields. Fifteen isolates of *P. porrigens* collected from rotting conifers, *Cryptomeria japonica* and *Pinus densiflora*, in different geographical areas in Japan were cultivated on potato dextrose agar (PDA) medium; large variation in growth rate and colony features was observed among these isolates. The five isolates with the best growth rates were then cultured in five kinds of liquid media, potato dextrose (PD) medium, malt extract · yeast extract (MY) medium, potato extract · carrot extract (PC) medium, Amazake medium, and Ohta's medium at 20°C in the dark. Dry biomasses of the isolates cultured in the liquid media were determined after 8 weeks of static cultivation. Among the tested liquid media, PD medium was the most suitable for biomass growth, followed by Ohta's, MY, Amazake and PC media. The average biomass growth of the isolates cultured in the synthetic medium (Ohta's medium) was 20-92% of that in PD medium. Remarkably large biomass variation was also observed among the isolates cultured on each liquid medium. Mycelia of this mushroom had abortive lateral branching at high frequency which could be one reason why this mushroom grows very slowly. Moreover, the Japanese population of *P. porrigens* has large variation in vegetative growth. Taken together, elucidation of the possible association between its chemical constituents and the onset of encephalopathy may be possible by culturing isolates with high growth ability on PD medium as a natural medium and Ohta's medium as a synthetic medium.

Keywords : abortive branching, growth variation, liquid medium, morphological variation, Sugihiratake

## I Introduction

A basidiomycetous wood rotting fungus, *Pleurocybella porrigens* (Pers.) Singer, is a well known, excellent edible mushroom and is named Sugihiratake in Japan based on its morphological and ecological features. This mushroom is most frequently encountered on the conifer Sugi (*Cryptomeria japonica* D. Don), in addition to other conifers, such as pine (*Pinus* spp.) and spruce (*Picea* spp.), and rarely on Japanese beech (*Fagus crenata* Blume)[1]. Many Japanese people, especially those living on the Sea of Japan side of the Chubu region, which is located in the center of Honshu Island, Japan, and the Tohoku region, the northeast part of Honshu Island, have been favorably consuming it in processed foods, such as miso soup and tempura, before 2004. In September 2004, an outbreak of serious acute encephalopathy exclusively occurred in patients with chronic kidney diseases after the intake of this mushroom in many areas of Japan including Akita, Yamagata, and Niigata Prefectures[2-6]. Thereafter, many researchers have examined the factors involved in the onset of encephalopathy. Akiyama *et al.* showed that high amounts of cyanide were detected in some *P. porrigens*

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from 2004 and discussed the association between cyanide and encephalopathy[3, 6]. Amakura *et al.* also detected three novel conjugated long-chain fatty acids (1–3) in some *P. porrigens*[7]. Sasaki *et al.* detected metabolites of vitamin D-like compounds in some *P. porrigens* and suggested that these metabolites might induce hypocalcemia by working as vitamin D agonists or antagonists[8]. Kondo *et al.* specifically detected α-eleostearic acid, which induces the caspase-independent and apoptosis-inducing factor-initiated apoptotic death of neuronal cell lines[9]. Matsumoto *et al.* reported that the mushroom specimens from different geographical areas in Japan were grouped into two distinct clusters based on internal transcribed spacer (ITS) rDNA sequences, although no correlation was found with differences of substrates for fruiting and geographic origins of the specimens[1]. Thus, the exact factors that induced encephalopathy remain unclear.

We considered that the artificial culture of *P. porrigens* would be critical for investigating the chemical factors in *P. porrigens* that induced encephalopathy. In our preliminary experiments, we found that vegetative growth of *P. porrigens* was extremely slow even when cultured on natural media, leading to obstacles for application to various fields of research on this mushroom.

In the present study, we therefore attempted to find a suitable medium for more rapid vegetative growth of *P. porrigens* for studying the possible association between its chemical constituents and the onset of encephalopathy.

## II Material and Methods

### 1. Organisms

The fungus isolates used in this study are shown in Table 1. CHU0006, CHU0011, CHU0021, CHU0022, CHU0023, CHU0027, CHU0028, CHU0035, CHU0042, CHU0046, CHU0051, CHU0054, and CHU0057 were isolated from natural basidiomata. All were maintained on potato dextrose agar medium (PDA) at 20 ± 1°C in the dark.

### 2. Media

Solid media used in this study were PDA (24 g dehydrated potato dextrose broth (PD) (Difco, BD, MD, USA) and 15 g agar (Nacalai Tesque, Kyoto, Japan) in 1000 mL distilled water) and MY agar (10 g malt extract (Difco), 2 g yeast extract (Difco), and 15 g agar (Nacalai Tesque) in 1000 mL distilled water). PD medium was prepared by mixing 24 g dehydrated PD broth (Difco) in 1000 mL distilled water; MY medium by mixing 10 g malt extract (Difco) and 2 g yeast extract (Difco) in 1000 mL distilled water; potato extract・carrot extract (PC) medium by boiling 20 g peeled potato and 20 g peeled carrot in 1000 mL distilled water for 30 min and filtering with gauze (the mixture of potato and carrot extracts was adjusted the volume to 1000 mL with distilled water and pH 5.0 by the addition of 1 M KOH and 1 M HCl); Amazake medium by boiling 15 mL Amazake (a traditional sweet, low-alcoholic Japanese drink made from fermented rice) extract in 1000 mL distilled water, filtering with cotton tissue and adjusting with distilled water to 1000 mL[10]; and Ohta's medium by mixing 10 g glucose, 1 g citric acid, 1 g ammonium tartrate, 1 g $KH_2PO_4$, 1 g $MgSO_4 \cdot 7H_2O$, 1 g $CaCl_2 \cdot 2H_2O$, 7 g HEPES, 10 mL mineral solution (including 5 g $FeCl_3$, 50 mg $MnSO_4 \cdot 6H_2O$, 300 mg $ZnSO_4 \cdot 7H_2O$, 50 mg $CoSO_4 \cdot 7H_2O$, 100 mg $CuSO_4 \cdot 5H_2O$, 200 mg $NiSO_4 \cdot 6H_2O$, and 3 mL acetylacetone in 1000 mL distilled water), 10 mL vitamin solution (consisting of 300 mg thiamine hydrochloride, 5 mg nicotinic acid, 3 mg folic acid, 5 mg biotin, 0.5 mg pyridoxine

Table 1. List of *Pleurocybella porrigens* isolates and the abberevations used in this study

| Isolate no. | Abbreviation | Locality | Substratum* | Collection date |
|---|---|---|---|---|
| CHU0006 | 0006 | Unotoro, Murakami City, Niigata Prefecture, Japan | A fallen tree of *Pinus densiflora* | Oct. 2nd, 2009 |
| CHU0011 | 0011 | Unotoro, Murakami City, Niigata Prefecture, Japan | A fallen tree of *Pinus densiflora* | Oct. 2nd, 2009 |
| CHU0021 | 0021 | Natsui, Tainai City, Niigata Prefecture, Japan | A rotten wood of coniferous tree** | Oct. 1st, 2009 |
| CHU0022 | 0022 | Natsui, Tainai City, Niigata Prefecture, Japan | A rotten wood of coniferous tree** | Oct. 1st, 2009 |
| CHU0023 | 0023 | Natsui, Tainai City, Niigata Prefecture, Japan | A rotten wood of coniferous tree** | Oct. 1st, 2009 |
| CHU0027 | 0027 | Higashihouden, Mogamimachi, Yamagata Prefecture, Japan | A rotten wood of *Cryptomeria japonica* | Oct. 7th, 2009 |
| CHU0028 | 0028 | Higashihouden, Mogamimachi, Yamagata Prefecture, Japan | A rotten wood of *Cryptomeria japonica* | Oct. 7th, 2009 |
| CHU0035 | 0035 | Higashihouden, Mogamimachi, Yamagata Prefecture, Japan | A rotten wood of *Cryptomeria japonica* | Oct. 7th, 2009 |
| CHU0042 | 0042 | Mt. Hakkaisan, Minamiuonuma City, Niigata Prefeture, Japan | A rotten wood of *Cryptomeria japonica* | Oct. 11th, 2009 |
| CHU0046 | 0046 | Mt. Hakkaisan, Minamiuonuma City, Niigata Prefeture, Japan | A rotten wood of *Cryptomeria japonica* | Oct. 11th, 2009 |
| CHU0051 | 0051 | Higashihouden, Mogamimachi, Yamagata Prefecture, Japan | A fallen tree of *Cryptomeria japonica* | Oct. 16th, 2009 |
| CHU0054 | 0054 | Higashihouden, Mogamimachi, Yamagata Prefecture, Japan | A rotten stump of *Cryptomeria japonica* | Oct. 16th, 2009 |
| CHU0057 | 0057 | Higashihouden, Mogamimachi, Yamagata Prefecture, Japan | A rotten stump of *Cryptomeria japonica* | Oct. 16th, 2009 |
| NBRC30334 | 30334 | | A stump of *Cryptomeria japonica* | |
| NBRC30384 | 30384 | | A rotten branch of *Cryptomeria japonica* | |

*: Substratum on which the fruit body of *P. porrigens* used for the isolation was observed. **: Possibly *Pinus densiflora*, but was not able to identify definitely. Blank: no record.

hydrochloride, 1 mg carnitine chloride, 3 mg adenine sulfate · $2H_2O$, 3 mg choline chloride) and adjusting to 1000 mL with distilled water and pH 5.0-5.1 by 1 M KOH[11]. Ohta's medium was sterilized by autoclaving at 120°C for 7 min[11]. Other media were sterilized by autoclaving at 121°C for 10 min.

### 3. Inocula

Inoculate mycelial disks were cut with a cork borer 4 mm in diameter from the sub-peripheral region of a colony actively growing on the MY agar plate, aseptically after 12 weeks.

### 4. Mycelial expansion

The disk was placed on PDA in a 9.0-cm diameter Petri dish for mycelial expansion. The diameters of the mycelial colony crossing at right angles were measured separately after 12-week incubation at 20.0 ± 0.5°C in the dark. Mycelial expansion was determined by the average of the diameters of the mycelial colonies crossing at right angles. All mycelial colonies were in a linear growth phase at measurement. The mycelial expansion of each culture is presented as the average of five replicates.

### 5. Biomass experiments

The disk was placed aseptically into 30 mL sterilized liquid medium in a 50-mL conical flask and then sealed with a sterilized Silicosen (Shin-Etsu Finetech Co., Ltd., Tokyo, Japan). The mycelia were harvested after 8-week incubation at 20.0 ± 0.5°C in the dark. The dry weight of each mycelium was obtained after drying at 60°C for 24 h with an air blast. Biomass of each culture is presented as the average of dry weights of ten replicates with both standard deviation and standard error. The final pH of each medium was measured with a glass electrode pH meter (Horiba pH/Ion meter F-23, Horiba, Ltd., Kyoto, Japan).

An additional replicate of all tested media was separately prepared for the morphological observation of each mycelium. A piece of mycelium was mounted with each cultivated medium and observed by differential interference contrast microscope (Olympus BX51, Olympus Co., Tokyo, Japan).

### 6. Statistical analysis

Data was analyzed statistically by one-way analysis of variance (ANOVA) and post-hoc Tukey-Krammer test. All analyses were performed using Statcel2 statistical software (OMS Publishing Inc., Tokyo, Japan).

## Ⅲ Results

### 1. Growth and multiform colonies of *P. porrigens* on PDA

Fifteen isolates of *P. porrigens* collected from rotting conifers, *C. japonica* and *Pinus densiflora* Sieb. et Zucc., in different geographical areas in Japan grew extremely slow. Most tested isolates formed mycelia that look like those often observed in the cultivation of basidiomycetous ectomycorrhizal fungi. After 12-week cultivation, five isolates (0011, 0028, 0046, 30334, and 30384) showed larger mycelial colonies among the ten isolates chosen at the first screening of the original 15 isolates; five isolates were previously removed as they showed conspicuously poor growth abilities (Fig. 1; Table 2). Large variation in morphological features in mycelial colonies was associated with growth rate among the isolates cultured on solid medium (i.e., PDA). The shape and density of

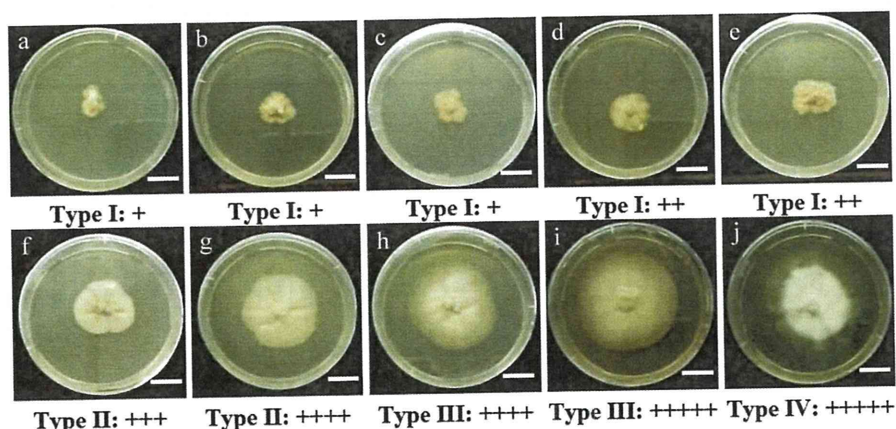


Fig. 1. Multiform colonies of *Pleurocybella porrigens* isolates. All mycelial colonies were cultivated for 12 weeks on PDA. Each dish is labeled with the colony type and growth ability. The mycelial growth ability was estimated from the lowest to the highest by the symbols, "+", "++", "+++", "++++" and "++++++", in which "+" indicates the colony square with a similar size to the inoculum and "+++++" indicates the colony square that almost covers the Petri dishes. (a) 0027; (b) 0035; (c) 0057; (d) 0006; (e) 0051; (f) 0028; (g) 0011; (h) 0046; (i) 30334; (j) 30384. Bars: 18 mm.

Table 2. Mycelial expansion of *Pleurocybella porrigens* isolates on PDA after 12-week cultivation

| Isolate | Diameter of mycelial colony (mm)* | Growth rate | Type |
|---|---|---|---|
| 0006 | $24.2 \pm 1.0^{a}$ | ++ | I |
| 0011 | $44.4 \pm 1.5^{b}$ | ++++ | II |
| 0027 | $14.9 \pm 0.8^{c}$ | + | I |
| 0028 | $33.3 \pm 1.1^{d}$ | +++ | II |
| 0035 | $20.5 \pm 1.3^{e}$ | + | I |
| 0046 | $47.6 \pm 1.3^{f}$ | ++++ | III |
| 0051 | $24.1 \pm 1.2^{a}$ | ++ | I |
| 0057 | $18.2 \pm 0.8^{e}$ | + | I |
| 30334 | $56.2 \pm 1.0^{g}$ | +++++ | III |
| 30384 | $60.6 \pm 0.5^{h}$ | +++++ | IV |

* Average ± standard deviation (SD). The different lowercase letters indicate significant difference (Tukey-Kramer test, $p < 0.05$) in average diameter of mycelial colonies between the isolates.

colonies of these isolates were multiform and thus temporarily separated into four types: I, II, III, and IV (Fig. 1). In Type I, the colony surface was rough and the aerial hyphal layer was equally thick, tending to grow in the margins in sub-medium. In Type II, the colony surface was smooth and the aerial hyphal layer was equally thick, with some striation from the center. In Type III, the colony surface was smooth and the aerial hyphal layer was thick in the center but thin with branching hyphae in the margins. In Type IV, colonies looked like callus and separated into two parts: thick in center and thin speckled in the margins.

We selected five isolates for the following experiments because growth rates of some isolates were quite slow on PDA and thus unsuitable.

## 2. Growth of *P. porrigens* mycelia in liquid media

The dry biomass obtained from 8-week cultivation of each isolate in *P. porrigens* was significantly different among media (Fig. 2). Large variation in dry biomass among the isolates was observed in MY, PD, Ohta's, and Amazake media, but not in PC medium which showed extremely small values. Among the five liquid media, PD medium was the most suitable medium for biomass growth, followed by Ohta's, MY, Amazake and PC media. The average biomass growth of the isolates cultured in the synthetic medium (Ohta's medium) was 20-92% of that in the natural medium (PD medium). Remarkable large biomass variation was also observed among the isolates cultured on the each liquid medium. The maximum dry biomass (126.9 mg) was obtained from isolate 0028 cultivated in PD medium whereas the minimum dry biomass (4.9 mg) was obtained from isolate 30334 cultivated in PC medium (Fig. 2).

From these results, we categorized the five isolates into three groups: isolates that have good growth both in PD medium and on PDA (isolates 0011, 0028, and 30384), isolate that has average growth in PD medium but good on PDA (isolate 0046), and isolate that has poor growth in PD medium

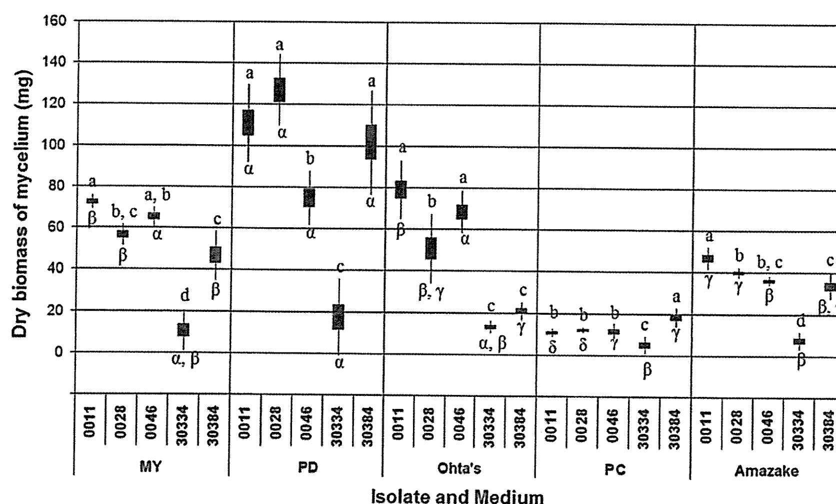


Fig. 2. Mycelial dry biomass of *Pleurocybella porrigens* isolates cultured for 8 weeks in five media: MY medium, PD medium, Ohta's medium, PC medium and Amazake medium (abbreviated in this figure as MY, PD, Ohta's, PC and Amazake). Vertical column: average ± standard error; vertical bar: average ± standard deviation. The different lowercase letters above each bar indicate significant difference of dry biomass of mycelium (Tukey-Kramer test, $p < 0.05$) when internally compared among the five isolates cultured in the same medium. The different symbols below each bar indicate significant difference of dry biomass of mycelium (Tukey-Kramer-test, $p < 0.05$) when compared with the same isolate cultivated in the five media: MY, PD, Ohta's, PC and Amazake.

but good on PDA (isolate 30334) (Figs. 1 & 2; Table 2).

The liquid media changed colors by 8-week cultivation of the five isolates (Fig. 3). Isolate 30334 grown in Amazake medium gradually became less turbid during 8-week cultivation. Liquid Amazake and Ohta's media used for the cultivation of isolate 0046 was stramineous yellow compared with that used for the cultivation of other isolates in the same medium.

### 3. Micro-characteristics of *P. porrigens* mycelia

Mycelia of *P. porrigens* had short lateral branching hyphae (abortive branching hyphae) at high frequency. The abortive branching hyphae projected from behind septa were common in the mycelia of all *P. porrigens* isolates in both the liquid media (Fig. 4a) and the solid medium (agar medium). Swollen hyphae at the tips and/or internodes were observed in the liquid media (Fig. 4b), but not on PDA. All isolates had many dark cotton-like parts, and were tentatively called dark bodies, only in the mycelia cultured in MY medium (Fig. 4c).

## Ⅳ Discussion

In 2005, Matsumoto *et al.* reported that a natural Japanese population of *P. porrigens* had diverged and grouped them into two distinct clusters based on nuclear ITS rDNA sequences[1]. In the present study, remarkable variation in growth ability and morphological features of mycelial colonies were noted among the isolates collected from rotting conifers, *C. japonica* and *P. densiflora*, in different geographical areas even when they were cultured on the same liquid medium. Average biomass growth of all isolates cultured in the same liquid medium showed similar tendencies among the tested isolates except for isolate 30384 (Fig. 2). No relationship was found between growth ability of the isolates and their collection location and inhabited substratum (Fig. 2; Table 1).

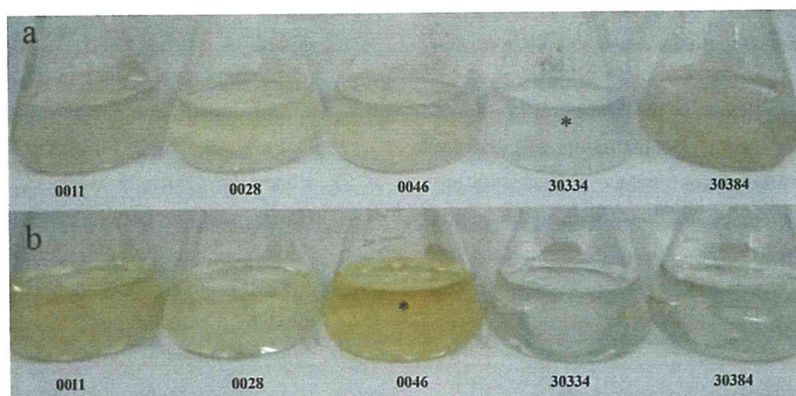


Fig. 3. Cultivation of five isolates (0011, 0028, 0046, 30334, and 30384) in liquid media after 8 weeks. a) Amazake medium. b) Ohta's medium. Isolates 0046 and 30334 (*) showed different color compared with other isolates in the same medium.

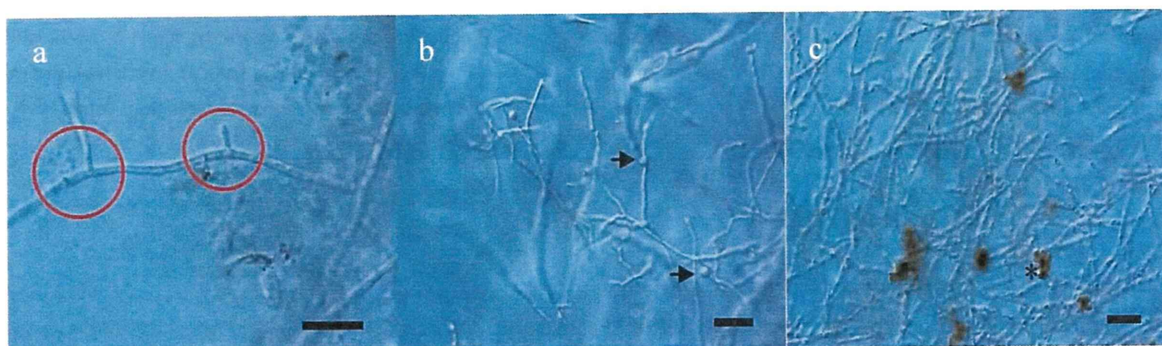


Fig. 4. Micro-characteristics of *Pleurocybella porrigens* mycelia. a) Branching hyphae adjacent to septa (circles) in the mycelium of isolate 30334 grown in Amazake medium. b) Mycelium with several swelling parts (arrows) like chlamydospores of isolate 0011 grown in Amazake medium. c) Dark bodies (*) combined with the mycelium of isolate 0011 grown in MY medium. Bars: 35 μm.

Ishihara reported that the vegetative growth of this fungus is quite slow and Amazake agar medium (solid medium) was the most suitable for the vegetative growth[10]. However, for the improvement of vegetative growth, Amazake medium (liquid medium) was inferior to other tested liquid media except for PC medium (Fig. 2). The difference between the results in Ishihara's experiments and ours could be derived from the solid media used for the former and liquid media used for the latter since isolates 0028 and 00334 showed prominent differences in biomass growth in PD medium (liquid medium) and on PDA (solid medium) (Fig. 2; Table 2). Of course, we cannot deny the possibility of differences in the isolates used in both experiments, as large variance in mycelial growth among isolates were observed in the present experiments, irrespective of the medium (Fig. 2; Table 2).

The extreme slow growth of this fungus compared with other saprobic fungi may at least in part be caused by the formation of short abortive branching of hyphae (Fig. 4a). The accelerated growth observed in the liquid cultures may be derived partly from the formation of the swelling parts, which look like clamydospores, allowing secondary colonization by dispersion followed by germination in the liquid medium (Fig. 4b).

In conclusion, further research to elucidate the possible association between the chemical constituents and the onset of encephalopathy may be possible when isolates with high growth ability are cultured in suitable liquid media such as PD medium as a natural medium and Ohta's medium as a synthetic medium.

## V Acknowledgements

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