Phylogenetic Relationship of Psychoactive Fungi Based on the rRNA Gene for a Large Subunit and Their Identification Using the TaqMan Assay

Takuro MARUYAMA,[a] Kazumasa YOKOYAMA,[b] Yukiko MAKINO,[c] and Yukihiro GODA*,[a]

[a] *National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan:* [b] *Faculty of Education, Shiga University; 2–5–1, Hiratsu, Otsu 520–0862, Japan: and* [c] *Kanto-Shin'etsu Regional Narcotic Control Office, Ministry of Health, Labor and Welfare; 2–4–14, Nakameguro, Meguro-ku, Tokyo 153–0061, Japan.*

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"Magic mushrooms" (MMs) are psychoactive fungi containing the and Psychotropics Control Law in Japan. Because there are many kinds of MMs and they are often sold even as dry powders in local markets, it is very difficult to identify the original species of the MMs by morphological observation. Therefore, we investigated the rRNA gene for a large subunit (LSU) of several MMs to classify them by a genetic approach. In this paper, we described the phylogeny of species of MMs based on the partial sequence (about 970 bp) of the LSU and the rapid identification of MMs using the TaqMan PCR assay.

Key words magic mushroom; rRNA large subunit; TaqMan polymerase chain reaction (PCR); genus *Psilocybe*; genus *Panaeolus*

"Magic mushroom" (MM) is the name most commonly given to psychoactive fungi, which contain the hallucinogenic compounds, psilocin (**1**) and psilocybin (**2**) (Fig. 1). The hallucinogenic activities of **1** and **2** are 1/200 of LSD (**3**) and 50 times higher than that of mescaline (**4**).[1,2] It is thought that the chemical similarity to serotonine (**5**), one of the neurotransmission substances in the brain, explains their hallucinogenic effects.[3] In recent years, MMs have appeared in the Japanese street markets, and caused various intoxication accidents. Therefore, since June 6, 2002, MMs have been regulated by the Narcotics and Psychotropics Control Law in Japan.[4]

In an earlier report,[6] we investigated the nucleotide sequence of the internal transcribed spacer (ITS) region of the MMs obtained in Japanese markets and found that the DNA sequence analysis was useful for the classification of MMs. However, a series of operations, such as extraction of genomic DNA, polymerase chain reaction (PCR) and nucleotide sequencing, is very troublesome and time-consuming. Therefore, in this study, we attempted to establish an easier identification system by TaqMan PCR method[7] which has been used in the detection of genetically modified foods and pathogens in food materials.[8—10]

Experimental

Fungus Materials MM samples used are listed in Table 1. Samples J1-J5 were collected in Japan, N1 and N2 were purchased in the markets of the Netherlands, and MM-1 through MM-6 were obtained in Japanese markets in 2001. Except for MM-2 through MM-6 whose original species were guessed from the nucleotide sequence of ITS and LSU,[6] all MM specimens were identified morphologically by K. Yokoyama (second author) and stored in the Laboratory of Biology, Faculty of Education, Shiga University. It is noted that MM-5 and MM-6 are not MMs, though they are psychoactive fungi containing ibotenic acid (**6**). Food mushrooms were kindly provided from a Japanese supplier and are listed in Table 2.

Sequence Analysis of the rRNA Gene for a Large Subunit (LSU) Genomic DNA was extracted and purified from about 50 mg of dried sample using DNeasy Plant Mini Kit (Qiagen). The 1300 bp DNA fragment composed of the complete sequence of ITS-2 and the partial sequence of the 5.8S rRNA gene and LSU (about 970 bp), was amplified by PCR from obtained genomic DNA with the primers (sense-1 and antisense-1) and KOD DNA polymerase (Toyobo), which shows high fidelity attributed to its 3′—5′ exonuclease activity. The PCR was performed on a TaKaRa PCR Thermal Cycler MP (Takara Shuzo) with the following temperature program: 94 °C,

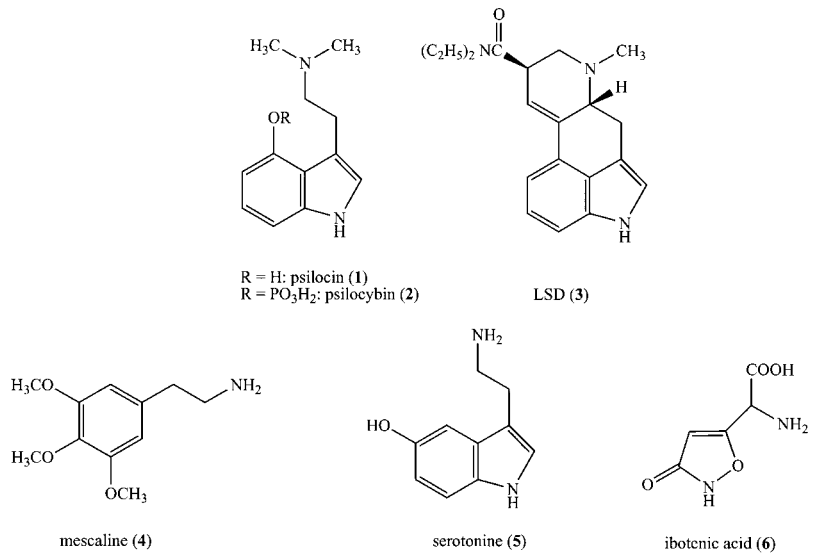

Fig. 1. Structures of Hallucinogenic Compounds and Serotonine

* To whom correspondence should be addressed. e-mail: goda@nihs.go.jp

Table 1. Sample Lists of *Psilocybe* and *Panaeolus* Genus and Their Data

Group	Scientific name	Japanese name	Date of collection	Fragment size of LSU (bp)	Voucher No.
J1	*Panaeolus sphinctrinus* (Fr.) Quél.	Hikagetake	1999.06.30	970	KY7130
J2	*Panaeolus subbalteatus* (Berk. *et* Br.) Sacc.	Senbonsaigyougasa	1983.07.06	969	KY4155
J3	*Psilocybe argentipes* K. Yokoyama	Hikageshibiretake	1983.07.06	971	KY3573
J4	*Psilocybe fasciata* Hongo	Aisenbontake	1974.05.03	Not determined	KY1837
J5	*Psilocybe subaeruginascens* Höhnel	Oshibiretake	1983.09.23	964	KY4097
N1	*Psilocybe subcubensis* Guzmán	—	2000.01.21	971	KY7054
N2	*Psilocybe tampanensis* Guzmán *et* Pollock	—	2000.12.04	971	KY7134
MM-1[a)]	*Psilocybe cubensis* (Ear.) Singer	Minamishibiretake	—	971	KY7156
MM-2-1[a)]	*Panaeolus cyanescens* (Berk. *et* Br.) Sacc.[b)]	Aizomehikagetake	—	970	—
MM-2-2[a)]	*Panaeolus cyanescens* (Berk. *et* Br.) Sacc.[b)]	Aizomehikagetake	—	970	—
MM-3[a)]	*Psilocybe semilanceata* (Fr. *ex* Sec.) Kummer[b)]	—	—	970	—
MM-4[a)]	*Psilocybe tampanensis* Guzmán *et* Pollock[b)]	—	—	971	—
MM-5[a)]	*Amanita* sp.-1[b)]	—	—	978	—
MM-6[a)]	*Amanita* sp.-2[b)]	—	—	978	—

a) Same as the group described in our previous report (ref. 6). *b*) guessed from the nucleotide sequence of ITS and LSU (ref. 6).

Table 2. Sample Lists of Food Mushroom and Their Data

Scientific name	Japanese name	Date of collection	Fragment size of LSU (bp)	Voucher No.
Flammulina velutipes (Curt. *ex* Fr.) Sing.	Enokitake	2002.05	969	TM001
Pleurotus eryngii (Dc. *ex* Fr.) Quél.	Eringi	2002.05	948	TM002
Lentinus edodes (Berk.) Sing.	Shiitake	2002.05	969	TM003
Pholiota nameko (T. Ito) S. Ito *et* S. Imai	Nameko	2002.05	970	TM004
Hypsizygus marmoreus (Peck) Bigelow	Bunashimezi	2002.05	971	TM005
Grifola frondosa (Dicks. *ex* Fr.) Sf. Gray	Maitake	2002.05	973	TM006

4 min; 30 cycles of 98 °C, 15 s, 60 °C, 5 s, 74 °C, 30 s; 74 °C, 4 min. After the removal of excess primers and dNTPs by microcon-PCR (Millipore), the amplified fragments were sequenced directly by the dideoxy chain termination method using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). In the case that the obtained PCR product was insufficient yield to direct sequencing due to highly damaged DNA, nested PCR was performed using the primers (sense-2 and antisense-1), then the amplicon was introduced into the pCR-Blunt vector using Zero Blunt PCR Cloning Kit (Invitrogen) for subsequent sequencing analysis. The sequence information obtained in this study were registered on the nucleotide sequence databases (DDBJ, EMBL, GenBank) with the accession no. shown in Fig. 2 except for MM-2 through MM-6. Alignment and phylogenetic analyses of the sequence data were carried out by Clustal W program.[11)] The sequence information of the primers used in this study is shown in Table 3 together with the information on the primers and the probes for TaqMan assay described below.

TaqMan Assay Positive probe and primers (MM-S1, MM-A1) were designed in the conserved region among fungi, and probes 1 and 2 were designed in the region specific to MMs (Fig. 3). All probes were designed based on the sequence of the antisense strand. The size of amplicon produced by the primer pair is about 220 bp. TaqMan assay was performed in a total reaction volume of 25 μl containing 1×Master mix, 200 nM primers, 200 nM TaqMan MGB probe(s)[12)] (The above all are from Applied Biosystems), and 1 μl of template (10 ng) except for the following samples: *Ps. tampanensis* (MM-4, 4.6 ng), *Ps. argentipes* (J3, 7.2 ng), *Ps. fasciata* (J4, 3.7 ng), and *Ps. subcubensis* (N1, 3.5 ng). Thermal cycling was performed in an ABI PRISM 7700 (Applied Biosystems) and consisted of two initial holds (50 °C for 2 min and 95 °C for 10 min) and 35 cycles of denaturation at 95 °C for 15 s and an anneal/extend step of 60 °C for 1 min. Each test was performed in quadruple in the same run. Data were analyzed using the SDS 1.7 application software (Applied Biosystems). The assay was divided into two steps; namely, the first step for the confirmation of amplification with the positive probe and the second step for the identification of MMs with probe-1 and probe-2.

Results and Discussion

Phylogenetic Relationship of Psychoactive Fungi Based on the LSU Sequence The rRNA gene for a large subunit (LSU) was selected as the target gene for the following reasons: 1) The gene has already been investigated in many fungi including the *Psilocybe* and *Panaeolus* genus.[13,14)] 2) The gene has stem and loop regions and nucleotide substitutions have occurred more frequently in the stem region than in the loop region.[15)] This feature is very suitable to establish the species identification method using the TaqMan assay because the set of the fluorescence probe in the variable stem region and the primers in the conserved loop region enable us to construct the examination system covering various species. The LSU partial sequence was amplified by PCR using genomic DNA and directly sequenced. Among all sequence data, the sequence of *Pa. cyanescens* (MM-2-1) was consistent with that of *Copelandia cyanescens* (=*Pa. cyanescens*, Accession no. AF261526 which contains only 925 bp of the LSU), but was different in only one position with that of *Pa. cyanescens* (MM-2-2). Furthermore, 2 and 4 nucleotide changes (substitutions or deletions) were observed in the data of *Ps. cubensis* (MM-1) and *Ps. semilanceata* (MM-3) when the sequence was compared with the corresponding known ones (AF261619, AF261616, respectively). These data indicate the presence of intra-specific mutations. On the other hand, the LSU sequence of two *Ps. tampanensis* samples (N2 and MM-4) is identical to each other although two nucleotide substitutions were observed in their ITS sequences.[6)] The ITS and LSU nucleotide sequences of *Ps. cubensis* (MM-1) and *Ps. subcubensis* (N1) are also identical to each other. Hence, sequence analysis of ITS and LSU is useless to distinguish between the two. The fragment size of LSU derived from *Psilocybe* and *Panaeolus* genus is shown in Table 1 and ranged between 969—972 bp except for one

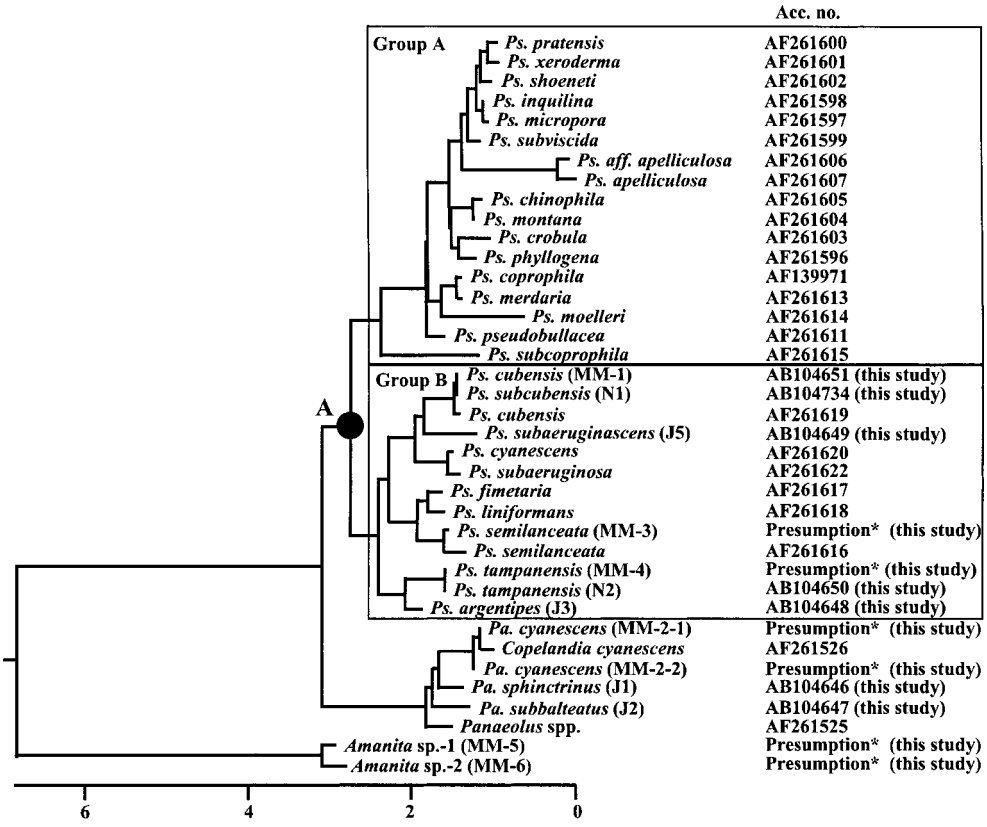

Fig. 2. Phylogenetic Tree Constructed from the Nucleotide Sequences of the rRNA Large Subunit Genes of Several Magic Mushrooms

*: Their original species are now being investigated on the basis of morphology by K. Y. (Second author).

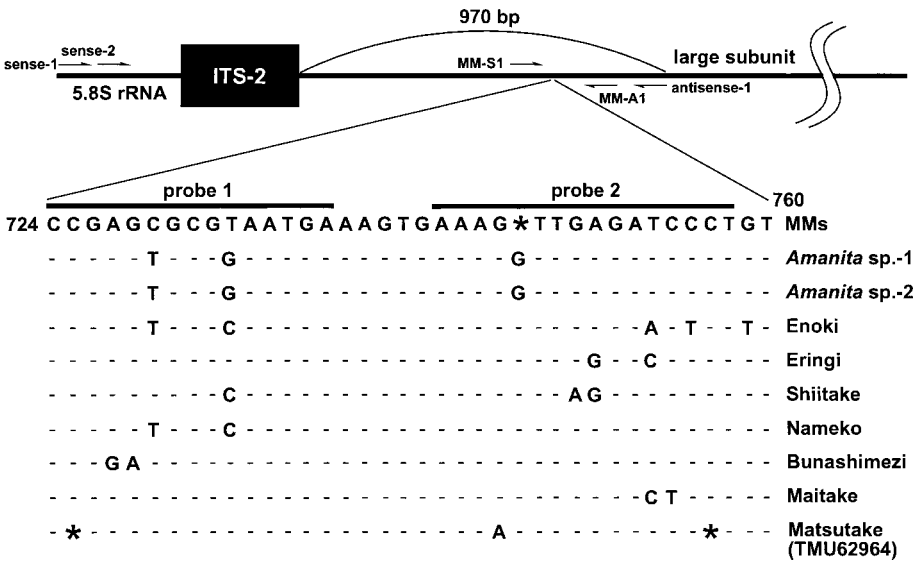

Fig. 3. Nucleotide Sequences of the LSU Region Amplified from Several Fungi

Number is the nucleotide position in *Psilocybe cubensis*. A hyphen '–' and an asterisk '*' indicate the same nucleotide as MMs and a gap, respectively.

species (*Ps. subaeruginascens*; J5).

A phylogenetic tree constructed from the LSU nucleotide sequences obtained in this study and registered on databases (DDBJ, EMBL and GenBank) is shown in Fig. 2. Three genera (*Psilocybe*, *Panaeolus*, and *Amanita*) used in this study were classified into distinct clusters, and its dividing pattern was similar to that obtained from the ITS sequences.[6] The cluster composed of the *Psilocybe* genus was furthermore divided into two groups (Group A and B, Fig. 2). Moncalvo *et al.* has already pointed out that psilocybin-containing species are distinguished from no psilocybin-containing species phylogenetically in the *Psilocybe* genus.[14] The former species is classified into Group B and our results are well in accord with theirs. When we considered the evolution route of the *Psilocybe* genus, the loss of main secondary metabolites seems to be very important. Namely, it means that some

events, such as loss of psilocybin biosynthetic enzymes or their transcription control factors, occurred at bifurcation A in Fig. 2. The consistency between phylogenetic classification and the distribution of major secondary metabolites was also observed between *Glycyrrhiza* plants and glycyrrhizin.[16)]

Identification of MM Using the TaqMan Assay Sequence alignments of the LSU of MMs and food mushrooms allowed us to find the specific sequence to MMs as shown in Fig. 3 except for the case of the non-psilocybin mushroom, *Ps. subcoprophila*, whose sequence has an additional guanine at position 748. Furthermore, we found the corresponding sequences of other fungi, especially edible mushrooms, from databases and checked them. As a result, it was confirmed that the above region was almost specific to MMs among the mushroom kingdom. However, the length of this region was exceedingly long for designing a specific probe which could clearly detect a mutation in this region. Recently, a TaqMan MGB probe, which is shorter in length and has higher sequence specificity than the ordinarily used probe, was developed.[12)] In the newest TaqMan assay, two distinct fluorescent signals were detectable simultaneously in each run by using a differential reporter dye in their 5′-ends. Therefore, we designed a set of two different TaqMan MGB probes (probe-1 and probe-2) for the region (Fig. 3) to discriminate MMs from the other fungi by TaqMan PCR. In addition, we constructed a third probe (positive probe) for the confirmation of the PCR amplification in the conserved region (Table 3).

Table 3. The Sequences of Primers and TaqMan MGB Probes Used in This Study

Primer	Sense-1	5′-TCGATGAAGAACGCAGCG-3′
	Sense-2	5′-CATCGAATCTTTGAACGCACCTTG-3′
	Antisense-1	5′-ATCCTGAGGGAAACTTCGGCA-3′
	MM-S1	5′-CGTCTTGAAACACGGACCAA-3′
	MM-A1	5′-CACCAGAGTTTCCTCTGGCTTC-3′
Probe	Probe-1	5′-FAM-TCATTACGCGCTCGG-MGB-3′
	Probe-2	5′-VIC-AGGGATCTCAACTTT-MGB-3′
	Positive probe	5′-FAM-TTCAGGCATAGTTCAC-MGB-3′

FAM and VIC are reporter dyes. MGB is the minor groove binder ligand.

Considering nucleotide sequences, it is expected that all of the MMs, except for *Ps. subcoprophila*, afford the fluorescent signals derived from the set of probes in the TaqMan PCR assay. On the other hand, it is expected that at least one fluorescent signal is missing in the case of food mushrooms. Namely, “Enoki” and “Shiitake” afford no signal, and “Eringi” and “Maitake”, and “Bunashimezi” and “Nameko” afford one signal from probe-1 and probe-2, respectively.

The data of the TaqMan assay are summarized in Table 4. All samples, except for *Ps. fasciata*, afford a fluorescent signal in the confirmation of PCR amplification using the positive probe. The fungus, *Ps. fasciata*, was collected about 28 years before (Table 1). Therefore, the genomic DNA of *Ps. fasciata* was degraded so heavily that the PCR product was not amplified. The examination with probe-1 and -2 gave the same results as expected before. The results indicate that these probes have high sequence specificity and that an established assay can clearly distinguish the MMs from the other fungi.

The results of assay with probes-1 and -2 are graphed in Fig. 4. MMs were classified into two groups. Namely, MMs of Group 1 show high fluorescence, and those of Group 2 show relatively low fluorescence. All samples in Group 1 were collected or purchased in 1999 to 2001, and all samples in Group 2 were collected in 1983. These data indicate that the fluorescent intensity at the endpoint of the TaqMan assay comparatively correlates to the preserved time of post-harvest.

In Fig. 4, MMs were plotted on the function, $y=1.175x$ ($R=0.954$) as shown with dashed line, but others were on either axes. This means that the value of Fprobe-1/Fprobe-2 is

Table 4. Data of the TaqMan PCR Assay

Sample name	Positive probe		Probe-1		Probe-2	
	$\Delta\Delta$Rn	Detection	$\Delta\Delta$Rn	Detection	$\Delta\Delta$Rn	Detection
MM-1	**2.96**	+	**1.12**	+	**1.47**	+
MM-2-1	**2.87**	+	**1.5**	+	**1.74**	+
MM-2-2	**2.93**	+	**1.4**	+	**1.75**	+
MM-3	**2.72**	+	**1.36**	+	**1.5**	+
MM-4	**2.53**	+	**1.24**	+	**1.28**	+
MM-5	3.24	+	5.56E-02	−	0.65	+
MM-6	2.86	+	2.57E-02	−	0.56	+
Pa. sphinctrinus	**2.67**	+	**1.43**	+	**1.7**	+
Pa. subbalteatus	**1.70**	+	**0.75**	+	**0.68**	+
Ps. argentipes	**1.74**	+	**0.63**	+	**0.81**	+
Ps. fasciata	**4.77E-02**	−	**n.d.**	**n.d.**	**n.d.**	**n.d.**
Ps. subaeruginascens	**1.78**	+	**0.71**	+	**0.83**	+
Ps. subcubensis	**2.57**	+	**1.1**	+	**1.39**	+
Ps. tampanensis	**2.34**	+	**0.99**	+	**1.21**	+
Enoki	3.15	+	−6.93E-03	−	3.18E-02	−
Eringi	2.93	+	0.66	+	3.77E-02	−
Shiitake	2.98	+	2.82E-02	−	−1.39E-03	−
Nameko	3.08	+	8.07E-03	−	1.43	+
Bunashimezi	3.46	+	6.87E-02	−	1.51	+
Maitake	3.26	+	0.82	+	1.07E-01	−

Rn=fluorescent intensity from the reporter dye/fluorescent intensity from the reference dye. ΔRn=Rn−R (baseline), $\Delta\Delta$Rn=ΔRn (template)−ΔRn (no template), n.d.=not data. The fungi containing psilocin and their results are indicated in bold letters.

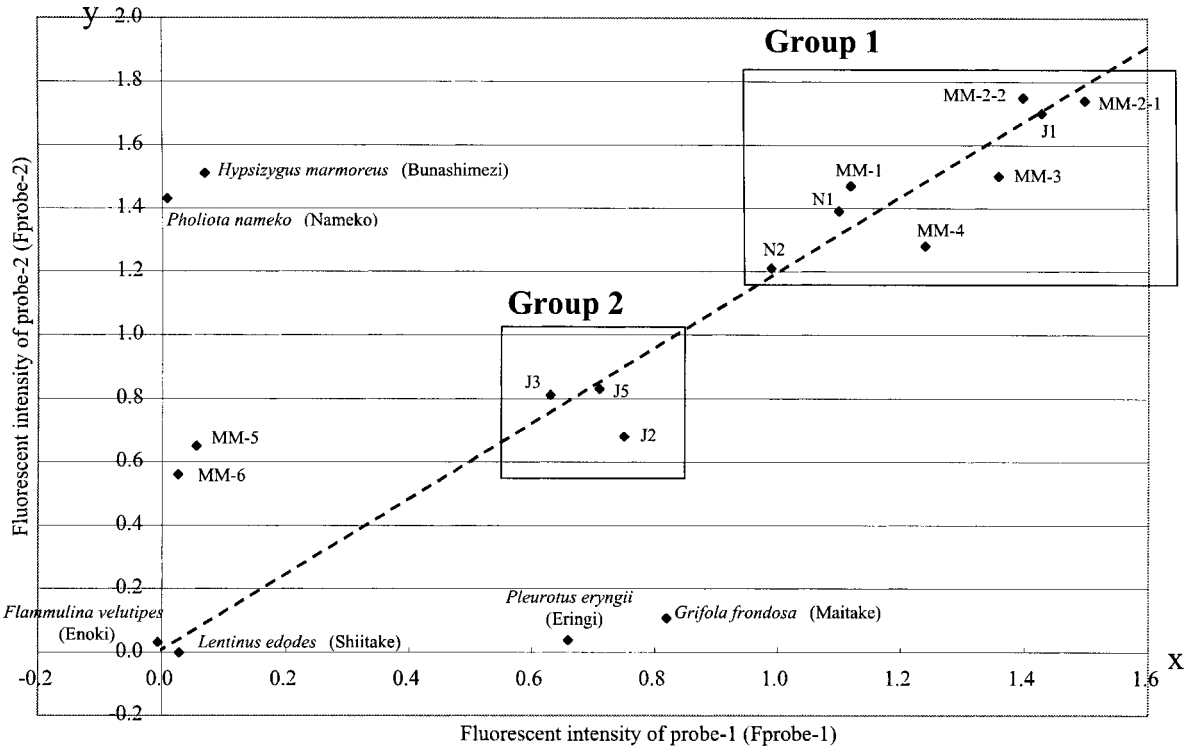

Fig. 4. The Results of the TaqMan Assay Using Probe-1 and -2

Group 1 was collected or purchased from 1999 to 2001. Group 2 was collected in 1983. Dashed line indicates the function, $y=1.175x$.

constant in all MMs regardless of absolute fluorescent intensity.

The TaqMan PCR method has the advantage in terms of not requiring post-PCR operations such as the separation of the amplicon by electrophoresis and sequencing. Therefore, we can say that our established methods principally save assay time for the identification of MMs. In addition, the assay seemed to provide information about the collection period of MMs from the fluorescent intensity of signals, though further studies are needed to clarify that aspect.

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