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Phylogenetic relationship of psychoactive fungi based on rRNA gene for a large subunit and their identification using the TaqMan assay (II)

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Abstract

"Magic mushroom (MM)" is the name most commonly given to psychoactive fungi containing the hallucinogenic components: psilocin (**1**) and psilocybin (**2**). We investigated the rRNA gene (internal transcribed spacer (ITS) and large subunit (LSU)) of two *Panaeolus* species and four *Psilocybe* species fungi (of these, two are non-psilocybin species). On the basis of sequence alignment, we improved the identification system developed in our previous study. In this paper, we describe the new system capable of distinguishing MMs from non-psilocybin *Psilocybe* species, its application data and the phylogeny of MM species.

Keywords: Magic mushroom; rRNA gene; TaqMan PCR; Genus *Psilocybe*; Genus *Panaeolus*

1. Introduction

"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 that of LSD (**3**) and 50 times 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 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].

The fungi containing **1** and **2** mainly belong to the genus *Psilocybe* and *Panaeolus* and their number exceeds 50 species of which 11 are found in Japan [3,5]. Because there are many kinds of MMs and they are often sold even as dry powders or capsules, it is very difficult to identify the species of the MMs by morphological observation. Therefore, we planned to develop an identification method based on a genetic approach.

In the earlier reports [6,7], we investigated the nucleotide sequence of the rRNA gene (internal transcribed spacer

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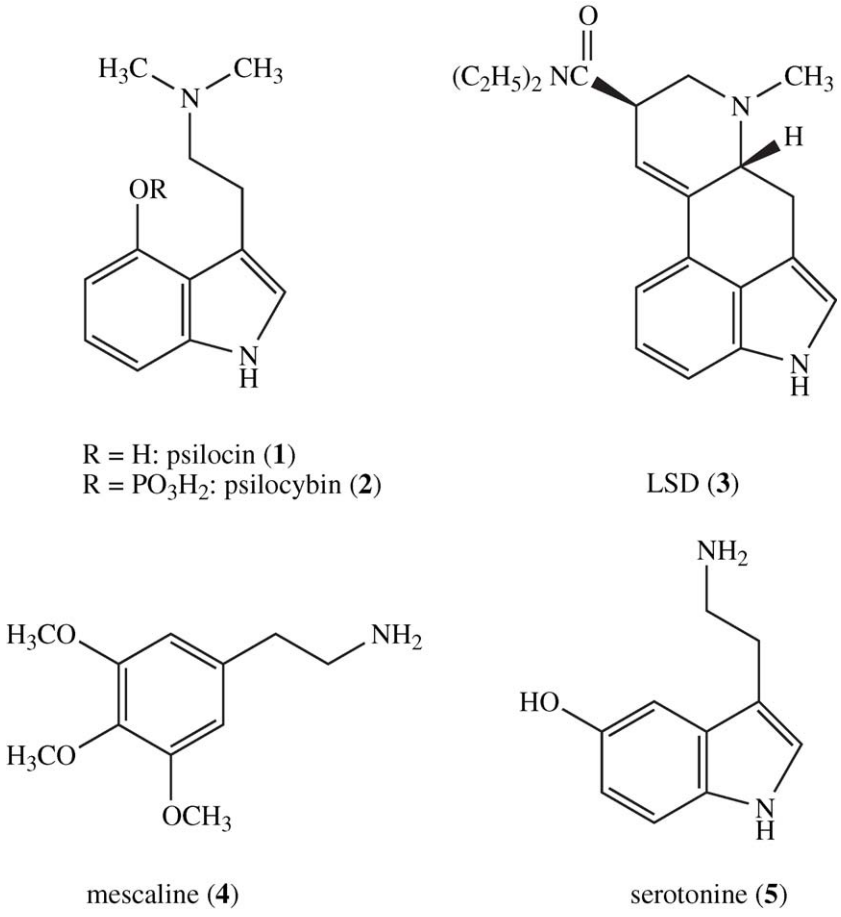

Fig. 1. Structures of hallucinogenic compounds and serotonine.

(ITS) and large subunit (LSU)) of several MMs, and based on the results, we classified the original species of MMs obtained in the Japanese markets. Furthermore, we found the MMs' specific sequence in the LSU region and developed a rapid identification system for MMs using the TaqMan PCR method [8] which has been used in the detection of genetically modified foods and pathogens in food materials [9–11].

Some species are said to contain neither **1** nor **2** in the *Psilocybe* genus [5], and our identification system could not distinguish these species from other psilocybin mushrooms. In this study, four hallucinogenic fungi (*Panaeolus cambodginiensis*, *Panaeolus retirugis*, *Psilocybe fasciata*, *Psilocybe subcaerulipes*) and two non-psilocybin *Psilocybe* species (*Psilocybe coprophila* and *Psilocybe merdaria*) were newly employed in the nucleotide sequence analysis for the purpose of developing a new system capable of discrimination between the MMs and other fungi including the non-psilocybin *Psilocybe* species. As a result, we found a distinguishable sequence between psilocybin and non-psilocybin mushrooms in the LSU region, and our previous identification system for MMs was slightly changed to discriminate the MMs from other fungi including non-psilocybin *Psilocybe* species. In this

Table 1
The sequences of primers and TaqMan MGB probes used in this study FAM and VIC are reporter dyes

Primer	ITS1	5′-GGAAGTAAAAGTCGTAACAAGG-3′
	ITS2	5′-TTCTCACCCTCTATGACGCTCC-3′
	LSU1	5′-CAAGGATTCCCCTAGTAACTGCGA-3′
	LSU2	5′-ATCCTGAGGGAAACTTCGGCA-3′
	MM-S1	5′-CGTCTTGAAACACGGACCAAO-3′
	MM-A1	5′-CACCAGAGTTTCCTCTGGCTTC-3′
Probe	Probe-1	5′-FAM-TCATTACGCGCTCGG-MGB-3′
	Probe-2	5′-VIC-CGCGGATCCGTC-MGB-3′
	Positive probe	5′-FAM-TTCAGGCATAGTTCAC-MGB-3′

MGB is the minor groove binder ligand.

paper, we describe the improved system, its application to wild fungi collected in Japan and the phylogeny of MM species.

2. Materials and methods

2.1. Fungus materials

Four MM samples (I1–I4 in Table 2) and two non-hallucinogenic *Psilocybe* specimens (I5 and I6 in Table 2) were obtained as fungous strains from the Biological Resource Center, Department of Biotechnology, National Institute of Technology and Evaluation (Japan). The origin of other MM samples (J1–J5, N1–N2, MM1–MM6 in Table 2) and edible mushrooms (E1–E6 in Table 2) was described in the previous paper [7]. Japanese wild fungi (F1–F30 in Table 3) were collected and identified by the fifth author. These are stored in the Natural History Museum and Institute, Chiba. The scientific names and voucher numbers of each sample are indicated in Tables 2 and 3 together with the results of the TaqMan assay.

2.2. Chemical reagents

Unless otherwise noted, chemical reagents were purchased from Kanto Chemicals. Authentic samples of **1** and **2** were synthesized in the Division of Pharmacognosy and Narcotics, National Institute of Health Sciences [12].

2.3. Extraction of genomic DNA

Except for I1–I6, the extraction of nucleic acid was performed as described in the previous paper [7]. Fungous strains of I1–I6 were incubated in potato–dextrose–agar medium (PDA; Nissui) at 25 °C for 4 weeks. A part of the growing mycelia was transferred into potato–dextrose liquid medium (PD; Becton Dickinson) and incubated at 27 °C for a week. After the removal of medium by centrifugation, the mycelia were crushed with a mixer mill, MM300 (Qiagen), and then genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen).

2.4. Sequence analysis of rRNA gene (ITS and LSU)

The ITS and LSU region of the DNA obtained from I1–I6 was amplified by PCR using the primer sets (ITS1–ITS2 for ITS and LSU1–LSU2 for LSU) to afford the amplicon in lengths of 800 and 900 bp, respectively. The PCR was performed on a DNA Engine® PTC-200 (MJ research) using KOD DNA polymerase (Toyobo) which has high fidelity due to its 3′–5′ exonuclease activity. The temperature program is given as follows: 94 °C, 4 min; 30 cycles of 98 °C, 15 s; 55.2 °C (for ITS) or 62 °C (for LSU), 5 s; 74 °C,

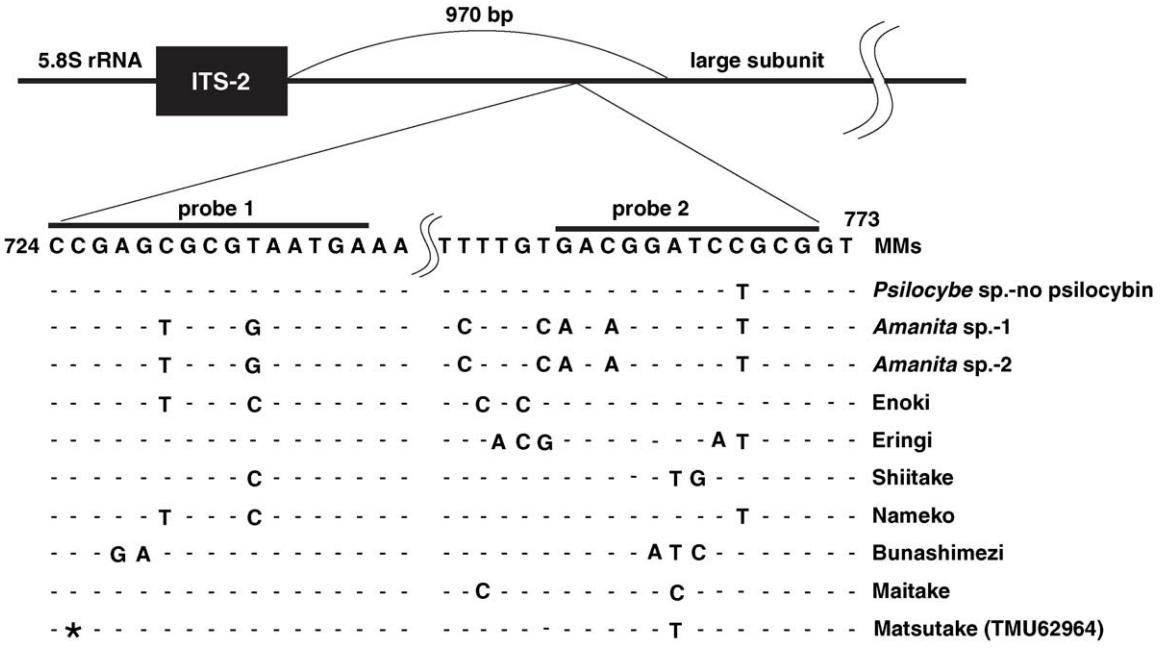

Fig. 2. Nucleotide sequences of the LSU region of several fungi. The number is the nucleotide position in *Psilocybe cubensis*. A hyphen (-) and an asterisk (*) indicate the same nucleotide as in the MMs and a gap, respectively.

Table 2
The results of the TaqMan assay for MMs and edible mushrooms

Number	Sample name	Voucher number	Positive probe			Probe-1			Probe-2		
			ΔR_n		±	ΔR		±	ΔR		±
			Mean	S.D.		Mean	S.D.		Mean	S.D.	
MM-1	***Psilocybe cubensis* (Ear.) Sing.**	**KY7156**	**2.95**	**0.16**	**+**	**1421**	**183**	**+**	**1075**	**120**	**+**
MM-2-1	***Panaeolus cyanescens* (Berk. et Br.) Sacc.**	**–**	**2.86**	**0.21**	**+**	**1499**	**135**	**+**	**1138**	**106**	**+**
MM-2-2	***Pa. cyanescens* (Berk. et Br.) Sacc.**	**–**	**2.92**	**0.21**	**+**	**1049**	**266**	**+**	**736**	**163**	**+**
MM-3	***Ps. semilanceata* (Fr. ex Sec.) Kumm.**	**–**	**2.71**	**0.01**	**+**	**992**	**86**	**+**	**731**	**47**	**+**
MM-4	***Ps. tampanensis* Guzmán et Pollock**	**–**	**2.52**	**1.23**	**+**	**1230**	**370**	**+**	**850**	**223**	**+**
MM-5	*Amanita* sp.-1	–	3.23	1.14	+	248	46	–	129	21	–
MM-6	*Amanita* sp.-2	–	2.85	0.11	+	215	31	–	71	6	–
J1	***Pa. sphinctrinus* (Fr.) Quél.**	**KY7130**	**2.66**	**0.39**	**+**	**1059**	**688**	**+**	**773**	**506**	**+**
J2	***Pa. subbalteatus* (Berk. et Br.) Sacc.**	**KY4155**	**1.69**	**0.33**	**+**	**1042**	**232**	**+**	**609**	**88**	**+**
J3	***Ps. argentipes* K. Yokoy.**	**KY3573**	**1.73**	**0.17**	**+**	**1084**	**240**	**+**	**696**	**113**	**+**
J4	***Ps. fasciata* Hongo**	**KY1837**	**0.04**	**0.01**	**–**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
J5	***Ps. subaeruginascens* Höhnel**	**KY4097**	**1.77**	**0.08**	**+**	**400**	**401**	**–**	**258**	**255**	**–**
N1	***Ps. subcubensis* Guzmán**	**KY7054**	**2.56**	**0.03**	**+**	**974**	**89**	**+**	**793**	**57**	**+**
N2	***Ps. tampanensis* Guzmán et Pollock**	**KY7134**	**2.33**	**0.19**	**+**	**1387**	**247**	**+**	**834**	**86**	**+**
I1	***Pa. cambodginiensis* Ola'h and Heim**	**NBRC30222**	**2.17**	**0.09**	**+**	**1565**	**189**	**+**	**1001**	**114**	**+**
I2	***Pa. retirugis* (Fr.) Gillet**	**NBRC31867**	**2.20**	**0.07**	**+**	**1521**	**203**	**+**	**992**	**123**	**+**
I3	***Ps. fasciata* Hongo**	**NBRC30190**	**2.38**	**0.24**	**+**	**1676**	**130**	**+**	**1084**	**45**	**+**
I4	***Ps. subcaerulipes* Hongo**	**NBRC30189**	**2.94**	**0.44**	**+**	**1472**	**100**	**+**	**988**	**76**	**+**
I5	*Ps. coprophila* (Bull. ex Fr.) Kumm.	NBRC31869	2.49	0.16	+	1618	119	+	108	30	–
I6	*Ps. merdaria* (Fr.) Ricken	NBRC30568	2.60	0.03	+	1542	178	+	137	14	–
E1	*Flammulina velutipes* (Curt. ex Fr.) Sing.	TM001	3.14	0.29	+	82	74	–	902	126	+
E2	*Pleurotus eryngii* (DC. ex Fr.) Quél	TM002	2.92	0.03	+	1763	162	+	118	18	–
E3	*Lentinus edodes* (Berk.) Sing.	TM003	2.97	0.01	+	198	39	–	53	18	–
E4	*Pholiota nameko* (T. Ito) S. Ito et S. Imai	TM004	3.07	0.15	+	116	21	–	56	8	–
E5	*Hypsizygus marmoreus* (Peck) Bigelow	TM005	3.45	0.34	+	154	23	–	30	5	–
E6	*Grifola frondosa* (Dicks. ex Fr.) SF. Gray	TM006	3.25	0.09	+	1037	30	+	77	10	–

The data of magic mushrooms are indicated in bold style.

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). The process of PCR-sequencing was performed twice to confirm no mis-incorporation of the nucleotide by DNA polymerase. Alignment and phylogenetic analyses of the sequence data were carried out by the Clustal W program [13]. The sequence information of the primers used in this study is shown in Table 1 together with the information on the primers and the probes for the TaqMan assay described below.

2.5. TaqMan assay

The positive probe and primers (MM-S1 and MM-A1) were designed in the conserved region among fungi, and probes-1 and -2 were designed in the region specific to MMs (Fig. 2). Except for probe-2, all probes and primers are the same as those in the previous report [7], and all probes were designed based on the sequence of the antisense strand. The size of the amplicon produced by the primer pair is about 220 bp. The TaqMan 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 probes-1 and -2. The assay with the positive probe was performed as described in the previous report [7], but the template amount was changed to 5 ng for I1–I6. On the other hand, the assay with probes-1 and -2 failed to make these probes emit a fluorescent signal in the primitive experiment using *Psilocybe cubensis* (MM1) as a positive control. When this reaction mixture was subjected to electrophoresis analysis, no PCR product was observed. The reaction with only probe-1 was trouble-free but not the reaction with probe-2. So, it was considered that probe-2 prevents the reaction for some reason. Nevertheless, the reason remains unclear. When Gene Taq NT DNA polymerase (Nippon Gene) was used as the PCR enzyme, the PCR proceeded in an ordinary manner and fluorescence emission from these probes was also detected. Therefore, this enzyme was applied to the assay with probes-1 and -2. A 25 μl reaction mixture composed of 1× Gene Taq Universal Buffer, 1.5 unit of Gene Taq NT, 250 nM dNTPs (all of the above are from Nippon Gene), each 200 nM primers and the TaqMan MGB

Table 3
The results of the TaqMan assay for Japanese wild mushrooms

Number	Sample name	Voucher number	Positive probe			Probe-1			Probe-2		
			ΔR_n		±	ΔR		±	ΔR		±
			Mean	S.D.		Mean	S.D.		Mean	S.D.	
F1	*Amanita citrina* (Schaeff.) Pers. var. *citrtna*	FB-31832	1.74	0.04	+	105	7	–	56	9	–
F2	*Kuehneromyces mutabilis* (Schaeff. ex Fr.) Sing. et A.H.Sm.	FB-31833	2.00	0.27	+	1146	157	+	959	108	+
F3	*Amanita spissacea* Imai	FB-31919	1.79	0.20	+	67	10	–	38	2	–
F4	*Amanita vaginata* (Bull. ex Fr.) Vitt. var. punctata (Cleland et Cheel) Gilb.	FB-31924	0.86	0.15	+	260	312	–	170	259	–
F5	*Astraeus hygrometricus* (Pers.) Morgan	FB-31927	2.22	0.06	+	147	39	–	57	21	–
F6	*Amanita javanica* (Corner et Bas) Oda, Tanaka, Tsuda	FB-32703	2.19	0.15	+	312	75	–	165	47	–
F7	*Amanita neoovoidea* Hongo	FB-32704	1.91	0.11	+	71	9	–	23	4	–
F8	*Suillus granulatus* (L. ex Fr.) O. Kuntze	FB-32705	2.03	0.08	+	893	17	+	78	9	–
F9	*Macrolepiota neomastoidea* (Hongo) Hongo	FB-32706	2.26	0.98	+	1029	88	+	667	35	+
F10	*Amanita imazekii* Oda, Tanaka, Tsuda	FB-32707	1.17	0.25	+	74	20	–	26	13	–
F11	*Amanita hemibapha* (Berk. et Br.) Sacc. Subsp. *hemibapha*	FB-32708	1.83	0.38	+	77	21	–	24	9	–
F12	*Tylopilus virens* (Chiu) Hongo	FB-32709	1.41	0.13	+	293	80	–	60	40	–
F13	*Phallus impudicus* L. ex Pers.	FB-32710	1.46	0.09	+	1238	12	+	124	11	–
F14	*Pulveroboletus ravenelii* (Berk. et Curt.) Murr.	FB-32711	2.36	0.11	+	227	21	–	86	4	–
F15	*Amanita farinosa* Schw.	FB-32712	1.78	0.19	+	221	18	–	133	12	–
F16	*Amanita pseudaporphyria* Hongo	FB-32713	2.32	0.12	+	155	30	–	80	14	–
F17	*Calvatia craniiformis* (Schw.) Fr.	FB-32714	2.93	0.19	+	676	135	+	865	191	+
F18	*Amanita esculenta* Hongo et Matsuda	FB-32715	2.68	0.32	+	102	8	–	50	4	–
F19	*Amanita gemmata* (Fr.) Bertillon	FB-32716	2.86	0.26	+	118	24	–	62	16	–
F20	*Amanita ceciltae* (Berk. et Br.) Bas	FB-32717	1.19	0.03	+	153	26	–	87	12	–
F21	*Amanita pantherina* (DC. ex Fr.) Krombh.	FB-32718	3.39	0.35	+	90	42	–	32	27	–
F22	*Boletus violaceofuscus* Chiu	FB-32719	1.86	0.02	+	86	32	–	45	5	–
F23	*Amanita virgineoides* Bas	FB-32720	2.66	0.04	+	121	21	–	66	20	–
F24	*Amanita virosa* (Fr.) Bertillon	FB-32721	2.10	0.05	+	98	8	–	59	7	–
F25	*Amanita spissacea* Imai	FB-32722	2.39	0.01	+	82	22	–	47	9	–
F26	*Tylopilus eximius* (Peck) Sing.	FB-32723	3.08	0.08	+	125	15	–	47	14	–
F27	*Amanita rubescens* Pers. ex Fr.	FB-32724	2.13	0.15	+	84	3	–	50	3	–
F28	*Tylopilus neofelleus* Hongo	FB-32725	2.41	0.29	+	81	9	–	37	4	–
F29	*Amanita vaginata* (Bull. ex Fr.) Vitt.	FB-32726	1.64	0.13	+	176	6	–	119	5	–
F30	*Chlorophyllum molybdites* (G. Meyer ex Fr.) Massee	FB-32727	2.98	0.04	+	1200	222	+	1008	170	+

probes [14] (Applied Biosystems) and template DNA (about 5 ng) was used for the exam with probes-1 and -2. 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 92 °C for 15 s and an anneal/extend step of 60 °C for 1 min. Each test was performed in quadruplicate in the same run. Data were analyzed using the SDS 1.7 application software (Applied Biosystems). In using TaqNT, dispersion of the fluorescence intensity becomes large due to the absence of the passive reference pigment, ROX. However, this is not a problem because the presence/absence of fluorescence emission at the endpoint of the PCR was utilized for identification of the MMs in this study (Tables 2 and 3).

2.6. LC–MS analysis

One hundred milligrams of dried fruit body was powdered with a mortar and pestle. This powder was suspended in 75% MeOH and shaken with a shaker, SR-2w (Taitec). After the centrifugation, 1 ml of the supernatant was filtered with a GL chromatodisc (non-aqueous, 0.45 μm; GL Sciences). The filtrate was used as the sample solution for LC–MS analysis. The analysis was performed using a JMS-T100LC AccuTOF spectrometer (JEOL) equipped with an Inertsil ODS-3, 4.6 mm × 250 mm, 5 μm (GL Sciences). The conditions were as follows: acetonitrile and 10 mM ammonium formate buffer (pH 3.0) were used as the mobile phase at a flow rate of 0.5 ml/min. The composition gradient started with 10% acetonitrile and 90% ammonium formate

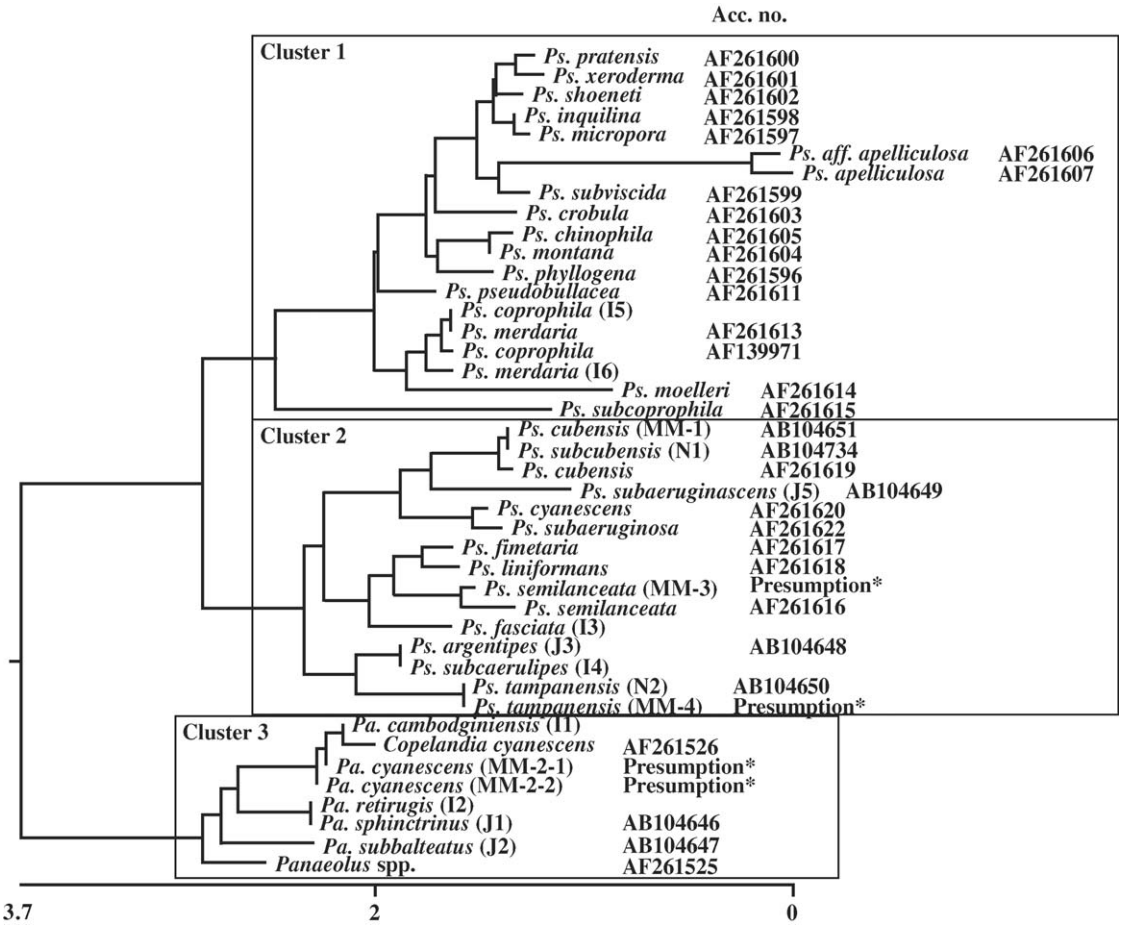

Fig. 3. Phylogenetic tree constructed from the nucleotide sequences of the RNA large subunit genes of magic mushrooms. *Their original species are now being investigated on the basis of morphology by the third author.

buffer; the acetonitrile content was then increased to 20% (0–15 min) and then decreased to 10% (15–20 min). This level was held constant for 5 min as post-time. The temperature of the column oven was set at 40 °C and the injection volume was 10 μl. For mass spectrometry, the positive electrospray ionization (ESI) mode was used and pseudomolecular ion peaks at *m/z* 205 and 285 were confirmed for the identification of **1** and **2**, respectively. The retention times of **1** and **2** in this condition were 8.9 and 11.9 min, respectively.

3. Results and discussion

3.1. Phylogenetic relationship of psychoactive fungi based on the LSU sequence

Genomic DNA was extracted from six fungi (I1–I6) and the rRNA gene (ITS and LSU) was amplified by PCR using this DNA as template and directly sequenced. The LSU sequence of *Pa. cambodginiensis* (I1) was consistent with that of *Panaeolus cyanescens* (accession no. AF261526). Therefore, some of the 15 samples (MM-2) which were estimated to be *Pa. cyanescens* based on the LSU sequence in our previous study [7] may be *Pa. cambodginiensis*. Additionally, the ITS1 sequence of II was also consistent with that of MM-2-1. On the other hand, *Pa. retirugis* was identical with *Panaeolus sphinctrinus* in the LSU region (accession no. AB104646), but not in ITS1 (accession no. AY152728). The LSU sequences of *Ps. coprophila* (15) and *Ps. merdaria* (16) were very similar to each other, but were not identical with the corresponding sequence (accession nos. AF139971 and AF26I613, respectively) registered on databases. These data indicate the existence of intra-specific mutation as described in our previous paper [7]. *Psilocybe* (14) and *Psilocybe argentipes* (accession nos. AB092792 and AB104647) had the same sequence in both regions.

A phylogenetic tree was constructed from the LSU nucleotide sequences of *Psilocybe* and *Panaeolus* fungi which were obtained in our studies and/or are registered on databases (Fig. 3). Three clusters were found in this tree and each cluster represented non-psilocybin *Psilocybe* spe-

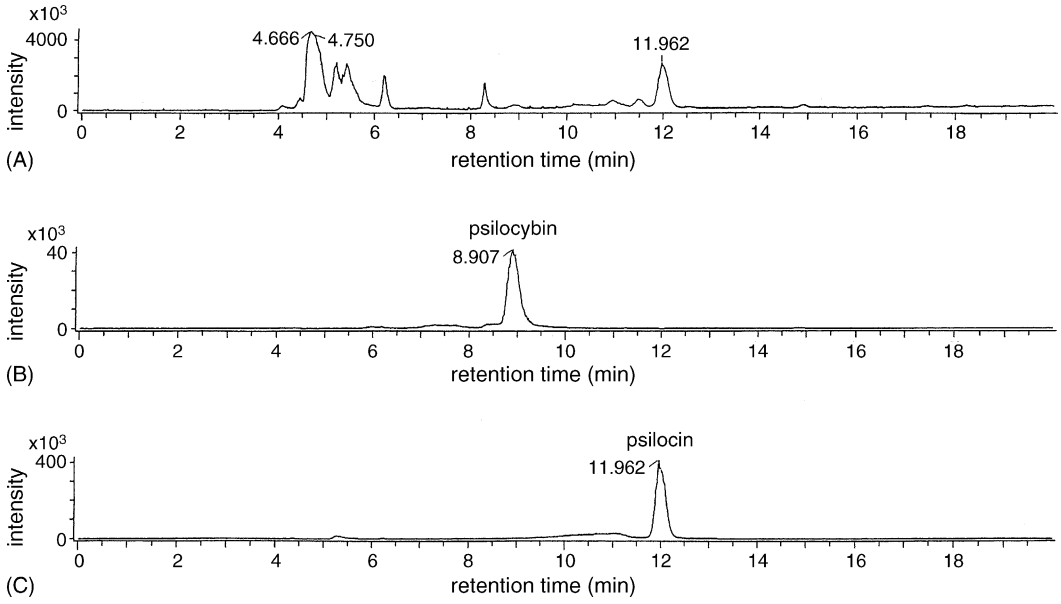

Fig. 4. Chromatogram of extract from a *Psilocybe cubensis* mushroom. (A) Total ion chromatogram, (B) mass chromatogram at *m/z* 284.5–285.5 and (C) mass chromatogram at *m/z* 204.5–205.5.

cies (cluster 1), hallucinogenic *Psilocybe* species (cluster 2) and *Panaeolus* species (cluster 3). Moncalvo et al. [15] has already pointed out that hallucinogenic species are distinguished from non-psilocybin species phylogenetically in the *Psilocybe* genus. Our results are well in accord with theirs.

3.2. Identification of MM using TaqMan PCR

Based on the comparison of nucleotide sequence data, we found the region where hallucinogenic mushrooms were distinguishable from non-psilocybin *Psilocybe* species and other edible mushrooms (Fig. 2). The probe-2 can hybridize with MMs but dose not hybridize with non-psilocybin *Psilocybe* species. So, unlike the previous system, the combination of these two probes can achieve the discrimination between MMs and non-psilocybin *Psilocybe* species. Probe-1 is the same as that in the previous report; therefore, only probe-2 was newly designed to improve the identification system for MMs.

The assay results for MMs (MM1–MM6, J1–J5, N1–N2 and I1–I4), non-psilocybin *Psilocybe* species (I5–I6) and

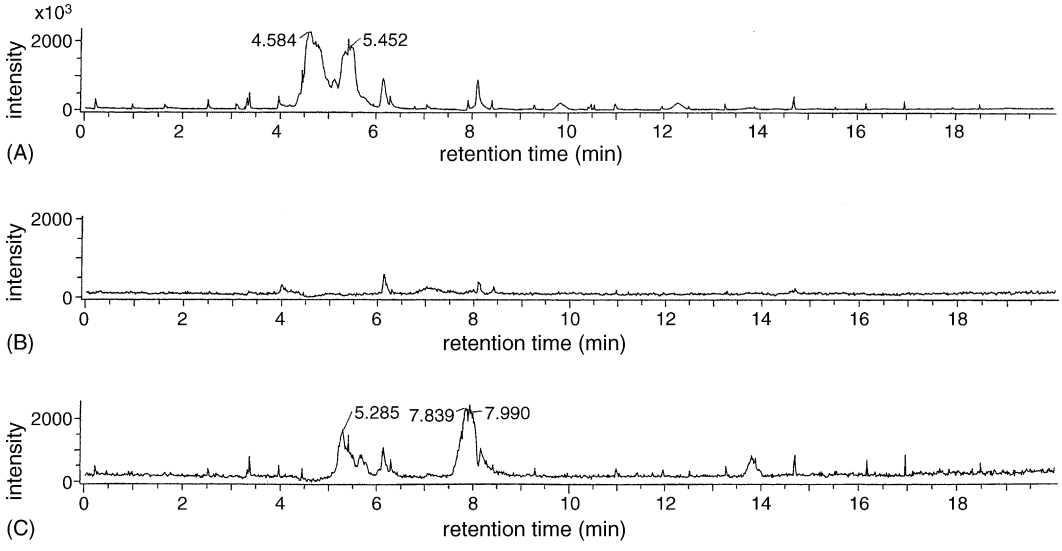

Fig. 5. Chromatogram of extract from a *Macrolepiota neomastoidea* mushroom (F9). (A) Total ion chromatogram, (B) mass chromatogram at *m/z* 284.5–285.5 and (C) mass chromatogram at *m/z* 204.5–205.5.

edible mushrooms (E1–E6) are shown in Table 2. As expected from the nucleotide sequence alignment in Fig. 2, fluorescence emission from both probes was observed only in the *Panaeolus* species and *Psilocybe* species containing **1** and **2**, and at least one fluorescent signal is missing in the others. Thus, this improved identification system is estimated to detect MMs more precisely than the previous one in terms of the discrimination among *Psilocybe* species.

On the other hand, among 30 Japanese wild mushrooms, *Kuehneromyces mutabilis* (F2), *Macrolepiota neomastoidea* (F9), *Calvatia craniiformis* (F17) and *Chlorophyllum molybdites* (F30) were regarded as magic mushrooms in this assay (Table 3). However, their chemical analysis on LC–MS proved that these fungi contain neither **1** nor **2** (Figs. 4 and 5), showing they were not MM. This is probably attributed to the fact that they are biologically close to MMs. Because F30 appears to contain **1** and **2** depending on the growing conditions, these exceptional mushrooms may contain **1** and **2** in other case.

4. Conclusion

The rRNA gene sequence data from six members of two fungus genera, *Panaeolus* and *Psilocybe*, were obtained and a phylogenetic tree was constructed on the basis of these data. The improved identification system could discriminate the MMs from other fungi including non-psilocybin *Psilocybe* species. Although, a few exceptions were found in the application data, they were excluded from MMs by chemical analysis. Thus, we can say that our established methods were useful for the first screening in MM identification.

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