DNA sequencing analysis of ITS and 28S rRNA of Poria cocos

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Summary
We determined the DNA sequences of the internal transcribed spacer 1 and 2 (ITS 1and 2), the 5.8S rRNA gene and most of the 28S rRNA gene of *Poria cocos* for the first time, and conducted analysis of 20 samples including cultured mycelias and crude drug materials obtained from various localities and markets. Direct sequencing of the ITS 1 and 2 regions of the samples, except for four wild samples, showed that they had identical DNA sequences for ITS 1 and 2 with nucleotide lengths of 997 bps and 460 bps, respectively. By cloning, the four wild samples were found to have combined sequences of common ITS sequences with 1 or 2-base-pair insertions. Altogether both ITS 1 and 2 sequences were substantially longer than those of other fungal crude drugs such as *Ganoderma lucidum* and *Polyporus umbellatus*. Thus, *Poria cocos* could be distinguished from these crude drugs and fakes by comparing the nucleotide length of PCR products of ITS 1 and 2. Contrary to the basic homogeneity in ITS 1 and 2, three types (Group 1, 2, and 3) of the 28S rRNA gene with distinctive differences in length and sequence were found. Furthermore, Group 1 could be divided into three subgroups depending on differences at nucleotide position 690. Products with different types of 28S rRNA gene were found in crude drugs from Yunnan and Anhui Provinces as well as the Korean Peninsula, suggesting that the locality of the crude drugs does not guarantee genetic uniformity. The result of DNA typing of *Poria cocos* may help discrimination of the quality of the crude drug by genotype.

Keywords; *Poria cocos* WOLF (syn. *Wolfiporia cocos*); internal transcribed spacer 1 and 2; 28S rRNA gene

Introduction

Hoelen, a dried sclerotium of *Poria cocos* Wolf (*Polyporaceae*) has been used as a crude drug in both Chinese and Japanese traditional medicines (Kampo). In addition to the traditional use, recent studies have shown the therapeutic potential of Hoelen as a cytokine secretion modulator[1)] and an anticancer agent[2)]. In Kampo medicine, Hoelen is prescribed in many important formulations, and about 700 tons per year is consumed in the Japanese market, mostly imported from China and some from the Korean Peninsula [3)]. Recently, cultivated Chinese Hoelens accounted for most of Japanese market. However, the harvests of cultivated Hoelens decreased greatly in China in 2004. The reason for this decrease was due to the forest conservation activities by the government of Anhui Province, one of the main cultivation centers of Hoelen [4)]. Therefore, it is thought that fake Hoelen will emerge in the market. Furthermore, the locality of the crude drug has long been believed to specify its quality. Hoelen produced in Yunnan province, for example, is renowned for its medicinal properties[5) 6)]. However, the relationship between the quality of the crude drug and its locality has not yet been clarified. Moreover, there is no evidence that Hoelens from the same locality have the same genetic background. Molecular genetic criteria have been introduced to classify the crude drug species and intraspecific variation in recent years. DNA sequences of appropriate regions in the *Poria cocos* genome could be ultimate molecular markers of the crude drug. There was no difference between Chinese and Japanese *Poria cocos* in the nucleotide sequence of the 18S rRNA gene, while mutual aversion phenomena were observed between them [7)]. Nucleotide analysis of the nuclear ribosomal ITS region and 28S rRNA gene may provide more information about inter- and intraspecific variations of the fungus. In the present paper, we report the DNA sequences of the complete ITS region and most of the 28S rRNA gene sequences of *Poria cocos*, and the results of the analysis of 20 *Poria cocos* samples including cultured mycelia and crude drug materials obtained from various localities.

MATERIALS AND METHODS

Fungal Materials

The 20 samples used in this study including cultured mycelia of *Poria cocos* and Hoelens obtained from various localities are summarized in Table 1. The cultured mycelia were derived from fresh Hoelen samples. Fresh Japanese Hoelen samples were collected by us. Two Hoelen samples from the Korean Peninsula, Korea-1 and Korea-2, were purchased in Kyungdong market. Fresh Hoelens used to derive cultured mycelia Sekkou-1 and -2, and crude drug Korea-3 (Korean Peninsula) were obtained from Tochimoto-Tenkaido Co. Ltd. A Hoelen sample from Guizhou, Kishu, was purchased in Chong-Qing-Zhong-Yao-Zhuan-Ye market. A Hoelen sample from Yunnan, Yunnan-2, was purchased from Xing-Lin Co. Ltd. in the An-Guo crude drug market. Hoelen samples, Yunnan-1, Yunnan-3, Yunnan-4 (Yunnan), and Shisen (Sichuan) were obtained from He-Hua-Chi via Kanebo Pharmaceutical Co. Ltd. Anhui Hoelen samples Anki-1, -2, and -3 were obtained from Uchida Wakanyaku Co. Ltd. All of these crude drug specimens were stored in the Herbarium of the Laboratory of the Herbal Medicine and Natural Resources of the Graduate School of Natural Science and Technology, Kanazawa University. Ganoderma *(Ganoderma lucidum* (Leiss. ex. Fr.) Karst. and Polyporus (*Polyporus umbellatus* (Pers.) Fries.) were purchased from Uchida Wakanyaku.

Isolation of Total DNA

Total DNA was extracted from 2-4 g of crude drug material ground into fine powder. The resulting crude drug powder was washed with a 1 ml of washing buffer containing 0.1 M Tris-HCl (pH 8.0), 0.35 M Sorbitol, 10% polyethylene glycol, 2% mercaptoethanol, and total DNA was extracted using a DNeasy Plant Mini Kit according to the manufacturer's protocol. Total DNA was extracted from 200-400 mg of fresh cultured mycelia material using a DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's protocol with minor modifications**.**

PCR Amplification

Polymerase chain reaction (PCR) was performed using 30-100 ng of total DNA as the template in 25 μl of a reaction mixture containing 2.5 l 10×PCR buffer for KOD -Plus-, 0.2 mM of each dNTP, 1.0 M of $MgSO_4$, 0.5 units KOD -Plus- polymerase (Toyobo), and 0.4 mM of each primer. Primers used are shown in Table 2. Amplification was carried out under the following conditions: pre-heating at 94℃ for 2 min; 30 cycles of denaturation at 94℃ for 15 s, annealing at 55℃ for 30 s and elongation at 68℃ for 2 min; a final elongation at 68℃ for 5 min. One tenth volume of the PCR products was analyzed by agarose gel electrophoresis and then the remaining part was purified using a QIAquick PCR Purification Kit (Qiagen).

Sequencing Reaction

The purified PCR product was subjected to direct sequencing using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) using an ABI PRISM 310 sequencer (Applied Biosystem). The DNA sequences were aligned using 'DNASIS' version 3.0 (Hitachi).

Cloning of PCR products

Total DNA of Shikoku and Yunnan-1, were amplified by using a tagged primer set, **p** and **q.** Those of L2 and Korea-2, were amplified by using primer set, **p** and **i**, and those with combined sequences in the 28S rRNA gene, L2, Shikoku and Yunnan-1, were amplified by using primer sets **h** and **j** or **r** and **s**. After purification using a QIAquick PCR purification kit, the PCR products (100-200 ng) were digested with *Bam*HI and *Hind*III (TaKaRa) in a 10 L-reaction reaction mixture containing 20 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 M KCl at 37℃ for 1 hr, and then the reaction mixture was heated to 70℃ for 15 min. Four L of the reaction mixtures were combined with 5 ng of pBluescript SK(-)plasmid digested with the same enzymes and 5 L of DNA Ligation Kit Ver.2.1 (TaKaRa). The ligation reaction was performed at 16 ℃ overnight. Competent cells (Competent high DH5α, TOYOBO) were transformed with the ligated product according to the manufacturer's protocol. The transformed cells were spread on LB / Amp plates (2% tripton, 1% yeast extract, 2% NaCl, 3% agar, and 10 g/mL ampicillin) and incubated at 37℃ overnight. Bacterial colonies were picked up separately, and sub-cultured in liquid LB / Amp medium. After overnight-incubation, the bacteria were collected by centrifugation and plasmids were obtained according to a standard protocol[8)].

Restriction fragment length polymorphism experiment for the discrimination of Group-1, -2, and -3

PCR products amplified by using primer sets of **k** and **o** or **k** and **l** were purified using a QIAquick PCR purification kit and then digested with 15 units of restriction enzyme either *Bam*HI or *Dra*I (Takara) at 37℃ for 1 hour. Reaction mixtures were separated by electrophoresis in 2% agarose gels with 1×TAE buffer. After 1 hour immersion in 0.5 ng/mL ethidium bromide solution, the digested products were visualized under UV light and photographed.

RESULTS

Sequencing Analysis of the ITS Regions

Direct sequencing of PCR products of the ITS 1 and 2 regions revealed that 16 of 20 *Poria cocos* samples (the exceptions were L2, Shikoku, Korea-2, and Yunnan-1) had identical sequences with the nucleotide lengths of 997 bps and 460 bps, respectively (Table 3). By cloning the PCR products of L2, Shikoku, Korea-2, and Yunnan-1, they were found to have overlapping of the common sequence and sequences with 1 or 2 base-pair-insertions; a 2 base-pair-insertion at nucleotide positions 637-638 of ITS1 in Yunnan-1, and 1 base-pair-insertions at nucleotide position 419 of ITS2 in L2, at nucleotide position 657 of ITS1 in Shikoku, and at nucleotide position 453 of ITS2 in Korea-2. Altogether, the nucleotide lengths of ITS 1 and 2 of these *Poria cocos* samples were substantially longer in comparison with other fungus crude drugs, such as *Ganoderma lucidum* and *Polyporu umbellatus*; the lengths of their ITS 1 and 2 sequences were reported within the ranges of 190-220 bps and 170-200 bps, respectively[9) 10) 11) 12)]. When the PCR products amplified from the ITS 1 region using primer set **a** and **d** of the *Poria cocos* samples and those of *Ganoderma lucidum* and *Polyporus umbellatus* were compared, they could be clearly distinguished from each other by agarose gel electrophoresis (Fig. 1).

Sequencing analysis of 28S rRNA Gene

About nine tenths of the 28S rRNA gene sequence was determined by the nucleotide sequence alignment of PCR products amplified using primer sets **g** and **j, k** and **m,** and **n** and **o**. The results revealed that the nucleotide length of this region varied as shown in Table 3. By cloning the PCR products, the 28S rRNA gene sequence of 2 Japanese samples, L2 and Shikoku, and Yunnan-1 (Yunnan) were found to have overlapping of the common sequence with 2865 bps and sequences with insertions, a 3-base- pair insertion at nucleotide positions 2166-2168 in L2 and Shikoku, and a 1 base–pair insertion at nucleotide positions 611 in Yunnan-1. Some examples of deletions and mutations are shown in Table 4. The sequences of 14 samples listed from the top in Table 4, including 6 Japanese samples, 1 Korean sample, and 7 Chinese samples had almost identical sequences of 2865 bps. On the other hand, 6 samples had shortened sequences due to deletions. Two of them, Korea-3 (Korean Peninsula) and Yunnan-2 (Yunnan), had a identical sequence of 2823 bps, and the four other samples, Korea-1 (Korean Peninsula), Shisen (Sichuan), and Anki-1 and -3 (Anhui), also had an identical sequence of 2821 bps. Based on this result, we could divide these samples into 3 groups, as indicated in Table 3. In addition to deletions, a considerable number of nucleotide substitutions were found. Among these substitutions, the nucleotide positions

1130-1135 of Group III were TTTAAA, the recognition site of *Dra*I, and that of Group I and II were TTTGAA. Furthermore, the nucleotide positions 2001-2006 of Group I were AGATCC, whereas those of Group II and III were GGATCC, the recognition site of *Bam*HI. Using *Bam*HI or *Dra*I, the restriction fragment length polymorphisms (RFLP) of the groups can be observed, as shown in Fig. 2 and 3. Thus, the discrimination of 3 groups could be achieved by the combination of RFLPs. Group I could be further classified by a notable substitution at nucleotide position 690. All Japanese samples, Korea-2 (Korean Peninsula), and Yunnan-1 (Yunnan) had adenine at this position, which made a restriction site for *Dra*I (TTTAAA), whereas Sekkou-1 and -2 (Zhejiang) and Kishu (Guizhou) had guanine, and Anki-2 (Anhui), Yunnan-3, and Yunnan-4 (Yunnan) had adenine and guanine (Table 4). When PCR products amplified from the samples using primer set **h** and **i** were digested with *Dra*I, these subgroups of Group I with adenine were digested whereas those with guanine remained intact and with adenine and guanine showed digested and intact signals. Thus, the subgroups could be also distinguished by this RFLP (Fig. 4).

Discussion

We determined the DNA sequences of ITS 1, the 5.8S rRNA gene, ITS 2, and most of the 28S rRNA gene of *Poria cocos* for the first time. Other fungal crude drugs that belong to the *Polyporaceae*, *Ganoderma lucidum,* and *Polyporus umbellatus,* were reported to have various substitutions in ITS 1 and 2[9) 10) 11) 12)]. Although the nucleotide lengths of ITS 1 and 2 of *Poria cocos* samples were remarkably longer than *Ganoderma* and *Polyporus*, they preserved almost identical sequences in these regions. Some of the *Poria cocos* samples possessed combined sequences, their sequences were combinations of the common sequences and those with insertions of 1-3 bps at single sites. Thus, ITS 1 and ITS 2 of *Poria cocos* were quite different from *Ganoderma lucidum* and *Polyporus umbellatus* in nucleotide length as well as in homogeneity. The first fifth of the 28S rRNA gene of the *Poria cocos* samples, except position 690, was also homologous and was similar to the *Ganoderma* sequence. On the other hand, the other part of the 28S rRNA gene could be divided in 3 groups (Group 1, 2, and 3) with distinct differences in length and in nucleotide sequence. All of the Japanese cultured mycelia samples were classified in Group 1, while the crude drugs from China and Korean Peninsula included products of different genotypes. Among the Yunnan samples, for example, Yunnan-1, Yunnan-3, and Yunnan-4 had their genotypes classified in Group 1, whereas Yunnan-2 was in Group 2. Similarly, 3 cultivated Anhui samples had genotypes classified in different groups; Anki-2 in Group 1, and Anki-1 and -3 in Group 3. These results suggest not only that there are crude drugs with a variety of genotypes but also that the current locality of the crude drugs does not guarantee genetic uniformity. The inhibitory activity of beta-glucan of Hoelen on cancer cells, one of the newly recognized therapeutic potentials of the crude drug, seemed to be different between strains[13)]. The traditional concept of quality as well as recently recognized efficacy of the crude drug must be discussed when considering their genotype. These results of DNA typing of *Poria cocos* and the methods we propose here will help to discriminate of the quality of the crude drug by genotype.

References

1) Spelman K, Burns J, Nichols D, Winters N, Ottersberg S, Tenborg M. Modulation of cytokine expression by traditional medicines: a review of herbal immunomodulators. Altern Med Rev., Review, 11, 128-50 (2006).

2) Yance DR Jr, Sagar SM., Targeting angiogenesis with integrative cancer therapies. Integr Cancer Ther. Review, 5, 9-29 (2006)

3) Editorial committee of guidebook to Japanese Pharmacopoeia 15th (eds.), Guidebook to Japanese Pharmacopoeia 15th pp, D-582-D585, Hirokawa Shoten, Tokyo, 2006.

4) Implementation opinions of Anhui province Committee of the Communist Party of China and The people's government of Anhui province about 'The Central Committee of the Communist Party of China and The state council of the People's Republic of China's decision to accelerate the development of forestry'. No.5 Act, issued on March 9, 2004

5) Zhang, B., Clinic. J. Chinese Med., 23, 2-6 (2002)

6) Zheng, H.-Z, Dong, Z.-H., She, J., (eds), Modern study of traditional Chinese medicine, pp3114-pp3136, Beijing Xue Yuan Press, Beijing (1998)

7) Kozuka, A., Master's Thesis in Kanazawa University, the Graduate School of Natural Science and Technology, 1999.

8) Sambrook, J., Fritsch, E. F., and Maniatis, T., (eds.), Chapter 1, Molecular Cloning, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989)

9) Singh,S.K., Yadav,M.C., Upadhyay,R.C., Kamal,S., Rai,R.D. and Tewari,R.P. Molecular characterization of specialty mushroom germplasm of the National Mushroom Repository, Mushroom Res. 12 (2), 67-78 (2003)

10) Edwards,I.P., Cripliver,J.L., Gillespie,A.R., Johnsen,K.H., Scholler,M. and Turco,R.F, Nitrogen availability alters macrofungal basidiomycete community structure in optimally fertilized loblolly pine forests. New Phytol. 162 (3), 755-770 (2004)

11) Thon,M.R. and Royse,D.J. Evidence for two independent lineages of shiitake of the americas (Lentinula boryana) based on rDNA and beta-tubulin gene sequences Mol. Phylogenet. Evol. 13 (3), 520-524 (1999)

12) Moncalvo,J.M., Wang,H.H. and Hseu,R.S. Phylogenetic relationships in Ganoderma inferred from the internal transcribed spacers and 25S ribosomal DNA sequences Mycologia 87, 223-238 (1995)

13) Jin Y, Zhang L, Zhang M, Chen L, Cheung PC, Oi VE, Lin Y. Antitumor activities of heteropolysaccharides of Poria cocos mycelia from different strains and culture media. Carbohydr Res. 338, 1517-21 (2003).

Legends for Figures

Fig. 1. PCR amplification of ITS1 using primer set **a** and **d**.
Lane M : DNA molecular weight marker XIV (Roche). Lane 1: Gano-1, Lane 2: Gano-2, Lane 3:Chorei, Lane 4: K1, Lane 5: Shikoku, Lane 6: Mikawa, Lane 7: Korea-1, Lane 8: Korea-2, Lane 9: Korea-3, Lane 10: Anki-2, Lane 11: Anki-3, Lane 12: Yunnan-2, Lane 13: Yunnan-1, Lane 14: Shisen, Lane 15: Sekkou-1.

Fig. 2.
The PCR products amplified using primer set **k** and **l**, about 600 bps were digested with *Dra*I.
Lane M: DNA molecular weight marker XIV (Roche). Lane 1: K1, Lane 2: Mikawa, Lane 3: Shikoku, Lane 4: Korea-2, Lane 5: Yunnan-3, Lane 6: Yunnan-4, Lane 7: Kishu, Lane 8: Sekkou1, Lane 9: Anki-2, Lane 10: Korea-3, Lane 11: Yunnan-2, Lane 12: Korea-1, Lane 13: Shisen, Lane 14: Anki-1, Lane 15: Anki-3

Fig. 3 Restriction enzyme digestion of PCR products of the 28S rRNA gene. The PCR products amplified using primer set **k** and **o**, about 2000 bps, were digested with *Bam*HI.
Lane M: DNA molecular weight marker XIV (Roche). Lane 1: K1, Lane 2: Mikawa, Lane 3: Shikoku, Lane 4: Korea-2, Lane 5: Yunnan-3, Lane 6: Yunnan-4, Lane 7: Kishu, Lane 8: Sekkou1, Lane 9: Anki-2, Lane 10: Korea-3, Lane 11: Yunnan-2, Lane 12: Korea-1, Lane 13: Shisen, Lane 14: Anki-1, Lane 15: Anki-3

Fig. 4 Restriction enzyme digestion of PCR products of the 28S rRNA gene of Group I. The PCR products amplified using primer set **h** and **i**, about 450 bps, were digested with *Dra*I.
Lane M: DNA molecular weight marker XIV (Roche). Lane 1: K1, Lane 2: L2, Lane 3:Mikawa , Lane 4: Shikoku, Lane 5: Korea-2, Lane 6: Yunnan-1, Lane 7: Kishu,
Lane 8: Sekkou1, Lane 9: Sekkou2, Lane 10: Anki-2, Lane 11: Yunnan-3, Lane 12: Yunnan-4.

Fig. 1

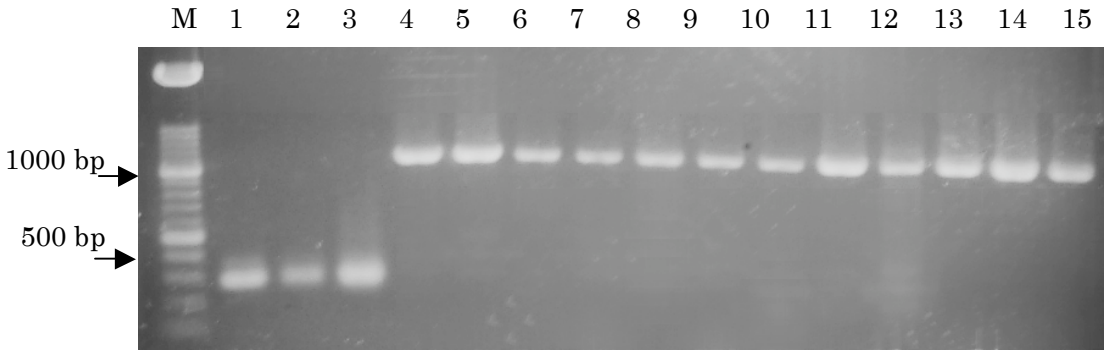

Fig. 2.

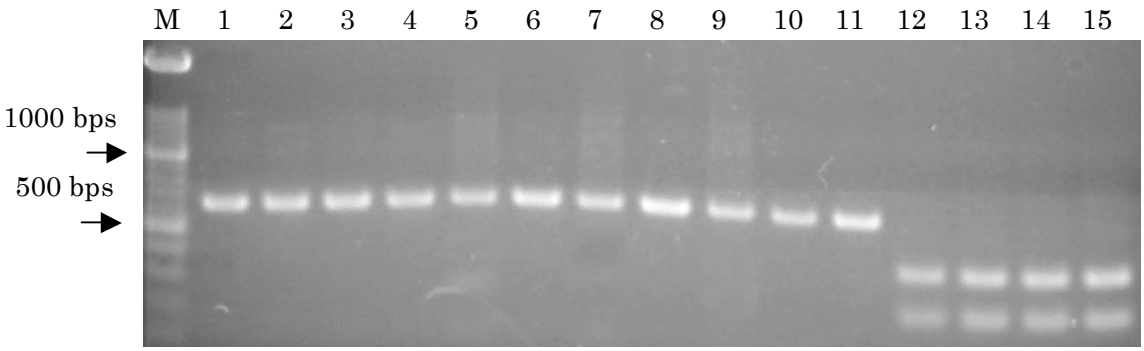

Fig. 3

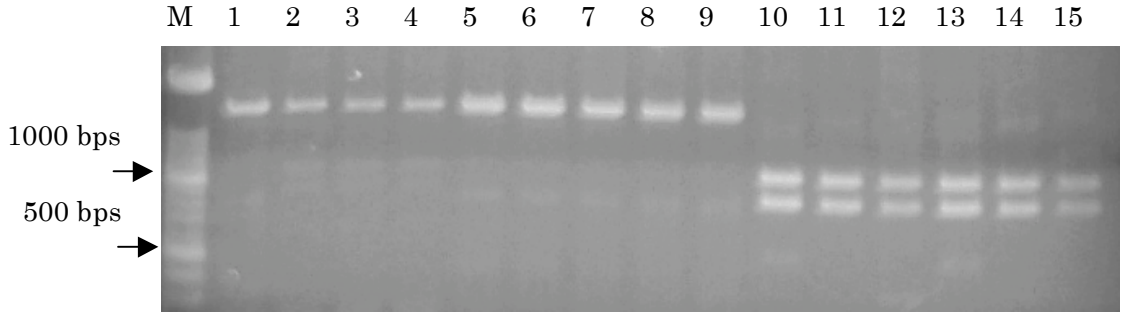

Fig.4

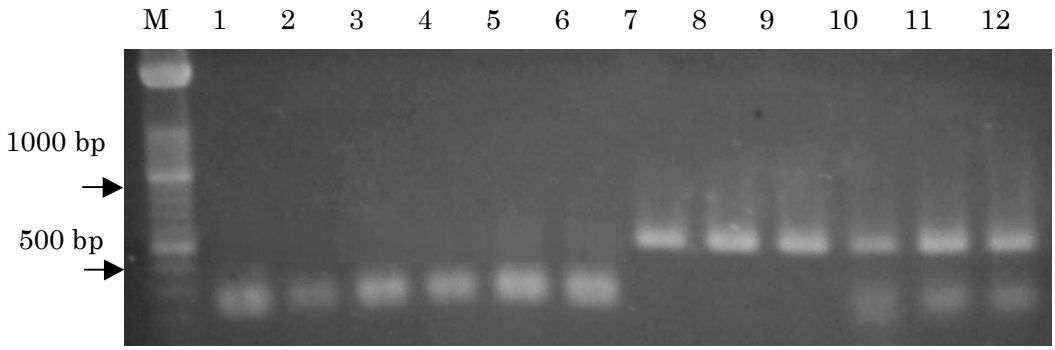

Table 1. Materials Used in This Study

Species	Sample ID	Locality	Collection Date	Voucher No.	Status	Origin
Poria cocos Wolf	K1	Minowa, Nagano, Japan	Nov. 1992		cultured mycelia	wild
	L2	Minowa, Nagano, Japan	Nov. 1992		cultured mycelia	wild
	I2	Matsumoto, Nagano, Japan	May. 1992		cultured mycelia	wild
	Nagano 15	Shiojiri, Nagano, Japan	April. 2005		cultured mycelia	wild
	Mikawa	Mikawa, Ishikawa, Japan	May. 1993		cultured mycelia	wild
	Shikoku	Matsuyama, Ehime, Japan	April. 1992		cultured mycelia	wild
	Korea-1	Korea	Nov. 2004	KANP No.6565	crude drug	wild
	Korea-2	Inje, GangWonDo, Korea	Jul. 2005	KANP No.6763	crude drug	wild
	Korea-3	North Korea	Feb. 2006	KANP No.7152	crude drug	wild
	Anki-1	Anhui, China	Jun. 2005	KANP No.7233	crude drug	cultivated
	Anki-2	Anhui, China	Jun. 2005	KANP No.7234	crude drug	cultivated
	Anki-3	Anhui, China	Jun. 2005	KANP No.7235	crude drug	cultivated
	Kishu	Guizhou, China	Jul. 2003	KANP No.6254	crude drug	ns
	Sekkou-1	Zhejiang, China	April. 1994		cultured mycelia	cultivated
	Sekkou-2	Zhejiang, China	April. 1994		cultured mycelia	cultivated
	Shisen	Sichuan, China	May 2006	KANP No.7156	crude drug	wild
	Yunnan-1	Yunnan, China	May 2006	KANP No.7153	crude drug	wild
	Yunnan-2	Yunnan, China	Mar. 2006	KANP No.7067	crude drug	ns
	Yunnan-3	Yunnan, China	May 2006	KANP No.7154	cultured mycelia*	cultivated
	Yunnan-4	Yunnan, China	May 2006	KANP No.7155	cultured mycelia*	cultivated
Ganoderma spp.	Gano-1	Ohchi, Shimane, Japan	Jan. 2005		cultured mycelia	cultivated
Ganoderma lucidum	Gano-2	Zhejiang, China	Dec. 2006	VPKNR	crude drug	cultivated
Polyporus umbellatus	Chorei	Shanxi and Gansu, China	Dec. 2006	US312502	crude drug	cultivated

*: Cultured mycelia derived from crude drug, ns: not specified.

Table 2 Primers used in this study

ID	Primer name	Sequence
a	Poria ITS1F	5’-CCGTAGGTGAACCTGCAGAA-3’
b	Poria ITS1W	5’-CGACATTCTCTCTTGAACTCGG-3’
c	Poria ITS1 850F	5’-GGTACCGTCTTTCTCGATAG-3’
d	Poria 5.8SR	5’-CGCATTTCGCTGCGTTCTTC-3’
e	Poria ITS2 200F	5’-GTTGAACGGGAACCCTAGAA-3’
f	Poria ITS2 nes.R	5’-GGTAGTCCTGCCTGATCTGA-3’
g	Poria 28SF	5’-AAGATCTCGACTCCGGTTTG-3’
h	BH Poria 28S500F	5’-ATAAGGATCCAAACGCTTGAAGTCAGTCGC-3’
i	HD Poria 28S950R	5’-ATATAAGCTTCCTATTCAGGCATAGTTCAC-3’
j	Poria 28S cent.R	5’-CGATCGATTTGCACGTCAGA-3’
k	Poria 28S 1000F	5’-CGAAAGATGGTGAACTATGCC-3’
l	Poria 28S 1600R	5’-GCTACTACCACCAAGATCTG-3’
m	Poria 28S 2000R	5’-TGCCGACTTCCCTTATCTAC-3’
n	Poria 28S 2000F	5’-TACTCATAACCGCAGCAGGT-3’
o	Poria 28S 3000R	5’-AGCCAGTTATCCCTGTGGTA-3’
p	Poria ITS1 BH 520F	5’-TTTTGGATCCTCTACAGCCATCTTCCGAGT-3’
q	Poria ITS1 HD 1040R	5’-AAAAAAGCTTTCGCTGCGTTCTTCATCGAT-3’
r	Poria 28S BH 1900F	5’-ATAAGGATCCAATCGGTTCGTCCGGAGCTA-3’
s	Poria 28S HD 2900R	5’-ATTTAAGCTTCCAAAGGATCGATAGGCCAC-3’

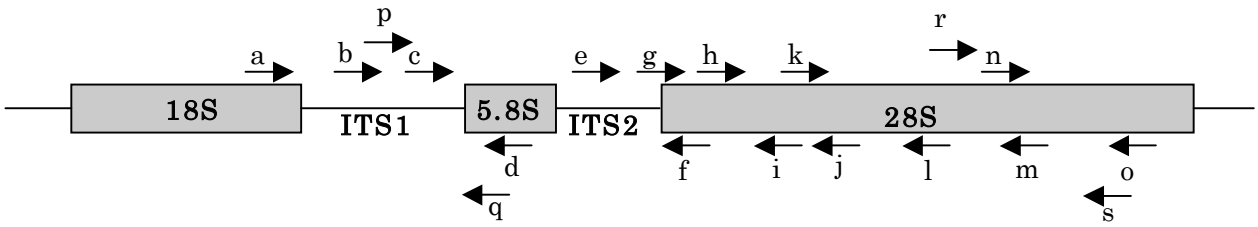

Table 3 Nucleotide Lengths of the ITS Regions and rRNA Genes

Group	Locality (region)	Sample IDs	ITS1 a)	5.8S b)	ITS2 c)	28S d)
Group Ⅰ	Japan	K1, I2, Nagano15, Mikawa	997 e)	160 f)	460 g)	2865 h)
		L2	997	160	460 i)	2865 i)
					461 i)	2868 i)
		Shikoku	997 i)	160	460	2865 i)
			998 i)			2868 i)
	Korean Peninsula	Korea-2	997	160	460 i)	2865
					461 i)	
	China	Anki-2, Kishu, Sekkou-1, Sekkou-2, Yunnan-3, Yunnan-4	997	160	460	2865
		Yunnan-1	997 i)	160	460	2865 i)
			999 i)			2866 i)
Group Ⅱ	Korean Peninsula	Korea-3	997	160	460	2823 j
	China	Yunnan-2	997	160	460	2823
Group Ⅲ	Korean Peninsula	Korea-1	997	160	460	2821
	China	Anki-1, Anki-3, Shisen	997	160	460	2821 k)

a): Result of sequencing of PCR products amplified using primer sets **a** and **d,** b): result of sequenceing of PCR products amplified using primer sets **c** and **f, c):** result of sequencing of PCR products amplified using primer sets **c** and **f,** d): result of sequence alignment of PCR products amplified using primer sets **g** and **j, k** and **m,** and **n** and **o**, e): DDBJ/EMBL/GenBank accession No. EF397596, f): DDBJ/EMBL/GenBank accession No.EF397595, g): DDBJ/EMBL/GenBank accession No. EF397597, h): DDBJ/EMBL/GenBank accession No. EF397598, i): determined by cloning, j): DDBJ/EMBL/GenBank accession No. EF397599, k): DDBJ/EMBL/GenBank accession No. EF397600

Table 4 Examples of deletions and mutations in the 28S rRNA gene

Sample name	611	690	1058	1106	1168	1467	1642-1643	1654-1655	1714	1785-1787	2082
K1	-	A	C	T	-	T	TC	CC	G	CGC	T
I2	-	*	*	*	-	*	**	**	*	***	*
L2	-	*	*	*	-	*	**	**	*	***	*
Nagano 15	-	*	*	*	-	*	**	**	*	***	*
Shikoku	-	*	*	*	-	*	**	**	*	***	*
Mikawa	-	*	*	*	-	*	**	**	*	***	*
Korea-2	-	*	*	*	-	*	**	**	*	***	*
Yunnan-1	-(T)	*	*	*	-	*	**	**	*	***	*
Anki-2	-	G	*	*	-	*	**	**	*	***	*
Kishu	-	G	*	*	-	*	**	**	*	***	*
Sekkou-1	-	G	*	*	-	*	**	**	*	***	*
Sekkou-2	-	R	*	*	-	*	**	**	*	***	*
Yunnan-4	-	R	*	*	-	*	**	**	*	***	*
Yunnan-3	-	R	*	*	-	*	**	**	*	***	*
Korea-3	-	R	*	*	-	*	--	--	-	---	-
Yunnan-2	-	R	*	*	-	*	--	--	-	---	-
Korea-1	-	R	-	-	T	-	--	--	-	---	-
Anki-1	-	R	-	-	T	-	--	--	-	---	-
Anki-3	-	R	-	-	T	-	--	--	-	---	-
Shisen	-	R	-	-	T	-	--	--	-	---	-

Sample name	2103-2114	2149-2152	2166-2168	2569-2571	2632	2641-2643	2670-2676
K1	TTCGACGGACTC	TCCT	---	TTA	G	GGC	CTCACCG
I2	**********	****	---	***	*	***	*******
L2	**********	****	---(GGG)	***	*	***	*******
Nagano 15	**********	****	---	***	*	***	*******
Shikoku	**********	****	---(GGG)	***	*	***	*******
Mikawa	**********	****	---	***	*	***	*******
Korea-2	**********	****	---	***	*	***	*******
Yunnan-1	**********	****	---	***	*	***	*******
Anki-2	**********	****	---	***	*	***	*******
Kishu	**********	****	---	***	*	***	*******
Sekkou-1	**********	****	---	***	*	***	*******
Sekkou-2	**********	****	---	***	*	***	*******
Yunnan-4	**********	****	---	***	*	***	*******
Yunnan-3	**********	****	---	***	*	***	*******
Korea-3	----------	----	GGC	---	-	---	-------
Yunnan-2	----------	----	GGC	---	-	---	-------
Korea-1	----------	----	GGC	---	-	---	-------
Anki-1	----------	----	GGC	---	-	---	-------
Anki-3	----------	----	GGC	---	-	---	-------
Shisen	----------	----	GGC	---	-	---	-------

*: same as the top column, -: deletion, R: adenine/guanine.