Cultivation and Antioxidative Activities of a New Functional Mushroom, *Pholiota* spp.

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Abstract: This study was carried out to develop a new functional mushroom with focus on its antioxidation activity. According to the analysis of ribosomal DNA sequences, they were classified into five clusters containing *Pholiota adiposa, P. squarrosa, P. nameko* and other *Pholota* species. Their spacer regions were 644-700 nucleotides in length. Using the nitro blue tetrazolium method, superoxide dismutase (SOD) activity in mycelia extracts of collected strains ranged from 11.5 to 37.9%. The strains with over 30% of SOD activity were ASI 24009, 24013, 24015, 24017, 24018 and 24028. All were classified in the same group (*P. adiposa*) according to sequence data. All strains tested showed only Mn-SOD activity. They were induced to produce fruit bodies in 850 ml bottles filled with poplar sawdust and in 40 × 40 cm^2 plastic boxes with cotton waste. Their fruit body productivity and morphological characteristics were measured.

Key words: Antioxidative activity, cultivation, *Pholiota* spp., superoxide dismutase, ribosomal DNA

1 Introduction

Mushrooms provide potentially beneficial effects for several of the most common diseases afflicting human beings, including cancer.[1] Furthermore, a lot of attention has been recently directed to the development of natural antioxidants as biologically active substances that can exert considerable protection against aging and cancer caused by free-radicals in humans.[2]

Superoxide (O_2^-) is very toxic in cells due to their highly reactive nature. Oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis.[3] However, most cells are able to protect themselves to a certain extent from the toxicity of superoxide ions by superoxide dismutases (SODs). This antioxidant supplement may be used to help the human body reduce oxidative damage. SODs are metalloproteins containing iron, manganese, copper plus zinc or nickel as the prosthetic groups.[4]

Pholiota genus belongs to division Basidiomycota, class Homobasidiomycetes, order Agaricales, family Strophariaceae.[5] A species of *Pholiota, P. nameko*, is not only cultured for edible mushroom, but also well known healthy food in Japan. Recently, various *Pholiota* species components possessing biological characteristics such as anticarcinogens, antioxidatives, antitumor, high quality protein, organic acid and vitamin have received much attention from many researchers.[6]

The taxonomy of this edible mushroom, however, is still confusing apparently due to expansion of special cultivation techniques and incorrect naming of newly cultivated strains.[7] Collected strains of *Pholiota* species were so far ambiguously or incorrectly named, leading to erroneous identification. Therefore, analysis of ITS (Internal Transcribed Spacer) is regarded as a recently useful method to discriminate species.[8] Because the strong preservation of functioning genomic zones (5.8s, 18s, 28s) does not permit an intergeneric discrimination, the large variability of the ITS region justified the choice of this intergene for collected *Pholiota* strains.

Cultivation of *Pholiota nameko* is well established in Japan. Cultural characteristics and cultivation method of *P. adiposa* were recentry reported.[9, 10] Optimal incubation temperature for hyphal growth was 25°C and fruitbody

yield was higher at 15°C than 20°C. In this study, phylogenic relationships and antioxidative activities among collected *Pholiota* strains were investigated. For the commercial production of fruitbody in the farm and to supply basic information for breeding functional mushrooms, they were cultivated and characterized.

2 Materials and Methods

2.1 Strains

Forty-five collected strains were used to investigate phylogenic relationships, antioxidative activities and cultural characteristics (Table 1). These strains were cultured and maintained on potato dextrose agar (PDA).

2.2 DNA preparation and ITS sequencing

Total genomic DNA was prepared by a modification of the simplified method described by Graham.[11] For PCR-amplification of ITS I ∏ region, the sequences of two primers ITS 1 and ITS 4 (Primer ITS 1 : 5'-TCC GTA GGT GAA CCT GCG G-3', ITS 4 : 5'-TCC TCC GCT TAT TGA TAT GC-3') were chosen from the known sequence of the 3'-end of 18s rRNA gene and the 5'-end of 28s rRNA gene of rDNA repeat unit and were synthesized using an automatic DNA synthesizer (Applied Biosystems Model/9600).[12] The PCR-amplified fragments were subcloned into pGEM T vector (Promega) and the nucleotide sequences were determined by dideoxy chain termination method with forward sequencing and reverse sequencing primer.[13] Alignment of nucleotide sequences was determined using DNA Star program.

2.3 Measurement of superoxide dismutase (SOD) activity

Mycelia and fresh mushroom fruit body from each strain were freeze-dried and then ground using liquid nitrogen. After a fine powder was obtained, the sample (0.2g) was suspended in 2mL extraction buffer at pH 7.0 containing 100mM potassium phosphate, 10mM sodium ascorbate and 5mM EDTA with 0.4g polyvinylpolypyrrolidone (PVP).[14] After the homogenate was centrifuged at 15,000rpm for 20 min, the clarified supernatant solution was purified using a PD-10 column of Sephadex G-25 (Amersham Pharmacia Biotech AB). Protein was determined spectrophotometrically according to Bradford[15] using bovine serum albumin (BSA) as the standard.

SOD activity of collected strains was measured by the Nitro Blue Tetrazolium(NBT) reduction method.[16] The mixture contained in test tubes with 3mL of assay buffer, 60μL of crude enzyme and 30μL of riboflavin were illuminated for 7min in a box lined with aluminum foil containing two 20-W Sylvania Groux Fluorescent lamps at 25°C. After reaction, the absorbance of the blank solution and reaction solution was measured at 560nm. One unit of SOD was defined as the amount of enzyme that inhibited NBT reduction by 50%. SOD activities were calculated using the following equation: SOD activity (%) = {1- (A/B)} × 100, A : absorbance of sample, B : absorbance of blank

Measurement of radical scavenging activity : Mushroom fruit body powder (5g) was extracted with 100mL of 80% methyl alcohol for 24h in a water bath at room temperature with stirring. The extracts were then filtered through Whatman No. 4 filter paper. The filtrates were taken to dryness on a rotary vacuum evaporator below 30°C and in a freeze-drier at – 40°C. The dried crude samples were dissolved in 80% methyl alcohol (1% w/v solution), and then used for the measurement of 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH) and electron spin resonance (ESR). The test for free radical scavenging activity was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH), according to the method of Yoshida et al.[17] A reaction solution of 2.5mL on 0.35mM DPPH with 50% ethyl alcohol and 0.25mL of each 1% sample solution were mixed. The DPPH value was estimated at an absorbance of 517nm after response for 10min at room temperature. The activities were calculated against the control, which used 80% methyl alcohol. The activity was calculated as an inhibition percentage. The superox

ide radical scavenging activity of *Pholiota* spp. was also evaluated using electron spin resonance (ESR) spectrometry. Superoxide radicals were generated from a hypoxanthine-xanthine oxidase system.[18] Hypoxanthine (25μ, 2mM)was mixed with 25μ of samples, xanthine oxydase (25μ, 0.5U) and DMPO (10μ, 0.67M). The reaction mixture was transferred into a 100μL flat quartz cell. After 45s, the ESR spectrum was recorded using an ESR spectrometer. Manganese oxide was used as an internal standard. Experimental conditions were as follows: Magnetic field, 337.100±5 mT; power, 5.00 mW; modulation frequency, 100kHz; frequency, 9.422GHz; amplitude, 5.0×100; sweep time, 1min; time constant, 0.1sec.

2.4 Determination of MnSOD activity

SOD activity was analyzed by non-denaturing polyacrylamide gel electrophoresis (PAGE). Activity staining for SOD on a non-denaturing polyacrylamide gel was performed according to the modified method proposed by Beauchamp & Fridovich.[19] The gels were incubated in the dark for 30min in 80mL reaction mixture containing 0.1M potassium-phosphate buffer (pH 7.8), 1mM EDTA, 33μM riboflavin, 245μM NBT, 17mM TEMED. The gel was then incubated in 0.1M potassium-phosphate buffer (pH 7.8) with 1mM EDTA and exposed to light for 30min. SOD activity was detected by the appearance of transparent bands, representing the inhibition of NBT reduction by superoxide anions, on a blue background. CuZnSOD activity is sensitive to both H_2O_2 and cyanide. In contrast, MnSOD activity is not inhibited by either. To distinguish it from CuZnSOD, MnSOD activity was assayed in all cases in the presence of 5mM KCN. CuZnSOD from bovine erythrocytes (Sigma) and MnSOD from *E. coli* (Sigma) were used as controls.

2.5 Mushroom cultivation

Cultivation of mushrooms was conducted by the method described by Chang et al.[10] For bottle cultivation, 550-600 g poplar sawdust media containing 20% rice bran packed in 850ml plastic bottle was autoclaved for 90 minutes at 121°C/15psi. After spawn inoculation, they were incubated at 20-23°C for 30 days. When the spawn had grown well throughout the medium, surface mycelia in the culture bottle was removed to induce budding. Cultures were then transferred to cultivation rooms maintained at 15°C and over 90% relative humidity to initiate fruit body formation. For box cultivation, about 6 kg cotton waste adjusted to 70% moisture were packed in the plastic box sized 45 × 45 cm and sterilized at 65°C for 10 hours and 55°C for three days. Spawns were inoculated on the bottom and surface of the medium and then incubated at 20-23°C for 15 days. After incubation, they were moved to cultivating room maintained at 15°C and over 90% relative humidity to initiate frui body formation.

3 Results

3.1 Phylogenic relationships of collected strains of *Pholiota* spp.

Forty-five collected strains of *Pholiota* spp. were used in this study (Table 1). According to the phylogenic tree (Fig.1), the collected strains of *Pholiota* spp. were classified into five groups. Based on ITS analysis, the length variation for the entire ITS region (including 5.8S) ranged from 644-700bp. The nucleotide sequence similarity of ITS region among *Pholiota* spp. tested was 49.6-99.9%. The nucleotide sequence homology was 95.3-100% among strains in group A, only one strain ASI 24019 in group B, 98.8-99.9% in group C, 76.3-99.9% in group D and 97.6% in group E, respectively. In case of comparison between groups, the genetic homologies of ITS sequences were 52.9% between group A and B, and 49.6% between group A and E, respectively. The components of group A, D and E consisted of several species. However, the species of group C was the same with *P. nameko*. This result showed that the phylogenic analysis might give us a criterion to classify species in our

collected strains, especially, *P. nameko*. In the confirmed sequences tested, ASI 24027 (Genbank. AY251300), 24032 (Genbank. AY251301), 24038 (Genbank. AY251302), 24040 (Genbank. AY251303), 24036 (Genbank. AY251304), 24028 (Genbank. AY251305), 24034 (Genbank. AY251306) were registered to National Center for Biotechnology Information (NCBI) in U.S.A.

3.2 SOD activity in *Pholiota* spp.

Mycelia of collected strains of *Pholiota* spp. were used to analyze SOD activity by the NBT reduction method (Table 2).The SOD activities differed significantly among strains tested. The high SOD activity strains, over 30% of SOD activity, ASI 24008, 24009, 24013, 24015, 24017, 24018 and 24028, were selected by NBT method. The SOD activity of ASI 24008 (37.9%) was highest when compared with the other strains. Conversely, the SOD activity of ASI 5008 was the lowest. The selected strains were interestingly included in group A (Figure 1). This group was classified *P. adiposa* according to the result of ITS sequence data. They will be used for further study for breeding new commercial strains.

Table 1. The list of the strains used in this study

Strain No. (ASI)*	Scientific name	Source	Strain No. (ASI)	Scientific name	Source
24001	*P. adiposa*	Korea	24026	*P. flammans*	Korea
24002	*P. adiposa*	Korea	24021	*P. highlandensis*	Korea
24003	*P. adiposa*	Korea	24034	*P. lucifera*	CBS 280.29
24004	*P. adiposa*	Korea	24035	*P. lucifera*	CBS 595.82
24005	*P. adiposa*	Korea	24014	*P. malicola*	USA MW58
24007	*P. adiposa*	Korea	24036	*P. nameko*	CBS 360.51
24008	*P. adiposa*	Korea	24037	*P. nameko*	CBS 801.91
24010	*P. adiposa*	Korea	5008	*P. nameko*	Japan
24012	*P. adiposa*	Korea	5011	*P. nameko*	Japan
24013	*P. adiposa*	Korea	5019	*P. nameko*	Korea
24015	*P. adiposa*	USA MW59	5020	*P. nameko*	China
24017	*P. adiposa*	Korea	24006	*P. squarrosa*	Korea
24018	*P. adiposa*	Korea	24038	*P. squarrosa*	CBS 109.11
24022	*P. adiposa*	Korea	24039	*P. squarrosa*	CBS 319.82
24024	*P. adiposa*	Korea	24009	*P. squarrosa-adiposa*	Korea
24025	*P. adiposa*	Korea	24011	*P. squarrosa-adiposa*	Korea
24027	*P. adiposa*	Korea	24020	*P. squarrosa-adiposa*	Korea
24016	*P. aggericola*	USA WC19	24040	*P. squarrosa-adiposa*	CBS 911.72
24028	*P. aurivella*	CBS 318.82	24019	*P.* sp	Korea
24030	*P. aurivella*	CBS 322.85	24023	*P.* sp	Korea
24031	*P. aurivella*	CBS 118.18	24029	*P.* sp.	CBS 279.29
24032	*P. carbonaria*	CBS 213.39	5010	*P.* sp	Japan
24033	*P. carbonaria*	CBS 579.81			

* ASI : Agricultural Science Institute, Suwon, Korea

Table 2. SOD activity in mycelia of 34 *Pholiota* strains

Strain No. (ASI)	SOD activity (%)	Strain NO. (ASI)	SOD activity (%)
24001	21.7	24018	31.5
24002	14.9	24019	25.1
24003	22.0	24021	25.6
24004	17.7	24022	24.7
24005	28.8	24023	28.5
24006	26.6	24024	20.3
24007	25.0	24025	19.4
24008	37.9	24027	20.1
24009	35.3	24028	30.8
24010	24.5	24029	25.7
24011	28.3	24033	20.4
24012	26.4	24035	17.1
24013	30.2	24037	29.5
24014	20.3	24040	26.9
24015	31.7	5008	11.5
24016	19.1	5011	25.1
24017	31.6	5019	25.5
L.S.D	7.0	L.S.D	7.0

3.3 Antioxidative activity test

Mushroom fruit body was used to test antioxidative activities (NBT, ESR, DPPH test). Table 3 shows the DPPH values of the 16 strains (ITS group A). The highest DPPH activity (above 8%) were exhibited by strains ASI 24010 and 24013. Lower activity strains (below 4%) were ASI 24008, 24018 and 24025. There appeared to be low electron donating ability by DPPH. Superoxide radical scavenging activity was evaluated using ESR. The superoxide radical scavenging activity was calculated by comparison of the average relative peak height among 16 *Pholiota* strains. The strain of ASI 24007 showed the highest SOD radical scavenging activity (60. 1%), whereas ASI 24027 and 24022 showed low antioxidant activities (40.6% and 45.7%, respectively).

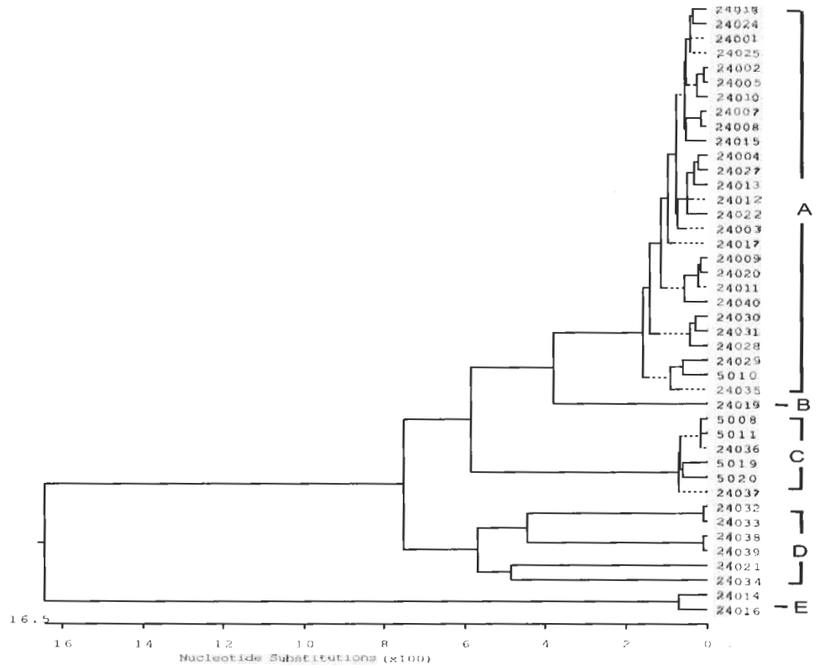

Figure 1. The phylogenetic tree based on ITS region sequences of ribosomal DNA of 42 *Pholiota* strains

3.4 Determination of MnSOD activity

The only SOD activity detectable in cell extracts of *Pholiota* spp. was that of MnSOD. SOD proteins were electrophoresed on 10%(w/v) polyacrylamide gels and stained for SOD activity with NBT and riboflavin; 5mM KCN was added to inactivate CuZnSOD. CuZnSOD activity was not detected in those extracts. The strains tested exhibited only MnSOD activity, as no band disappeared when the gels were incubated in the presence of 5mM KCN (Fig. 2). Similar results were obtained when H_2O_2 was added to the incubation solution (data not shown).

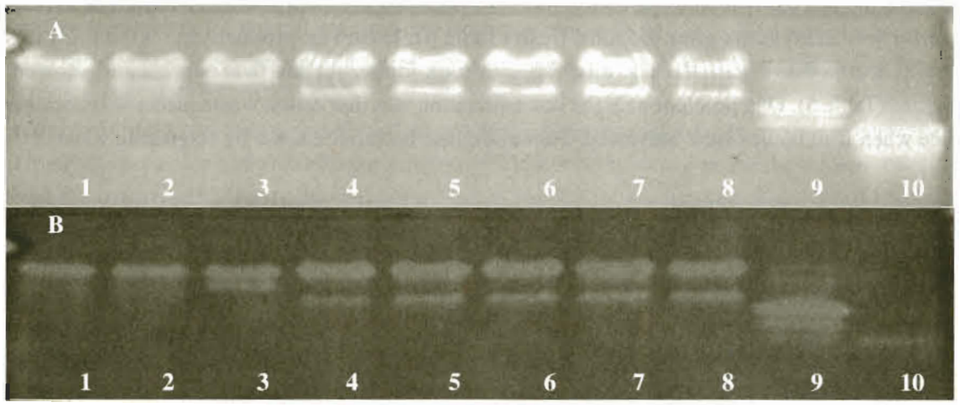

Figure 2. Native PAGE of SOD proteins stained for SOD activity

A is KCN-, B is KCN +; The lane order is 24002, 24008, 24009, 24013, 24015, 24017, 24018, 24028, MnSOD, CuZnSOD from 1 to 10, respectively.

Table 3. Antioxidative activity in fruit bodies of *Pholiota* strains

Strain No. (ASI)	SOD(NBT)	SOD(ESR)	DPPH	Mean
24001	17.0	53.3	5.9	21.2
24002	26.9	46.9	7.5	31.5
24003	19.8	47.9	7.7	24.4
24004	28.7	57.5	4.9	29.8
24007	23.2	60.1	7.4	29.4
24008	24.4	54.4	3.9	27.2
24010	25.8	59.4	8.7	31.3
24012	22.8	53.9	4.7	27.3
24013	21.9	56.4	8.8	27.6
24015	20.3	55.7	4.9	24.6
24017	26.2	52.0	4.3	28.2
24018	29.4	48.1	3.7	27.0
24022	26.9	45.7	6.4	29.9
24024	21.0	54.2	5.7	24.9
24025	25.6	48.4	3.5	27.7
24027	27.3	40.6	5.8	26.7
L.S.D	6.7	10.8	2.9	

3.5 Mushroom cultivation

Since the tested strains were composed of several species, some species did not produce fruit bodies except *P. adiposa, P. squarrosa, P. nameko* and a few *Pholiota* sp. in bottle cultivation. Since previous results had shown that antioxidative activities were higher in *P. adiposa* strains, as we focused on *P. adiposa* cultivation. Productivity of *P. adiposa* was different according to the strains although fruit body characteristics were almost the same (Table 4). ASI 24012, 24020 and 24027 showed good productivities. The color of fruit body pilei was reddish yellow and the shape was convex. The surface of pilei was subviscid and there were many white scales. On the other hand, other species strains showed low productivities under these cultivation conditions (data not shown). The color of *P. squarrosa* fruit body pilei was dark brown and had a few long stipes and some scaly pilei. The color of *P. nameko* fruit body pilei was brown or dark brown and had short stipes and very viscid pilei (Figure 3). For the box cultivation using cotton waste, the incubation time was only 15 days. The average yield of *P. adiposa* was 2200 g/box after the third flush (Table 5). However, productivity in the third flush was dramatically decreased (Figure 4). The other species took longer for the first harvest and showed lower productivities (Table 5). One problem with the box cultivation was that cotton waste media was detached from the surface when fruit bodies were harvested. This will cause bacterial disease by stagnating water.

Figure 3. Fruit bodies produced by the bottle cultivation method (*P. adiposa, P. squarrosa, P. nameko*)

Table 4. Fruit body characteristics of *Pholiota* strains

Strain No. (ASI)	Pilei(mm)			Stipes(mm)			No. of fruitbody	Yield (g/850cc)
	Color*	Shape**	Size	Thickness	Length	Diameter		
24001	RW	C	65	5	81	9	11	87
24002	RW	C	48	6	95	16	2	32
24004	RW	C	43	8	70	10	13	78
24005	RW	C	47	9	126	15	1	31
24006	DB	PC	36	4	60	17	5	55
24007	RW	C	73	12	110	16	2	64
24008	RW	C	33	7	65	13	1	12
24009	DB	PC	45	4	95	11	6	79
24010	RW	C	40	6	76	9	7	46
24012	RW	C	35	5	90	7	20	117
24013	RW	C	29	3	79	9	21	92
24015	RW	C	43	3	78	8	14	111
24017	RW	C	37	3	75	9	12	65
24018	RW	C	37	3	80	5	27	110
24020	LY	C	55	5	85	10	11	118
24024	RW	C	28	4	68	7	14	41
24025	LY	C	75	10	60	16	2	62
24027	RW	C	43	3	78	8	22	127
24028	LY	C	60	7	78	15	5	70
24029	RW	C	38	6	45	11	9	65
24035	LY	C	32	3	60	10	9	42

* Color : RW-Reddish yellow, DB-Dark brown, LY-Light yellow

** Shape C-Convex, PC-Plano-convex

4 Discussion

The health benefits of mushroom have been well known for a long time and are widely recognized around the world.[20] Nowadays, the demands for "healthy food" or "functional food", are increasing in many countries. Also, bioactive substances for natural antioxidants from mushroom SOD plays an essential role in allowing organisms to survive in the presence of molecular oxygen. In plants, as well as in mammalian and fungal cells, CuZnSOD is a major SOD.[21]

However, only MnSOD was assayed in the white-rot fungi *Pleurotus florida, P. chrysosporium, Lentinula edodes, Trametes versicolor* and *Ganoderma lucidum*.[22] As has been shown in earlier studies, *Pholiota* spp. also showed the same result. Especially, the expression of MnSOD is essential for the survival of aerobic life and for the development of cellular resistance to oxygen radical-mediated toxicity. Therefore, we could expect that SOD activity was an important selection marker for breeding medicinal mushrooms. Besides, there is a significant difference among the *Pholiota* strains tested. In order to develop the new functional strains, we should first classify the collected strains. According to our results, the use of ITS sequencing is a powerful method to accurately discriminate groups. *P. nameko* is very popular edible mushroom in Japan. The other species in *Pholiota* are not well known yet, even though they have many functional benefits. This study will give some functional information about the *P. adiposa* fruit body. The income from the commercial production of *P. adiposa* fruit bodies in farms is expected to increase. More accurate and detailed isolation, identification and extraction of antioxidative substances are required to produce better results for future research.

Figure 4. Yield of *P. adiposa* according to the stages (1st flush and 3rd flush) in cotton waste box cultivation

Table 5. Yields of *Pholiota* spp. at different stages in cotton waste box cultivation

Strain No. (ASI)	Species	1st flush		2nd flush		3rd flush		Total yield (g/box)
		Days	Yield(g)	Days	Yield(g)	Days	Yield(g)	
24001	*P. adiposa*	10	923	25	795	42	700	2418
24002	*P. adiposa*	12	1307	29	1016			2323
24003	*P. adiposa*	10	1461	26	1068	45	305	2834
24004	*P. adiposa*	12	1890	34	856			2746
24005	*P. adiposa*	12	1241	29	465	45	447	2153
24008	*P. adiposa*	12	1261	31	1376			2637
24010	*P. adiposa*	12	356	31	931			1287
24012	*P. adiposa*	12	1377	31	571			1948
24013	*P. adiposa*	12	977	32	568			1545
24015	*P. adiposa*	18	1080	43	151			1231
24017	*P. adiposa*	10	1077	26	904			1981
24018	*P. adiposa*	12	1453	34	578			2031
24022	*P. adiposa*	12	1983	29	841			2824
24024	*P. adiposa*	10	1065	25	881	42	1139	3085
24025	*P. adiposa*	12	1140	29	778	45	507	2425
24027	*P. adiposa*	10	981	25	1101	43	500	2582
24041	*P. adiposa*	10	800	32	554			1354
Mean		11	1198	30	790	43	600	2200
Distribution ratio (%)			54.50		35.90		27.20	
24006	*P. squarrosa*	14	1467	34	716			2183
24009	*P. squarrosa*	14	209	22	269	31	528	1006
24011	*P. squarrosa*	14	231	25	156	29	238	625
24020	*P. squarrosa*	14	1083	34	922			2005
24028	*P. aurivella*	12	337	39	446			783
24029	*Pholiota.* sp	14	757	34	921			1678
Mean		14	681	31	572		383	1380

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