Bull. Natl. Mus. Nat. Sci., Ser. B, 43(2), pp. 33–44, May 22, 2017

DNA Extraction, PCR and Sequencing were largely Unsuccessful from the Type Specimens of Mushrooms but Some 50-year-old or Older Specimens produced Authentic Sequences

Kentaro Hosaka

Department of Botany, National Museum of Nature and Science,
Amakubo 4–1–1, Tsukuba, Ibaraki 305–0005, Japan
E-mail: khosaka@kahaku.go.jp

(Received 16 March 2017; accepted 22 March 2017)

Abstract DNA extraction, PCR and sequencing of the nuclear ribosomal internal transcribed spacer region from a total of 293 type specimens of Agaricomycotina (Basidiomycota, Fungi) were conducted. Although more than 50% of those specimens produced positive PCR bands, a total of 114 specimens from National Museum of Nature and Science (TNS) and 21 specimens from Field Museum of Natural History (F), respectively, produced sequences of low quality with no discernable peaks. All of the 43 good sequences obtained from the specimens at TNS were demonstrated to be from apparent contamination. In contrast, out of the 16 good sequences obtained from the specimens at F, only 2 sequences were from apparent contamination and the remaining 14 were considered authentic. The oldest specimen from which authentic sequence has been obtained was from 1940, but the PCR and sequencing success rate from older specimens was general low. This study clearly demonstrates the need of immediate DNA extraction from type specimens. Alternatively, small pieces of type specimens should be preserved separately from the herbarium for future DNA studies.

Key words: BLAST, herbarium, ITS, mushrooms, taxonomy, type specimens.

Introduction

Fungi in general have limited morphological characters and mushrooms with macroscopic fruit bodies are no exception. It has often been demonstrated that mushrooms with similar or indistinguishable fruit body morphology represent phylogenetically distantly related taxa (e.g., Kasuya *et al.*, 2012; Wilson *et al.*, 2016). For this reason, DNA sequence data, especially from the type specimens, are essential to establish stable taxonomic system of mushrooms.

It is generally appreciated that the nuclear ribosomal internal transcribed spacer (ITS) region is an official DNA barcode region for fungi (Schoch *et al.*, 2012). Although the ITS sequences cannot discriminate some closely related species, especially among Ascomycota, the presence of "universal" primers and high success rate of PCR and sequencing make this region widely applicable to many studies, such as taxonomy and ecology (Dentinger *et al.*, 2010; Nagy *et al.*, 2011; Schoch *et al.*, 2012).

In contrast to the rapid accumulation of the ITS sequences in GenBank, the number of sequences from the type specimens is not increasing at the same rate. The study by Nagy *et al.* (2011) showed that a number of unidentifiable sequences from environmental sequencing projects matched with the sequences from the type strains of *Mortierella*. Similarly, Brock *et al.* (2009) estimated that ca. 70% of taxonomic diversity at the fungal herbarium of Kew Gardens alone was not yet represented in GenBank. These studies clearly demonstrated the need of obtaining ITS sequences from the type speci-

mens to appropriately link fungal names and DNA sequence data.

Molecular studies using the type specimens, however, face a number of challenges. In general, DNA in specimens degrades with time (Erkens *et al.*, 2008; Willerslev and Cooper, 2005), but the majority of type specimens are "old". In addition, storage conditions such as temperature, humidity, and type of fumigation all affect the quality of DNA (Willerslev and Cooper, 2005). It has also been demonstrated that older specimens tend to be more contaminated by other fungi (Brock *et al.*, 2009).

In this study, the type specimens of diverse groups of mushrooms housed at two different herbaria collected in different times (from 1880's to 1990's) were tested for assessing the DNA quality. All specimens were extracted for genomic DNA, PCR amplified using general primers of the ITS, and sequence data were obtained by direct sequencing of PCR products. The aforementioned challenges are further emphasized, and a potential solution for future studies is presented.

Materials and Methods

Type specimens used in this study

A total of 235 specimens housed at the fungal herbarium, Department of Botany, National Museum of Nature and Science, Tsukuba, Japan (TNS), and a total of 58 specimens housed at the Field Museum of Natural History, Chicago, USA (F) were studied. All of them were dried specimens with various ages (collection years ranged from 1882–1992). Specimens collected more recently (within 20 years) were intentionally excluded from this study because one of the main purposes was to assess the DNA quality from old specimens.

All specimens belong to the subphylum Agaricomycotina (mushrooms) of various families and orders (Table 2, 3). Although the original specimen labels all indicate they are type specimens (including holotype, isotype and paratype), the nomenclatural status could not be confirmed for some specimens. For example, "*Ganoderma spathulatum*" (TNS-F-201590) was indicated on the specimen label as "Typus" but no such names could be found either from Index Fungorum (http://www.indexfungorum.org/) or a cumulative list of Japanese fungi by Katsumoto (2010). Such species of uncertain type status are indicated with quotation marks throughout the text and tables (Table 2, 3).

All dried specimens, including the specimen labels and packets, were photographed for later assessing the quality of specimens, such as presence of fungal or insect damage. Some representative photographs and specimen data of type specimens were uploaded at Type Specimen Database of National Museum of Nature and Science, and they are publicly available from http://www.type.kahaku.go.jp/TypeDB/.

DNA preparation, PCR, sequencing, and BLAST search

DNA was extracted from hymenial tissues of dried specimens (~200 mg) stored overnight in DMSO buffer (Seutin *et al.*, 1991; Hosaka, 2009). Tissues were first ground under liquid nitrogen using a mortar and pestle. Ground tissues were then transferred to new 1.5 ml tubes using clean spatulas, and CTAB buffer was added. DNA was extracted using the modified CTAB extraction protocol (Doyle and Doyle, 1987) followed by glass milk purification methods as summarized by Hosaka (2009) and Hosaka and Castellano (2008). Briefly, ground samples were incubated in CTAB buffer at 65°C for 1 hour, and proteins were removed using the mixture of chloroform: isoamylalcohol (24 : 1). The materials were further purified using 6M sodium iodine buffer with glass milk, washed with ethanol/buffer solution, and finally eluted in 100 μl of TE buffer. All samples of extracted genomic DNA were deposited in the molecular laboratory, Department of Botany, National Museum of Nature and Science, Tsukuba, Japan, under the DNA voucher numbers denoted in Tables 2 and 3.

DNA sequence data were obtained from the

ITS region using the primers ITS5 and ITS4 for amplifying ca. 600 bp region (White *et al.*, 1990). PCR reactions were carried out using 20 μl reaction volumes each containing: 1 μl genomic DNA, 1 μl dNTPs (4 mM), 1 μl of each primer (8 μM), 0.5 units of Taq polymerase (TaKaRa, Tokyo, Japan), 2 μl $MgCl_2$ (25 mM), 2 μl Bovine Serum Albumin (BSA). Cycling parameters were 1 cycle of 94℃ for 3 min, 30 cycles of 94℃ for 1 min, 51℃ for 30 s and 72℃ for 1 min, with a final extension at 72℃ for 15 min. PCR products were electrophoresed in 1% agarose gels stained with ethidium bromide and visualized under UV light. When amplification bands were confirmed, the results were scored either as "successful (O)" (with single, clear band) or "failed (X)" (no bands or only smears were observed) (Tables 2, 3). PCR products were then purified using the ExoSap-IT (Millipore, Molsheim, France) and directly sequenced using the Big Dye Terminator Cycle Sequencing Kit on ABI3500 (Applied Biosystems Inc., Foster City, CA, USA), following the manufacturer's instructions.

Before editing raw sequence data, quality of sequences were scored as "good (O)" (readily editable sequences with clear chromatograms), "hetero (H)" (chromatograms with hetero-peaks), or "messy (X)" (sequences that are not editable with no discernable peaks), using Sequence Scanner version 1.0 (Applied Biosystems Inc., Foster City, CA, USA) (Table 2, 3). Only the sequences scored as "good" were further edited using ATGC version 7.1.0 (GENETYX Corporation, Tokyo, Japan).

Edited sequences were analyzed using the GenBank BLAST search (Altschul *et al.*, 1990). Default settings of blastn option were used such as "Database" = "Others (nr etc.)" and "Program Selection" = "Highly similar sequences (megablast)". No parameters, such as "Max target sequences" and "Expect threshold", were changed from the default settings. The top hit (regardless of its taxonomic level) and the next fully identified hit (to species level) were recorded. When those hits could be considered erroneous (e.g., the top hit was ascomycetes but all the other hits showed basidiomycetous taxa), the "erroneous" hits were discarded and the results of the remaining hits were recorded.

Table 1. Comparison of the results of PCR and sequencing from the specimens housed at two herbaria

	Herbarium	
	TNS*1	F*2
Numbers of specimens tested	235	58
Year collected (oldest)	1891	1882
Year collected (latest)	1992	1991
PCR successful	169	42
Sequences with low quality	114	21
Sequences with hetero-peaks	12	5
Sequences from apparent contamination	43	2
Authentic sequences	0	14

*1 Department of Botany, National Museum of Nature and Science, Tsukuba, Japan.

*2 Department of Botany, Field Museum of Natural History, Chicago, USA.

Because sequence data from the majority of species tested in this study were expected not to be in GenBank database yet, BLAST results matched at generic level (or synonym of such) were considered "authentic" (DNA sequences were truly obtained from the specimens, not from the other sources). All the other matches were considered sequences from "apparent contamination" (Table 1, 2, 3).

Results and Discussion

Most specimens used in this study were in "good" condition macroscopically, without obvious insect damage (Fig. 1). However, some specimens had obvious fungal growth on the surface of fruit bodies, presumably by ascomycetous molds (Fig. 1A, D). The results of BLAST search indicated that sequences obtained from more than 20 specimens at TNS herbarium were from apparent contamination of ascomycetes (Table 1, 2). The visible presence of molds on fruit bodies was, however, not necessarily corresponding to the BLAST results. For example, "molds" on "*Ganoderma spathulatum*" (Fig. 1A) and *Gas-*

Table 2. Type specimens tested for this study housed at National Museum of Nature and Science (TNS) with the results of PCR and sequencing

Herbarium Voucher Nos.	DNA Voucher Nos.*1	Order	Family	Taxon names in the original label*2	Year collected	PCR*3	Sequencing*4	BLAST results*5
TNS-F-201590	A3016	Polyporales	Ganodermataceae	"*Ganoderma spathulatum*"	1891	X	–	–
TNS-F-180124	A3056	Boletales	Boletaceae	*Boletus hiratsukae*	1896	O	H	–
TNS-F-201999	A3023	Thelephorales	Thelephoraceae	*Thelephora komabensis*	1898	O	X	–
TNS-F-206977	A3057	Polyporales	Ganodermataceae	*Ganoderma subumbraculum*	1908	O	X	–
TNS-F-202252	A3025	Polyporales	Polyporaceae	*Daedalea kusanoi*	1909	O	X	–
TNS-F-202482	A3001	Polyporales	Polyporaceae	*Fomes olivaceus*	1911	X	–	–
TNS-F-202550	A3037	Polyporales	Polyporaceae	*Fomes angularis*	1912	O	H	–
TNS-F-202243	A3061	Polyporales	Polyporaceae	*Daedalea dickinsii*	1912	O	X	–
TNS-F-201565	A3006	Polyporales	Polyporaceae	*Polyporus pubertatis*	1912	X	–	–
TNS-F-201364	A3031	Polyporales	Polyporaceae	*Polyporus sendaiensis*	1913	O	H	–
TNS-F-202551	A3033	Polyporales	Polyporaceae	*Fomes pusillus*	1913	X	–	–
TNS-F-203004	A2336	Russulales	Stereaceae	*Stereum roseum*	1913	X	–	–
TNS-F-202818	A3028	Thelephorales	Thelephoraceae	*Thelephora papillosa*	1914	O	H	–
TNS-F-202337	A3026	Polyporales	Meruliaceae	*Merulius castaneus*	1914	X	–	–
TNS-F-201423	A2998	Polyporales	Polyporaceae	*Polyporus versisporus*	1914	X	–	–
TNS-F-202805	A3027	Hymenochaetales	Hymenochaetaceae	*Hymenochaete boninensis*	1915	O	H	–
TNS-F-203033	A3040	Russulales	Stereaceae	*Stereum japonicum*	1915	O	H	–
TNS-F-201758	A3002	Polyporales	Polyporaceae	*Polystictus nipponicus*	1915	O	X	–
TNS-F-201801	A3000	Polyporales	Polyporaceae	*Polystictus glabratus*	1915	O	X	–
TNS-F-202823	A3034	Thelephorales	Thelephoraceae	*Thelephora japonica*	1915	O	X	–
TNS-F-203077	A3029	Polyporales	Meruliaceae	*Irpex iyoensis*	1916	O	H	–
TNS-F-203063	A3035	Polyporales	Meruliaceae	*Irpex purpureus*	1916	O	X	–
TNS-F-202071	A3011	Polyporales	Polyporaceae	*Trametes sensitivus*	1916	O	X	–
TNS-F-202757	A3067	Russulales	Stereaceae	*Aleurodiscus japonicus*	1916	O	X	–
TNS-F-203006	A3007	Russulales	Stereaceae	*Stereum sendaiense*	1916	O	X	–
TNS-F-203069	A2271	Polyporales	Meruliaceae	*Irpex tabacinoides*	1916	X	–	–
TNS-F-202783	A3017	Russulales	Stereaceae	*Stereum liratum*	1916	X	–	–
TNS-F-201575	A3032	Polyporales	Ganodermataceae	*Ganoderma tsunodae*	1917	O	H	–
TNS-F-201448	A3036	Polyporales	Polyporaceae	*Polyporus profissilis*	1917	O	H	–
TNS-F-207812	A3018	Agaricales	Pterulaceae	*Pterula fusispora*	1917	O	X	–
TNS-F-202077	A3060	Polyporales	Polyporaceae	*Trametes japonica*	1917	O	O	uncultured ascomycetes
TNS-F-195151	A2215	Cantharellales	Cantharellaceae	*Cantharellus pallidus*	1917	X	–	–
TNS-F-202751	A3063	Russulales	Stereaceae	*Aleurodiscus orientalis*	1918	O	X	–
TNS-F-202075	A3059	Polyporales	Polyporaceae	*Trametes symploci*	1918	O	O	*Rhodosporidium*
TNS-F-201771	A2218	Hymenochaetales	Hymenochaetaceae	*Coltriciella pusilla*	1918	X	–	–
TNS-F-204262	A2272	Polyporales	Meruliaceae	*Irpex tabacinoides*	1918	X	–	–
TNS-F-201309	A2995	Polyporales	Polyporaceae	*Polyporus greenii*	1918	X	–	–
TNS-F-201566	A3012	Polyporales	Polyporaceae	*Polyporus kanehirae*	1918	X	–	–
TNS-F-202941	A3070	Russulales	Stereaceae	*Coniophora matsuzawae*	1918	X	–	–
TNS-F-202748	A3062	Russulales	Stereaceae	*Aleurodiscus tsugae*	1919	O	X	–
TNS-F-202774	A3069	Russulales	Stereaceae	"*Aleurodiscus suborientalis*"	1919	O	X	–
TNS-F-202073	A3024	Polyporales	Polyporaceae	*Trametes minutissima*	1920	O	X	–
TNS-F-202754	A3064	Russulales	Stereaceae	*Aleurodiscus reflexus*	1920	O	X	–
TNS-F-202755	A3065	Russulales	Stereaceae	*Aleurodiscus sendaiensis*	1920	O	X	–
TNS-F-202756	A3066	Russulales	Stereaceae	*Aleurodiscus stereoides*	1920	O	X	–
TNS-F-201365	A2996	Polyporales	Polyporaceae	*Polyporus sendaiensis*	1921	O	X	–
TNS-F-201366	A2997	Polyporales	Polyporaceae	*Polyporus sendaiensis*	1921	X	–	–
TNS-F-203018	A3039	Russulales	Stereaceae	*Stereum hamamelidis*	1922	O	H	–
TNS-F-203017	A3038	Russulales	Stereaceae	*Stereum kurilense*	1923	O	H	–
TNS-F-201532	A3010	Polyporales	Polyporaceae	*Polyporus liukiuensis*	1923	O	O	*Psathyrella*
TNS-F-244375	A2179	Agaricales	Strophariaceae	*Agrocybe media*	1925	O	X	–
TNS-F-201260	A3004	Polyporales	Ganodermataceae	*Ganoderma hainanense*	1928	O	X	–
TNS-F-200234	A2261	Hymenochaetales	Hymenochaetaceae	*Hymenochaete yasudai*	1929	X	–	–
TNS-F-200021	A3058	Polyporales	Polyporaceae	"*Polyporus kinkazanensis*"	1931	O	X	–
TNS-F-203362	A3030	Hymenochaetales	Hymenochaetaceae	*Hymenochaete rufo-marginata*	1932	O	H	–
TNS-F-200828	A2236	Polyporales	Fomitopsidaceae	"*Fomitopsis laevigata*"	1934	X	–	–
TNS-F-200762	A3003	Polyporales	Ganodermataceae	*Ganoderma neojaponicum*	1935	O	X	–
TNS-F-200900	A2306	Phallales	Protophallaceae	*Protubera nipponica*	1935	O	O	*Laccaria*
TNS-F-172699	A3019	Agaricales	Agaricaceae	*Cyathus badius*	1936	O	O	*Psathyrella*
TNS-F-193009	A2197	Auriculariales	Auriculariaceae	*Auricularia leucochroma*	1936	X	–	–
TNS-F-203372	A3021	Polyporales	Polyporaceae	*Fomes mangrovicus*	1937	X	–	–
TNS-F-206958	A3005	Polyporales	Polyporaceae	*Daedaleopsis nipponica*	1938	O	O	*Psathyrella*
TNS-F-206965	A3013	Hymenochaetales	Hymenochaetaceae	*Inonotus sciurinus*	1938	X	–	–
TNS-F-208290	A2278	Polyporales	Polyporaceae	*Lentinus palauensis*	1939	O	X	–
TNS-F-207357	A3071	Hymenochaetales	Hymenochaetaceae	*Hymenochaete yasudai*	1939	O	O	*Pleurotus*
TNS-F-207357	A2262	Hymenochaetales	Hymenochaetaceae	*Hymenochaete yasudai*	1939	X	–	–
TNS-F-190105	A2200	Auriculariales	Auriculariaceae	*Auricularia semipellucida*	1940	O	X	–
TNS-F-208513	A2294	Agaricales	Marasmiaceae	*Marasmius aurantiacus*	1941	O	X	–
TNS-F-6563	A2199	Auriculariales	Auriculariaceae	*Auricularia polytricha*	1941	X	–	–

Table 2. Continued

Herbarium Voucher Nos.	DNA Voucher Nos.*1	Order	Family	Taxon names in the original label*2	Year collected	PCR*3	Sequencing*4	BLAST results*5
TNS-F-212095	A3014	Hymenochaetales	Hymenochaetaceae	*Cryptoderma cercidiphyllum*	1941	X	–	–
TNS-F-213570	A2237	Polyporales	Fomitopsidaceae	"*Fomitopsis tenuis*"	1943	O	O	uncultured ascomycetes
TNS-F-237517	A2414	Russulales	Russulaceae	*Russula kansaiensis*	1945	O	X	–
TNS-F-237667	A2318	Agaricales	Entolomataceae	*Rhodophyllus cyanoniger*	1950	O	X	–
TNS-F-234247	A2217	Agaricales	Omphalotaceae	*Collybia ozeensis*	1950	X	–	–
TNS-F-237485	A2303	Agaricales	Pleurotaceae	*Pleurotus incarnatus*	1952	O	X	–
TNS-F-237560	A2339	Agaricales	Strophariaceae	*Stropharia rugosoannulata*	1952	O	X	–
TNS-F-237621	A2344	Agaricales	Tricholomataceae	*Tricholoma muscarium*	1952	O	X	–
TNS-F-237369	A2284	Agaricales	Agaricaceae	*Lepiota clypeolaria*	1953	O	X	–
TNS-F-237379	A2291	Agaricales	Lyophyllaceae	*Lyophyllum musashiense*	1953	O	X	–
TNS-F-237296	A2216	Agaricales	Omphalotaceae	*Collybia neofusipes*	1953	X	–	–
TNS-F-237333	A2257	Agaricales	Hygrophoraceae	*Hygrophorus pseudococcineus*	1954	O	X	–
TNS-F-237490	A2309	Agaricales	Tricholomataceae	*Pseudohiatula ohshimae*	1954	O	X	–
TNS-F-237518	A2329	Russulales	Russulaceae	*Russula metachroa*	1954	O	X	–
TNS-F-237522	A2332	Russulales	Russulaceae	*Russula niigatensis*	1954	O	X	–
TNS-F-237523	A2333	Russulales	Russulaceae	*Russula omiensis*	1954	O	X	–
TNS-F-237524	A2334	Russulales	Russulaceae	*Russula subnigricans*	1954	O	X	–
TNS-F-237307	A2229	Agaricales	Cortinariaceae	*Cortinarius subalboviolaceus*	1954	O	O	uncultured ascomycetes
TNS-F-237325	A2249	Agaricales	Hygrophoraceae	*Hygrophorus dichrous*	1954	O	O	uncultured ascomycetes
TNS-F-237319	A2244	Boletales	Hygrophoropsidaceae	*Hygrophoropsis bicolor*	1954	O	O	uncultured ascomycetes
TNS-F-237304	A2223	Agaricales	Cortinariaceae	*Cortinarius claricolor*	1954	X	–	–
TNS-F-237328	A2252	Agaricales	Hygrophoraceae	*Hygrophorus imazekii*	1954	X	–	–
TNS-F-237347	A2263	Agaricales	Inocybaceae	*Inocybe ammophila*	1954	X	–	–
TNS-F-237373	A2286	Agaricales	Agaricaceae	*Lepiota subcitrophylla*	1955	O	X	–
TNS-F-237480	A2297	Agaricales	Mycenaceae	*Mycena umbilicata*	1955	O	X	–
TNS-F-237491	A2311	Agaricales	Hymenogastraceae	*Psilocybe fasciata*	1955	O	X	–
TNS-F-237519	A2330	Russulales	Russulaceae	*Russula nauseosa*	1955	O	X	–
TNS-F-237375	A2287	Agaricales	Agaricaceae	*Leucocoprinus denudatus*	1955	O	O	*Laccaria*
TNS-F-237273	A2178	Agaricales	Bolbitiaceae	*Agrocybe farinacea*	1956	O	X	–
TNS-F-237278	A2186	Agaricales	Amanitaceae	*Amanita lutescens*	1956	O	X	–
TNS-F-237276	A2183	Agaricales	Amanitaceae	*Amanita citrina*	1956	O	X	–
TNS-F-237281	A2189	Agaricales	Amanitaceae	*Amanita pseudoporphyria*	1956	O	X	–
TNS-F-237479	A2296	Agaricales	Mycenaceae	*Mycena roseomarginata*	1956	O	X	–
TNS-F-237619	A2343	Agaricales	Tricholomataceae	*Tricholoma maculatipus*	1956	O	X	–
TNS-F-237359	A2275	Russulales	Russulaceae	*Lactarius circellatus*	1956	O	X	–
TNS-F-237229	A2228	Agaricales	Cortinariaceae	*Cortinarius shigaensis*	1956	O	O	uncultured ascomycetes
TNS-F-237321	A2245	Agaricales	Hygrophoraceae	*Hygrophorus cantharellus*	1956	O	O	uncultured ascomycetes
TNS-F-237385	A2288	Agaricales	Agaricaceae	*Leucocoprinus otsuensis*	1956	O	O	*Laccaria*
TNS-F-237494	A2316	Agaricales	Entolomataceae	*Rhodophyllus ater*	1956	O	O	uncultured ascomycetes
TNS-F-237301	A2220	Agaricales	Cortinariaceae	*Cortinarius aurantiofulvus*	1956	X	–	–
TNS-F-237284	A2201	Agaricales	Bolbitiaceae	*Bolbitius incarnatus*	1957	O	X	–
TNS-F-237367	A2282	Agaricales	Agaricaceae	*Lepiota aurantioflava*	1957	O	X	–
TNS-F-237625	A2345	Agaricales	Tricholomataceae	*Tricholomopsis bambusina*	1957	O	X	–
TNS-F-237309	A2231	Agaricales	Cortinariaceae	*Cortinarius subdelibutus*	1957	O	O	uncultured ascomycetes
TNS-F-237324	A2248	Agaricales	Hygrophoraceae	*Hygrophorus cruentus*	1957	O	O	uncultured ascomycetes
TNS-F-237352	A2267	Agaricales	Inocybaceae	*Inocybe macrosperma*	1957	O	O	uncultured ascomycetes
TNS-F-237492	A2312	Agaricales	Hymenogastraceae	*Psilocybe subcaerulipes*	1957	O	O	*Laccaria*
TNS-F-237332	A2256	Agaricales	Hygrophoraceae	*Hygrophorus pantoleucus*	1957	X	–	–
TNS-F-237351	A2266	Agaricales	Inocybaceae	*Inocybe kobayasii*	1957	X	–	–
TNS-F-237365	A2280	Agaricales	Agaricaceae	*Lepiota alborubescens*	1958	O	X	–
TNS-F-237368	A2283	Agaricales	Agaricaceae	*Lepiota cinnamomea*	1958	O	X	–
TNS-F-237495	A2317	Agaricales	Entolomataceae	*Rhodophyllus babingtonii*	1958	O	X	–
TNS-F-237629	A2347	Agaricales	Tricholomataceae	*Tricholomopsis sasae*	1958	O	X	–
TNS-F-237322	A2246	Agaricales	Hygrophoraceae	*Hygrophorus conicus*	1958	O	O	uncultured ascomycetes
TNS-F-237366	A2281	Agaricales	Agaricaceae	*Lepiota atrosquamulosa*	1958	O	O	*Laccaria*
TNS-F-237487	A2305	Boletales	Boletaceae	*Porphyrellus subvirens*	1958	O	O	*Laccaria*
TNS-F-237337	A2259	Agaricales	Hygrophoraceae	*Hygrophorus subcinnabarinus*	1958	X	–	–
TNS-F-237354	A2269	Agaricales	Inocybaceae	*Inocybe subvolvata*	1958	X	–	–
TNS-F-237277	A2184	Agaricales	Amanitaceae	*Amanita griseofarinosa*	1959	O	X	–
TNS-F-237508	A2323	Agaricales	Entolomataceae	*Rhodophyllus omiensis*	1960	O	X	–
TNS-F-237303	A2222	Agaricales	Cortinariaceae	*Cortinarius cinnamomeoides*	1960	X	–	–
TNS-F-237329	A2253	Agaricales	Hygrophoraceae	*Hygrophorus lilacinogriseus*	1960	X	–	–
TNS-F-237645	A2270	Agaricales	Inocybaceae	*Inocybe subvolvata*	1960	X	–	–
TNS-F-237647	A2349	Boletales	Boletaceae	*Tylopilus areolatus*	1961	O	X	–
TNS-F-237641	A2361	Boletales	Boletaceae	*Xerocomus nigromaculatus*	1961	O	X	–
TNS-F-237310	A2232	Agaricales	Cortinariaceae	*Cortinarius watamukiensis*	1961	O	O	uncultured ascomycetes
TNS-F-237481	A2298	Agaricales	Strophariaceae	*Naematoloma gracile*	1961	O	O	*Laccaria*
TNS-F-237338	A2260	Agaricales	Hygrophoraceae	*Hygrophorus suzukaensis*	1961	X	–	–
TNS-F-237353	A2268	Agaricales	Inocybaceae	*Inocybe quercina*	1961	X	–	–
TNS-F-237637	A2356	Boletales	Boletaceae	*Tylopilus otsuensis*	1962	O	X	–

Table 2. Continued

Herbarium Voucher Nos.	DNA Voucher Nos.*1	Order	Family	Taxon names in the original label*2	Year collected	PCR*3	Sequencing*4	BLAST results*5
TNS-F-237652	A2363	Boletales	Boletaceae	*Xerocomus parvulus*	1962	O	X	–
TNS-F-237525	A2335	Russulales	Russulaceae	*Russula virescens*	1962	O	X	–
TNS-F-193006	A2235	Agaricales	Mycenaceae	*Dictyopanus orientalis*	1962	O	O	uncultured ascomycetes
TNS-F-174776	A2355	Boletales	Boletaceae	*Tylopilus otsuensis*	1962	O	O	*Laccaria*
TNS-F-237350	A2265	Agaricales	Inocybaceae	*Inocybe kasugayamensis*	1962	X	–	–
TNS-F-237362	A2276	Russulales	Russulaceae	*Lactarius subpiperatus*	1963	O	X	–
TNS-F-237231	A2230	Agaricales	Cortinariaceae	*Cortinarius subarmillatus*	1963	O	O	uncultured ascomycetes
TNS-F-237382	A2293	Agaricales	Marasmiaceae	*Marasmius aurantioferrugineus*	1963	O	O	*Rhodosporidium*
TNS-F-237297	A2219	Agaricales	Psathyrellaceae	*Coprinus aokii*	1963	O	–	–
TNS-F-237355	A2273	Agaricales	Strophariaceae	*Kuehneromyces castaneus*	1963	X	–	–
TNS-F-237557	A2337	Agaricales	Strophariaceae	*Stropharia aeruginosa*	1964	O	X	–
TNS-F-237558	A2338	Agaricales	Strophariaceae	*Stropharia aeruginosa*	1964	O	X	–
TNS-F-174775	A2365	Boletales	Boletaceae	*Xerocomus nigromaculatus*	1964	O	X	–
TNS-F-237228	A2224	Agaricales	Cortinariaceae	*Cortinarius galeroides*	1964	X	–	–
TNS-F-244373	A2191	Agaricales	Amanitaceae	*Amanita rufoferruginea*	1965	O	X	–
TNS-F-237348	A2264	Agaricales	Inocybaceae	*Inocybe atroumbonata*	1965	O	X	–
TNS-F-237330	A2254	Agaricales	Hygrophoraceae	*Hygrophorus olivaceoviridis*	1965	X	–	–
TNS-F-237489	A2308	Agaricales	Bolbitiaceae	*Pseudoconocybe nodulosospora*	1966	O	X	–
TNS-F-174771	A2354	Boletales	Boletaceae	*Tylopilus neofelleus*	1966	O	O	*Laccaria*
TNS-F-237305	A2225	Agaricales	Cortinariaceae	*Cortinarius neoarmillatus*	1966	X	–	–
TNS-F-237331	A2255	Agaricales	Hygrophoraceae	*Hygrophorus olivaceoviridis*	1966	X	–	–
TNS-F-237287	A2207	Boletales	Boletaceae	*Boletus griseus*	1966	X	–	–
TNS-F-237288	A2209	Boletales	Boletaceae	*Boletus laetissimus*	1966	X	–	–
TNS-F-237289	A2210	Boletales	Boletaceae	*Boletus obscureumbrinus*	1966	X	–	–
TNS-F-244377	A2192	Agaricales	Amanitaceae	*Amanita sphaerobulbosa*	1967	O	X	–
TNS-F-237510	A2326	Russulales	Russulaceae	*Russula bella*	1967	O	O	*Laccaria*
TNS-F-237226	A2205	Agaricales	Amanitaceae	*Amanita neoovoidea*	1967	X	–	–
TNS-F-237293	A2214	Boletales	Boletaceae	*Boletus umbiriniporus*	1967	X	–	–
TNS-F-237655	A2213	Boletales	Boletaceae	*Boletus subfuscus*	1967	X	–	–
TNS-F-237372	A2285	Agaricales	Agaricaceae	*Lepiota neomastoidea*	1968	O	X	–
TNS-F-237504	A2320	Agaricales	Entolomataceae	*Rhodophyllus kansaiensis*	1968	O	X	–
TNS-F-237651	A2348	Boletales	Boletaceae	*Tylopilus alutaceoumbrinus*	1968	O	X	–
TNS-F-50254	A2315	Gomphales	Gomphaceae	*Ramaria zippelii*	1968	O	X	–
TNS-F-237306	A2226	Agaricales	Cortinariaceae	*Cortinarius nigrosquamosus*	1968	O	O	uncultured ascomycetes
TNS-F-237323	A2247	Agaricales	Hygrophoraceae	*Hygrophorus croceoluteus*	1968	O	O	uncultured ascomycetes
TNS-F-237274	A2181	Agaricales	Amanitaceae	*Amanita alboflavescens*	1969	X	–	–
TNS-F-237488	A2307	Agaricales	Psathyrellaceae	*Psathyrella pennata*	1969	O	X	–
TNS-F-234519	A2196	Auriculariales	Auriculariaceae	*Auricularia fibrillifera*	1969	O	X	–
TNS-F-172603	A2353	Boletales	Boletaceae	*Tylopilus hongoi*	1969	O	X	–
TNS-F-237283	A2193	Agaricales	Amanitaceae	*Amanita sychnopyramis*	1970	O	X	–
TNS-F-244379	A2208	Agaricales	Amanitaceae	*Amanita pseudogemmata*	1970	X	–	–
TNS-F-237302	A2221	Agaricales	Cortinariaceae	*Cortinarius aureobrunneus*	1970	X	–	–
TNS-F-237290	A2211	Boletales	Boletaceae	*Boletus pseudocalopus*	1970	X	–	–
TNS-F-228004	A2194	Agaricales	Physalacriaceae	*Armillariella fellea*	1971	O	X	–
TNS-F-237377	A2289	Agaricales	Agaricaceae	*Leucocoprinus subglobisporus*	1971	O	X	–
TNS-F-228005	A2295	Agaricales	Omphalotaceae	*Micromphale pacificum*	1971	O	X	–
TNS-F-228018	A2299	Agaricales	Strophariaceae	*Naematoloma papuanum*	1971	O	X	–
TNS-F-176271	A2304	Agaricales	Mycenaceae	*Poromycena rubra*	1971	O	X	–
TNS-F-228000	A2346	Agaricales	Tricholomataceae	*Tricholomopsis elata*	1971	O	X	–
TNS-F-225511	A2195	Boletales	Boletaceae	*Aureoboletus novoguineensis*	1971	O	X	–
TNS-F-228299	A2301	Boletales	Boletaceae	*Boletus oksapminensis*	1971	O	X	–
TNS-F-228001	A2279	Polyporales	Polyporaceae	*Lentinus papuanus*	1971	O	X	–
TNS-F-225521	A2274	Russulales	Russulaceae	*Lactarius austrovolemus*	1971	O	X	–
TNS-F-228002	A2300	Polyporales	Polyporaceae	*Panus verruciceps*	1971	O	O	*Laccaria*
TNS-F-225479	A2328	Russulales	Russulaceae	*Russula eburneoareolata*	1971	O	O	*Laccaria*
TNS-F-237513	A2327	Russulales	Russulaceae	*Russula castanopsidis*	1971	O	O	*Laccaria*
TNS-F-228066	A2241	Agaricales	Strophariaceae	*Gymnopilus novoguineensis*	1971	X	–	–
TNS-F-225485	A2203	Boletales	Boletaceae	*Boletus erythropus*	1971	X	–	–
TNS-F-237275	A2182	Agaricales	Amanitaceae	*Amanita castanopsidis*	1972	X	–	–
TNS-F-237364	A2277	Boletales	Boletaceae	*Leccinum subradicatum*	1972	O	O	uncultured ascomycetes
TNS-F-237503	A2319	Agaricales	Entolomataceae	*Rhodophyllus kansaiensis*	1973	O	X	–
TNS-F-237313	A2234	Agaricales	Agaricaceae	*Cystoderma neoamianthinum*	1973	O	O	uncultured ascomycetes
TNS-F-237314	A2238	Agaricales	Hymenogastraceae	*Galerina fasciculata*	1973	X	–	–
TNS-F-182277	A2212	Boletales	Boletaceae	"*Boletus subcinnamomeus*"	1973	X	–	–
TNS-F-228435	A2310	Agaricales	Hymenogastraceae	*Psilocybe argentipes*	1975	O	X	–
TNS-F-174773	A2360	Boletales	Boletaceae	*Tylopilus vinosobrunneus*	1975	O	X	–
TNS-F-237648	A2352	Boletales	Boletaceae	*Tylopilus eximus*	1975	O	X	–
TNS-F-237285	A2206	Boletales	Boletaceae	*Boletus fuscopunctatus*	1975	X	–	–
TNS-F-198224	A2187	Agaricales	Amanitaceae	*Amanita miculifera*	1976	O	X	–
TNS-F-198225	A2188	Agaricales	Amanitaceae	*Amanita miculifera*	1976	O	X	–

Table 2. Continued

Herbarium Voucher Nos.	DNA Voucher Nos.*1	Order	Family	Taxon names in the original label*2	Year collected	PCR*3	Sequencing*4	BLAST results*5
TNS-F-237633	A2359	Boletales	Boletaceae	*Tylopilus ruguloso-reticulatus*	1976	O	X	–
TNS-F-237326	A2250	Agaricales	Hygrophoraceae	*Hygrophorus hahashimensis*	1977	O	X	–
TNS-F-237653	A2362	Boletales	Boletaceae	*Xerocomus obscurebrunneus*	1977	O	X	–
TNS-F-237657	A2364	Boletales	Boletaceae	*Xerocomus subcinnamomeus*	1977	O	X	–
TNS-F-237511	A2324	Russulales	Russulaceae	*Russula alboareolata*	1977	O	X	–
TNS-F-237311	A2233	Agaricales	Inocybaceae	*Crepidotus roseus*	1977	O	O	uncultured ascomycetes
TNS-F-237378	A2290	Agaricales	Amanitaceae	*Limacella olivaceobrunnea*	1977	O	O	*Laccaria*
TNS-F-174748	A2258	Agaricales	Hygrophoraceae	*Hygrophorus subacutus*	1977	X	–	–
TNS-F-237327	A2251	Agaricales	Hygrophoraceae	*Hygrophorus hypohaemactus*	1977	X	–	–
TNS-F-237505	A2321	Agaricales	Entolomataceae	*Rhodophyllus kujuensis*	1978	O	X	–
TNS-F-174772	A2358	Boletales	Boletaceae	*Tylopilus ruguloso-reticulatus*	1978	O	X	–
TNS-F-174774	A2357	Boletales	Boletaceae	*Tylopilus rigens*	1978	O	X	–
TNS-F-237520	A2331	Russulales	Russulaceae	*Russula neoemetica*	1978	O	X	–
TNS-F-237512	A2325	Russulales	Russulaceae	*Russula alboareolata*	1978	O	O	*Laccaria*
TNS-F-237282	A2190	Agaricales	Amanitaceae	*Amanita pseudovaginata*	1980	O	X	–
TNS-F-237650	A2202	Boletales	Boletaceae	*Boletus aokii*	1980	X	–	–
TNS-F-237646	A2350	Boletales	Boletaceae	*Tylopilus argillaceus*	1981	O	X	–
TNS-F-182343	A2242	Agaricales	Hymenogastraceae	*Hebeloma radicosoides*	1983	O	X	–
TNS-F-237654	A2351	Boletales	Boletaceae	*Tylopilus castaneiceps*	1983	O	X	–
TNS-F-237506	A2322	Agaricales	Entolomataceae	*Rhodophyllus kujuensis*	1983	O	O	*Laccaria*
TNS-F-237562	A2342	Agaricales	Tricholomataceae	*Tricholoma aurantiipes*	1985	O	X	–
TNS-F-237381	A2292	Agaricales	Lyophyllaceae	*Lyophyllum sykosporum*	1986	O	X	–
TNS-F-237316	A2243	Agaricales	Hygrophoraceae	*Hygrocybe miniatoaurantiaca*	1987	O	O	uncultured ascomycetes
TNS-F-237670	A2180	Agaricales	Hymenogastraceae	*Alnicola lactariolens*	1988	O	X	–
TNS-F-200842	A3009	Polyporales	Polyporaceae	*Polyporus tuberaster*	1992	O	X	–
TNS-F-171953	A2240	Boletales	Boletaceae	*Gastroboletus doii*	1992	X	–	–

*1 DNA sampes are stored under the DNA voucher numbers at Department of Botany, National Museum of Nature and Science, Tsukuba, Japan.
*2 Species names with uncertain nomenclatural status were indicated with quotation marks.
*3 PCR amplification was condidered "successful" with clear single band (O) or "failed" with no bands or smears (X).
*4 Direct sequencing resulted in clear, readily editable sequences was considered "good" (O). Sequences were considered low quality or "messy" with no discernable peaks (X). Sequences with hetero-peaks are indicated with "H".
*5 The results of the top hit are shown.

troboletus doii (Fig. 1D) were not amplified (PCR amplification was negative for both cases). On the other hand, some specimens without visible molds on the surface also resulted in "apparent contamination" (Table 1, 2) using BLAST. Many ascomycetous molds can secondarily colonize on mushroom specimens and old specimens have much higher risk of such contamination. When dealing with old specimens, visual inspection of specimens by stereo microscope and PCR amplification using basidiomycetes specific primers can minimize the risk of contamination, but we always need to recognize that old specimens are always contaminated by other (micro-) organisms by certain extent.

Such "apparent contamination" was caused not only by ascomycetes, but also by other basidiomycetous mushrooms. For example, the sequence obtained from *Amanita aestivalis* F-1099363 (Amanitaceae) matched with *Pholiota* (Strophariaceae) (Table 3). Similarly, the sequence from *Leccinum albellum* F-1015573 (Boletales) matched with *Skeletocutis* (Polyporales) (Table 3). It is noteworthy that the sequences from various TNS specimens matched with unrelated mushroom *Laccaria* (Hydnangiaceae) (Table 2). During this study, the project on *Laccaria* phylogeography was on-going at the same time, and a large number of *Laccaria* specimens were processed in the same molecular lab. I believe "apparent contamination" of *Laccaria* was partially caused by genomic DNA accidentally mixed in some reagents commonly used in our molecular lab. It should be emphasized that no such *Laccaria* contamination has been detected in any other studies conducted in our molecular lab ever since.

Although the contamination by ascomycetes tends to be more frequent, mushroom contamination by other mushrooms is also widespread as shown here. Such contamination at molecular lab can be avoided by always using fresh reagents,

Table 3. Type specimens tested for this study housed at Field Museum of Natural History (F) with the results of PCR and sequencing

Herbarium Voucher Nos.	DNA Voucher Nos.*1	Order	Family	Taxon names in the original label*2	Year collected	PCR*3	Sequencing*4	BLAST results*5
F-1037060	A2771	Russulales	Russulaceae	*Lactarius mamorensis*	1882	O	X	–
F-332202	A2768	Boletales	Boletaceae	*Boletus debeauxii*	1884	O	X	–
F-1295888	A2785	Agaricales	Agaricaceae	*Lepiota denudata*	1892	X	–	–
F-1174531	A2739	Agaricales	Agaricaceae	*Lepiota naucinoides*	1893	O	X	–
F-1247332	A2760	Russulales	Russulaceae	*Lactarius rubrifulvus*	1904	X	–	–
F-497510	A2795	Agaricales	Amanitaceae	*Amanita protecta*	1920	O	X	–
F-1001353	A2777	Russulales	Stereaceae	*Aleurodiscus scutellatus*	1926	X	–	–
F-1001210	A2764	Russulales	Russulaceae	*Arcangeliella scissilis*	1933	X	–	–
F-1201600	A2737	Agaricales	Agaricaceae	*Lepiota fischeri*	1938	O	X	–
F-1152706	A2784	Agaricales	Agaricaceae	*Lepiota cystidiosa*	1940	O	O	*Cystolepiota cystidiosa*
F-1001332	A2736	Agaricales	Marasmiaceae	*Lactocollybia angiospermarum*	1942	O	X	–
F-1330205	A2782	Agaricales	Agaricaceae	*Lepiota aurora*	1948	O	X	–
F-1330212	A2790	Agaricales	Amanitaceae	*Amanita tenuifolia*	1948	O	X	–
F-1099363	A2747	Agaricales	Amanitaceae	*Amanita aestivalis*	1953	O	O	*Pholiota*
F-1020987	A2767	Boletales	Boletaceae	*Boletus guadelupae*	1957	O	O	*Boletus erythropus*
F-1037071	A2772	Russulales	Russulaceae	*Lactarius paulensis*	1964	X	–	–
F-1014447	A2786	Agaricales	Agaricaceae	*Lepiota xanthophylloides*	1966	X	–	–
F-1016880	A2773	Agaricales	Hydnangiaceae	"*Laccaria carbonophila*"	1967	O	O	*Laccaria*
F-1013501	A2794	Agaricales	Hygrophoraceae	*Botrydina lobata*	1968	O	H	–
F-1015825	A2765	Boletales	Boletaceae	*Boletus fuligineotomentosus*	1968	O	X	–
F-1018786	A2758	Agaricales	Hydnangiaceae	*Laccaria laccata var. vulcanica*	1968	O	O	*Laccaria*
F-1080297	A2799	Boletales	Boletaceae	"*Boletus ixoflavus*"	1969	X	–	–
F-1080296	A2803	Boletales	Boletaceae	*Boletus subluridellus*	1969	O	H	–
F-1018284	A2734	Boletales	Boletaceae	*Boletus michoacanus*	1969	O	X	–
F-1015573	A2759	Boletales	Boletaceae	*Leccinum albellum*	1969	O	O	*Skeletocutis*
F-1016998	A2801	Agaricales	Hydnangiaceae	"*Laccaria moelleri*"	1970	O	O	*Laccaria*
F-1017120	A2743	Agaricales	Hydnangiaceae	*Laccaria pussilla*	1970	O	O	*Laccaria*
F-1015677	A2752	Agaricales	Strophariaceae	*Agrocybe amara*	1970	O	O	*Agrocybe praecox*
F-1016843	A2762	Agaricales	Hydnangiaceae	*Laccaria montana*	1971	X	–	–
F-1018209	A2738	Agaricales	Bolbitiaceae	*Bolbitius mesosporus*	1973	X	–	–
F-1019126	A2774	Agaricales	Hydnangiaceae	*Laccaria laccata*	1974	X	–	–
F-1018595	A2778	Agaricales	Hydnangiaceae	*Laccaria laccata var. pusilla*	1974	O	X	–
F-1021123	A2757	Agaricales	Hydnangiaceae	*Laccaria laccata var. tatrensis*	1974	O	O	*Laccaria*
F-1030847	A2766	Agaricales	Bolbitiaceae	*Boletellus sp.*	1976	O	X	–
F-1030894	A2791	Boletales	Boletaceae	*Boletellus ananas var. crassotunicatus*	1976	O	X	–
F-1037055	A2749	Russulales	Russulaceae	*Lactarius amazonensis*	1977	O	H	–
F-1097232	A2754	Boletales	Boletaceae	*Boletellus ananas*	1978	O	X	–
F-1037054	A2761	Russulales	Russulaceae	*Lactarius subpallidipes*	1978	O	X	–
F-1031387	A2742	Agaricales	Physalacriaceae	*Armillariella viridiflava*	1979	X	–	–
F-1030749	A2770	Russulales	Russulaceae	*Lactarius igapoensis*	1980	O	X	–
F-1059109	A2744	Agaricales	Hydnangiaceae	"*Laccaria kauaiensis*"	1981	X	–	–
F-1036989	A2781	Polyporales	Polyporaceae	*Lentinus prancei*	1981	O	H	–
F-1037041	A2792	Russulales	Russulaceae	*Lactarius costaricensis*	1981	O	O	*Lactarius fumosus*
F-1052251	A2769	Russulales	Russulaceae	*Lactarius guanacastensis*	1982	X	–	–
F-1052236	A2800	Boletales	Boletaceae	*Boletellus ananas*	1982	X	–	–
F-1051926	A2793	Agaricales	Hydnangiaceae	*Laccaria gomezii*	1982	O	O	*Laccaria*
F-1073280	A2740	Agaricales	Amanitaceae	*Amanita flavoconia*	1983	O	X	–
F-1053721	A2796	Agaricales	Amanitaceae	*Amanita nauseosa*	1983	O	X	–
F-1073299	A2763	Boletales	Boletaceae	*Boletus heterodermus*	1983	O	X	–
F-1160662	A2751	Agaricales	Amanitaceae	*Agaricus pleropus*	1984	X	–	–
F-1091979	A2735	Agaricales	Hydnangiaceae	*Laccaria longipes*	1984	O	X	–
F-1315642	A2802	Agaricales	Agaricaceae	*Agaricus yucatanensis*	1985	O	X	–
F-1068563	A2797	Agaricales	Physalacriaceae	*Armillariella affinis*	1986	O	H	–
F-1074629	A2745	Boletales	Boletaceae	*Leccinum andinum*	1986	O	O	*Boletus bicolor*
F-1075916	A2798	Boletales	Paxillaceae	*Austrogaster marthae*	1988	X	–	–
F-1097223	A2787	Boletales	Boletaceae	*Laccinum roseoscabrum*	1988	O	O	*Leccinum talamancae*
F-1100021	A2741	Boletales	Boletaceae	"*Boletus pini-oocarpae*"	1991	O	O	*Boletus subvelutipes*
F-1097225	A2746	Boletales	Boletaceae	*Leccinum roseoscabrum*	1991	O	O	*Leccinum talamancae*

*1 DNA sampes are stored under the DNA voucher numbers at Department of Botany, National Museum of Nature and Science, Tsukuba, Japan.
*2 Species names with uncertain nomenclatural status were indicated with quotation marks.
*3 PCR amplification was condidered "successful" with clear single band (O) or "failed" with no bands or smears (X).
*4 Direct sequencing resulted in clear, readily editable sequences was considered "good" (O). Sequences were considered low quality or "messy" with no discernable peaks (X). Sequences with hetero-peaks are indicated with "H".
*5 The results of the top hit are shown.

Fig. 1. Some representatives of the type specimens tested in this study.
A. "*Ganoderma spathulatum*" (TNS-F-201590) from 1891. B. *Boletus hiratsukae* (TNS-F-180124) from 1896. C. *Cystoderma neoamianthinum* (TNS-F-237313) from 1973. D. *Gastroboletus doii* (TNS-F-171953) from 1992. E. *Lactarius mamorensis* (F-1037060) from 1882. F. *Lepiota denudata* (F-1295888) from 1892. G. *Lepiota cystidiosa* (F-1152706) from 1940. H. *Agrocybe amara* (F-1015677) from 1970. Bars = 2.5 cm.

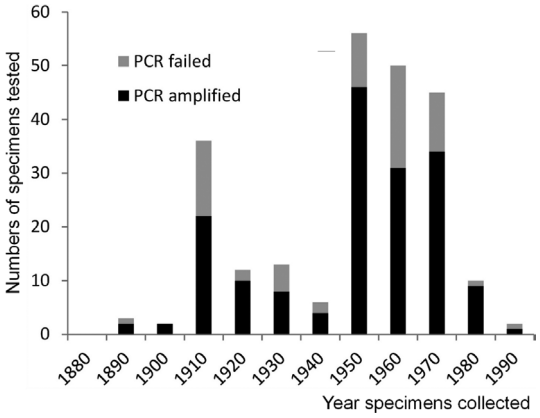

Fig. 2. Success rate of PCR amplification from the specimens stored at TNS herbarium.

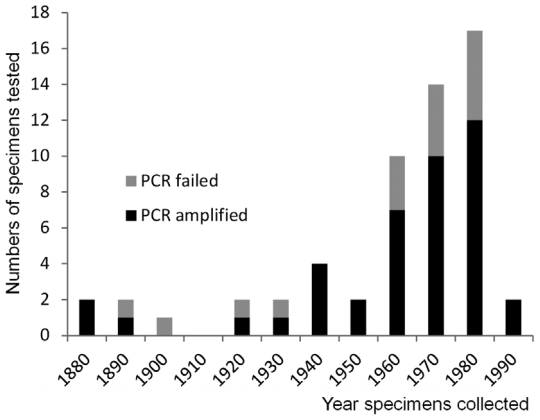

Fig. 3. Success rate of PCR amplification from the specimens stored at F herbarium.

but the contamination in the herbarium (e.g., mushroom specimens covered with spores of other mushrooms) is usually unavoidable. Researchers attempting to obtain DNA from old mushroom specimens should always be careful when interpreting the sequence data.

PCR amplification from more than 50% of specimens housed at TNS and F herbaria was successful (Table 1, Fig. 2, 3). Some specimens of more than 50 year old also had positive amplification (Fig. 2, 3). Although all successfully obtained sequences from TNS specimens were demonstrated to be from "apparent contamination", the majority of direct sequencing was unsuccessful due to low quality (114) and hetero-peaks (12), which may contain authentic sequences (Table 1). Similarly, out of 42 PCR products obtained from F specimens, only 14 produced authentic sequences and the remaining ones (21 low quality sequences and 5 hetero-peaks) may contain authentic sequences as well (Table 1). This study is an initial screening of old type specimens and the next obvious step is to use taxon-specific primers and/or to sequence by cloning.

It is well documented from the previous studies that many specimens at TNS have very fragmented DNA due to fumigation (Hosaka and Uno, 2013). Most specimens of 30 year old or older have been fumigated by methyl bromide and the average lengths of DNA fragments were demonstrated to be 150 bp. or shorter (Hosaka and Uno, 2013). More recently, TNS herbarium has been fumigated by sulphuryl fluoride, which is claimed not to affect DNA molecules (Kigawa *et al.*, 2003; Whitten *et al.*, 1999). Although some positive PCR products with "failed" sequencing may contain authentic sequences, the possibility of obtaining ca. 600 bp. sequences using the primers ITS5 and ITS4 (White *et al.*, 1990) is apparently low. The clear contrast in success rate of obtaining authentic sequences between TNS (0) and F (14) herbaria indicates that the effect of fumigation is not trivial. Although no detailed records of fumigation at F herbarium are currently available, the results of this study indicate that major fungal herbaria in the USA and Japan may have different fumigation history.

It is obvious that DNA in specimens degrades with time (Erkens *et al.*, 2008; Willerslev and Cooper, 2005). The current study shows that no authentic sequences were obtained from the specimens collected in 1930's or before (Fig. 4). The oldest specimen from which we could PCR amplify the ITS region (ca. 600 bp.) and successfully sequence was from 1940 (*Lepiota cystidiosa* F-1152706) (Table 3, Fig. 1G, 4). Although other factors such as humidity, temperature and fumigation also affect the quality of DNA (Willerslev and Cooper, 2005), this study clearly dem-

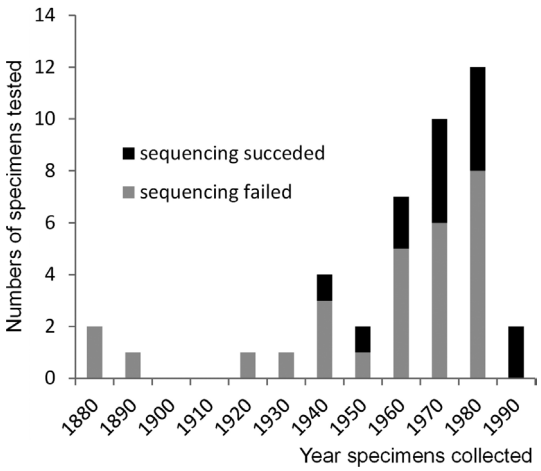

Fig. 4. Success rate of direct sequencing from the specimens stored at F herbarium.
Sequencing reactions producing "authentic" sequences were scored as success, and all the other sequences (apparent contamination, hetero-peaks, and "messy" sequences) were scored as failed.

onstrates the need of immediate DNA extraction from historically/taxonomically important specimens, i.e., type specimens. Historical records of fumigation in major fungal herbaria should be investigated for higher success rate of PCR and sequencing from such specimens. Alternatively, as suggested by Hosaka and Uno (2013), small pieces of type specimens should be preserved in deep-freezer, organic solvent (Fukatsu, 1999) or DNA preservation buffer (Dawson *et al.*, 1998; Laulier *et al.*, 1995; Nagy, 2010) for future molecular studies.

For future studies, the protocol for each step of DNA extraction, PCR and sequencing need to be re-examined. For example, fragmented DNA molecules are known to less effectively bind to silica particles (glass milk), but the binding efficiency can be improved by lowering the pH of the binding buffer (GENECLEAN Kit®, MP Biomedicals, Solon, OH, USA; protocol available from https://www.funakoshi.co.jp/data/datasheet/GEN/1001-200.pdf). The PCR efficiency can certainly be improved by designing taxon-specific primers and targeting shorter regions of DNA. As noted above, some "failed" sequences in this study may contain authentic sequences, which can be obtained by cloning.

As initially expected, an attempt to obtain sequence data from the type specimens, especially from old specimens, faced a lot of challenges. As long as the fungal nomenclature is based on the principle of typification, however, further attempts should be made to obtain "authentic" sequence data from most, if not all, type specimens. If no such attempts are successful or type specimens are apparently absent, epitypification from more recently collected specimens accompanied with good sequence data should be conducted (Hyde and Zhang, 2008).

Acknowledgments

The author thanks Kunihiko Uno and Kyung-Ok Nam for assisting my molecular work. I also thank Dr. Robert Luecking and other herbarium staffs for facilitating the loan of the type specimens from Field Museum of Natural History. This study was financed in part by a JSPS Grant-in-Aid for Young Scientists (A) (no. 24680085) and JSPS Grant-in-Aid for Scientific Research (B) (no. 40414362).

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. 1990. Basic local alignment search tool. Journal of Molecular Biology 215: 403–410.

Brock, P. M., Döring, H. and Bidartondo, M. I. 2008. How to know unknown fungi: the role of a herbarium. New Phytologist 181: 719–724.

Dawson, M. N., Raskoff, K. A. and Jacobs, D. K. 1998. Field preservation of marine invertebrate tissue for DNA analyses. Molecular Marine Biology and Biotechnology 7: 145–152.

Dentinger, B. T. M., Margaritescu, S. and Moncalvo, J.-M. 2010. Rapid and reliable high-throughput methods of DNA extraction for use in barcoding and molecular systematics of mushrooms. Molecular Ecology Resources 10: 628–633.

Doyle, J. J. and Doyle, J. L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bulletin 19: 11–15.

Erkens, R. H. J., Cross, H., Maas, J. W., Hoenselaar, K. and Chatrou, L. W. 2008. Assessment of age and green-

ness of herbarium specimens as predictors for successful extraction and amplification of DNA. Blumea 53: 407–428.

Fukatsu, T. 1999. Acetone preservation: a practical technique for molecular analysis. Molecular Ecology 8: 1935–1945.

Hosaka, K. 2009. Phylogeography of the genus *Pisolithus* revisited with some additional taxa from New Caledonia and Japan. Bulletin of the National Museum of Nature and Science, Series B 35: 151–167.

Hosaka, K. 2015. Successful PCR amplification of Bacteria from mushroom fruit bodies. Bulletin of the National Museum of Nature and Science, Series B 41: 1–7.

Hosaka, K. and Castellano, M. A. 2008. Molecular phylogenetics of Geastrales with special emphasis on the position of *Sclerogaster*. Bulletin of the National Museum of Nature and Science, Series B 34: 161–173.

Hosaka, K. and Uno, K. 2013. Assessment of the DNA quality in mushroom specimens: a recovery of the whole ITS sequence from fragmented DNA of the type specimen. Bulletin of the National Museum of Nature and Science, Series B 39: 53–60.

Hyde, K. D. and Zhang, Y. 2008. Epitypification: should we epitypify? Journal of Zhejiang University Science B 9: 842–846.

Kasuya, T., Hosaka, K., Uno, K. and Kakishima, M. 2012. Phylogenetic placement of *Geastrum melanocephalum* and polyphyly of *Geastrum triplex*. Mycoscience 53: 411–426.

Katsumoto, K. 2010. List of fungi recorded in Japan. The Kanto Branch of Mycological Society of Japan, Funabashi, Chiba, Japan.

Kigawa, R., Nochide, H., Kimura, H. and Miura, S. 2003. Effects of various fumigants, thermal methods and carbon dioxide treatment on DNA extraction and amplification: a case study on freeze-dried mushroom and freeze-dried muscle specimens. Collection Forum 18: 74–89.

Laulier, M., Pradier, E., Bigot, Y. and Périquet, G. 1995. An easy method for preserving nucleic acids in field samples for later molecular and genetic studies without refrigerating. Journal of Evolutionary Biology 8: 657–663.

Nagy, Z. T. 2010. A hands-on overview of tissue preservation methods for molecular genetic analyses. Organisms Diversity & Evolution 10: 91–105.

Nagy, L. G., Petkovits, T., Kovács, G. M., Voigt, K., Vágvölgyi, C. and Papp, T. 2011. Where is the unseen fungal diversity hidden? A study of *Mortierella* reveals a large contribution of reference collections to the identification of fungal environmental sequences. New Phytologist 191: 789–794.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W. and Fungal Barcoding Consortium. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Sciences of the United States of America 109: 6241–6246.

Seutin, G., White, B. N. and Boag, P. T. 1991. Preservation of avian blood and tissue samples for DNA analyses. Canadian Journal of Zoology 69: 82–90.

White, T. J., Bruns, T., Lee, S. and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. (eds.), PCR protocols, pp. 315–322, Academic Press, New York.

Whitten, W. M., Williams, N. H. and Glover, K. V. 1999. Sulphuryl fluoride fumigation: effect on DNA extraction and amplification from herbarium specimens. Taxon 48: 507–510.

Willerslev, E. and Cooper, A. 2005. Ancient DNA. Proceedings of the Royal Society B 272: 3–16.

Wilson, A. W., Hosaka, K. and Mueller, G. M. 2016. Evolution of ectomycorrhizas as a driver of diversification and biogeographic patterns in the model mycorrhizal mushroom genus *Laccaria*. New Phytologist 213: 1862–1873.