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Successful PCR Amplification of Bacteria from Mushroom Fruit Bodies

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Abstract Bacterial and archaeal DNA as well as fungal DNA was extracted and PCR amplified from mushroom fruit bodies of phylogenetically and ecologically diverse taxa. Out of 36 mushroom taxa examined, 35 produced positive PCR bands using bacterial/archaeal specific primers. No obvious contamination or unspecific amplification was detected. The study demonstrated that various bacterial taxa were consistently present in mushroom fruit bodies and more extensive studies need to be conducted for such relatively unexplored habitat.

Key words: Archaea, Ascomycota, bacteria, Basidiomycota, fruit bodies, metagenomics, symbiotic, 16S.

Introduction

Fungi of diverse groups produce fruit bodies of visible sizes, ranging from less than 1 mm to larger than 1 m. Fruit bodies produced by Agaricales (many gilled mushrooms) tend to be ephemeral, fruiting only several days per year, but "polypores" or "conks" (many belong to Polyporales, but Hymenochaetales and other groups also produce similar types of fruit bodies) produces perennial fruit bodies. Many animals, especially rodents and small marsupials, consume significant amount of mushroom fruit bodies as part of their diet (Castellano *et al.*, 1989). It is also shown that mushroom fruit bodies are important nutrient source for insects, as summarized by Hosaka and Uno (2012).

In addition to macro-organisms mentioned above, diverse micro-organisms utilize mushroom fruit bodies, but this phenomenon has relatively been unexplored. Recent studies have extensively investigated diversity, distribution and community structures of bacteria from various habitat, such as soil, water and insects (Fierer *et al.*, 2011; Fierer and Jackson, 2006; Nemergut *et al.*, 2010), but fungal substrates were largely neglected. The situation is not trivial because the estimated number of fungal species ranges from at least 600,000 (Mora *et al.*, 2011) to more than 5 million (Blackwell, 2011). This means that fungi may be the second largest kingdom on earth.

The patterns of global bacterial distribution are intriguing. In general, they do not show elevational and latitudinal gradients, which typically predict plant and animal diversity (Fierer and Jackson, 2006; Fierer *et al.*, 2011). Fungi, on the other hand, appear to be most species-rich in tropical rain forests, the same pattern observed in plant and animals (Tedersoo *et al.*, 2014). However, some major groups of fungi show unique patterns, i.e., most diverse in temperate forests or arctic biome (Tedersoo *et al.*, 2014). It is therefore critical to investigate bacterial diversity directly associated with fungi.

Recently, bacterial communities associated with lichens (Bates *et al.*, 2011), endophytes (Hoffman and Arnold, 2010), and hyphae of mycorrhizal fungi (Bertaux *et al.*, 2005) were investigated. It is noteworthy that some bacteria

seem to promote mycorrhizal formation (Schrey *et al.*, 2005). Among such studies, bacterial communities in mushroom fruit bodies have been surprisingly scarcely reported. The only extensively studied mushroom is the genus *Tuber* (Barbieri *et al.*, 2005), and the rest of diverse mushroom taxa remain uninvestigated.

This study aimed to confirm the presence of bacteria in mushroom fruit bodies from diverse Basidiomycota and Ascomycota. The specific primers for bacterial/archaeal 16S rDNA were used for PCR amplification of DNA samples which were extracted from internal tissues of mushroom fruit bodies.

Materials and Methods

Collecting sites, collecting scheme, and curation of specimens

Fieldwork was conducted by the author in November and December, 2013. The collecting sites were located in the Tsukuba Botanical Garden, Ibaraki, Japan (36°06′N, 140°06′E). The garden occupies ca.14 ha (36 acres), and has ca. 7000 taxa of domestic and exotic plants. It is divided into several sections simulating natural habitat, such as "Evergreen broad-leaved forest section", "Temperate coniferous forest section", and "Warm-temperate deciduous broad-leaved forest section".

A general collection scheme for mushroom-forming fungi was followed, as summarized by Hosaka and Uno (2012). At each collecting site, fruit bodies were collected and the specific location (section) in the Tsukuba Botanical Garden and habitat information were recorded. If presumable ectomycorrhizal or wood decomposing mushrooms were collected, potential ectomycorrhizal host trees or woody substrates near fruit bodies were identified to species. If alternative ectomycorrhizal hosts were present near fruit bodies, those were recorded as well.

Each specimen was photographed and macroscopic observation was conducted. All specimens were cut into half and dried with low heat and good air circulation, using a food dehydrator (Snackmaster Express FD-60, Nesco/American Harvest, WI, USA) for 24 to 48 hours. In addition to dried materials, small fragments of clean, internal tissue (e.g., gleba, pileus or stipe tissue) from freshly collected samples were cut using a clean, sterile razor blade. Contamination of visible soil particles and other materials was strictly avoided. The tissue fragments were soaked in DMSO buffer (Seutin *et al.*, 1991) with an addition of 100 mM Tris-HCl (pH 8.0) and 0.1 M sodium sulfite (Na_2SO_3) under 4°C, following the procedures of Hosaka (2009) and Hosaka and Castellano (2008).

Specimens collected during the fieldwork were deposited at the fungal herbarium of the National Museum of Nature and Science, Tsukuba, Japan (TNS). All tissue samples were stored in freezers (−80°C) at the Center for Molecular Biodiversity Research, National Museum of Nature and Science.

DNA preparation, PCR, and sequencing

DNA was extracted from the tissue fragments stored in DMSO buffer. Tissues were ground under liquid nitrogen using a mortar and pestle. DNA extractions used a modified CTAB extraction followed by glass milk purification methods as summarized by Hosaka (2009) and Hosaka and Castellano (2008).

DNA sequence data of mushrooms were obtained from the internal transcribed spacer region (ITS) of the nuclear ribosomal DNA. For amplifying the ITS, the primer combination of ITS5 and ITS4 (White *et al.*, 1990) was used. 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). PCR products were electrophoresed in 1% agarose gels stained with ethidium bromide and visualized under UV light. When amplification bands were confirmed, PCR products were then purified using the ExoSap-IT (Millipore, Molsheim, France) and directly sequenced using the Big Dye Terminator Cycle

Table 1. Mushroom samples investigated for presence/absence of bacteria in this study

Sample Nos.*	Herbarium Voucher Nos.	Order	Family	*Lentinula*	Collected on: (year/month/day)
1	TNS-F-59072	Agaricales	Omphalotaceae	*Lentinus edodes*	2013/11/6
2	TNS-F-59074	Agaricales	Agaricaceae	*Agaricus* sp.	2013/11/6
3	TNS-F-59076	Agaricales	Entolomataceae	*Entoloma rhodopolium*	2013/11/6
4	TNS-F-59078	Agaricales	Tricholomataceae	*Lepista nuda*	2013/11/6
5	TNS-F-59081	Agaricales	Agaricaceae	*Lycoperdon* sp.	2013/11/6
6	TNS-F-59084	Geastrales	Geastraceae	*Geastrum* sp.	2013/11/6
7	TNS-F-59090	Agaricales	Pleurotaceae	*Pleurotus ostreatus*	2013/11/6
8	TNS-F-59091	Boletales	Diplocystidiaceae	*Astraeus hygrometricus*	2013/11/6
9	TNS-F-59093	Polyporales	Polyporaceae	*Lenzites betulina*	2013/11/13
10	TNS-F-59094	Agaricales	Schyzophyllaceae	*Schizophyllum commune*	2013/11/13
11	TNS-F-59095	Agaricales	Lyophyllaceae	*Lyophyllum decastes*	2013/11/13
12	TNS-F-59096	Agaricales	Physalacriaceae	*Flammulina velutipes*	2013/11/13
13	TNS-F-59101	Agaricales	Agaricaceae	*Calvatia craniiformis*	2013/11/13
14	TNS-F-59104	Agaricales	Pluteaceae	*Pluteus leoninus*	2013/11/13
15	TNS-F-59110	Russulales	Russulaceae	*Russula* sp.	2013/11/14
16	TNS-F-59115	Agaricales	Amanitaceae	*Amanita* sp.	2013/11/14
17	TNS-F-59120	Auriculariales	Auriculariaceae	*Exidia* sp.	2013/11/14
18	TNS-F-59121	Russulales	Stereaceae	*Stereum* sp.	2013/11/14
19	TNS-F-59129	Phallales	Phallaceae	*Phallus impudicus*	2013/11/14
20	TNS-F-59135	Agaricales	Strophariaceae	*Hypholoma fasciculare*	2013/11/14
21	TNS-F-59136	Russulales	Stereaceae	*Xylobolus spectabilis*	2013/11/14
22	TNS-F-59140	Boletales	Suillaceae	*Suillus* sp.	2013/11/14
23	TNS-F-59142	Agaricales	Hygrophoraceae	*Hygrocybe* sp.	2013/11/14
24	TNS-F-59144	Polyporales	Polyporaceae	*Pycnoporus* sp.	2013/11/14
25	TNS-F-59148	Agaricales	Psathyrellaceae	*Psathyrella* sp.	2013/11/20
26	TNS-F-59159	Pezizales	Pezizaceae	*Peziza* sp.	2013/11/20
27	TNS-F-59186	Phallales	Protophallaceae	*Kobayasia nipponica*	2013/12/1
28	TNS-F-59187	Auriculariales	Auriculariaceae	*Auricularia polytricha*	2013/12/1
29	TNS-F-59199	Boletales	Sclerodermataceae	*Scleroderma* sp.	2013/12/5
30	TNS-F-59211	Pezizales	Morchellaceae	*Morchella* sp.	2013/12/16
31	TNS-F-59215	Polyporales	Fomitopsidaceae	*Daedalea* sp.	2013/12/19
32	TNS-F-59220	Phallales	Clathraceae	*Linderia bicolumnata*	2013/12/19

*Sample numbers correspond to those used in Fig. 2.

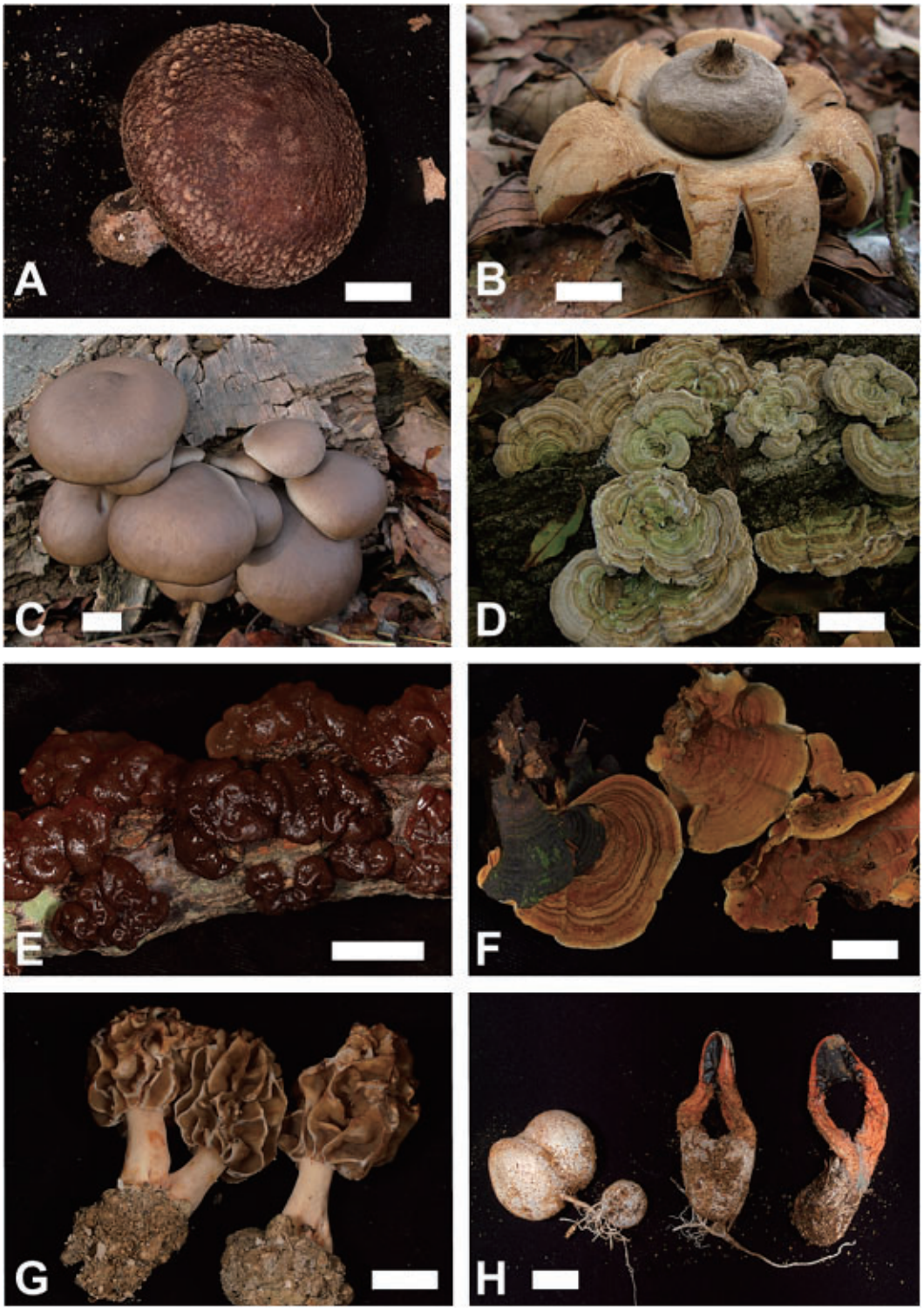
Sequencing Kit (Applied Biosystems Inc., Norwalk, CT, USA), following the manufacturer's instructions.

PCR amplification of a portion of the 16S small subunit rDNA was also conducted to check presence/absence of bacteria and archaea in mushroom fruit bodies. The primer combination of F515 and R806 (Bates *et al.*, 2010, 2011) was used following the PCR conditions described above. PCR products were electrophoresed in 1% agarose gels stained with ethidium bromide and visualized under UV light. All extracted DNA samples were stored in freezers (−80°C) at the Center for Molecular Biodiversity Research, National Museum of Nature and Science.

Results

Mushroom specimens examined

During the collecting period in November and December, 2013, a total of 32 mushroom specimens were selected for further analyses (Table 1). To cover a wide range of ecological and phylogenetic diversity, a total of 32 species belonging to two phyla (Basidiomycota and Ascomycota), 8 orders, and 27 families were selected (Table 1, Fig. 1). Ecologically, ectomycorrhizal, wood-rotting, and various saprotrophic fungi were all included. Some mushroom fruit bodies of inferior quality (e.g., moldy, insect damage, etc.) were also collected, but not included for further analyses. Some examples of mushroom fruit bodies included in this study are shown in Fig. 1. Although mushroom fruit bodies were often cov-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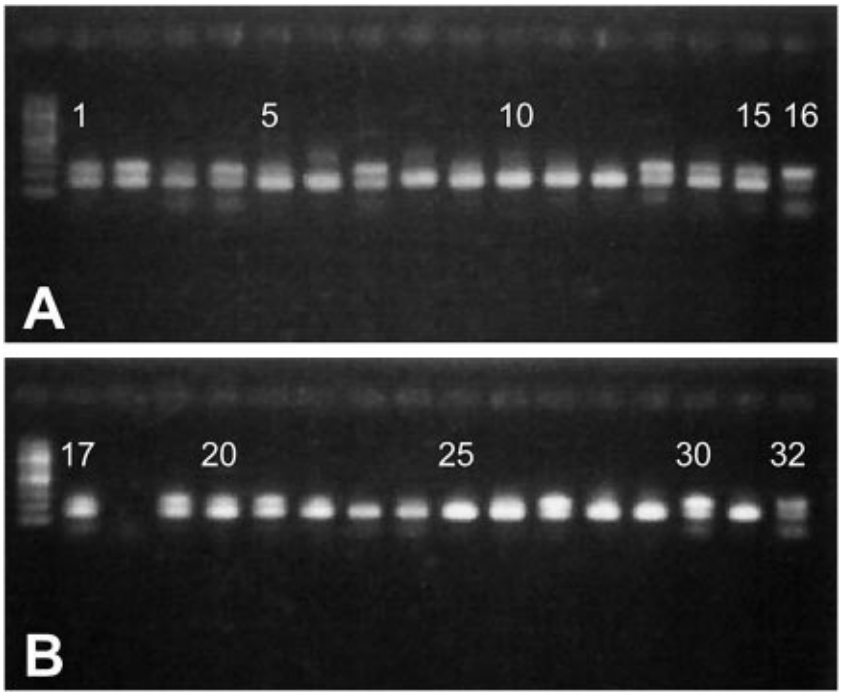

Fig. 2. PCR bands of 16S rDNA using bacteria specific primers observed on agarose gel.
A: Sample numbers 1 to 16. B: Sample numbers 17 to 32. Samples numbers correspond to those in Table 1. Bands on the left are from the DNA size markers.

ered by soil particles (Fig. 1H), tissue fragments were collected only from interior parts of fruit bodies (Fig. 3) to avoid contamination. All specimens were identified to genus or species level morphologically, and the identification was further confirmed by DNA barcode (ITS region). DNA extraction, PCR and sequencing from all mushroom specimens were successful.

PCR amplification of bacterial DNA

Using the primers specifically designed for bacterial and archaeal 16S rDNA (Bates *et al.*, 2010, 2011), successful amplification was confirmed from 31 out of a total of 32 mushroom fruit bodies (Fig. 2). Most samples produced multiple PCR bands, and some were apparently two or more clearly separated bands, for example, sample number 2, 7, 13 (Fig. 2A), 19, 21, and 30 (Fig. 2B). The only sample which did not produce a visible PCR band was sample 18; *Stereum* sp. (Basidiomycota, Agaricomycetes, Russulales, Stereaceae) (Fig. 1F, 2B). No PCR bands of significantly longer or shorter sizes than the rest of samples were observed.

Discussion

This study indicates that bacteria (and/or archaea) are consistently present in mushroom fruit bodies (Fig. 2). The fact that multiple PCR bands were frequently observed (Fig. 2) also indicates diverse bacterial communities exist in

Fig. 1. Representatives of mushrooms used in this study. A. *Lentinula edodes* (TNS-F-59072). B. *Geastrum* sp. (TNS-F-59084). C. *Pleurotus ostreatus* (TNS-F-59090. D. *Lenzites betulina* (TNS-F-59093). E. *Exidia* sp. (TNS-F-59120). F. *Stereum* sp. (TNS-F-59121). G. *Morchella* sp. (TNS-F-59211). H. *Linderia bicolumnata* (TNS-F-59220). Bars = 1 cm.

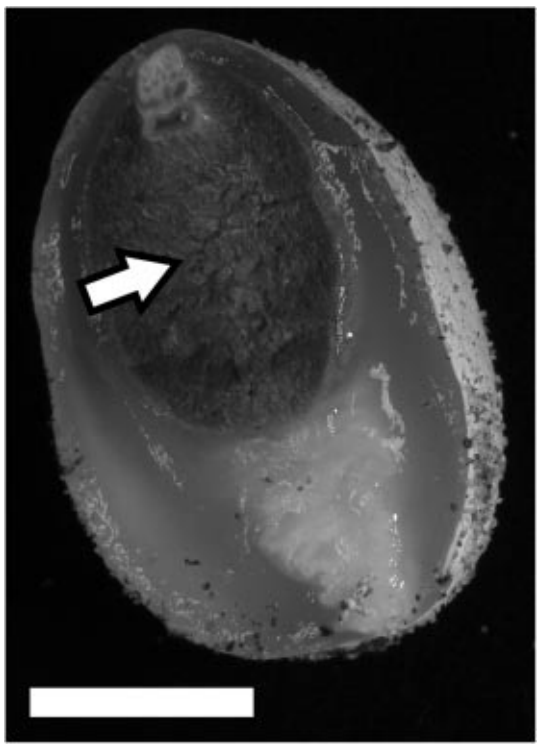

Fig. 3. An example of internal tissue investigated in this study (*Linderia bicolumnata*, TNS-F-59220). Fruit bodies were covered by soil particles, but internal glebal tissue (arrow) was clean without visible contaminants. Bar = 1 cm.

mushroom fruit bodies. The presence/absence of bacteria appears unrelated to taxonomic/phylogenetic positions of mushrooms because the only taxon which did not produce a positive band of bacterial DNA was *Stereum* (Basidiomycota, Russulales; Fig. 1F), but all other Basidiomycota (including Russulales) and Ascomycota consistently produced visible bands (Fig. 2). The sizes of PCR bands were mostly consistent, without having extremely long or short bands, indicating that the primers successfully amplified bacterial/archaeal 16S (Fig. 2), without unspecific amplification of fungal DNA or the other genes in genomic DNA.

It is possible that opportunistic contamination, such as bacteria from atmosphere and mushroom surface, produced the PCR bands. Such contamination cannot be completely avoided from the procedure used in this study. However, extra caution was made to obtain tissue fragments only from clean, internal parts of fruit bodies (Fig. 3). Many samples were further used for tissue isolation and clean cultures (without fungal or bacterial contamination) were successfully obtained (data not shown). I therefore consider that if opportunistic bacterial contamination occurred during this study, the impact was minimal or absent.

As described above, *Stereum* (Fig. 1F) was the only taxon produced no visible PCR bands (Fig. 2). In addition to taxonomic/phylogenetic position, ecological features do not appear to be related to this pattern. The other taxa of wood-rotting habit, such as *Pycnoporus*, *Lenzites*, and *Auricularia* all produced positive PCR bands. *Stereum* typically produces very thin fruit bodies, of less than 1 mm thick. The physical structure of their fruit bodies may be a limiting factor for some bacteria. However, it is well known that diverse bacteria can inhabit within or surface of hyphae of various fungal groups, including mycorrhizal mushrooms (Bertaux *et al.*, 2005; Barbieri *et al.*, 2005), endophytes (Hoffman and Arnold, 2010), and lichens (Bates *et al.*, 2011). The biomass of fruit bodies *per se*, therefore, is probably unrelated to presence/absence of bacteria.

This study demonstrated that bacteria (and/or archaea) are consistently present in mushroom fruit bodies, surprisingly unexplored habitat for microbiological research. The species composition of bacteria detected in this study should be investigated in near future using metagenomic approaches. Because mushrooms (and other fungi) are distributed in all continents with various levels of diversity (Tedersoo *et al.*, 2014), bacterial communities in mushroom fruit bodies may reveal new insight into the patterns already detected from soil, water, or plant substrates (Fierer and Jackson, 2006; Fierer *et al.*, 2011; Nemergut *et al.*, 2010).

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