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A Preliminary Survey on Larval Diversity in Mushroom Fruit Bodies

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Abstract We investigated diversity of insect larvae in mushroom fruit bodies. A total of 24 larvae from nine species of mushrooms (7 genera, 3 orders) were collected and DNA sequences were obtained. The BLAST searches could not identify these larvae to species but they all belong to Diptera. Phylogenetic analyses revealed the presence of a total of nine presumable species of flies in mushroom fruit bodies. Each species of flies was found from a single fruit body, except for the maggots of Group 2 and 3, which were collected from two species of mushrooms. Although our sampling effort is very limited for this study, the following conclusions can be drawn: (1) Different species of mushrooms can be inhabited by the same Diptera species. (2) Different species of larvae can be present in the same mushroom fruit bodies. (3) Same species of larvae can be present in the different mushroom fruit bodies at different times.

Key words: COI, Diptera, DNA barcode, maggots, mycophagy.

Introduction

Mycophagy (fungal consumption) by mammals and arthropods has attracted researchers for centuries. Among which, mycophagy by small animals, especially by rodents and small marsupials, has been well documented as an example of truffle-animal interaction (Claridge and May, 1994; Lehmkuhl *et al.*, 2004; Meyer *et al.*, 2005). Because fruit bodies of truffle-like fungi are produced below ground, the spores are not disseminated by wind, as is the case in many above-ground mushroom-forming species, which have potential of long distance spore dispersal (Thiers, 1985; Bruns *et al.*, 1989). Instead, they produce a unique aroma to attract small animals, which rely on truffle-like fungi as a large part of their diet (Thiers, 1985; Castellano *et al.*, 1989). Truffle-like fruiting bodies are eaten by small animals and the fungal spores are disseminated with the animal feces (Castellano *et al.*, 1989). This association between small animals and truffle-like fungi can be seen in many areas of the world (Malajczuk *et al.*, 1987). Because of the tight interaction between mycophagous animals and truffle-like fungi, some sort of co-evolution scenario is a possibility. For example, one could hypothesize that the origin of mycophagous animals and hypogeous fungi occurred at the same time.

The other well documented examples of interactions include mycophagy by arthropods, which was reported from 1800's or even earlier (Fulton, 1889). Many reports came from the order Phallales (commonly known as stinkhorns) as one of the prime examples of insect mycophagy (Smith, 1956; Shorrocks and Charlesworth, 1982; Tuno, 1998). Most notably, spore mass (gleba) of Phallales becomes slimy and emits strong odor when matured, which attracts small insects, especially flies, and their spores are dispersed by those insects (Fulton, 1889). There are also large numbers of truffle-like taxa, in addition to stinkhorn-like taxa, in the order Phallales (Hosaka *et al.*, 2006). Interestingly, however, spores of truffle-like taxa in Phallales have not

been recorded from animal feces in previous studies. This may indicate that that spore dispersal of Phallales, including truffle-like taxa, is entirely dependent on arthropods. It is therefore possible, as in the case of truffle-like fungi, that mycophagous insects and stinkhorns have co-evolved.

Mycophagy by arthropods is known not only from Phallales, but also from a wide variety of mushrooms (Martin, 1979; Pacioni *et al.*, 1991). At the same time, a wide variety of arthropods, mostly insects, mites, and springtails (Collembola) has been reported as mycophagous (Tuno, 1999; Sawahata *et al.*, 2002, 2008; Takahashi *et al.*, 2005; Yamashita and Hijii, 2007; Kadowaki, 2010). It is a general trend that insect fauna of perennial fruit bodies (polypores or conks) is composed of a wide variety of groups, often dominated by Coleoptera, but mushrooms with ephemeral, soft fruit bodies are mostly fed by Collembola and Diptera, often dominated by the family Drosophilidae (Atkinson and Shorrocks, 1981; Takahashi *et al.*, 2005; Kadowaki, 2010; Tuno, 1999; Yamashita and Hijii, 2007).

Among mycophagous insects, the diversity and behavior of drosophilid flies have most extensively been studied (Kimura *et al.*, 1977; Jeanike, 1978; Grimaldi, 1985; Worthen, 1989; Worthen *et al.*, 1996; Toda and Kimura, 1997; Toda *et al.*, 1999; Tuno *et al.*, 2007). For example, spore dispersal of stinkhorns by flies has been documented for centuries (Fulton, 1889; Tuno, 1998). Most studies, however, are based on field observation of adult flies, or rearing experiments from field-collected mushrooms (Jaenike, 1978; Grimaldi, 1985; Toda and Kimura, 1997; Yamashita and Hijii, 2007; Kadowaki, 2010), both of which depend on the identification of the adult flies.

We argue that those methods may significantly bias results because not all species of larvae can become adults under the experimental settings, and we therefore may miss the true diversity of larvae in fruit bodies. In this study, we attempted to reveal such "hidden" diversity of larvae in mushroom fruit bodies using DNA barcode data directly sequenced from larval stage. We focused on collecting only larval stage of flies (maggots), and the identification of each fly was entirely dependent on DNA sequence data. By this way, we also attempted to link the sequence data with species names, and to reveal the presence of cryptic or new species of flies.

Materials and Methods

Collecting Sites, Collecting Scheme, and Curation of Specimens

Fieldwork was conducted in the year of 2011 (July–September). The main collecting sites were located in Tsukuba Botanical Garden, Tsukuba, Japan (36°06′N, 140°06′E). The garden occupies ca. 14 ha. (36 acres), and has ca. 5000 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. 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 mushrooms were collected, potential ectomycorrhizal host trees near fruit bodies, mostly Pinaceae and Fagaceae, were identified to species. If alternative ectomycorrhizal hosts were present near fruit bodies, those were recorded as well.

All fruit bodies were collected, photographed and dried 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 hymenial tissue from freshly collected samples were soaked in 1.5 ml tubes with 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). Voucher numbers were assigned to representative speci-

mens.

When maggots were found, they were collected under dissecting microscope using clean forceps, and stored in 1.5 ml tubes with DMSO buffer. At least ten maggots were collected from a single fruit body, and the collection data were recorded by assigning the voucher number according to the mushrooms from which maggots were found. If maggots were detected from multiple fruit bodies of the same specimens (under the same voucher numbers), they were stored in separate tubes. The exact positions of maggots found (e.g., cap, gills, stalk, etc) were not recorded for this study.

Specimens collected during the fieldwork were deposited at the fungal herbarium of the National Museum of Nature and Science, Tsukuba, Japan (TNS). All maggot samples were stored in refrigerator at the laboratory of KH.

DNA Preparation, PCR, and Sequencing

DNA of mushrooms and maggots was extracted from the samples stored in DMSO buffer. Tissues of mushrooms were ground under liquid nitrogen using a mortar and pestle. Maggots were first transferred to new 1.5 ml tubes using clean forceps, and CTAB buffer was added. Maggots were then ground using plastic pestle by hands. Only one maggot was used for each extraction. DNA was extracted using the modified CTAB extraction protocol 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℃ for 1 hour, and proteins were removed using the mixture of chloroform : isoamylalcohol (24 : 1). The materials were further purified using 6 M sodium iodine buffer with glass milk, washed with ethanol/buffer solution, and finally eluted in 100 μl of TE buffer.

DNA sequence data of mushrooms were obtained from the internal transcribed spacer regions (ITS) of the nuclear ribosomal DNA. For amplifying the ITS region, the primer combination of ITS5 and ITS4 (White *et al.*, 1990) was used. DNA sequence data of maggots were obtained from the cytochrome c oxidase I gene (COI). For amplifying the COI, the combination of LCO1490 and HCO2198 (Folmer *et al.*, 1994; Hebert *et al.*, 2003) was used. PCR reactions were carried out using 20 μl reaction volumes each containing: 1 μl genomic DNA, 1 μl dNTP, (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, PCR products were then purified using the ExoSap-IT (Millipore, Molsheim, France) and directly sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Norwalk, CT, USA), following the manufacturer's instructions.

Phylogenetic Analyses

DNA sequences of the COI were aligned manually using the data editor of BioEdit ver. 7.0.1 (Hall, 1999). Ambiguously aligned regions were excluded from the analyses. The dataset was then analyzed by maximum parsimony (MP) and neighbor-joining (NJ) analyses. MP analyses were conducted under the equally weighted parsimony criterion using PAUP* version 4.0b10 (Swofford, 2002), with heuristic search option (with TBR and Multrees on) with MAXTREES set to 10,000. Support for the individual nodes was tested with bootstrap (BS) analysis under the equally-weighted parsimony criterion. BS analysis was based on 1,000 BS replicates using the heuristic search option (TBR and Multrees options off), with ten random addition sequences. NJ analysis was conducted using PAUP* (Swofford, 2002) with the Kimura-2-parameter model, with a gamma shape set to 0.5. Support for individual nodes was tested by BS analysis based on 1,000 BS replicates under the same settings.

Results

Collections of Mushrooms and Larvae

A total of nine species of mushrooms (7 genera, 3 orders) contained multiple maggots in their fruit bodies (Table 1). We intentionally avoided collecting rotten fruit bodies because maggots found in such materials could simply be sapro-

Table 1. Maggots sequenced for this study with host mushrooms and collection information

Vocuher nos. of maggots	Host mushrooms of maggots (orders in parentheses)	Herbarium nos. of mushrooms	Collection date	Specific location in the Tsukuba Botanical Garden
375-1	*Tylopilus* sp. (Boletales)	TNS-F-41193	July 7, 2011	Cool temperate deciduous broad-leaved forest section
375-2	*Tylopilus* sp. (Boletales)	TNS-F-41193	July 7, 2011	Cool temperate deciduous broad-leaved forest section
375-3	*Tylopilus* sp. (Boletales)	TNS-F-41193	July 7, 2011	Cool temperate deciduous broad-leaved forest section
375-4	*Tylopilus* sp. (Boletales)	TNS-F-41193	July 7, 2011	Cool temperate deciduous broad-leaved forest section
535-1	*Boletus* sp. (Boletales)	TNS-F-41353	August 11, 2011	Evergreen broad-leaved forest section
535-2	*Boletus* sp. (Boletales)	TNS-F-41353	August 11, 2011	Evergreen broad-leaved forest section
535-3	*Boletus* sp. (Boletales)	TNS-F-41353	August 11, 2011	Evergreen broad-leaved forest section
566-1	*Amanita* sp. (Agaricales)	TNS-F-41384	August 12, 2011	Cool temperate deciduous broad-leaved forest section
566-2	*Amanita* sp. (Agaricales)	TNS-F-41384	August 12, 2011	Cool temperate deciduous broad-leaved forest section
566-3	*Amanita* sp. (Agaricales)	TNS-F-41384	August 12, 2011	Cool temperate deciduous broad-leaved forest section
566-4	*Amanita* sp. (Agaricales)	TNS-F-41384	August 12, 2011	Cool temperate deciduous broad-leaved forest section
576-1	*Calvatia craniiformis* (Agaricales)	TNS-F-41393	August 12, 2011	Cool temperate deciduous broad-leaved forest section
580-1	*Russula* sp. (Russulales)	TNS-F-41397	August 12, 2011	Warm temperate deciduous broad-leaved forest section
761-1	*Amanita hemibapha* (Agaricales)	TNS-F-42118	September 22, 2011	Warm temperate deciduous broad-leaved forest section
761-2	*Amanita hemibapha* (Agaricales)	TNS-F-42118	September 22, 2011	Warm temperate deciduous broad-leaved forest section
761-3	*Amanita hemibapha* (Agaricales)	TNS-F-42118	September 22, 2011	Warm temperate deciduous broad-leaved forest section
588-1	*Strobilomyces* sp. (Boletales)	TNS-F-41945	August 18, 2011	Evergreen broad-leaved forest section
593-1	*Xerocomus* sp. (Boletales)	TNS-F-41950	August 18, 2011	Warm temperate deciduous broad-leaved forest section
593-2	*Xerocomus* sp. (Boletales)	TNS-F-41950	August 18, 2011	Warm temperate deciduous broad-leaved forest section
593-3	*Xerocomus* sp. (Boletales)	TNS-F-41950	August 18, 2011	Warm temperate deciduous broad-leaved forest section
675-1	*Russula* sp. (Russulales)	TNS-F-42032	September 2, 2011	Warm temperate deciduous broad-leaved forest section
675-2	*Russula* sp. (Russulales)	TNS-F-42032	September 2, 2011	Warm temperate deciduous broad-leaved forest section
675-3	*Russula* sp. (Russulales)	TNS-F-42032	September 2, 2011	Warm temperate deciduous broad-leaved forest section
675-4	*Russula* sp. (Russulales)	TNS-F-42032	September 2, 2011	Warm temperate deciduous broad-leaved forest section

The classification at the ordinal level was based on Kirk *et al.* (2008).

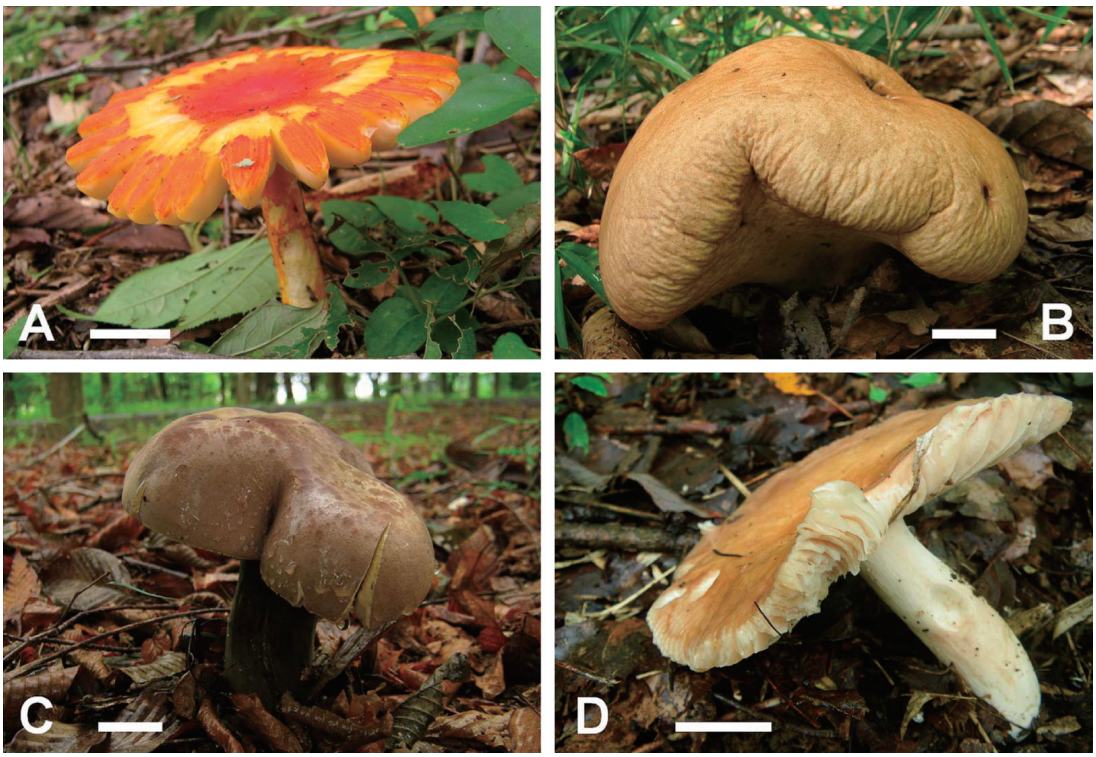

Fig. 1. Representative fruit bodies of mushrooms from which maggots were isolated. A. *Amanita hemibapha* (Agaricales), TNS-F-42118. B. *Calvatia craniiformis* (Agaricales), TNS-F-41393. C. *Tylopilus* sp. (Boletales), TNS-F-41193. D. *Russula* sp. (Russulales), TNS-F-42032. Bars = 2 cm.

trophic not specific to mushrooms, and therefore not necessarily mycophagous. Most of the collected fruit bodies had no apparent insect damage judging from the outside appearance (Fig. 1). Typically, maggots were found only when fruit bodies were cut in half prior to drying. At least ten maggots from each fruit body were successfully collected without damaging the outer layer of their bodies, and stored in DMSO buffer for further molecular studies.

DNA Preparation

The number of maggots sequenced varied among samples (Table 1). Only one maggot was extracted for DNA each from *Calvatia craniiformis* (TNS-F-41393; Fig. 1B), *Russula* sp. (TNS-F-41397), and *Strobilomyces* sp. (TNS-F-41945). On the other hand, three or four maggots were extracted from *Amanita hemibapha* (TNS-F-42118; Fig. 1A), *Amanita* sp. (TNS-F-41384), *Boletus* sp. (TNS-F-41353), *Russula* sp. (TNS-F-42032; Fig. 1D), *Tylopilus* sp. (TNS-F-41193; Fig. 1C), and *Xerocomus* sp. (TNS-F-41945). DNA was extracted from a total of 24 maggots, and PCR and sequencing of the COI were successful for all samples. The BLAST searches of the COI sequences only resulted in the identification at the ordinal level (Diptera) and could not lead to the species names. All mushroom samples were successfully extracted and sequenced, and the identification at the generic level was confirmed by the BLAST searches.

Phylogenetic Analyses

The initial alignment resulted in 680 bp long, and the beginning and end regions were deleted from the analyses. This resulted in the dataset of 455 characters without gaps or ambiguously aligned regions. Out of 455 characters, 317 were parsimony uninformative and the remaining 138

characters were used for the MP analyses. The MP analyses produced four equally parsimonious trees with a tree length of 271 steps, CI of 0.6052, RI of 0.8333, and RC of 0.5043. Because the NJ analyses produced the trees with almost identical topology, only one of the most parsimonious trees is shown (Fig. 2).

A total of nine groups, all of which were supported by 100% bootstrap values, were detected (Fig. 2). Most groups were represented by the maggots from a single fruit body, but the group 2 and 3 were represented by the maggots from different species of mushrooms. The group 2 represents the maggots from *Strobilomyces* sp. (TNS-F-41945) and *Xerocomus* sp. (TNS-F-41950), both of which belong to the order Boletales. Furthermore, they were both collected on the same day (August 18, 2011), but were from different sections in the garden (Table 1). The group 3 represents the maggots from *Russula* spp., but one was collected on August 12, 2011, and the other was collected on September 2, 2011. Those two species of *Russula* were both from the same section of the garden (Warm temperate deciduous broad-leaved forest section). Most species of mushrooms hosted only one group of maggots, but *Russula* sp. (TNS-F-42032; Fig. 1D) hosted the maggots of groups 3 and 5, and *Xerocomus* sp. (TNS-F-41950) hosted the maggots of groups 2 and 8.

Discussion

The primer combinations of the COI successfully amplified and sequenced the DNA from all 24 maggots we investigated. Those primers (LCO1490 and HCO2198) were originally designed for amplifying a wide variety of animals (Folmer *et al.*, 1994; Hebert *et al.*, 2003), but have been shown to be inappropriate for certain groups

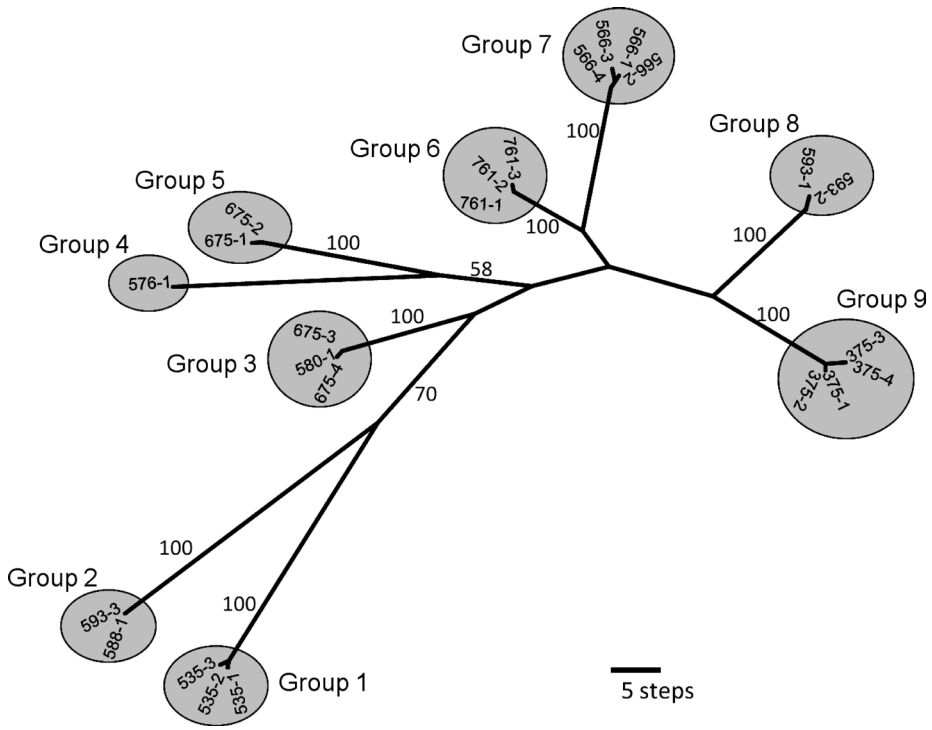

Fig. 2. An unrooted network of the COI phylogeny of maggots based on parsimony analyses. One of four equally parsimonious trees is shown with bootstrap values on branches. The numbers in the circles indicate the individual maggot corresponding to Table 1.

(Lohman *et al.*, 2009; Park *et al.*, 2010). Our study showed that those primers can be used for a wide variety of mycophagous Diptera, but more studies are needed to conclude if they can be applied to other mycophagous insects.

All maggot sequences were tentatively identified as "Diptera" by the BLAST searches, without lower taxonomic units identified. In addition, phylogenetic analysis using the COI sequences of selected *Drosophila* subg. *Drosophila* (http://kyotofly.kit.jp/ehime/genome2007/COI_Drosophila.html) showed that maggots sampled for this study did not form a cluster with any *Drosophila* species (data not shown). This indicates that species of *Drosophila*, which includes several mycophagous species, were not collected during this study. Other genera with known mycophagous species, such as *Megaselia*, *Cordyla*, *Allodia*, *Rondaniella*, and *Mycetophila* should also be included in phylogenetic analyses for better understanding of maggot diversity.

Although we do not have species names, we tentatively considered each group in Fig. 2 (Group 1 to 9) as species because each group was supported by 100% of bootstrap values, and they were separated by ten or more substitutions. This reflects the current situation of DNA barcoding in insect taxonomy, clearly indicating the need of sequencing more well-identified specimens. The problem of absence of reliable link between species names and DNA sequence data is also critical in mushroom identification (Brock *et al.*, 2008; Nagy *et al.*, 2011). The accumulation of reliable DNA data in the barcode database takes more time, but we have sequenced the barcoding regions both from insects (as maggots) and mushroom hosts (as mature fruit bodies), and we keep the voucher specimens for all of them. These data and specimens can therefore be used for future studies.

We focused on collecting the larval stage of flies because they clearly use mushroom fruit bodies as food source. The adult flies and beetles are also known to feed on mushroom spores and hyphae (Tuno, 1998, 1999; Takahashi *et al.*, 2005; Kadowaki, 2010), but it is often difficult to distinguish truly mycophagous insects from opportunistic visitors. Furthermore, fresh fruit bodies without apparent insect damage (Fig. 1) are often inhabited by numerous maggots. This indicates that a variety of insects may exist in mushroom fruit bodies from earlier developmental stages, as shown by Yamashita and Hijii (2007).

There are extensive studies on mycophagous insect fauna based on field observation and/or rearing experiments from field-collected mushrooms (Jaenike, 1978; Grimaldi, 1985; Toda and Kimura, 1997; Yamashita and Hijii, 2007; Kadowaki, 2010), both of which depend on the identification of the adult flies. Because competitive coexistence, instead of competitive exclusion, was proposed as a mechanism of mycophagous flies in mushroom fruit bodies (Grimaldi, 1985), previous studies may already have revealed a vast majority of insect diversity in mushroom fruit bodies. However, we argue that true diversity of mycophagous insects cannot be understood by collecting adults alone. This is somewhat analogous to the study of mushroom diversity where true mushroom diversity cannot be understood by collecting fruit bodies alone.

Our sampling for this study is clearly limited, and more data are necessary both for mushrooms and insects. Based on such limited sampling, however, the following conclusions can be drawn:

(1) Different species of mushrooms can be inhabited by the same Diptera species. This clearly indicates that the interactions between mycophagous flies and mushrooms are not species specific. At this stage, however, we cannot conclude if the maggot groups found only from a single mushroom species (Groups 1, 4–9; Fig. 2) have specific interactions.

(2) Different species of larvae can be present in the same mushroom fruit bodies. This study showed only two species of mushrooms hosted multiple species of flies. However, we have not sequenced all maggots we sampled. We believe more mushrooms actually host multiple species of flies in a single fruit body, which can only be answered with increasing sampling and sequenc-

ing effort of maggots and mushrooms.

(3) Same species of larvae can be present in the different mushroom fruit bodies at different times. It is important information to understand the biology of flies as well as mushrooms. There was a three-week gap between the first (voucher number 580-1; Fig. 2) and last (voucher number 675-3, 4) collections of maggots of the same species (Group 3; Fig. 2). This may indicate maggots sampled at different times represent different developmental stages (the ones from September were more mature), or the fly species lay eggs over a three-week span.

Some of basic questions remain to be answered: What are the Diptera species found in this study? What is the true diversity of larvae in mushrooms? Are there any host preferences? Are fungal spores dispersed by larvae? Are fungal spores stimulated to germinate by larval consumption? These questions can only be answered by the combination of increasing sampling effort of both adult and larval stages, developing experimental designs, including rearing experiments, and a good taxonomy based on morphology and DNA barcoding data.

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