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Metagenomic Analysis of Fungal Communities Inhabiting the Fairy Ring Zone of *Tricholoma matsutake*

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Tricholoma matsutake, an ectomycorrhiza that has mutual relationships with the rootlet of *Pinus denisflora*, forms a fruiting body that serves as a valuable food in Asia. However, the artificial culture of this fungus has not been successful. Soil fungi, including *T. matsutake*, coexist with many other microorganisms and plants; therefore, complex microbial communities have an influence on the fruiting body formation of *T. matsutake*. Here, we report on the structures of fungal communities associated with the fairy ring of *T. matsutake* through the pyrosequencing method. Soil samples were collected inside the fairy ring zone, in the fairy ring zone, and outside the fairy ring zone. A total of 37,125 sequencing reads were obtained and 728 to 1,962 operational taxonomic units (OTUs) were observed in the sampling zones. The fairy ring zone had the lowest OTUs and the lowest fungal diversity of all sampling zones. The number of OTUs and fungal taxa inside and outside the fairy ring zone was, respectively, about 2 times and 1.5 times higher than the fairy ring. Taxonomic analysis showed that each sampling zone has different fungal communities. In particular, out of 209 genera total, 6 genera in the fairy ring zone, such as *Hemimycena*, were uniquely present and 31 genera, such as *Mycena*, *Boletopsis*, and *Repetophragma*, were specifically absent. The results of metagenomic analysis based on the pyrosequencing indicate a decrease of fungal communities in the fairy ring zone and changes of fungal communities depending on the fairy ring growth of *T. matsutake*.

Keywords: *Tricholoma matsutake*, fungal communities, metagenomic analysis, pyrosequencing

Introduction

Tricholoma matsutake, an ectomycorrhizal (ECM) fungus, forms a symbiotic relationship with the root tips of living *Pinus densiflora*. Its fruiting bodies, called Pine mushrooms, have great value as a commercial food in Asia because of its unique flavor and many medicinal effects [2, 8, 16, 17, 21, 29, 33, 53]. However, the annual production of this fungus is very limited and unpredictable because it is affected by many ecological conditions, especially rainfall and temperature [4, 30, 44].

Over the past few decades, many researchers have investigated the optimal growth conditions and artificial culturing methods for the Pine mushroom (*T. matsutake*) [19, 20, 32, 34, 45, 47, 51, 52]. Despite these continued efforts, however, artificial cultivation has not been successful. In addition, many questions still remain about the ecological interactions between *T. matsutake* and other factors influencing its growth, such as microbial communities, the host plant, edaphic qualities, and climate. Among these factors, the microbial communities that coexist with *T. matsutake* especially influence the development of *T. matsutake* fruiting bodies; however, there have been few studies on the microbial diversity and their interactions in habitats of *T. matsutake* [18, 25, 46].

Natural soil ecosystems have a great variety of microbial communities. In terrestrial ecosystems, fungi tend to dominate over other microorganisms (such as bacteria)

under acidic soil conditions since they have an ability to tolerate the acidic pH [38, 39]. Soil fungi are classified into three functional groups based on how they gain energy: (1) saprophytic fungi (decomposers of dead organic matter), (2) mutualists or mycorrhizal fungi, and (3) pathogenic fungi. Mycorrhizal fungi, the most typical mutualists, are also categorized into four types: arbuscular (VAM), ectomycorrhizal (ECM), ericoid, and orchid. The ECM fungi, the major group of mycorrhizae (which includes *T. matsutake*), have mutually beneficial relationships with plants and a strong preference for their host plants [23]. This fungal group especially plays a key role in plant growth by colonizing around living plant rootlets. There, the fungi help host plants absorb water and mineral nutrients effectively, while obtaining essential carbon from their plant roots [1, 7, 12, 13]. *T. matsutake* stimulates the growth of host plant seedlings as an ectomycorrhizal symbiont [10]. In the soil environment, ECM fungi generally coexist with countless other soil fungi, as well as plants, and their interactions are too various for biologists to understand perfectly [1].

To date, approximately 99.8% of the microorganisms that exist in various natural environments have not been cultured. Although a number of culturing techniques have been developed for the identification of microorganisms over the past years, it is very difficult to evaluate the true microbial diversity on Earth, since there are still limits to estimating the number and type of uncultured microbes [3, 43]. For studies on microbial communities, the sequencing of ribosomal DNA (rDNA) has generally been used as a DNA barcode to identify microbes [14, 22, 35, 48, 54]. Metagenomics, a novel and innovative technology, now enables the study of both culturable and unculturable microorganisms. Next-generation sequencing (NGS) technology for metagenomics, more useful than other previous sequencing methods, is able to rapidly analyze massive amounts of DNA sequence data for all microorganisms at a relatively low cost, including unculturable species [11, 41, 43]. Among the several tools for NGS, recently conducted studies have shown that Roche 454 GS-FLX pyrosequencing is an appropriate method for detecting enormous DNA sequences directly from environmental samples, such as soil, forest matter, and marine ecosystems. Therefore, this tool is effective at analyzing microbial communities [5, 6, 9, 27, 42].

Currently, the diversity of microbial communities associated with *T. matsutake* has been studied using various methods, such as culture-based assays and denaturing gradient gel electrophoresis (DGGE) based on the Sanger sequencing [18, 25, 46]. These methods have some limits, including their inability to identify unculturable microbes and the difficulty in extracting each DNA band from a gel. Therefore, in this study, to determine the different distribution of fungal communities in soil where *T. matsutake* occurs, we grouped sampling sites into areas inside, beneath, and outside the fairy ring zone of *T. matsutake*, and investigated the soil fungal communities at each sampling site using the 454 GS-FLX pyrosequencing platform.

Materials and Methods

Soil Sampling Strategy

The sampling site was in the mountains near Gachang-myeon, Dalseong-gun, Daegu, South Korea. In September 2012, the three sampling sites were selected based on their location relative to *T. matsutake* fairy rings as follows: (i) inside the fairy ring zone, (ii) in the zone of mycorrhizae for fruiting bodies (the zone of the fairy ring), and (iii) outside the fairy ring zone. By using a soil sampler, ten soil cores (depth: 15 cm, diameter: 2 cm) were collected in each region (30 soil cores total) at about 30~40 cm intervals, because the fairy ring of *T. matsutake* generally expands outward 10~15 cm every year according to its growth on pine rootlets [28]. Subsequently, the ten soil cores collected in each region were homogenized (depending on their groups) for further experiments.

DNA Extraction, Purification, and Pyrosequencing

To perform pyrosequencing, metagenomic DNA was first extracted from each 5~10 g of soil samples with the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). The resulting DNA for each soil sample was amplified using barcoded fusion primers targeting the D1/D2 regions of large-subunit rRNA genes. The primers consisted of the 454 adapter, key sequence (4 bp), barcode (only in the reverse primer, 7~9 bp), linker sequence (2 bp), and a universal primer (LR0R and LR3) [49]. The forward primer sequence was 5′-CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-TG-ACCCGCTGAAYTTAAGCATAT-3′ and the reverse primer sequence was 5′-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-X-GA-CTTGGTCCGTGTTTCAAGAC-3′; the "X", representing the barcode sequence, differs between the samples and was used to separate samples for further analysis. The PCR was carried out on the PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA, USA) under the following conditions: initial denaturation (5 min at 94°C), and 30 cycles of denaturation (30 sec at 94°C), annealing (45 sec at 55°C), and extension (1 min 30 sec at 72°C). All PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and the cleaned DNA products were quantified using both the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) and TBS-380 Mini-Fluorometer (Turner BioSystems, Sunnyvale, CA, USA). Equal amounts of the resulting PCR products from each sample were pooled and electrophoresed on an agarose gel to select the DNA samples longer than 300 bp. In the end, the pooled sample was checked

Table 1. Summary of results from pyrosequencing and statistical analysis.

	Inside the fairy ring zone	The fairy ring zone	Outside the fairy ring zone
Number of total reads	10,604	15,433	13,724
Number of validated reads	9,635	15,154	12,336
Number of normalized reads	9,000	9,000	9,000
Mean read length (bp)	480.93	479.6	480.36
Maximum read length (bp)	582	544	550
Number of OTUs[a]	1,726	728	1,962
Chao1 estimation[b]	3,306	1,204	4,175
Shannon index[c]	6.227	4.948	6.668
Evenness index	0.835	0.751	0.879
Good's coverage[d]	0.896	0.964	0.881

[a]OTUs: operational taxonomic units.

[b]Chao1 estimation: species richness.

[c]Shannon index: species diversity.

[d]Good's coverage: proportions of non-singleton phylotypes in total sequences.

again for fragment length using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) [36].

The pyrosequencing was carried out by Chunlab Inc. (Seoul, South Korea) using the 454 GS-FLX Titanium Sequencing System (Roche, Branford, CT, USA), based on the manufacturer's instructions. All pyrosequencing data were submitted to the EMBL Sequence Read Archive (SRA) database under the study accession number PRJEB4170 (http://www.ebi.ac.uk/ena/data/view/PRJEB4170).

Taxonomic Assignment of Sequencing Reads and Statistical Data Analysis

All pyrosequencing reads from different soil samples were processed by trimming barcodes and primers and by filtering sequences with low quality. Taxonomic assignments of all pre-processed sequences were performed using the Ribosomal Database Project (RDP) Naïve Bayesian rRNA Classifier (ver. 2.5) with a default confidence threshold (80%) [26, 37, 50].

All statistical analyses of the fungal communities were performed using CLcommunity software (Chunlab, Inc., Seoul, South Korea). As the total number of initial validated reads varied depending on three samples, random subsampling (9,000 reads per sample) was conducted for statistical data analysis. The OTUs were defined with the CD-HIT program at a 99% sequence similarity [24]. The rarefaction curve [15] and diversity indices, such as Chao1 and Shannon, were calculated by the Mothur platform [40].

Results and Discussion

Pyrosequencing and Statistical Data Analysis

A total of 39,761 reads were pyrosequenced and 37,125 reads passed the criteria for taxonomical assignment. The number of validated reads ranged from 9,635 to 15,154 per sample, and the mean length of normalized read was approximately 480 bp. In all, 728 to 1,962 OTUs per soil sample were obtained at a 99% similarity level. The richness of fungal OTUs differed, depending on the type of sampling sites (Table 1). The rarefaction curves, which reveal the species richness of each sample, indicated that the outside of the fairy ring zone had a greater fungal community than other soil samples, whereas the fairy ring zone had the lowest number of OTUs in all soil samples (Fig. 1). The Chao1 estimation also showed that species richness significantly decreased, in the following order:

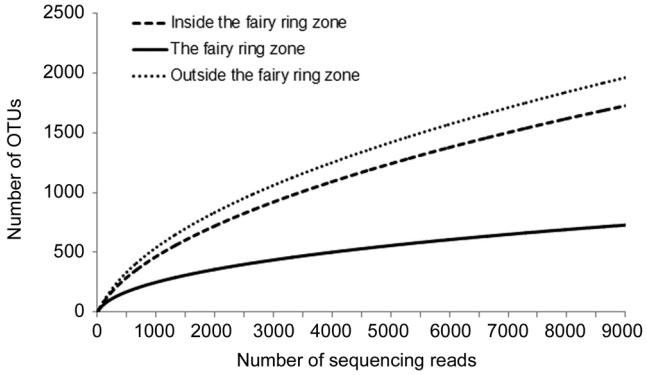

Fig. 1. Rarefaction curves for operational taxonomic units (OTUs) at each sampling site around the fairy ring of *T. matsutake* (the cutoff value is a 99% similarity).
In the rarefaction curves, the number of OTUs increases with sequencing reads. Sampling site (x, number of sequencing reads; y, number of OTUs): inside the fairy ring zone (9,000; 1,726); the fairy ring zone (9,000; 728); outside the fairy ring zone (9,000; 1,962).

outside the fairy ring zone (4,175) > inside the fairy ring zone (3,306) > the fairy ring zone (1,204). In addition, the outside of the fairy ring zone had the most diverse fungal community (Shannon index = 6.668). In contrast, the fairy ring zone had the lowest fungal diversity (4.948). The species evenness, indicating the distribution of each species in a community, indicated that the fairy ring zone had a relatively uneven distribution (Evenness index = 0.751) compared with the inside (0.835) and the outside of the fairy ring zone (0.879). Consequently, the fairy ring zone had the lowest number of OTUs (728) and fungal diversity (Shannon index = 4.948) in all sampling zones, despite having the greatest number of sequencing reads (Table 1). The results from the fairy ring zone (the mycorrhizal zone for fruiting bodies) were considered due to the predominance of *T. matsutake*. Therefore, the farther away the forest soil fungal communities were from the fairy ring, the more diverse was their composition.

Diversity and Relative Abundance of Fungal Communities

Each sequence of fungal large-subunit rRNA gene was classified from phylum down to the genus level using the RDP Naïve Bayesian rRNA Classifier. The proportion of sequences assigned to unclassified fungi was 3.72% (inside the fairy ring zone), 2.17% (in the fairy ring zone), and 7% (outside the fairy ring zone). Taxonomic composition analysis was conducted at the phylum level (Fig. 2). A total of five phyla were identified: Basidiomycota, Ascomycota, Chytridiomycota, Glomeromycota, and Zygomycota; but the fairy ring zone only had three of these phyla (lacked Glomeromycota and Zygomycota) and inside the fairy ring zone only had four (lacked Zygomycota). The Basidiomycota and Ascomycota were the dominant phyla in all soil samples tested. Inside the fairy ring zone, the taxonomic composition was Basidiomycota (54.95%), Ascomycota (41.03%), Chytridiomycota (0.26%), and Glomeromycota (0.04%). Phyla from outside the fairy ring zone consisted of Ascomycota (51.21%), Basidiomycota (41.23%), Chytridiomycota (0.48%), Glomeromycota (0.07%), and Zygomycota (0.01%). In the fairy ring zone, the Basidiomycota significantly dominated the community (92.20%), whereas Ascomycota (5.50%) and Chytridiomycota (0.13%) occupied a small proportion of the total fungal community. The relative abundance of Basidiomycota in the fairy ring zone was especially high (92.20%) compared with the inside (54.95%) and outside of the fairy ring zone (41.23%), whereas the Ascomycota was the dominant phylum outside the fairy ring zone (51.21%) compared with inside the fairy ring zone (41.03%) and in the fairy ring zone (5.50%). Overall, the inside and outside of the fairy ring zone seemed to have similar relative abundances across the phylum level, except for some differences; for instance, the abundance of Chytridiomycota outside the fairy ring zone was about two times higher than inside the fairy ring zone.

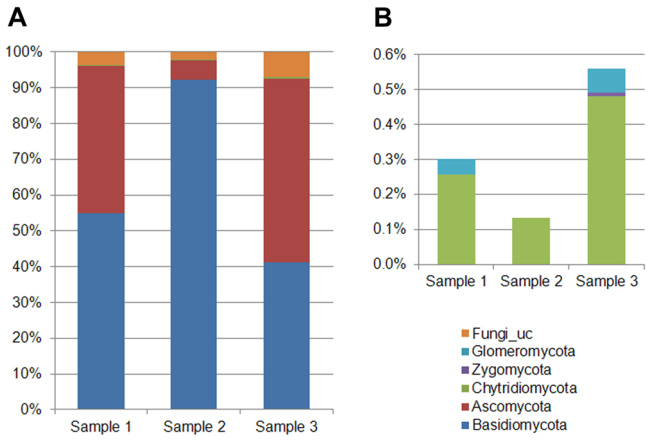

Fig. 2. Taxonomic composition analysis at the phylum level.
(**A**) The total phyla, and (**B**) a focus on Chytridiomycota, Glomeromycota, and Zygomycota. The Ascomycota and Basidiomycota are dominant phyla around the fairy ring of *T. matsutake*, whereas Chytridiomycota, Glomeromycota, and Zygomycota are rare phyla. In Sample 2 (from the fairy ring zone), members of the phylum Glomeromycota and phylum Zygomycota are not found. (Sample 1: inside the fairy ring zone; Sample 2: the fairy ring zone; Sample 3: outside the fairy ring zone; and "uc": unclassified into sublevel)

The relative abundance at the class level for Basidiomycota and Ascomycota, the most frequently observed phyla in all samples, showed distinct differences between three zones (Fig. 3). Of the total 22 classes, 10 classes from Ascomycota and eight classes from Basidiomycota were observed. The seven classes of the phylum Ascomycota commonly shared were the Leotiomycetes (inside the fairy ring zone, 12.99%; in the fairy ring zone, 2.01%; outside the fairy ring zone, 24.94%), Eurotiomycetes (4.94%; 1.21%; 14.46%), unclassified Ascomycota (9.60%; 1.49%; 6.49%), Dothideomycetes (7.85%; 0.49%; 2.16%), Sordariomycetes (3.01%; 0.16%; 2.46%), *incertae sedis* within phylum Ascomycota (2.37%; 0.04%; 0.07%), and Lecanoromycetes (0.14%; 0.04%; 0.07%). In contrast, members of Pezizomycetes were not found outside the fairy ring zone and members of Saccharomycetes were not found inside the fairy ring zone. In addition, members of Arthoniomycetes were only observed at low proportions inside the fairy ring zone (0.01%). At the level of phylum Basidiomycota, all sampling zones shared the following five classes: Agaricomycetes (53.60%; 91.29%; 34.88%), unclassified Basidiomycota (0.91%; 0.81%; 3.36%), Tremellomycetes

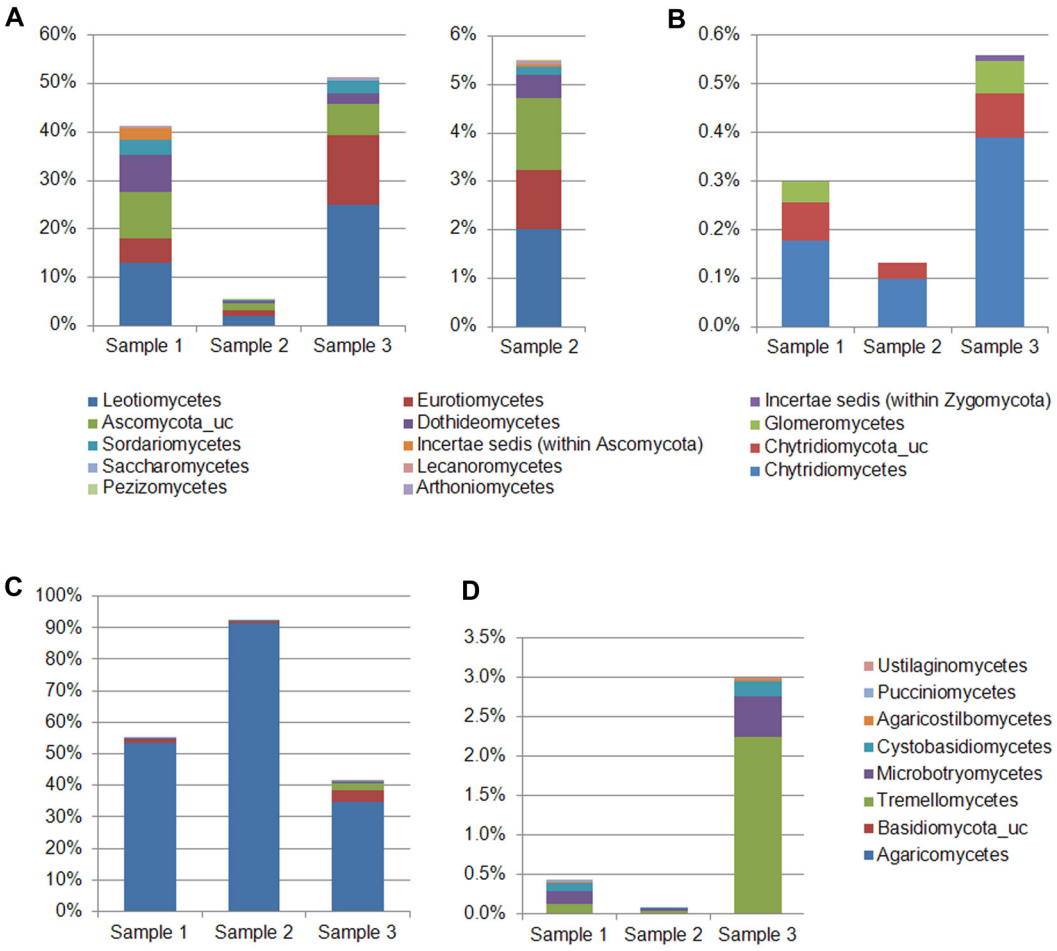

Fig. 3. Taxonomic composition analysis at the class level.

Out of a total of 22 classes, 14 classes are shared between all samples. The Agaricomycetes of the phylum Basidiomycota is the dominant class in all sampling zones, followed by Leotiomycetes of the phylum Ascomycota. (**A**) shows 10 fungal classes of the phylum Ascomycota, (**B**) shows fungal classes from the phyla Chytridiomycota, Glomeromycota, and Zygomycota, and both (**C**) and (**D**) show eight fungal classes of the phylum Basidiomycota. (Sample 1: inside the fairy ring zone; Sample 2: the fairy ring zone; Sample 3: outside the fairy ring zone; and "uc": unclassified into sublevel)

(0.13%; 0.04%; 2.24%), Microbotryomycetes (0.16%; 0.02%; 0.52%), and Cystobasidiomycetes (0.10%; 0.02%; 0.18%). In contrast, members of Agaricostilbomycetes were not found in the fairy ring zone. Ustilaginomycetes (0.01%) was only observed outside the fairy ring zone and Pucciniomycetes (0.03%) was only observed inside the zone. The other three phyla, Chytridiomycota (2), Glomeromycota (1), and Zygomycota (1), had fewer classes than Ascomycota and Basidiomycota. The sum of relative abundance for these four minor classes comprised less than 1%. For members of the phylum Chytridiomycota, both Chytridiomycetes (0.18%; 0.10%; 0.39%) and unclassified Chytridiomycota (0.08%; 0.03%; 0.09%) were shared classes. However, Glomeromycetes of Glomeromycota and an unknown class of Zygomycota (genus *Piptocephalis*) were not found in the fairy ring zone. Moreover, the latter unknown class was not found inside the fairy ring zone. The dominant class found (Agaricomycetes) was consistent with results from two previous studies. In one study of the underground community structure of ECM fungi around the *T. matsutake* fairy ring, the frequently observed species, *Russula* sp. and *Craterellus* sp. (both inside and outside the fairy ring), and *T. matsutake* (beneath the fairy ring), belonged to class Agaricomycetes [25]. In the other study, pyrosequencing analysis of fungal diversity in forest soil revealed that Agaricomycetes was the dominant fungal class [5].

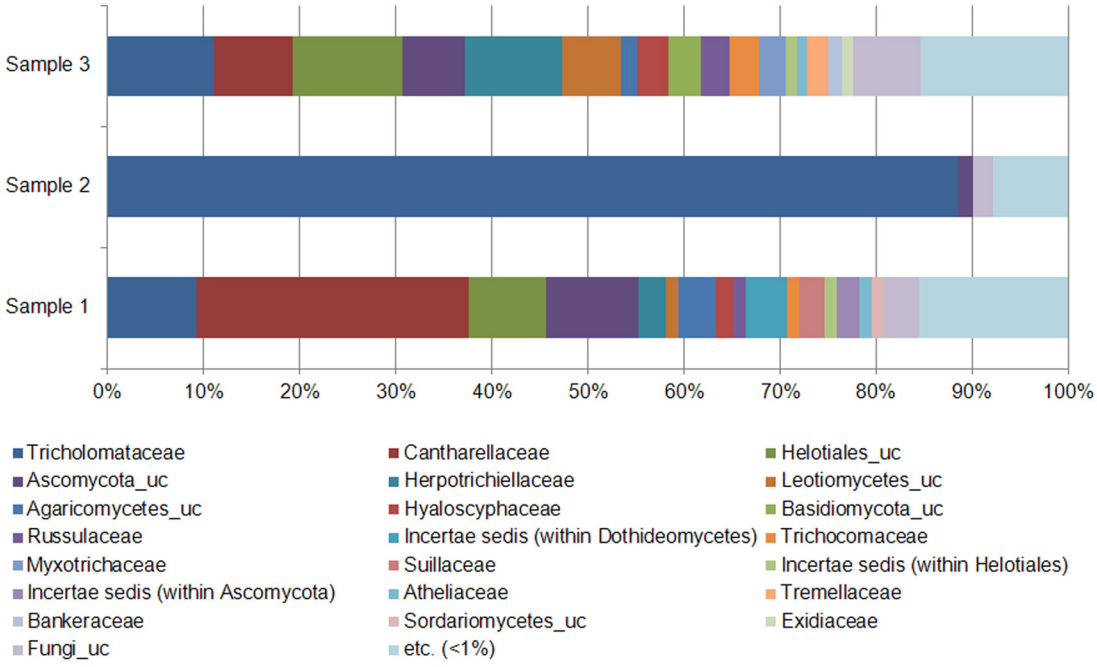

Fig. 4. Taxonomic composition analysis at the family level (>1% of total sequences in each sample).
Out of 128 families, 53 families are shared between all sampling zones. There are significant differences in family composition relative to the fairy ring of *T. matsutake*. The dominant family is Cantharellaceae (28.40%) in Sample 1, Tricholomataceae (88.57%) in Sample 2, and unclassified Helotiales (11.47%) in Sample 3. (Sample 1: inside the fairy ring zone; Sample 2: the fairy ring zone; Sample 3: outside the fairy ring zone; and "uc": unclassified into sublevel)

A total of 128 families were observed and 53 families overlapped in all samples (Fig. 4). The total number of families was roughly similar between the inside of the fairy ring zone (100) and the outside of the fairy ring zone (98), but there was a significantly different composition between the two zones. The fairy ring zone had 61 families. Among them, Myriangiaceae (0.01%), unknown family of genus *Nigrospora* (0.01%), and unclassified Rhizophydiales (0.01%) occurred exclusively in the fairy ring zone (Table 2); however, 20 families were not in the fairy ring zone and these families were also rarely observed both inside and outside the fairy ring zone. The 20 families were Amanitaceae, Amphisphaeriaceae, Astraeaceae, Bankeraceae, Bionectriaceae, Chionosphaeraceae, Dothioraceae, Glomeraceae, Gomphidiaceae, Helotiaceae, Microthyriaceae, Montagnulaceae, Mycosphaerellaceae, Pleosporaceae, *incertae sedis* within class Leotiomycetes, *incertae sedis* within class Sordariomycetes, unclassified Glomeromycetes, unclassified Pleosporales, unclassified

Table 2. List of genera unique to the fairy ring zone of *T. matsutake*.

Phylum	Family	Genus	Fr[a]	Relative abundance
Basidiomycota	Tricholomataceae	*Hemimycena* spp.	10	0.11%
Ascomycota	Trichocomaceae	*Eurotium* sp.	1	0.01%
Ascomycota	Myriangiaceae	*Myriangium* sp.	1	0.01%
Ascomycota	*Incertae sedis*	*Nigrospora* sp.	1	0.01%
Ascomycota	Verrucariaceae	*Verrucaria* sp.	1	0.01%
Chytridiomycota	Rhizophydiales_uc[b]	Rhizophydiales_uc[b]	1	0.01%

A total of six genera and 15 fungal strains are uniquely present in the fairy ring zone. Among them, *Hemimycena* spp., which belong to the family Tricholomataceae of the phylum Basidiomycota, comprise a large proportion of total fungal strains.

[a]Frequency of fungi detected in the fairy ring zone.

[b]Unclassified into sublevel.

Russulales, and unclassified Sebacinales. The top five families inside the fairy ring zone were Cantharellaceae (28.40%), unclassified Ascomycota (9.60%), Tricholomataceae (9.27%), unclassified Helotiales (7.99%), and *incertae sedis* within class Dothideomycetes (4.34%). In the fairy ring zone, Tricholomataceae (88.57%) accounted for the vast majority of families, followed by unclassified Ascomycota (1.49%), unclassified Helotiales (0.90%), unclassified Basidiomycota (0.81%), and unclassified Agaricomycetes (0.80%) at very low proportions. Outside the fairy ring zone, unclassified Helotiales (11.47%), Tricholomataceae (11.15%), Herpotrichiellaceae (10.07%), Cantharellaceae (8.14%), and unclassified Ascomycota (6.49%) were the dominant families.

A total of 209 genera were observed, with the following number in each zone: 158 (inside the fairy ring zone), 88 (in the fairy ring zone), and 143 (outside the fairy ring zone). The results of classification on the genus level showed some fungal genera specifically present or absent in the fairy ring zone (Tables 2 and 3). In total, six genera were present and 31 genera were absent. The genera present, in order of relative abundances, were *Hemimycena* (0.11%), *Eurotium* (0.01%), *Myriangium* (0.01%), *Nigrospora* (0.01%), *Verrucaria* (0.01%), and unclassified Rhizophydiales (0.01%). The top three genera absent (Table 3) were the same both inside and outside the fairy ring zone: *Boletopsis*, *Mycena*, and *Repetophragma*. The genus *Mycena* of the family Tricholomataceae had a relatively high proportion between the two zones (inside, 3.60%; outside, 0.44%). The dominant genera were different in each zone. The uncultured Cantharellaceae (28.36%) and unclassified Ascomycota (9.60%) were frequently found inside the fairy ring zone. The unclassified Helotiales (11.47%) and uncultured Cantharellaceae (7.33%) dominated outside of the fairy ring zone. In the fairy ring zone, uncultured Tricholomataceae (46.80%) and unclassified Tricholomataceae (41.62%), both belonging to the family Tricholomataceae, were the dominant genera (as expected owing to *Tricholoma matsutake*), whereas other fungi were observed as very low compositions. These results for dominant genera at the fairy ring coincided with several studies [25, 46].

In this study, the total number of OTUs and fungal taxa inside and outside the fairy ring zone were, respectively, about 2 times and 1.5 times higher than the fairy ring zone of *T. matsutake*. The results corroborate a previous study that investigated the decrease of fungal populations in the fairy ring zone of *T. matsutake*, and indicated that the mycelia of *T. matsutake* form fruiting bodies under little competition with other microorganisms [25, 31]. Previous studies have only reported the structure of ECM fungal communities associated with *T. matsutake* or have only

Table 3. List of genera specifically absent in the fairy ring zone of *T. matsutake*.

Genus	Inside the fairy ring zone		Outside the fairy ring zone	
	Fr[a]	Relative abundance	Fr[a]	Relative abundance
Amanita spp.	40	0.45%	1	0.01%
Anungitopsis spp.	1	0.01%	2	0.02%
Aspergillus spp.	1	0.01%	4	0.04%
Astraeus spp.	3	0.03%	10	0.11%
Bionectria spp.	2	0.02%	8	0.09%
Boletopsis spp.	57	0.63%	129	1.44%
Chroogomphus spp.	1	0.01%	1	0.01%
Collophora spp.	6	0.07%	8	0.09%
Cudoniella spp.	1	0.01%	5	0.06%
Glomus spp.	1	0.01%	4	0.04%
Gomphidius spp.	14	0.16%	1	0.01%
Hilberina spp.	3	0.03%	1	0.01%
Hypomyces spp.	1	0.01%	9	0.10%
Lecanicillium spp.	2	0.02%	1	0.01%
Microthyrium spp.	1	0.01%	1	0.01%
Mycena spp.	323	3.60%	39	0.44%
Phaeomoniella spp.	1	0.01%	2	0.02%
Pyrenochaeta spp.	6	0.07%	5	0.06%
Repetophragma spp.	83	0.92%	22	0.25%
Sydowia spp.	13	0.14%	1	0.01%
Tomentellopsis spp.	5	0.06%	14	0.16%
Uncultured Atheliaceae	1	0.01%	1	0.01%
Amphisphaeriaceae_uc[b]	6	0.07%	1	0.01%
Ceratobasidiaceae_uc[b]	19	0.21%	18	0.20%
Glomeromycetes_uc[b]	3	0.03%	2	0.02%
Helotiaceae_uc[b]	2	0.02%	1	0.01%
Montagnulaceae_uc[b]	11	0.12%	1	0.01%
Pleosporales_uc[b]	6	0.07%	8	0.09%
Russulales_uc[b]	2	0.02%	9	0.10%
Sebacinales_uc[b]	23	0.26%	15	0.17%
Verrucariaceae_uc[b]	2	0.02%	1	0.01%

A total of 31 genera were present both inside and outside the fairy ring zone and not in the zone of mycorrhizae for fruiting bodies. Among them, *Mycena* spp., which belongs to the family Tricholomataceae, exists between the two zones with the highest frequency count, followed by *Boletopsis* spp. and *Repetophragma* spp.

[a]Frequency of fungi detected in each sampling zone.

[b]Unclassified into sublevel.

applied the Sanger sequencing method for all fungal communities [25, 46]. This study investigated overall fungal communities related to the fairy ring of *T. matsutake* using pyrosequencing technology. Like earlier studies, the pyrosequencing results suggest considerable changes in fungal communities depending on *T. matsutake* mycelia. In a previous study, there were no significant differences in ECM communities between the inside and outside of fairy rings [25]; however, in this study, there were differences in fungal communities between the inside and outside of the fairy ring zone. These results may be caused by a difference in methods applied, since pyrosequencing technology enables massive microbes to be directly identified from environmental samples. Therefore, it is an appropriate method for acquiring high-throughput data more quickly and accurately for the assessment of microbial communities, compared with the Sanger sequencing method. From a our pyrosequencing results, although a number of OTUs were obtained, most sequences were unclassifiable. For more accurate taxonomic assignment, not only analyzing sequences targeting other regions of rRNA, but also expanding identification databases are needed.

In summary, the results of our metagenomic analysis show that the fairy ring zone of *T. matsutake* had the lowest OTUs, fungal diversity, and evenness in all sampling zones and that the taxonomic composition of fungi differed according to their relative location from the fairy ring of *T. matsutake*. A total of five phyla (Basidiomycota, Ascomycota, Chytridiomycota, Glomeromycota, and Zygomycota) were observed outside the fairy ring zones, whereas inside the fairy ring zone had four of these phyla (lacked Zygomycota) and in the fairy ring zone only had three (lacked Glomeromycota and Zygomycota). The dominant fungal class was Agaricomycetes in all sampling zones tested. A total of 128 families were observed and 53 families overlapped in all samples. The fairy ring zone had 61 families, including three exclusive families: Myriangiaceae, unknown family of genus *Nigrospora*, and unclassified Rhizophydiales. A total of 209 genera were classified and the number of genera inside, beneath, and outside the fairy ring zone was 158, 88, and 143, respectively. Specifically, in the fairy ring zone, six genera, such as genus *Hemimycena*, were uniquely present, and 31 genera, such as *Mycena*, *Boletopsis*, and *Repetophragma*, were specifically absent. These results are expected to contribute toward an expanding foundation for scientific knowledge on the fungal community in habitats of *T. matsutake*. Further studies need to investigate how these differences in fungal communities influence the formation of fruiting bodies of *T. matsutake*.

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