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# Detecting Nonculturable Bacteria in the Active Mycorrhizal Zone of the Pine Mushroom *Tricholoma matsutake*

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**The fungus *Tricholoma matsutake* forms an ectomycorrhizal relationship with pine trees. Its sporocarps often develop in a circle, which is commonly known as a fairy ring. The fungus produces a solid, compact, white aggregate of mycelia and mycorrhizae beneath the fairy ring, which in Japanese is called a 'shiro'. In the present study, we used soil dilution plating and molecular techniques to analyze the bacterial communities within, beneath, and outside the *T. matsutake* fairy ring. Soil dilution plating confirmed previous reports that bacteria and actinomycetes are seldom present in the soil of the active mycorrhizal zone of the *T. matsutake* shiro. In addition, the results showed that the absence of bacteria was strongly correlated with the presence of *T. matsutake* mycorrhizae. The results demonstrate that bacteria, especially aerobic and heterotrophic forms, and actinomycetes, are strongly inhibited by *T. matsutake*. Indeed, neither bacteria nor actinomycetes were detected in 11.3% of 213 soil samples from the entire shiro area by culture-dependent methods. However, molecular techniques demonstrated that some bacteria, such as individual genera of *Sphingomonas* and *Acidobacterium*, were present in the active mycorrhizal zone, even though they were not detected in soil assays using the dilution plating technique.**

***Keywords***: bacterial communities, bacterial populations, PCR-DGGE, Shiro, *Tricholoma matsutake*

## Introduction

The mycorrhiza-forming fungus *Tricholoma matsutake* is mainly associated with Japanese red pine (*Pinus densiflora* Sieb. et Zucc.). The mycorrhizae and hyphae of *T. matsutake* form a whitish mycelium-soil aggregate zone, which in Japanese is called a 'shiro'. The shiro is characteristic of this microbial community and normally occurs 10–20 cm below the soil surface in the B horizon of mineral soil (Yamada *et al.*, 2006). The 'shiro' occurs under a ring of sporocarps known as a fairy ring, which spreads 10–15 cm outwards every year and corresponds to mycorrhizal development on the pine rootlets. Therefore, the outermost peripheral layer of the shiro consists of young and active mycorrhizae, i.e. an active mycorrhizal zone (AMZ) from which the 'matsutake' mushrooms form in the following year. The mycorrhizae degenerate inside the active mycorrhizal zone (AMZ), where *T. matsutake* and any other mushrooms seldom occur.

Bacteria have many and varied effects on mycorrhizal symbioses, ranging from beneficial to deleterious effects (Fitter and Garbaye, 1994; Garbaye, 1994). For instance, some mycorrhizal fungi inhibit bacterial populations (Olsson *et al.*, 1996). Ohara (1966) showed that fresh mycorrhizae of *T. matsutake* aseptically obtained from the AMZ inhibited *Arthrobacter simplex* and *Agrobacterium radiobacter* on culture media. As well, the inhibitory effect on bacterial growth occurred only with black and branched mycorrhizae originating from the AMZ. In a *Pinus densiflora* forest, Ohara and Hamada (1967) investigated the bacterial community inside, beneath, and outside the AMZ using a dilution plating technique. They found that *T. matsutake* had antagonistic effects on soil bacteria, giving rise to the assumption that bacteria and actinomycetes rarely occur in the mycorrhizosphere soil of the *T. matsutake* AMZ. Considering the abundance of bacteria in the surrounding soil, it is very unusual that there are no bacteria in the AMZ. One possible explanation is that the dilution plating method, as a culture-dependent method, can only detect culturable bacteria. Those bacteria that require other nutrients or vitamins, or those that are in a particular physiological condition (e.g., in a sublethal or injured state) fail to grow (Rantsiou *et al.*, 2005), and therefore, are not detected.

Recent advances in molecular techniques enable detection of bacteria that are seldom, if ever, cultured *in vitro*. In the present study, we used denaturing gradient gel electrophoresis (DGGE) as a culture-independent molecular method to analyze the bacterial community within, beneath, and outside the AMZ of *T. matsutake*. This method is useful for detecting any bacterium, regardless of its culturability. As such, it may allow the detection of novel bacteria that would go undetected by traditional, culture-based approaches. Such a molecular technique might permit the detection of bacteria in the soil of AMZ. The objects of the present

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work were (1) to confirm the antagonistic effects of the AMZ using the methods reported by Ohara and Hamada (1967), and (2) to use PCR-DGGE to detect previously undetected bacteria in the soil of the *T. matsutake* AMZ.

## Materials and Methods

### Study site

This site was conducted at long-term *T. matsutake* (S. Ito et Imai) research sites in a pine forest. We examined three *T. matsutake* fairy rings (No. 1, 2, and 3) in a *Pinus densiflora* forest at Sakai, Kyotanba, Kyoto, Japan (35°11′N, 135°20′E) (Fig. 1A). The distances between No. 1, and 2, between No. 1 and 3, and between No. 2 and 3 were ~ 87.1, 123.2, and 205.8 m, respectively. The mean annual temperature and precipitation at the site were 13.5°C and 1324 mm. The soil texture is clay, and 40–50-year-old *P. densiflora* (originating from natural regeneration) was the main vegetation.

### Sporocarp sampling

In 2006, 13, 15, and 57 sporocarps of *T. matsutake* occurred at fairy ring No. 1, No. 2, and No. 3, respectively. In 2007, 18, 18, and 11 sporocarps occurred at the corresponding fairy rings. The positions of sporocarps at each fairy ring are shown in Fig. 1A.

### Soil sampling to estimate distribution of *T. matsutake* mycorrhizae and bacteria

In each fairy ring, two transect lines were placed from the center of the ring outwards. Along each line, six locations were marked out at 10-cm intervals to collect soil samples (Fig. 1B). There were three locations within each of the three fairy rings (A, B, and C), one situated on the AMZ where sporocarps occurred in 2007 (D) and 10 cm (E), and 20 cm outside of the AMZ (F) (Fig. 1C). To determine mycorrhizal distribution, soil blocks (10×10×5 cm) were collected at each location on 15 November, 2007, at soil depths of 0–5, 5–10, 10–15, 15–20, 20–25, and 25–30 cm. For each sample, all the *P. densiflora* roots were washed carefully to remove adhering soil, and sorted into non-ectomycorrhizal and ectomycorrhizal fine roots (≤2 mm in diameter) colonized by both *T. matsutake* (hereafter referred to as *T. matsutake* mycorrhizae), and other types of ectomycorrhizal fine roots, based on their morphotype (Kikuchi and Futai, 2003). The samples were dried in a forced-draft oven at 60°C for >48 h and weighed. To sample for bacteria, a sterilized, 2-ml centrifuge tube was inserted at a right angle into the vertical wall of each hole that had been dug to sample mycorrhizae. Bacterial samples were taken at depths of 0–5, 5–10, 10–15, 15–20, 20–25, and 25–30 cm. This method prevented the samples from becoming contaminated with organic matter from the soil surface (Ohara and Hamada, 1967). Bacteria were isolated from the samples using the dilution plating technique and their abundance was estimated. The bacteria were cultured on three different media: GPYS agar medium containing (w/v) 1.0% glucose, 1.0% peptone, 0.5% yeast extract, 100 ml soil extract, and 1.5% agar (Ohara and Hamada, 1967); Thornton's asparagine-mannitol agar medium containing (w/v) 0.1% $K_2HPO_4$, 0.02% $MgSO_4$, 0.01% $CaCl_2$, 0.01% NaCl, $FeCl_3$, 0.05% $KNO_3$, 0.05% asparagine, 0.1% mannitol, and 0.025% yeast extract (Ohara and Hamada, 1967); and nutrient agar medium containing (w/v) 1% peptone, 1% beef extract, 0.5% NaCl, and 1.5% agar. Under aseptic conditions, a 0.2 g subsample was taken from each soil sample and added to 4.5 ml sterile, distilled water. After vortexing the soil suspension, 50 µl of each soil solution was pipetted onto each of the three culture media and incubated at 25°C for 72 h (Ohara and Hamada, 1967). There were three replicates for each medium. After 72 h, the bacterial colonies were counted to estimate bacterial distribution and abundance in the shiro. The distribution of actinomycetes was unique in the shiro and only *Streptomyces* sp. were detected in some soil samples.

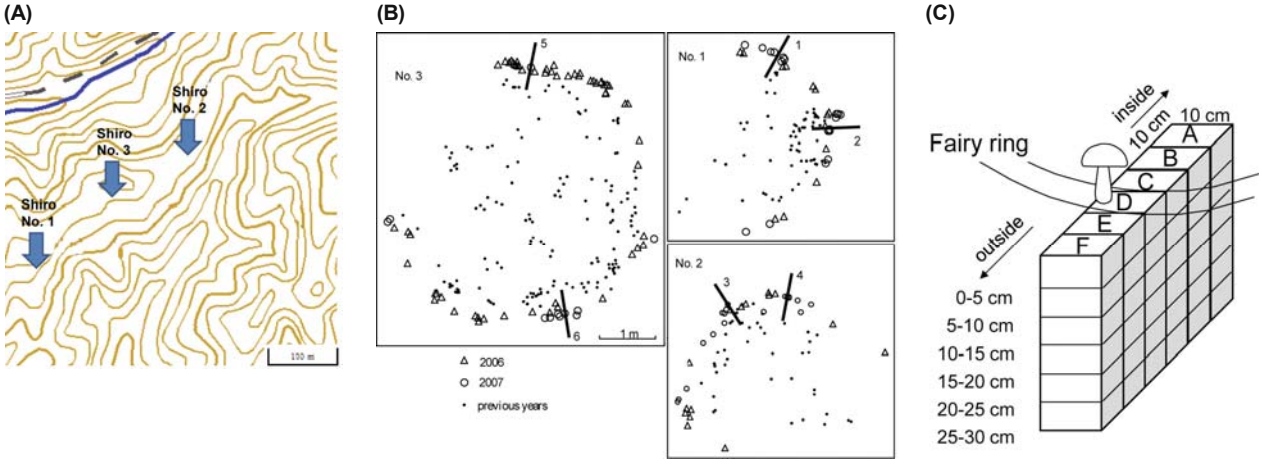


**Fig. 1. (A) Map of the study area (long-term *T. matsutake* research sites in a pine forest of the Kyoto Forest Research Institute).** (B) Spatial distribution of *T. matsutake* sporocarps in each fairy ring at the study sites. Open circle, open triangle, and closed circle represent a *T. matsutake* sporocarp that occurred in 2007, 2006, and before 2006 (previous years), respectively. (C) Schematic image of horizontal [A–F] and vertical (up to 25–30 cm in depth) locations of soil sampling.

Therefore, the actinomycetes colonies and other bacterial colonies were counted separately, based on their morphologically distinct forms, i.e., hard or non-hard and filamentous or non-filamentous forms, while it was difficult to distinguish between some bacteria and actinomycetes that formed similar colonies. As well, *Mortierella* species were also counted separately, based on their morphological features. *Mortierella* species appeared on the media as a unique fungus in some soil samples (Fig. 2D).

### Community diversity and abundance of culturable bacteria in *T. matsutake* shiro

To determine the profile (diversity and abundance per phylogenetic type) of culturable bacteria in the *T. matsutake* shiro, we studied in detail the soil samples from fairy ring No. 1. This fairy ring was selected because it was very active, with as many as 18 fruiting bodies produced in 2007. We used a random number table to randomly select 300 bacterial colonies from all of the dilution plates (>500 plates from 84 soil samples obtained from all soil depths ranging from 0 to 30 cm). After extracting the DNA from each of the bacterial colonies (Kataoka and Futai, 2009), the 16S rRNA region was PCR-amplified using the primers 9F (GAGTTTGATCCTGGCTCAG) and 1541R (AAGGAGGTGATCCAGCC) (Lane, 1991). The PCR amplification system and conditions were as follows: Gene Amp PCR System 9700 (Applied Biosystems, USA), with 1 cycle of 95°C for 5 min, 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min. The PCR products were

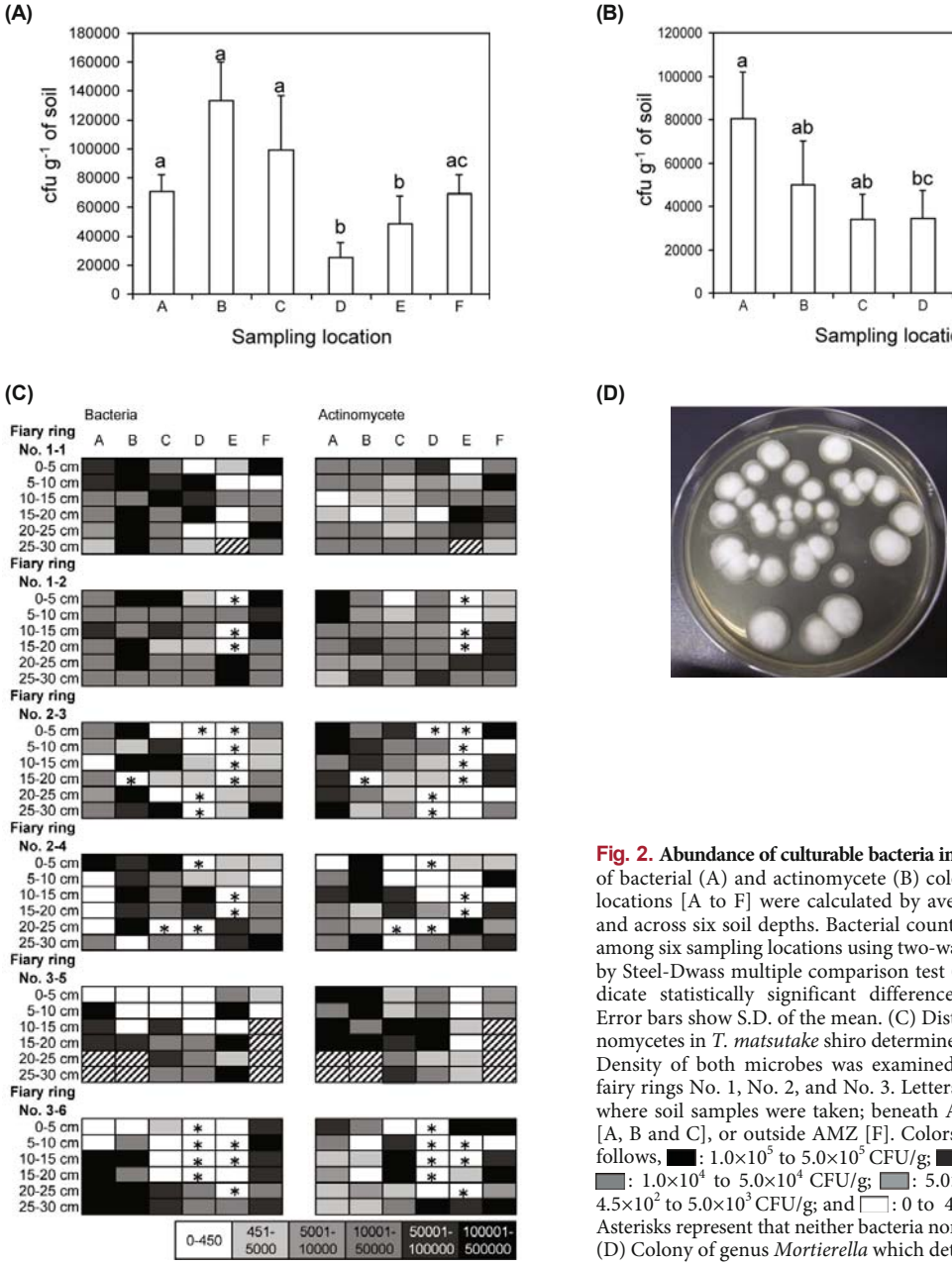


**Fig. 2. Abundance of culturable bacteria in *T. matsutake* shiro.** Numbers of bacterial (A) and actinomycete (B) colonies at each of six sampling locations [A to F] were calculated by averaging over three fairy rings and across six soil depths. Bacterial counts were statistically compared among six sampling locations using two-way ANOVA ($P<0.05$) followed by Steel-Dwass multiple comparison test ($P<0.05$). Different letters indicate statistically significant differences among sample locations. Error bars show S.D. of the mean. (C) Distribution of bacteria and actinomycetes in *T. matsutake* shiro determined by dilution plating method. Density of both microbes was examined along two transect lines in fairy rings No. 1, No. 2, and No. 3. Letters [A to F] represent locations where soil samples were taken; beneath AMZ [D and E], inside AMZ [A, B and C], or outside AMZ [F]. Colors indicate bacterial density as follows, ■: $1.0\times10^5$ to $5.0\times10^5$ CFU/g; ■: $5.0\times10^4$ to $1.0\times10^5$ CFU/g; ■: $1.0\times10^4$ to $5.0\times10^4$ CFU/g; ■: $5.0\times10^3$ to $1.0\times10^4$ CFU/g; □: $4.5\times10^2$ to $5.0\times10^3$ CFU/g; and □: 0 to $4.5\times10^2$ CFU/g. ▨: bedrock. Asterisks represent that neither bacteria nor actinomycetes were detected. (D) Colony of genus *Mortierella* which detected from the soil of AMZ.

digested with two restriction enzymes, *Alu*I and *Hin*fI, and then separated by electrophoresis on 1.5% (w/v) agarose gels. The resulting banding patterns allowed classification of bacteria into 25 types. We conducted sequencing analysis to identify each of the 25 bacterial types at the genus level. The 25 bacterial types were re-amplified by PCR as described above using the 341f and 907r primer set (app. 600 bp). The PCR-amplified DNA fragments were purified and directly sequenced using the Big Dye Terminator v.1.1 Cycle Sequencing kit (Applied Biosystems) and either of the primers 341f or 907r for 16S rRNA, according to the manufacturer's instructions. Nucleotide sequences were determined with an Applied Biosystems 3300 sequencer (Applied Biosystems). BLAST searches were carried out to compare sequences with those in the GenBank database. The accession numbers were obtained from the DNA data bank of Japan (DDBJ) for each bacterium.

### Detection of nonculturable bacteria using PCR-DGGE

There were 24 soil samples in which no bacteria were detected by the dilution plating method. From these 24 samples, we randomly selected nine for further analysis: two, three, and four samples from fairy rings No. 1 (location D), No. 2 (location D), and No. 3 (location C, D), respectively. As controls, six soil samples were collected from locations A to F of fairy ring No. 1 (depth; 0–5 cm) and another soil sample was collected 3 m away from the fairy ring. The DNA was directly extracted from 0.1 g soil using an ISOIL DNA Isolation kit (Nippon Gene Co. Ltd., Japan). The primers used were 341f (CCTACGGGAGGCAGCAG) and 907r (CCGTCAATTCCTTTGAGTTT), which correspond to positions 341–357 to 907–926 in *Escherichia coli* (Muyzer *et al.*, 1993), were used. To facilitate DGGE separation, a GC-rich sequence (5′-CGCCCGCCGCGCCCCGCGCCC GTCCCGCCGCCCCCGCCCG-3′) was attached to the 5′ end of the 341f primer. The PCR system and conditions were as follows: Gene Amp PCR System 9700, with 1 cycle at 95°C for 5 min, 20 cycles at 94°C for 30 sec, 65 to 55°C for 30 sec with a 1°C touchdown every second cycle and 72°C for 1 min, and 15 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min with a final extension at 72°C for 10 min. The amplicons were separated on 1.5% (w/v) agarose gels, stained with ethidium bromide (1 mg/L) for 30 min, and visualized using an UV light transilluminator to check the success of the PCR amplification.

DGGE analysis of PCR-amplified 16S rDNA was carried out using 6% (w/v) acrylamide gels (37.5/1 acrylamide/bis-acrylamide), with a denaturant gradient of 20–60% (100% denaturant consisted of 7 M urea and 40% (v/v) formamide). PCR amplicons were loaded at 5.0 μg DNA per lane. The gel electrophoreses were conducted in 0.5× Tris-acetate-EDTA (TAE) buffer at 60°C at a constant voltage of 150 V for 4 h using the Dcode system (Bio-Rad Laboratories, USA). The gels were stained for 60 min with GelRed (Wako Laboratory Chemicals, Japan) (1 mg/L) and photographed using a UV light transilluminator. The resulting banding patterns were compared to those of the controls.

### Evaluation of community diversity from the DGGE banding patterns

The DGGE gel was digitized and the background removed using Image J software (http://rsb.info.nih.gov/ij/). The community diversity of the bacteria in each sample was evaluated by the Shannon-Wiener diversity index (H'), which was calculated as follows: $H' = - \Sigma(P_i \ln P_i)$, where $P_i$ is the importance probability of the bands in a gel lane, calculated using the formula $P_i = n_i/N$, where $n_i$ is the intensity of a band and N is the sum of intensities of all bands (Hoshino and Matsumoto, 2007).

### Recovery of bands from DGGE gels and sequence analysis for bacteria

The prominent DGGE bands were interpreted as those that reflected dominant bacteria. These bands were selected for determining partial nucleotide sequences to identify the bacteria to the genus level. Each band selected was excised and placed in a 0.5 ml micro-tube and 0.1 ml of TE buffer was added. The tubes were incubated at 4°C overnight. A 1-μl aliquot of TE buffer containing DNA was used as the template for a PCR (1 cycle at 95°C for 5 min, 30 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min, with a final extension at 72°C for 10 min). The PCR-amplified DNA fragments were purified and directly sequenced using the Big Dye Terminator v.1.1 Cycle Sequencing kit (Applied Biosystems) and either the primers 341f or 907r for 16S rRNA according to the manufacturer's instructions. Nucleotide sequences were determined with an Applied Biosystems 3300 sequencer (Applied Biosystems). BLAST searches were carried out to compare sequences with those deposited in the GenBank database. The accession numbers were obtained from the DNA Data Bank of Japan (DDBJ) for each bacterium.

### Statistical analyses

For each soil depth at each sampling location, six replicates of data (= two transect lines×three fairy rings) were obtained for the bacterial colonies. These data were statistically compared among the six sampling locations (A to F) and among six soil depths at each sampling location using two-way ANOVA ($P<0.05$) followed by Steel-Dwass tests to distinguish the significance of mean differences ($P<0.05$) (Dwass, 1960; Steel, 1960).

## Results

### Abundance of culturable bacteria in *T. matsutake* shiro

Using the data obtained by the dilution plating technique, we compared the abundance of actinomycetes (Gram-positive bacteria) and other bacteria separately. Neither bacteria nor actinomycetes were detected in 11.3% of 213 soil samples collected from the entire shiro area and the shiro itself. Most of those samples were collected beneath the fruiting bodies (location D) or 10 cm outside the fruiting bodies (location E). There was no significant difference ($p<0.05$) in bacterial abundance among the three fairy

**Table 1. Relative abundance of bacteria obtained from a *T. matsutake* shiro using the dilution plating technique.** Microorganisms were identified to genus level using PCR-RFLP and sequencing analysis. Capital letters indicate soil sampling locations; A, B, and C within the active mycorrhizal zone (AMZ), D and E at the AMZ, and F outside the AMZ. Asterisk indicates occurrence of the fungus *Mortierella* sp., which was frequently detected outside the AMZ. Values are percentages.

| DDBJ ribosomal database sequence similarity to next related isolates | Similarity (%) | Overlap (bp) | Isolate | Accession number | Frequency of bacteria in the fairy ring No. 1 (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | (/37) | (/31) | (/29) | (/40) | (/43) | (/34) |
| | | | | | A | B | C | D | E | F |
| *Streptomyces thioluteus* (AJ781360) | 99 | 516 | *Streptomyces* sp.1 | AB449081 | 11 | 0 | 0 | 0 | 0 | 6 |
| *Streptomyces* sp. (Y15505) | 99 | 498 | *Streptomyces* sp.2 | AB449082 | 8 | 6 | 14 | 8 | 0 | 24 |
| *Streptomyces cinnamoneus* subsp. Albosporus (EF654100) | 99 | 483 | *Streptomyces* sp.3 | AB449075 | 54 | 35 | 41 | 8 | 14 | 18 |
| *Streptomyces* sp. (Y15498) | 99 | 485 | *Streptomyces* sp.4 | AB449076 | 3 | 0 | 0 | 25 | 0 | 0 |
| *Streptomyces atroaurantiacus* (DQ026645) | 100 | 499 | *Streptomyces* sp.5 | AB453389 | 0 | 0 | 0 | 3 | 0 | 0 |
| *Burkholderia phenazinium* (AB021394) | 98 | 500 | *Burkholderia* sp.1 | AB449088 | 8 | 3 | 7 | 8 | 7 | 9 |
| *Burkholderia* sp. CB2 (AY178059) | 100 | 500 | *Burkholderia* sp.2 | AB449087 | 11 | 29 | 7 | 0 | 0 | 0 |
| *Burkholderia* sp. SM-30 (EF197740) | 98 | 498 | *Burkholderia* sp.3 | AB449086 | 0 | 6 | 3 | 0 | 0 | 6 |
| *Burkholderia* sp. ESR108 (EF602567) | 99 | 513 | *Burkholderia* sp.4 | AB449085 | 0 | 3 | 0 | 5 | 12 | 12 |
| *Burkholderia glathei* (AY154379) | 98 | 499 | *Burkholderia* sp.5 | AB449084 | 0 | 3 | 17 | 0 | 0 | 0 |
| *Burkholderia* sp. Yws-02 (AJ704380) | 99 | 496 | *Burkholderia* sp.6 | AB449068 | 0 | 0 | 0 | 15 | 0 | 0 |
| *Burkholderia* sp. CCBAU 11189 (EF149008) | 98 | 765 | *Burkholderia* sp.7 | AB449069 | 0 | 0 | 0 | 0 | 2 | 0 |
| *Bacillus thuringiensis* (Z84594) | 99 | 508 | *Bacillus* sp.1 | AB449090 | 5 | 6 | 3 | 18 | 2 | 0 |
| *Bacillus* sp. cryopeg_4b (AY660700) | 99 | 505 | *Bacillus* sp.2 | AB449089 | 0 | 3 | 0 | 0 | 0 | 0 |
| *Bacillus cereus* (EF690431) | 99 | 782 | *Bacillus* sp.3 | AB449066 | 0 | 0 | 3 | 3 | 0 | 0 |
| *Bacillus* sp. BS19 (EU031769) | 99 | 733 | *Bacillus* sp.4 | AB449067 | 0 | 0 | 0 | 0 | 2 | 0 |
| *Rhizobium* sp. CCBAU 43112 (DQ993272) | 98 | 513 | *Rhizobium* sp. | AB449083 | 0 | 3 | 0 | 0 | 0 | 0 |
| *Paenibacillus* sp. NR1004 (EF585246) | 99 | 494 | *Paenibacillus* sp.1 | AB449070 | 0 | 0 | 0 | 3 | 0 | 0 |
| *Paenibacillus assamensis* (AY884046) | 97 | 497 | *Paenibacillus* sp.2 | AB449071 | 0 | 0 | 0 | 3 | 0 | 0 |
| *Paenibacillus* sp. YO4-16 (AM162349) | 100 | 506 | *Paenibacillus* sp.3 | AB449072 | 0 | 0 | 3 | 0 | 0 | 0 |
| *Paenibacillus* sp. BCRC 17757 (EU179327) | 99 | 502 | *Paenibacillus* sp.4 | AB449073 | 0 | 0 | 0 | 0 | 2 | 0 |
| *Paenibacillus wynnii* (DQ870708) | 99 | 759 | *Paenibacillus* sp.5 | AB449074 | 0 | 0 | 0 | 0 | 0 | 3 |
| *Arthrobacter* sp. 17a-1 (AY561559) | 99 | 494 | *Arthrobacter* sp.1 | AB449065 | 0 | 0 | 0 | 3 | 0 | 0 |
| *Arthrobacter* sp. Ellin178 (AF409020) | 99 | 485 | *Arthrobacter* sp.2 | AB453388 | 0 | 0 | 0 | 0 | 0 | 3 |
| rhizosphere bacterium Ptrs1 (AB301921) | 99 | 508 | *Ralstonia* sp. | AB301921 | 0 | 0 | 0 | 3 | 0 | 0 |
| *Mortierella* sp. olrim89 (AY354282)* | 99 | 513 | *Mortierella* sp. | AB449080 | 0 | 0 | 0 | 0 | 58 | 21 |

rings. Therefore, the differences were analyzed among the six sampling depths and among the six locations (A to F). There were statistically significant differences both in the numbers of bacterial and actinomycete colonies among the six sampling locations, but there were no significant differences among the six sampling depths (two-way ANOVA, $p<0.05$). We then used the Steel-Dwass multiple comparison test ($p<0.05$) to determine the significance of differences among the six sampling locations (A to F) using values averaged across the six sampling depths. There were significantly fewer bacterial colonies in samples from locations D and E, compared with other locations (Fig. 2A). There were significantly fewer actinomycete colonies in samples from E than in samples from A, B, C, and F (Fig. 2B).

### Community composition of culturable bacteria in *T. matsutake* shiro

The 300 bacterial colonies obtained by dilution plating and then identified using PCR-RFLP and sequencing analysis belonged to 25 types (Table 1). Among them, *Streptomyces* spp. (Accession no. AB449081, AB449082, AB449075, AB449076, AB453389 for species 1, 2, 3, 4, 5, respectively) were the most common actinomycetes, occurring in almost all soil samples. The next most common were *Burkholderia* spp. (Accession no. AB449088, AB449087, AB449086, AB449085, AB449084, AB449068, AB449069 for species 1, 2, 3, 4, 5, 6, 7, respectively), *Bacillus* spp. (Accession no. AB449090, AB449089, AB449066, AB449067 for species 1, 2, 3, 4, respectively), and *Paenibacillus* spp. (Accession no. AB449070, AB449071, AB449072, AB449073, AB449074 for species 1, 2, 3, 4, 5, respectively). Two species of *Arthrobacter* (Accession no. AB449065 and AB453388 for species 1 and 2, respectively) and a species of *Ralstonia* (Accession no. AB301921) were detected at location D, where *T. matsutake* fruit bodies formed (Table 1). In addition, the fungus *Mortierella* sp. was frequently detected outside, but never inside, the AMZ.

### Nonculturable bacteria detected by DGGE analysis

The DGGE analysis confirmed that there were bacteria in those samples for which no bacteria were detected by the dilution plating technique (Fig. 3). Five in nine samples performed in this study were represented (Table 2), and remaining four samples were same banding pattern with them. A single species of each of the bacterial genera *Sphingomonas* and *Acidobacterium* was detected by this analysis (Table 2). In those locations (D and E), therefore, the di-

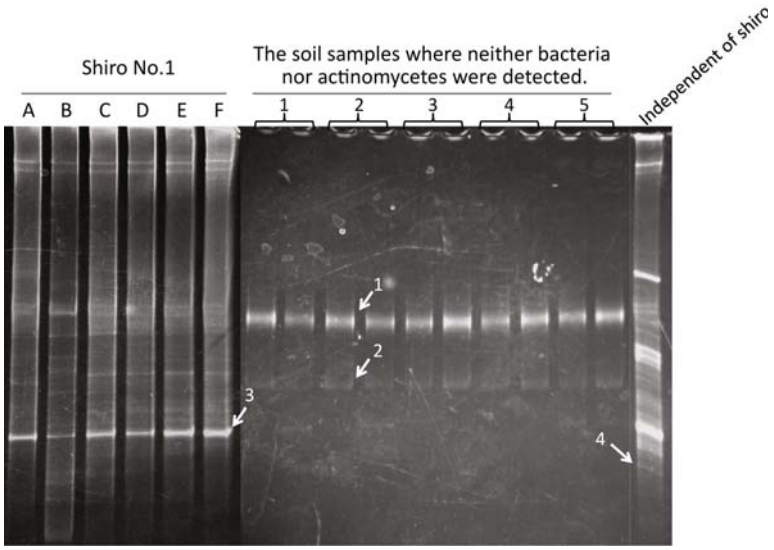


**Fig. 3. Culture-independent denaturing gradient gel electrophoresis (DGGE) image of bacterial PCR-amplified 16S rDNA.** Lanes A to F show bacterial DNA banding pattern of samples collected from 0–5 cm depth in shiro No. 1. Lanes 1–5 (two replicates per sample) show DNA samples of bacteria extracted from soils in which no bacteria were detected by the dilution plating technique. All of these soil samples were collected from beneath the AMZ. Sample in lane 17 is from a soil sample taken at a distance from the fairy ring.

versity index of bacteria was very low (0.55). This contrasts with an index value of 4.01 calculated for the soil outside the fairy ring. Thus, the diversity indices showed that the bacterial community had a much simpler structure in the AMZ. This was especially true for samples from which neither bacteria nor actinomycetes were detected, even when the molecular technique was used to detect bacteria. In contrast, the bacterial diversity inside the shiro seemed to have recovered, but the individual bands were weak compared with those in samples taken from outside the shiro.

### Relationship between bacterial density and *T. matsutake* mycorrhizae biomass

There was a significant negative correlation ($P<0.05$) between the density of culturable bacteria and the biomass of *T. matsutake* mycorrhizae, with the abundance of culturable bacteria decreasing as the biomasss of *T. matsutake* mycorrhizae increased (Fig. 4). The Pearson correlations (r) were -0.2798 ($p<0.05$), -0.2546 ($p<0.05$), and -0.4729 ($p<0.05$) in fairy ring No. 1, 2, and 3, respectively.

## Discussion

Ohara and Hamada (1967) and Ohara (1966) found that in the active mycorrhizal zone (AMZ) of *T. matsutake*, bacteria, especially aerobic and heterotrophic types and actinomycetes, were strongly inhibited. However, it has been stated elsewhere that bacteria are common inhabitants in the mycorrhizosphere, and that bacterial density in the mycorrhizosphere is higher than that in bulk soil (Mogge *et al.*, 2000; Khetmalas *et al.*, 2002; Uroz *et al.*, 2007). In the present study, the dilution plating technique detected no bacteria or actinomycetes in 24 soil samples from the AMZ. Also, the bacterial density in each fairy ring was very low beneath *T. matsutake* sporocarps (location D) and at 10 cm outside of the fairy ring (location E).

Ohara (1980) isolated some bacteria (individual species of *Sarcina* and *Micrococcus*) and actinomycetes (*Streptomyces* spp.) from inside of the AMZ of *T. matsutake*. Our results also showed that the bacterial community includes several species of *Streptomyces*, *Burkholderia*, *Bacillus*, and *Paenibacillus*, which were uniformly distributed inside the AMZ (Table 1). For example, *Streptomyces* spp. were prevalent in all locations in the *T. matsutake* shiro, representing approximately 75, 42, 55, 43, 14, and 47% of the microorganisms at locations A, B, C, D, E, and F, respectively. As shown in Fig. 2, however, the abundance of culturable bacteria, including *Streptomyces* spp., was very low in the AMZ (locations D and E), probably because of the strong antibacterial activity of *T. matsutake* mycorrhizae. Moreover, the Shannon-Wiener diversity index (H') calculated from DGGE banding intensities across sampling depths was only 0.55 at the AMZ, compared with 4.01 at 3 m away from the shiro. This result suggested that the bacterial community in the AMZ was much simpler than those at other locations in the shiro. Thus, bacterial populations and the abundance of *T. matsutake* mycorrhizae appear to be negatively related under field conditions. In fact, bacteria were hardly

**Table 2. Bacterial types detected by denaturing gradient gel electrophoresis (DGGE) that were otherwise undetectable using the dilution plating method**

| DGGE band | DDBJ ribosomal database sequence similarity to next related isolates (Accession number) | Similarity (%) | Over lap (bp) | Accession number |
|---|---|---|---|---|
| 1 | *Sphingomonas insulae* (EF363714) | 99 | 508 | AB449077 |
| 2 | *Sphingomonas insulae* (EF363714) | 99 | 508 | AB449077 |
| 3 | *Acidobacteria bacterium* Ellin7184 (AY673350) | 95 | 509 | AB449078 |
| 4 | *Acidobacteria bacterium* Ellin7184 (AY673350) | 98 | 521 | AB449078 |

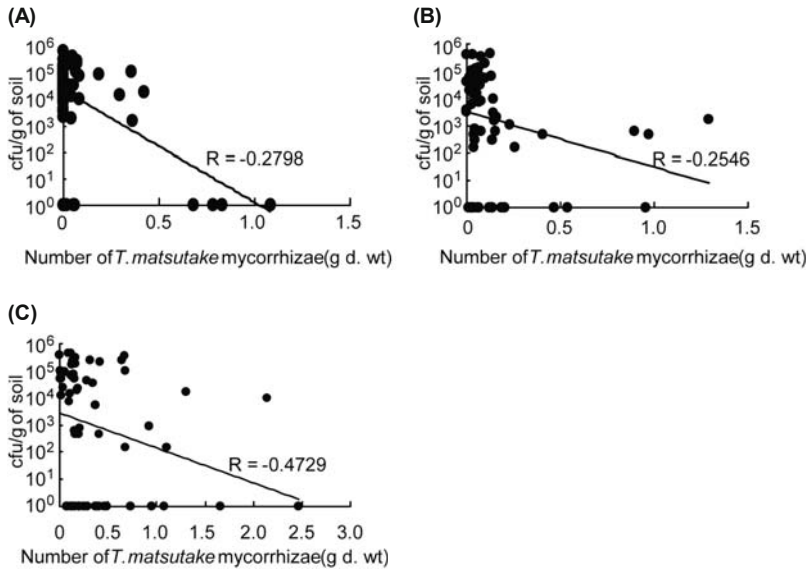


**Fig. 4. Correlations between *T. matsutake* mycorrhizae and bacterial abundance obtained from dilution plating.** (A), (B), and (C) show significant linear relationship ($P<0.05$) between bacterial abundance and mycorrhizal density at fairy ring No. 1, No. 2, and No. 3, respectively.

detected from the soil at depths of 0 to 20 cm in the AMZ (Fig. 2C), perhaps reflecting the abundance of mycorrhizal roots within that zone. The Pearson correlations (r) calculated for statistical comparison between bacterial densities and *T. matsutake* mycorrhizae abundance showed that the bacterial population decreased as *T. matsutake* mycorrhizae increased (Fig. 4). Therefore, the absence of bacteria, especially aerobic and heterotrophic, as well as actinomycetes, is closely related to the mycelial activity of *T. matsutake* or to mycorrhizal development on the pine rootlets under field conditions.

The DGGE method enabled us to detect individual types of *Sphingomonas* and *Acidobacterium* bacteria in soil samples from the AMZ of *T. matsutake* from which the dilution plating technique was not able, or barely able, to detect bacteria. Surprisingly, only two bands were detected from AMZ soil by the DGGE method. There were very few bands compared with those amplified from soil samples taken from outside the shiro. Thus, *T. matsutake* strongly inhibits bacteria, but a single type of each of the bacterial genera *Sphingomonas* and *Acidobacterium* was present in the soil of the AMZ.

Sphingomonad bacteria are ubiquitous both in non-mycorrhizosphere and mycorrhizosphere soils (Uroz *et al.*, 2007). Ohara (1980) detected Flavobacteria (including some species of *Sphingomonas*) from outside the AMZ, but not from within the AMZ of *T. matsutake*. Leys *et al.* (2004) detected *Sphingomonas* communities from polluted soils at the species level using a newly designed *Sphingomonas*-specific PCR-DGGE detection technique. The ability of these microbes to use polycyclic aromatic hydrocarbons (PAH) as their sole source of carbon and energy might explain why Sphingomonads can thrive in polluted soils. The results presented here show that *Sphingomonas* species are in the AMZ of *T. matsutake*. This indicates that they are resistant to, or are able to degrade, the antibiotics produced by *T. matsutake*.

The physiology and ecology of *Acidobacterium* spp. are poorly understood, because bacteria in the phylum Acidobacteria have only recently been discovered using molecular techniques, and most have not been cultured *in vitro*. There are over 3,000 sequences for this phylum in public databases. Almost all of these sequences were obtained from nonculturable organisms from very diverse environments (Barns *et al.*, 2007), such as soil contaminated with 2,4,6-trinitrotoluene (George *et al.*, 2009), tuberculate ectomycorrhizae of *Rhizopogon* spp. (Kretzer *et al.*, 2009), and uranium-contaminated subsurface sediments (Barns *et al.*, 2007). In the present study, an *Acidobacterium* sp. was detected from the AMZ, but its role is unknown.

Although there are many reports on the physiological effects of the host plant on *T. matsutake*, little is known about the effects of bacteria on *T. matsutake*. Our results show that there are individual species of *Sphingomonas* and *Acidobacterium* present in the AMZ of *T. matsutake*. This information might lead to new insights about the ecological roles of bacteria related to *T. matsutake*. Despite nearly a century of research (Ogawa, 1975a, 1975b, 1977), attempts to cultivate 'matsutake' have been unsuccessful. A deeper understanding of the biology and fruit body production of *T. matsutake* in pine forests, and the associations between this fungus and other microorganisms, especially bacteria, is very important for successful regeneration and management of *T. matsutake* both to protect shiro and also to ensure adequate production of 'matsutake' mushrooms.

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