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# Diversity of cultivable microorganisms of *Davidia involucrate* in rhizosphere soil

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**The purpose of this study was to assay the cultivable microorganisms of *Davidia involucrate* in rhizosphere soil and to analyze their diversity using colony and individual morphology and genetic profiling methods. A total of 118 strains were obtained after colony characterization and a microscopic examination, including 52 bacteria strains, 27 actinomycetes strains and 39 fungi strains. Fifty-seven (57) of these strains were randomly selected for rDNA analysis and phylogenetic identification, including 23 rhizobaceria isolates, 29 fungi isolates, and five streptomyces isolates. The selected isolates were accurately identified at the genus level with consistent results using a morphology examination and phylogenetic identification. From the study, we concluded the following: (1) *Bacillus*, *Lactobacillus*, *Azotobacter*, and *Streptomyces* are predominant microorganisms of *D. involucrate* in rhizophere soil and *Bacillus* is a dominant rhizobacteria that might have potential in host growth promotion; (2) the physiological activity of dovetree could significantly influence the microorganism biomass of the rhizophere soil; and (3) the dovetree is a highly complex rhizosphere ecosystem, and additional research on this topic should be carried out in the future.**

**Key words:** Rhizosphere microorganism, *Davidia involucrate*, plant growth promotion, soil.

## INTRODUCTION

Rhizophere microorganisms play a central role in the regulation of soil structure (Aira et al., 2010), formation of symbiosis (Filippi et al., 1995), controlling plant pathogens (Yang and Cao, 2012) and nutrient cycling (Cambardella and Eliott, 1992; Collins et al., 1992). Helman et al. (2011) summarized that among the microorganisms inhabiting the rhizosphere, several are plant growth promoting rhizobacteria (PGPR), such as genera of *Azospirillum*, *Herbaspirillum*, *Gluconacetobacter*, *Burkholderia*, *Pseudomonas*, and *Paenibacillus*. Arun et al. (2012) reported that some bacteria isolated from the rhizosphere of *Cassia occidentalis* exhibited significant growth promoting activities that could enhance root length in *Vigna radiate* and *Vigna mungo*.

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**Abbreviations: PGPR,** plant growth promoting rhizobacteria; **PCR,** polymerase chain reaction; **DGGE,** denaturing gradient gel electrophoresis; **SSCP,** single strand conformation polymorphism; **RFLP,** restriction fragment length polymorphisms; **T-RFLP,** terminal restriction fragment length polymorphisms; **q-PCR,** quantitative PCR; **NA,** nutrition agar; **ISSA ,** inorganic salt starch agar; **PDA ,** potato dextrose agar; **CFU,** colony forming units; **BLAST,** Basic local alignment search tool; **NCBI,** National Center of Biotechnology Information.

For assaying the genetic microorganism diversity of rhizophere soils, different culture-independent methods have been developed since Pace et al. (1985) proposed the direct cloning of environmental DNA. Most of those methods are based on the technology of polymerase chain reaction (PCR), such as denaturing gradient gel electrophoresis (DGGE) (Muyzer, 1999), single strand conformation polymorphism (SSCP) (Lee et al., 1996), restriction fragment length polymorphisms (RFLP) (Laguerre et al., 1994), terminal restriction fragment length polymorphisms (T-RFLP), (Dunbar et al., 2000), and quantitative PCR (q-PCR) (Takai and Horikoshi, 2000). Those methods have been widely used in assays of the genetic diversity of environment microbial.

*Davidia involucrate* is a tertiary relic species endemic to China and is reputed to be a “living fossil” in the plant kingdom. It is also an ornamental tree known as Chinese dovetree by virtue of the large pair of white bracts surrounding the small flower, which looks like a dove. In past studies, the dovetree has been well recognized for the diversity of its population, its gene expression and its distribution (Song and Bao, 2006; Wu et al., 2004; Li et al., 2002). However, the rhizophere microorganisms and their relationship with the dovetree have been infrequently studied. Therefore, in this study, we assay the cultivable microorganism biomass and diversity in the rhizosphere soil of *D. involucrate* by identifying isolations using colony and individual morphology examinations, and rDNA PCR methods. It was expected to isolate some cultivable strains that have potential to increase soil fertility or to promote host growth and that could be used as growth promotion candidate strains in further studies.

## MATERIALS AND METHODS

### Study site

The study site is located in the Wolong Nature Reserve of Wenchuan County, Sichuan, China (E102°56’ N30°51’ 1,718 m a.s.l.). Annual mean precipitation is 931 mm. Annual mean temperature is 8.9 ± 0.5 °C with a maximum of 29.2 ± 1.1°C (July) and a minimum of -8.5 ± 1.3°C (January). A 50-years-old natural *D. involucrate* forest was chosen to conduct this study. Canopy vegetation is dominated by *D. involucrate*, *Phoebe neuraratha*, and *Cornus controversa* with some understory shrubs (*Rhododendron violaceumv*, *Sibiraea angustata*, and *Sinarundinaria chungii*) and grass (*Polygonum viviparum*, *Cystopteris Montana*, and *Meconopsis* spp.). Soil is classified in the mountain brown soil series. The soil at a 0 to 20 cm depth had basic chemical properties of pH 5.8±0.2, 130.3±12.8 g total organic C $kg^{-1}$ DW, 20.2±1.9 g total N $kg^{-1}$ DW, and 3.2±0.4 g total P $kg^{-1}$ DW.

### Soil collection

Eight plots in the study site were selected and marked as GT1, GT2, GT3, GT4, and FGT1, FGT2, FGT3, FGT4. The GT plots were set as positive controls with *D. involucrate* grown. Conversely, the FGT plots were set as negative controls without *D. involucrate* grown. Soil samples were collected at depths of 0 to 20 cm after removing the surface soil (3 mm) at intervals of 1 m from the tree trunk and three soil samples were collected at each plot. The rhizosphere soil was defined as the soil attached to the root after gentle crushing and shaking of the collected roots. A total of 24 soil samples were collected. These samples were stored in freezer boxes at 4°C and transported to the laboratory within 24 h.

### Rhizosphere microorganism cultivation and isolation

Rhizosphere cultivable heterotrophic microorganisms were quantified by the plate dilution method. Nutrition agar (NA) was used as selection medium for isolating bacteria (except for actinomycete), inorganic salt starch agar (ISSA) for actinomycete and potato dextrose agar (PDA, Hangzhou Microbial Reagent CO., LTD, China) for fungi, respectively. In brief, 5 g of mixed fresh soil was extracted with 45 ml sterile physiological saline solution (0.85% NaCl) in an Erlenmeyer flask by shaking the mixture for 30 min at about 150 rpm/min. immediately after shaking, the suspension experienced a series of tenfold dilution by pipetting 1 ml aliquots into 9 ml sterilized water. The final dilution was $10^9$-fold, and 0.2 ml of each dilution of the series was placed onto a Petri dish. Three replicate dishes were made for each dilution. NA plates were incubated at 37°C for 48 h, ISSA and PDA plates were incubated at 28°C for 5-7 days. After incubation, the colony forming units (CFU) of each plate were counted and each typical rhizosphere microbial isolate was sub cultured on appropriate medium to obtain pure colony. Each selected typical colony was characterized according to the properties of shape, surface, color and transparency (Stainer et al., 1987), as well as an individual morphology examination through Gram’s staining. All selected strains were stored at -80°C in broth with 10% glycerol.

### Colony PCR of rhizobacteria

The colony PCR method was used to amplify 16S rDNA of rhizobacteria as described by Sheu et al. (2000). Colony PCR was performed using a TIANGEN 2×Taq PCR MasterMix Kit (TIANGEN Biotech, Beijing, China) according to the manufacturer’s introduction by following universal primers: 5'-AGA GTT TGA TCC TGG CTC AG-3' (27F) and 5'-TAC GGY TAC CTT GTT ACG ACT T-3'(1492R). The colony PCR was performed in a volume of 50 µl containing 25 µl TIANGEN 2×Taq PCR MasterMix, 1 µl of 20 mmol/l 27F primer, 1 µl of 20 mmol/l 1492R primer, 23 µl sterile water, and approximately $10^3$ bacterial cells. PCR conditions were performed in a DNA thermal cycler (Bio-Rad, Hercules, CA) as previous reported (Luo et al., 2012); 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1.5 min, 72°C for 10 min and 4°C for completion. The expected size of PCR products were 1500 bp. PCR amplicons were electrophoresed in 1% agarose gels and stained with Gold-View (0.005% v/v).

### Genomic DNA extraction and PCR amplification of fungi

The genomic DNA of isolated fungi was extracted by a double-sedimentation method (Wu et al., 2003). PCR was performed using TIANGEN 2×Taq PCR MasterMix Kit by two universal primers: 5’-TCC GTA GGT GAA CCT GCG G-3’ ($ITS_1$) and 5’-TCC TCC GCT TAT TGA TAT GC-3’ ($ITS_4$). PCR reaction was carried out in a volume of 50 µl containing 25 µl TIANGEN 2×Taq PCR MasterMix, 1 µl of 20 mmol/l $ITS_1$ primer, 1 µl of 20 mmol/l $ITS_4$ primer, 1 µl of template DNA, 22 µl sterile water. PCR conditions were as follows: 3 min at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 52°C, 1 min at 72°C, 72°C for 10 min, and 4°C for completion. The expected size of PCR products were 800 bp. PCR amplicons were electrophoresed in 1% agarose gels and stained with Gold-View (0.005% v/v).

**Table 1.** Colony forming units counting results of each soil sample.

| Plot | Colony count results (CFU/g) | | |
|---|---|---|---|
| | Bacteria | Actinomycetes | Fungi |
| GT1 | $(6.25\pm2.09)\times10^6$ | $(1.15\pm0.39)\times10^4$ | $(2.05\pm0.34)\times10^2$ |
| GT2 | $(45.86\pm5.33)\times10^6$ | $(1.13\pm0.21)\times10^4$ | $(1.88\pm0.29)\times10^2$ |
| GT3 | $(4.75\pm2.15)\times10^6$ | $(0.98\pm0.24)\times10^4$ | $(1.69\pm0.33)\times10^2$ |
| GT4 | $(6.79\pm1.31)\times10^6$ | $(1.05\pm0.18)\times10^4$ | $(1.98\pm0.37)\times10^2$ |
| FGT | $(5.84\pm1.87)\times10^6$ | $(0.95\pm0.34)\times10^4$ | $(1.58\pm0.49)\times10^2$ |

**Sequencing and analysis of PCR amplicons**

The complete sequencing of PCR amplicons were performed by Sangon Biotech (Shanghai, China). Closest known relatives of the isolates were determined by performing Basic Local Alignment Search Tool (BLAST) program at National Center of Biotechnology Information (NCBI) website (Altschul et al., 1990). Phylogenetic and molecular evolutionary analyses of the sequences were conducted using MEGA version 4 (Tamura et al., 2007). Multiple sequence alignments were performed and drawing of phylogenetic tree was carried out by Neighbor-Joining method (Saitou and Nei, 1987). Correction with 1000 replicates to produce bootstrap values (Felsenstein, 1985) and the phylogenetic tree was confirmed by maximum-parsimony method (Kluge and Farris, 1969) and maximum-likelihood method (Cavalli-Sforza and Edwards, 1967).

## RESULTS

### Colony counting results

The colonies counting results of plates after incubation are shown in Table 1. The counting results revealed that rhizobacteria was the dominant microorganisms in 24 tested soil samples. There were no significant differences in the population of rhizosphere microorganisms except for GT2. However, it is difficult to calculate the fungi colonies of tested soil samples since rare fungi colonies grew on the PDA plates, indicating that the fungi biomass was very low in each plot.

### Colony and morphology characterization

A total of 118 strains were obtained after colony characterization and microscope examination, including 52 strains isolated by NA medium (labeled as NA-isolated), 27 strains isolated by ISSA medium (labeled as ISSA-isolates) and 39 strains isolated by PDA medium (labeled as PDA-isolates). The 52 NA-isolates were grouped into nine genera as shown in Table 2; genus *Bacillus* (36.53%, n=19) and *Lactobacillus* (26.92%, n=14) were dominant microorganisms in tested soil samples. It wass noticeable that there were significantly more Gram-positive strains (69.23%, n=36) than Gram-negative strains (30.78%, n=16). The 27 ISSA-isolates were identified as streptomyces genera by means of an individual morphology examination according to Taddei's reports (Taddei et al., 2006).

Colony and morphology characterization results of 39 PAD-isolates are summarized in Table 3. The isolates were divided into 11 genera as described by Wei (1979); genus *Penicillium* (15.38%, n=6), *Trichodema* (15.38%, n=6), *Aspergillus* (12.82%, n=5) and *Mucor* (12.82%, n=5) were detected as dominant fungi genera. The morphology results indicated that fungi species diversity of the tested soil samples is variable, although fungi biomass was low (only 39 isolates).

### rDNA analysis and phylogenetic identification

57 isolates were randomly selected to perform rDNA analysis and phylogenetic identification, including 23 NA-isolates and 29 PDA-isolates (two isolates selected from the group which contains less than three isolates and three from those that contained more than three isolates), and 5 ISSA-isolates. Electrophoresis indicated that the expected sizes of PCR products were obtained (Figure 1). Each rDNA sequence was analyzed by BLAST program on NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) after complete sequencing. Phylogenetic trees were constructed by using the sequences of randomly selected isolates and three typical strain (ten for streptomyces genera) sequences of each closely related genus for phylogenetic identification (Figures 2, 3 and Figure 4). The phylogenetic analysis results revealed that each group of 57 randomly selected isolates was clearly separated into individual branches, which coincided with the morphology examination results as described above.

## DISCUSSION

To our knowledge, it is the first time that cultivable microorganisms were assayed in *D. involucrate* rhizosphere soil. In this study, we found that the biomass of rhizobacteria in GT2 was much higher than the other plots (Table 1). According to previous studies, physiological activity of plant could influence the rhizobacteria biomass. Wang et al. (2010) found that plants are able to regulate the soil microbial community in their immediate vicinity through the secretion from *Rehmannia glutinosa* roots to the rhizosphere. Arun et al. (2012) reported that the

**Table 2.** Colony and morphology characterization of rhizobacteria isolates.

| Group | Strains number | Gram staining | Spore | Colony characterization | | | | Individual morphology | Genera |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Shape | Surface | Color | Transparency | | |
| 1 | 19 | + | + | Radiciform | Rough | Milk-white | Translucent | Rod | *Bacillus* |
| 2 | 3 | - | - | Round | Smooth | Purple | Opaque | Rod | *Chromobacterium* |
| 3 | 2 | - | - | Round | Smooth | Greenyellow | Opaque | Short rod | *Vibrio* |
| 4 | 2 | - | - | Round | Smooth | - | Transparent | Rod | *Agrobacterium* |
| 5 | 2 | - | - | Round | Smooth | Milk-white | Translucent | Rod | *Photobacterium* |
| 6 | 3 | + | - | Round | Smooth | - | Translucent | Cocci | *Micrococcus* |
| 7 | 2 | - | - | Round | Smooth | Pink | Opaque | Rod | *Serratia* |
| 8 | 14 | + | - | Round | Smooth | Milk-white | Opaque | Rod | *Lactobacillus* |
| 9 | 5 | - | - | Irregular | Mucoid | - | Translucent | Rod | *Azotobacter* |

+, positive; -, negative.

**Table 3** Colony and morphology characterization of fungi isoltes.

| Group | Strains number | Colony color | Hyphae | Septum | Spore | | | | Genera |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Type | Shape | Size (μm) | Color | |
| 1 | 3 | White | Flocculence | - | Sporangiospore | Reniform | 13-19 | Transparent | *Pythium* |
| 2 | 2 | Brown | Stolon | - | Sporangiospore | Oval | 4-10 | Gray | *Rhizopus* |
| 3 | 5 | Gray | Flocculence | + | Sporangiospore | Oval | 4-9 | Faint yellow | *Mucor* |
| 4 | 2 | Blue purple | Stolon | + | Sporangiospore | Sphericity | 2-7 | Black | *Absidia* |
| 5 | 2 | Brown green | Flocculence | + | Sporangiospore | Sphericity | 6-13 | Black | *Circinella* |
| 6 | 3 | Brown | Panniform | + | Sporangiospore | Oval | 8-16 | Faint yellow | *Thamnidium* |
| 7 | 2 | Gray | Panniform | + | Sporangiospore | Oval | 11-13 | Dark brown | *Chaetomium* |
| 8 | 5 | Blue | Panniform | + | Conidium | Rod | 1-3 | Transparent | *Aspergillus* |
| 9 | 6 | Dark green | Panniform | + | Conidium | Chain | 2-7 | green | *Penicillium* |
| 10 | 6 | Green | Panniform | + | Conidium | Sphericity | 2-5 | green | *Trichodema* |
| 11 | 3 | Discrepant | Panniform | + | Conidium | Oval | 2-9 | Gray | *Cephalosporium* |

+, positive; -, negative.

bacteria diversity of *Cassia occidentalis* rhizosphere differed at the different stages of the plant. The physiological activity of plant is generally considered to be closely related to the stage of the plant. Therefore, we considered that the reason for the different biomass of rhizobacteria in plot GT2 might have been due to the special physiological activity of the dovetree growing in GT2, although further researches should be carried out to investigate the physiological activity of the dovetree at different stages or using different tissue.

Strain identification is a time-consuming work although several molecular methods have been established. In the present study, both conven-

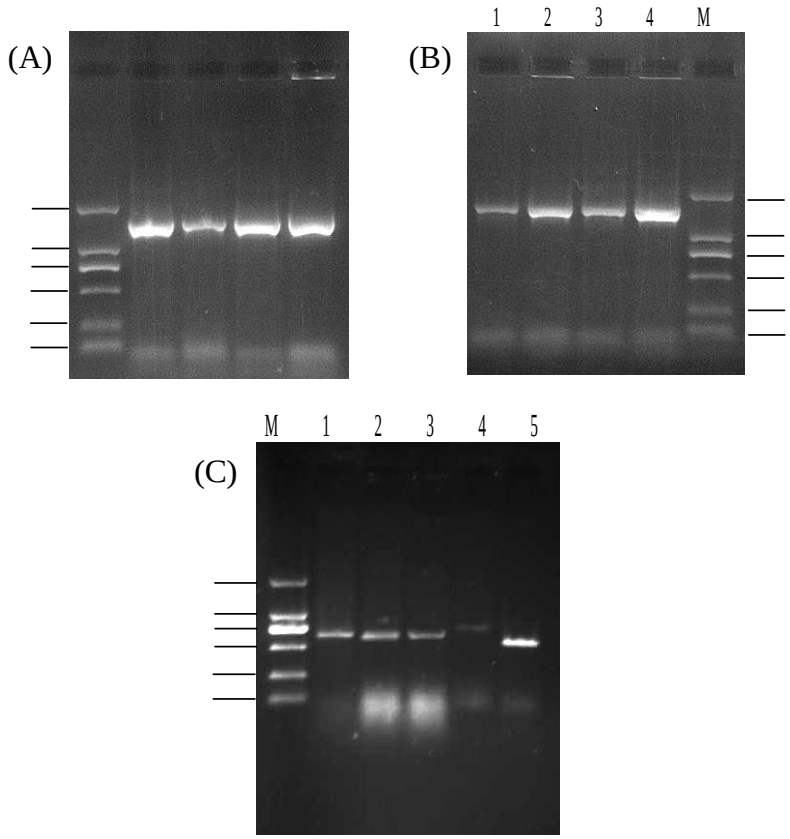



**Figure 1.** Electrophoretic analysis of colony PCR products. **(A)** lane M, D2000 DNA marker (TINAGEN); Lane 1-4, partial 16s rDNA amplicons of rhizobacteria isolates; **(B)** lanes M: D2000 DNA marker; lane 1-4, partial 16s rDNA amplicons of streptomyces isolates; **(C)** lane M, D2000 DNA marker; lane 1-5, partial 16s rDNA amplicons of fungi isolates. The electrophoresis picture of other colony PCR products was not showed in the paper.

tional methods and molecular methods were used to identify the isolates at the genus level for the assessment of diversity as previously reported (Marie et al., 2010). The results reveal that genus identification can be done using morphology examination and the phylogenetic identification method.

The importance of rhizosphere microbial diversity for maintenance of root health, nutrient uptake, and tolerance of environmental stress has been well recognized (Bowen and Rovira, 1999; Cook, 2002). In this study, a genera of *Bacillus* (36.54%, n=19) was detected as the dominant rhizobacteria. A number of investigators have reported that the genus *Bacillus* has good potential in promoting plant growth (Khurram et al., 2012; Sgroy et al., 2009), which is probably because the *Bacillus* provides a double benefit to the plant: (1) It gradually releases phosphorus (P) from insoluble P complexes though P-solubilization activity, and (2) it improves root growth and root surface area for better uptake of P and other nutrients through ACC deaminase activity. Based on the benefit mechanisms of *Bacillus*, we have concluded that the huge mass of *Bacillus* in the dovetree rhizophere soil should be beneficial to the growth of the dovetree. In this study, 21 cultivable genera were isolated, including nine bacteria genera, 11 fungi genera and one streptomyces genus. Those rhizophere microorganisms constitute a complex rhizophere ecosystem that could significant influence the soil fertility and structure (Lynch and Bragg, 1985). Moreover, it is estimated that 99% of microorganisms observed in nature are typically not cultivated using standard techniques (Amann et al., 1995). Consequently, the rhizophere symbiosis is highly complex and its plant growth promotion mechanism is not fully understood.

In summary, we have concluded that (1) the *Bacillus*, *Lactobacillus*, *Azotobacter*, and *Streptomyces* are the dominant microorganism's genera in the rhizophere soil of the dovetree and that *Bacillus* is the dominant rhizobacteria that might have potential in host growth promotion; (2) the physiological activity of the dovetree could significantly influence the microorganism biomass of the rhizophere soil; and (3) the dovetree has a highly complex rhizosphere ecosystem and further study should be carried out in this area.

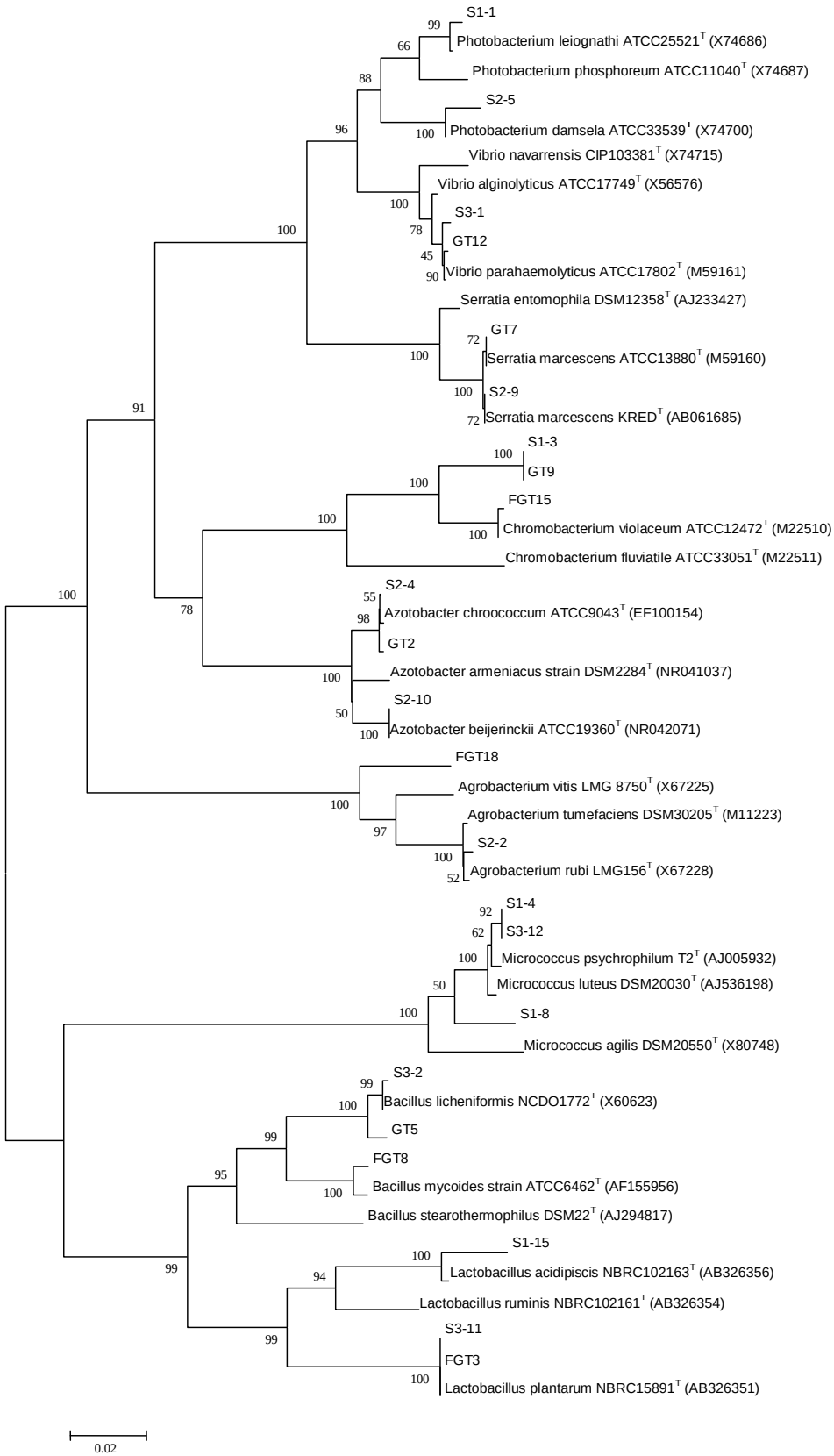
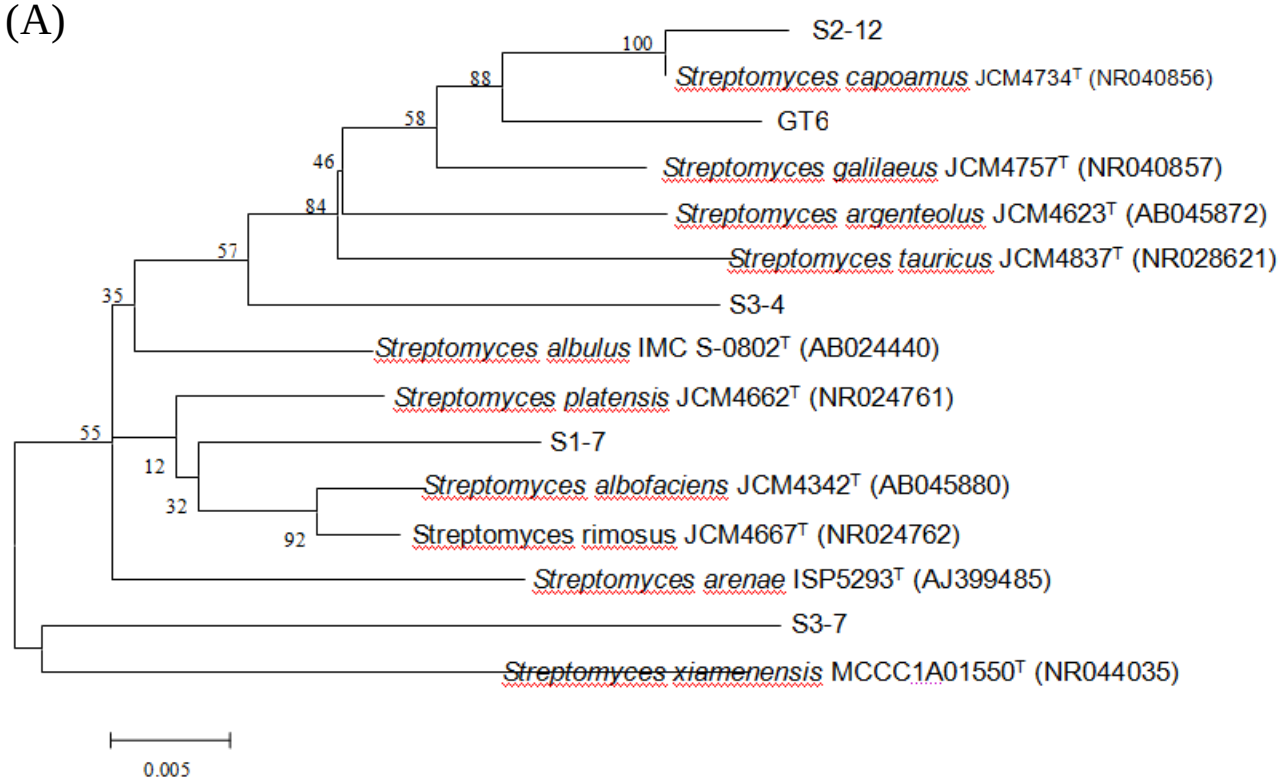



**Figure 2.** Phylogenetic tree of rhizobacteria isolates. The phylogenetic tree was constructed by Kimura 2-parameter model and Neighbor-Joining method. The robustness of individual branches was estimated by using bootstrapping with 1000 replications and the phylogenetic tree was conformed by the maximum-parsimony method and maximum-likelihood method.

(A)




(B)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. GT6 | | | | | | | | | | | | | | | |
| 2. S2-12 | 0.022 | | | | | | | | | | | | | | |
| 3. S1-7 | 0.045 | 0.044 | | | | | | | | | | | | | |
| 4. S3-4 | 0.043 | 0.043 | 0.047 | | | | | | | | | | | | |
| 5. S3-7 | 0.058 | 0.066 | 0.048 | 0.070 | | | | | | | | | | | |
| 6. AB045880 | 0.044 | 0.043 | 0.023 | 0.034 | 0.047 | | | | | | | | | | |
| 7. AB024440 | 0.040 | 0.040 | 0.027 | 0.029 | 0.050 | 0.017 | | | | | | | | | |
| 8. AJ399485 | 0.043 | 0.034 | 0.041 | 0.047 | 0.057 | 0.037 | 0.035 | | | | | | | | |
| 9. AB045872 | 0.030 | 0.037 | 0.039 | 0.035 | 0.057 | 0.037 | 0.033 | 0.044 | | | | | | | |
| 10. NR040856 | 0.018 | 0.005 | 0.040 | 0.039 | 0.061 | 0.038 | 0.035 | 0.029 | 0.031 | | | | | | |
| 11. NR040857 | 0.022 | 0.024 | 0.042 | 0.033 | 0.061 | 0.040 | 0.028 | 0.031 | 0.020 | 0.018 | | | | | |
| 12. NR024761 | 0.040 | 0.036 | 0.027 | 0.038 | 0.044 | 0.019 | 0.021 | 0.030 | 0.036 | 0.030 | 0.038 | | | | |
| 13. NR024762 | 0.039 | 0.041 | 0.024 | 0.037 | 0.042 | 0.008 | 0.020 | 0.035 | 0.037 | 0.036 | 0.042 | 0.015 | | | |
| 14. NR028621 | 0.036 | 0.037 | 0.040 | 0.040 | 0.064 | 0.040 | 0.034 | 0.040 | 0.029 | 0.032 | 0.027 | 0.039 | 0.038 | | |
| 15. NR044035 | 0.058 | 0.066 | 0.054 | 0.053 | 0.061 | 0.044 | 0.048 | 0.049 | 0.060 | 0.061 | 0.059 | 0.048 | 0.044 | 0.065 | |

**Figure 3.** Phylogenetic tree of streptomyces isolates (A) and Pairwise distance of Streptomyces (B).

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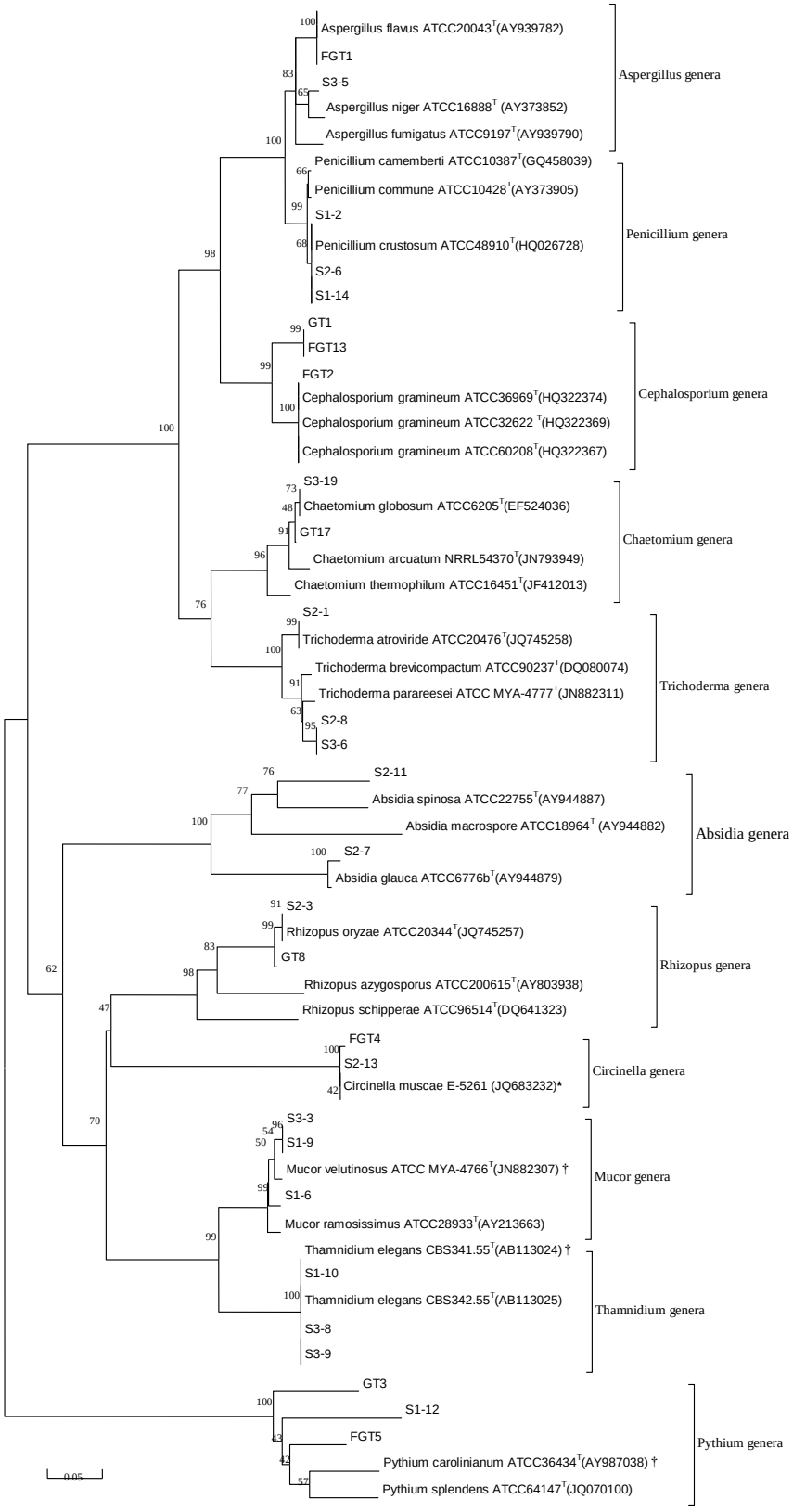


**Figure 4.** Phylogenetic tree of fungi isolates. The phylogenetic tree was constructed by Kimura 2-parameter model and Neighbor-Joining method. The robustness of individual branches was estimated by using bootstrapping with 1000 replications and the phylogenetic tree was confirmed by the maximum-parsimony method and maximum-likelihood method. *, no typical circinella genera strain is searched in Genbank database, circinella muscae E-5261 isolate was selected to construct the pylogentic tree; †, there were only two typical strains were searched in Genbank database.

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