

Research Paper

Diversity of Microorganisms Isolated from the Soil Sample surround *Chroogomphus rutilus* in the Beijing Region

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Abstract

Artificially cultivating *Chroogomphus rutilus* is too inefficient to be commercially feasible. Furthermore, isolating *C. rutilus* mycelia in the wild is difficult. Thus, it is important to determine the natural habitat of its fruiting body. This study focused on the ecology of the *C. rutilus* habitat to isolate and classify beneficial microorganisms that could affect its growth, which could be used in future research on artificial cultivation. In total, 342 isolates were isolated from soil samples collected around a *C. rutilus* colony in the Beijing region. Of these, 22 bacterial and 14 fungal isolates were selected for sequencing and phylogenetic analysis, based on their growth characteristics and colony morphology. Using 16S rRNA gene sequence analysis, the bacterial isolates were divided into two monophyletic clusters which had significant hits to the genera *Bacillus* and *Pseudomonas*, respectively. Using internal transcribed spacer (ITS) sequence analysis, fungal isolates were divided into four monophyletic clusters: *Penicillium*, *Trichoderma*, *Mortierella*, and *Bionectria*. Moreover, the phylogenetic diversity of these isolates was analysed. The results indicated that numerous microorganisms were present in *C. rutilus* habitat. This was the first reported examination of the microbiological ecology of *C. rutilus*.

Key words: *Chroogomphus rutilus*, isolation, phylogenetic diversity, 16S rRNA, ITS

Introduction

Chroogomphus rutilus (Schaeff.) O.K. Mill. is a type species in the genus *Chroogomphus*. The common names pine-spike and spike-cap are based on its shape and close growth association with pine trees. Based on records and our recent investigation, *Chroogomphus* spp. inhabit the northern hemisphere, including Asia, North America, and Europe [1]. *C. rutilus* is edible and especially flavourful when

dried. Furthermore, it has been investigated as a source of antibiotics and other potentially useful secondary compounds. The fruiting body is economically important for food or herbal medicine use [2].

Since artificially cultivating *C. rutilus* is difficult, making it too expensive to be a common commodity. There have been many attempts to find an efficient method of cultivating *C. rutilus*. Researchers have

attempted to imitate its natural habitat to promote mycelia growth, such as using soil collected near wild *C. rutilus* colonies and using pine tree tissue culture medium [3]. Pure mycelia cultures have been isolated; however, artificial cultivation is still inefficient and the fruiting body is difficult to obtain under artificial conditions [4]. Given the short time frame of research and lack of knowledge concerning the niche of *C. rutilus* in its ecosystem, it is not surprising that cultivating *C. rutilus* artificially is difficult, even outdoors.

Artificial cultivation may have failed due to the complex habitat *C. rutilus* requires. *C. rutilus* may require specific soil chemical composition and the presence of certain plants and microbes in order to grow. Some researchers have examined the plants living in *C. rutilus* habitats [5]; however, little research has been done to investigate the microorganisms present. Because no organism lives entirely isolated, soil microorganisms could directly or indirectly affect spore germination and fruiting body formation [6]. Furthermore, organisms often establish biological interactions, such as symbiosis, competition, and autoecism. *C. rutilus* interacts with other fungi and bacteria in its habitat, and may affect each other's growth or life cycles [7].

Based on the known requirements of casing-soil for the half artificial cultivation of *Morchella*, Shen *et al.* (2008) examined the bacterial community contained in the fruiting body of *Morchella* using a denaturing gradient gel electrophoresis (DGGE) technology. To some extent, the results of sequencing the 16S rRNA genes of 7 kinds of bacteria reflect the bacterial community structure inside *Morchella* [8]. Shen *et al.* (2009) also reported that the investigation of fungal communities inhabiting *Morchella* growth soil using DGGE. Sequence analysis of 12 recovered dominant bands revealed the presence of 10 fungi whose growth may have been inhibited by the dominant *Morchella* [6]. All of the above studies suggest that co-occurring microbes play an important role in fungal growth.

This study was initiated to investigate the diversity of microorganisms isolated from soil near a *C. rutilus* colony in the Beijing region. Bacteria and fungi were obtained selectively from soil samples collected around *C. rutilus* growing areas in the Beijing region based on their growth characteristics and colonial morphology. The 16S rRNA gene sequences of the bacteria isolates and the ITS gene sequences of the fungi isolates were amplified and sequenced respectively. The phylogenetic diversity of the isolated bacteria and fungi was determined based on the 16S rRNA genes and ITS sequences, respectively. This was the first demonstration of the microbe surrounding *C. rutilus*.

Materials and Methods

Soil sample collection and microbe isolation

Soil samples about 10 cm under the fruiting body of a *C. rutilus* colony in the Beijing region of the People's Republic of China were collected at an altitude of 790 m. The GPS coordinate was N40 57.186 E116 29.923. The samples were immediately transported to the laboratory for gradient dilution using autoclaved water and aliquots were transferred to appropriate growth medium. Three consecutive propagations using beef extract-peptone agar slants for bacteria and PDA media for fungi were conducted. After a 2-3-day incubation period at 28°C, single colony of per propagation was selected for observation. Out of the colonies owing the same growth characteristics and morphology, only one colony of each morpho-species was selected for further genetic sequencing. In this study, growth characteristics mainly included growth rate and colony morphologies of the isolated strains included size, color, diaphaneity of the colony, the degree of smoothness and so on. All strains in this study (Table 1) were preserved at -80°C in their respective liquid media with glycerol added to a final concentration of 15% (v/v).

Genomic DNA extraction from the isolates

Bacterial genomic DNA was extracted according to the methods described by Sambrook (1989) [9] for further 16S rRNA amplification. Fungal genomic DNA was extracted following the methods described by Doyle and Doyle (1987) [10], with some modifications. These pure DNAs extracted from the isolates were then sequenced for phylogenetic analyses.

16S rRNA gene amplification, cloning, and sequencing

The nearly complete 16S rRNA gene sequence, about 1,500-bp long, was amplified using the universal forward primer P1 and the universal reverse primer P6 [11] (Table 2). The PCR reactions were run using the methods described by Chouari *et al.* (2003, 2005) [12, 13]. In this study, we used high fidelity *Ex taq* polymerase (Takara, Japan). The 16S rRNA gene PCR products were purified following the Nucleic Acid Recycling and Purification Kit protocol (Tiangen Biotech, P. R. China). Purified nucleotide sequences and pGEM-T plasmid vectors (Promega, USA) were ligated overnight at 4°C. Recombined plasmids were transformed into *Escherichia coli* DH10B by electroporation (BTX ECM830, USA) at 500 V for 17 ms and evaluated using blue-white screening. Plasmids containing 16S rRNA gene sequences were extracted and purified according to the method described by Sam-

brook (1989) [6]. In the present study, three positive clones were selected for sequencing by using the primers RV-M/M13-47 (Table 2) in both directions

with an ABI377 automatic sequencer (Applied Biosystems, USA).

Table 1. Strains isolated in this study

Strain number	Genus	16S rRNA sequences GenBank accession number	ITS sequences GenBank accession number
I2X	<i>Bacillus</i> sp.	HQ727951	-
I3X	<i>Bacillus</i> sp.	HQ727952	-
I4X	<i>Bacillus</i> sp.	HQ727953	-
II2X	<i>Bacillus</i> sp.	HQ727954	-
II3X	<i>Bacillus</i> sp.	HQ844041	-
III1X	<i>Bacillus</i> sp.	HQ727955	-
III3X	<i>Bacillus</i> sp.	HQ727956	-
IV1X	<i>Bacillus</i> sp.	HQ727957	-
IV3X	<i>Bacillus</i> sp.	HQ727958	-
V1X	<i>Bacillus</i> sp.	HQ727959	-
V2X	<i>Bacillus</i> sp.	HQ727960	-
V3X	<i>Bacillus</i> sp.	HQ727961	-
V4X	<i>Bacillus</i> sp.	HQ727962	-
VI2X	<i>Bacillus</i> sp.	HQ727963	-
VII1X	<i>Bacillus</i> sp.	HQ727964	-
VII2X	<i>Bacillus</i> sp.	HQ727965	-
VII3X	<i>Bacillus</i> sp.	HQ727966	-
II4X	<i>Pseudomonas</i> sp.	HQ727967	-
III4X	<i>Pseudomonas</i> sp.	HQ727968	-
IV2X	<i>Pseudomonas</i> sp.	HQ727969	-
VI3X	<i>Pseudomonas</i> sp.	HQ727970	-
I1X	<i>Pseudomonas</i> sp.	HQ844040	-
I1Z	<i>Penicillium</i> sp.	-	HQ730121
I2Z	<i>Trichoderma</i> sp.	-	HQ730122
II1Z	<i>Penicillium</i> sp.	-	HQ730123
II2Z	<i>Bionectria</i> sp.	-	HQ730124
II3Z	<i>Trichoderma</i> sp.	-	HQ730125
III1Z	<i>Mortierella</i> sp.	-	HQ730126
III2Z	<i>Penicillium</i> sp.	-	HQ730127
V2Z	<i>Penicillium</i> sp.	-	HQ730128
VI1Z	<i>Mortierella</i> sp.	-	HQ730129
VI2Z	<i>Trichoderma</i> sp.	-	HQ730130
VII1Z	<i>Trichoderma</i> sp.	-	HQ730131
VII2Z	<i>Mortierella</i> sp.	-	HQ730132
IV2Z	<i>Trichoderma</i> sp.	-	HQ844042
V3Z	<i>Mortierella</i> sp.	-	HQ844043

Table 2. Primers used in this study

Primers	Sequence (5'→3')	Reference
16S P1	AGAGTTTGATCCTGGTCAGAACGCT	Yanagi and Yamasato, 1993
16S P2	TACGGCTACCTTGTTACGACTTCACCCC	Yanagi and Yamasato, 1993
ITS1	TCCGTAGGTGAACCTGCGG	White et al, 1990
ITS4	TCCTCCGCTTATTGATATGC	White et al, 1990
RV-M	GAGCGGATAACAATTCACACAGG	Takara, Code No. D3880, Japan.
M13-47	CGCCAGGGTTTCCAGTCACGAC	Takara, Code No. D3887, Japan.

ITS gene amplification, cloning, and sequencing

Recently, the ITS region of the ribosomal operon has become preferentially used for fungi classification instead of 18S ribosome RNA sequences [14]. The entire stretch of ITS1-5.8S-ITS2, approximately 750-bp long, was amplified using the universal forward primer ITS1 and the universal reverse primer ITS4 [15]. *Ex taq* polymerase (Takara, Japan) was used. The purification and sequencing procedures were the same as those described for the 16S rRNA sequences.

Sequence analysis and phylogenetic tree construction

The 16S rRNA and ITS gene sequences of the isolated strains were compared with sequences in DDBJ/EMBL/GenBank using the basic local alignment search tool (BLAST) [16]. The isolates had significant hits to the genus which owed the lowest e-value in the results of BLAST. When more than one sequence had the same e-value, only the first sequence was selected. Further, sequences were aligned using the Clustal_X software [17]. The distances were calculated according to two-parameter method [18]. The phylogenetic tree was inferred using the neighbour-joining methods [19]. Bootstrap analysis was based on 1,000 re-samplings. The MEGA4.0 package [20] was used for all analyses. All 16S rRNA sequences of the *Bacillus* and *Pseudomonas* type strains were obtained from the websites at <http://www.bacterio.cict.fr/b/bacillus.html> and <http://www.bacterio.cict.fr/p/pseudomonas.html>, respectively. The ITS sequences of the fungi type strains were obtained from DDBJ/EMBL/GenBank.

Results

Bacteria and fungi isolation from the soil samples

In total, 342 isolates were selectively obtained from soil samples collected near a *C. rutilus* colony in the Beijing region. Of these, 22 bacterial and 14 fungal isolates were selected out of 342 isolates based on the methods described in Materials and Methods.

Sequencing and phylogenetic analysis of the 16S rRNA gene sequences

The nearly complete 16S rRNA gene sequences of the 22 bacteria isolates were amplified using the universal primers for the 16S rRNA sequence shown

in Table 2 and sequenced. Comparing the 16S rRNA sequences with those in DDBJ/EMBL/GenBank, the strains labeled I2X, I3X, I4X, II2X, II3X, III1X, III3X, IV1X, IV3X, V1X, V2X, V3X, V4X, VI2X, VII1X, VII2X, and VII3X had significant hits to *Bacillus* spp. and those labeled I1X, II4X, III4X, IV2X, and VI3X had significant hits to *Pseudomonas* spp.. The 16S rRNA sequence GenBank accession numbers of these strains are indicated after the bacterial names in Table 1.

A phylogenetic tree based on the *Bacillus* spp. listed in the website <http://www.bacterio.cict.fr/b/bacillus.html> and the 17 sequenced *Bacillus* isolates was established (data not shown). Based on genetic distances, *Bacillus* spp. that had high similarities with the isolates were selected to construct the phylogenetic tree. The 17 *Bacillus* isolates formed three clusters (Fig. 1). Cluster I included eight isolates. The strains labeled V1X, VII1X, V4X, VII3X, and I3X were clustered with *B. simplex*, exhibiting 99.9, 99.9, 99.9, 100, and 100% 16S rRNA sequence similarity with *B. simplex*, respectively. Strains VI2X, V3X, and IV1X clustered with *B. sp. LMG 20238*, all exhibiting 99.9% 16S rRNA sequence similarity with *B. sp. LMG 20238*. Cluster II included strains II3X and III1X, which had 99.9% 16S rRNA sequence similarity with *B. aryabhattai*. Cluster III consisted of seven strains. Strains I2X, V2X, I4X, VII2X, II2X, and III3X were clustered with *B. thuringiensis*, exhibiting 99.9, 99.9, 100, 99.9, 100, and 100% 16S rRNA sequence similarity, respectively. Strain IV3X showed the highest 16S rRNA sequence similarity, 99.9%, with *B. cereus*.

The phylogenetic diversity of the *Pseudomonas* isolates was analyzed as described above for *Bacillus*. A phylogenetic tree was constructed based on the *Pseudomonas* spp. in the website (<http://www.bacterio.cict.fr/p/pseudomonas.html>) and the five isolates (data not shown). Using genetic distances, the *Pseudomonas* spp. which had high similarities with these five isolates were selected to construct the phylogenetic tree. The *Pseudomonas* isolates formed two clusters (Fig. 2). The *Pseudomonas* isolates labeled II4X, VI3X, IV2X, and III4X clustered with *P. koreensis*, exhibiting 99.8, 99.7, 99.8, and 99.8% 16S rRNA sequence similarity, respectively. In the other cluster, strain I1X had 99.8% similarities with *P. umsongensis*.

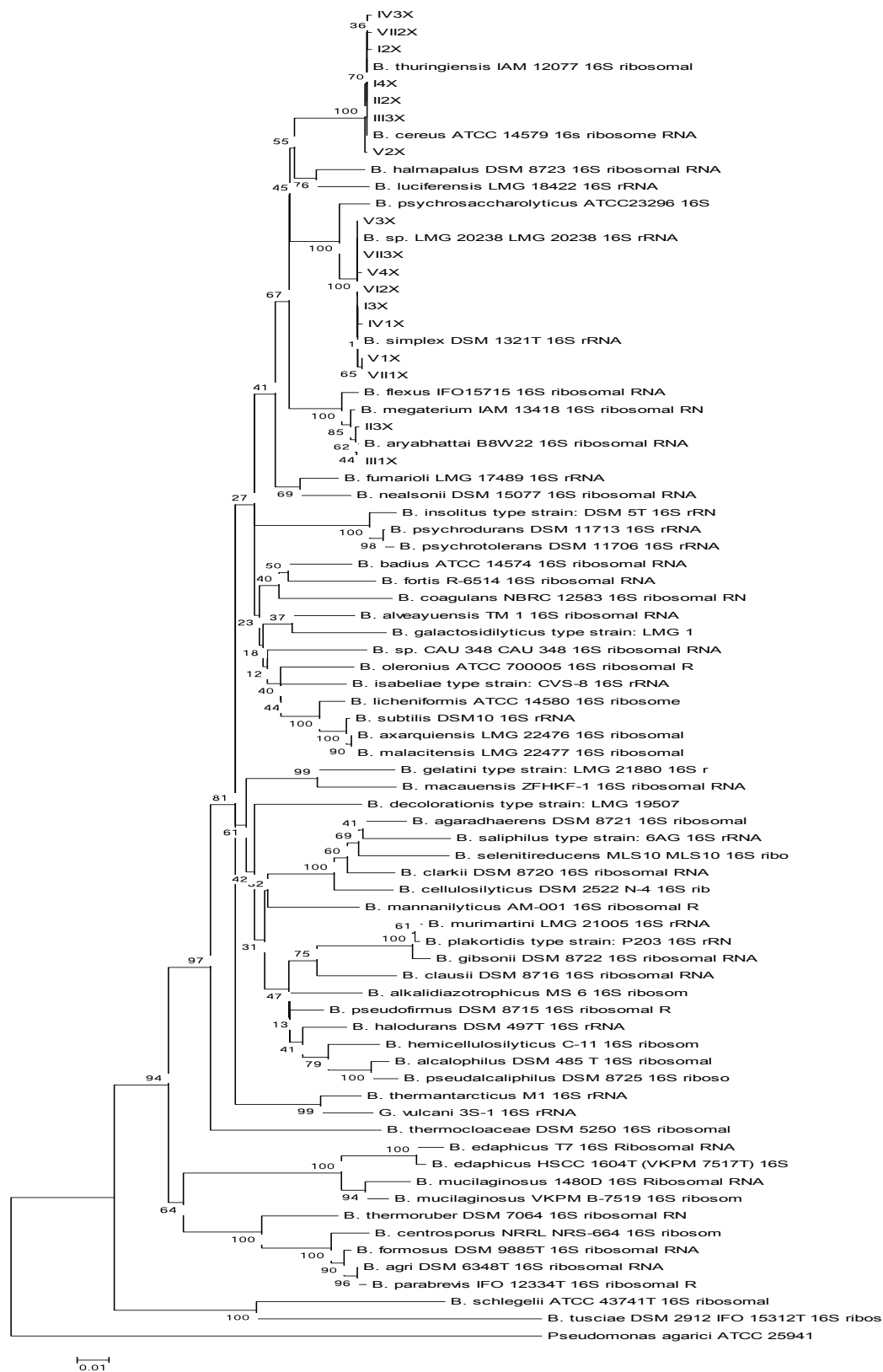


Fig. 1 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the distance of isolated strains with the nearest species of the genus *Bacillus*. *Pseudomonas agarici* ATCC25941 was used as an out group. Bootstrap percentage values as obtained from 1,000 resamplings of the date set are given at the nodes of the tree. Bar, 0.01 substitutions per nucleotide position.

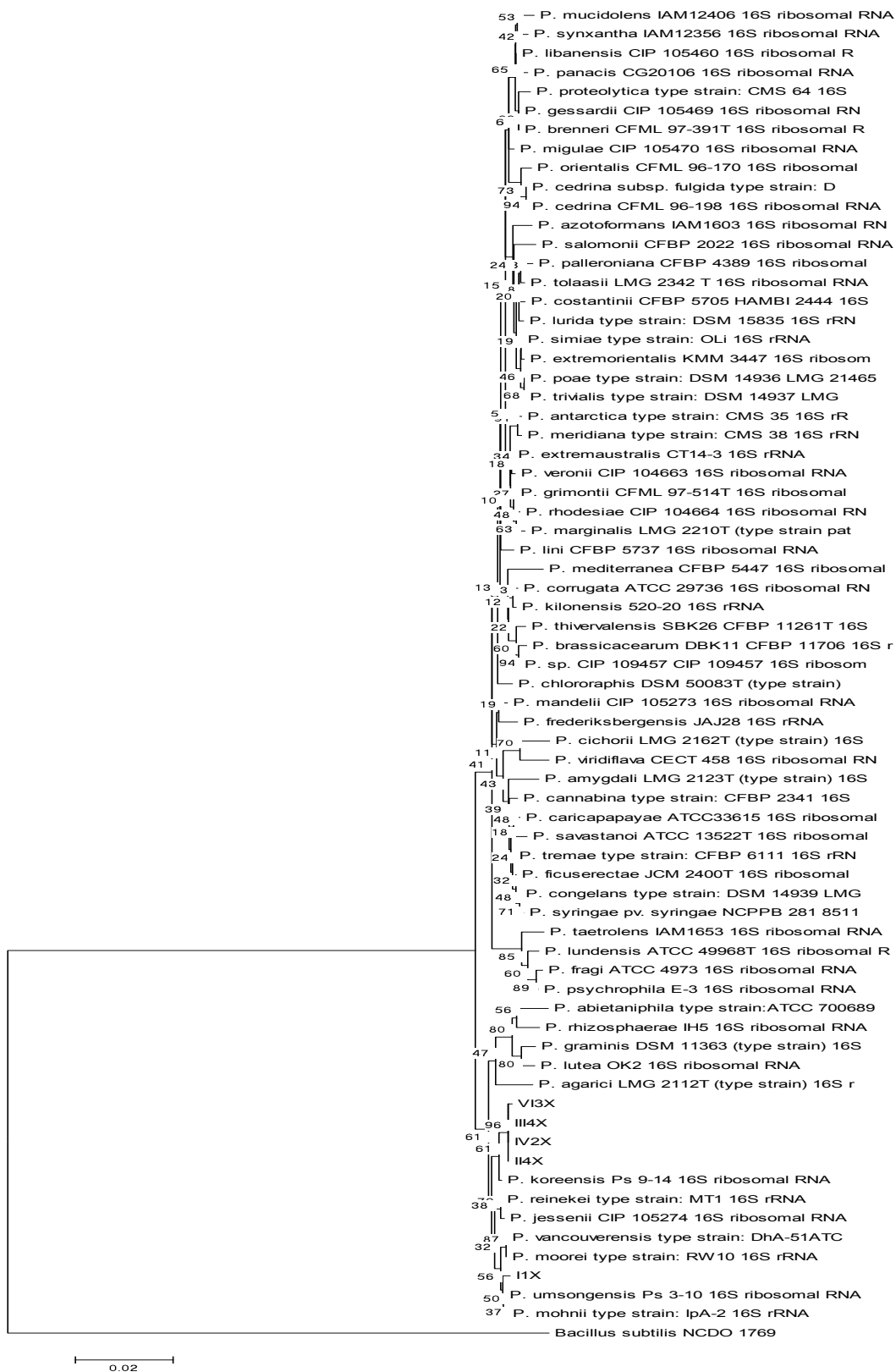


Fig. 2 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the distance of isolated strains with the nearest species of the genus *Pseudomonas*. *Bacillus subtilis* NCDO 1769^T was used as an out group. Bootstrap percentage values as obtained from 1,000 resamplings of the data set are given at the nodes of the tree. Bar, 0.02 substitutions per nucleotide position.

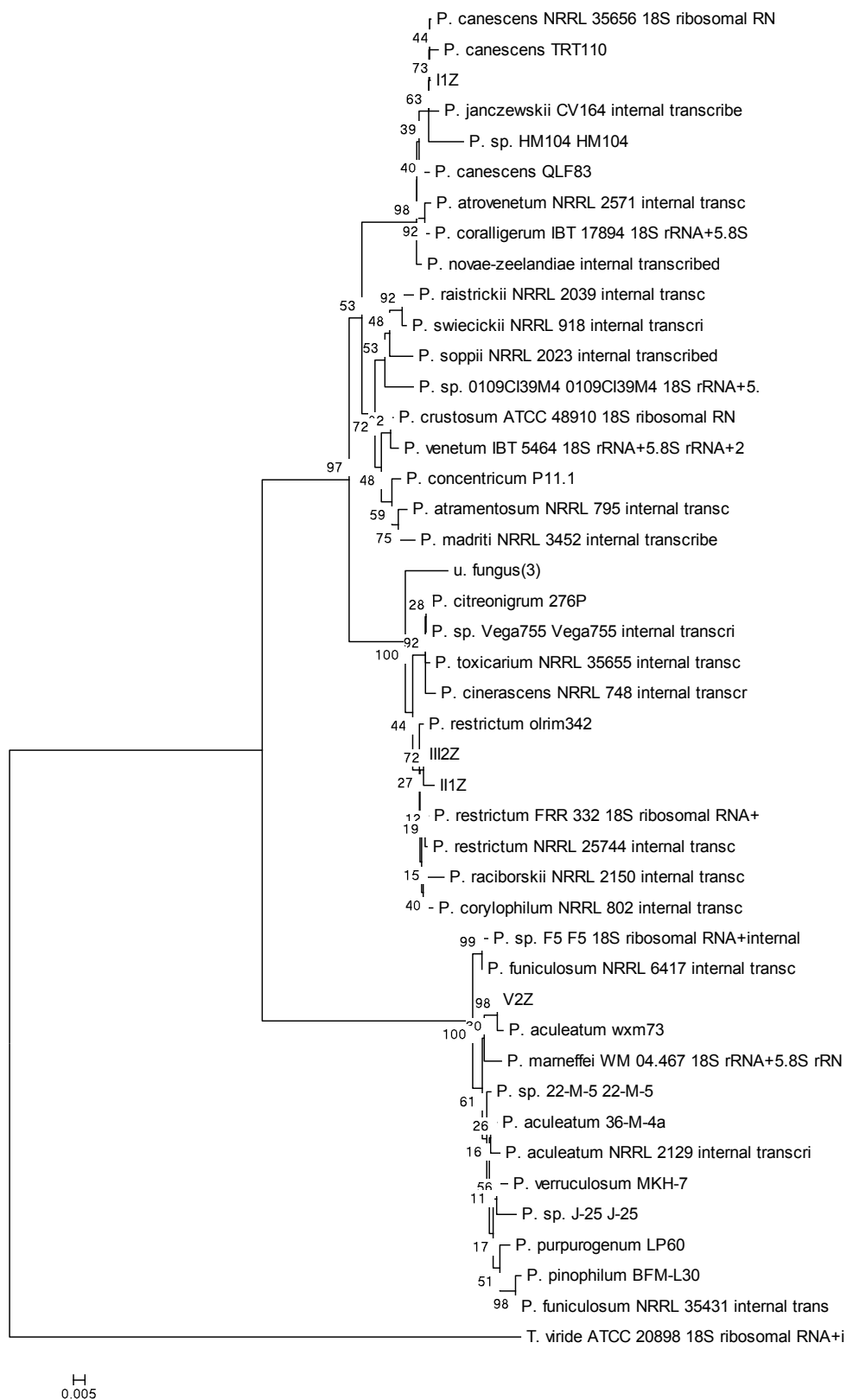


Fig. 3 Neighbour-joining phylogenetic tree based on ITS sequences showing the distance of isolated strains with the nearest species of the genus *Penicillium*. *Trichoderma viride* ATCC20898 was used as an out group. Bootstrap percentage values as obtained from 1,000 resamplings of the data set are given at the nodes of the tree. Bar, 0.005 substitutions per nucleotide position.

Sequencing and phylogenetic analysis of the ITS sequences

Because ITS sequences can accumulate mutations at a faster rate than the 5.8S, 18S, and 28S rRNA genes, the ITS region is typically most useful for molecular systematics at the species or within species level [21, 22]. The ITS1-5.8S-ITS2 gene sequences of the 14 isolates were compared with sequences listed in DDBJ/EMBL/GenBank, indicating that they had significant hits to the genera *Penicillium*, *Trichoderma*, *Mortierella* and *Bionectria*. The strains labeled I1Z, II1Z, III2Z, and V2Z had significant hits to *Penicillium*. Strains I2Z, II3Z, IV2Z, VI2Z and VII1Z had significant hits to *Trichoderma*. Strains III1Z, V3Z, VI1Z and VII2Z had significant hits to *Mortierella*. Strain II2Z had significant hits to *Bionectria*. The ITS gene sequence GenBank accession numbers of the isolated fungi are indicated in Table 1.

A phylogenetic tree using the *Penicillium* spp.

which had high similarities to the isolated fungi was constructed based on genetic distance (Fig. 3). The four *Penicillium* spp. isolates formed three clusters (Fig. 3). Cluster I consisted of strain I1Z, which exhibited 100% ITS sequence similarity with *P. canescens*. Cluster II contained strains II1Z and III2Z, which had the highest ITS sequence similarity to *P. restrictum*, 99.3% and 99.8%, respectively. Cluster III consisted of V2Z, with 100% similarity to *P. aculeatum*, and I1Z, with 100% similarity to *P. canescens*.

Using the method described for *Penicillium*, strains I2Z, II3Z, VI2Z, and VII1Z had 100, 99.9, 100, and 100% similarity with *Trichoderma koningiopsis*, respectively (Fig. 4). Strain IV2Z had 99% similarity with *T. atroviride* (Fig. 4). The fungi labeled III1Z, VI1Z, VII2Z, and V3Z had 100, 100, 100 and 99.8% similarity with *Mortierella alpina*, respectively (Fig. 5). The fungus labeled II2Z had 100% similarities with *B. ochroleuca* (Fig. 6).

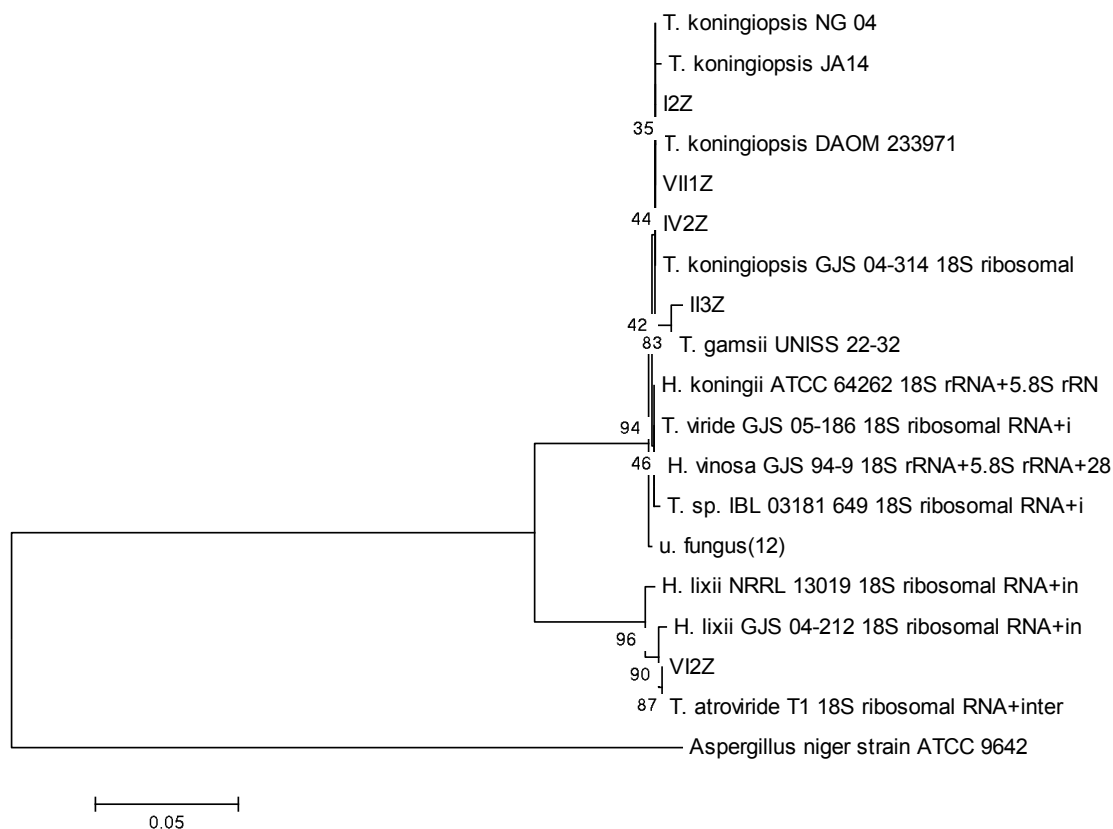


Fig. 4 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the distance of isolated strains with the nearest species of the genus *Trichoderma*. *Aspergillus niger* ATCC9642 was used as an out group. Bootstrap percentage values as obtained from 1,000 resamplings of the data set are given at the nodes of the tree. Bar, 0.05 substitutions per nucleotide position.



Fig. 5 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the distance of isolated strains with the nearest species of the genus *Mortierella*. *Aspergillus niger* ATCC9642 was used as an out group. Bootstrap percentage values as obtained from 1,000 resamplings of the data set are given at the nodes of the tree. Bar, 0.05 substitutions per nucleotide position.

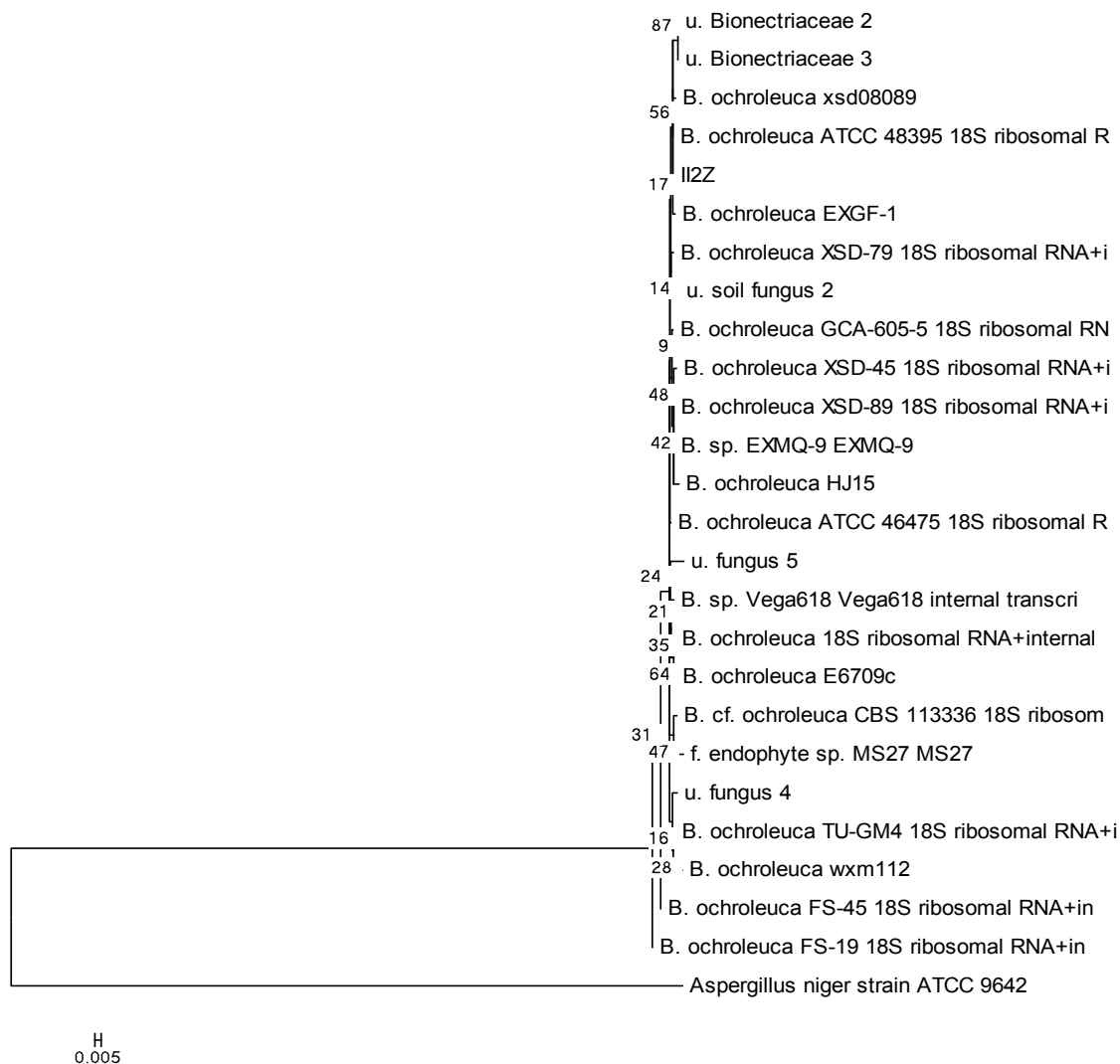


Fig. 6 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the distance of isolated strains with the nearest species of the genus *Bionectria*. *Aspergillus niger* ATCC9642 was used as an out group. Bootstrap percentage values as obtained from 1,000 resamplings of the data set are given at the nodes of the tree. Bar, 0.005 substitutions per nucleotide position.

Discussions

To date, the artificial cultivation of some edible wild fungi, including *Chroogomphus* spp., *Morchella* spp., and *Matsutake* spp., has not been successful. The slow mycelia growth rate on nutrient medium and the difficulty in fruiting body formation is due to the lack of information on the mechanisms of spore germination and fruiting body formation. Fruiting body formation could be affected by factors within the mycelia or externally, in the environment. Many microorganisms secrete beneficial secondary metabolites, which could be important for spore germination or fruiting body formation in wild mushrooms [23]. Rainey *et al.* (1991) reported that casing was required for the in-

duction of fruiting body formation, which is caused by the presence of saprophytic bacteria in the casing medium [24]. Ntougias *et al.* (2004) reported that Gram-negative bacteria existed in spent mushroom compost and in mushroom cultivation substrate analyzed immediately after pasteurisation and cooling. The biological properties of compost appear to be important for the induction of fruiting body formation [25]. The microorganisms that exist in proximity to *C. rutilus* require further detailed examination to determine important interactions, which could advance the artificial cultivation of *C. rutilus*.

In the present study, 10 gram soil sample were resolved and serial diluted. Only a portion of the solution was spread on the appropriate media. Finally,

36 isolates were obtained for further classification and phylogenetic analysis. Limited by the sample size, there was the chance that some key microbes needed for *C. rutilus* might be missed. Moreover, traditional cultural method exhibit its limitations in the phylogenetic analysis, since only a small proportion of microbe can be obtained in laboratory condition [26]. Culture-independent methods, such as metagenomic approach, could overcome these difficulties and limitations [27]. To solve these existing problems, metagenomic approach will be applied in the future study. Except of these, several important results from this investigation were summarized below.

First, of the 22 bacteria strains, 17, or about 80%, were *Bacillus* spp. It was reported that *Bacillus* spp. are abundant and widely distributed in agro-ecosystem [28, 29]. This could be because these *Bacillus* strains form a thick outer spore coat increasing resistance to extreme conditions, such as heat, ultraviolet radiation, toxic chemicals, and desiccation. Effects of *Bacillus* sp. on growth on mushroom was reported by Bis'ko *et al.* (1995) [30].

Second, four *Pseudomonas* spp. were isolated from the soil sample. Reports indicated that the presence of saprophytic bacteria, such as *Pseudomonas* spp., in the casing medium could induce fruiting body formation [31]. Moreover, secondary products excreted by *Pseudomonas*, such as insecticides and germicides, could affect the growth of *C. rutilus* mycelia in its natural habitat. Kloepper *et al.* (2004) reported that *Pseudomonas* spp. were one of the familiar plant growth-promoting rhizobacteria (PGPR) [32]. This explained the isolation of *Pseudomonas* spp. in the soil sample in this study. Further research on *Pseudomonas* spp. and *C. rutilus* interactions should be conducted, because this interaction might be the key point to artificial cultivation.

Third, we observed that some bacteria isolates could secrete much extracellular polysaccharides in the medium. Extracellular polysaccharides are important in inter-microorganism signaling [33, 34] and are involved in selection recognition, which is important for symbiosis [35]. In this study, the fact that soil samples were collected where *C. rutilus* grew implied that these extracellular polysaccharides could be important for recognition and signalling between bacteria and *C. rutilus*. By understanding the molecular mechanisms of these hypothesized interactions, we could enhance the interactions and expand the use of these bacteria.

Finally, it is notable that the fungus strain labeled II2Z had significant hits to the genus *Bionectria*. *Bionectria* are endophytic fungi that can colonize living, internal plant tissue without any immediate or

overt negative effects [36]. In fact, endophytic fungi were detected and reported in many plants. These fungi showed positive role to help protecting the plants against insects, nematodes, pathogens and so on [37, 38]. In the present study, *Bionectria* spp. was isolated from the soil sample together with the mycelium of *C. rutilus*. It could be deduced that *Bionectria* spp. might interact with the organism nearby. The organism perhaps was the nearby pine tree or the mycelium of *C. rutilus*. Based on the results obtained in the present study, the detail interaction between *C. rutilus* and *Bionectria* sp. II2Z requires more research to determine if this strain played the role on the fruit body formation of *C. rutilus*.

In this study, microorganisms were isolated and classified in *C. rutilus* habitat. This laid the foundation of the artificial cultivation of *C. rutilus*. This report is the first reported examination of the microbiological ecology of *C. rutilus*. Based on the results of this study, we will examine the exact interactions between *C. rutilus* and these isolated microorganisms to reveal how these microorganisms assist in the artificial cultivation of *C. rutilus*. If this research path is successful, the applications of *C. rutilus* will broaden.

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Conflict of Interests

The authors have declared that no conflict of interest exists.

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