Diversity of Ascomycetes Mushroom in Para Rubber Plantation, Thailand

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Abstract

Ascomycetes mushrooms are fungi that produce ascospores in asci and some with perithecia. Not only they have a role of decomposer in ecology but also produced some bioactive compound, anti-microbial activity, and cytotoxicity. This study aims to explore the diversity of ascomycetes mushroom species in para rubber plantations and to identify them by morphological and sequence analysis of the internal transcribed spacer (ITS) region. The results found ascomycetes mushroom consist of *Trichoderma pezizoides* (RP1, % identity 98.79, DQ835513.1), *Daldinia eschscholtzii* (RP2, % identity 100, MN310384.1), *Cookeina sulcipes* (RP3, % identity 98.44, KY094620.1), *Cookeina garethjonesii* (RP4, % identity 99.06, KY094622.1), *Cookeina tricholoma* (RP5, % identity 100, KY094619.1) and *Xylaria terricola* (RP6, % identity 88.42, MF577038.1). Most of the ascomycetes in this study have previously been described in Thailand except *Xylaria terricola*. Additionally, phylogenetic analysis of ascomycetes mushroom showed high genetic relatedness with reference strains. Therefore, the sequence similarity and phylogenetic analysis confirmed the identity of six ascomycetes mushroom species, and further study of bioactive compound from these mushrooms may be investigated for other applications.

Introduction

Para rubber (*Hevea brasiliensis*) plantation is one of the most favorite careers in the Eastern of Thailand such as Trat province. Although these areas using for para rubber agriculture but also revealed diversity in several organisms including biodiversity of mushrooms. However, the study of mushroom species in para rubber plantations very little. Mushrooms are macrofungi with a different form of fruiting bodies and can be classified in basidiomycetes and ascomycetes based on sexual spore production and development patterns in the life cycle. Ascomycetes mushrooms are fungi that produce ascospores in asci and some with perithecia. The family of ascomycetes fungus such as Xylariaceae is widespread and diverse of species. These mushrooms have an important role in the ecosystem as decomposers and know to produce several classed of bioactive compounds (Adnan et al. 2018; Noppawan et al. 2020; Sodngam et al. 2014). Recently, chemical compounds and their bioactive potential were found in *Xylaria primorskensis* (Adnan et al. 2018).

The mushroom identification by morphology methods such as characteristic of shape, size, and spore is easy to confuse, time-consuming, and low accuracy. It is difficult to differentiate closely related species. However, molecular techniques such as polymerase chain reaction (PCR) for mushroom identification have proved to be fast, specific, and sensitive, especially, inconsistent morphology and indiscrimination among species of mushrooms (Cho et al. 2015; Kim et al. 2016; Lee et al. 2000; Persoh et al. 2009; Raja et al. 2017). The conserved region such as Internal Transcribed Spacer (ITS) of nuclear ribosomal DNA (rDNA) usually has been targeted because it is highly conserved among interspecies but variable between intraspecies (Raja et al. 2017). This study aims to explore the diversity of ascomycetes mushroom species in para rubber plantations and to identify them by morphological and sequence analysis of the ITS region.

Materials And Methods

1. Study area and sample collection

The ascomycetes mushrooms were collected from 40 rubber plantation farms (204.32 acres) in Santoong, Trat province, Thailand (Latitude 12°27'16" N to 12°18'49" N and longitude 102°24'40" E to 102°22'27" E). The mushroom samples were randomly collected from decaying wood, soil, and rubber tree between July 2019 to September 2019. Mushroom samples were harvested and placed in a sterile paper bag or plastic box. All samples were transferred to the laboratory and kept in a refrigerator.

2. Presumptive morphology study

Mushroom samples were presumptively identified by morphology as previously described (Ekanayaka et al. 2016; Jaklitsch 2009; Jaklitsch 2011; Jaklitsch et al. 2008; Weinstein et al. 2002; Wongkanoun et al. 2020; Wongkanoun et al. 2019). For mushroom preservation, they were dried in an oven.

3. DNA extraction

DNA extraction from fruiting bodies or mycelia was performed by methods as previously described (Izumitsu et al. 2012; Plaza et al. 2004). The microwave method, a piece of the mushroom sample was transferred to a 1.5 ml microcentrifuge tube. The tubes were microwaved (600W) for 1 min and incubated at room temperature for 30 sec. After that, the tubes were microwaved again (600W, 1 min) and were incubated at -20°C for at least 10 min. All tubes were centrifuged at 10,000 rpm for 5 min. The supernatants were used for PCR amplification (Izumitsu et al. 2012). The DNA extraction method by Plaza, et al. (2004) with some modification was performed. Briefly, mushroom mycelium was added to 1.5 ml microcentrifuge tubes and suspended in 500 µl of a bead beating solution (0.1M NaCl, 0.5M Tris-HCl, pH8.0, and 5% sodium dodecyl sulfate). Glass beads with a mixed diameter (1.0mm/0.5mm/0.1mm) were added for crushing of cell walls. The tubes were mixed by a vortex mixture and homogenized for 10 min at maximum speed. Subsequently, the tubes were centrifuged for 10 min at 11,000 g. DNA extraction step, the supernatants were removed into new tubes. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Invitrogen, USA) was added to each sample, vortexed briefly, and centrifuged for 5 min. The aqueous layer was transferred to a new tube and extracted again with phenol:chloroform:isoamyl alcohol. DNA precipitation step, 2.5 volumes of isopropanol were added and the tubes were incubated in a refrigerator (1 hour) and centrifuged (4°C, 10 min, 14,000 g). The pellets were washed with cold 70% ethanol, air-dried, and then resuspended in sterile water PCR grade (Invitrogen, USA) (Plaza et al. 2004).

4. PCR amplification of internal transcribed spacer (ITS) region

The ITS region was amplified by PCR with two primers, ITS1 (5’- TCCGTAGGTGAACCTGCGG-3’) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’) as previously described (Cho et al. 2015; Kim et al. 2016; Lee et al. 2000; Persoh et al. 2009; Raja et al. 2017). PCR reactions were performed in a total volume of 20 µl consists of 6 µl distilled water, 10 µl 2x PCR master mix (iNtRONbiotechnology, Korea), 1 µl of each 10 µM primer (0.5 µM) and 2 µl template DNA. The PCR amplification was performed in thermal cycler under the

following condition: initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 51°C for 1 min and extension at 72°C for 1 min, and lastly, a final extension at 72°C for 1 min.

The PCR products were analyzed by 2% agarose gel electrophoresis with RedSafe (iNtRONbiotechnology, Korea) The DNA ladders (100 bps) (BIO-HELIX, Taiwan) and PCR product was loaded in agarose gel and then allowed to run at 100V, 30 min. The DNA fragments from agarose gel were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany)

5. DNA sequencing and ITS region analysis

Sequencing was performed at 1st base (Selangor, Malaysia). The DNA sequences were BLAST (Basic Local Alignment Search Tool) in GenBank to determine percent similarity and the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 2.28607013 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2500 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. This analysis involved 15 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 514 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

Results

Fifty-two mushroom samples were collected from rubber plantation farms, Santoong, Trat province, Thailand in 2019. Among mushroom specimens, 6 mushroom samples were classified in ascomycetes mushroom by the presence of ascospore in asci namely, RP1, RP2, RP3, RP4, RP5, and RP6. The morphological characteristic classified into genus *Trichoderma* sp. (RP1), *Daldinia* sp. (RP2), *Cookeina* sp. (RP3-5), and *Xylaria* sp. (RP6) (Figure 1). According to mushroom identification by morphology is low accuracy and time-consuming, therefore, the molecular identification by amplification of ITS region was performed and revealed PCR product approximately 600-700 bp (Figure2). The BLAST results of ITS sequences showed that ascomycetes mushrooms were identified (collection number, %identity, accession number) as *Trichoderma pezizoides* (RP1, % identity 98.79, DQ835513.1), *Daldinia eschscholtzii* (RP2, % identity 100, MN310384.1), *Cookeina sulcipes* (RP3, % identity 98.44, KY094620.1), *Cookeina garethjonesii* (RP4, % identity 99.06, KY094622.1), *Cookeina tricholoma* (RP5, % identity 100, KY094619.1) and *Xylaria terricola* (RP6, % identity 88.42, MF577038.1) (Table 1, Figure 3). The phylogenetic tree indicated that *Cookeina sulcipes* (RP3) was closely related to *Cookeina garethjonesii* (RP4) but *Cookeina tricholoma* (RP5) was distinct. A close relationship was also observed between *Trichoderma pezizoides* (RP1) and *Xylaria terricola* (RP6), while *Daldinia eschscholtzii* (RP2) showed a distant relationship (Figure 4). Moreover, phylogenetic analysis of ascomycetes mushroom

showed high genetic relatedness with reference strains. Therefore, sequence similarity and phylogenetic analysis confirmed the identity of six ascomycetes mushroom species found in rubber plantation farms.

Discussion

This study was the first report of ascomycetes mushroom that was collected from para rubber plantation areas in Santoong, Trat, Eastern Thailand. Although these areas were used for agriculture, the biodiversity of macrofungi was observed. We found 6 ascomycetes mushrooms included *Trichoderma pezizoides, Daldinia eschscholtzii, Cookeina sulcipes, Cookeina garethjonesii, Cookeina tricholoma,* and *Xylaria terricola.* Surprisingly, *Xylaria terricola* has not been previously described in Thailand. To confirmed the species identity, the molecular identification method was performed by analysis of ITS sequence. In this study, amplification of ITS region revealed PCR product approximately 600-700 bp (Figure2) but several studies in mushroom identification by amplification of ITS using ITS1 and ITS4 primers reported PCR product size between 400 and 850 bp (Adeniyi et al. 2018; Appiah et al. 2017; Das et al. 2013). Additionally, 350-880 bp of PCR product also reported in fungal identification by Fujita et al (2001) (Fujita et al. 2001). The variability of the fungal ITS region and variations in the quality of DNA may be responsible for this (Gomes et al. 2002; Krimitzas et al. 2013; Lee et al. 2012; Lorenz 2012).

In Thailand, several studies of ascomycetes were investigated especially Xyalariaceae. These studies provide evidence involve biodiversity, bioactive compound, and cytotoxicity

(Noppawan et al. 2020; Okane et al. 2008; Osono et al. 2011; Srihanant et al. 2015; Velmurugan et al. 2013; Wongkanoun et al. 2020). Interestingly, The new species of Xyalariaceae such as *Xylaria thailandica* were discovered by Srihanant et al. (2015) (Srihanant et al. 2015) and *Daldinia flavogranulata, D. phadaengensis, D. chiangdaoensis* were explored by Wongkanoun et al. (2020) (Wongkanoun et al. 2020). In present study, *Trichoderma pezizoides*, *Daldinia eschscholtzii*, *Cookeina sulcipes*, *Cookeina garethjonesii*, *Cookeina tricholoma*, the ascomycetes mushroom found in para rubber plantation have previously described in Thailand but the evidence of *Xylaria terricola* has not been reported.

In this study, six ascomycetes mushrooms were found in Eastern Thailand. The climate of this area is tropical but the summer rains are more abundant, especially in Trat province, they are plentiful in most portion up to 3,500 mm (138 in) of rainfall per year. These climates maybe support the growth and distribution of *these ascomycetes fungi.*

In conclusion, although the para rubber plantation area was used for agriculture, the biodiversity of mushrooms was observed, especially ascomycetes fungi. Additionally, the combination of morphological and molecular approaches is important for accuracy and specificity in mushroom identification. The further study of ascomycetes mushroom found in this study may be investigated such as their bioactive compound, wood-decaying activity, anti-microbial activity, and cytotoxicity for application in industrial, agricultural, and medicine.

Declarations

Authors' contributions

S.S., S.N., C.K., and L.K. collected the mushroom sample, performed morphological study, and molecular identification by PCR techniques. P.K., M.Y. analyzed the nucleotide sequences and phylogenetic tree analysis.

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Competing interests

Authors state no competing interests.

Availability of data and materials

All data generated and analyzed during this study are included in this article.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Ethical statement

This article does not contain any study with human participants or animals performed by any of the authors.

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Tables

Table 1. BLAST results and accession number of ascomycetes mushroom found in this study

Species	Collection No.	Best match (Accession No.)		
		ITS	Similarity (%)	Accession No.
Trichoderma pezizoides	RP1	*Trichoderma pezizoides* (DQ835513.1)	98.79	MW659098
Daldinia eschscholtzii	RP2	*Daldinia eschscholtzii* (MN310384.1)	100	MW659100
Cookeina sulcipes	RP3	*Cookeina sulcipes* (KY094620.1)	98.44	MW659101
Cookeina garethjonesii	RP4	*Cookeina garethjonesii* (KY094622.1)	99.06	MW680773
Cookeina tricholoma	RP5	*Cookeina tricholoma* (KY094619.1)	100	MW680771
Xylaria terricola	RP6	*Xylaria terricola* (MF577038.1)	88.42	MW659104

Figures

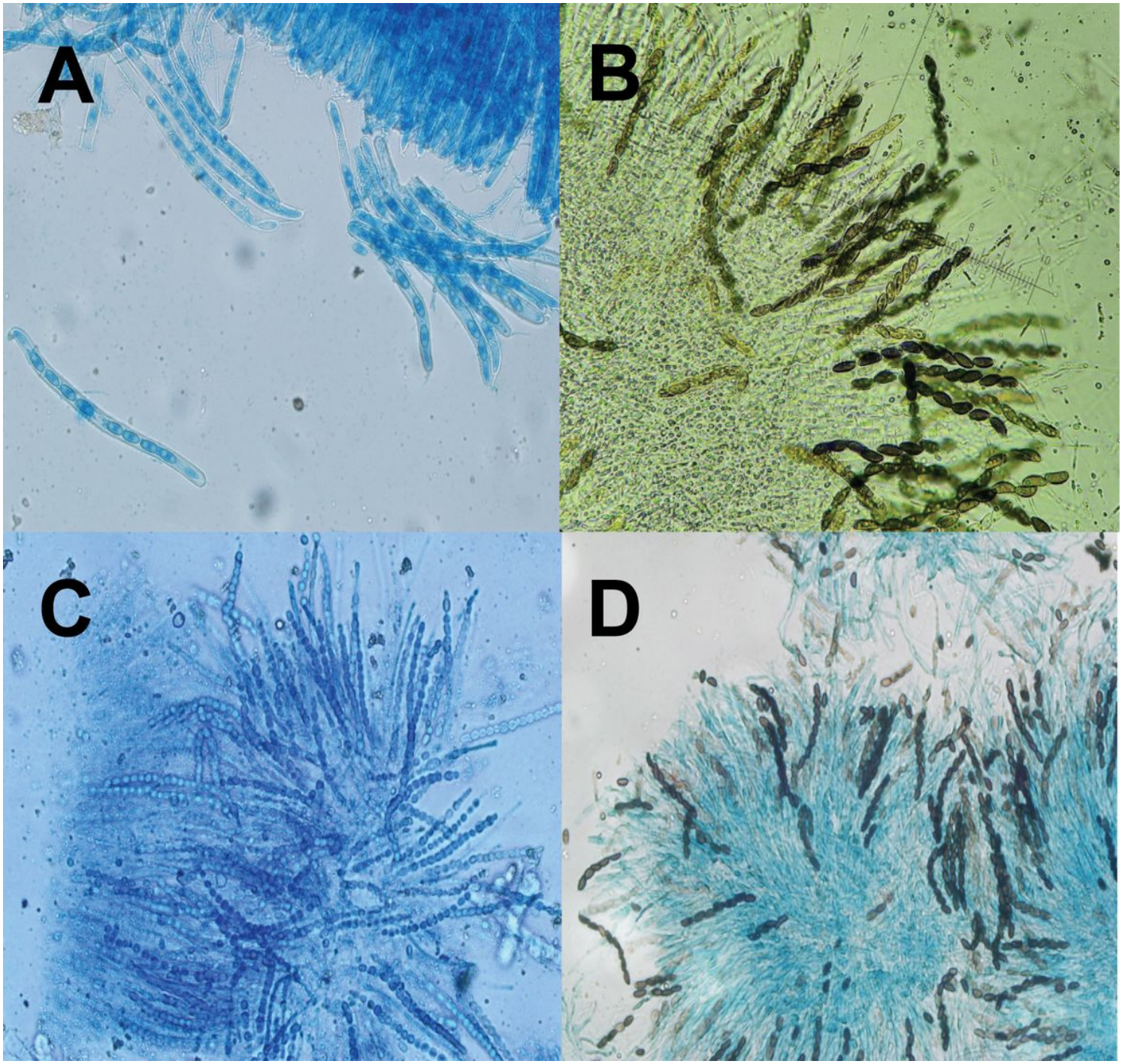

Figure 1

Representative ascospore of ascomycetes mushrooms from rubber plantation. A: Cookeina sp.; B: Daldinia sp.; C: Trichoderma sp.; D: Xylaria sp.

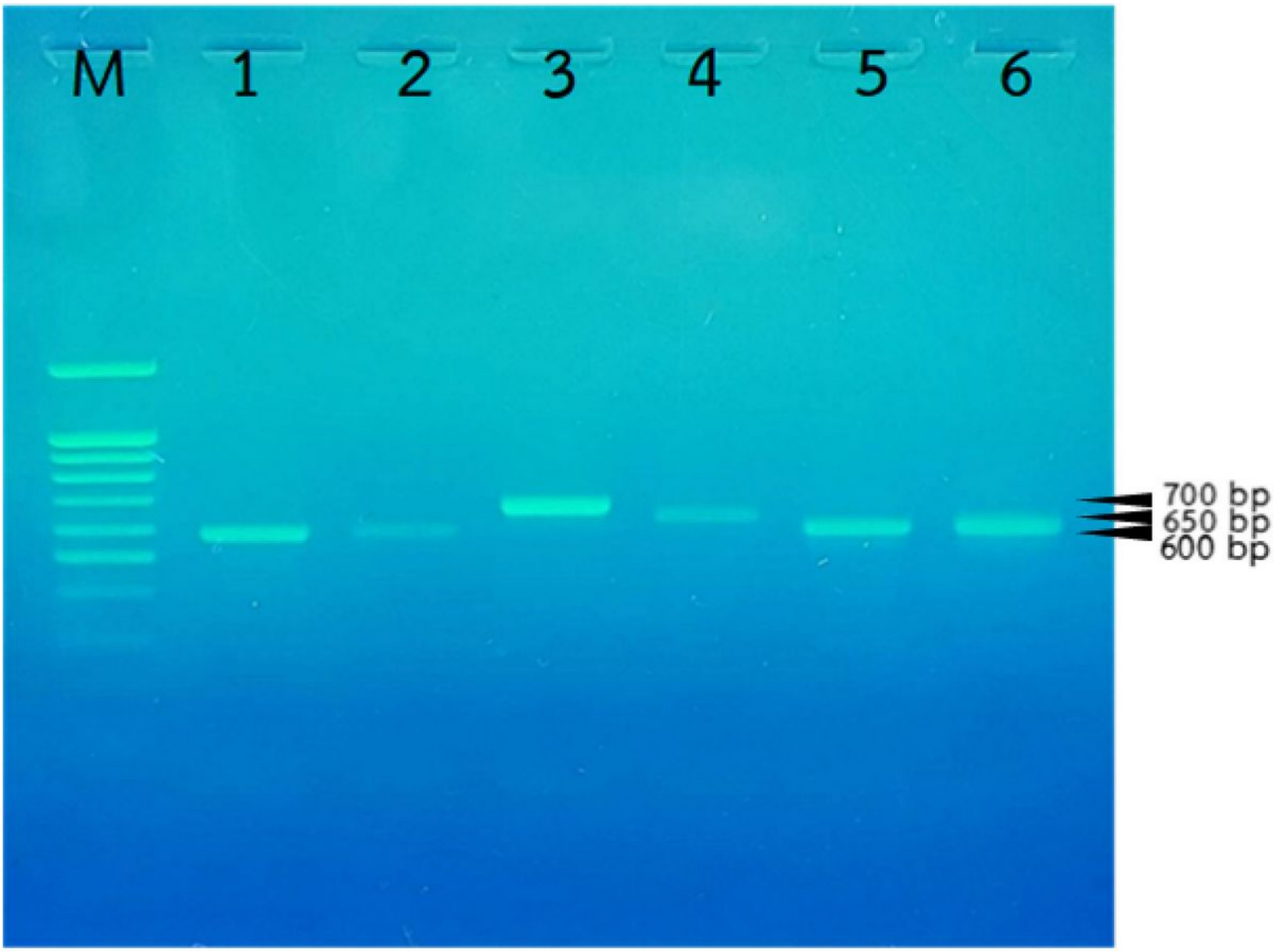

Figure 2

Agarose gel electrophoresis of ITS region amplification in ascomycetes mushroom. Lane M: DNA ladder (100 bp); Lane 1: RP1 (600 bp); Lane 2: RP2 (600 bp); Lane 3: RP3 (700 bp); Lane 4: RP4 (650 bp); Lane 5: PR5 (600 bp); Lane 6: RP6 (600 bp)

Figure 3

Ascomycetes mushroom identified in this study. A: Trichoderma pezizoides (RP1); B: Daldinia eschscholtzii (RP2); C: Cookeina sulcipes (RP3); D: Cookeina garethjonesii (RP4); E: Cookeina tricholoma (RP5); F: Xylaria terricola (RP6)

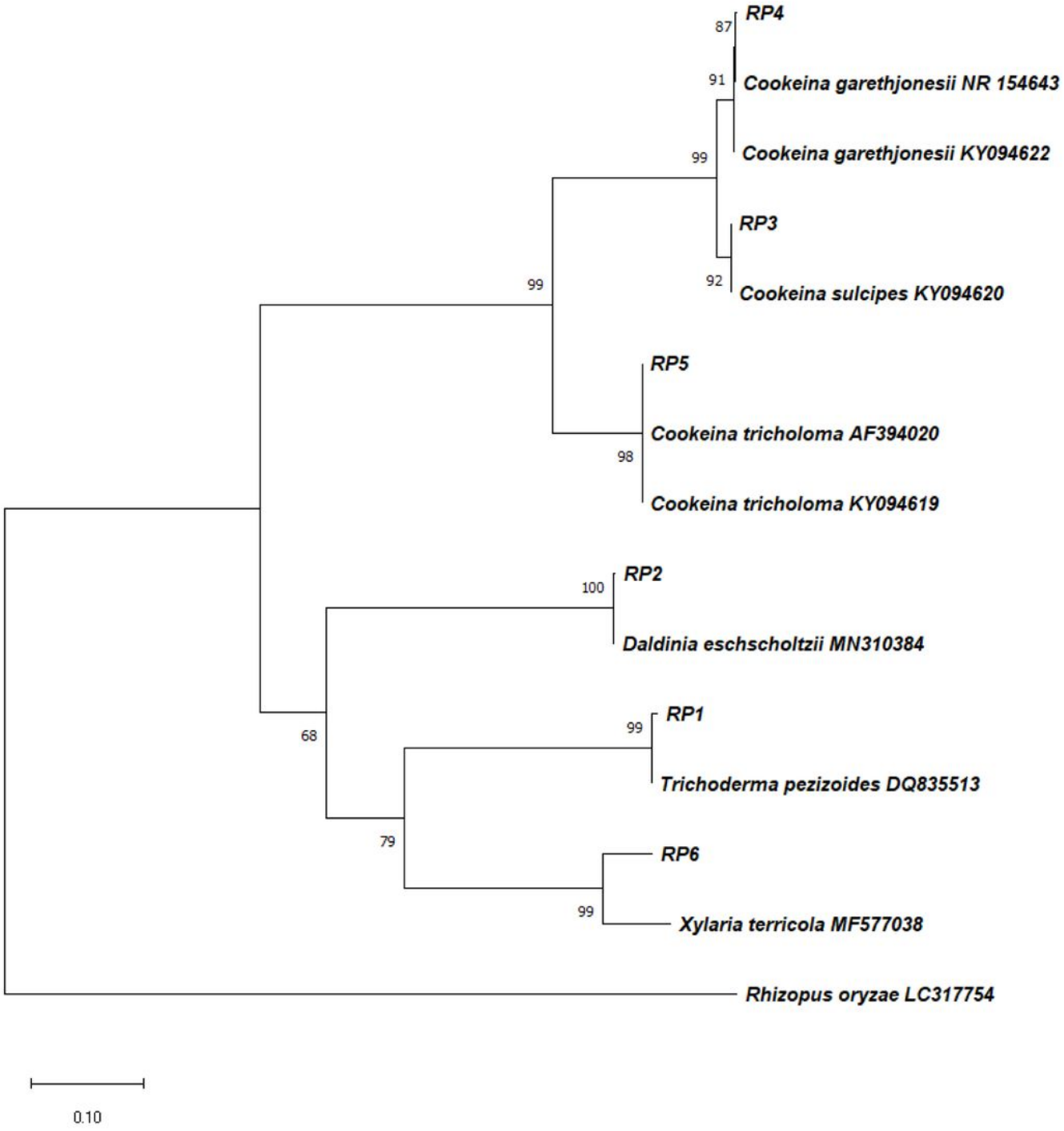

Figure 4

Phylogenetic analysis based on ITS sequences of 6 ascomycetes mushroom and 9 other references sequences from the GenBank.