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Research Article
Diversity of *Suillus* Fungi from Pine (*Pinus merkusii*) Stands at Various Locations in Bandung Area, Indonesia

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Abstract

Background and Objective: Ectomycorrhyza is associated with most trees, including the pine tree. The interaction between ectomycorhizzal fungi with pine trees has been reported in some species from other regions but none from Indonesia, especially in Bandung (West Java). This study aims to determine the diversity of ectomycorrhizal fungi found under pine (*Pinus merkusii*) stands at various locations in the Bandung area. **Material and Methods:** The method used in this study includes exploration, collection of the fungal fruiting body, isolation and identification using morphological and molecular approaches. The exploration site was based on the presence of the pine tree in seven locations in Bandung. **Results:** Seven samples of fruiting bodies representing each location were collected from under stands of pine tree, namely BK-01 (Bojong Koneng), GS-02 (Ganesha campus), TC-03 (Taman Cibeunying), CK-04 (Cikutra), SK-05 (Sukaluyu), CS-06 (Cisitu) and PP-07 (Perum Pahlawan). **Conclucion:** The identification results based on morphology and the ITS DNA character show that all ectomycorhizal samples belong to the order of Boletales, family of Suillaceae and genera of *Suillus*. The ectomycorrhizal fungi from Bojong Koneng, Ganesha campus and Perum Pahlawan are identified as *Suillus placidus,* while the ectomycorrhizal fungi from Taman Cibeunying, Cisitu, Cikutra and Sukaluyu are identified as *Suillus granulatus*. This study has clearly shown that the diversity of ectomycorrhizal fungi were found under the pine trees in the area of Bandung is a fungi with the name of the species *Suillus placidus* and *Suillus granulatus*.

Key words: Ectomycorrhizal diversity, phylogenetic, pine tree, *Pinus merkusii*, boletales, suillaceae, *Suillus placidus*, *Suillus granulatus*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

One of the microbial groups that has important functions in the rhizosphere are fungi. They can help plants to grow through various mechanisms, such as an increasing the absorption of nutrients, acting as a biological control against pathogens and also producing growth hormone for plants[1,2]. One group of fungi that play an important role in the absorption of water and nutrients is mycorrhiza[3]. Mycorrhizal fungi has a form of mutualistic symbiotic relationship between fungi (mykes) and the roots (rhiza) of higher plants[4]. Mycorrhizal fungi are classified into three groups i.e endomycorrhiza (vesicular-arbuscular mycorhizal), ectomycorrhiza and ectendomycorrhiza which is an intermediate form of the 2 types[5]. Ectomycorrhizal fungi can associate with an estimated number of 6,000-10,000 species of vascular plants[4,6], Mainly from of the family Fagaceae, Pinaceae, Betulaceae and Dipterocarpaceae[7]. Meanwhile, endomychorrizal fungi can associate with nearly 80% of plant species[6,8].

Ectomycorrhizal fungi are able to interact with plants roots and help to absorb water and nutrients in the soil. Among others roles, ectomycorrhizal fungi play an important role in solubilizing phosphorus and aiding its the absorption as well as other inorganic nutrients including nitrogen and potassium[3,9]. In general, the association of plants and ectomycorhizal fungi results in a better plant growth compared to non-associated plants. The formation of ectomycorrhizal fungi is a series of fairly complex processes that involves both physiological and morphological changes in plant roots and soil fungi[10,11]. Several factors are required in association mycorrhizal fungi that appropriate environmental conditions, the host root compatible and viable inoculum.

Pine is an example of a host plant for ectomycorrhizal fungi which forms a mutually beneficial symbiosis when its roots are colonized by the fungi[12-16]. Some species of ectomycorhizal fungi have been found beneath stands of pine forest in North Sumatra (Indonesia) is only known by the name of the genera, namely *Boletus* sp., *Russula* sp., *Suillus* sp., *Inocybe* sp. and *Lactarius* sp.[17]. Information about the species name of ectomycorhizal fungi has not been reported in Indonesia, especially in Bandung and surrounding areas. This paper documents for the first time, the species name of ectomycorhizal fungi found under pine trees (*Pinus merkusii*) in Indonesia especially in the Bandung areas.

MATERIALS AND METHODS

Exploration and isolation of ectomycorrhizal fungi: The study was done by exploring ectomycorrhizal fungi, which grow beneath pine stands in several locations in the Bandung area. Pine trees are mainly planted as shedding plant in city parks or housing complexes. Fungal fruiting bodies were collected in plastic bags after being wrapped in a wet tissue paper. Isolation was done by culturing sterile tissue on Modified Melin-Norkrans (MMN) agar media and modified PDA[18]. The MMN agar contains $(NH_4)_2HPO_4$ (0.25 g L^{-1}), KH_2PO_4 (0.5 g L^{-1}), $MgSO_4.7H_2O$ (0.15 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.05 g L^{-1}), NaCl (0.025 g L^{-1}), $FeCl_3 \cdot 6H_2O$ (liquid 1%) (1.2 mL L^{-1}), D-glucose (10 g L^{-1}), thiamine (0.0001 g L^{-1}), malt extract (3 g L^{-1}) and agar (15 g L^{-1}) while modified PDA contains potato extract (200 g L^{-1}), dextrose (20 g L^{-1}), bacto agar (15 g L^{-1}), K_2HPO_4 (2 g L^{-1}) and thiamin (0.01 g L^{-1}). Isolation was done by cutting a piece of sterile tissue after cutting apart by hand the fruiting body apart by hands near the pileus and stem. Inoculated media were then incubated at room temperature in an incubation chamber over 7-14 days.

Characterization and identification of ectomycorrhizal fungi: Characterization of ectomycorrhizal fungi was conducted by observing morphological, macroscopic and microscopic characters based on the Brundrett method[19]. Morphological characterization was done by observing the size, shape, color and texture of the cap, hymenium and fruiting body. Macroscopic characterization was done by observing the size, shape and color of the mycelium colony, while microscopic characterization was done by observing the size, shape and color of the spores and hyphae of certain isolates that can grow on the culture medium of certain isolate. Preparation of genomic DNA for molecular identification of ectomycorrhizal fungi was as follows: Genomic DNA was extracted from the fungi's fruiting bodies as well as from the hyphae of the succesfully cultured isolate by using a slightly modified Cationichexadecyl Trimethyl Ammonium Bromide (CTAB) method[20]. The Internal Transcribed Spacer (ITS) region was amplified using a pair of primer, ITS4 (5'-CCCGCCTGACCTGGGGTCGC-3') as reverse primer and ITS5 (5'-TAGAGGAAGGAGAAGTCGTAACAA-3') as forward primer. Determination of the base sequence of DNA was carried out in Macrogen (South Korea) and sequence similarity was analyzed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The DNA base sequences derived from the ITS region was aligned by using a computer program CLUSTALW[21]. The phylogenetic analysis was performed using DNA characters from the nucleotide sequence of the ITS region. The phylogenetic tree was created by using a computer program mega[21] 5.05.

RESULTS AND DISCUSSION

Diversity of ectomycorrhiza fungi: The fruiting bodies of 7 ectomycorrhizal fungi found under pine stands from various locations in Bandung were collected. They were coded as BK-01 from Bojong Koneng, GS-02 from Ganesha campus, TC-03 from Taman Cibeunying, CK-04 from Cikutra, SK-05 from Sukaluyu, CS-06 from Cisitu and PP-07 from Perum Pahlawan (Fig. 1). All of the 7 isolates have a common characteristic, which is a fruiting body that is shaped like an umbrella. This is a is typical feature of Basidiomycetes class. In general, ectomycorrhizal fungi found under pine stands in Indonesia has been reported to belong in the Basidiomycetes class[17], ours are not an exception. In addition to the umbrella shape, the spore bearing hymenium of all the fungal isolates were in the form of pores instead of gills. According to Binder and Hibbett[22] this kind of characteristics belong to several family within the order of Boletales i.e., Boletaceae, Boletinellaceae, Gyroporacease, Paxillaceae and Suillaceae.

The ectomycorrhizal fungi found in Bojong Koneng (BK-01), Ganesha campus (GS-02) and Perum Pahlawan (PP-07) have similar fruiting body shape as well as the color and texture of the mushroom cap (Table 1). The ectomycorrhizal fungi from Taman Cibeunying (TC-03), Cisitu

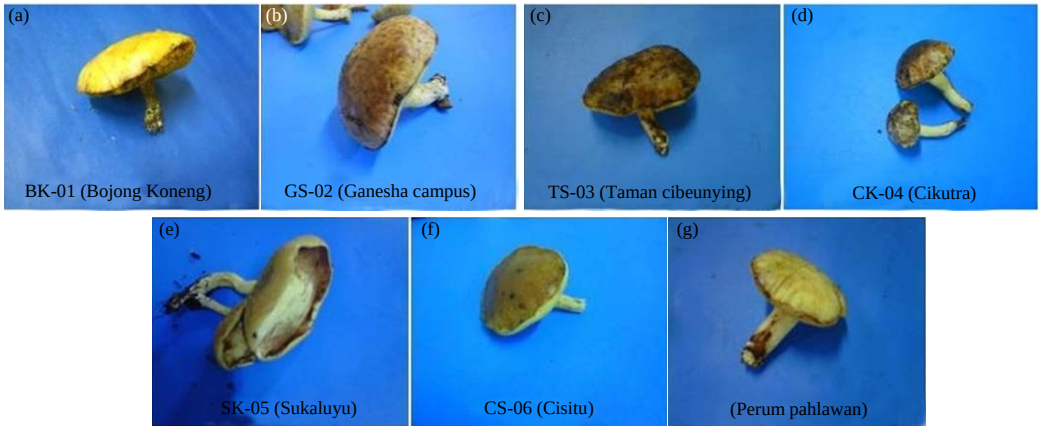

Fig. 1(a-g): Morphological diversity of ectomychorrhizal fungi's fruiting body from Bandung areas, (a) BK-01 (Bojong Koneng), (b) GS-02 (Ganesha campus), (c) TS-03 (Taman cibeunying), (d) CK-04 (Cikutra), (e) SK-05 (Sukaluyu), (f) CS-06 (Cisitu) and (g) (Perum pahlawan)

Table 1: Morphological characteristics of ectomycorrhizal fungi collected from under pine stands in Bandung areas

Location	Characteristics	Size (cm)	Shape	Color	Texture
Bojong	Cap	Φ 6-8.6	Convex	Yellow	Rough
Koneng	Hymenium	Φ 0.4-0.7	Porous	Yellow	Smooth
(BK-01)	Stem	↑ 3-3.5	Cylindrical	White	Rough
Ganesha	Cap	Φ 7-9	Convex	Yellow	Rough
campus	Hymenium	Φ 0.3-0.5	Porous	Yellow	Smooth
(GS-02)	Stem	↑ 2-3.5	Cylindrical	White	Rough
Taman	Cap	Φ 5-8.5	Convex	Yellow to brown	Rough
Cibeunying	Hymenium	Φ 0.2-0.6	Porous	Yellow	Smooth
(TC-03)	Stem	↑ 2-3.5	Cylindrical	White	Rough
Cikutra	Cap	Φ 2-4	Convex	Brown	Rough
(CK-04)	Hymenium	Φ 0.2-0.4	Porous	Yellow	Smooth
	Stem	↑ 1.5-3	Cylindrical	White	Rough
Sukaluyu	Cap	Φ 6-9.5	Concave	Brown	Rough
(SK-05)	Hymenium	Φ 0.4-0.7	Porous	Yellow	Smooth
	Stem	↑ 3-3.5	Cylindrical	White	Rough
Cisitu	Cap	Φ 5-7	Convex	Yellow to brown	Rough
(CS-06)	Hymenium	Φ 0.4-0.7	Porous	Yellow	Smooth
	Stem	↑ 2-3.5	Cylindrical	White	Rough
Perum	Cap	Φ 5.5-8	Convex	Yellow	Rough
Pahlawan	Hymenium	Φ 0.3-0.5	Porous	Yellow	Smooth
(PP-07)	Stem	↑ 2.5-3.5	Cylindrical	White	Rough

Description: φ: Diameter, ↑: Height

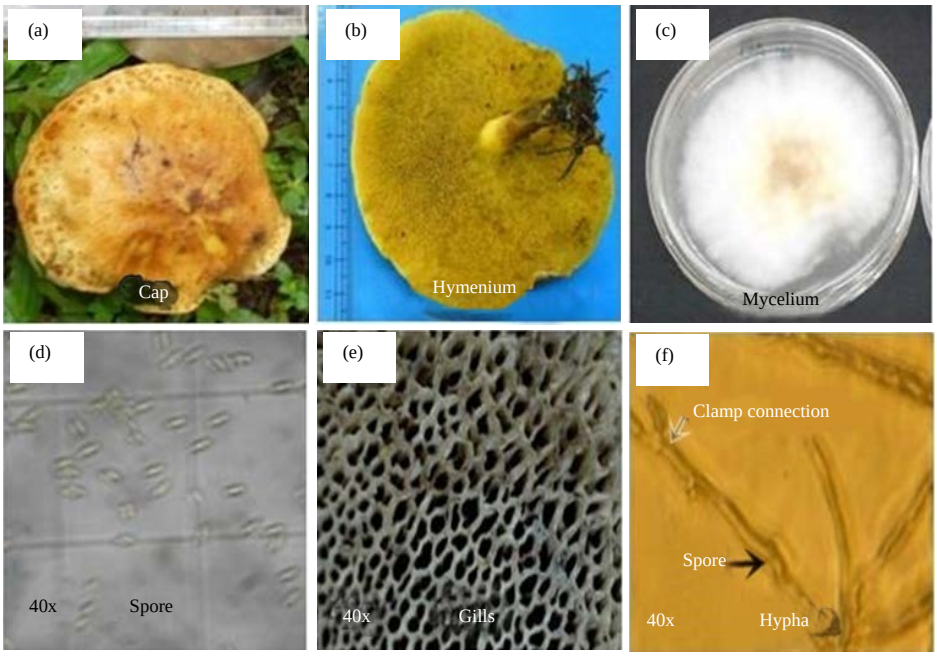

Fig. 2(a-f): Macroscopic and microscopic characteristics of ectomycorrhizal fungi from Bojong Koneng (BK-01) molecular identification, (a) Cap, (b) Hymenium, (c) Mycelium, (d) Spore, (e) Gills and (f) Hypha

(CS-06) and Cikutra (CK-04) was similar in morphology, although the cap color of CK-04 is brown (Table 1). Meanwhile, the ectomycorrhizal fungi from Sukaluyu (SK-05) is the only one a concave cap (Table 1). These varieties of morphological characters indicate the diversity of ectomycorrhizal fungi found under pine stands in the Bandung area.

The ectomycorrhizal fungal isolates from the of Bojong Koneng area (BK-01) could be cultured in MMN medium whereas the other 6 isolates could. Some ectomycorrhizal fungi were reported to be somewhat difficult to grow (cannot be cultured) *in-vitro*, since they could only grow well only when associated with their the host plant. Microbial interaction in the rizosphere area apparently also influence the physiology of ectomycorrhizal fungi to grow *in vitro.* Some studyers reported that the root exudates of host plants played a role in stimulating the growth of ectomycorrhizal fungi[1,23,24]. Several studies have also reported that the majority of ectomycorrhizal fungi were obligate symbionts, so it is difficult grown them in a culture medium *in vitro*. Because these fungi are typically obligate symbionts, their isolation into a pure culture medium is extremely difficult. Nevertheless, the isolation was reported possible, both from sporocarp tissue and from colonized roots but the growth rate was very slow *in vitro*[25-27].

Macroscopic and microscopic observations were able to be conducted on isolate BK-01 (Fig. 2). The isolate has white colonies of mycelium, which is somewhat thick in texture (Fig. 2c). The spores is oval, with size ranging between 50-200 mm. The amount of spores is 1.93×10^6 spores g^{-1} of fruiting body and the color is hyaline (Fig. 2d). The number of pores (gills) on hymenium was 876 mm^{-2} (Fig. 2e). The hyphae formed a clamp connection, which is an extensive structure shaped like small buds on bamboo nodes (Fig. 2f). This structure as well as the presence of septa are characteristic spossessed by most ectomycorrhizal fungi from the Basidoimycetes class[19].

Molecular identification: Based on the result of amplification, the size of the ITS region of the 7 ectomycorrhizal isolates range between 500 and 750 bp (Fig. 3). According to Porter and Golding[28] the full ITS region in fungi has an average length of 500 and 600 bp for Ascomycetes and Basidiomycetes, respectively and an average length of 600 bp across all fungal lineages. The size of the base pairs was also varies due to variation in the length of each area of ITS rDNA and also because there might be a deletion, insertion or substitution in the ITS region between species[29].

The ITS region sequence of the 7 isolates was analyzed using BLAST to compare their similarity with other sequence available in the GenBank data base. The BLAST results showed that isolate BK-01, GS-02 and PP-7 are similar to *Suillus placidus* at a similarity of 97, 99 and 96%,

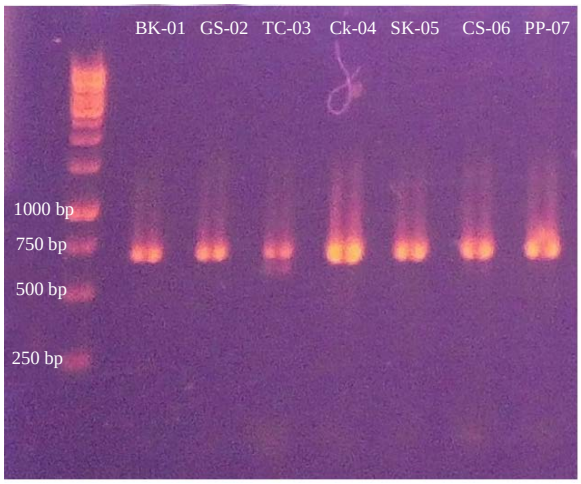

Fig. 3: Electropherogram of amplification product profile of ITS region of the seven isolates of ectomycorrhizal fungi

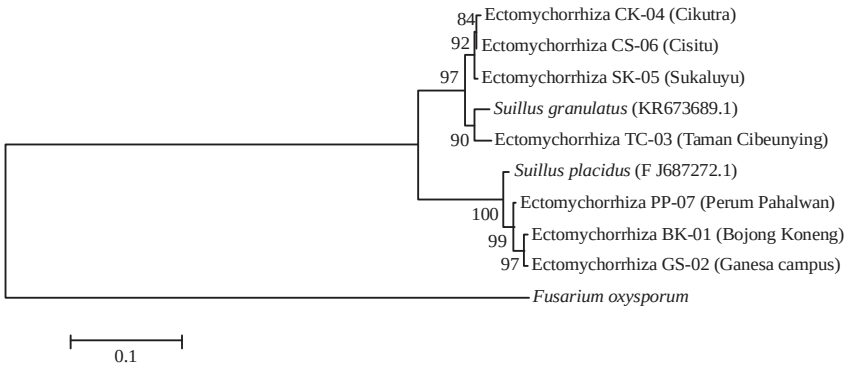

Fig. 4: Phylogenetic analysis of ectomycorrhizal fungi based on ITS sequences using the neighbour joining method with 1000x bootstrap

respectively. While, isolate TC-03, CK-04, SK-05 and CS-06 are similar to *Suillus granulatus* at similarity of 99, 97, 81 and 99%. Since, all the ITS sequences of the seven isolates showed high similarity with that of other fungi in the GenBank data base, this suggested that the PCR amplification of the ITS region was successful[30].

Identification of genotypes based on the phylogenetic tree construct: The phylogenetic tree is a logical approach to show the evolutionary relationships between organisms[31]. One purpose for preparing the phylogenetic tree is to establish the proper relationship between organisms as well as estimate the difference from a common ancestor to the offspring[32]. To illustrate the relationship between the ectomycorrhizal fungi isolate, we compared their ITS gene sequence to the other ectomycorrhizal fungi species found in the NCBI GeneBank, using ClustalW program[21]. The result showed that the genotypic character of isolates TC-03 was similar to *S. granulatus* with a bootstrap value 90 as well as CK-04, SK-05 and CS-06 were similar to *S. granulatus* with a bootstrap value of 97. All four isolates were similar to *S. granulatus* (GenBank accession number: KR673689.1) from Korea (Fig. 4). Based on this tree topology, they are identified as *S. granulatus*. The genotypic character of isolates BK-01, GS-02 and PP-07 were similar to each other and they were also similar to *S. placidus* (GenBank accession number: FJ687272.1) from China with a bootstrap value of 100 (Fig. 4). This shows that the three isolates can be identified as *S. placidus*.

CONCLUSION

Results of morphological and molecular identification through ITS DNA analysis indicated that all ectomycorhizal isolates found from seven locations around belong in the class of Basidiomycetes, order of Boletales, family of Suillaceae and

genera of *Suillus*. Based on the phylogenetic tree constructed from the genotypes character the ectomycorrhizal fungi from Bojong Koneng (BK-01), Ganesha campus (GS-02) and Perum Pahlawan (PP-07) are identified as *S. placidus*, while ectomycorrhizal fungi from Taman Cibeunying (TC-03), Cikutra (CK-04), Sukaluyu (SK-05) and Cisitu (CS-06) are identified as *S. granulatus*.

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