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The Unpopular Edible Bolete (*Phlebopus portentosus*) in Indonesia

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ABSTRACT

Phlebopus portentosus **(Berk. & Broome) Boedijn was firstly constructed from the collection of Indonesia in 1951. To date, the subsequent collection of this ectomycorrhizal (ECM) fungi has not been done in Indonesia. In addition, the utilization information of this edible mushroom as food is not popular for the country. The goal of our work was to update the current collection, provide the morphological and molecular data, and promote the utilization of this edible mushroom in Indonesia. Fresh fruiting bodies were evaluated for morphological and molecular evidence. The basidiomata were analysis on the basis of the morphological and molecular evidence. The phylogenetic tree was constructed following the rDNA-ITS 1/2 sequence.** ***P. portentosus*** **was verified by morphological and phylogenetic studies combined. The indigenous people in the research site use this wild edible mushroom for self-consumption and sell it to local market.** ***Phlebopus portentosus*** **BO24626 was solitary to connate, boletoid basidioma, yellowish to greenish brown pileus and stipe, yellowish hymenophore, clavate stipe, club shaped basidia, oval to subglobose basidiospores, cystidia present. The absence of sponge-like tissues and the occurrence of sterigmata distinguished our specimens from the similar morphological species** ***P. spongious*****. The lack of reddish stipe distinguished our specimen from** ***P. roseus*****. In addition, the presence of hymenial cystidia delimits our specimens from** ***P. colossus*****. The inferred phylogenetic tree nested our specimen in the group of** ***P. portentosus*** **(sister to** ***P. spongiosus*****). The ITS sequence of our specimen is now deposited at GenBank and can be applied to upcoming research of** ***P. portentosus*****.**

1. Introduction

Phlebopus was proposed as part of the genus Boletus to accommodate *B. colossus* (Heim 1936). Subsequently, *Phlebopus* was raised to genus and typified by *P. colossus* (Singer 1936). Kirk *et al.* (2008) accounted 12 species of *Phlebopus* worldwide. Another author settled *Phlebopus* in the Boletinellaceae (Binder and Bresinsky 2002). This genus can be recognized by boletoid basidiomata, brown shade of spore print, ellipsoidal spores, and clamped in all tissues (Singer 1986; Watling 1999). Currently, Index Fungorum (2023) accepted 23 epithets of *Phlebopus* worldwide with 20 species include: *P. beniensis*, *P. braunii*, *P. bruchii*, *P. brunneoruber*, *P. colossus*, *P. cystidiosus*, *P. harleyi*, *P. hemichrysus*, *P. latiporus*, *P. lignicola*, *P. brasiliensis*, *P. marginatus*, *P. mexicanus*, *P. portentosus*, *P. silvaticus*, *P. spongiosus*, *P. sudanicus*, *P. sulfureus*, *P. tropicus*, *P. viperinus*, and *P. xanthopus*. Of those species, only *P. portentosus* has been described from Indonesia by Boedjin (1951).

Phlebopus portentosus is extensively allotted across many countries including Indonesia (McKenzie *et al.* 2000; Watling 2001; Lumyong *et al.* 2007; Lei *et al.* 2009; Sanmee *et al.* 2010; Kumla *et al.* 2012). This species is usually located in natural habitats where mycorrhizae formed (Ji *et al.* 2011). Morphologically, *P. portentosus* is morphologically close to *P. spongiosus* (Pham *et al.* 2012). The latter species is claimed to be endemic and consumed in Vietnam and Thailand (Kumla *et al.* 2020). *P. portentosus* is a commonly

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eaten mushroom in China and Thailand due to its high nutrient content and popularity in the local market (Ji *et al.* 2011; Kumla *et al.* 2012; Kumla *et al.* 2020). In addition, this species holds the distinction of being the initial species within the Boletaceae order to undergo large-scale industrial cultivation (Ji *et al.* 2011; Kumla *et al.* 2015).

Although *P. portentous* is acknowledged as a delicacy (Sanmee *et al.* 2010; Kumla *et al.* 2012; Zhang *et al.* 2017) and originally validated from Indonesia (Boedjin 1951), the consumption of this species is less popular in Indonesia. During surveys of Basidiomycota in Kuningan (West Java, Indonesia), some basidiomata of yellowish bolete were collected. At a glance, we identified it as *P. portentosus* by macromorphological features in situ. In addition, after consulting with the indigenous people, this mushroom was usually consumed and traded. The goal of this research was to update the current information on *P. portentosus* (with herbarium collection) in Indonesia.

2. Materials and Methods

2.1. Specimen Collection

The materials were acquired from Kuningan, West Java, Indonesia during our mycobiota survey in 2023. The mushrooms were observed in their natural habitat and relevant ecological data was documented. Some basidiomata were stored to Herbarium Bogoriense (BRIN), Indonesia. The utilization information including time of foraging and price of mushrooms was obtained through discussion with the indigenous people in the research site.

2.2. Morphological Identification

The macromorphological features observed in situ include habitat, growth pattern, basidiomata texture, cap features (shape, surface, color, margin, wetness), hymenophore attributes (type, color, arrangement), context, and stipe characters. The microscopic features comprising basidia, cystidia, spores, and clamp connection were examined using a optical microscope. Specimens were further analyzed using scanning electron microscopy (SEM), as described by Goldstein *et al.* (1992). The lamellae were sliced into small pieces measuring 5 × 5 mm and then treated with a solution of 2.5% glutaraldehyde in a cacodylate buffer with a pH of 8.4 at a temperature of 27°C for a duration of two days. Following this, the samples underwent pre-fixation in a 2% tannic acid solution for a period of six hours and were subsequently washed using four different cacodylate buffers. Dehydration was carried out using a series of ethanol concentrations ranging from 50% to 100%, followed by two rounds of infiltration with t-butanol for 10 minutes each. The samples were then freeze-dried and mounted on an aluminum stub using double-sided carbon tape. Finally, a gold coating was applied before observations were conducted using the JSM IT 200 SEM system manufactured by JEOL in Tokyo, Japan. Morphological identification was based on several references, including Boedjin (1951), Kumla *et al.* (2012), Pham *et al.* (2012), Kumla *et al.* (2020), and Baroni *et al.* (2015).

2.3. Molecular Analyses

Chromosomal DNA was extracted from a fresh basidiocarp at iLab Laboratory, Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Indonesia, using the Qiagen Dneasy Plant Mini Kit following the manufacturer's instructions. The extraction method described by Putra *et al.* (2023) was employed to extract fresh basidiomata. DNA amplification was carried out using the Thermo Scientific Arktik Thermal Cycler (Thermo Fisher Scientific) with the primer pairs ITS 5 and ITS 4, as described by White *et al.* (1990). The PCR reaction mixture consisted of 9 µL ddH2O, 1.5 µL of 10 pmol of each primer, 25 µL PCR mix from 2× PCR buffer for KOD FX Neo (Toyobo), and 2 µL of 100 ng template DNA in a total volume of 50 µL. The PCR conditions included an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 45 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The PCR product was then subjected to 1.5% agarose gel electrophoresis using TAE buffer (40 mm Tris-acetate, pH 8.0 1 mM EDTA), stained with FloroSafe DNA stain, and visualized using the Gel Doc EZ Gel Documentation System (Biorad). Finally, the PCR products were sent to 1st Base Malaysia for sequencing.

The ChromasPro software was utilized to assemble the sequences, and subsequently, the final generated sequences were deposited in GenBank to acquire the accession number. To determine the homology with existing data, the sequences underwent Basic Local Alignment Search Tool (BLAST) analysis in NCBI. The BLAST results (Table 1) were then used to select published sequences (Kumla *et al.* 2020) for phylogenetic tree analyses, with *Leucoagaricus medioflavoides* serving as the outgroup. The alignment of the sequences was performed using Clustal X Ver. 2.1 software (Larkin *et al.* 2007) and saved as PHYLIP format files. The phylogenetic tree was generated using the Randomized Axelerated Maximum Likelihood (RAxML) Black Box on CIPRES (Stamatakis 2014). Bootstrap values (BS) of 70% or higher were indicated on the branches of the phylogenetic trees.

3. Results

3.1. Taxonomy

Phlebopus portentosus (Berk. & Broome) Boedijn (Figure 1-3), Sydowia 5(3-6): 218 (1951).
Basionym:
Boletus portentosus Berk. & Broome 1873

Basidiomata solitary to connate. Pileus up to 12 cm broad, convex to plano-convex when young, applanate when mature, yellowish to greenish brown, with some darker spots, olive tints, smooth, dry to moist, centrally depressed, margin inrolled at young stage and entire in maturity, sometimes with brown latex near margin. Context 0.4-2.0 cm thick, fleshy, pale yellow, slowly bruised olive green after injury. Tubes decurrent around the stipe, yellowish brown, subangular, up to 1 mm in diam. Stipe central, yellowish brown to blackish brown, cylindrical to clavate, enlarge at bottom, 3-6 cm length, 1.5-3.5 cm wide at base, 1.0-1.6 cm near apical, interior solid, bruised olive green after cut, surface dry, tomentose, longitudinally striate toward the base, basal mycelium blackish brown. Basidia and basidioles club-shaped. Basidia 23-32 × 3-4 µm, 4-spored, sterigmata 3-4 µm length. Basidiospores oval to subglobose, yellowish brown, with large central gutta, thin-walled, smooth, 4-6 × 3-4 µm. Hymenial cystidia present. Hymenial and stipe trama with densely interwoven hyphae, thin-walled. Pileipellis consists of appressed interwoven hyphae.

Specimen examined: under *Albizia chinensis* (Osbeck) Merr., Dusun Calingcing, Desa Cileuya, Kecamatan Cimahi, Kabupaten Kuningan, West Java, Indonesia, 7°02'38.9"S 108°42'00.6"E, 768 m a.s.l, collected by EK December 2023, *Phlebopus portentosus* BO246256.

3.2. Molecular Analyses

The final ITS sequence was registered at GenBank with reference number ITSON015653. The BLAST search revealed that *P. portentosus* BO246256 exhibits a high query cover with *P. portentosus* in all hits. The phylogenetic tree (Figure 4) was constructed from four species of *Phlebopus* with available ITS sequences. The phylogenetic tree displayed *P. portentosus* BO246256 in the same taxonomical group to *P. portentosus* with 99% BS values. In addition, *P. spongiosus* was a sister clade to *P. portentosus* clade.

Table 1. Selected species, voucher information, origin, and GenBank accession of sequences used in this study

Species	Collection code	GenBank accesion number of ITS region
Boletus edulis	Voucher Can der Linde SvdL15	EU417873
Phlebopus beniensis	Voucher MAN 357	MG996747
Phlebopus beniensis	Voucher VO1891	MT939284
Phlebopus braunii	Isolate PH57105	KM236049
Phlebopus braunii	Isolate PH57106	KM236050
Phlebopus braunii	Isolate PH57103	KM236047
Phlebopus marginatus	Isolate REH8883	EU718190
Phlebopus mexicanus	Voucher FCME J Cifuentes and S Cappello 2009 233	KM675999
Phlebopus portentosus	Isolate REH8795	EU718111
Phlebopus portentosus	Isolate FG YB3	MT272130
Phlebopus portentosus	**Voucher BO 246256**	**ON015653**
Phlebopus roseus	Voucher MY2017277	MK734174
Phlebopus roseus	Voucher MY2017275	MK734171
Phlebopus roseus	Voucher MY2017274	MK734175
Phlebopus sp.	Isolate php1	EU718110
Phlebopus spongiosus	Strain CMUB39826	KX57661
Phlebopus spongiosus	Strain CMUB39824	KX575660

Figure 1. Macrocopic characters of *Phlebopus portentosus* BO246256. (A) Basidiomata grow near *Albizia chinensis*, (B) the stipe showing enlarge at base, (C) context detail, (D) vertical section of the hymenophore showing no sponge-like tissue. Bar: A–B: 5 cm, C: 2 cm, D: 1 mm

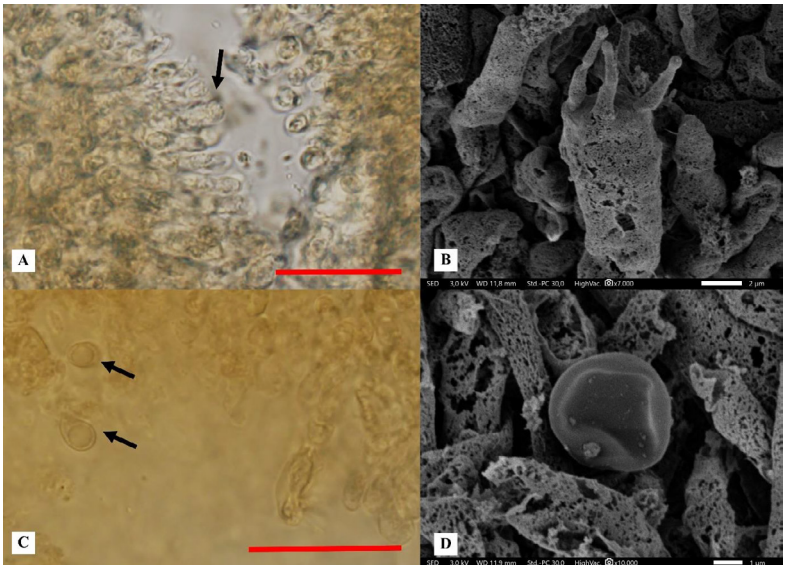

Figure 2. Basidia and basidiospores of *Phlebopus portentosus* BO246256. (A and B) Club-shaped basidia with 4 sterigmata, (C and D) oval to subglobose basidiospores with central gutta. Bar: A, C: 20 µm, B: 2. µm, D: 1 µm

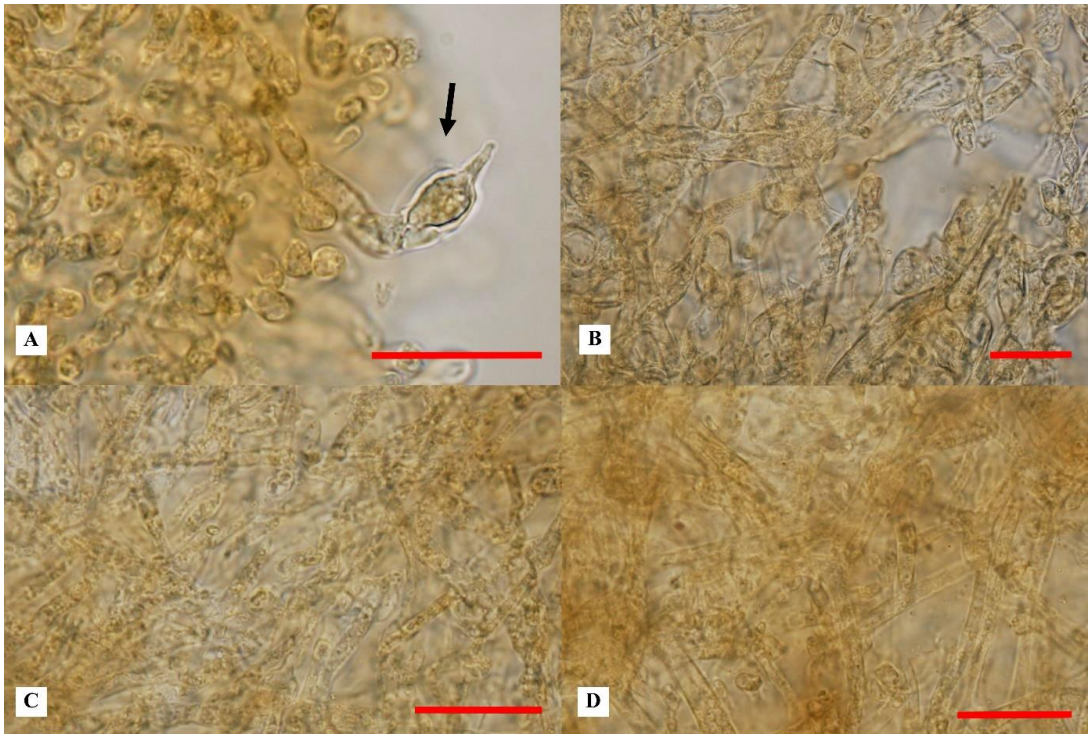

Figure 3. Cystidia, trama, and pileipellis of *Phlebopus portentosus* BO246256. (A) Hymenial cystidia, (B) hymenial trama, (C) stipe trama, (D) interwoven hyphae of pileipellis. Bars: 20 µm

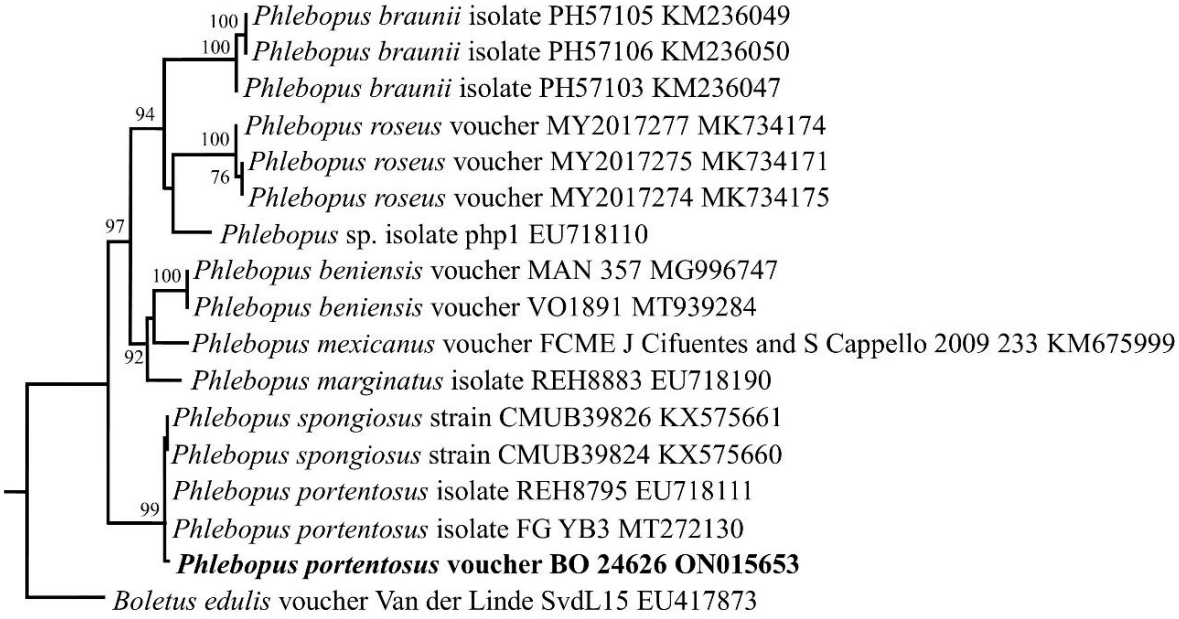

Figure 4. The phylogenetic tree of *Phlebopus portentosus* BO246256 inferred from ITS1/ITS2 rDNA sequence using RAxML HPC2 on XSEDE

4. Discussion

Currently, there are twenty species of *Phlebopus* recognized around the world (Index Fungorum 2023). The 'black bolete' which was previously described as *Boletus portentosus* from Sri Lanka, was validated as *P. portentosus* by Boedjin (1951) from the Indonesian collection. The current study reports the current collection of *P. portentosus* in Indonesia after more than seventy years of hiatus. *Phlebopus portentosus* BO246256 can be recognized by the large size of yellowish to greenish boletoid basidiomata. To some extent, our specimens were resembling the *P. spongiosus*. However, the absence of thin layer of tissue with sponge features, which covers the young pores, and spore prints that are deep brown in color, distinguished *P. portentosus* BO246256 from *P. spongiosus*. The dimension of basidiospores from the current study were smaller compared to those reported by Boedjin (1951). Mei *et al.* (2020) reported that the lack of reddish stipe in *P. portentosus* distinguished this species from *P. roseus*. In addition, the occurrence of hymenial cystidia was delimited our specimens from *P. spongiosus* as explained by Pham *et al.* (2012) and Kumla *et al.* (2020). Moreover, the presence of hymenial cystidia separated *P. portentosus* BO246256 from *P. colossus* based on the description of Americas *Phlebopus* (Baroni *et al.* 2015). Interestingly, this finding is contrary to Boedjin (1951) which reported the lack of hymenial cystidia from the Indonesian *P. portentosus*. We suggest that more field collection should be warranted to confirm the occurrence of hymenial cystidia in *P. portentosus.*

The BLAST outcome displayed that the current specimen has a close relation to *P. portentosus* (96%) as all hits. confirming the BLAST result, the phylogenetic tree showed that BO246256 as *P. potentosus* (99% BS value) and formed a sister clade with *P. spongiosus*. Prior molecular studies revealed that *P. portentosus* was placed in an inconsistent position. *Phlebopus portentosus* was placed in the same clade as *P. spongiosus* (Baroni *et al.* 2015), while the phylogenetic tree from Kumla *et al.* (2020) nested *P. portentosus* with *P. sudanicus*. Baroni *et al.* (2015) reported that *P. portentosus* is conspecific with *P. sudanicus,* but not with *P. marginatus.* Previously, some authors accepted *P. marginatus* Watling and N.M. Greg., described from Australia as a synonym of *P. portentosus* (Watling and Gregory 1988; Lei *et al.* 2009). In line with Baroni *et al.* (2015), *P. portentosus* was in a different clade with *P. marginatus* in our phylogenetic tree, supporting the fact that they are not conspecific. In addition, the ITS sequence of the current study is the only available sequence of *P. portentosus* from Indonesia. Schoch *et al.* (2012) reported that the ITS gene is an appropriate gene for fungal delimitation.

In the research site, *P. portentosus* BO246256 usually grows on the ground under *Albizia chinensis* (Osbeck) Merr. or sometimes near the base of *Musa* spp. Previously, Mei *et al.* (2020), explained that the basidiomata of *P. portentosus* appeared near the fruiting bodies of *P. roseous.* In other studies, *P. portentosus* often growing near tress include *Elaeocarpus hygrophilus, Dimocarpus longan, Syzygium cumini, Mimosa pigra, Mangifera indica*, and *Quercus* (Lei *et al.* 2009; Sanmee *et al.* 2010; Kumla *et al.* 2012). In addition, other studies reported that this species can be found near *Coffea arabica, Artocarpus heterophyllus, Eucalyptus* sp., *Citrus maxima* (Ji *et al.* 2011). Due to its habitat, prior works have proposed that this species as an ectomycorrhizal mushroom (Watling and Gregory 1988; Lumyong *et al.* 2007; Sanmee *et al.* 2010). However, Ji *et al.* (2011) proved that *P. portentosus* is a fungus that exhibits saprobic characteristics rather than engaging in symbiotic relationships and can be used for mushroom cultivation. The distinctive biotrophic nature of *P. portentosus* has been revealed in recent times and it forms the tripartite lifestyle between fungi, soil mealy bugs that form an insect gall, and plant roots (Zhang *et al.* 2017; Fang *et al.* 2020). However, we did not encounter the insect gall near the root of *A. chinensis* in this study.

Phlebopus portentous is considered as delicacy mushroom and only eaten in northern Thailand, China, Myanmar, and Lao (Ji *et al.* 2011; Kumla *et al.* 2012; Mortimer *et al.* 2012; Zhang *et al.* 2017; Łuczaj *et al.* 2021). Even though this species was originally validated from Indonesia in 1951, our study is the first information on the consumption of *P. portentosus* in Indonesia. The indigenous people (Sunda people) of Kuningan (West Java) usually collected this species around October. Kumla *et al.* (2012) reported that this species usually forms the fruiting bodies at May-July where the transition between hot and rainy season occurs and sometimes at the conclusion of the wet season (October) in northern Thailand. In the sampling site, the local people named *P. portentosus*

as 'babon' mushroom which refers to the big size of its basidiomata. This edible mushroom is known as 'Hed Har' or 'Hed Tub Tao Dam' in Thailand (Kumla *et al.* 2012), while in China it is called as 'Hei-ben-ma-lang' (Zhang *et al.* 2010). Due to its delicacy and high nutritional content, many reports considered *P. portentosus* as esteemed mushroom of Asia (Sanmee *et al.* 2010; Ji *et al.* 2011). In Thailand the market price for *P. portentosus* is 100–200 Baht/kg (Kumla *et al.* 2012), while it is US$10-15/kg in China (Zhang *et al.* 2017). However, in the research area this mushroom is only prized Rp. 15,000/kg or only US$1/kg. Considering the nutritional composition, taste, price, and biotrophic style of *P. portentosus*, the cultivation efforts of this species need a warrant in Indonesia.

Conflict of Interest

The authors declare no conflict of interest.

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