RECENT COLLECTION OF THE POISONOUS MUSHROOM, *CLARKEINDA TRACHODES* (BERK.) IN INDONESIA

IVAN PERMANA PUTRA[1*], OKTAN DWI NURHAYAT[2], WAHYU AJI MAHARDHIKA[3] AND OKTA YULIA SARI[3]

[1]*Mycology Division, Department of Biology, Faculty of Mathematic and Natural Sciences, IPB University, Bogor 16680, Indonesia*
[2]*Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Bogor 16911, Indonesia*
[3]*Microbiology of Post Graduate Program, Department of Biology, Faculty of Mathematic and Natural Sciences, IPB University, Bogor, 16680, Indonesia*

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ABSTRACT

Clarkeinda trachodes is a fascinating agaric recognized globally as one of the poisonous mushrooms. This species was discovered in the tropical regions of Asia, including Indonesia, but there has been no clear record of primary information and herbarium collection. During regular mushroom foraging in the forest of IPB University campus, Indonesia, the basidiomata of *C. trachodes* were encountered. Therefore, this study aimed to confirm the taxonomic position of the specimens using morphological and molecular analyses. Observation of fresh basidiomata was carried out to identify the macro- and micro-morphological features, followed by molecular analysis and phylogenetic tree construction based on the rDNA-ITS 1/2 sequence. The results confirmed the specimen BO24637 as *C. trachodes*. BO24637 was morphologically ascertained by a large basidiocarp of Agaricales, a prominent pellicle on disc pileus, a prominent ring, reddish brown context and stipe when injured, and a truncated apex of basidiospores. The BLAST result showed a high similarity (98.79%) to *C. trachodes*. In addition, the phylogenetic tree constructed using ITS sequence identified specimen BO24637 as *C. trachodes* with 100% Bootstrap value. In conclusion, the results provided a clear and accessible record of *C. trachodes* in Indonesia, including morphological and molecular information as well as herbarium documentation, which are relevant for future studies on this species.

Keywords: agaricales, *clarkeinda*, poisonus mushroom, taxonomy, toxic, west java

INTRODUCTION

Clarkeinda Kuntze is an unpopular and monotypic genus belonging to the family Agaricaceae (Basidiomycota, Agaricales). It is primarily found in Southeast Asia (Pegler 1985) and was discovered by Otto Kuntze (Kuntze 1891). The generic name *Clarkeinda* refers to the British botanist Charles Baron Clarke, and the suffix '*-inda'* suggests a connection to India (Pegler 1985). The Index Fungorum (2023) records 14 species of *Clarkeinda*, including *C. caparidensis, C. cellaris, C. Coprinus, C. gennadi, C. pedilia, C. pequinii, C. pervolvata, C. plana, C. poderes, C. rubrics,* and *C. trachodes*. Among these, *C. trachodes* is acknowledged globally as a poisonous macrofungus.

C. trachodes (Berk.) Singer has a long and dynamic history. It was originally identified in Sri Lanka as a new species called *Agaricus trachodes* Berk. (Berkeley 1847). The mushroom is known by several synonyms, including *A. trachodes* Berk. (1847) and *Chitoniella trachodes* (Berk.) Petch (1908). It is distinguished by a large basidiome, prominent chocolate or coffee brown to deep brown pellicle on the pileus disc surface, presence of an annulus, olive-brown to umber brown spore deposit, slightly thick-walled spores with a truncated apex, and a context that changes from white to reddish brown when cut. Furthermore, the species has dark-colored spores and two types of veils on its basidioma, partial and universal (Hosen and Ge 2011; Pegler 1985).

*Corresponding author, email: ivanpermanaputra@apps.ipb.ac.id

C. trachodes was reported to grow in Asian countries such as Thailand, Bangladesh, China, India, Malaysia, Indonesia, and Sri Lanka (Leelavathy *et al.* 1981; Yang 1991; Hosen and Ge 2011). Yang (1991) initially mentioned its distribution in Indonesia when describing an Agaric new to China. However, comprehensive details about the morphological description or herbarium collection of Indonesian specimens were not provided. During Mushroom Club regular survey at IPB University campus forest, West Java, Indonesia, basidiomata similar to *C. trachodes* was obtained. This study aimed to confirm the taxonomic position of the specimens through morphological and molecular analyses.

MATERIALS AND METHODS

Specimen Collection

The specimen was collected at IPB University Campus Forest, Bogor, West Java, Indonesia, in 2023 during regular foraging by the mushroom club of IPB University. The basidiocarp was photographed *in situ,* and ecological information, comprising coordinates, substrate, and vegetation, was noted. The specimen was contributed to the Herbarium Bogoriense, Indonesia, with collection number BO24637.

Morphological Identification

The morphological features were observed from fresh basidiocarp *in situ* and at the Mycology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Indonesia. Macroscopic characters, including color, size, pileus, stipe ornamentation, margin, and lamellae were observed directly. The microscopic features comprising basidia, cystidia, spores (shape, size, color, ornamentation), and clamp connection were examined using a light microscope. Specimens were further analyzed using scanning electron microscopy (SEM), as described by Goldstein *et al.* (1992). Lamellae were cut into small pieces (5 × 5 mm) and pre-fixed in 2.5% glutaraldehyde of a cacodylate buffer with a pH of 8.4 at 27°C for two days. Subsequently, the samples were pre-fixed in 2% tannic acid for six hours and washed with four different cacodylate buffers. Dehydration was carried out in 50%–100% ethanol series, infiltrated with t-butanol twice for 10 minutes, and freeze-dried. The freeze-dried samples were mounted on an aluminum stub with double-sided carbon tape and gold-coated. Observations were made with the JSM IT 200 SEM system (JEOL, Tokyo, Japan). The specimens were then identified using relevant identification references (Pegler 1985; Hosen and Ge 2011).

Molecular Analysis

Chromosomal DNA was isolated from a fresh basidiocarp using a Qiagen Dneasy Plant Mini Kit according to the manufacturer's instructions at iLab Laboratory, Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Indonesia. DNA amplification was conducted using a Thermo Scientific Arktik Thermal Cycler (Thermo Fisher Scientific). The amplification reaction was carried out using the primer pairs ITS 5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al.* 1990). The PCR amplification was performed in a 50 µL reaction mixture containing 9 µL ddH_2O, 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. The PCR condition comprised 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, an extension at 72°C for 1 minute, and a final extension set at 72°C for 10 minutes. The PCR product was analyzed using 1.5% agarose gel electrophoresis run with TAE buffer (40 mm Tris-acetate, pH 8.0 1 mM EDTA), stained with FloroSafe DNA stain, and visualized by the Gel Doc EZ Gel Documentation System (Biorad). PCR products were then sent to 1st Base Malaysia for sequencing.

The sequences were assembled using ChromasPro software, and the final generated sequences were deposited in GenBank (https://www.ncbi.nlm.nih.gov/) to obtain the accession number. These sequences were then subjected to Basic Local Alignment Search Tool (BLAST) in NCBI to compare the homology with existing data. Based on the BLAST results (Table 1), selected published sequences were used for phylogenetic tree analyses with *Leucoagaricus medioflavoides* as the outgroup. The phylogenetic tree was generated using MEGA X software (Kumar *et al.* 2018) and refined using TreeGraph Software version 2.9.2-622 beta. Bootstrap values (BS) of 70 % or higher were shown on the branches of the phylogenetic trees.

Table 1 Species, outgroup, herbarium voucher, and GenBank accession numbers used in this study

Species	Collection Code	ITS Accession Number
Agaricus cupressophilus	ATCC MYA-4431	NR_111346.1
A. desjardinii	HMAS WZR2012-8212	NR_158308.1
A. grandiomyces	HMAS 275728	NR_145005.1
A. lusitanicus	LIP 0001283	NR_158338.1
A. megacystidiatus	MFLU 12-0137	NR_119953.1
A. microvolvatulus	BR 5020002084476	NR_119952.1
A. pakistanicus	LAH 35299	NR_173276.1
A. sinodeliciosus	HMAS WZR2012-821	NR_164537.1
A. sinoplacomyces	HMAS 275724	NR_145009.1
A. subsubensis	ATCC MYA-4432	NR_137710.1
A. tibetensis	HMAS 275725	NR_145011.1
Clarkeinda trachodes	ECV3838	HM488751.1
C. trachodes	Isolate KUBOT-KRMK-2020-26	MW425600.1
C. trachodes	**Voucher BO24637**	**OR975901**
C. trachodes	Voucher HKAS122726	ON794453.1
C. trachodes	PS2011-12	MN099351.1
Leucoagaricus medioflavoides	Voucher MCVE:2324	GQ329055.1

RESULTS AND DISCUSSION

Taxonomy

Clarkeinda trachodes (Berk.) Singer, Lilloa 22: 413 (1951) [1949]
Synonym:
Agaricus trachodes Berk., London Journal of Botany 6: 487 (1847)
Chitoniella trachodes (Berk.) Petch, Annals of the Royal Botanic Gardens Peradeniya 4: 396 (1909)
Fungus trachodes (Berk.) Kuntze: 480 (1898)
Chitoniella poderes (Berk. & Broome) Henn., Die natürlichen Pflanzenfamilien nebst ihren Gattungen und wichtigeren Arten insbesondere den Nutzpflanzen: I. Tl., 1. Abt.: Fungi (Eumycetes): 240 (1898)
Clarkeinda poderes (Berk. & Broome) Kuntze, Revisio generum plantarum 2: 848 (1891)
Agaricus pedilius Berk. & Broome, Journal of the Linnean Society. Botany 14: 32 (1875)
Clarkeinda pedilia (Berk. & Broome) Kuntze, Revisio generum plantarum 3 (3) (1891)
Chitonis pedilis (Berk. & Broome) Clem, The genera of Fungi: 114 (1909)
Agaricus poderes Berk. & Broome, Journal of the Linnean Society. Botany 14: 32 (1875)

Basidiomata large, solitary, terrestrial. **Pileus** light brown to brown, 190-210 mm in diameter, applanate to plano-concave, with a straight margin. The surface is covered with numerous, small, revolute, loosely floccose brown squamules towards the margin. A dark brown, smooth to glabrous, cartilaginous patch is present at the center of the pileus. **Context** up to 7 mm wide at the pileus center, white to cream, turning reddish over time. **Lamellae** free, remote from stipe, 75 × 15 mm in diameter, crowded, dull yellow, concolorous, with series of lamellulae, margin entire. **Stipe** is central, 100 − 110 × 16 – 20 mm (apex) × 28 – 30 mm (base) mm, bulbous base tapering to the apex, hollow interior that turns reddish when injured. White to cream at apex, light brown toward the base, smooth toward annulus, densely furfuraceous squamules towards the base. **Annulus** membranous, superior, up to 9 cm wide, fragile, persistent to maturity, fragile and ragged with age, eroded margin, cream to pale brown. **Volva** present, white to cream. **Basidia** hyaline, 21 − 28 × 5 – 7 μm, clavate to subclavate, thin-walled, 4-spored. **Basidiospores** aseptate, ovoid to ellipsoid, 5.8 − 7.5 × 3.7 − 4.5 μm, smooth, thick-walled, with truncated apex and germ pore, hyaline to pale brown, interior with several lipid bodies. **Basidioles** narrow clavate. **Cheilocystidia** abundant, slightly thick-walled, 22 − 43 × 14 − 18 μm, clavate to broadly clavate, hyaline, thin-walled, smooth, septate at the base. **Hymenophore trama** interwoven, hyaline, thin-walled. **Pileipellis** are constructed by short chains of hyphae, slightly interwoven, and pale brown pigment inside the cells. **Pellicle** consists of short oblong cells mixed with oval to round cells, pigment inside cells darker than pileipellis. **Stipe** parallel to slightly interwoven, with hyaline hyphae.
Habitat: Mixed with bamboo leaves litter.

Distribution: China, India, Indonesia, Malaysia, Thailand, Laos, and Sri Lanka.
Specimen examined: Under bamboo trees, Darmaga, IPB University Campus Forest, West Java, Indonesia, S 6° 33' 14.22"E 106° 43' 26.418", 2023, collected by IP Putra, collection code BO24637.

Molecular Analysis

The final sequence was deposited in GenBank (https://www.ncbi.nlm.nih.gov/) with ITS accession number OR975901. The top BLAST result showed a 98-100% match with *C. trachodes*. The phylogenetic tree constructed from ITS sequence placed the specimen BO24637 in the same clade with *C. trachodes* reference strains (*C. trachodes* HM488751.1, *C. trachodes* PS2011-12, *C. trachodes* HKAS122726, and *C. trachodes* KUBOT-KRMK-2020-26) with 100% BS value (Figure 5). Moreover, *C. trachodes* BO24637 was fully separated from the clade of *Agaricus* species.

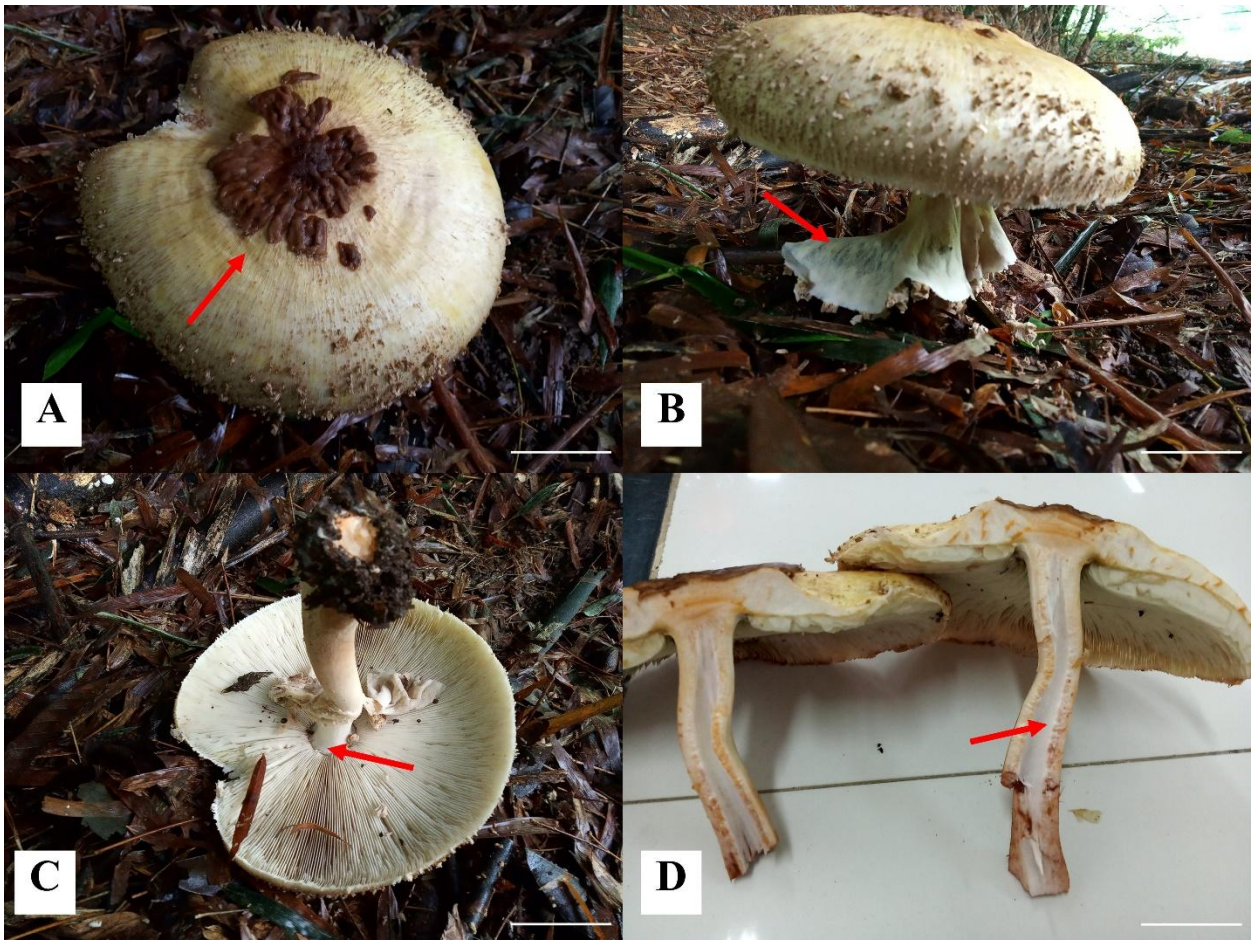

Figure 1 Field photograph of *C. trachodes* BO24637. A: Upper side of pileus with pellicle crown (arrow). B: Ring (arrow) at a superior position. C: Underside of pileus with free stipe to lamella. D: Hollow interior of stipe. Bars = 5 cm

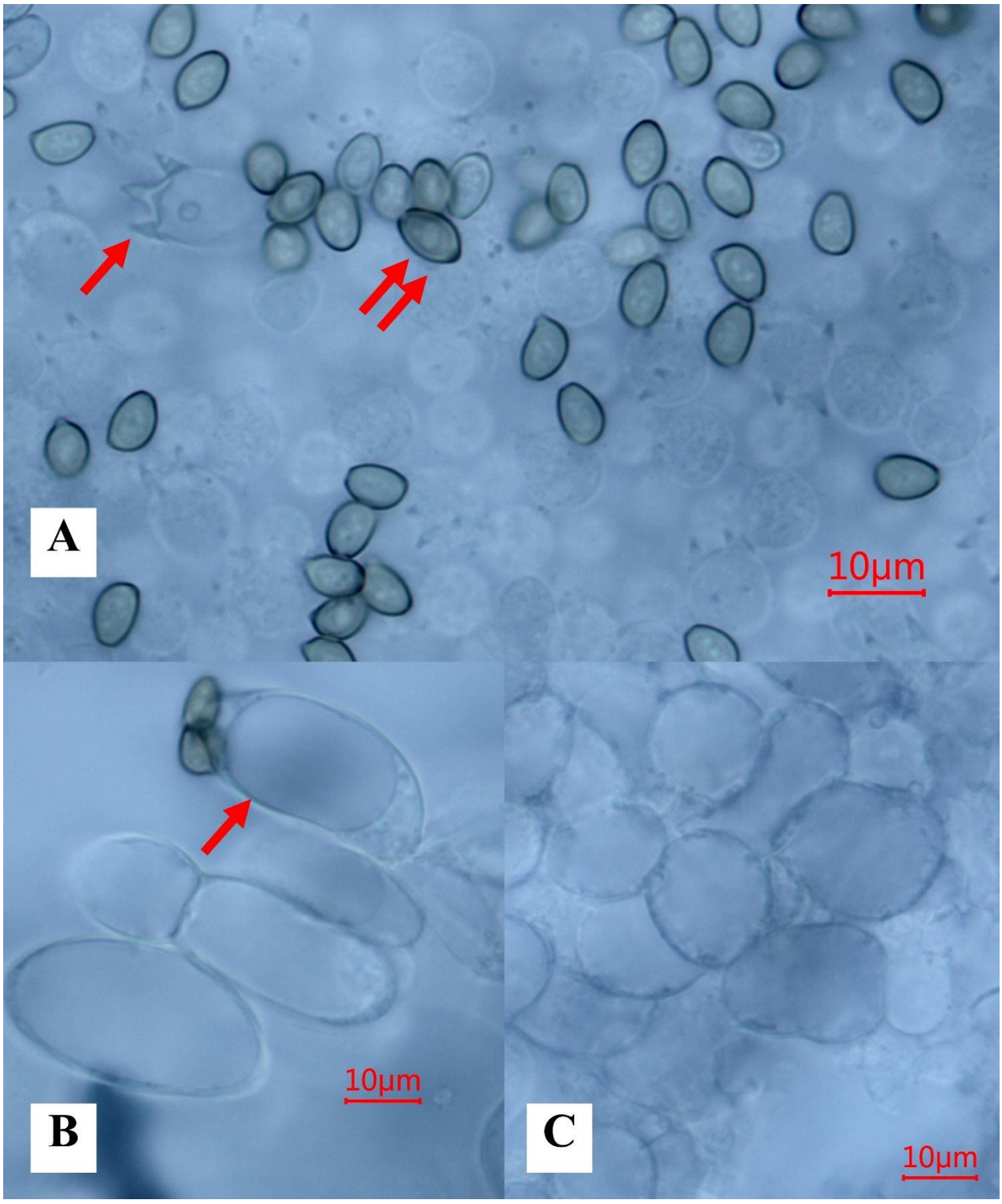

Figure 2 Microscopic characters of *C. trachodes* BO24637. A: Basidium with sterigmata (arrow), ovoid basidiospores (double arrow). B-C: Cheilocystidia

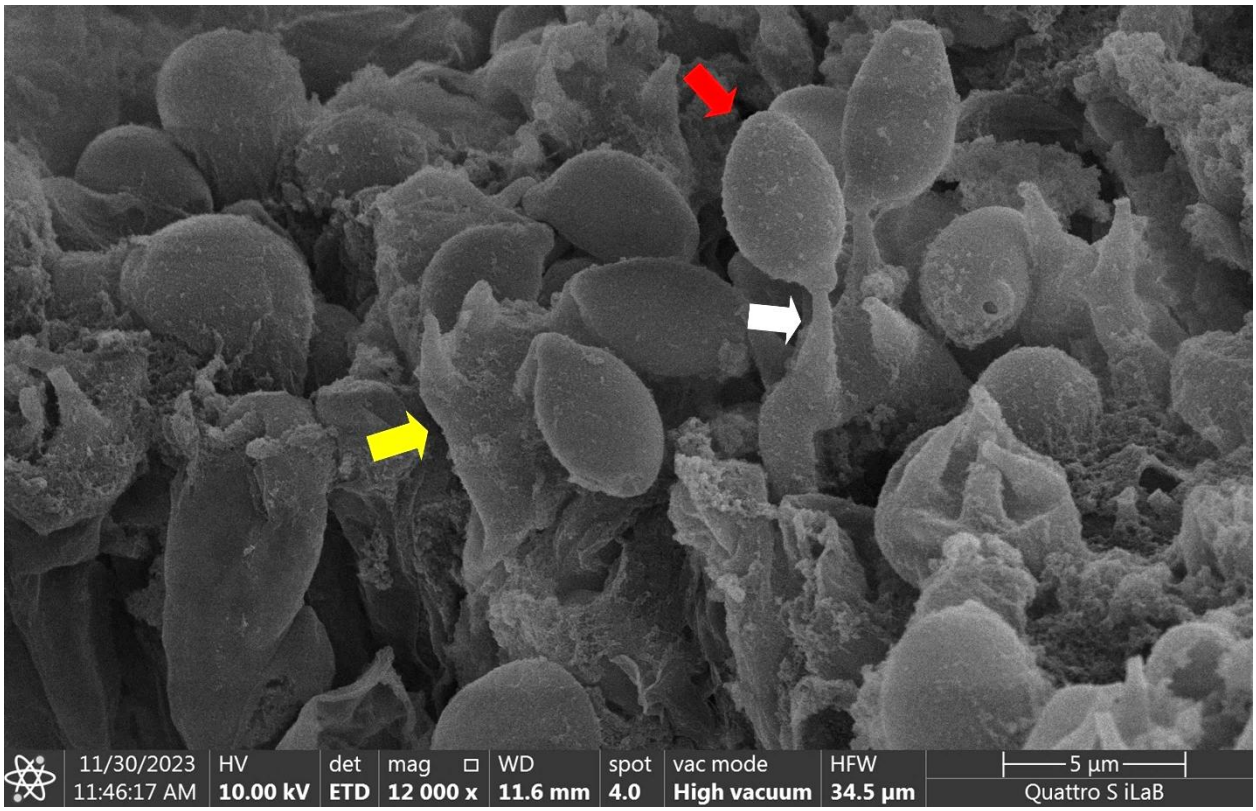

Figure 3 SEM image of *C. trachodes* BO24637. Spore (red arrow), sterigma (white arrow), and basidium (yellow arrow)

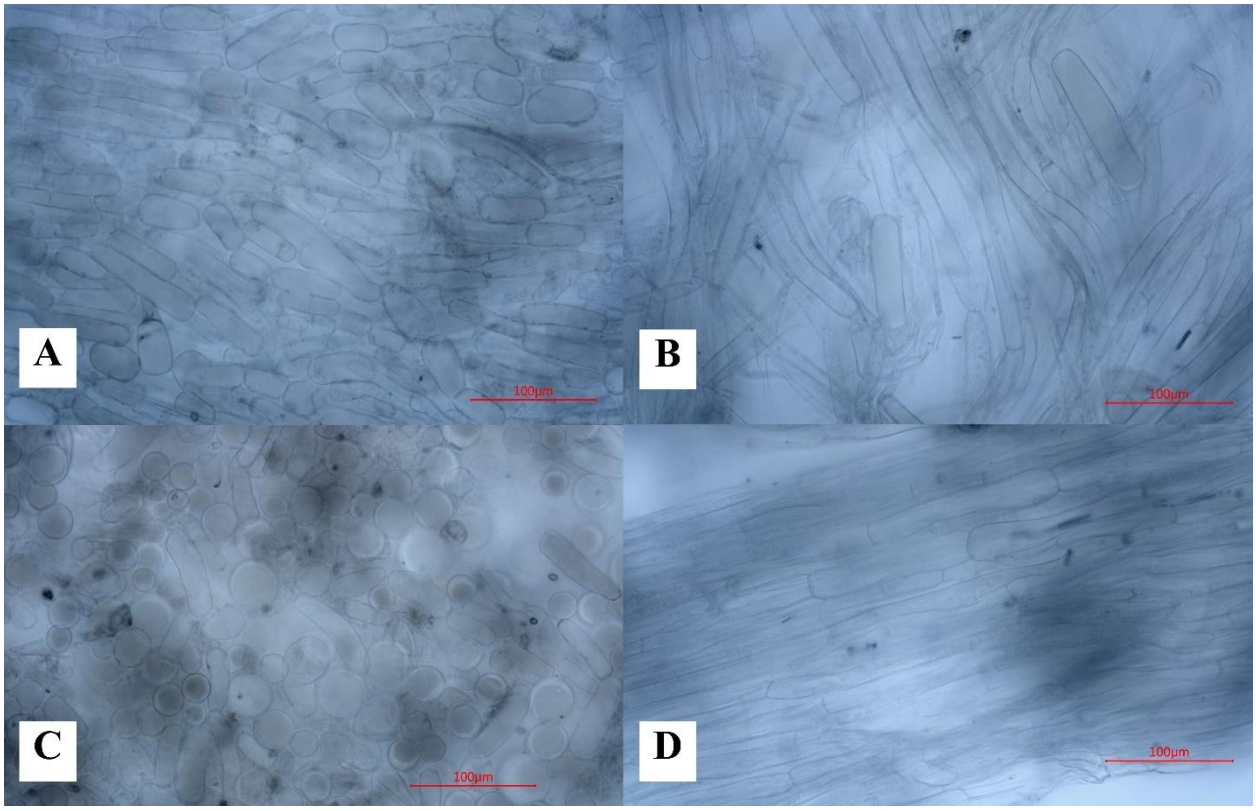

Figure 4 Microscopic characters of *C. trachodes* BO24637. A-B: Pileipellis. C: Pellicle crown section. D: Tissue of stipe

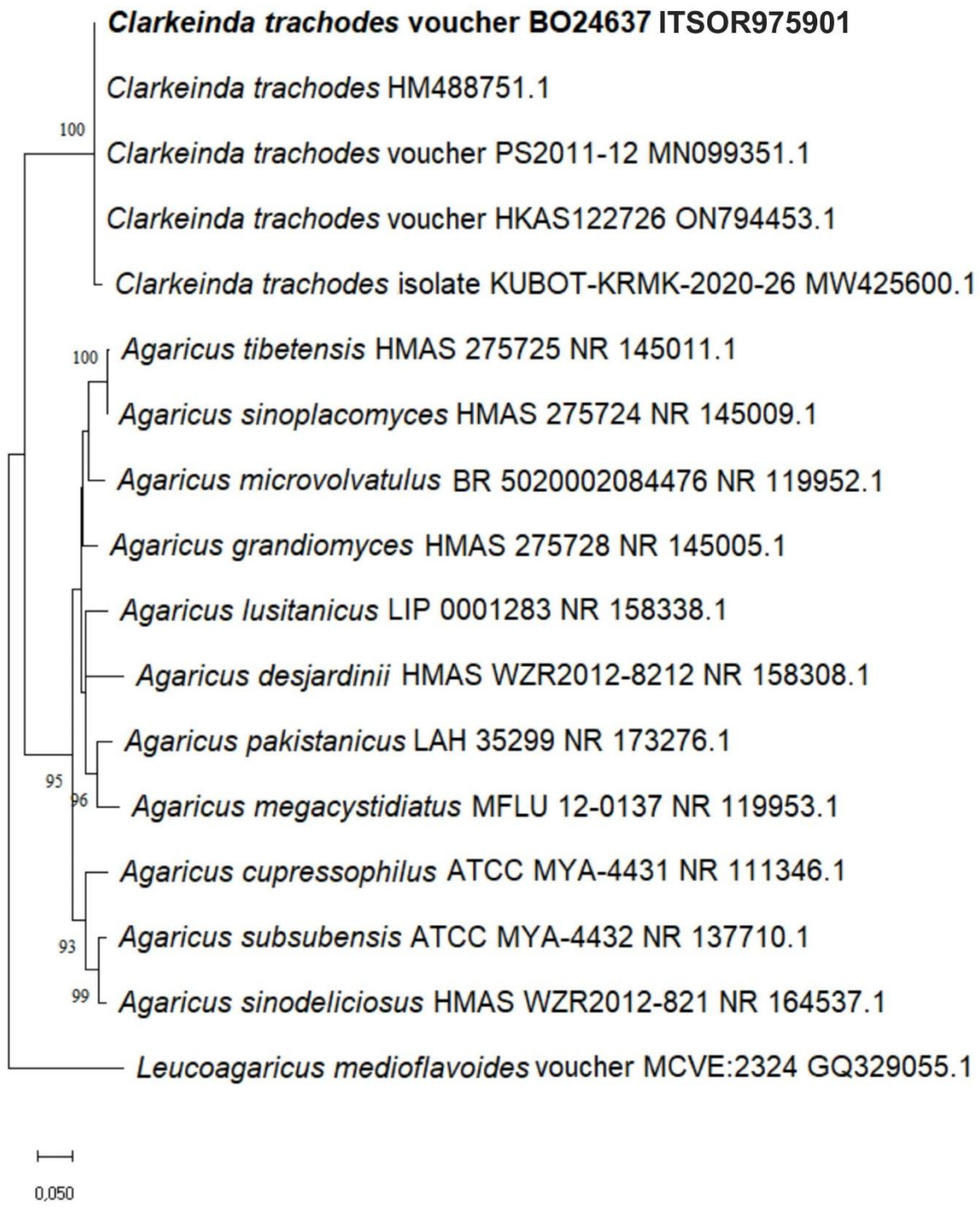

Figure 5 *Clarkeienda trachodes* BO24637 phylogenetic tree based on rDNA-ITS1/2 region using Maximum Likelihood with 1000 Bootstrap Analysis. The specimen in this study is in bold on the phylogenetic tree

This study provides comprehensive data and accessible information on *C. trachodes* in Indonesia. The species was initially described as *Chitoniella* by Boedjin from Java (1934). Several publications have since mentioned the distribution of *Chitoniella* (*C. trachodes*) in Indonesia (Pegler 1985; Yang 1991; Hosen and Ge 2011; Verma *et al.* 2016; Sysouphanthong 2021). However, there were no reports detailing the taxonomy of *C. trachodes* or herbarium materials in Indonesia. The materials from this study were submitted to Herbarium Bogoriense, Indonesia, under the collection number BO24637. The morphological and molecular information (DNA sequence) were also provided to facilitate future studies on *C. trachodes* in

Indonesia. Although recognized as poisonous, there has been no record of *C. trachodes* mushroom poisoning in Indonesia.

C. trachodes BO24637 is similar to *Chlorophyllum* species but can be distinguished by a large basidiome, brown pellicle of pileus disc, prominent ring, and volva (Pegler 1985; Hosen and Ge 2011; Kumar and Kaviyarasan 2011; Læssøe *et al.* 2019). *C. trachodes* BO24637 has a larger pileus dimension compared to previous reports of same species from China (Yang 1991), India (Verma *et al.* 2016), Bangladesh (Hosen and Ge 2011), Laos (Læssøe *et al.* 2019), and Thailand (Sysouphanthong *et al.* 2021). It is morphologically similar to specimens from India, Laos, and Thailand but differs from the basidiomata reported from Bangladesh, which have reddish-brown pileus and stipe. In addition, specimen BO24637 features thick-walled basidiospores similar to all collections discussed except the specimen from Bangladesh. Previous reports described *C. trachodes* as a terrestrial macrofungus with a habitat ranging from grasses (Verma *et al.* 2016), *Shorea robusta* (Dipterocarpaceae) forests (Hosen and Ge 2011), to nutrient-rich soils in deciduous rain forests (Sysouphanthong 2021). This study showed that basidomata grew solitary on bamboo litter, providing new insights into the habitat of this species.

Hosen and Ge (2011) reported that the record of *C. trachodes* in Italy by Lavorato & Contu (2002) was based on a misidentified *Agaricus* specimen. Therefore, in this study, molecular analysis was conducted alongside morphological characterization to increase the reliability of the results. The BLAST top hits result showed that the BO24637 was highly similar to *C. trachodes.* The phylogenetic tree also nested specimen BO24637 in the clade of *C. trachodes* from Thailand, India, and China with 100% BS value. The specimen was also placed in a different clade than *Agaricus,* consistent with reports by Sysouphanthong *et al.* (2021).

CONCLUSION

In conclusion, morphological and molecular analyses confirmed the collected specimen as *C. trachodes*. This study established a clear and accessible record of *C. trachodes* in Indonesia. In addition, the herbarium collection and ITS sequence of the species were contributed to Indonesia, which is essential for future studies globally.

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