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RESEARCH ARTICLE

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New Species of *Termitomyces* (Lyophyllaceae, Basidiomycota) from Sabah (Northern Borneo), Malaysia

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ABSTRACT

The genus *Termitomyces* (Lyophyllaceae, Basidiomycota) is often associated with fungus-feeding termites (Macrotermitinae) due to their strong symbiotic relationships. The genus is widely found exclusively in certain regions of Africa and Asia. They are recognized as edible mushroom within Southeast Asia as well. But it is often misidentified based on morphology by the local communities especially in Malaysia for *Chlorophyllum molybdites* which is a highly poisonous mushroom. Thus, it is necessary to study the genus for Malaysia with the synergy of using both morphological and molecular identification. In this study, we aim to describe another new species as an addition to the genus *Termitomyces* found within Sabah, Malaysia. We generated two new sequences (nrLSU and mtSSU) for the new species and a total of 28 nrLSU and mtSSU sequences were retrieved from GenBank for the phylogenetic analysis using maximum likelihood and Bayesian inferences. We identified that the new collection from Sabah province is a new species and named as *Termitomyces gilvus* based on the termites found in the mound. A phylogeny tree made from the concatenated genes of LSU and mtSSU suggests that *T. gilvus* is closely related to *T. bulborhizus* from China. According to our results, the combination of molecular and morphology proved to be a robust approach to re-evaluate the taxonomic status of *Termitomyces* species in Malaysia. Additional surveys are needed to verify the species diversity and clarify their geographic distribution.

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1. Introduction

The genus *Termitomyces* R. Heim, is a paleotropical and edible mushroom classified under the family Lyophyllaceae (Basidiomycota). The genus forms an obligate symbiotic or mutualistic association with the fungus-feeding termites [1,2]. The fruiting bodies of the *Termitomyces* are the main food source of the fungus-growing termites, under the family Macrotermitinae (Isoptera). The Macrotermitinae termites are exclusively found in Africa and Southeast Asia [1–3]. *Termitomyces* species are able to decompose and degrade lignin in order to utilize the cellulose more efficiently by termites [4]. *Termitomyces* mushroom also provide digestive enzymes and vitamins to their hosts [5,6].

Globally, about 30 species of *Termitomyces* have been estimated so far [7]. Based on the *Index Fungorum* database, there are currently 92 legitimate names available within this group. In addition, Mossebo [8] had revised the current distribution of *Termitomyces* taxa which they described two more additional new taxa and one new species combination. *Termitomyces* species have been widely documented from equatorial and throughout southern Africa and Southeast Asia [7,8]. All species within *Termitomyces* are delicious in flavor, edible, nutritious and consumed locally in many South-East Asian [9–11]. *Termitomyces* contain biologically active compounds that are potential uses as antioxidants, immunomodulators, antitumors, antimicrobials and treating neurodegenerative disorders [11–14].

Termitomyces and their host termite species relationships have been widely studied [1–3,15]. The fungi live symbiotically with different genera of termites including *Odontotermes*, *Microtermes*, *Macrotermes*, *Hypotermes*, *Protermes* and *Canthotermes* [16]. Different species of termites cultivate different species of *Termitomyces* [17]. Morphological characteristics alone are insufficient for *Termitomyces* species identification [15]. Thus, molecular identification is useful and widely has been accepted for species-level identification within the genus. For fungal identification, molecular markers such as the internal

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transcribed spacer (ITS) region, nuclear large subunit ribosomal DNA (nrLSU) region and mitochondrial small subunit ribosomal DNA (mtSSU) region have been mainly used in *Termitomyces* studies [18,19]. Some studies used ITS barcode for *Termitomyces* but all of them were unidentified strains of *Termitomyces* [8]. Recently, Moseebo et al. [8] have generated new sequences with a large number of species collections, sequences and an update information on *Termitomyces* from Africa and Asia using both nLSU and mtSSU.

In Malaysia, *Termitomyces* species is locally known as "*Cendawan busut*", "*Cendawan melukut*", "Cendawan *susu pelanduk*" (mousedeer hoof mushroom), "*Cendawan anai-anai* (termite mushroom)," "*Cendawan guruh*" (thunder mushroom), "*Kulat tahun*" (annual mushroom), "*Cendawan Tali*" or "*Kulat Taun*" [20–22]. The most common species such as the *T. eurhizus, T. heimii* and *T. clypeatus* are mainly found in the oil palm plantation areas and have been delicious for many local people of Malay Peninsula [20,23] and Malaysian Borneo [24,25]. Eight *Termitomyces* species have been reported in Malaysia: *Termitomyces clypeatus*, *T. entolomoides*, *T. heimii*, *T. eurhizus*, *T. microcarpus*, *T. aurantiacus, T. radicatus*, and *T. striatus*. There were mainly reported from Peninsular Malaysia [9,10,26].

Sabah (Northern Borneo), the eastern part of Malaysia (Borneo Island), is well known for its high biodiversity flora and fauna. Mycological studies in this region started in the early 1930–1990s [27–34]. However, there were no documentation of *Termitomyces* species from this region. As part of the Borneensis-Agaricomycetes DNA Barcoding project, we have started exploring the diversity of Bornean mushrooms and during the study; we encountered an interesting collection of *Termitomyces* from the surroundings of University Malaysia Sabah campus area (mainly secondary forest). The morphological features of the collected *Termitomyces* species are similar to those of *Termitomyces bulborhizus*, but they showed a clear difference by phylogenetic analyses based on nrLSU and mtSSU sequences. Therefore, we examined its taxonomic status using morphology and phylogenetic analysis of concatenated nrLSU-mtSSU sequences and provide detailed description of *Termitomyces* as a new species.

2. Materials and methods

2.1. Sample collections

The *Termitomyces* specimens were collected from Universiti Malaysia Sabah campus area, Kota Kinabalu, Sabah, Malaysia Borneo (6°2′ N, 116°6′ E) between May and June 2018. The fruit bodies of *Termitomyces* were collected and labeled with the field number. Information of the specimen habitat and location were recorded using the Global Positioning System (GPS). Fresh morphological characteristics were recorded and photographs were taken using Olympus Digital camera (Model TG-4 16MP). All color names and alphanumeric codes follow the Methuen Handbook of Color [35]. All samples were collected from the field and were brought to the laboratory for further identification and analysis. Specimens were dried using the food dehydrator around 40 °C for 1–2 days. The dried specimens were placed in a paper bag with silica gel and stored at BORNEENSIS (BORH) herbaria, Institute of Tropical Biology and Conservation (ITBC), Universiti Malaysia Sabah. As for termite species identification, the soldier and worker termites were collected. The sample was identified at the species level based on the identification key following Ahmad [36] and Eggleton [37].

2.2. Morphological studies

The macro-morphological characteristics of the fresh specimens were observed. The microscopic features were observed under microscope by using fresh specimens and dried specimen. Sections of pileus, lamellae, and context were prepared with a razor blade. They were rehydrated in 3% KOH and stained with 1% (w/v) Phloxine and Melzer's reagent [38] and then observed using an 80i compound light microscope (Nikon, Tokyo, Japan) at either 400× or 1000× magnification. A total of 20 basidiospores and basidia were measured. Q value denotes the length/width ratio of the basidiospores. Basidiospore statistics include: Xm, the arithmetic mean of the basidiospore length by basidiospore width (±standard deviation) for n basidiospores measured in a single specimen. Morphological identification was assisted using the literatures [8,10,11].

2.3. DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from fresh or dried fruit bodies of *Termitomyces* using CTAB method [39] with slight modifications. The fungal primer pair LR0R/LR5 [40] and SSUFW/SSUREV [1] were used to amplify the nrLSU region and the mtSSU, respectively. PCR reactions were performed in a C 1000 thermal cycler (Bio-Rad, Hercules, CA) using AccuPower PCR premix (Bioneer Co., Daejon, Korea) in a final volume of 20 µl containing 10 pmol of each primer and 1 µl of genomic DNA. PCR amplification was performed as described by Mossebo et al. [8]. The PCR products were electrophoresed through a 1% agarose gel stained with EcoDye DNA staining solution (SolGent Co.,

Daejeon, Korea) and purified with the Expin PCR Purification Kit (GeneAll Biotechnology, Seoul, Korea) according to manufacturer's instructions. DNA sequencing was performed in both directions using the PCR primers at Macrogen (Seoul, Korea) in an ABI3700 automated DNA sequencer.

2.4. Phylogenetic analysis

New sequences were generated for nrLSU and mtSSU. Sequences were manually corrected by reviewing the chromatograms of the DNA sequences. All sequences were assembled and edited using MEGA 6 software [41] and were deposited in GenBank (www.ncbi.nlm.nih.gov) with the accession number (GenBank accession numbers: MK472701 for nrLSU and MK478904 for mtSSU). A total of 28 nrLSU and 29 mtSSU sequences were retrieved from GenBank (Table 1). *Lyophyllum semitale* and *L. decastes* were selected as outgroup. All sequences were aligned using MAFFT v7 [42] with the default settings.

Maximum likelihood (ML) and Bayesian Analysis (BA) were performed with the following parameters (i) ML: the analysis was run in the RAxML v. 3.3 in CIPRES web portal (http://www.phylo.org/portal2/; [43]) under a GTR model with 1000 bootstrap replicates; (ii) BA: the analysis was run using MrBayes 3.2.2 [44] for 10 million generations, under a HKY + G + I and HKY + G model for nrLSU and mtSSU, respectively, with four chains, and trees sampled every 100 generations; after examining the graphic representation of the likelihood scores, using Tracer (http://tree.bio.ed.ac.uk/software/tracer/), the burn-in period was set to 1.5 million generations for all datasets. Bootstrap values (BS) ≥70% and posterior probability (PP) ≥90% values were considered significant. The edited alignment sequence dataset was deposited in TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S23814).

3. Results

The sequencing of nrLSU and mtSSU from the specimen was successful. Additional sequences of the two loci were retrieved from GenBank and using in constructing the alignment datasets. The concatenated alignment contained 30 specimens, of which sequences from the two loci were present. The alignments of nrLSU and mtSSU consisted of 566 and 393 characters, respectively.

A phylogeny tree made from the concatenated genes of LSU and mtSSU suggests that *Termitomyces* specimen is closely related to *T. bulborhizus* from China [45,46]. ML analysis revealed that *Termitomyces* specimen is monophyletic with strong support (BS = 100%; PP = 1.0) and distinctive compared to *T. bulborhizus* from China. The

Table 1. List of species, voucher number, geographic origin and GenBank accession numbers of nrLSU and mtSSU sequences used in the molecular analysis.

			GenBank accession numbers	
Species	Herbarium number	Geographic origin	nrLSU	mtSSU
T. aurantiacus	HUY1-DM 152E	Cameroon	KY809234	KY809186
T. brunneopileatus	K(M) 144300	Cameroon	KY809273	KY809225
T. bulborhizus	K(M) 128338	China	KY809261	KY809213
T. cartilagineus	K(M) 109565	South Africa	KY809259	KY809211
T. clypeatus	K(M) 128340	China	KY809262	KY809214
T. entolomoides	tgf103	Africa	AY232693	AY232680
T. eurrhizus	K(M) 142 419	Zambia	KY809266	KY809218
T. globulus	HUY1-DM 770	Cameroon	KY809252	KY809204
T. heimii	K(M) 16 528	Malaysia	KY809253	KY809205
T. heimii	K(M) 109 538	Pakistan	KY809257	KY809209
T. letestui	HUY1-DM 666A	Cameroon	KY809248	KY809200
T. gilvus	**BORH/FUMS-A03**	**Malaysia (Borneo)**	**MK472701**	**MK478904**
T. mammiformis	HUY1-DM 25G	Cameroon	KY809230	KY809183
T. mboudaeina	HUY1-DM 223E	Cameroon	KY809237	KY809189
T. medius	K(M) 16 685	Nigeria	KY809254	KY809206
T. medius	HUY1-DM 372G	Cameroon	KY809243	KY809195
T. microcarpus	HUY1-DM 268E	Cameroon	/	KY809191
	PRU3900	/	AF042587	AF357092
T. robustus	HUY1-DM 436	Tanzania	KY809265	KY809217
T. sagittaeformis	K(M) 109566	South Africa	KY809260	KY809212
T. schimperi	HUY1-DM 24E	Cameroon	KY809228	KY809181
T. singidensis	tgf74	Tanzania	AY232713	AY232687
T. striatus	K(M) 142436	Malawi	KY809267	KY809219
T. striatus f. *bibasidiatus*	HUY1-DM 280	Cameroon	KY809240	KY809192
	HUY1-DM 280B	Cameroon	KY809241	KY809193
T. subumkowaan	HUY1-DM 260B	Cameroon	KY809275	KY809227
	HUY1-DM 260F	Cameroon	KY809239	KY809190
T. titanicus	K(M) 142 416	Zambia	KY809264	KY809216
Lyophyllum semitale	CBS 369.47	–	AF223207	AF357124
L. decastes	JM 87/16	–	AF042583	AF357136

Accession numbers of the newly generated sequences are indicated in bold. BORH-BORNEENSIS Herbarium, Universiti Malaysia Sabah.
Bold sequences indicate new sequences produced in this study.

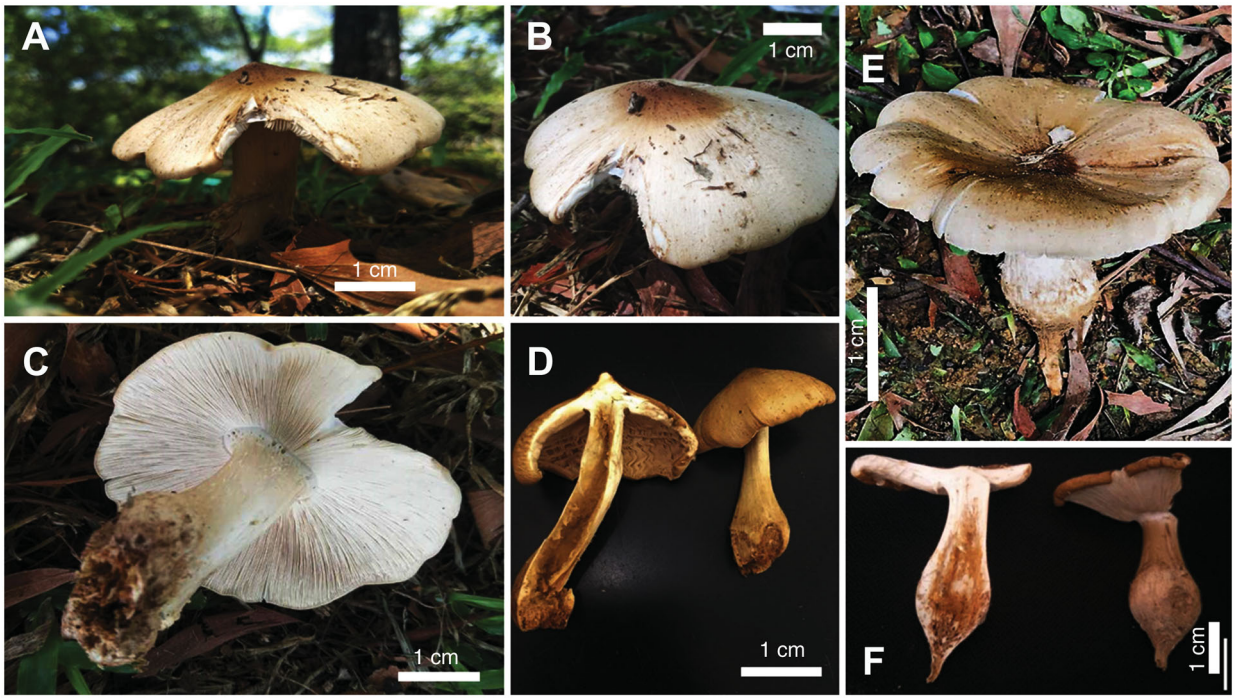

Figure 1. Fruit body of *Termitomyces gilvus* sp. nov. (BORH/FUMS-A03, holotype). (A–B, E) surface of pileus; (C) lamellae; (D, F) stipe.

same topology was observed for single gene analysis using nrLSU and mtSSU (not shown here).

4. Taxonomy

***Termitomyces gilvus* C.S. Yee, and J.S. Sathiya Seelan, sp. nov.** (Figures 1 and 2)

MycoBank MB829475.

Diagnosis: *Termitomyces gilvus* differs morphologically from the closely related species of *T. bulborhizus* by pileus size and color, length of bulbous stipe, short pseudorrhiza, and larger basidia and pleurocystidia.

Type: Malaysia. Sabah, Kota Kinabalu, Universiti Malaysia Sabah campus (UMS), 50 m elev., N6˚2′ 116˚6′ E, 10 May 2018, leg. J.S Sathiya Seelan, collected by J.S. Sathiya Seelan, BORH/FUMS-A03 (Holotype, BORH!).

rDNA and mtDNA sequence ex holotype: MK472701 (nLSU); MK478904 (mtDNA)

Pileus 80–130 mm in diam, fleshy, at first convex becoming convexo-applanate with strongly blunt pointed perforatorium and irregularly lobed margin; Surface brownish orange (5C6) to dark brown at the center, brownish yellow (5C7) to orange white (5A2) toward the margin, rough to slightly smooth; perforatorium usually dark brown (6F5) velar squamules; margin enrolled when young and expanding toward the age, never uplifting, often crenulate. *Lamellae* free, white to pinkish (11A2) to 11 mm wide, densely crowded, with two series of lamellulae between lamellae. Context up to 40 mm thick, white, comprising repent, thin-walled hyphae, 2.7–7.7 µm diam, inflating to 28 µm. Spore print pink. *Odor* pleasant. *Stipe* 9–13 cm long above ground, 5–11 cm thick, enlarged to 1–6 cm diam. at ground level and usually abruptly forming a prominent globose bulb below ground, solid, robust, fibrous, surface above and pale brown on the bulb; with concolorous, sparsely distributed floccules. *Partial veil* ephemeral and often absent after the initial stage. *Pseudorrhiza* to 3 cm long, narrowing to 30–60 mm immediately below the bulb or tapering toward the base, surface white to pale brown, with longitudinal grooves and cracks.

Basidiospores 6–8.5 × 3.7–5.4 µm ($Q = 1.5–1.6(1.8)$, $n = 20$), ovoid to ellipsoid, subhyaline, thin-walled. *Basidia* 21.8–29.2 × 6.1–8.3 µm, clavate, bearing four sterigmata. *Hymenophoral trama* regular, 8.0–20 µm wide, of hyaline hyphae, 2.5–22.0 µm diam. Subhymenial layer 12.0–30.0 µm diam. wide, repent hyphae, 2.4–2.9 µm diam. *Lamella-edge* heteromorphous, with crowded cheilocystidia, dispersed with basidia. *Cheilocystidia* clavate to pyriform, 36.8–51.1 × 12.7–24.8 µm, thin-walled. *Pleurocystidia* abundant, clavate to pyriform, occasionally turbinate, 47.0–66.3 × 21.2–31.5 µm, thin-walled. *Pileipellis* a repent epicutis of narrow, radial hyphae, 14.2–14.9 µm. *Clamp connection* present.

Habitat: Scattered to gregarious around termite (*Macrotermes gilvus*) mounds or on soil within campus area.

Etymology: The species epithet is named after the host termite species that was found.

Comments: *Termitomyces gilvus* mainly found scattered to gregarious near termite mound (colonies of *Macrotermes gilvus*). The species is morphologically and phylogenetically similar to *T.*

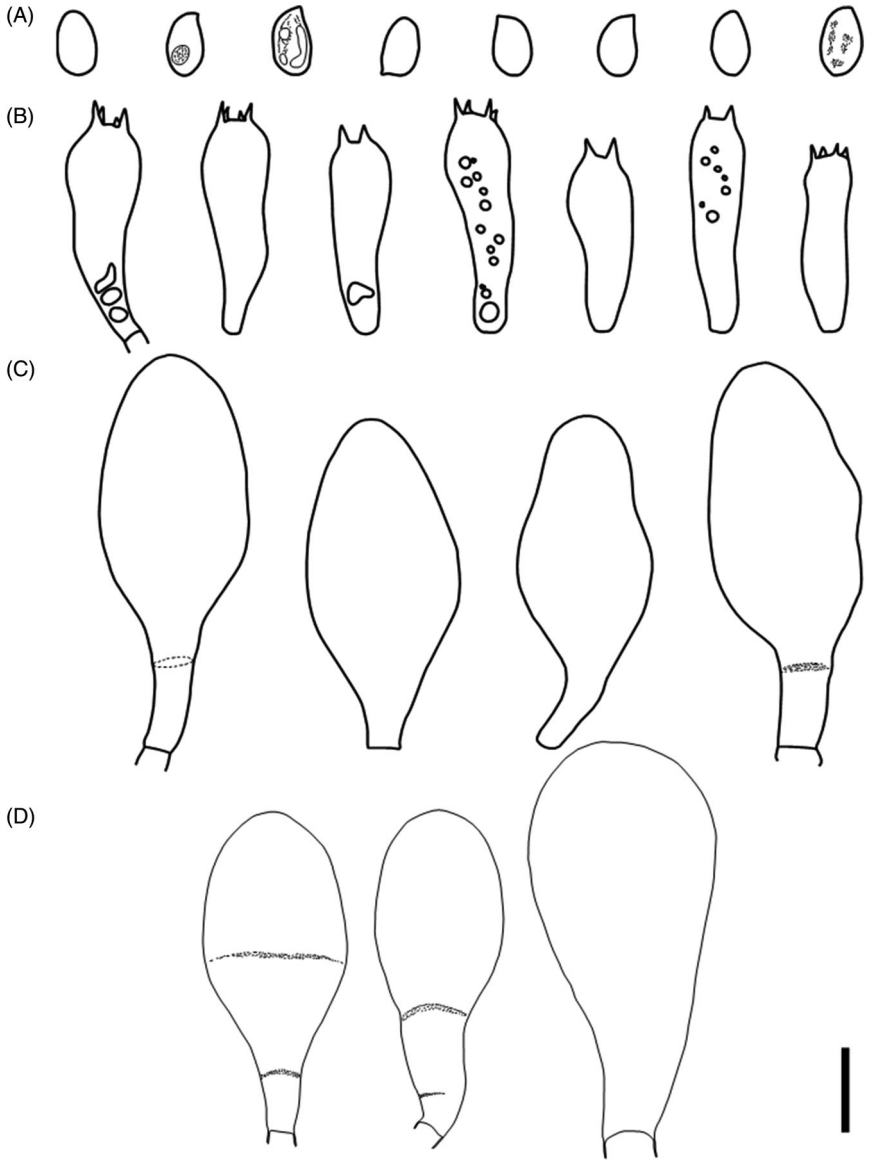

Figure 2. Microscopic features of *Termitomyces gilvus* sp. nov (BORH/FUMS-A03, holotype). (A) basidiospores; (B) basidia; (C) cheilocystidia; (D) pleurocystidia. Scale bar = 10 µm.

bulborhizus. The pileal surface color of *T. gilvus* is brownish orange compared to *T. bulborhizus* (KM128338, China) which has a darker reddish brown in color. *Termitomyces gilvus* has smaller bulbous stipe and shorter pseudorrhiza compared to *T. bulborhizus*. Microscopically, *T. gilvus* has slightly larger basidia and pleurocystidia compared to *T. bulborhizus*.

5. Discussion

Morphological characters and concatenated analyses of nrLSU-mtSSU of the novel edible *T. gilvus* are produced herein (Figure 3). The type specimen BORH/F-UMSA03 *T. gilvus* is different from *T. bulborhizus* specimens from China and Thailand based on their pileus size and color, lamellae, stipe, pseudorrhiza, basidia, pleurocystidia and the termite host (Table 2). *Termitomyces gilvus* is mainly characterized by brownish orange to dark brown colored dry pileus, thick stipe, wide lamellae, shorter pseudorrhiza with floccose, and larger basidia and pleurocystidia. Meanwhile, *T. bulborhizus* is mainly distinguished by the large basidioma, bulbous stipe base and floccose stipe surface [45,46].

The most prominent morphological characters for *T. gilvus* in comparison to *T. bulborhizus* are presented in Table 2. Morphological features of *T. gilvus* mainly distinguished by the golden orange pilei, shorter pseudorrhiza, larger basidia and pleurocystidia. *Termitomyces bulborhizus* possess sub-globulose base at the end of the stipe similar to *T. bulborhizus* [45,46]. However, the bulbous stipe of type specimen BORH/FUMS-A03 tend to be smaller in size than the *T. bulborhizus* (Table 2). Pileus size were smaller (80–130 mm) and lamellae with 2-series were observed in the Bornean mushroom (BORH/FUMS-A03). However, *T. bulborhizus* from China tend to have larger pileus size (100–220 mm) and without lamellulae. Although similar morphology were observed as

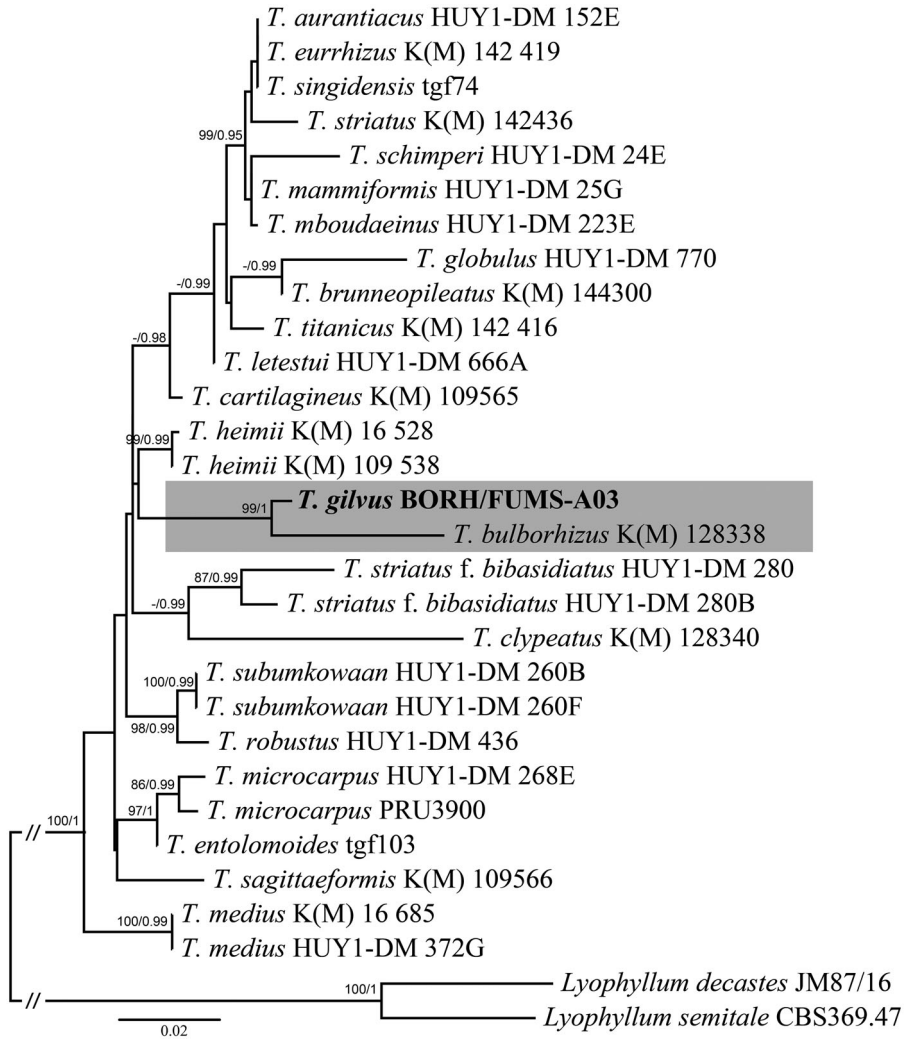

Figure 3. Phylogenetic position of *Termitomyces* species inferred from concatenated nrLSU-mtSSU sequences using maximum likelihood analysis. Bootstrap and posterior probability values (ML/PP: 100%/1.0) are indicated above/below branches. The new species in in bold.

Table 2. Macroscopic, microscopic features, termite hosts and distribution of *Termitomyces gilvus* and closely related species of *T. bulborhizus* from China and Thailand.

Characters/Host	***T. gilvus* (Malaysia)** (This study)	*T. bulborhizus* (China) [46,47]	*T. bulborhizus* (Thailand) [19]
Pileus size	8−13 cm	10−22 cm	9.2−21 cm
Pileus color	Surface brownish orange (5C6) to dark brown at the center, brownish yellow (5C7) to orange white (5A2) toward the margin	Surface reddish brown to dark brown at center; pale brown to brown toward the margin	Surface dark brown at the center, elsewhere pale brown to brown, paling toward the margin
Pileus shape	Convex to convexo-applanate	Convex to convexo-applanate	Convex then expanding to convexo-applanate
Lamellae	White to pink; 11 mm wide; densely crowded; lamellulae 2 series	Free; white to pink; 8 mm wide; densely crowded; lamellulae	White, to 9 mm wide, crowded, with lamellulae
Stipe	9–13 cm long; 5−11 cm thick, enlarged to 1−6 cm diam.	3–13 cm long; 0.8−6 cm thick; enlarged 3−9 cm diam.	5−9 cm long; 6−8 cm thick; enlarged to 8.7−14.5 cm diam.
Pseudorrhiza	3 cm long	80 cm long	1−4 cm long
Spore size	6−8.5 × 3.7−5.4 µm	6−9 × 4−6 µm	5−8.5 × 3.5−6 µm
Basidia	21.8−29.2 × 6.1−8.3 µm	17.5−27 × 5.5−9 µm	16−25 × 4−9 µm
Cheilocystidia	36.8−51.1 × 12.7−24.8 µm	19−60 × 12−34 µm	NA
Pleurocystidia	47.0−66.3 × 21.2−31.5 µm	19−78 × 10−32 µm	NA
Termite Host	*Macrotermes gilvus*	*Odontotermes formosanus; Macrotermes barneyi*	*Hypotermes makhamensis*
Distribution	Sabah (Malaysia)	South and southwest of (China)	Sai Yok district (Thailand)

Characters with bold font are distinguishing features between the species.
NA: not available.

in *T. bulborhizus*, somehow, the bulbous stipe of *T. gilvus* is smaller and the presence of floccose stipe surface when it was freshly collected. Pseudorrhiza of *T. bulborhizus* were longer (80 cm long) for Chinese specimen however the Bornean specimen tend to be smaller in size (<3 cm long) which is similar to the

pseudorrhiza of *T. bulbohizus* found in Thailand (1–4 cm long) [19].

Termitomyces bulborhizus were first recorded and described in Sichuan and Yunnan province in China. Later, it was recorded in the central region of Thailand [19,47]. Based on the ITS sequences, Sawhassan et al. [19] have reported that the Thai specimen was *T. bulborhizus* and similar to the Chinese specimen. In terms of host, *T. clypeatus* and *T. bulborhizus* are usually associated with *Macrotermes gilvus* Sawhassan et al. [19]. They recorded *T. bulborhizus* was associated with *Hypotermes makhamensis* in the central region of Thailand. This species is a fairly rare species in the area, and only one fruiting body was found. Besides, it occurs only nearby the termite mound of *Macrotermes gilvus* colonies below the *Acacia mangium* tree.

Phylogenetic analysis showed that the Bornean collection clusters within *Termitomyces*, and is closely related to *T. bulborhizus*. Sawhassan et al. [19] produced ITS gene of *T. bulborhizus* from Thailand specimen. Later, Mossebo et al. [8] reported that ITS collections for *Termitomyces* specimens were mostly limited and unidentified strains. The African and Asian specimens have been revised by Mossebo et al. [8] using the nrLSU and mtSSU for *Termitomyces* taxa which includes the *T. bulborhizus* specimen (KM 128338) from China in their study. The most representation of *Termitomyces* sequences (nrLSU and mtSSU) from Mossebo et al. [8] were used in this study for the phylogenetic position of the Bornean specimen, *T. gilvus*. Thus, *T. gilvus* is the first report for Sabah, using both morphology and with molecular data. Previously, *T. clypeatus* and *T. eurrhizus* were reported in Sabah [25,34] without any molecular work. So far, there are eight recognized *Termitomyces* species in Malaysia, but the results of this study suggest that there are more novel species awaiting for discovery. Further studies of *Termitomyces* using both morphology and phylogenetic analysis will be revised on this genus for Malaysia.

In this study, we propose that *Termitomyces gilvus* is a new species based on the differences in morphology and their phylogenetic placement, which is closely related to *T. bulborhizus* from China. *Termitomyces gilvus* is easily recognized due to their short bulbous stipe, golden orange pileus, large sized basidia and pleurocystidia. To our knowledge, this is the first report for Sabah (Northern Borneo) on this genus with adequate morphological description and molecular data. The erection of the new species has prompted the interest on how many species of *Termitomyces* are actually distributed within Borneo. The new species discovery within this genus suggests that more studies and sampling should be conducted to revise the Malaysian *Termitomyces* since it is regarded as one of the seasonal delicacy in Malaysia. The cultivation of *Termitomyces* could sustain the economic development among the local people in Sabah. In the long run through Borneensis-Agaricomycetes 2020–2025 project, many novel species discoveries are possible and this will be a major contribution to tropical mushrooms study. Thus, this study could serve as a baseline information to gather more information on this genus in Malaysia especially in Borneo.

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Disclosure statement

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