

# Discovery of a rare medicinal mushroom, *Lignosus hainanensis* (Polyporaceae, Basidiomycota) in Sabah (Northern Borneo), Malaysia

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**Abstract:** *Lignosus hainanensis* was discovered in the Hainan Island, China, a decade ago, marking the only known occurrence of this species worldwide. This study provides the first report of *Lignosus hainanensis* from the Kadamaian Forest in Sabah (Northern Borneo), Malaysia, including detailed macro- and micromorphological descriptions, supported by molecular analyses. The morphological characteristics of the Malaysian collection was matched with the description of *Lignosus hainanensis* from the Hainan Island, China. Despite similarities in basidiocarps, Malaysian *L. hainanensis* showed similar hymenophore and basidiospore in terms of size, but with slightly different basidiospore shapes. Phylogenetic analysis of the Internal Transcribed Spacer (ITS) region strongly supported the phylogenetic placement of the Malaysian collection alongside the Chinese collections. This study confirmed the occurrence of *Lignosus hainanensis* in Sabah, Malaysia, marking its first recorded presence in the region.

**Keywords:** Borneo, medicinal mushroom, phylogeny, Sabah, tiger's milk mushroom

## INTRODUCTION

Tiger's milk mushroom, classified within the genus *Lignosus* from family Polyporaceae, Order Polyporales and phylum Basidiomycota, which is recognised for its effective and valuable medicinal prospectus, and has been used throughout Southeast Asia and South China (Ng *et al.* 2023). *Lignosus* is referred to as cendawan susu harimau (Malaysia), jamur susu harimau or Ndurabi' (Indonesia), huru lingzhi (China), and hijiritake (Japan) in local languages (Wei *et al.* 2007; Nallathamby *et al.* 2018). Traditionally, this valuable mushroom has been used to treat ailments such as fever, cough, and nasal allergies (Lee *et al.* 2012; Lau *et al.* 2015; Tan *et al.* 2021; Yap *et al.* 2023). Due to its medicinal properties, it has gained popularity and significance as a consumable medicinal mushroom in various Asian countries (Borneo Post Online 2009; Lee *et al.* 2009). Numerous scientific studies have supported the traditional use of the tiger's milk mushroom as a treatment for a variety of illnesses (Lee *et al.* 2012; Kong *et al.* 2016; Fung *et al.* 2019; Tan *et al.* 2021; Yap *et al.* 2023). Furthermore, this mushroom has been successfully commercialised and introduced to the market as a consumable product in Malaysia (Fung and Tan 2023).

*Lignosus* is characterised by its stalked basidiomata with circular caps, a unique hyphal system containing generative hyphae with clamp connections, and nearly spherical basidiospores (Ryvarden 1991; Kües and Navarro-Gonzalez 2015). This genus possesses distinctive characteristics, including a hard, inedible fruiting body, and a soft sclerotium for consumption (Lau *et al.* 2015; Fung and Tan 2019). The sclerotium, which grows underground before the emergence of the fruiting body above the soil, requires a long time for maturation, typically between six months to a year, before it can be collected (Abdullah *et al.* 2013). The growth rate of this mushroom is relatively slow (Usuldin *et al.* 2020).

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According to the Global Biodiversity Information Facility (GBIF), the occurrence records of the *Lignosus* is notably scarce, with localities being infrequently documented (GBIF 2023). *Lignosus* is native to tropical regions, including the southern regions of China, Sri Lanka, Thailand, the Philippines, Indonesia, Papua New Guinea, Australia, India, and Malaysia (Fui *et al.* 2018; Alugoju and Tencomnao 2023). As of 2021, there are a total of eight recognised species within the *Lignosus* genus according to the Index Fungorum's listing. Among these, *Lignosus rhinocerus* and *L. sacer* are frequently reported across various regions (Lau *et al.* 2015). On the other hand, *L. ekombitii*, *L. dimiticus* and *L. goetzii* are primarily found in Africa, while *L. cameronensis* and *L. tigris* are predominantly found in Malaysia, and *L. hainanensis* is native to China (Ryvarden and Johansen 1980; Cui *et al.* 2011; Tan *et al.* 2013).

In Malaysia, occurrences of three *Lignosus* species have been reported, namely *Lignosus rhinocerus*, *L. cameronensis*, and *L. tigris* (Tan *et al.* 2013; Fung *et al.* 2019). Most of these *Lignosus* records are concentrated in Peninsular Malaysia. While in Bornean Malaysia, information on *Lignosus* remains scarce. *Lignosus rhinocerus* was reported in Sabah and Sarawak from previous studies (Fui *et al.* 2018; Yamashita *et al.* 2018). Hence, the *Lignosus* collection described in this study represents one of the few rare collections from the Borneo Geographic Expedition at Kadamaian-Kinabalu Park in 2019. This study provides the first comprehensive account of the *L. hainanensis* in Malaysia, incorporating detailed morphological descriptions alongside molecular evidence.

## MATERIALS & METHODS

### Sample collection

The collection point is located on the side of Wasai Waterfall at the Kadamaian-Kinabalu Park Forest Area (6°13'22.83"N, 116°30'13.36"E), at an altitude of 417 m above sea level. This region is characterised by highland mixed dipterocarp forest, which experience a high precipitation, with a recorded rainfall rate of about 409.3 mm in October 2019 according to the Sabah Meteorological Department. The annual temperature in this area ranges from 22°C to 34°C (TimeandDate 2024).

The specimen of a tiger's milk mushroom was designated with the field code KD094 and subsequently brought back to the Molecular Mycology Laboratory, Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah for further analysis.

### Morphology observation

The morphological characteristics of the collected specimen were initially documented in the field. The specimen was then subjected to drying using a laboratory dryer. The Methuen Handbook of Colour (1967) was used to note the color. The hymenophore of the mushroom was observed and measured using a portable standalone LCD Digital Microscope 5M 500X, with measurements calibrated against a centimeter ruler through ToupTek ToupView 3.7 (Karich *et al.* 2024). Microscopic features were observed and measured using MShot Image Analysis System software connecting to the microscope LED2800. The specimens were stained using aqueous solutions containing 1% Congo red, Melzer's reagent, followed by mounting in a 5% aqueous solution of potassium hydroxide (KOH) (Khan *et al.* 2020). The characteristics of hyphae (n = 47), basidiospores (n = 2) and sclerids (n = 30) were measured (Krishnapriya and Kumar 2022).

## DNA extraction, PCR amplification and sequencing

The complete genomic DNA was extracted from the sclerotium by employing an E.Z.A. DNA Fungal Kit, following the manufacturer's instructions provided by Omega Bio-Tek, USA. Primers pairs of ITS1F (5'- CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3') were used to amplify the Internal Transcribed Spacer (ITS) regions (White *et al.* 1990; Gardes and Bruns 1993). The polymerase chain reaction (PCR) protocol for ITS involved an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, concluding with a final extension at 72°C for 10 minutes (Tan *et al.* 2013). The PCR products were sent to Apical Scientific Sdn. Bhd., Selangor, Malaysia, for sequencing. The resulting sequence was subjected to NCBI databases using BLAST program to determine the primary identity of the specimen and was subsequently submitted to GenBank (Accession No.: OQ954761).

## Phylogenetic analyses

The newly generated sequence was combined with 15 ITS GenBank sequences, with two *Daedaleopsis* spp. serving as the outgroup for phylogenetic analyses, employing both Maximum Likelihood and Bayesian Inference probability (Table 1). The dataset was aligned in MAFFT v. 7 and manually adjusted in BioEdit v. 7.2.5 (Krishnapriya and Kumar 2022). Maximum likelihood, JModel Test and Bayesian Inference analyses were performed on the CIPRES Portal v. 3.1 (Asif *et al.* 2024).

Maximum Likelihood (ML) analysis was performed using RAxML-HPC2 on ACCESS v. 8.2.12, with 1000 nonparametric bootstrap iterations (Kazerooni *et al.* 2021). Bootstrap value that is equal to or higher than 70% is considered significant support for ML analysis. Then, JModelTest2 on XSEDE v. 2.1.6 determined TrNef+G as the best-fit models of nucleotide substitution for the dataset with the following parameters: lsetnst = 6; rates = gamma; prset revmatpr = fixed 1.0000, 1.7639, 1.0000, 1.0000, 3.1308, 1.000; shapepr = fixed 0.725 (Song *et al.* 2023). Lastly, Bayesian Inference (BI) analysis was performed using MrBayes on XSEDE v. 3.2.7a (Asif *et al.* 2024). Four Markov chains were run for 5,000,000 generations, and trees were sampled every 1000 generations. The first 25% of trees, which represented the burn-in phase of the analysis, were discarded, resulting in 1000 trees used for calculating posterior probabilities (PP) in the consensus tree. Probability value that are equal to or higher than 0.90 are considered significant support for BI analysis.

The aligned sequence data matrix was deposited in TreeBase (Submission ID: 30461, Reviewer access: <http://purl.org/phylo/treebase/phylovs/study/TB2:S30461?x-access-code=f40751c76d3e482c0ba96a6929be7182&format=html>). FigTree v. 1.4.4 was used to display the phylogenetic tree, and exported for further editing in Inkscape v. 1.3.2.

**Table 1.** Taxa information and GenBank accession numbers of the sequences used in this study.

Species	Voucher Number	Origin	ITS	LSU	Reference
<i>Lignosus hainanensis</i>	KD094	Malaysia	OQ954761	PP770733	This study
<i>Lignosus hainanensis</i>	Y.C. Dai 10670 _fruit body	China	NR154112/ GU580883	GU580885	Cui <i>et al.</i> 2011
<i>Lignosus hainanensis</i>	Y.C. Dai 10670 _sclerotium	China	GU580884	GU580886	Cui <i>et al.</i> 2011
<i>Lignosus sacer</i>	Isolate 11329	Zimbabwe	GU001674	N/A	Cui <i>et al.</i> 2011
<i>Lignosus sacer</i>	Isolate 15572	Kenya	GU001675	N/A	Cui <i>et al.</i> 2011

<i>Lignosus tigris</i>	Strain A	Malaysia	JQ409480	JQ681743	Unpublished
<i>Lignosus tigris</i>	Strain K	Malaysia	JQ409481	N/A	Tan <i>et al.</i> 2013
<i>Lignosus tigris</i>	Stain T	Malaysia	JQ409482	N/A	Tan <i>et al.</i> 2013
<i>Lignosus rhinocerus</i>	Isolate CH2	Malaysia	FJ380871	FJ899145	Tan <i>et al.</i> 2013
<i>Lignosus rhinocerus</i>	Strain CH31	Malaysia	FJ899143	FJ899146	Tan <i>et al.</i> 2013
<i>Lignosus rhinocerus</i>	PEN94	Malaysia	JQ409359	AB368074	Sotome <i>et al.</i> 2008
<i>Lignosus rhinocerus</i>	Strain TM02	Malaysia	JQ409479	JQ681742	Unpublished
<i>Lignosus cameronensis</i>	Strain T1	Malaysia	JQ409483	N/A	Tan <i>et al.</i> 2013
<i>Lignosus cameronensis</i>	Strain T8	Malaysia	JQ409484	JQ681744	Tan <i>et al.</i> 2013
<i>Lignosus cameronensis</i>	Strain T12	Malaysia	JQ409485	N/A	Unpublished
<i>Daedaleopsis confragosa</i>	Strain 4	Germany	FR686551	N/A	Schmidt <i>et al.</i> 2012
<i>Daedaleopsis sinensis</i>	Cui3700	China	FJ627256	N/A	Unpublished

N/A = not available

## RESULTS

### Taxonomy

*Lignosus hainanensis* B. K. Cui (2011)

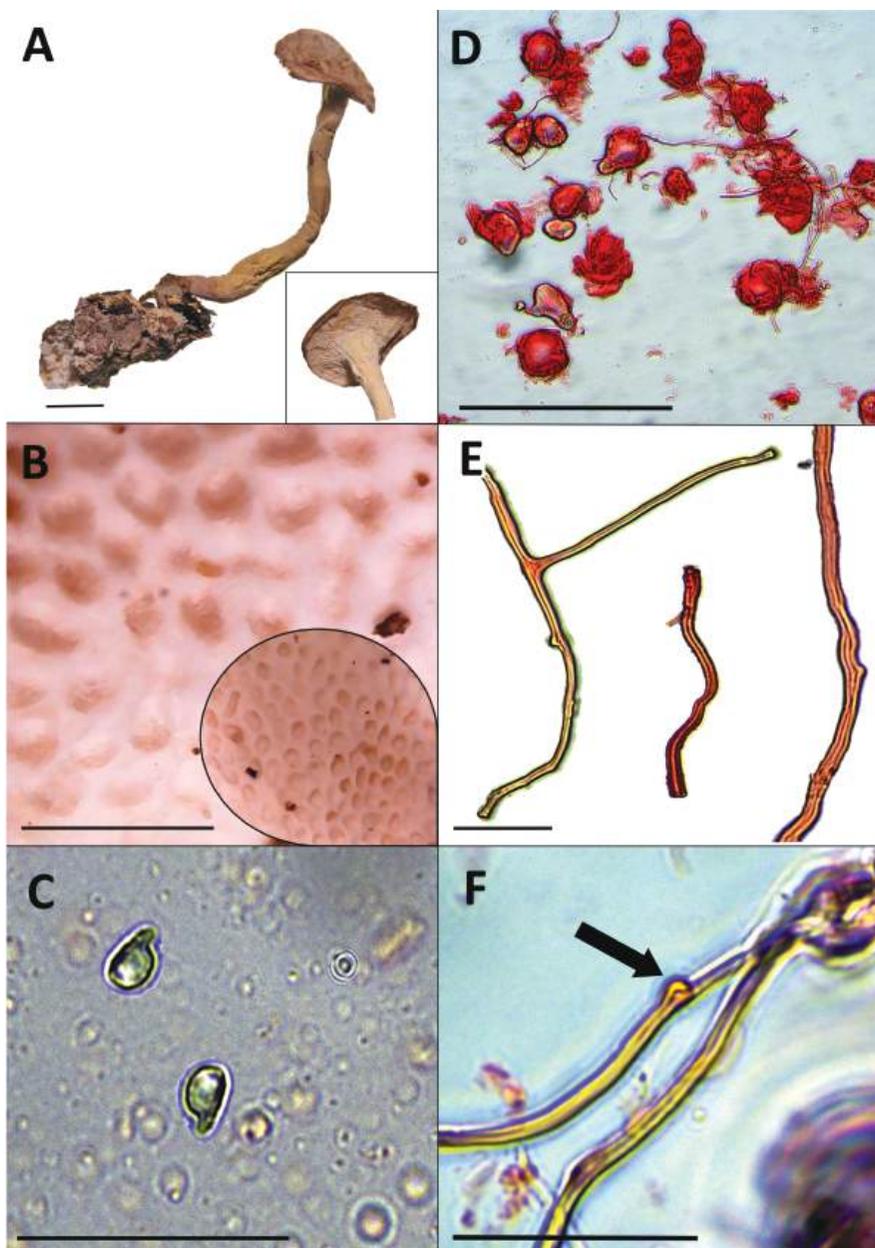
**Description:** —**Basidiomata** single, solitary, terrestrial, centrally stipitate with a circular pileus connecting to a sclerotium, tough, without odor or taste when fresh, hard corky to woody hard when dry (Figure 1). **Pileus** 5.5 cm in diameter, 0.1 cm thick at the center, circular, slightly conic in the center, concentrically zonate and brownish grey (8C2-E2) to dark brown (8EF7-8), rugose; margin, marginate, wrinkled, slightly incurved when dry, 0.1 cm thick, context pure white. **Stipe** 4 cm long, 0.3 cm diameter, brownish grey (5C2-3) on the surface, single, central, terete, woody hard and smooth, solid flesh, pure white, skeletal hyphae with 2.5 – 3.9  $\mu\text{m}$  in diameter, connecting the pileus to sclerotium. **Sclerotium** 4 cm height 2 cm wide, irregular, elongated, very light weighted when dry, wrinkled, brownish beige surface, hard corky, context pure white, soft, buried in soil. **Hymenophore** 0.15 – 0.39  $\mu\text{m}$  diameter, pale yellow (4A3), 3 – 4 pores per mm, pores round to angular, dissepiments, not reach pilei, corky upon drying. **Tube** 50  $\mu\text{m}$  long, 1.1–3.9  $\mu\text{m}$  in diameter, creamy white, skeletal hyphae.

**Basidiospores** 2.3 – 2.4  $\times$  5.3 – 5.4  $\mu\text{m}$  (n=20), clavate, oblong ellipsoid to cylindrical, hyaline, thin-walled, smooth, negative in Melzer's reagent. **Cystidia** and **cystidioles** absent. No observation on **basidia**. **Hyphal system** 0.9 – 3.9  $\pm$  0.894  $\mu\text{m}$  (n=47), trimitic, dominant skeletal hyphae with thick wall, 1.9 – 3.9  $\mu\text{m}$  in diameter, often branched, interwoven, with clamp connections, tissue unchanged in KOH, negative in Melzer's reagent. **Generative hyphae** infrequent, 1.8–3.3  $\mu\text{m}$  in diameter. **Binding hyphae** present occasionally, 1.5 – 2.5  $\mu\text{m}$  in diameter, branched. **Sclerids** 19.2 – 36.9  $\times$  29.6 – 66.8  $\mu\text{m}$  (n=30), globose, pear-shape to irregular. **Skeletal hyphae** dominant, 1.9 – 3.9  $\mu\text{m}$  in diameter, thick wall with narrow lumen, frequently branched, interwoven, found only in sclerotium with hyphae.

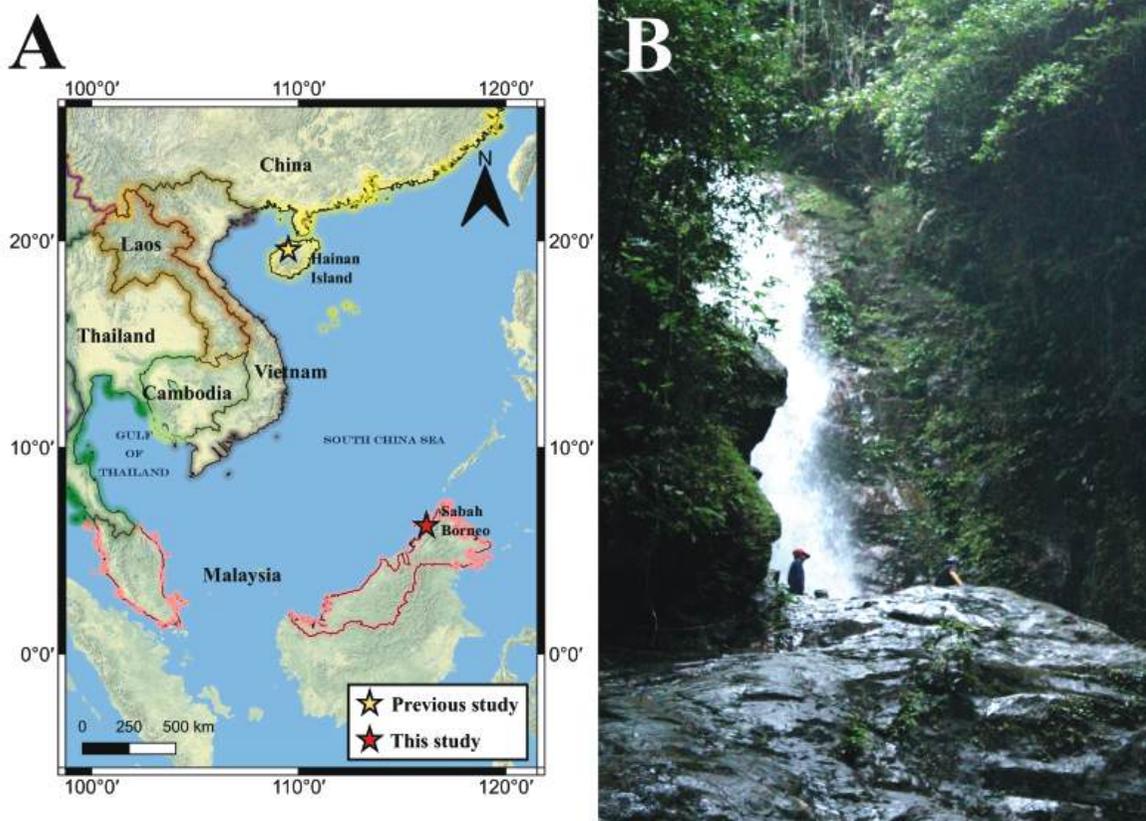
**Known geographical distribution:** —Angiosperm forest, Jian Feng Ling Nature Reserve, Ledong County, Hainan Province, China (Cui *et al.* 2011).

**Material examined:** —MALAYSIA, Sabah, Kota Belud District, Kadamaian Forest, on the ground next to the Wasai Waterfall in a lowland mixed dipterocarp forest (Figure 2), elev. 417 m.a.s.l., (6°13'22.83" N, 116°30'13.36"E), 17 October 2019, Jaya Seelan Sathiya Seelan, (KD094, BORH(F)00523). GenBank accession: ITS = OQ954761, LSU = PP770733.

**Remarks:** —Basidiospore shape of Bornean specimen was more spherical at the bottom as compared to the *L. hainanensis* from China.



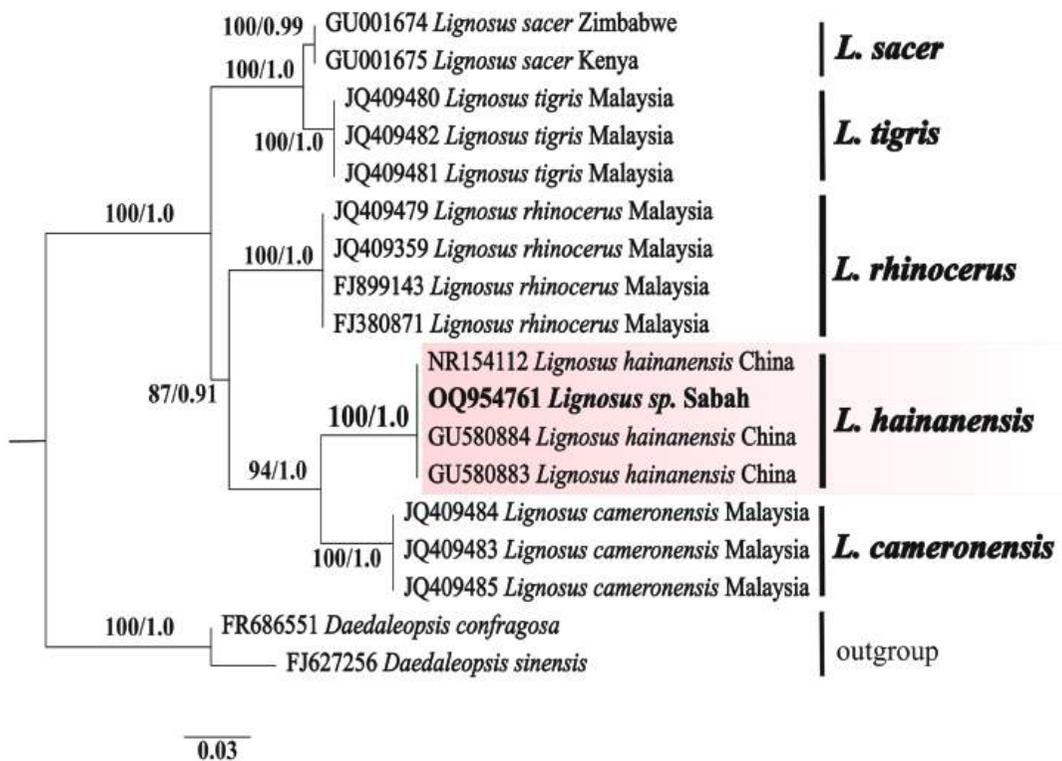
**Figure 1.** Morphological characteristics of *Lignosus hainanensis* collected in Sabah. **A.** The fresh sample of *L. hainanensis* found at Kadamaian Forest. Scale bar: 1 cm. **B.** The hymenophores of *L. hainanensis*. Scale bar: 1 mm. **C.** The spores. Scale bar: 30 μm. **D.** The sclerids from sclerotium. Scale bar: 200 μm. **E.** The dominant skeletal hyphae with narrow lumen. Scale bar: 30 μm. **F.** Clamp connection. Scale bar: 30 μm.



**Figure 2.** Specimen collection sites related to *Lignosus hainanensis*.  
**A.** The location of *Lignosus hainanensis* holotype at the Hainan Island of China (yellow star) and the location of *L. hainanensis* collected in this study at Sabah, Borneo (red star).  
**B.** Wasai Waterfall in the Kadamaian Forest where the *L. hainanensis* specimen was collected.

### Phylogenetic analyses

BLAST search using the ITS sequence of the specimen KD094 revealed sequence identities of 99.03% and 99.18% with two accession numbers of *L. hainanensis* from China, NR154112 and GU580884, respectively. The tree topologies of the Maximum Likelihood and the Bayesian Inference analyses were similar (Figure 3). The phylogenetic analyses included 18 sequences (including one new sequence data and two outgroup sequences) of the ITS region consisting of 740 nucleotide sites. Of which, 531 were constant, 191 parsimony informative sites, and 18 parsimony uninformative sites. Phylogenetic analysis positioned the newly generated sequence from this study (KD094) within the *Lignosus hainanensis* clade. The phylogenetic tree showed high support for the new specimen KD094, which is closely related to the isolates from China (NR154112, GU580883, and GU580884) (Bootstrap value = 100% / Posterior probability = 1.0). Out of the eight *Lignosus* species, only five were incorporated in this study, as the other three species, *L. dimiticus*, *L. ekombitii* and *L. goetzii*, were not available in GenBank database. Additionally, the LSU phylogenetic tree (Bootstrap value = 90% / Posterior probability = 1.0) and concatenated phylogenetic trees (Bootstrap value = 89% / Posterior probability = 1.0) further confirmed the identification of the collection as *L. hainanensis*.



**Figure 3.** Phylogram of *Lignosus hainanensis* obtained from Maximum Likelihood (RAxML) of ITS dataset. Bootstrap values (BS) greater than 70% from Maximum Likelihood and Bayesian posterior probabilities (PP) greater than 0.95 are indicated above the nodes as BS/PP. Newly generated sequence is indicated in black bold.

## DISCUSSION

This study presents a comprehensive documentation of the morphological characteristics and molecular attributes of *Lignosus hainanensis*, establishing a new geographical record for this species in Malaysia. The morphological and phylogenetic analyses confirmed that the Malaysian specimen is consistent with Cui *et al.* (2011), who reported *L. hainanensis* from China as holotype specimen.

Cui *et al.* (2011) first discovered *L. hainanensis* in the Nature Reserve of Angiosperm Forest in Hainan Province, China. Nearly a decade later, during research expeditions in 2019, this species was found in Kadamaian-Kinabalu Park beside the Wasai Waterfall in Sabah, Malaysia, as a new record. Notably, both locations share similar geographical and climatic conditions. Both Hainan Island and Sabah have similar elevation profile, transitioning from coastal area to hills to mountainous terrain (Guo *et al.* 2021). The specimen of *Lignosus hainanensis* collected from Sabah, Borneo is situated within mixed highland dipterocarp forests, sharing geographical characteristics with the tropical regions of Hainan Province, China (Hartemink *et al.* 2008).

Morphologically, the size of pores and basidiospores are the main characteristics to distinguish species within the genus *Lignosus*. In this study, the morphological features of *L. hainanensis* in the current research correspond closely with those described in the specimen from China, with the exception of the size of pileus. The diameter of pileus was

5.5 cm and 0.1 cm thick at the center, whereas *L. hainanensis* reported from China was larger, with pileus diameter of 10 cm and a thickness of 0.5 cm at the center. Importantly, the size of pores and basidiospores confirmed the similarity of the specimen to *L. hainanensis* documented in China. The pore size of the *L. hainanensis* in this study features 3 – 4 pores per mm and round in shape.

The *L. hainanensis* specimens collected in this study were at its younger development stage. Only two basidiospores were observed, with their size ( $2.3 - 2.4 \times 5.3 - 5.4 \mu\text{m}$ ) falling within the range reported for the specimens from China ( $2.2 - 2.9 \times 4.9 - 6.0 \mu\text{m}$ ). However, the shape of basidiospores observed in this study was more spherical on the bottom as compared with the shape of basidiospores reported from China. Sclerids, measuring  $19.2 - 36.9 \times 29.6 - 66.8 \mu\text{m}$ , are globose, pear-shaped to irregular form. The skeletal hyphae were predominant,  $1.9 - 3.9 \mu\text{m}$  in diameter, has thick walls with narrow lumen, frequently branched, interwoven, and found only in sclerotium with hyphae. These features align with those reported by Cui *et al.* (2011).

The *Lignosus hainanensis* specimen collected in this study demonstrates solitary ecological traits, utilising soil as substrate and exhibiting the structure of polypore with cap, stipe, and sclerotium (Abdullah *et al.* 2013). In addition, specimen in present study shares high similarity to other *Lignosus* species in their microscopic characteristics, such as the trimitic hyphae system (dominate skeletal hyphae, infrequent generative hyphae, and binding hyphae), as well as the presence of clamp connections (Cui *et al.* 2011; Vinjusha and ArunKumar 2021; Ng *et al.* 2023).

The pore size of *L. hainanensis* (3 – 4 pores per mm) was comparable to that of *L. cameronensis* (2 – 4 pores per mm), yet it is smaller than that of *L. tigris* (1– 2 pores per mm), and larger than that of *L. rhinocerus* (6 – 7 pores per mm). The pore shape of *L. hainanensis* was similar to that of *L. rhinocerus* (round), but different from that of *L. tigris* and *L. cameronensis* (isometric). In terms of basidiospore morphology, *L. hainanensis* is distinguished by its oblong ellipsoid to cylindrical shape, compared to *L. rhinocerus* which is subcylindrical to broadly ellipsoid in shape, or *L. tigris* and *L. cameronensis* which is ellipsoid to subglobose in shape. The sclerid of *L. hainanensis* was globose, pear-shaped to irregular, which was similar to *L. cameronensis*, but differs from *L. rhinocerus* (globose to round) and *L. tigris* (subglobose, oblong to irregular). In summary, *L. hainanensis* demonstrates a closer similarity to *L. cameronensis*, particularly in pore size and sclerid shape.

Cui *et al.* (2011) generated both ITS and nLSU sequences from the fruiting body and sclerotium of China's *L. hainanensis* specimen (Holotype: Y. C. Dai 10670). In this study, two sequences were generated, each for the ITS and nLSU regions. The phylogenetic analysis using the ITS region showed high degree of support, evidenced by a rapid bootstrap value (BS = 100%) and posterior probability (PP = 1.0), indicating a close similarity with *L. hainanensis* reported from China (Figure 3). The phylogram of *L. hainanensis* (Figure 3) provides a clearer insight into the phylogenetic relationships among *Lignosus* genus as compared with the phylogenetic result mentioned in Tan *et al.* (2013). *Lignosus sacer* was a sister group to *L. tigris* with a 100% bootstrap value and a 0.99 posterior probability value, while *L. rhinocerus* was a sister group with a high support value (BS = 87% / PP = 0.91) to *L. hainanensis* and *L. cameronensis*. In other words, *L. rhinocerus*, *L. cameronensis*, and *L. hainanensis* were close to Asian strains, while *L. tigris* lies closer to the African strain (*L. sacer*) in molecular characterisation. Particularly, *L. hainanensis* was the closest sister group to *L. cameronensis* with 94% bootstrap value and 1.0 posterior probability value. This corresponds to the higher similarity in morphology characteristics between *L. hainanensis* and *L. cameronensis*. Both morphology and molecular data strongly supported the identity of the collected specimen as *L. hainanensis*.

## Dichotomous key to *Lignosus* species

1. Pileus white to ochraceous.....2  
Pileus light brown to dark brown.....3
2. Pores large, 0.5 – 2 pores per mm.....*L. goetzii*  
Pores small, 6 – 8 pores per mm.....*L. dimiticus*
3. Pores  $\geq$  7 pores per mm.....*L. rhinocerus*  
Pores  $<$  6 pores per mm .....4
4. Basidiospores broadly ellipsoid to subglobose .....5  
Basidiospores oblong ellipsoid to cylindrical .....7
5. Basidiospores 5 – 7  $\mu$ m long .....*L. sacer*  
Basidiospores 2.5 – 5.5  $\mu$ m long .....6
6. Pores 1 – 2 per mm.....*L. tigris*  
Pores 2 – 4 per mm.....*L. cameronensis*
7. Basidiospores  $>$  6  $\mu$ m long.....*L. ekombitii*  
Basidiospores  $<$  6  $\mu$ m long.....*L. hainanensis*

## CONCLUSION

In this study, *Lignosus hainanensis* was identified from the Kadamaian Forest in Sabah using both morphological and molecular approaches. This marks the first recorded presence of *L. hainanensis* in Sabah (Northern Borneo), Malaysia. With this discovery, Malaysia now has four recognised *Lignosus* species: *L. rhinocerus*, *L. cameronensis*, *L. tigris*, and *L. hainanensis*.

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