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# First record of *Borofutus dhakanus* (*Boletaceae*, *Leccinoideae*) in Thailand

Sutheera Thongkantha[1], Sulichet Thongklam[2], Wasu Pathom-aree[1], Saisamorn Lumyong[1] and Boonsom Bussaban[1,*]

[1] *Department of Biology, Faculty of Science, Chiang Mai University*, Chiang Mai 50200, Thailand
[2] *Highland Research and Development Institute* (*Public Organization*), Chiang Mai 50200, Thailand

***Corresponding author**: bbussaban@gmail.com

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**Abstract:** A new record of *Borofutus dhakanus* Hosen & Zhu L. Yang is reported from Chiang Mai Province, northern Thailand. The phylogenetic relationships of the Thai specimen were compared with those of the type species and allied genera from seven major subfamilies of *Boletaceae*. The trees were constructed using sequences of partial 28S rDNA combined with internal transcribed spacer (ITS) gene regions. The morphological characteristics of the fungus and its edibility are also discussed. Although many boletes have been documented from Thailand, most previous records are based only on morphological characters and there is an urgent need for taxonomic revision.

**Keywords:** *Basidiomycete*; *Boletales*; new record; phylogeny; taxonomy

## INTRODUCTION

Over the past 100 years, the diversity of macrofungi in Thailand has been investigated all over the country by foreign as well as Thai mycologists [1-11]. The most up-to-date checklist contains 1978 species of Thai mushroom (*Basidiomycetes*) and was published in 2011 [12]. Approximately 35 genera and 230 species of *Boletales* were recorded. This included 19 genera in the family *Boletaceae*: *Aureoboletus* Pouzar (3 spp.), *Austroboletus* (Corner) Wolfe (3 spp.), *Boletellus* Murrill (7 spp.), *Boletus* L. (104 spp.), *Buchwaldoboletus* Pilát (2 spp.), *Chalciporus* Bataille (2 spp.), *Heimioporus* E. Horak (5 spp.), *Ixechinus* R. Heim (1 sp.), *Leccinellum* Bresinsky & Manfr. Binder (3 spp.), *Leccinum* Gray (15 spp.), *Phylloporus* Quél. (8 spp.), *Porphyrellus* E-J. Gilbert (2 spp.), *Pulveroboletus* Murrill (3 spp.), *Retiboletus* Manfr. Binder & Bresinsky (1 sp.), *Rubinoboletus* Pilát & Dermek (1 sp.), *Spongiforma* Desjardin, Manfr. Binder, Roekring & Flegel (1 sp.), *Strobilomyces* Berk. (6 spp.), *Tylopilus* P. Karst (25 spp.) and *Xerocomus* Quél. (3 spp.). However, most reports have been based on morphological identification and they lack supporting molecular data. Some genera and species require revision with the aid of molecular techniques, alongside an examination of new collections.

During a field survey in 2010, small- to medium-sized basidiomata of a bolete with distinctly broad pores were collected from the forest area of Huai Hongkhrai Royal Development Study Center. The unique macro- and microcharacters, in combination with the molecular sequence of the large subunit ribosomal deoxyribonucleic acid (28S rDNA) and the internal transcribed spacer (ITS) gene region, indicate that the fungus is representative of the genus *Borofutus* Hosen & Zhu L. Yang, which was recently introduced as a new genus in the *Boletaceae* [13]. The type species of the genus, *Borofutus dhakanus* Hosen & Zhu L. Yang, is known only from the Dhaka division of Bhawal National Park in Bangladesh. Description, illustrations, phylogenetic analysis within the *Boletaceae* as well as comparison of the nucleotide sequence alignment with the allied taxa are presented herein.

## MATERIALS AND METHODS

### Fungal collection and morphological identification

Specimens of boletes were collected during early rainy season (July) from the dry dipterocarp forest area of Huai Hongkhrai Royal Development Study Center,

Chiang Mai, Thailand. Macroscopic characters were described from fresh basidiomata, and documented by photographs. Codes of color follow Kornerup and Wanscher [14]. Basidiomes were sectioned using a sharp razor blade and revived in lactoglycerol. One hundred basidiospores were measured and their dimensions are presented as n=number of measured spores, $L^m$=average length, $W^m$=average width, E=length/width ratio range, and Q=average of length/width ratio from all spores measured. The amyloid reaction was examined by Melzer's reagent. Dried material of the fungus was deposited at the Research Laboratory for Excellence in Sustainable Development of Biological Resources, Department of Biology, Faculty of Science, Chiang Mai University (SDBR) and the Biology Department's Herbarium of Chiang Mai University (CMUB).

**DNA extraction**

A small piece of fruit body, cut from the flesh section between the pileus and stipe, was placed into a 1.5-ml centrifuge tube containing 300 µl 2× cetyltrimethylammonium bromide (CTAB) buffer and stored at -20°C for subsequent DNA extraction using a modified procedure of Doyle [15]. The small piece of tissue was ground with a mini pestle and sterilized quartz sand (200 mg). The extraction buffer was adjusted to 600 µl and placed in a 60°C incubator for 30 min, with gentle swirling. The solution was then extracted until no interface was visible with an equal volume of chloroform:isoamyl alcohol (24:1) at 13000 rpm for 30 min, two or three times. The supernatant phase containing the DNA was precipitated by the addition of 2.5 volumes of absolute ethanol and kept at -20°C overnight. The DNA pellet was washed with 70% ethanol twice, dried under vacuum, resuspended in TE buffer (1 mM EDTA, 10mM Tris-HCl, pH 8) and mixed with RNase A (1 mg $ml^{-1}$). Genomic DNA was checked by electrophoresis on 1% agarose gels stained with ethidium bromide and visualized under UV illuminator.

**PCR amplification and sequencing**

The partial 28S rDNA gene region was amplified by LROR (5'-ACCCGCTGAACTTAAGC -3') and LR5 (5'-TCCTGAGGGAAACTTCG-3') [16]. The ITS region contained a pair of ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') sequences [17]. Genomic DNA (50 ng/1 µl) was used in a standard 50 µl PCR mixture (25 mM $MgCl_2$, 10 Mg-free buffer, 2.5 µM dNTPs, 1.5 µM primers, and 1.5 unit of *Taq* DNA Polymerase-BioLabs M0267-S). The thermal conditions were as follows: 94°C for 30s, 35 cycles of 94°C for 30 s, 50°C (ITS) or 60°C (28S rDNA) for 30 s, and 72°C for 1.5 min. Amplicons were checked on 1% agarose gels. Negative control reactions without DNA were included in all sets of amplifications to monitor for potential contamination by exogenous DNA. PCR products were purified using the NucleoSpin® Extract II PCR clean-up kit (Macherey-Nagel, USA) according to the manufacturer's protocol. The amplified 28S rDNA and ITS fragments were directly sequenced. Sequencing reactions were performed and sequences determined automatically by the Pacific Science Company, Canada, using the PCR primers mentioned above.

**Sequence alignment and phylogenetic analysis**

The 28S rDNA and ITS sequences of the bolete CMUB39815 were compared with those available in the GenBank database. The sequences were 99% matches with all sequences of the same gene region of *Borofutus dhakanus* (28S rDNA: JQ928615-JQ928617, NG042663, ITS: JQ928606, JQ928607, NR120117). Both gene region sequences of CMUB39815 and all those from GenBank (*B. dhakanus*, 99% and *Spongiforma* spp., 94%) were checked in detail by multiple base pair alignment. In addition, the sequence datasets reported by Hosen et al. [13] and some other allied genera in the family *Boletaceae* were retrieved from GenBank and combined with our own sequences to analyze their phylogenetic relationship.

A total of 24 bolete taxa were included in the final dataset analyses (Table 1). Nucleotide sequences of the 28S rDNA and ITS gene regions were initially aligned and combined using BioEdit 7.2.5 [18] and/or Clustal X 1.83 with default parameter settings [19]. The alignments were then manually optimized using BioEdit. Maximum likelihood (ML), maximum parsimony (MP) and neighbor-joining (NJ) analyses were conducted using MEGA6 [20] and PAUP*4.0b10 [21]. Tree searches were carried out using the heuristic method with a random stepwise addition and tree bisection and reconstruction branch-swapping algorithm.

**Table 1** Specimens used in the ITS and 28S rDNA phylogenetic study and their GenBank accession numbers.

| Fungal name | Specimen no. | ITS | 28S rDNA |
|---|---|---|---|
| ***Austroboletoideae*** | | | |
| Austroboletus fusisporus | HKAS75207 | JX889719 | JX889720 |
| ***Boletoideae*** | | | |
| Boletus edulis | BD380 | EU231984 | HQ161848 |
| Boletus satanas | Bs2 | DQ534567 | AF336242 |
| Boletus sinicus | HKAS56304 | KJ605666 | KJ605673 |
| Porphyrellus porphyroporus | MB 97023 | DQ534563 | DQ534643 |
| Strobilomyces floccopus | AFTOL 716 | AY854068 | AY684155 |
| ***Chalciporoideae*** | | | |
| Chalciporus piperatus | HKAS 50214 | JQ928610 | JQ928621 |
| Chalciporus piperatus | MB04 001 | AF074922 | DQ534648 |
| ***Leccinoideae*** | | | |
| Borofutus dhakanus | HKAS73785 | NR120117 | JQ928615 |
| Borofutus dhakanus | HKAS73789 | JQ928606 | JQ928616 |
| Borofutus dhakanus | HKAS73792 | JQ928607 | JQ928617 |
| ***Borofutus dhakanus* (this study)** | CMUB39815 | KU168045 | KU168044 |
| Leccinum scabrum | KPM-NC0017840 | KC552012 | JN378515 |
| Octaviania asterosperma | Trappe 23377 | JN257998 | JN378497 |
| Octaviania tasmanica | OSC 132097 | KP222909 | JN378494 |
| Rossbeevera vittatispora | MEL 2329434 | KJ001084 | KJ001097 |
| Spongiforma squarepantsii | LHFB 14 | HQ724511 | HQ724509 |
| Spongiforma thailandica | DED7873 | EU685113 | NG042464 |
| ***Xerocomoideae*** | | | |
| Boletellus projectellus | AFTOL 713 | AY789082 | AY684158 |
| ***Zangioideae*** | | | |
| Zangia roseola | HKAS52661 | JQ928614 | JQ928623 |
| **Other group of *Boletaceae*** | | | |
| Bothia castanella | 28003 | DQ867111 | DQ867118 |
| Bothia fujianensis | HKAS82694 | KM269195 | KM269193 |
| **Out group (*Paxillaceae*)** | | | |
| Gyrodon lividus | REGGl 1 | DQ534568 | AF098378 |
| Paxillus vernalis | AFTOL 715 | DQ647827 | AY645059 |


**Fig. 1.** *Borofutus dhakanus* CMUB39815 **A** – Mature basidiomata with broad-pored hymenophores in its natural habitat. **B** – Unchanging context after section. **C, D** – Pileus, stipe and hymenophoral surface. **E** – Boletoid to amygdaliform basidiospores. **F, G** –Basidiocarps for sale at roadside market in northeastern provinces. Scale bar: A-D=1 cm, E=10 µm.

## RESULTS AND DISCUSSION

### Morphological studies

*Borofutus* Hosen & Zhu L. Yang

*Borofutus dhakanus* Hosen & Zhu L. Yang

Basidiomata epigeous, stipitate-pileate with tubular hymenophore, small to medium-sized (Fig. 1). Pileus 25-50 mm in diameter, convex when young becoming plane when mature, becoming rimose with age, margin occasionally uplifted, dry, slightly viscid, covered with light brown (6D3-4) to cocoa brown (6E5-6) or grayish brown (6E3-4) squamules, which become grayish black (6F1) at maturity. Hymenophore broadly tubular subdecurrent, pallid to creamy to pale yellow (4A1-3), turning pale reddish to pale reddish purple on exposure to air for long time (1-2 h), becoming light brown (6D6-8) to golden brown (5D7-8) at maturity; pores 4-11 mm long, 2-4 mm wide, mostly hexagonal, with reddish powdery mass inside the tubes when aged. Stipe 24-62×4-8 mm, central, cylindrical, occasionally slightly swollen at the base, covered with purplish to grayish to cocoa brown (6E5-6) squamules, basal mycelium whitish. Context 4-12 mm thick in the center of the pileus, solid, creamy to yellowish (4A1-2), usually unchanging in color when cut but turning pale reddish to pale reddish purple in some areas after 1-2 h. Taste slightly bitter, odor mild. Basidiospores (8.6)10.3-12.0(13.8)×(4.4)4.8-5.9(7.3) µm (n=100; $L^m$=10.2±0.87 µm; $W^m$=5.4±0.62 µm; E=1.7–2.3; Q=2.0), boletoid to amygdaliform with slightly thickened wall (up to 0.5 µm), surface finely verrucose, brown-violet to purplish red (11E6-8, 11F5-8) in lactoglycerol (Fig. 1E), amyloid. Basidia (25)31-33(38)×(7)8-10(12) µm, narrowly clavate to clavate, hyaline to pale yellowish, thin-walled, 4-spored. Hymenophoral trama 100-140 µm wide, bilateral; hyphae 7-13 µm wide, cylindrical, hyaline. Cheilocystidia (50)70-80(97)×7-12 µm, lageniform to broadly lageniform, slightly thickened

**Table 2** The 28S rDNA sequence length of *Borofutus dhakanus* CMUB39815, HKAS73785, HKAS73789 and HKAS73792 compared with those of two *Spongiforma* species.

| No. | Name | GenBank accession No. | Specimens No. | Sequence length (a/b) |
|---|---|---|---|---|
| 1 | ***Borofutus dhakanus*** | KU168044 (this study) | CMUB39815 | 913/910 |
| 2 | *Borofutus dhakanus* | JQ928615 | HKAS73792 | 848/846 |
| 3 | *Borofutus dhakanus* | JQ928616 | HKAS73789 | 806/804 |
| 4 | *Borofutus dhakanus* | JQ928617 | HKAS73785 | 858/856 |
| 5 | *Spongiforma squarepantsii* | HQ724509 | LHFB01 | 498/497 |
| 6 | *Spongiforma squarepantsii* | HQ724510 | LHFB14 | 498/497 |
| 7 | *Spongiforma thailandica* | NG042464 | DED7873 | 913/908 |

a – total bases including gap on the align sequences
b – total bases of each sequences without gap

wall (0.7-1.2 μm thick), with an attenuate appendage, sometimes with a secondary septum. Pleurocystidia 80-108×11-18 μm, slightly thickened wall (0.5 μm), narrowly lageniform to broadly lageniform with an appendage-like apex. Pileipellis, a trichoderm when young, becoming a subcutis at maturity. Stipe trama composed of vertically arranged hyphae, hyphae 7-13 μm wide. Stipitipellis, a sterile hymenium-like structure composed of subclavate or clavate to fusiform cells with projecting cystidia and yellowish brown to pale brown vacuolar pigmentation.

Specimens examined: Thailand, Chiang Mai Province, Doi Saket District, dry dipterocarp forest in Huai Hongkhrai Royal Development Study Center, 10 July 2010, S. Thongkantha CMUB39815 (SDBR-CMU-ST58-001); Thailand, Ubonratchathanee Province, Don Mot Daeng District, roadside market, 13 September 2014, S. Vadthanarat SDBR-CMU-SV057.

Habitat: mostly solitary or often in small groups, usually found growing on red clay soil in dry dipterocarp forest dominated by *Dipterocarpus* spp. and *Shorea* spp.

## Molecular studies

Comparison of the highest percentage matched 28S rDNA sequence of the bolete CMUB39815 with three sequences of the type species of *Borofutus dhakanus* [13] (99%: JQ928615-JQ928617) and *Spongiforma* spp. (94%: *S. squarepantsii*: HQ724509, HQ724510 and *S. thailandica*: EU865108, NG042464) from GenBank showed that a good quality nucleotide sequence of CMUB39815 (910 bases) is longer than those of *B. dhakanus* JQ928617 (856), JQ928615 (846) and JQ928616 (804) with 54, 64 and 106 bases, respectively. In addition, all are similar throughout their sequence alignment and only at a few positions the bases are not the same. The differentiation between the sequences of *B. dhakanus* and the two species of *Spongiforma* were also investigated and the results are summarized in Table 2 and Fig. 2.

The combined dataset consisted of 851 and 652 nucleotides (including gaps) for 28S and ITS, respectively. In this alignment, 463 characters were constant, while 1040 characters were variable, of which 1343 were informative. For both datasets, phylogenetic trees generated from ML, MP and NJ analyses were almost identical, with minimal variation of the statistical support values. Phylogenetic trees generated from both 28S (data not shown) and the combined datasets (Fig. 3) showed that *Borofutus* formed an independent clade in the family *Boletaceae*, clearly separated by genetic distance, and clustered with the gasteroid bolete *Spongiforma*. The current phylogenetic relationship study of *B. dhakanus* CMUB39815 agrees well with the molecular analyses reported by Hosen et al. [13] and Wu et al. [22]. These results inply that *Borofutus* is a member of *Boletaceae* with 99% bootstrap support in ML (Fig. 3) and it is sister to *Spongiforma* with high bootstrap support (100%).

The species complex of *B. dhakanus*/*S. squarepantsii*/*S. thailandica* clade with subfamily *Austroboletoideae* (*Austroboletus fusisporus*)/*Boletoideae* (*Porphyrellus porphyrosporus* and *Strobilomyces floccopus*)/*Leccinoideae* (*Leccinum scabrum*, *Octaviania asterosperma*, *O. tasmanica*, *Rossbeevera vittatispora*)/ *Zangioideae* (*Zangia roseola*) had 72% bootstrap support in the ML analysis (Fig. 3) and 83% in the NJ analysis (data not shown). These results are similar to those of Wu et al. [22], who placed both *Borofutus* and *Spongiforma* in

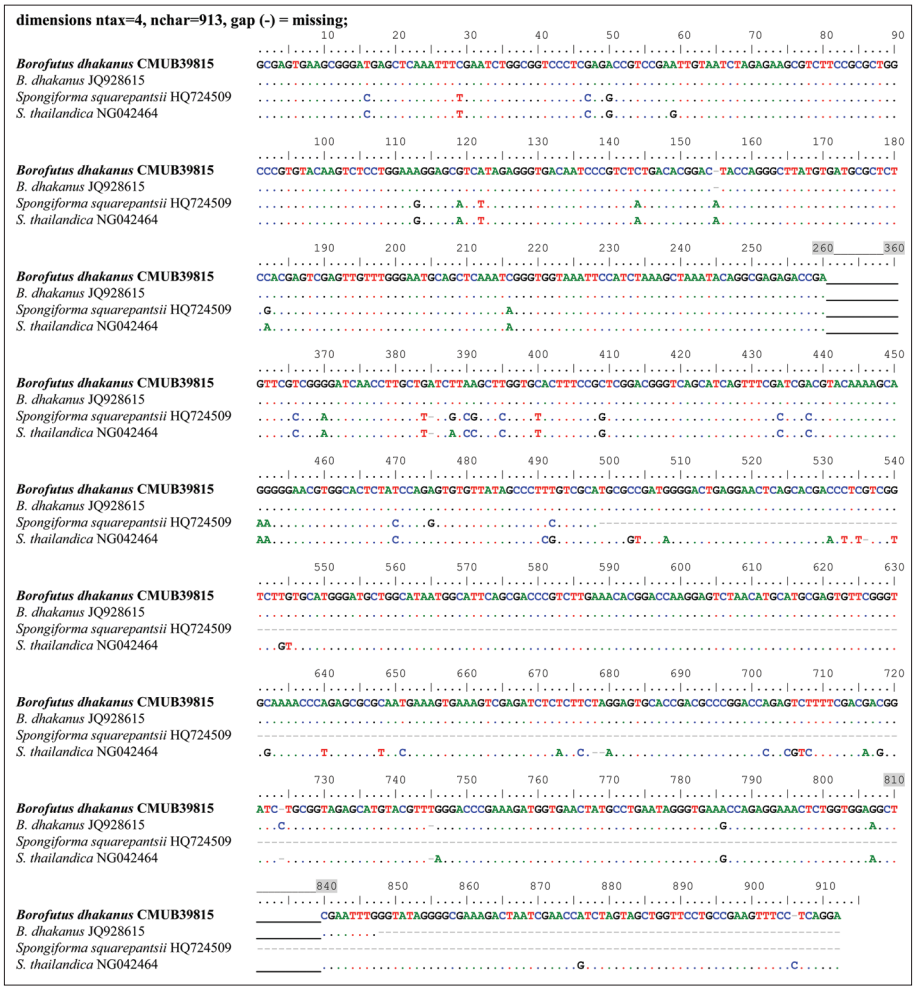


**Fig. 2.** Multiple base pair indels in 28S rDNA sequence of *Borofutus dhakanus* CMUB39815 (910 bases), which is very similar to a sequence of JQ928615 but different from those of two *Spongiforma* species. Numbers refer to the positions in the alignment (260-360 and 810-840 are not shown because they are the same bases).

the subfamily *Leccinoideae*, one of seven subfamilies or major clades of *Boletaceae* supported by the multilocus gene of 28S, translation elongation factor 1-alpha (*tef1-α*), RNA polymerase largest subunit 1 and 2 (*rpb 1*) and (*rpb 2*) relationship analysis.

However, the ITS sequences of some other species of *Austroboletus*, *Porphyrellus* and *Zangia*, as well as those of *Retiboletus* spp. (*Leccinoideae*), need to be combined with 28S sequences to resolve their phylogenetic placement within subfamilies. Analysis of some other gene regions such as atp6, *tef1-α*, *rpb 1*, *rpb* 2 and/or the small subunit (18S) might be needed to clarify their genetic lineage within the complex species of *Boletaceae* as well as at a higher taxonomic level [13, 22, 23-27].

Multiple base pairing of the ITS sequence of *Borofutus dhakanus* CMUB39815 (800 bases) with those of three taxa, as reported by Hosen et al. [13], was observed: *B. dhakanus* JQ928606 (800), JQ928607 (745) and NR120117 (746) (Fig. 4). There is one site on the nucleotide sequence alignment that is shown as a deletion/insertion region at position 714-720.

The general morphological features combined with molecular evidence from the Thai specimen (CMUB39815) show that it is a best fit to *B. dhakanus*, which was described by Hosen et al. [13]. This species is characterized by a small- to medium-sized fruit body with grayish brown to cocoa brown pileus; subdecurrent hymenophore that changes from cream

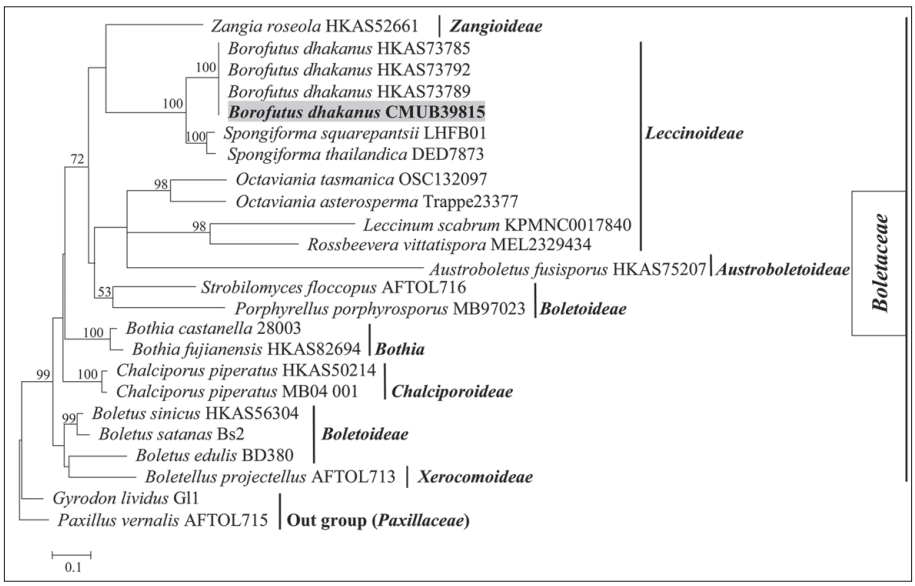


**Fig. 3.** Phylogenetic relationship among representative specimens of *Boletaceae* (*Leccinoideae*) and *Borofutus dhakanus* CMUB39815 inferred from a combined 28S rDNA and ITS dataset analyzed by ML method with the Kimura 2-parameter model. There was a total of 1343 positions in the final dataset (1503 bases including gaps).

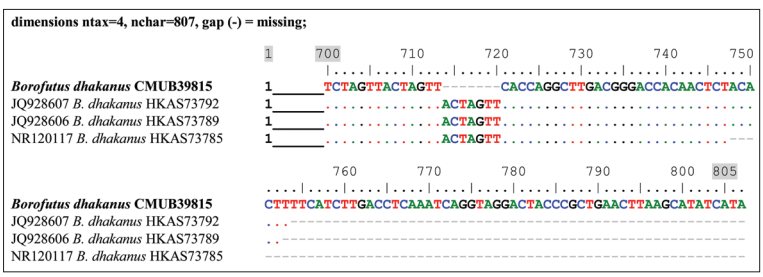


**Fig. 4.** Multiple base pairs of ITS sequences of *Borofutus dhakanus* CMUB39815, JQ928606, JQ928607 and NR120117. Numbers refer to the position in the alignment from position 700-807 (1-700 are not shown because they are the same bases).

to golden brown, with broad and nearly hexagonal pores; basidiospores that are purple to purplish red in water [13] or brown-violet to purplish red in lactoglycerol (this study), ornamented with irregular to regular shallow pits; cystidia lageniform and thick-walled. The Thai specimens have a slightly smaller pileus (25-50 mm vs. 30-65 mm) and the basidiospores are slightly shorter but equal in width (10.3-12.0×4.8-5.9 µm Q=2.0 vs. 10-14×4.5-6.5 µm, Q=2.3). The Thai specimen was found on red clay soil in a forest dominated by various species of *Shorea* and *Dipterocarpus*, features similar to the collection site of the holotype. However, the type specimen was reported as a mycorrhizal bolete associated with *Shorea robusta*.

Tropical forests support a wide diversity of macrofungi, often including ectomycorrhizal fungi, which play a major role in nutrient cycling by mobilizing nitrogen and phosphorus [28]. Additionally, many species are also edible, for example, *Boletus edulis*, *B. griseus*, *B. nobilis*, *Phlebopus portentosus*, *Tylopilus balloui* and *T. virens*. Edible mushrooms are not only appreciated for their texture, flavor and nutritional properties, but also for their bioactive compounds that have been demonstrated to possess antitumor, antimicrobial and other activities [29-32]. The present study also found that *B. dhakanus* is an edible species, an attribute not mentioned by Hosen et al. [13]. It is collected for cooking and for sale in northeastern

Thailand (Fig. 1F and G). Further collections of *B. dhakanus* are needed for additional studies on its edibility as well as nutrition and content of bioactive compounds.

## CONCLUSION

Basidiocarps of a small- to medium-sized bolete were collected during the rainy season (July, 2010) from dry dipterocarp forest areas of Huai Hongkhrai Royal Development Study Center in Chiang Mai Province. Based on both morphological and molecular analyses (28S rDNA and ITS sequences) this specimen (CMUB39815) was confidently identified as *Borofutus dhakanus*.

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