
Spongiforma, a new genus of gasteroid boletes from Thailand

Desjardin, D.E.^{1*}, Binder, M.², Roekring, S.³ and Flegel, T.⁴

¹Department of Biology, San Francisco State University, 1600 Holloway Ave., San Francisco, CA 94132

²Department of Biology, Clark University, 950 Main St., Worcester, MA 01601

³Asia Star Lab Co., Ltd., Research and Development, 9 Soi Prachanimitr, Pradipat Road, Samsennai Phayathai, Bangkok 10400, Thailand

⁴Centex Shrimp, 4th Floor Chalermprakit Bldg., Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand

Desjardin, D.E., Binder, M., Roekring, S. and Flegel, T. (2009). *Spongiforma*, a new genus of gasteroid boletes from Thailand. *Fungal Diversity* 37: 1-8.

Based on morphological and molecular characters, *Spongiforma* is described as a new genus of gasteroid boletes belonging in the Boletineae. It is represented by a single species, *S. thailandica*, that is putatively mycorrhizal with dipterocarp trees in central Thailand. Unusual morphological features include a sponge-like, astipitate, epigeous basidiome with large exposed locules and a strong coal tar odor, and rugulose, reddish brown basidiospores with an apical pore that become smooth and violet grey in 3% potassium hydroxide solution. A description, illustrations, phylogenetic analysis and comparison with allied taxa are presented.

Key words: Agaricomycotina, Basidiomycota, Boletineae, molecular phylogenetics, taxonomy.

Article Information

Received 27 October 2008

Accepted 4 March 2009

Published online 1 August 2009

*Corresponding author: Dennis E. Desjardin; email: ded@sfsu.edu

Introduction

Although Southeast Asia is home to numerous genera of boletes (Basidiomycota, Agaricomycotina, Boletales), few secotioid or gasteroid taxa have been reported from the region (Corner and Hawker, 1953; Corner, 1972; Watling and Lee, 1995, 1998; Lee *et al.*, 2002; Halling *et al.*, 2007). Recently, specimens of an unusual, epigeous, astipitate and sponge-like species were collected from Khao Yai National Park in central Thailand. Basidiomes have a rubbery-gelatinous texture, emit a strong coal tar odor, and are associated with *Shorea henryana* Pierre and *Dipterocarpus gracilis* Blume (Dipterocarpaceae) in a primary forest. A unique set of macro- and micromorphological features in combination with molecular sequences of the nuclear large subunit (nuc-18S) gene region indicate that the organism represents a new genus in the Boletineae. A description, illustrations, phylogenetic analysis and comparison with allied taxa are presented herein.

Materials and Methods

Morphology

Macromorphological data were derived from fresh specimens, whereas micromorphological data were derived from dried specimens rehydrated in ethanol followed by distilled water, 3% KOH or Melzer's reagent. Duplicate specimens are deposited in BBH and SFSU.

DNA extraction, PCR and DNA sequencing

Collections used in this study for molecular analyses are listed in Table 1. Genomic DNA from herbarium specimens was isolated following the phenol/chloroform procedure by Lee and Taylor (1990). The crude DNA extracts were diluted up to 1000-fold with deionized water for use as PCR templates. PCR reactions were performed for two nuclear rDNA regions using the primer combinations ITS1-F-ITS4 (ITS region including the 5.8S gene) and LR0R-LR5 (nuc-18S). Sequences of primers used in this study have been described elsewhere (Vilgalys and Hester, 1990; White

Table 1. Isolate numbers, location, date, collector and accession numbers for fungal isolates sequenced for use in this study.

Species	Isolate	Origin	Date	leg./det.	GenBank accession numbers	
					ITS	nuc-lsu
<i>Chalciporus rubinellus</i>	191/81	U.S.A., ME	8/1981	W. Steglich	EU685111	EU685106
<i>Porphyrellus pseudoscaber</i>	RH8716	U.S.A., CA	11/15/2005	R. Halling	EU685112	EU685107
<i>Spongiforma thailandica</i>	DED7873	Thailand	7/7/2005	D. Desjardin	EU685113	EU685108
<i>Strobilomyces</i> sp.	RH4514	Australia	2/1992	R. Halling	---	EU685109
<i>Tylophilus atronicotianus</i>	Both s.n.	U.S.A., NY	9/18/2004	E. Both	EU685114	EU685110

et al., 1990; Gardes and Bruns, 1993). The amplifications were run in 35 cycles on a PTC-200 thermal cycler (MJ Research, Waltham, Massachusetts) using the following parameters: denaturation 94°C (1 min), annealing 50°C (45 sec), extension 72°C (1.5 min). PCR products were purified using Pellet Paint (Novagen, EMB Biosciences, San Diego, California). All PCR products were sequenced using BigDye terminator sequencing 3.1 chemistry (Applied Biosystems, Foster City, California), and run on an Applied Biosystems 3130 genetic analyzer. Contiguous sequences were assembled and edited using Sequencher 4.7 (GeneCodes Corp., Ann Arbor, Michigan). Automated alignments that were generated with ClustalX (Thompson *et al.*, 1997) were manually adjusted in MacClade 4.08 (Maddison and Maddison, 2005).

Datasets and phylogenetic analyses

Initial blast searches (Altschul *et al.*, 1997) using the ITS sequence of *S. thailandica* as a query produced exclusively hits in the Boletaceae, however, without providing significant results at the generic rank. The newly generated nuc-lsu rDNA data were first blasted and then, along with the best blast hits, aligned into the dataset of Binder and Hibbett (2006), which was narrowed down to 85 taxa after several rounds of consecutive bootstrapping and pruning of redundant sequences. The final alignment included 933 positions and the data were analyzed using maximum-parsimony (MP), maximum-likelihood (ML) and Bayesian methods. A non-parametric MP bootstrap analysis was performed using 1000 replicates in PAUP*4.0b10 (Swofford, 2002), all characters equally weighted, one random taxon addition sequence, and tree bisection

reconnection (TBR) branch swapping. RAxML v. 2.2.3 (Stamatakis, 2006) was used to run 100 ML replicates under the GTRMIX model and, in addition, 1000 ML bootstrap replicates under the GTRCAT model. A 50% majority-rule consensus tree was built from the resulting 1000 trees in PAUP* to estimate confidence values. Finally, posterior probabilities (PP) were determined in two independent analyses by running one cold and three incrementally heated Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analyses for 3×10^6 generations using MrBayes v3.0b4 (Ronquist and Huelsenbeck, 2003), saving trees every 100th generation. The GTR+ Γ +I model was specified as prior, assuming equal probability for all trees and unconstrained branch length. The final burn-in period determining the stationary proportion of trees saved after likelihood scores converged to a stable equilibrium was estimated using Tracer v1.4 (Rambaut and Drummond, 2007). A 50% majority-rule consensus tree was generated from the stationary trees in PAUP* to estimate confidence intervals. The results of the analyses are summarized in Fig. 1.

Results

Phylogenetic analyses

The complete ITS sequence obtained from *Spongiforma thailandica*, consisting of the two spacer regions and 5.8S gene, has a size of 682 bp. Blast searches using the entire ITS sequence and the three regions separately as queries produce only matches for the 5.8S gene, and place the species in the Boletaceae.

Inferences on the nuc-lsu dataset under parsimony, likelihood, and Bayesian methods yielded highly similar tree topologies,

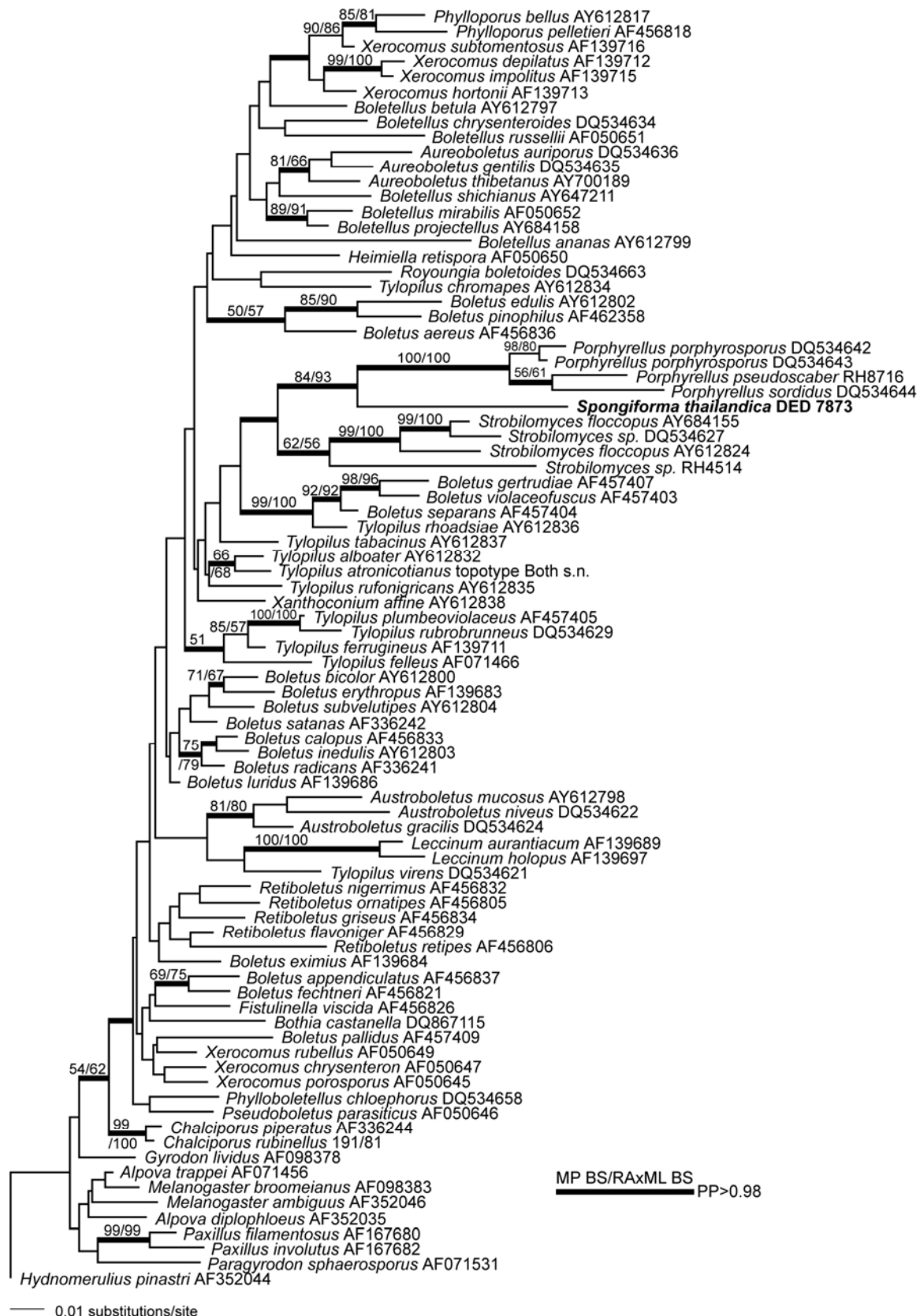


Fig. 1. Phylogenetic relationships of *Spongiforma* inferred from nuc-18S rDNA data using RAxML. One of 100 trees is shown here ($-\ln L = 10299.869$) and BS support values $> 50\%$ are indicated along nodes, while PP > 0.98 are marked as thickened branches. A GenBank accession number is provided for published sequences and the isolate number is provided for newly generated data.

suggesting that the new genus *Spongiforma* is the sister group of *Porphyrellus*. Long branches produced by species in both genera seem to obscure this relationship to some degree. Nevertheless, high statistical support received from three different methods suggests that this finding is based on a true phylogenetic signal and not a result of long-branch attraction. In addition, the independent Bayesian runs under a GTR+ Γ +I model, which was predetermined (Binder & Hibbett 2006) without testing the suitability of simpler models for this analysis, repeatedly and quickly converged after 230 000 generations. Thus, the clade including *Spongiforma* and *Porphyrellus* is consistently resolved in all analyses presented here (Fig. 1).

Taxonomy

Spongiforma Desjardin, Manf. Binder, Roekring & Flegel **gen. nov.**
MycoBank: MB 512541

Etymology: Referring to the sponge-like basidiomes.

Type species: *Spongiforma thailandica* Desjardin, Manf. Binder, Roekring & Flegel

Basidiomata epigaea, sessilia, cerebriformia. *Peridium* nulla. *Gleba* loculis labyrinthiformibus, 2-20 mm lata, brunneis vel rubrobrunneis; columella irregularis, pyriformis, cremea-alba. *Basidiospores* amygdaliformae, symmetricae, rugulosae, apiculatae cum poro, inamyloideae, cyanophileae. *Basidia* statismosporicae, 4-sporigera. *Cystidia* cylindrica vel ventricosa-rostrata. *Trama* glebae gelatinosae. *Fibulae* nulla.

Basidiomes epigeous, sessile, cerebriform to sponge-like with rubbery-gelatinous texture; *peridium* absent; *locules* 2-20 mm diam., irregular in outline, lined with a smooth, greyish orange to brown or reddish brown hymenium, sterile ridges white to cream-coloured; *columella* poorly developed, pyriform, cream-coloured, attached to white rhizomorphs. *Basidiospores* brown to vinaceous brown in mass, amygdaliform, bilaterally symmetrical, rugulose, with a central apiculus and small apical pore, reddish brown in water, violet grey in 3% KOH, inamyloid, cyanophilic. *Basidia* statismosporic, 4-sterigmate. *Cystidia* common on sterile locule edges, cylindrical to ventricose-rostrate, hyaline. *Tramal hyphae* gelatinous, inamyloid. *Clamp connections* absent.

Spongiforma thailandica Desjardin, Manf. Binder, Roekring & Flegel **sp. nov.**

(Fig. 2a-f)

MycoBank: MB 512542

Etymology: Referring to the country in which the species grows.

Basidiomata epigaea, sessilia, cerebriformia, 50-100 × 40-70 mm, globosa vel ovoidea. *Peridium* nulla. *Gleba* loculis labyrinthiformibus, 2-20 mm lata, brunneis vel rubrobrunneis; columella irregularis, pyriformis, cremea-alba. *Basidiospores* (9-)10-11.5(-12.5) × 5.5-7(-7.5) μ m, amygdaliformae, symmetricae, rugulosae, apiculatae cum poro, inamyloideae, cyanophileae. *Basidia* statismosporicae, 25-32 × 6.5-9.5 μ m, cylindrica vel subclavata, 4-sporigera, sterigmata usque 9.5 μ m. *Cystidia* 25-48 × 5-10 μ m, cylindrica vel ventricosa-rostrata. *Trama* glebae gelatinosae. *Fibulae* nulla. Holotypus hic designatus: Thailand, DED 7873 (BBH)

Basidiomes (Fig. 2a-b) epigeous, 50-100 mm diam × 40-70 mm tall, astipitate, irregularly globose to ovoid, cerebriform to sponge-like, rubbery-pliant (not brittle; can be squeezed like a sponge to remove water and it will spring back to its original shape). *Peridium* absent. *Hymenophore* composed of ridges or folds delimiting empty locules; locules 2-15(-20) mm diam, irregular in outline, lined with a well-developed hymenium, pale greyish orange (5B3-4) to brownish grey (6C3) when young, becoming light brown (6D4-5) to brown (6E5-8), dark brown (6F5-8) or reddish brown (8-9E6-8) at maturity; sterile ridges paler, white to cream when dried; all tissues immediately deep purple to purplish black in 10% KOH. *Spores* brown (6E5-6) to light brown (6D5-6) or vinaceous brown (8E-F6). *Columella* 10-15 mm tall × 8-10 mm diam (apex) × 3-4 mm diam (base), obconical to pyriform, apex lacunose, base folded, cream buff (4A3) to pale greyish orange (5B3); attached to fine, reticulate-branched, white rhizomorphs. Odor mild and pleasant when young, but soon becoming strong and unpleasant, of coal tar or burned rubber (like *Tricholoma sulphureum*), can be detected from 10 m distant. *Peridium* absent. *Sterile ridges* composed of erect chains of cells terminated by cystidia. *Cystidia* 25-48 × 5-10 μ m, cylindrical to subclavate or ventricose-rostrate, obtuse, hyaline or a few with pale brown cellular contents, inamyloid, thin-walled; abundant on the sterile locule edges and scattered amongst basidia near the

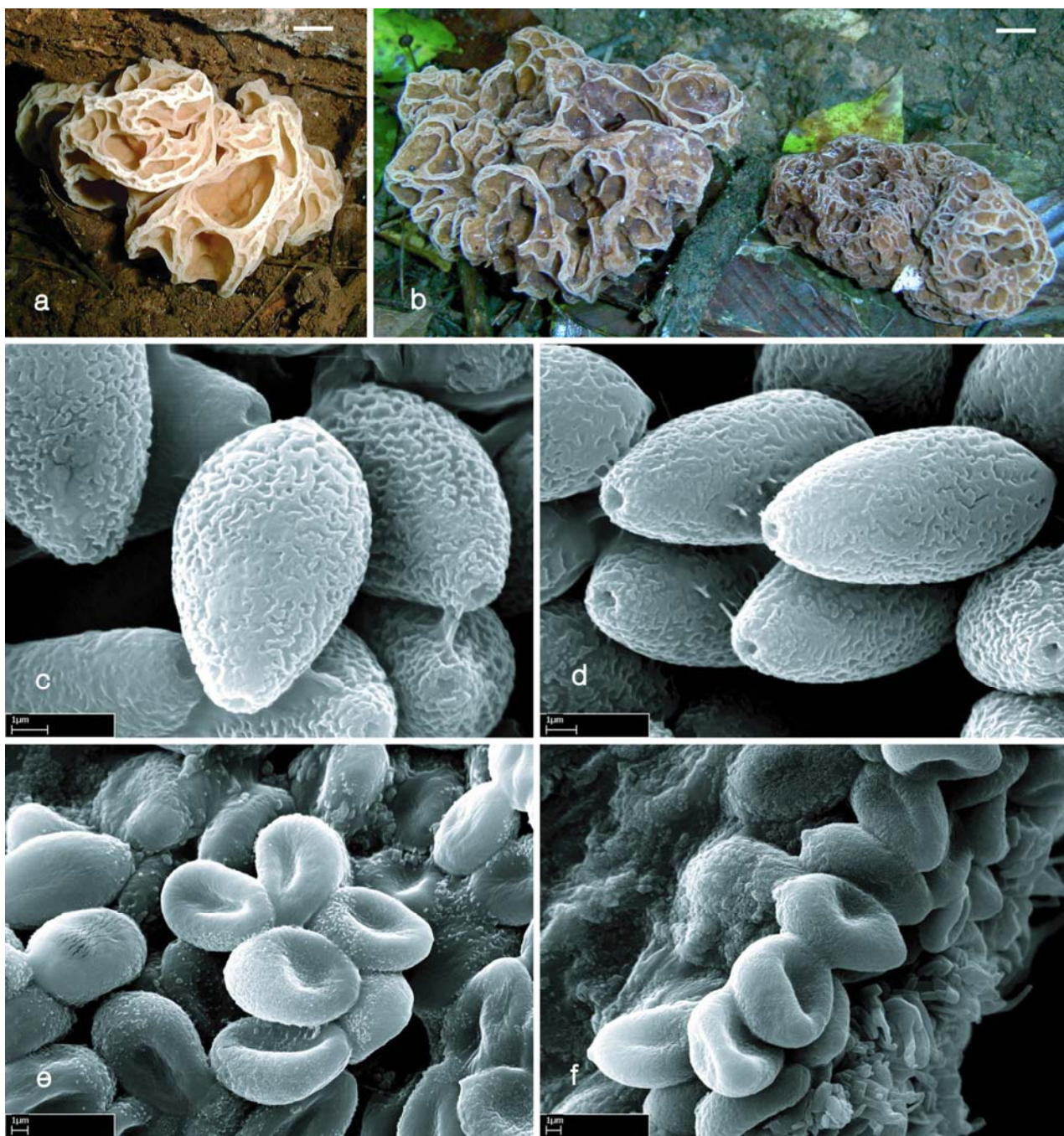


Fig. 2. *Spongiforma thailandica*. a-b. Basidiomes (DED 7485). c-d. SEM of air dried basidiospores (DED 7873, holotype). Note the distinct ornamentation and obvious apical pore. e-f. SEM of basidiospores mounted in 3% KOH (DED 7873, holotype). Note the loss of an apical pore and nearly smooth surface. — Scale bars: a-b = 10 mm; c-f = 1 μ m.

locule edges but absent elsewhere. *Hymenophoral trama* of subparallel to slightly interwoven hyphae 3–10 μ m diam, cylindrical, branched, septa often inflated (like an elbow joint), gelatinous, hyaline, inamyloid, thin-walled. *Subhymenium* of inflated to vesiculose cells 9–20 \times 9–14 μ m, hyaline, inamyloid, thin-walled, non-gelatinous. *Hymenial cystidia* absent (except as noted above). *Basidia* statismosporic, 25–32 \times 6.5–9.5 μ m, cylindrical to subclavate, 4-spored with straight

sterigmata up to 9.5 μ m long, unclamped. *Basidioles* cylindrical to subclavate. *Basidiospores* (Fig. 2c-f) (9–)10–11.5(–12.5) \times 5.5-7(–7.5) μ m [x_{mr} = 10.7–11.1 \times 5.9–6.5 μ m, x_{mm} = 11 \pm 0.3 \times 6.2 \pm 0.4, Q = 1.5–2.1, Q_{mr} = 1.7–1.8, Q_{mm} = 1.76 \pm 0.09, n = 20–25 spores per 2 specimens], amygdaliform, broadest at proximal end and gradually narrowed towards distal end, bilaterally symmetrical, with a small central apiculus, subtruncate at distal end and forming a tiny, narrow pore that may have a

small lip, walls thick ($-0.5\ \mu\text{m}$), thinner at the distal end, roughened to rugulose overall and reddish brown in water (Fig. 2c-d), appearing nearly smooth and violet grey in 3% KOH (Fig. 2e-f), inamyloid, cyanophilic. *Clamp connections* absent in all tissues.

Habit and habitat: Solitary, in soil under *Dipterocarpus* sp. and *Shorea* sp. in primary forest.

Known distribution: Thailand.

Material examined: THAILAND, Nakorn Nayok Province, Khao Yai National Park, Princess Trail ca 2 km from Visitor Center, N14°26.142', E101°23.080', elev. ca 750 m, 9 July 2002, E. Horak, T. Flegel and D.E. Desjardin as DED 7485 (BBH, SFSU); same location, 7 July 2005, D.E. Desjardin, DED 7873 (**holotype** BBH, **isotype** SFSU).

Notes: Diagnostic features of *Spongiforma thailandica* include the following: a relatively large, sponge-like and rubbery basidiome colored pale brownish grey to brown or reddish brown with irregular, relatively large locules lined with sporogenous tissue; no stipe but a small columella that is attached to copious, fine white rhizomorphs; a strong coal tar odor (like *Tricholoma sulphureum*); tissue that turns purple in 3-10% KOH; amygdaliform basidiospores with a tiny apical pore that are reddish brown and rugulose-roughened in water (Fig. 2c-d) but become violet grey, collapsed and nearly smooth in KOH (Fig. 2e-f); and the absence of clamp connections.

Discussion

The new Thai taxon is superficially similar to *Gymnopaxillus nudus* Claridge, Trappe & Castellano, a species described from material associated with *Eucalyptus* in Australia (Claridge *et al.*, 2001). In common with *Spongiforma*, *Gymnopaxillus nudus* forms astipitate, cerebriform, bright ferruginous basidiomes that lack a peridium and have relatively large locules, a basal columella, white rhizomorphs and cyanophilic, bilaterally symmetric basidiospores. However, the genus *Gymnopaxillus* forms smooth basidiospores that are ellipsoid to subfusoid, golden yellow in KOH and lack a tiny apical pore. In addition, *G. nudus* differs in forming primarily hypogeous basidiomes, forms a larger and more well-developed columella, lacks a strong odor, lacks a purple KOH reaction, has longer

basidiospores (11-16 μm), and is mycorrhizal with *Eucalyptus*. Molecular data indicate that *G. nudus* is allied with *Austropaxillus* species (Jarosch 2001) and together they are sister of *Serpula* in the Serpulaceae, all distantly related to *Spongiforma* (Binder and Hibbett, unpubl. data).

Our results drawn from the nuc-18S dataset indicate that *Spongiforma* belongs in the Boletaceae where it is sister of *Porphyrellus* (with 84% MP BS and 93% RAxML BS; PP = 1.0), although on a long branch. Together, this group forms the sister clade of *Strobilomyces* (Fig. 1), which is resolved in all analyses but is only supported by PP = 0.98. As noted in previous studies, ITS sequences of *Porphyrellus* and *Strobilomyces* spp. are highly divergent (Lutzoni *et al.*, 2004), and we were not able to align both taxa to the *S. thailandica* sequence or the latter sequence to any other taxa in the Boletaceae.

Bolete species with reddish brown to vinaceous brown, rugulose to perforate-punctate basidiospores have been placed historically in a number of taxa including *Boletus* subgen. *Tylopilus* (Corner, 1972), *Tylopilus* subgen. *Porphyrellus*, *Austroboletus* (both Wolfe, 1979), or *Porphyrellus* (Singer, 1945; Wolfe and Petersen, 1978). Two Southeast Asian species with basidiospore features very similar to those of *Spongiforma thailandica* are currently named *Austroboletus tristis* (Pat. & C.F. Baker) Wolfe and *A. longipes* (Masse) Wolfe. All three latter species form basidiospores with rugulose to rugulose-punctate surface that turn purple to violaceous grey in KOH. SEM photos of the type specimens of *A. tristis* and *A. longipes* (Figs 1, 2 and 4, respectively in Wolfe and Petersen, 1978) clearly show ornamentation similar to that of *S. thailandica* (Fig. 2c-d) although an apical pore was not reported on basidiospores of any *Austroboletus* species. Recently collected material of *A. longipes* from Malaysia confirms these observations (Chan & Halling, NYBG, pers. comm.). Sequences of *A. tristis* and *A. longipes* were not available for inclusion in our analyses. It is interesting to note, however, that in our analyses (Fig. 1), *Spongiforma* was more closely allied with the smooth-spored *Porphyrellus* (*P. porphyrosporus* (Fr.) E.-J. Gilbert, *P. pseudoscaber* Secr.

ex Singer, *P. sordidus* (Frost) Snell) than with the rugulose-punctate-spored *Austroboletus* (*A. mucosus* (Corner) Wolfe, *A. niveus* (G. Stev.) Wolfe, *A. gracilis* (Peck) Wolfe). An apical pore at the distal end of basidiospores is a rare feature in the boletes. Thiers (1975) reported the basidiospores of *Porphyrellus amylosporus* A.H. Sm. as dark reddish brown, smooth and truncate with a thin-walled depression. Several Old World species of *Heimioporus* (= *Heimiella*) form basidiospores with apical pores, such as *Heimiella* sp. 2 as illustrated in two SEM photographs by Watling and Hollands (Figs 11-12; 1989), and *H. fruticicola* (Berk.) E. Horak (R. Halling, NYBG, pers. comm.). In our molecular analyses (Fig. 1), *Heimioporus* is distantly related to *Spongiforma*. Another interesting similarity is that the pileipelli of *Austroboletus* and *Porphyrellus* species is a (ixo-) trichodermium formed from chains of short, cylindrical to submoniliform cells with cylindrical to clavate terminal cells. This anatomy is similar to that of the sterile edges of the tissues that delimit the locules in *Spongiforma*, which may represent vestigial pileipellis (peridium) tissue.

Spongiforma thailandica was first reported from Thailand as *Hymenogaster* sp. and illustrated with two color photographs in Ruksawong and Flegel (2001: 248).

Acknowledgments

This research was funded in part by National Science Foundations grants DEB-0118776 (PEET) to D.E. Desjardin and DEB-0444531 to M. Binder. We are indebted to Drs. Both, Halling and Steglich who provided materials for this study. Moreover, we thank Dr. Roy Halling (New York Botanical Garden) for discussions concerning generic concepts in the boletes and for providing descriptions and SEMs for comparison. A special thank you to the California Academy of Sciences, San Francisco, for allowing us to use their SEM facility and to Scott Serata (CAS) for producing the SEMs of basidiospores.

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