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Morphological and Molecular Studies of Selected *Termitomyces* Species Collected from 8 Districts of Kanchanaburi Province, Thailand

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Abstract

Kanchanaburi forests are well known for high diversity of *Termitomyces*, an un-culturable and economic mushroom in Thailand, but their systematics are limited and unorganized. We, therefore, identified 28 *Termitomyces* isolates collected from 8 districts in Kanchanaburi province based on morphological characteristics and ITS1-5.8S-ITS2 rDNA sequences. Nine species were identified as *T. albiceps*, *T. bulborhizus*, *T. cylindricus*, *T. heimii*, *T. microcarpus*, *T. radicatus*, *T. entolomoides*, *T. fuliginosus*, and *T. clypeatus*. Analysis of ITS1-5.8S-ITS2 rDNA sequences of these *Termitomyces* species revealed that morphological characteristics of *T. clypeatus* represented the most extremely variations that had not been described in any identification references. The inferred Neighbor-Joining phylogram of ITS1-5.8S-ITS2 rDNA sequences showed that 13 selected *Termitomyces* isolates were monophyletic and diverged into 2 clades with no common characteristic that can be shared in each clade. In addition, the phylogenetic study demonstrated the monophyletic tree from pure Kanchanaburi *Termitomyces* isolates and mixture of Asia and African *Termitomyces* samples implied that both Asia and African *Termitomyces* species have evolved from the same ancestor.

Keywords: classical classification, molecular systematic, morphological variation, phylogeny, *Termitomyces*

Introduction

In contrast to knowledge of Africa *Termitomyces*, information concerning systematics of Thailand *Termitomyces* species is limited and unorganized (De Fine Licht, et al. 2005; Heim, 1977; Pegler, 1977). Some diverse and novel forms of *Termitomyces* in Thailand have never been studied because locations for these mushrooms are kept secret by the local people due to the high value leading to the difficulty in *Termitomyces* taxonomic classification based on basidiocarp structures. Additionally, *Termitomyces* taxonomic classification based on few identification keys resulted in several synonyms of *Termitomyces* species found in Asia and Africa, but their morphological characteristics are quite different (Heim, 1977; Mossebo et al., 2002; Pegler, 1977; Tang et al., 2006; Wei et al., 2004; Wei et al., 2006).

Eighteen species of Thai *Termitomyces* were identified and reported using previously established keys (Bels and Pataragetvit, 1982; Pitchayangkul, 1998; The Royal Institute, 1996). Kosakul et al. (2007) described 20 *Termitomyces* species collected from the central region of Thailand, and determined the genetic diversity of *T. auranticus*, *T. entolomoides*, *T. heimii*, *T. clypeatus* and *T. cylindricus* using isozyme markers.

Sa-uangsub (2003) reported 4 species of fungus-growing termites found in mixed deciduous forest, Kanchanaburi. These termites were *Macrotermes carbonarius* Hagen, *M. gilvus* Hagen, *M. annandalei* Silvestri and *Hypotermes makhamensis* Ahmad. Among the 4 termite species, *H. makhamensis* and *M. gilvus* are highly prevalent. In general, 5 termite genera, *Ancistrotermes, Hypotermes, Macrotermes*, *Microtermes* and *Odontotermes*, were found to be symbiotic with 8 *Termitomyces* species namely *T. aurantiacus*, *T. cylindricus, T. clypeatus, T. fuliginosus, T. globules, T. microcarpus*, *T. striatus* and *Sinotermitomyces* sp. (Sornnuwat and Thienhirun, 2005). In addition, *T. clypeatus* can be isolated from several combs of fungus-growing termites such as *A. pakistanicus* Ahmad, *H. makhamensis* and *Macrotermes gilvus*.

The number of correctly identified *Termitomyces* species is unclear due to the problems previously mentioned even though house-keeping genes, such as ribosomal RNA gene, are now being used by mycologists to aid in constructing African *Termitomyces* taxonomy (Aanen et al., 2002; Aanen and Boomsma, 2005; Aanen and Eggleton, 2005; De Fine Licht et al., 2007; Froslev et al., 2003; Rouland-Lefevre et al., 2002). Only few presumptive molecular phylogeny data of *Termitomyces* species and their hosts are available in Thailand. Taprab et al. (2002) classified *Termitomyces* species from different districts of Thailand into 8 groups according to clades based on internal transcribed sequences (ITS) of nodules without description of morphological features.

Kanchanaburi forests are located in the western region of Thailand. These areas are well known for high diversity of *Termitomyces* species, unfortunately, there is a need to understand the taxonomy and molecular phylogeny of this mushroom. The objectives of this study are to combine morphological classification with molecular studies to solve the taxonomic problem of *Termitomyces* species in 8 districts of Kanchanaburi, and to study their evolutionary relationships based on sequences of internal transcribed spaces (ITS).

Materials and Methods

Sample Collection, Growth and Storage

Twenty-eight samples of *Termitomyces* mushrooms, 15 termite hosts and fungus combs were collected from 8 districts in Kanchanaburi province, Thailand as showed in Table 1. Pure cultures and dried specimens of *Termitomyces* were stored at the culture collection of Mahidol University, Kanchanaburi campus, Thailand. The inner tissues of *Termitomyces* mushroom stipe were removed using sterile forceps and stored at -20°C for DNA extraction. Each mushroom stipe tissue and single spore was cultivated on potato-dextrose-agar (PDA, Pronadisa S.A, Spain) at room temperature. Termites were removed from fungus combs and stored at -20°C for morphological identification.

Morphological Identification of *Termitomyces* and Its Termite Hosts

Twenty-eight *Termitomyces* isolates and 15 termite hosts were identified based on their morphological features. Photographs of mushrooms were recorded by a Canon IXUS 800 IS Digital Camera (Japan). The morphological characteristics including basidiocarps, caps, stalks and fifty basidiospores of 28 *Termitomyces* isolates were measured under a bright field microscope (Olympus, Japan) and recorded. Each sample was identified at the species level based on the morphological identification keys (Heim, 1977; Pegler and Vanhaecke, 1994; Pitchayangkul, 1998; Tang et al., 2006; Wei et al., 2004).

For the termite hosts, morphological features such as head, labrum, mandible and antenna of 15 termite soldiers were examined and measured under a bright field microscope (Olympus, Japan). Photo of termites were taken by a Canon IXUS 800 IS Digital Camera (Japan). Each sample was identified at the species level based on identification key of Ahmad (1965).

Table 1 List of *Termitomyces* isolates used in this study.

Isolate name	Identified molecular species	Identified morphology species	Identified termite host	Date of collection	Geographical origin and habitat	GenBank accession numbers
69/46	*T. albiceps*	*T. albiceps*	ND	4 October 2003	Lao Khwan district	HM230658**
1/51	*T. bulborhizus*	*T. bulborhizus*	*Hypotermes makhamensis*	26 June 2008	Sai Yok district	HM230663**
80/46	*T. clypeatus*	*T. robustus*	ND	4 October 2003	Maung district	EF091680
98/46		*T. robustus*	ND	7 October 2003	Srisawat district	EF091681
169/46		*T. robustus*	ND	13 October 2003	Sai Yok district	EF091687
217/46		*T. albiceps*	ND	27 October 2003	Phanom thuan district	EF091688
13/48		*T. clypeatus*	*Macrotermes gilvus*	20 July 2005	Sai Yok district	HQ702545
25/49		*T. fuliginosus*	*H. makhamensis*	21 October 2006	Sai Yok district, the teak forest, under the teak trees	HQ702547
26/49		*T. fuliginosus*	*H. makhamensis*	21 October 2006	Sai Yok district, the teak forest, under the teak trees	GU967667
27/49		*T. fuliginosus*	*H. makhamensis*	21 October 2006	Sai Yok district, the teak forest, under the teak trees	GU967668
18/50		*T. fuliginosus*	*H. makhamensis*	20 October 2007	Sai Yok district, the teak forest, under the teak trees	GU573958**
20/50		*T. fuliginosus*	*H. makhamensis*	20 October 2007	Sai Yok district, the sugar apple garden, under the death mango trees	HQ702552
5/50z1		*T. clypeatus*	*M. gilvus*	10 April 2007	Sai Yok district, the mixed tree garden	HQ702548
5/50z2		*T. clypeatus*	*M. gilvus*	10 April 2007	Sai Yok district, the mixed tree garden	HQ702549
5/50z4		*T. clypeatus*	*M. gilvus*	10 April 2007	Sai Yok district, the mixed tree garden	HQ702550
5/50z5		*T. clypeatus*	*M. gilvus*	10 April 2007	Sai Yok district, the mixed tree garden	HQ702551
19/50		*T. clypeatus*	*H. makhamensis*	20 October 2007	Sai Yok district, the sugar apple garden, under the sugar apple tree	FJ147329**
3/51		*T. clypeatus*	*H. makhamensis*	16 October 2008	Mahidol university, Kanchanaburi campus, Sai Yok district	GU967669
4/51		*T. clypeatus*	ND	16 October 2008	Sai Yok district	GU967670**
7/51		*T. clypeatus*	*H. makhamensis*	4 November 2008	Mahidol university, Kanchanaburi campus, Sai Yok district	GU967671
106/46	*T. cylindricus*	*T. cylindricus*	ND	8 October 2003	Mahidol university, Kanchanaburi campus, Sai Yok district, Lawn	EF091682**
67/46	*T. entolomoides*	*T. entolomoides*	ND	4 October 2003	Sangkhlaburi district	EF091679**
110/46		*T. entolomoides*	ND	9 October 2003	Srinakarin Dam, Srisawat district	EF091683**
38/46	*T. fuliginosus*	*T. cylindricus*	ND	15 September 2003	Mahidol university, Kanchanaburi campus, Sai Yok district , straw mushroom (*Volvariella volvacea*) field	EF091678**
97/46		*T. fuliginosus*	ND	7 October 2003	Srisawat district	HM230659**
204/46	*T. heimii*	*T. heimii*	ND	22 October 2003	Thong pha phum district, The teak forest	HM230662**
195/46	*T. microcarpus*	*T. microcarpus*	ND	16 October 2003	Sai Yok district, Plant debris	HM230661**
164/46	*T. radicatus*	*T. entolomoides*	ND	12 October 2003	Nongprue district	HM230660**

* ND = Not determine, ** ITS sequences were used for a phylogenetic reconstruction.

Sequence Analysis of *Termitomyces* ITS1-5.8S-ITS2 Regions

DNA was isolated from stipe inner tissues of *Termitomyces* basidiocarp using the method of Lee and Taylor (1990). The internal transcribed spacer (ITS) 1 and 2 regions of 28 *Termitomyces* isolates were amplified using two pairs of universal primers. Thirteen samples collected in year 2003 were amplified using primer ITS4 and ITS5 (White et al., 1990) while the samples collected after 2003 were amplified using primers NSA3 and NLC2 (Martin and Rygiewicz, 2005).

The polymerase chain reactions (PCR) using the primers ITS4 and ITS5 were performed in 50-µL volume containing 4 µL template DNA (50 ng μL^{-1}), 4 µL dNTP mixture (2 mM each), 2 µL of each primer (25 µM), 5 µL of 10X *Taq* buffer and 0.2 µL i-Taq DNA polymerase (5 unit μL^{-1}). The PCR reaction was performed with the program modified from Kong et al. (2003). An initial denaturation and enzyme activation step of 2 min at 96°C was followed by 35 cycles at the following conditions: 30s at 96°C, 45s at 55°C and 1 min at 72°C. A final 10 min extension at 72°C completed the protocol. The PCR of the primers NSA3 and NLC2 were conducted in 50-µL volume containing 4 µL template DNA (50 ng μL^{-1}), 5 µL dNTP mixture (2 mM each), 0.4 µL of each primer (25 µM), 5 µL of 10X *Taq* buffer and 0.2 µL of i-Taq DNA polymerase (5 unit μL^{-1}). DNA was amplified as described by Martin and Rygiewicz (2005). The PCR amplifications were conducted in a Mastercycle Eppendorf thermocycler (Eppendorf, Germany). PCR products were sequenced by Macrogen (Korea). ITS sequences were compared with other fungal sequences using BLAST 2.1 search (http://www.ncbi.nlm.nih.gov/BLAST).

Molecular Phylogenic Analysis of ITS1-5.8S-ITS2 Sequences

To study molecular phylogeny of *Termitomyces* species in Kanchanaburi, 13 *Termitomyces* strains isolated from Kanchanaburi were used as terminal taxa for an inferred phylogenetic tree reconstruction (Table 1). These 13 selected strains of 9 *Termitomyces* species included *T. albiceps* (69/46), *T. bulborhizus* (1/51), *T. clypeatus* (18/50, 19/50 and 4/51), *T. cylindricus* (106/46), *T. entolomoides* (67/46 and 110/46), *T. fuliginosus* (38/46 and 97/46), *T. heimii* (204/46), *T. microcarpus* (195/46) and *T. radicatus* (164/46) (Table 1). Outgroup taxa that retrieved from GenBank were *Tricholoma portentosum* (AF241517) and *Clytocibe lateritia* (U66431). The ITS sequences of 15 taxa for phylogenetic tree reconstructions had aligned 651 characters including gap for inserting/deleting (indel) positions and missing data. To study evolutionary relationships between Asian and African *Termitomyces*, the ITS sequences of 13 *Termitomyces* strains isolated from Kanchanaburi and 6 *Termitomyces* species retrieved from GenBank were aligned with the same 2 outgroup taxa by the ClustalX program. These six GenBank *Termitomyces* species were *T. eurhizus* (AF321366), *T. fuliginosus* (AF321374), *T. medius* (AF321365), *T. striatus* (AF321367) (Rouland-Lefevre et al., 2002), *T. heimii* (AF357022) and *T. microcarpus* (AF357023) (Hofstetter et al., 2002). Four species of *T. eurhizus, T. fuliginosus, T. medius* and *T. striatus* were isolated from African. Original geographic locations of *T. microcarpus* and *T. heimeii* were unknown. Both *T. microcarpus* and *T. heimeii* were collected by mycologists at the Duke University, USA. Total of 651 characters including gap for indel positions and missing data were used as matrix data to build a phylogenetic tree.

All three methods of Distance, Maximum Likelihood and Parsimony measurements were used to re-built inferred phylogenetic trees with the PAUP 4.0 beta 10 program (Swofford, 2000). Neighbor-joining setting was used for searching inferred phylogenetic trees. For the distance method, Kimura 2-parameter was set for inferred phylogenetic trees. For the Likelihood method the settings correspond to the HKY85 model that were: number of substitution types=2 (HKY85variant), transition/transversion ratio=2 (kappa=4.0381717), Assumed nucleotide frequencies (empirical frequencies): A=0.22645, C=0.21557, G=0.22725, and T=0.33072. Among-site rate variation, proportion of invariable sites was assumed as zero, and distribution of rates at variable sites was equal. To confirm reliability of each clade in inferred phylogenetic trees, the Bootstrap method was used with a neighbor-joining search. Number of bootstrap replicates was 1000. The TREEVIEW program version 1.6.6 provided by Institute of Biomedical and Life Sciences, University of Glasglow, Scotland, UK was used to demonstrate the inferred phylogenetic trees.

Results

Taxonomic Description of *Termitomyces* Collected from Kanchanaburi

Based on morphological characteristics, twenty-eight *Termitomyces* samples were identified to 9 species as shown in Table 1 and described as following.

***Termitomyces albiceps* S. C. He (Synonym of *T. eurhizus* R. Heim)**

Morphological characteristics of *Termitomyces* isolate 69/46 and 217/46 were same as the description of *T. albiceps* described by Pegler and Vanhaecke (1994) and Tang et al. (2006); *Pileus* 5.8-10.5 cm diameter, applanate with a round perforatorium; surface brown at centre, elsewhere yellowish brown and paling toward margin, radially striate; margin straight, radially splitting. *Lamellae* free, 6 mm wide, greyish white; crowded, with lamellulae. *Stipe* 7.5-13 cm long, 2-5 cm thick, central, cylindrical and thickening slightly at ground level; surface greyish to brownish, smooth; solid and fibrous. *Pseudorhiza* 5-10 cm long, tapering, terminating with pale yellowish sclerotic disk; surface dark brown in upper part and black below, longitudinally striate. *Partial veil* absent. *Spore deposit* ivory. *Basidiospores* 5.5-6.6 ×4-4.5 µm, ovoid to ellipsoid. *Basidia* 16-20.5×6.5-7 µm, clavate, tetrasporic.

***Termitomyces bulborhizus* T.Z. Wei**

Morphological characteristics of the *Termitomyces* isolate 1/51 matched with the description of *T. bulborhizus*, a recently reported novel species from China (Wei et al., 2004); *Pileus* 9.2-21 cm diameter, at first convex then expanding to convexo-applanate, broadly round or blunt pointed perforatorium; surface dark brown at the centre, elsewhere pale brown to brown, paling towards the margin; margin straight or incurved. *Lamellae* free, white, to 9 mm wide, crowded, with lamellulae. *Stipe* 5-9 cm long above ground, 6-8 cm thick, enlarged to 8.7-14.5 cm diameter at ground level and frequently forming a large globosely bulb below ground, solid, robust, fibrous; surface white above and pale brown on the bulb; scabrous above ground level. *Partial veil* absent. *Pseudorhiza* 1-4 cm long, tapering towards the base; surface white to pale brown, with longitudinal grooves and cracks. *Spore deposit* ivory. *Basidiospores* 5-8.5×3.5-6 mm, ovoid to ellipsoid, sub-hyaline, thin-walled. *Basidia* 16-25×4-9 mm, clavate, tetrasporic.

***Termitomyce clypeatus* R. Heim**

Basidiocarps of the *Termitomyces* isolates 5/50 (zone 1, 2, 4, and 5), 19/50, 3/51, 4/51 and 7/51 were same as the description of *T. clypeatus* described by Heim (1977) and Pegler and Vanhaecke (1994); *Pileus* 6.5-8 cm diameter, at first pointed-conical, later expanding to convex with a prominent dark spiniform perforatorium, surface orchraceous brown becoming paler towards the margin, fibrillose silky, margin incurved, and irregularly lobed . *Stipe* 10-6.6 cm long, 1.5-3 cm thick, solid, cylindrical then swollen slightly at the base before developing a long tapering pseudorrhiza, 3-8 cm long and 0.2-1 cm thick, longitudinally fibrillose. *Spore deposit* ivory. *Basidiospore* 2.5-6.3×5-8.8 µm, *Basidia* 19×4-6 µm, clavate, tetrasporic.

***Termitomyces cylindricus* S.C. He (Synonym of *T. aurantiacus* R. Heim)**

Basidiocarps of the *Termitomyces* isolates 38/46 and 106/46 were same as the description of *T. cylindricus* described by Pegler and Vanhaecke (1994) and Tang et al. (2006); *Pileus* up 3.6-5.8 cm diameter, conical-applanate, with a small and bluntly pointed perforatoium; surface brown to blackish and darker at the centre, glabrous; margin straight, radially striate and splitting. *Lamellae* free, 3-4 mm wide, greyish white; crowded, with lamellulae. *Stipe* 2.5-6.5×2.1-4.2 cm, central, cylindrical and slightly thickened at ground level; surface greyish white to brownish, smooth and glabrous; solid. *Pseudorhiza* 2-8 cm, tapering downward from ground level, cylindrical base; surface brownish; solid. *Partial veil* absent. *Spore deposit* ivory. *Basidiospores* 6.6-7.7×4.5-5 µm, ovoid to ellipsoid, thin-walled and subhyaline. *Basidia* 17-22×6-7.5 µm, clavate, tetrasporic, thin-walled and subhyaline.

Termitomyces entolomoides R. Heim

Morphological characteristics of *Termitomyces* isolates 67/46, 110/46 and 164/46 matched with description of *Termitomyces entolomoides* (Heim, 1977; Pegler and Vanhaecke, 1994); *Pileus* 3.2-5.1 cm diameter, obtusely conical to mucronate, finally expanding with a reflexed margin, with and ridged, pointed perforatorium; surface blackish gray with bluish tints, glabrous, finely striate, and rimose. *Lamellae* ivory, up to 8 mm wide, crowded, with lamellulae. *Stipe* 2.5-5 cm long, 1.8-4.5 cm thick, cylindrical but abruptly swollen at ground level, solid; surface white, fibrous-scaly, ridged. *Pseudorhiza* 2-6.5 cm long, tapering downward, grey. *Partial veil* absent. *Spore deposit* ivory. *Basidiospores* 5.7-7 ×3.2-4.2 µm, ovo-ellipsoid, hyaline, with granular content. *Basidia* 22-35×7-8 µm, elongate-clavate, tetrasporic.

Termitomyces fuliginosus R. Heim

Morphological characteristics of the *Termitomyces* isolates 97/46, 25/49, 26/49, 27/49, 18/50, and 20/50 were corresponded with the *T. fuliginosus*' description (Heim, 1977); *Pileus* 10-20 cm diameter, conical then convex or convexo-applanate; surface grayish brown to yellow brown, perforatorium spiniform, radially fibrillose, sometimes radially striate; margin finally lacerate. *Lamellae* free, white, crowded. *Stipe* 6-11 cm long, 1.5-5.5 cm thick, solid, cylindrical or slightly tapering downward; surface white above and pale brown on the bulb, fibrous. *Partial veil* absent. *Pseudorhiza* 10-21 cm long, terminating with sclerotic disk. *Spore deposit* ivory. *Spore deposit* ivory. *Basidiospores* 4-10×2-8 µm, ovoid to sub-cylindrical, hyaline, smooth. *Basidia* 26-34×5-9 µm, clavate, tetrasporic.

Termitomyces heimii Narajan

Morphological characteristics of the *Termitomyces* isolate 204/46 matched the description of *T. heimii* (Pegler and Vanhaecke, 1994); *Pileus* 4-8 cm diameter, convex to plano-convex with a broad, umbronate perforatorium, surface silky white, scarcely striate, smooth, at the centre gray becoming brownish, margin at first incurved, later fissile. *Lamelle* free, white, crowed, with lamellulae of two lengths, edge entire, of the splitting. *Stipe* 5-9× 1.5-2 cm, cylindrical, solid, surface white, scabrous below the annulus, smooth-striate elsewhere. *Pseudorhiza* 7-36 cm long, white, smooth, hollow. *Partial veil* fleshy, persistent, double, attached to upper quarter of stipe. *Basidiospores* 5-7×3.5-4.5 µm, ovoid to ellipsoid, a slightly thickened wall, smooth, imamyloid. *Basidia* 15-22×6-7 µm, clavate, tetrasporic.

Termitomyces microcarpus R. Heim

Morphological characteristics of the *Termitomyces* isolate 195/46 were corresponded with the description of *T. microcarpus* (Heim, 1977; Pegler and Vanhaecke, 1994); *Pileus* 0.5-1cm diameter, campanulate to convex then expanding, often umbronate with a papillate projection, upper surface whitish to cream, darkening at the centre, dry, glabous. Margin incurved or straight, entire to lobed. *Lamelae* subfree to adnexed, thin, white, 1-2 mm wide, modulately crowed with few lamellulae. *Stipe* 2.5-4.5 cm×1-3 mm, slender, solid, cylindrical, surface whitish, fibrous, smooth, glabrous. *Partial veil* absent. *Spore deposit* ivory. *Basidiospore* 6-8.5×3.7-4.8 µm, ovoid to ellipsoid, hyaline, inamyloid, thin-walled, with one or more refractive guttules, *Basidia* 20-30×6-8 µm, clavate, tetrasporic.

Termitomyces robustus R. Heim

Morphological characteristics of the *Termitomyces* isolates 80/46, 98/46 and 169/46 matched the description of *T. robustus* (Heim, 1977); *Pileus* 7-11 cm diameter, at first conical then expanding to convex but retaining a prominant, pointed to obtuse perforatorium, surface grayish brown to dark brown with scrobiculate, irregular lobe. *Lamellae* free, ivory 5-6 mm wide. *Stipe* 6-8 cm long, 3-7 cm thick, white, solid, central, cylindrical slightly tapering downward. *Spore deposit* ivory. *Basidiospore* 4-8×3-9 µm ovoid to sub-cylindrical.

Termite Morphological Identification

Based on morphological identification, termite soldiers associated with 15 *Termitomyces* isolates were classified into two species, which were *Macrotermes gilvus* and *Hypotermes makhamensis*. Termite hosts of *Termitomyces* isolate 13/48, 14/48 and 5/50 (zones 1, 2, 4 and 5) matched characteristics of *M. gilvus*, while termite hosts of *Termitomyces* isolate 25/49, 26/49, 18/50, 19/50, 20/50, 1/51, 3/51 and 7/51 were recognized as *H. makhamensis* (Table 1).

Termitomyces ITS1-5.8S-ITS2 Sequences Analysis

Primers NSA3 and NLC2 contributed about 996-1095 bp of PCR products covering partial small subunit (SSU) rDNA, complete ITS1-5.8S-ITS2 regions, and partial large subunit (LSU) rDNA regions. The blast search results using NCBI database confirmed that all 28 *Termitomyces* sequences were classified as *Termitomyces* genus by virtue of 83-100% identity. Based on positions of partial SSU rDNA -ITS1-5.8S-ITS2-LSU rDNA sequence of *Termitomyces* sp. Group 6 submitted in the Genbank by Taprab et al. (2002), the complete ITS1-5.8S-ITS2 regions of 28 *Termitomyces* isolates had 509-734 bp that comprised of 139-256 bp of ITS1, 158-161 bp of 5.8S, and 191-317 bp of ITS2. The SSU and LSU rDNA regions were 230-250 bp. The end of SSU rDNA regions demonstrated two kinds of nucleotides which were CATTA and TTTTG. The conserved nucleotides of 5.8S always started with TACAA and frequently ended with CATTA. Only *T. entolomoides* isolate 110/46 showed the deletion of CA at the conserved CATTA region of the 5.8S ending. The conserved nucleotides of LSU rDNA region frequently started with TTGACC.

Based on ITS1-5.8S-ITS2 rDNA sequences, 9 species of *Termitomyces* were identified as *T. albiceps* (69/46), *T. bulborhizus* (1/51), *T. clypeatus* (80/46, 98/46, 169/46, 217/46, 13/48, 25/49, 26/49, 27/49, 5/50 (zone 1, 2, 4 and 5), 18/50, 19/50, 20/50, 3/51, 4/51 and 7/51), *T. cylindricus* (106/46), *T. entolomoides* (67/46 and 110/46), *T. fuliginosus* (38/46 and 97/46), *T. heimii* (204/46), *T. microcarpus* (195/46) and *T. radicatus* (164/46) (Table 1). Eighteen ITS1-5.8S-ITS2 rDNA sequences of isolates 80/46, 98/46, 169/46, 217/46, 13/48, 25/49, 26/49, 27/49, 5/50 (zone 1, 2, 4 and 5), 18/50, 19/50, 20/50, 3/51, 4/51 and 7/51 that were morphologically identified as different species had 98-100% identity. Consequently, all members of this group should be classified as a species *T. clypeatus* with a variation of its morphological characteristic as shown in Figure 1.

Molecular Phylogeny Analysis of *Termitomyces* ITS1-5.8S-ITS2 Sequences

Phylogenetic analysis of ITS1-5.8S-ITS2 sequences of 13 Kanchanaburi *Termitomyces* species using Distance, Maximum likelihood and Parsimony measurements gave the similar inferred phylogenetic trees. The inferred maximum likelihood cladogram that branched into 2 clades, clade A and clade B, was shown in Figure 2. Thirteen taxa of Kanchanaburi *Termitomyces* formed a well-supported monophyletic group in the cladogram with bootstrap value of 98%.

Clade A comprised of *T. albiceps* (69/46), *T. cylindricus* (106/46), *T. entolomoides* (67/46 and 110/46), *T. fuliginosus* (38/46 and 97/46), *T. heimii* (204/46) and *T. microcarpus* (195/46). Two isolates of *T. entolomoides* (67/46 and 110/46) grouped together with a bootstrap value of 100% (Figure 2, clade A). They represented the recent ancestor of clade A. *T. entolomoides* is the smallest *Termitomyces* specie that has the bluish black pileus. Two isolates of *T. fuliginosus* (38/46 and 97/46) formed a well-supported grouped (bootstrap 100%). The isolate of *T. heimii* (204/46) was a sister group of *T. albiceps* (69/46) with a bootstrap value of 92%. The result of pairwise alignment showed 92.7% identity supporting their closely clades. The morphology of *T. heimii* (204/46) and *T. albiceps* (69/46) were the large basidiocarp with the persistent annulus and the large basidiocarp lacking annulus at all stages, respectively. The isolate of *T. microcarpus* (195/46), the lacking pseudorhizae *Termitomyces*, grouped with *T. cylindricus* (106/46), but their identity based on the pairwise alignment was 88.2%.

All three samples of *T. clypeatus* (18/50, 19/50 and 4/51) as well as *T. bulborhizus* (1/51) and *T. radicatus* (164/46) belonged to clade B with well-supported group with a bootstrap value of 98% (Figure 2, clade B). The isolate of *T. bulborhizus* (1/51) was a recent ancestor of clade B with a bootstrap value of 100%. Three *T. clypeatus* isolates (18/50, 19/50 and 4/51) with different morphological characteristics had identical sequences and formed a well-supported sub-group within clade B (bootstrap 97%). These *T. clypeatus* nested with *T. radicatus* (164/46) with a bootstrap value of 96%. The result of pairwise alignment demonstrated 89.2 % identity while the result of

Figure 1 The morphological variation of *Termitomyces clypeatus* basidiocarps: A) isolate 80/46, B) isolate 98/46, C) isolate 169/46, D) isolate 217/46, E) isolate 25/49, F) isolate 26/49, G) isolate 27/49, H) isolate 5/50 zone 1, I) isolate 5/50 zone 2, J) isolate 5/50 zone 3, K) isolate 5/50 zone 4, L) isolate 18/50, M) isolate 19/50, N) isolate 20/50, O) isolate 3/51, P) isolate 4/51.

sequences comparison between *T. clypeatus* (18/50, 19/50 and 4/51) and *T. bulborhizus* (1/51) showed only 58.4% identity. Figure 3 showed an evolutionary relationships between Asian and African *Termitomyces* reconstructed from the ITS sequences of 13 *Termitomyces* strains isolated from Kanchanaburi and six *Termitomyces* species retrieved from GenBank. Those six *Termitomyces* species (*T. eurhizus* (AF321366), *T. fuliginosus* (AF321374), *T. heimii* (AF357022), *T. medius* (AF321365), *T. microcarpus* (AF357023) and *T. striatus* (AF321367)) clustered into clade A that was mentioned previously whereas clade B composed of same species of Kanchanaburi *Termitomyces* which were *T. bulborhizus* (1/51), *T. clypeatus* (18/50, 19/50 and 4/51) and *T. radicatus* (164/46).

Clade A could be divided into 2 minor clades which were A1 and A2 by excluding the isolates of *T. albiceps* 69/46 and *T. entolomoides* (67/46 and 110/46) that did not group with any *Termitomyces* species. Clade A1 comprised of *T. cylindricus* (106/46), *T. heimii* (204/46 and AF357022) and *T. microcarpus* (195/46 and AF357023). The isolate of *T. heimii* (204/46) collected from Kanchanaburi was a sister group of *T. heimii* (AF357022) with 97% identity of ITS sequences that well supported 100% bootstrap value. Their result of pairwise alignment revealed 97% identity. The isolate of *T. microcarpus* (195/46) collected from Kanchanaburi nested with *T. microcarpus* (AF357023), but their ITS sequence similarity showed 87%.

All four isolates of African *Termitomyces* species and 2 isolates of Kanchanaburi *Termitomyces* formed a sub-group within group A with a well-supported bootstrap value of 91%. This group was assigned as clade A2 (Figure 3, clade A2). The *T. striatus* (AF321367) and *T. eurhizus* (AF321366)

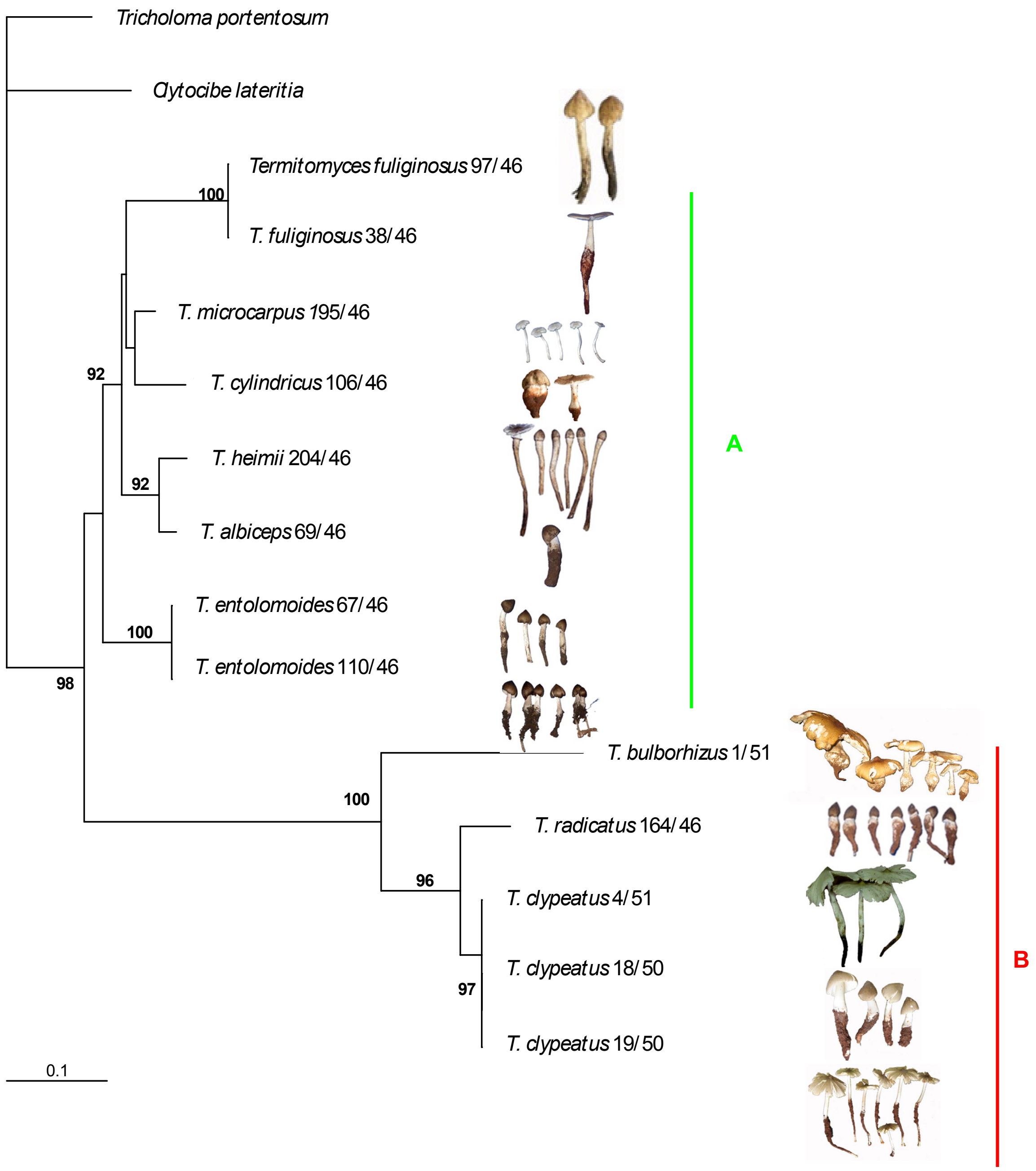

Figure 2 The inferred maximum likely hood cladogram based on neighbor-joining searching criteria of the ITS1-5.8S-ITS2 sequences (690 bp) from 13 selected strains of 9 *Termitomyces* species collected from Kanchanaburi. The outgroup taxa were *Tricholoma portentosum* (AF241517) and *Clytocibe lateritia* (U66431). Bootstrap values more than 50% were showed as bold number at each node. Scale bar represents branch length corresponding to different nucleotides at 10%. Morphological characteristics were illustrated at each species.

isolates grouped together with a bootstrap value of 99%. These 2 species were collected from Congo, Africa. Their result of pairwise alignment showed 96% identity. The *Termitomyces* sp. AS1 nested with the isolates of Kanchanaburi *T. fuliginosus* (38/46 and 97/46) with a bootstrap value of 79%. *T. fuliginosus* of Kanchanaburi and African *Termitomyces* sp. AG1 (AF321374) were in the same A2 clade but not grouped together. It is notably that 2 African *Termitomyces* species, *T. fuliginosus* AF321374 and *T. medius* AF321365, mention by Rouland-Lefevre et al. (2002) were recently revised their species names to *Termitomyces* sp. AG1 and *Termitomyces* sp. AS1, respectively.

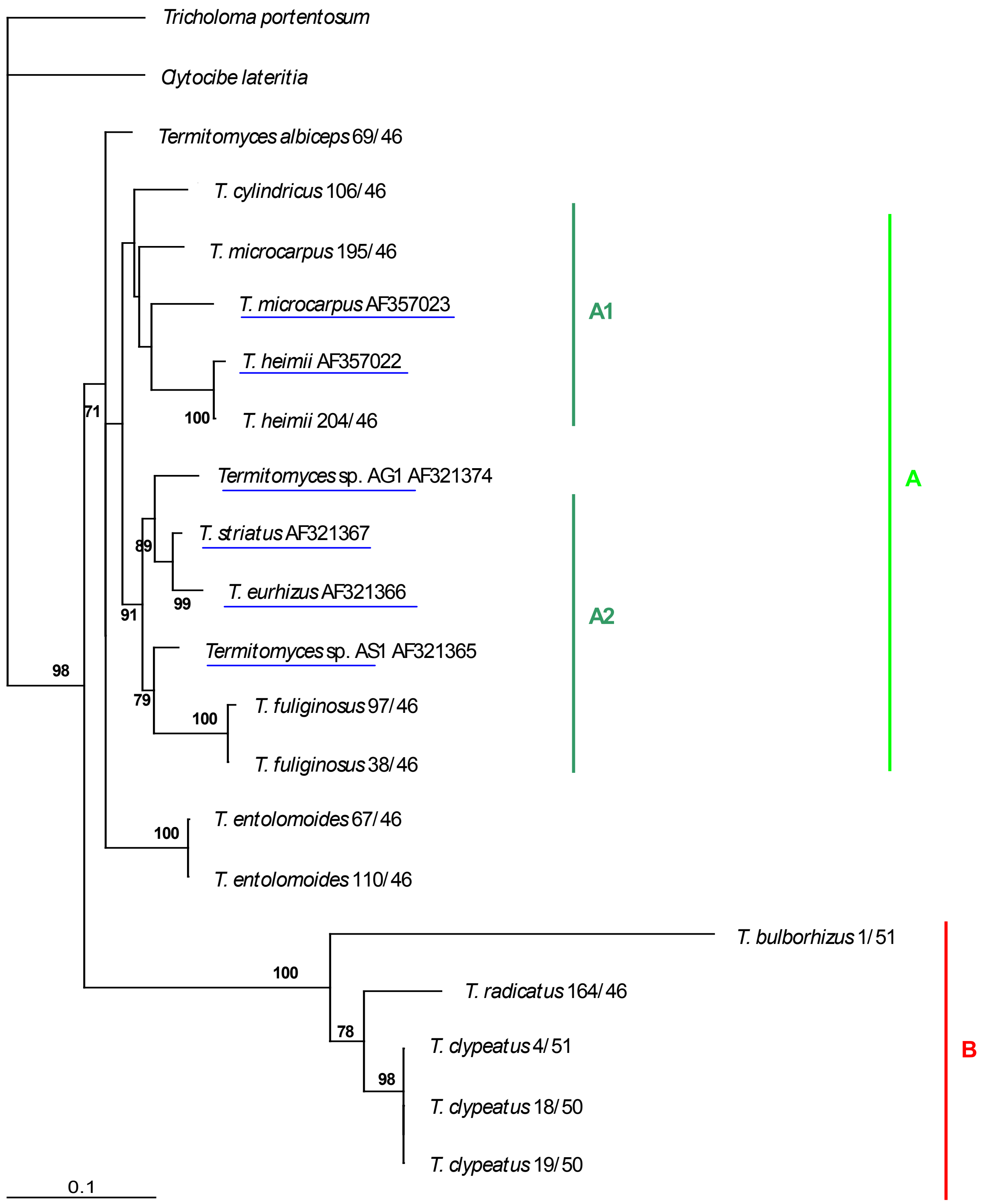

Figure 3 The inferred maximum likely hood cladogram based on neighbor-joining searching criteria of the ITS1-5.8S-ITS2 sequences (690 bp) from six species of *Termitomyces* retrieving from Genbank (represented by underline species) and 13 selected strains of 9 *Termitomyces* species collected from Kanchanaburi. Bootstrap values more than 50% were showed as bold number at each node. Scale bar represents branch length corresponding to different nucleotides at 10%.

Discussion

Among 28 *Termitomyces* isolates, 11 isolates had been misidentification based on morphological characteristics. Based on the results of ITS sequence comparison, the species with similar basidiocarps morphological characteristics demonstrated distinct ITS sequences, whereas those with distinct morphological characteristics of basidiocarps showed similar ITS sequences. For example, *T.*

entolomoides isolates 67/46, 110/46 and 164/46 which are closely similar in their morphological characters but demonstrated dissimilar ITS sequences where the isolate 164/46 showed only 76-76.5% identity with the isolates 67/46 and 110/46. The isolates 67/46 and 110/46 showed more distinctive characteristics of *T. entolomoides* as compared to isolate 164/46 (a clear dark color pileus with bluish black tint, rimose pileus and the swollen stipe at ground level). In addition, the isolate 164/46 had small pileus (1.5-3.5 cm diameter) and grayish/dark brown pileus. Consequently, we putatively re-identified the isolate 164/46 to *T. radicatus*.

The identifications of *T. heimii* and *T. clypeatus* based on morphological characteristics and ITS1-5.8S-ITS2 rDNA sequences are strongly valid since the molecular results supported the classical classification. The result of ITS pairwise alignment between the isolate of *T. heimii* (204/46) collected from Kanchanaburi and *T. heimii* (AF357022) retrieved from GenBank revealed 97% identity. Moreover, both isolates were a sister group with well supported 100% bootstrap value in the inferred molecular phylogeny trees. The pairwise alignment of the partial LSU rDNA sequences between *T. clypeatus* from Kanchanaburi and *T. clypeatus* AY127802 with 97.12% identity confirmed the correct identification based on morphological identification.

Results of ITS sequence alignment also showed that morphological characteristics of *T. clypeatus* found in different environments were extremely diverse leading to misidentification (Table 1). ITS sequences of 3 *T. robustus* isolates (80/46, 98/46 and 169/46) and 5 *T. fuliginosus* isolates (25/49, 26/49, 27/49, 18/50 and 20/50) which are morphologically different had 98-100% identical ITS sequences with *T. clypeatus*. The ITS sequence identification results reveal that some isolates of *T. clypeatus* do not have the strongly spiniform, dark perforatorium, the relatively slender habit as mentioned in the key of Pegler and Vanhaecke (1994) but their features should cover some of morphological characteristics of *T. robustus* and *T. fuliginosus* that mentioned in the key of Pegler and Vanhaecke (1994). Thus, a new key to identify *Termitomyces* mushrooms should be re-described in the combination with analysis of ITS sequences and molecular phylogeny.

The identifications of *T. microcarpus* isolate 195/46 and *T. fuliginosus* isolates 38/46 and 97/46 based on the ITS sequences in this study are not consistent with ITS sequences of *T. microcarpus* (AF357023) and *T. fuliginosus* (AF321374) retrieved from Genbank of the NCBI database. For the *T. microcarpus* species, ITS sequences of *T. microcarpus* (AF357023) was the *Termitomyces* from unknown source but the *T. microcarpus* 195/46 had basidiocarp characteristics matched description of the Southeast Asian *Termitomyces* species (Heim, 1977; Pegler and Vanhaecke, 1994). This species is easy to distinguish by the small basidiocarp lacking a pseudorhiza whereas *T. microcarpus* 195/46 from Kanchanaburi differs from previous researches by the cream spore deposit instead of the pink spore deposit. For the 2 strains of *T. fuliginosus* from Kanchanaburi, they had a large pileus, concentric scrobiculate stipe not annulate, obtuse perforatorium in which the features of *T. fuliginosus* described by Heim (1977). In addition, the name of African *T. fuliginosus* AF321374, mentioned by Rouland-Lefevre et al. (2002), was recently changed to *Termitomyces* sp. AG1 indicating uncertain classification. The difficulties of *Termitomyces* species identification have been mentioned in several researches (Froslev et al., 2003; Rouland-Lefevre et al., 2002; Tang et al., 2006). The abundant of unidentified *Termitomyces* species was demonstrated in several databases such as GenBank, EMBL and DDBJ.

Among 9 species of *Termitomyces* species found in 8 districts of Kanchanaburi, 8 species with the exclusion of *T. bulborhizus* were commonly found in the central region of Thailand as reported by Kosakul et al. (2007) and Sa-uangsub (2003). *T. bulborhizus* that was a recently reported novel species from China (Wei et al., 2004) was also distributed in Thailand as well. *T. bulborhizus* and *T. clypeatus* were found to be symbiotic with *Macrotermes gilvus* and *Hypotermes makhamensis*. Our study was consistent with the study of Rouland-Lefevre et al. (2006) that these mushroom species are symbiont with different termite species.

The ITS1-5.8S-ITS2 genomic analysis of 28 *Termitomyces* isolates in this study varied from 509 to 734 bp. The ITS1-5.8S-ITS2 size of African *Termitomyces* species was 650 bp (Rouland-Lefevre et al., 2002). However, this value is in accordance with 600-800 bp reported for fungi in general (Gardes and Bruns, 1996). The complete ITS1 and ITS2 regions showed the huge variation from 139-256 bp and 191-317 bp, respectively. The complete 5.8S region demonstrated the low variation from 158-161 bp. This result corresponding with the hypothesis that the 5.8S, SSU and LSU rDNA genes have a strong preservation of functioning genomic zones and do not permit an intergeneric discrimination (Rouland-Lefevre et al., 2002).

Our study demonstrated the monophyletic tree from both pure Kanchanaburi *Termitomyces* and mixture of Asia and African *Termitomyces* samples. This result was corresponding with the previous molecular phylogenetic studies (Aanen et al., 2002; Froslev et al., 2003; Rouland-Lefevre et al., 2002). The monophyletic result suggested that the cultivation of *Termitomyces* by termites has a single origin, and that no species of fungi has reverted from termite symbiont to a nonsymbiotic state.

The phylogenetic tree of Kanchanaburi *Termitomyces* and African *Termitomyces* retrieving from Genbank demonstrated that the clade B revealed pure Kanchanaburi *Termitomyces* while the African *Termitomyces* resolved into four different apical clades within subclade of A1 and A2. The African fungus growing-termite has been purposed to be an ancestor from the evidence that the Asian fungus growing-termite resolved into four different apical clades (Aanen and Eggleton, 2005). From our result the African *Termitomyces* did not represent as the ancestor like African fungus growing-termite. The African *Termitomyces* resolved into four different apical clades suggested that the evolution of African and Asian *Termitomyces* was equivalent.

This studies corresponding with the previous phylogenetic researches where the classical circumscriptions of the genus *Termitomyces* demonstrated problematic (Froslev et al., 2003). Several identical sequences were obtained from *Termitomyces* basidiocarps assigned to different species, even though these *Termitomyces* species were quite well-known. On the other hand, some sequences from samples assigned to the same species were different sequences. Therefore, analysis of conserved genes such as the ITS1-5.8S-ITS2 and molecular phylogeny is the better way to identify *Termitomyces* species.

In addition, the ITS analysis and molecular phylogeny showed that morphological patterns cannot be traced in the phylogenetic tree of Kanchanaburi *Termitomyces* because there is no common characteristic which can be shared in each clade. For example, clade A contained taxa with basidiocarps that varied from the smallest basidiocarp, 0.5 cm of *T. microcarpus* to 9.5 cm of *T. fuliginosus*. Clade B comprised taxa with basidiocarps ranging from 3.2 cm of *T. radicatus* to 21 cm of *T. bulborhizus*. Our results are consistent with Rouland-Lefevre et al. (2002). They also showed that the molecular phylogeny of *Termitomyces* did not support the subgroup classification on the basis of the basidiocarp morphology and fruiting behavior that were described by Heim (1977). Nonetheless, Froslev et al. (2003) reported that a few basidiocarp morphological patterns such as size of basidiocarp can be traced in the phylogenetic of African *Termitomyces*.

Our results demonstrated that there were no identical sequences between African and Kanchanaburi samples, suggesting a major geographical differentiation. *T. microcarpus* (195/46) collected from Kanchanaburi and African *T. microcarpus* (AF357023) are not synonym due to low identity of ITS sequence (87%), but they form a sister group. This result consisted with the previous research studied on African and Asian *Termitomyces* (Froslev et al., 2003).

In conclusion, based on a polyphasic identification approach, 9 *Termitomyces* species of 28 collected isolates were identified from 8 different locations of Kanchanaburi. They were *T. albiceps*, *T. bulborhizus*, *T. cylindricus*, *T. heimii*, *T. microcarpus*, *T. radicatus*, *T. entolomoides*, *T. fuliginosus*, and *T. clypeatus*. *T. clypeatus* was the species that frequently found and showed the most extremely variation of morphological characteristics originated in different environments. This study also confirmed that analysis and molecular phylogeny of ITS1-5.8S-ITS2 rDNA sequences is a useful approach help identify *Termitomyces* species in a combination with morphological classification. In addition, the monophyletic tree from pure Kanchanaburi *Termitomyces* isolates, mixture of Asia and African *Termitomyces* samples suggested that both Asia and African *Termitomyces* species have evolved from the same ancestor.

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