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Contributed Paper

Two Species of *Clitopilus* (Entolomataceae, Agaricales) from Northern Thailand

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ABSTRACT

As part of our efforts to study the saprobic mushrooms in Asia, we made three collections of *Clitopilus* in northern Thailand. Two of the three collections were identified, based on morphological and molecular analyses, as *Clitopilus chalybescens* described from Khao Yai National Park, Thailand. The third collection is a new species, *Clitopilus doimaesalongensis*, which we introduce in this paper. The new species *C. doimaesalongensis* and *C. chalybescens* are documented with line drawings, photographs and description and compared with similar taxa and molecular data is provided for *C. chalybescens* for the first time.

Keywords: basidiomycetes, molecular analyses, mushrooms, new species, saprobes

1. INTRODUCTION

Clitopilus (Fr. ex Rabenh.) P.Kumm. is a small genus in the family Entolomataceae, order Agaricales traditionally including saprotrophic or mycoparasitic species, characterized by basidiomes with omphaloid or clitocyboid to pleurotoid habits, subcurrent to decurrent lamellae, pink spore prints, and basidiospores distinctively furrowed by 3-12 longitudinal ribs [1, 2]. *Clitopilus* species can easily be recognized by a characteristic farinaceous odor. The type species of *Clitopilus* was described by J.A. Scopoli in 1772 as

Agaricus prunulus Scop., but in 1871, P. Kummer transferred it to the new genus *Clitopilus* and designated the type as *Clitopilus prunulus* (Scop.) P. Kummer [3].

The genus *Clitopilus* has a widespread distribution, especially in northern temperate areas, and was earlier included within *Rhodocybe* [4]. Although the 2008 estimate suggested that there are about 30 species in the genus [5], a more recent publication in 2009 using molecular phylogenetics suggests that the genus should be redefined to include many

former *Rhodocybe* species [4]. A good dichotomous key to species of the genus *Clitopilus* in Tasmania has recently been provided [6].

In Thailand, there have been very few studies on *Clitopilus*, with only *C. apalus* (Berk. & Br.) Petch, *C. prunulus* [7] and *C. chalybescens* T.J. Baroni & Desjardin [8] known. In this study, we describe a new species of *Clitopilus* from Thailand and a collection of *C. chalybescens* with the support of morphological characters and ITS rDNA sequence data.

2. MATERIALS AND METHODS

2.1 Sample Collection and Macro-morphological Character Examination

Three specimens of *Clitopilus* were made during numerous field excursions during 2012 to 2013 in Chiang Rai and Lampang provinces, Thailand, indicating the genus is

not very common. The basidiomata were photographed *in situ* (Figure 1). Specimens were placed in a plastic box after encasing with aluminium foil to prevent damage and were taken to the laboratory for further studies. Significant information about the specimens and macromorphological characteristics of specimens were annotated in the laboratory and then the specimens were dried in a standard food dryer (MARTA MT-1941) at 40°C for 24-48 hours. The colour terminology used for macromorphological identification is from Kornerup and Wanscher [9]. The specimens were sealed in Zip lock plastic bags containing dehydrated silica gel to control humidity. All herbarium specimens are deposited in the Mae Fah Luang University Herbarium (MFLU), Chiang Rai, Thailand. Faces of Fungi and Index Fungorum numbers were obtained as in Jayasiri et al. [10] and Index Fungorum [11].



Figure 1. Basidiomata in the field A, B). *Clitopilus chalybescens*, C). *Clitopilus doimaesalongensis*, Scale bars: A, B=25 mm, C=10 mm.

2.2 Micro-morphological Character Examination

Microscopic work was carried out with the dried specimens by taking free hand sections under a dissecting microscope (Motic SMZ-171). Slide preparation was implemented with 3-5% KOH and Congo red. A Nikon Eclipse 80i microscope was used for microscopic evaluation of basidiospores, basidia, and pileipellis. Image acquisition was made with a Canon 550D

digital camera. All these evaluations were carried out with a Nikon Eclipse 80i microscope at 200x, 400x and 1000x magnification and drawings made. In order to chronicle basidiospores (spore quotient), Q is used to mean “length/width ratio” of a basidiospore in side view. Basidiospore dimensions are based on the measurements of at least 25 basidiospores made in side view and measured excluding the hilar appendix or apiculus at 1000x magnification.

2.3 DNA Extraction, PCR and Sequencing

Genomic DNA was extracted from dried specimen using the Biospin Fungus Genomic DNA Extraction Kit (Bioer Technology Co., Ltd., Hangzhou, P.R. China). The Internal Transcribed Spacer (ITS) region of the ribosomal DNA was amplified and sequenced using primers ITS5 and ITS4. PCR reactions were completed in 25 μ l volumes containing 1.0 μ l template DNA, 9.5 μ l double distilled water, 1.0 μ l of each primer and 12.5 μ l of 2x power Taq PCR Master Mix (Genomics BioSci & Tech.) [A premix and ready to use solution, including 0.1 Units/ μ l Taq DNA Polymerase, 500 μ M dNTP Mixture each (dATP, dCTP, dGTP, dTTP), 20 mM Tris-HCL pH8.3, 100 mM KCl, 3 mM MgCl₂, stabilizer and enhancer]. The reaction was carried out with 35 cycles using the following conditions: denaturation (95°C, 30 s), annealing (52°C, 30 s), extension (72°C, 1 min) and final extension (72°C, 10 min). The primers used for sequencing were the same as those for amplification. Amplified products were confirmed with 1% agarose gel electrophoresis stained with ethidium bromide. Sequencing was performed in a commercial sequencing

provider (Beijing Bai Mai Hui Kang Biological Engineering Technology Co., P.R. China).

2.4 Sequence Alignment and Molecular Phylogenetic Analysis

Sequenced data were checked and assembled using BioEdit 7.0.9.0 [12], and blasted with those available in GenBank. The taxa information and GenBank accession numbers used in the molecular work are listed in Table 1. Sequences for each strain were aligned using Clustal X [13]. Alignments were manually adjusted to allow maximum sequence similarity. Gaps were treated as missing data. Phylogenetic analysis were performed using PAUP* 4.0b10 [14]. Ambiguously aligned regions were excluded from all analyses. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple parsimonious trees were saved. Clade stability of the trees resulting from the parsimony analyses were assessed by bootstrap analysis with 5000 replicates, each with 100 replicates of random stepwise addition of taxa [15]. Trees were figured in Treeview.

Table 1. Taxon information used for molecular analyses.

Taxon	Country of origin	GenBank accession numbers (ITS)	Reference
<i>Clitopilus giovanellae</i>	Madrid, Spain	EF413030	[16]
<i>C. scyphoides</i>	Bristol BS8 1UG, UK	FJ770390	[17]
<i>C. passeckerianus</i>	Bristol BS8 1UG, UK	FJ770406	[17]
<i>C. abortivus</i>	Bristol BS8 1UG, UK	FJ770404	[17]
<i>C. truncate*</i>	Bristol BS8 1UG, UK	FJ770393	[17]
<i>C. crispus</i>	Guangdong, China	JQ281489	[17]
<i>C. scyphoides</i>	Bristol BS8 1UG, UK	FJ770401	[17]
<i>C. hobsonii</i>	Bristol BS8 1UG, UK	FJ770402	[17]
<i>C. kamaka</i>	New Zealand	KJ461903	[18]
<i>C. scyphoides</i>	Bristol BS8 1UG, UK	KC176282	[19]

Table 1. Continued.

Taxon	Country of origin	GenBank accession numbers (ITS)	Reference
<i>C. cystidiatus</i>	Switzerland	HM623130	[20]
<i>C. chrischonensis</i>	Switzerland	HM623128	[20]
<i>C. austroprunulus</i>	Tasmania, Australia	KC139084	[21]
<i>C. fallax</i> *	Vaud, Switzerland	AF357018	[22]
<i>C. prunulus</i>	Jiangsu, China	EU273512	Unpublished
<i>C. brunnescens</i>	TN, USA	HQ222033	Unpublished
<i>C. amarus</i>	Leide, Netherlands	KC885963	Unpublished
<i>C. birneolus</i>	Leide, Netherlands	KC710132	Unpublished
<i>C. chalybescens</i>	Lampang, Thailand	KP938184	This study
<i>C. chalybescens</i>	Lampang, Thailand	KP938185	This study
<i>C. doimaesalongensis</i>	Chiang Rai, Thailand	KP938183	This study
<i>Lyophyllum decastes</i>	Vaud, Switzerland	AF357060	[22]

Note: * in GenBank these taxa are placed under the genus *Rhodocybe*.

3. RESULTS

3.1 Phylogenetic Analyses

To obtain an understanding on the phylogenetic relationships of our novel species *C. doimaesalongensis*, with other known *Clitopilus* species, we retrieved ITS sequence data from GenBank. The *Clitopilus* dataset comprised 21 collections (Table. 1), with 18 from GenBank, including the type species of the genus, *C. prunulus*, and the three *Clitopilus* collections from northern Thailand. *Lyophyllum decastes* (Fr.) Singer was chosen as the outgroup taxon. Of the 757 total characters, 441 characters are constant, of which 113 variable characters are parsimony-uninformative and 203 characters are parsimony-informative. The MP tree was produced after 238,115 rearrangements and the score of best tree found was 645 and other tree statistics include a consistency index (CI) of 0.4250, a

retention index (RI) of 0.638, and rescaled consistency index (RC) of 0.271. This most likely MP tree is presented and it was chosen to represent the phylogenetic position of *C. doimaesalongensis*.

Sequences were aligned and the alignment was subjected to Maximum parsimony analysis using PAUP to build the phylogenetic tree shown in Figure 2. The sequence of *C. doimaesalongensis* was grouped in a monophyletic clade supported by 100% bootstrap value. This clade was grouped together with the two sequences of *C. chalybescens* isolated in this study and also strongly statistical supported with 100% bootstrap value. Our new species is clearly showed that the *C. doimaesalongensis* clade distinct from the other *Clitopilus* species and distinct in having unique morphological characters (Table 2).

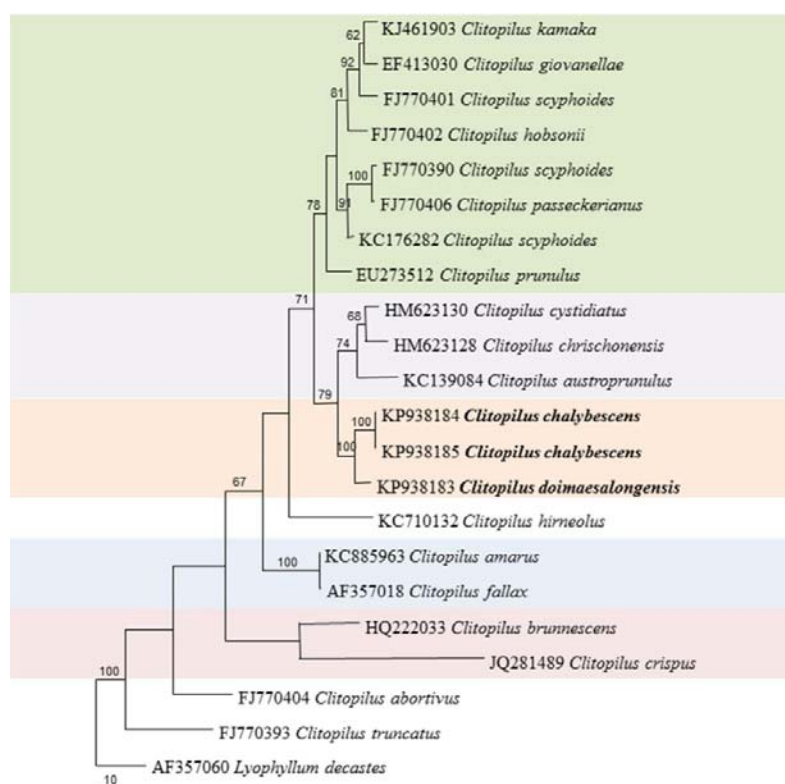


Figure 2. Maximum parsimony phylogram showing the phylogenetic position of *C. doimaesalongensis* sp. nov. with some selected *Clitopilus* species from GenBank based on ITS rDNA sequence data. Values above the branches are parsimony bootstrap ($\geq 50\%$). The tree is rooted with *Lyophyllum decastes*.

Taxonomy

1. *Clitopilus doimaesalongensis* Jatuwong, Karun. & K.D. Hyde, sp. nov. **Figure 3**

Etymology: The species epithet “doimaesalongensis” refers to the place where the fungus was collected.

Faces of fungi number: FoF 00712

MycoBank: MB 811415

Holotype: MFLU13-0519

Diagnosis: characterized by its white to chalk white, small basidiomata, with an eccentric stipe, absence of pleuro- and cheilocystidia and amygdaliform to limoniform basidiospores.

Pileus 11-19 mm in diameter, depressed with incurved margin; surface white to chalk white (A1), without staining, dull and sticky, winkle cover with the slightly matted silky-

fibrillose squamules. *Lamellae* decurrent, 0.7 cm wide, crowded, narrow, white (A1) when young. *Stipe* 17-25 × 7-4.5 mm, central, cylindrical, solid, white (A1) throughout surface, smooth with silky-fibrillose squamules, basal part with white (A1) cottony mycelium. *Odour* strong farinaceous.

Basidiospores 6.8-9.2 × 4.1-5.5 μm (Q=2.1-3.9, Qav=2.9, n=30), hyaline, ellipsoid in polar view, amygdaliform to limoniform in side view. *Basidia* 9.1-21.1 × 3.3-7 μm, clavate, 4-spored. *Pileipellis* a layer of loosely arranged, hyaline hyphae. *Cheilocystidia* absent. *Pleurocystidia* absent. *Clamp connections* absent in all tissues.

Habitat/Distribution: Gregarious on soil as small groups in disturbed forest, only known from Thailand.

Material examined: THAILAND, Chiang

Rai Province: Doi Mae Salong, 26 June 2013, Samantha C. Karunaratna (MFLU13-0519, **holotype**); ex-type living culture, MFLUCC 13-0806

Notes:

Clitopilus chalybescens was first described from Thailand [8]; and differs from *C. doimaesalongensis* by its dull, dry, opaque, silky-felty surface, pure white to pale greyish blue pileus, the central stipe, 5.5-7.5 × 3.6-4.8 µm ellipsoid basidiospores, and cylindrical hyphae in the pileipellis [8]. The other closely related species to our new species are *C. chrischonensis* Musumeci Vizzini & Contu, *C. cystidiatus* Hauskn & Noordel and *C. austoprunnulus* Morgado, G.M. Gates & Noordel. *Clitopilus chrischonensis* was described from Switzerland, and differs from *C. doimaesalongensis* in its smooth cap surface,

which is subglobose then irregularly convex and finally expanded without an umbo, cylindrical, 1-4 µm wide, 6-7 evident longitudinal ribs with 25-30 × 8-13 µm size basidiospores and, suprapellis consisting of cylindrical 1-4 µm wide hyphae [20]. *Clitopilus cystidiatus* was described from Austria, and differs significantly in the slightly grey or greyish ochre tinges of the pileus, efibuiata with 4-spored, ellipsoid or oblong of basidiospores and encrusted pileipellis hyphae [2, 23]. *Clitopilus austoprunnulus* differs from *C. doimaesalongensis* by its uniformly pale grey pileus which is convex when young then expanding to concave or infundibuliform and narrow, sometimes with a slight brown tinge at the centre, and the pileipellis hyphae is cylindrical, 4-8 µm wide with dark brown walls [21].

Table 2. Comparison of *C. doimaesalongensis* with the closely related species.

Scientific name	Basidiomata	Basidia	Basidiospores	Pileipellis
<i>Clitopilus doimaesalongensis</i>	11-19 mm in diameter, depressed with incurved margin, winkle cover with the slightly matted silky-fibrillose squamules	9.1-21.1 × 3.3-7.0 µm size, clavate, 4 spored	6.8-9.2 × 4.1-5.5 µm size, ellipsoid in polar view, amygdaliform to limoniform in side view, hyaline	loosely arranged, hyaline
<i>C. chrischonensis</i>	40 mm in diameter, smooth surface, first subglobose then irregularly convex, finally expanded without umbo	cylindrical, 1-4 µm wide	9.5-11.5 (-13.5) × 5.6 µm, hyaline with 6-7 evident longitudinal ribs	suprapellis consisting of cylindrical, 1-4 µm wide, hyphae
<i>C. cystidiatus</i>	20-70 mm in diameter, slightly grey or greyish, ochre tinges of the pileus, mealy-smelling context wider (4-7.5 µm), encrusted	4 spored, efibuiata	9.5-13.5 × 5.0-6.5 µm, ellipsoid or oblong	a cutis of cylindrical, 4.0-7.0 µm wide hyphae

Table 2. Continued.

Scientific name	Basidiomata	Basidia	Basidiospores	Pileipellis
<i>C. austoprunnulus</i>	40-90 mm in diameter, convex when young, expanding to concave or infundibuliform, involute margin	20-30× 4-8 μm 4 spored	(8-)9-11×4.5-6 μm occasionally, amygdaliform, distinctly ribbed lengthwise with 5-8 longitudinal ribs	a cutis of densely packed narrow cylindrical, 4-8 μm wide hyphae with dark brown coloured walls
<i>C. chalybescens</i>	15-45(-90) mm in diameter, infundibuliform	17.8-21× 6.4-8 μm, 4 streigmate, clavete sterigmata	5.5-7.5×3.6-4.8 μm, ellipsoid in profile and face view	hyaline, layer of loosy entangled cylindrical, hypha
<i>C. crispus</i> Pat. [24]	20-70 mm in diameter, convex to applanat, white to chalk white	20-30× 8-10 μm, subclavate, 4 spored, rarely 1, 2, or 3 spored	(5.5-)6.0-7.5× (4.0)4.5-5.5(-6.0) μm, ellipsoid to ellipsoid in side view, angular in apical view, 8-11 longitudinal	absent

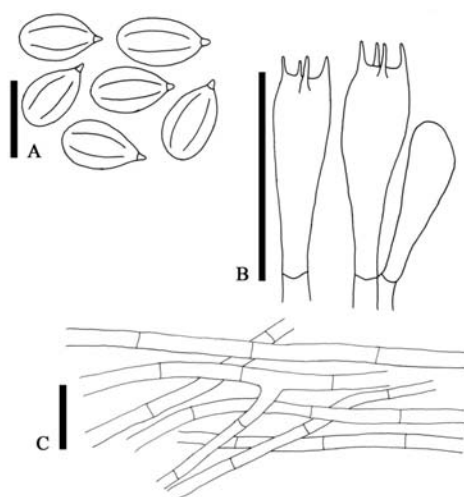


Figure 3. *Clitopilus doimaesalongensis* (MFLU13-0519) A. Basidiospores B. Basidia C. Pileipellis. Scale bars: A=10 μm, B=20 μm, C=10 μm.

2. *Clitopilus chalybescens* T.J. Baroni & Desjardin **Figure 4**

Pileus 21-26 mm in diameter, infundibuliform, depressed centre with undulate to lobed margin, splitting with age; surface dry, dull, silky-fibrillose; pure white, often changing colour to yellowish white (2A2) in age. *Lamellae* decurrent, heavily crowded, narrow, white when young becoming pinkish white (10A2) with age. *Stipe* 20-25 mm, central, cylindrical, glabrous, dry, brittle, hollow, white overall, becoming yellowish white (2A2) with age. *Odour* strong, farinaceous.

Basidiospores 5.3-7.1 × 3.7-5.0 μm, (6.2 × 4.2 μm, Q=1.3-2.2, Qav=1.6; n=30), hyaline, or pale pinkish in mass, ellipsoid in profile

and face views, with moderately prominent longitudinal ridges, angled in polar view. *Basidia* 15-21 × 5.1-7.7 μm, 4-spored, clavate. *Lamellar* trama of parallel or interwoven, hyaline, cylindrical hyphae. *Pileipellis* made up of loosely arranged, non-encrusted, hyaline, cylindrical hyphae. *Clamp connections* absent.

Habitat/Distribution: Gregarious on soil associated with mosses, only reported from Thailand.

Material examined: THAILAND, Lampang Province: Chaeson, Maecham Village 18°51'58.16"N, 99°27'45.34"E, alt.1010m, 13 June 2013, Kritsana Jatuwong & Thasanee Luangharn (MFLU13-0520); 13 June 2013, Kritsana Jatuwong & Thasanee Luangharn (MFLU13-0521).

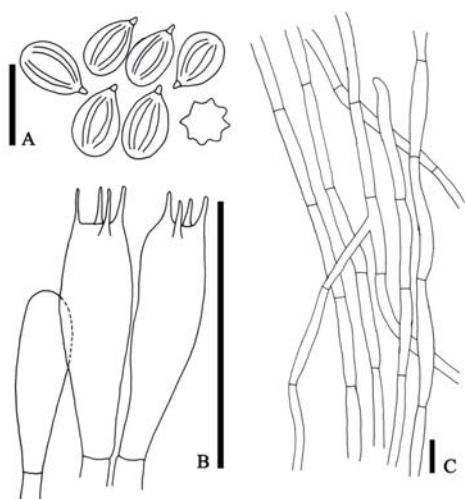


Figure 4. *Clitopilus chalybescens* (MFLU13-0520) A. Basidiospores B. Basidia C. Pileipellis. Scale bars: A=10 μm, B=25 μm, C=10 μm.

4. DISCUSSION

Clitopilus species are widespread in Europe, North America and Asia and could be grown in different biogeographical regions with several types of forests and habitats; for examples *C. austroprunulus* was seen growing in terrestrial in litter on wet sclerophyll forests in Kermadie Falls, Tasmania, Australia [21], while *C. amygdaliformis*

was found in broadleaved or coniferous forests in China (Taiwan and Yunnan). It was found from July to August [25], and *C. kamaka* was observed growing on well-rotted wood, soil, buried twigs and rock surfaces. This species was found from Little River, Okuti Reserve in Mid-Canterbury of New Zealand [18]. In this study, collections were found from northern part of Thailand in a disturbed forest. The morphological and phylogenetic analyses of the three *Clitopilus* collections from northern Thailand, one collection was identified as a distinct new species in the genus *Clitopilus*, with 100% bootstrap support and distinct morphological features. The new species is closely related to *C. chalybescens* which was also described from Thailand [8]. The comparison of macro- and micro- morphological characters (Table 2) and eight base pair differences between two sequences showed *C. doimaesalongensis* differs from *C. chalybescens* even though the phylogenetic analyses showed 100% bootstrap support. *Clitopilus chrischonensis*, *C. cystidiatus* and *C. austroprunulus* are also phylogenetically closely related to *C. doimaesalongensis*, but according to the morphological features (Table 2) our new species is distinct among the species in the genus.

Two of the three Thai collections were identified as *C. chalybescens* which was described from Khao Yai National Park, based on the macro- and micro-morphological features [8]. Thus we newly added sequence data for *C. chalybescens* to GenBank.

The main focus of this paper has been on biodiversity and taxonomy, however, it is worth noting that basidiomes of most *Clitopilus* species are potentially edible, and cultivatable, but studies have not been reported that carried out to cultivate *Clitopilus* [26]. The genus *Clitopilus* is also important for medicinally important secondary metabolites. *Clitopilus passeckerianus* produces a novel

polypeptides, diterpene synthase activity which is involved in the biosynthetic pathway for pleuromutilin, a tricyclic diterpene secondary metabolite [17, 27]. Future research should focus on the cultivation and secondary metabolite analyses of the genus *Clitopilus* including our new species *C. doimaesalongensis*.

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