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Two new species of *Helicascus* (Morosphaeriaceae) from submerged wood in northern Thailand

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Abstract

Two new *Helicascus* species *H. chiangraiensis* and *H. uniseptatus* from submerged wood in aquatic habitats in northern Thailand are introduced in this paper based on morphology and molecular analysis of DNA sequence data. Descriptions and illustrations of *H. chiangraiensis* and *H. uniseptatus* are provided. *Helicascus chiangraiensis* is characterized by its unilocular ascostromata, verrucose-walled ascospores with 2–4 large refractive guttules. *Helicascus uniseptatus* is characterized by its unilocular, small ascostromata, and uniseptate, smooth-walled ascospores lacking a mucilaginous sheath. Phylogenetic analysis based on combined ITS, LSU and *TEF1α* sequence data placed these species in Morosphaeriaceae (Pleosporales).

Key words: aquatic fungi, Dothideomycetes, Morosphaeriaceae, morphology, phylogeny

Introduction

Suetrong *et al.* (2009) introduced the family Morosphaeriaceae in Pleosporales for two *Massarina* species *M. ramunculicola* and *M. velatispora*, which did not group in Massarinaceae. Presently, *Morosphaeria* and *Helicascus* are accepted in this family, with some *Helicascus* species collected from freshwater habitats (Suetrong *et al.* 2009, Hyde *et al*. 2013, Wijayawardene *et al*. 2014, Zhang *et al*. 2013, 2014, 2015).

The genus *Helicascus* Kohlm. was introduced from intertidal mangrove wood in Hawaii and is typified by *H. kanaloanus* Kohlm. (Kohlmeyer 1969). Hyde (1991) introduced the second species, *H. nypae* collected from intertidal palm material in Brunei based on its morphological differences. *Helicascus kanaloanus* and *H. nypae* formed a well-supported clade within Morosphaeriaceae in a phylogenetic analysis of marine Dothideomycetes (Suetrong *et al*. 2009). Subsequently, two further freshwater species were added, i.e. *H. aegyptiacus* and *H. aquaticus* (Zhang *et al*. 2013). Meanwhile, *Massarina thalassioidea* and *Kirschsteiniothelia elaterascus* were also transferred to *Helicascus* as *H. thalassioideus* and *H. elaterascus* (Hyde *et al*. 2013, Zhang *et al*. 2013). Furthermore, *Helicascus gallicus* was described and illustrated from submerged wood collected from aquatic habitats in western and southern France (Zhang *et al*. 2014). Subsequently, Zhang *et al*. (2015) introduced a further *Helicascus* species, *H. unilocularis* collected from the Caribbean area. Previous research has shown that *Helicascus* is a monophyletic genus supported by molecular (Jones *et al*. 2009, 2015, Zhang *et al*. 2012) and morphological data (Kohlmeyer 1969, Hyde 1991).

Helicascus is characterized by its immersed ascostromata comprising several locules that share a common periphysate ostiole lying under a more or less conspicuous pseudostromatic tissue or solitary to clustered unilocular ascostromata, which may be immersed to almost superficial (Kohlmeyer 1969). It is a cosmopolitan genus which has been reported in Australia (Hyde & Goh 1998), Brunei (Ho *et al*. 2001), Chile (Shearer 1993), China (Tsui *et al*. 2000, Cai *et al*. 2002), France (Zhang *et al*. 2014), Lesser Antilles (Zhang *et al*. 2015), North America (Shearer 1993), Philippines (Cai *et al*. 2003), South Africa (Hyde *et al*. 1998) and Thailand (Zhang *et al*. 2013).

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This paper is part of a study on the taxonomy and diversity of microfungi on substrates in freshwater, along a north-south latitudinal gradient from China through to New Zealand (Hyde *et al*. 2016). The aim of this study is to introduce two new species of *Helicascus*, with descriptions and illustrations.

Materials and methods

Isolation and morphology

The specimens of decaying wood in freshwater were collected in October 2013 from a stream and pond in Chiang Rai Province, Thailand and returned to the laboratory in plastic bags. The samples were incubated in plastic boxes lined with moistened tissue paper at room temperature for one week. The samples were processed and examined following the methods described by Taylor & Hyde (2003). The morphological observations were taken under a Nikon SMZ-171 dissecting microscope and Nikon Eclipse 80i compound microscope with a Cannon EOS 600D camera.

Single spore isolations were made to obtain the pure cultures as described in Chomnunti *et al*. (2014). The cultures are deposited in Mae Fah Luang University Culture Collection (MFLUCC) and Dali University Culture Collection (DLUCC). Herbarium specimens are deposited at the herbarium of Mae Fah Luang University (MFLU) and the Herbarium of Cryptogams Kunming Institute of Botany Academia Sinica (HKAS). Facesoffungi and Index Fungorum numbers were obtained as in Jayasiri *et al*. (2015) and Index Fungorum (2016).

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from fresh fungal mycelium grown on PDA at room temperature. The EZ gene™ Fungal gDNA kit (GD2416) was used to extract DNA according to the manufacturer's instructions. ITS, LSU and *TEF1α* gene regions were amplified using the primer pairs ITS5/ITS4, LROR/LR7 and EF1-983F/ EF1-2218R. The final volume of the PCR reaction was 25 µL and contained 12.5 µL of 2×Power Taq PCR MasterMix (a premix and ready to use solution, including 0.1 Units/µl Taq DNA Polymerase, 500 µm dNTP Mixture each (dATP, dCTP, dGTP, dTTP), 20 mM Tris–HCl pH 8.3, 100MmKCl, 3 mMMgCl_2, stabilizer and enhancer), 1 µL of each primer (10 µM), 1 µL genomic DNA extract and 9.5 µL deionised water. The PCR thermal cycles for the amplification of the gene regions were as described in Su *et al*. (2015). PCR products were purified using minicolumns, purification resin and buffer according to the manufacturer's protocols (Amershamproduct code: 27–9602–01). The PCR products were observed on 1% agarose electrophoresis gels stained with ethidium bromide. Purification and sequencing of PCR products were carried at Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, P.R. China).

Phylogenetic analysis

Raw sequences were assembled with Sequencher 4.9 for Windows (Gene Codes Corp., Ann Arbor, Michigan). The consensus sequences were initially aligned using MAFFTv.7 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013) and optimised manually when needed.

A maximum likelihood analysis was performed using RAxMLGUI v. 1.3 (Silvestro & Michalak 2011). The optimal ML tree search was conducted with 1000 separate runs, using the default algorithm of the program from a random starting tree for each run. The final tree was selected among suboptimal trees from each run by comparing likelihood scores under the GTR+GAMMA substitution model.

Maximum-parsimony analyses were performed using the heuristic search option with 1000 random taxa additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Clade stability was assessed using a bootstrap (BT) analysis with 1000 replicates, each with 10 replicates of random stepwise addition of taxa (Hillis & Bull 1993).

Bayesian analyses were performed by using PAUP v.4.0b10 (Swofford 2002) and MrBayes v3.0b4 (Ronquist & Huelsenbeck 2003). The model of evolution was estimated by using MrModeltest 2.2 (Nylander 2004). Posterior probabilities (PP) (Rannala & Yang 1996) were performed by Markov Chain Monte Carlo Sampling (BMCMC) in MrBayes v. 3.0b4 (Liu *et al*. 2012). Six simultaneous Markov Chains were run for 1 m generations and trees were sampled every 100th generation (Resulting 10000 total trees) (Cai *et al*. 2006). The first 2000 trees representing the burn-in phase of the analyses were discarded and the remaining 8000 (post burning) trees used for calculating posterior probabilities (PP) in the majority rule consensus tree (Cai *et al*. 2006, Liu *et al*. 2012).

The phylogenetic analyses were carried out with the combined ITS, LSU and *TEF1α* sequence data alignment to

illustrate the placement of the isolates in Morosphaeriaceae. The single gene phylogenetic analysis showed same result with combined sequence data analysis. All new sequence data generated in this study are deposited in GenBank (Table 1). Phylogenetic trees were viewed in Treeview (Page 1996). The terminals of the tree (FIG. 1) are labeled with species and the isolates/culture collection codes as provided in GenBank.

TABLE 1. Isolates and sequences used in this study (newly generated sequences are indicated in red).

Species	Collection/Isolate no.	GenBank accession no.		
		LSU	ITS	*tef1*
Helicascus aegyptiacus	FWCC99	KC894853	–	–
H. aquaticus	MAFF 243866	AB807532	AB809627	AB808507
H. aquaticus	MFLUCC10–0918	KC886640	KC886639	–
H. chiangraiensis	MFLUCC 13–0883	KU900585	KU900583	KX455849
H. uniseptatus	MFLUCC 15–0057	KU900584	KU900582	KX455850
H. elaterascus	MAFF 243867	AB807533	AB809626	AB808508
H. elaterascus	CBS139689	LC014608	LC014552	LC014613
H. gallicus	BJFUCC200228	KM924832	KM924833–	–
H. gallicus	BJFUCC200224	KM924830	–	
H. gallicus	CBS 123118	KM924832	–	–
H. nypae	BCC 36751	GU479788		GU479854
H. nypae	BCC 36752	GU479789	–	GU479855
H. thalassioideus	MFLUCC10–0911	KC886636	KC886635	–
H. thalassioideus	JF14020–2	KP637165	KP637162	
H. thalassioideus	KH 242	AB807558	LC014554	AB808534
H. unilocularis	JF 14020	KP637166	KP637163	–
H. unilocularis	JF 14020–1	KP637167	KP637164	–
Morosphaeria ramunculicola	BCC 18404	GQ925853	–	–
M. ramunculicola	BCC 18405	GQ925854	–	–
M. ramunculicola	JK 5304B	GU479794	–	AB808530
M. ramunculicola	KH 220	AB807554	–	–
M. velatispora	BCC 17059	GQ925852	–	–
M. velatispora	KH 221	AB807556	LC014572	–
Montagnula opulenta	CBS 168.34	DQ678086	AF383966	

BCC: BIOTEC Culture Collection, Bangkok, Thailand; **BJFUCC:** Beijing Forestry University Culture Collection; **CBS:** Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; **MAFF:** Ministry of Agriculture, Forestry and Fisheries, Japan; **MFLUCC:** Mae Fah Luang University Culture Collection, ChiangRai, Thailand; **JF:** Jacques Fournier; **JK:** J. Kohlmeyer; **KH:** K. Hirayama

Results

Phylogeny

Combined analyses of ITS, LSU and *TEF1α* sequence data were used to determine the taxonomic placement of our strains. All the available sequence data of *Helicascus* species were included in our phylogenic analyses. The dataset comprised 24 taxa with *Montagnula opulenta* (CBS 168.34) as the out group. Phylogenetic analyses obtained from maximum likelihood (RAxML), maximum parsimony (MP) and Bayesian analyses showed similar topologies and were not significantly different. The best scoring RAxML tree was selected to represent the relationships among taxa and is shown in FIG. 1.

The phylogenic analyses obtained from maximum likelihood (RAxML), maximum parsimony (MP) and Bayesian analyses gave similar results as in previous studies (Zhang *et al*. 2013, 2014, 2015). *Helicascus chiangraiensis* clustered together with the other *Helicascus* species and formed a sister group with *H. gallicus*, but represent as a distinct clade, *H. uniseptatus* clustered with *H.elaterascus* in a sister group.

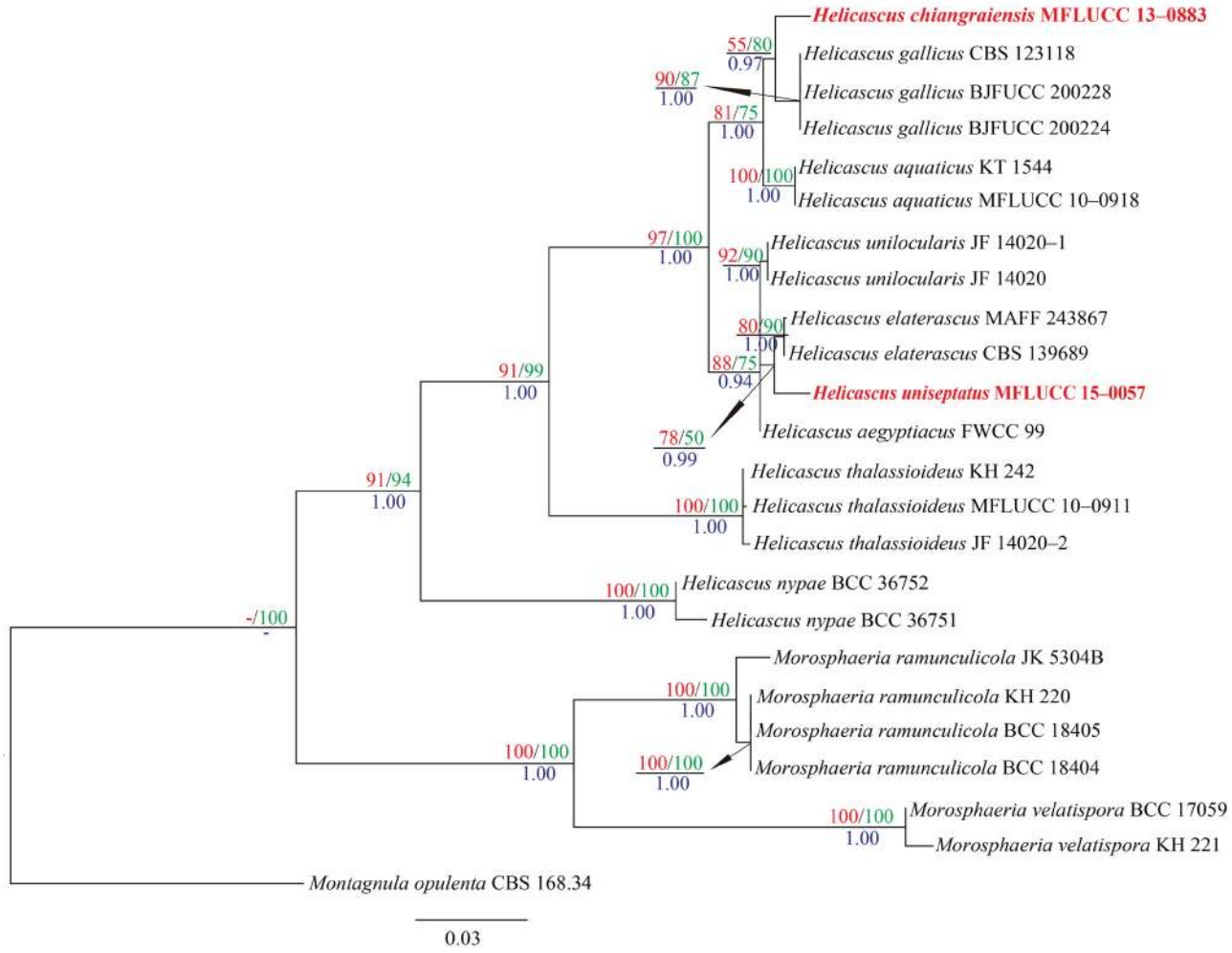

FIGURE 1. Phylogram generated from maximum likelihood analysis (RAxML) based on combined ITS, LSU and *TEF1α* sequenced data in the family Morosphaeriaceae. Bootstrap support values for maximum likelihood (ML, red) and maximum parsimony (MP, green) equal to or greater than 50% are given above the nodes. The values of the Bayesian posterior probabilities from MCMC analyses (BYPP, blue) equal or higher than 90% are given below the nodes. The tree was rooted to *Montagnula opulenta*. Newly generated sequences are indicated in red.

Taxonomy

Helicascus chiangraiensis Z.L. Luo, J.K Liu, H.Y. Su & K.D. Hyde, *sp. nov.* **FIGURE 2**

Index Fungorum: IF 552003; Facesoffungi number: FoF 02019

Etymology: With reference to the location of this taxon.

Saprobic on decaying, submerged wood in freshwater. ***Sexual morph***: *Ascostromata* 240–270 µm diam, 340–550 µm high, solitary, scattered, black, immersed, unilocular, globose to subglobose, ostiole central. *Peridium* 30–50 µm, subhyaline to dark brown, composed of several layers of pseudoparenchymatous cells, outer layer dark brown, with thick-walled cells, arranged in a *textura angularis*, inner layer hyaline with flattened, thin-walled cells. *Hamathecium* composed of septate, hypha-like pseudoparaphyses, 1.5–2.5 µm wide, slightly constricted at the septa, ramified above asci with free ends, embedded in a gel matrix. *Asci* 77–146 × 16–19 µm ($\bar{x}$ = 111.5 × 17.5 µm, n = 20), 8-spored, bitunicate, fissitunicate, clavate, apically rounded, dehiscence, endoascus narrow, coiled within ectoascus, ectoascus

forming a long tail extension. *Ascospores* 24.5–27.5 × 8.5–10.5 µm ($\overline{x}$ = 26 × 9.5 µm, n = 20), obliquely uniseriate and partially overlapping, ellipsoid-fusiform, verruculose, upper end narrowly rounded, lower end tapering, slightly curved in side view, with 2–4 large refractive guttules, 1-euseptate, septum submedian, hyaline when young, becoming brown when mature, thick-walled, verruculose, slightly constricted at the septum, surrounded by sheath. ***Asexual morph***: Undetermined.

Material examined:—THAILAND. Chiang Rai Province, saprobic on decaying wood submerged in a pond, October 2013, *Asanka Bandara*, ZL-11 (MFLU 15–0084, holotype); ex-type living culture, MFLUCC 13-0883, DLUCC; (HKAS 86459, isotype).

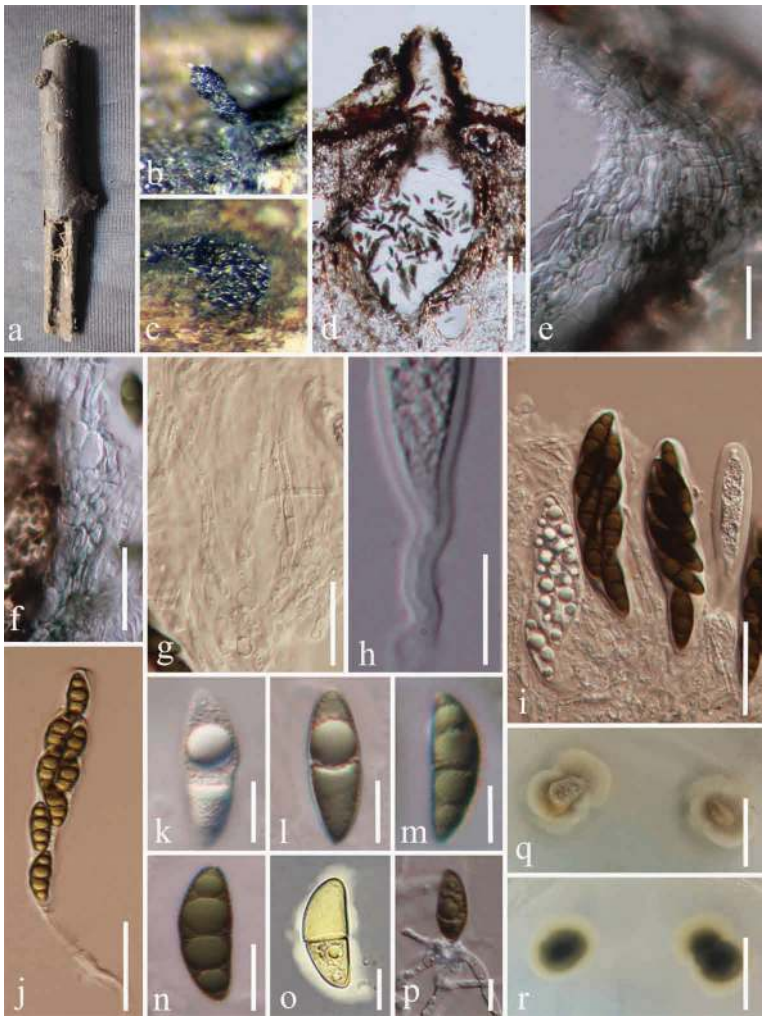

FIGURE 2. *Helicascus chiangraiensis* (MFLU 15–0084, holotype) a. Specimen; b. Appearance of black cirrhus of ascospores on surface of host; c. Section of ascoma; d. Longitudinal section of ascoma; e, f. Section of peridium; g. Paraphyses. h–j; Asci; k–o. Ascospores; p. Germinating ascospore; q–r. Culture grow on PDA after 3 weeks, q. upper side, r. reverse side. Scale bars: d = 150 µm; e–g = 25 µm; h–j = 30 µm; k–p = 10 µm; q, r = 10 mm.

Notes:—*Helicascus chiangraiensis* was collected from decaying wood submerged in a pond in Chiang Rai Province, Thailand. According to the key provided by Zhang *et al*. (2013), *H. chiangraiensis* is similar to *H. aegyptiacus* by its coiled endoascus, verruculose ascospores surrounded by a gelatinous sheath and both are collected from freshwater habitats. However, *H. chiangraiensis* differs from *H. aegyptiacus* in having unilocular, smaller ascostromata, while *H. aegyptiacus* has a longer ascostromata, pseudostromata with 2–3(4) dark locules (Table 2). In addition, the molecular analysis also showed that these two species are phylogenetically distinct from each other.

TABLE 2. Habitat and morphological comparison among species of *Helicascus* (data from Kohlmeyer 1969, Hyde 1991, Shearer 1993, Zhang *et al*. 2013, 2014, 2015).

Species	Habitats	Ascomata/Pseudostomata		Asci	Peridium (μm)	Ascospore		
		Size (μm)	Unilocular/ Locules			Size (μm)	Sheath	Ornamentation
Helicascus aegyptiacus	Freshwater	500–650×450–950	2–3(4) locules	Coiling	40–60 (upper region), 20–30 (basal region)	27–35×8–14	Present	Verruculose
H. aquaticus	Freshwater	200–300×800–1000	2 locules	Coiling	20–30	19–26×8–11	Present	ND
H. chiangraiensis	Freshwater	240–270×340–550	Unilocular	Coiling	30–50	24.5–27.5×8.5–10.5	Present	Verruculose
H. elaterascus	Freshwater	284–518×324–850	Unilocular	Coiling	60–70	22–33×6–13.5	Present	Verruculose
H. gallicus	Freshwater	340–420×580–920	2–3 locules	Coiling	27–35 (–70)	26–31.3×9.3–12	Absent	Smooth
H. kanaloanus	Marine	600–780×125–2750	3–4 locules	Coiling	ND	36.5–48.5×18–22.5	Present	Smooth
H. nypae	Marine	260–390×750–1500	3–4 locules	ND	ND	25–35×12–15	Present	Verruculose
Helicascus uniseptatus	Freshwater	100–200×300–500	Unilocular	Coiling	40–48	25–32×9–13	Absent	Smooth
H. thalassioideus	Freshwater	130–250×100–156	Lenticular locules	Coiling	Up to 70	25–31×7–10	Absent	Smooth
H. unilocularis	Freshwater	200–220×290–300	Unilocular	Coiling	50–65	23–25.8×10.2–11.4	Absent	Smooth

Note: ND: No documented

Helicascus uniseptatus J. Yang, J.K. Liu & K.D. Hyde, *sp. nov.* **FIGURE. 3**
Index Fungorum: IF 552251; Facesoffungi number: FoF 02018
Etymology: With reference to uniseptate ascospores.

Saprobic on decaying, submerged twigs in aquatic habitats, visible as masses of brown ascospores on the host surface. ***Sexual morph***: *Ascostromata* 100–200 μm diam, 300–500 μm high, solitary or aggregated in small groups, black, immersed, subglobose or obovate, appearing as slightly raised regions with black ostioles, with periphyses, papillate, black. *Peridium* 40–48 μm, comprising three fused layers, outer layer darker, with thick-walled cells of *textura globosa*, middle layer pale brown with thin-walled cells of *textura angularis*, and inner layer hyaline with flattened, thin-walled cells. *Hamathecium* composed of septate, hypha-like pseudoparaphyses, 2.2–4.2 μm wide, constricted at the septa, ramified above asci with free ends, embedded in a gel matrix. *Asci* 130–240 × 16–26 μm ($\overline{x}$ = 165 × 22 μm, n = 25), 8-spored, bitunicate, fissitunicate, clavate, long pedicellate, base of endoascus long, narrow and coiled within ectoascus, ectoascus uncoiling to form a long tail-like extension, apically rounded with a cylindrical ocular chamber. *Ascospores* 25–32 × 9–13 μm ($\overline{x}$ = 27 × 10 μm, n = 35), ellipsoid-fusiform, 2-seriate, uniseptate, constricted at the septum, apical cell usually longer than basal cell, hyaline when young, becoming brown when mature, guttulate, smooth-walled, lacking a mucilaginous sheath. ***Asexual morph***: Undetermined.

Material examined:—THAILAND, Chiang Rai Province, stream flowing near ThamLuang Nang Non Cave, on submerged wood, 25 November 2014, *Yang Jing*, YJ-4 (MFLU 15–1170, **holotype**); ex-type living culture, MFLUCC 15–0057.

Notes:**—***Helicascus uniseptatus* was collected from decaying wood submerged in a freshwater stream in Chiang Rai Province, Thailand. *H. uniseptatus* is similar to *H. unilocularis* by its unilocular ascostromata and ascospores with a smooth wall and lacking a mucilaginous sheath. However, *H. uniseptatus* differs from *H. unilocularis* in having smaller ascostromata (100–200 versus 200–220 μm), a thinner peridium (40–48 versus 50–65μm), and longer ascospores (25–32 versus 23–25.8 μm). In addition, *H. uniseptatus* is phylogenetically close to *H. elaterascus* in the phylogenetic analysis (FIG. 1), but *H. uniseptatus* differs from *H. elaterascus* in having smaller ascostromata, and smooth ascospore lacking a mucilaginous sheath, while *H. elaterascus* has verruculose ascospores with mucilaginous

sheath. Based on the morphological characters and phylogenetic analysis, we introduce this fungus as a new species in *Helicascus*.

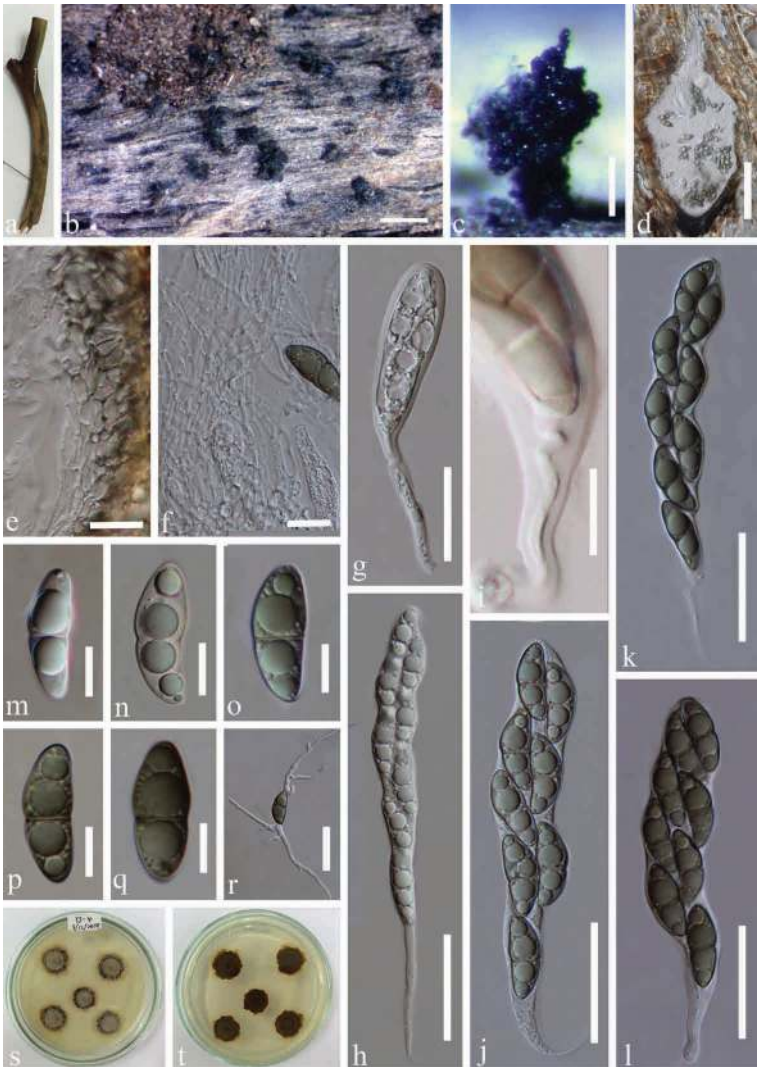

FIGURE 3. *Helicascus uniseptatus* (MFLU 15–1170, holotype) a. Specimen; b. Appearance of masses of ascospores on surface of host; c. Mass of spores on host surface; d. Section of ascoma; **e**. Section of peridium; f. Paraphyses; g–l. Asci; m–q. Ascospores; r. Germinating ascospore; s, t. Culture grow on PDA after 4 weeks, s. upper side, t. reverse side.. Scale bars: b = 1000 μm; c = 200 μm; d = 100 μm; e, f = 15 μm, g, i = 30 μm; h, j–l, r = 40 μm; m–q = 10 μm.

Discussion

The application of molecular data can bring genetic information to define species boundaries in taxonomic studies. Currently, there are eight species accepted in *Helicascus* (Zhang *et al*. 2013, 2014, 2015), all the available sequence data of *Helicascus* species were included in our phylogenic analyses, *Helicascus chiangraiensis* and *H. uniseptatus* are nested in the clade of *Helicascus* (FIG. 1), which is a well-supported clade within the family Morosphaeriaceae. All freshwater species of *Helicascus* clustered in a clade with strong support (91% ML, 99% MP and 1.00 PP) and the marine species *H. nypae* was in another clade in a basal position (FIG. 1).

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