A modern concept for *Helicascus* with a *Pleurophomopsis*-like asexual state

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Helicascus presently comprises two marine species, *H. kanaloanus* and *H. nypae*. During investigations of freshwater ascomycetes in Egypt, Malaysia and northern Thailand, we collected two new species of *Helicascus* from freshwater, which is a new habitat for the genus. *Helicascus aegyptiacus* sp. nov. is characterised by its smaller asci and ascospores with verruculose walls. *Helicascus aquaticus* sp. nov. is distinct in having 1–3-septate ascospores and producing a *Pleurophomopsis*-like anamorph when grown in water agar with sterilized pine needles. During this study we also collected *Massarina thalassioidea* from freshwater and molecular data confirm this as a species of *Helicascus*. Thus, we combine this species under *Helicascus*. All *Helicascus* species, *Massarina thalassioidea* and *Morosphaeria elaterascus* form a strongly supported monophyletic clade and the latter species is also transferred to *Helicascus*. A key to *Helicascus* species is provided.

Keywords: mangrove fungi, *Morosphaeria*, phylogeny, pseudostromata.

Helicascus Kohlm. is a monophyletic genus supported by molecular (Jones *et al.* 2009, Zhang *et al.* 2012 b) and morphological data (Kohlmeyer 1969, Hyde 1991) and presently comprises *H. kanaloanus* Kohlm. and *H. nypae* K. D. Hyde. Both species dwell in mangroves on decaying wood or palms.

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The genus is characterized by forming pseudostroma with several loculi uniting in the centre to form a single common ostiole and having endoasci with a coiling and stretching mechanism at the basal part (Kohlmeyer 1969, Hyde 1991, Zhang *et al.* 2012 b). *Helicascus kanaloanus* has subcylindrical asci, and thick-walled, one-septate ascospores surrounded by a gelatinous sheath, with a septum at the lower part (Kohlmeyer 1969). *Helicascus nypae* is distinct in having smaller asci and ascopores with verrucose walls and a persistent sheath (Hyde 1991).

As part of an ongoing study unraveling links between asexual coelomycetous states with sexual ascomycetes (Dai *et al.* 2012; Hodhod *et al.* 2012; Jami *et al.* 2012; Rungjindamai *et al.* 2012; Wijayawardene *et al.* 2012 a, c; Zhang *et al.* 2012 a, c), we establish the asexual state of a new species of *Helicascus* and show a likely link between *Helicascus nypae* and *Pleurophomopsis nypae* K. D. Hyde & B. Sutton (Hyde & Sutton 1992). In this paper we illustrate this link, introduce two new species of *Helicascus*, transfer *Massarina thalassioidea* and *Morosphaeria elaterascus* to *Helicascus* and amend the description of the genus. Our findings are supported by analysis of partial SSU and LSU rDNA sequence data which also support the monophyly of *Helicascus* and *Morosphaeria* in *Morosphaeriaceae*.

Materials and methods

Morphological study

Submerged wood was collected from Wachiratharn Waterfall (N 18° 31′, E 98° 29′), a lotic freshwater habitat in Chiang Mai Province, Thailand in November 2010 and from the River Nile in Egypt in September 2004 following the procedures described in Kurniawati *et al.* (2010). Samples were placed separately in Ziplock plastic bags with sterile moist tissue paper and transported to the laboratory. Collection site information was recorded in the field. In the laboratory, samples were placed in moist chambers and incubated at room temperature (~25 °C). The woody substrates were examined under a dissecting microscope for fruiting bodies after incubation for one week and up to 2 months (Shearer *et al.* 2004). Observations and photomicrographs were made from material mounted in water or lactic acid (85 %) using a Nikon ECLIPSE 80i microscope. Melzer's reagent (MEZ; 0.5 g iodine, 1.5 g IKI, 20 g chloral hydrate, 20 ml distilled water) and aqueous cotton blue were added to determine staining reactions of the ascus apical apparatus (Raja & Shearer 2008). India ink was used to reveal gelatinous sheaths on or around ascospores. Measurements were made with the Tarosoft (R) Image Frame Work.

Isolations were made from single ascospores and grown on 2 % water agar (WA) (Biolab, S.A.) with sterilized pine needles on the medium to induce sporulation (Chomnunti *et al.* 2011). Isolates were placed at ambient temperatures (about 25 °C) under 12/12 h (light/dark) conditions for establishing colony characteristics (Liu *et al.* 2010). Herbarium specimens have

been deposited at Mae Fah Luang University (MFLU), Chiang Rai, Thailand and cultures study are deposited at Mae Fah Luang University Culture Collection (MFLUCC), Thailand, International Fungal Research & Development Centre Culture Collection (IFRDCC), China and Centraalbureau voor Schimmelcultures (CBS), Netherlands.

DNA Extraction, PCR amplification and sequencing

Fungal isolates were grown on PDA for 14 d at 25 °C in the dark. Genomic DNA was extracted from the fresh mycelium using Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) according to the manufacturer's protocol (Hangzhou, P. R. China).

DNA amplification was performed by polymerase chain reaction (PCR). For nucleotide sequence comparisons fragments of two loci were analysed: LSU and SSU. Primer pairs LROR and LR5 (Vilgalys & Hester 1990) for LSU, NS7R and NS24 (http://www.lutzonilab.net/primers/page244.shtml) for SSU were utilized to amplify. The amplifications were performed in a 50 µl reaction: 1× PCR buffer, 0.2 mM dNTPs, 0.3 µM of each primer; 1.5 mM $MgCl_2$, 0.8 units Taq Polymerase and 5–10 ng DNA (Jeewon *et al.* 2004, Shenoy *et al.* 2007 b). The amplification conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, annealing at 55 °C for 2 min and elongation at 72 °C for 90 s, with a final extension period of 72 °C for 10 min (Liu *et al.* 1999). The PCR products were checked on 1% agarose electrophoresis gels stained with ethidium bromide.

PCR products were then purified using minicolumns, purification resin and buffer according to the manufacturer's protocols (Amersham Biosciences, Buckinghamshire, UK; product code: 27–9602–017). The sequencing of the PCR products was carried out by Shanghai Sangon Biological Engineering Technology & Services Co. Shanghai, P. R. China.

Sequence alignment and phylogenetic analysis

The large and small subunits of the nuclear ribosomal RNA genes (LSU, SSU) were included in the analysis. All additional sequences used in the analysis follow major phylogenies published in Schoch *et al.* (2009) and Zhang *et al.* (2012 b) and were obtained from GenBank (Tab. 1). Sequences were aligned using Bioedit version (Hall 1999) and ClustalX v. 1.83 (Thompson *et al.* 1997). The alignments were checked visually and improved manually where necessary. Phylogenetic analyses were carried out using PAUP v. 4.0b10 (Swofford 2002) for Maximum-parsimony (MP) and MrBayes v. 3.0b4 (Ronquist & Huelsenbeck 2003) for Bayesian analyses. Maximum-parsimony analysis was performed in order to obtain the most parsimonious tree. Trees were inferred using the heuristic search option with 1000 random sequence additions. Maxtrees were setup to 500 and branches of zero length were collapsed and all multiple parsimonious trees were saved. Descriptive tree statistics for parsimony (Tree Length [TL], Consistency Index [CI], Retention

Tab. 1. Species and sequences database accession numbers used in this study (newly generated sequences are indicated in bold).

Name	Voucher/ Culture	GenBank accession no.		
		SSU	LSU	ITS
Aigialus grandis	BCC 18419	GU479838	GU479774	
Aigialus parvus	BCC 32558	GU479843	GU479779	
Amniculicola immersa	CBS 123083	GU456295	FJ795498	
Amniculicola parva	CBS 123092	GU296134	FJ795497	
Ascocratera manglicola	BCC 09270	GU479846	GU479782	
Bimuria novae-zelandiae	CBS 107.79	AY016338	AY016356	
Cochliobolus heterostrophus	CBS 134.39	AY544727	AY544645	
Didymella exigua	CBS 183.55	EU754056	EU754155	
Dothidotthia aspera	CPC 12933	EU673228	EU673276	
Dothidotthia symphoricarpi	CBS119687	EU673224	EU673273	
Eremodothis (Westerdykella) angulata	CBS 610.74	DQ384067	DQ384105	
Falciformispora lignatilis	BCC 21118	GU371835	GU371827	
Helicascus aegyptiacus	**FWCC99**	**KC894852**	**KC894853**	
Helicascus aquaticus	**MFLUCC10-0918**	**KC886640**	**KC886638**	**KC886639**
Helicascus elaterascus	HKUCC 7769	AY787934		
Helicascus kanaloanus	A237	AF053729		
Helicascus nypae 1	BCC 17058	GQ925840	GQ925851	
Helicascus nypae 2	BCC 36752	GU479755	GU479789	
Helicascus thalassioideus	**MFLUCC10-0911**	**KC886636**	**KC886637**	**KC886635**
Kalmusia scabrispora	MAFF 239517	AB524452	AB524593	
Katumotoa bambusicola	CBS 123099	GU296156	GU301823	
Leptosphaerulina australis	CBS 317.83	GU296160	GU301830	
Lentithecium aquaticum	CBS 122367	GU296158	GU301825	
Lentithecium fluviatile	CBS 122367	GU301825	GU296158	
Massarina eburnea	CBS 473.64	GU296170	GU301840	
Melanomma pulvis-pyrius	CBS 371.75	GU349019	GU301845	
Monotosporella tuberculata	CBS 256.84		GU301851	
Montagnula opulenta	CBS 168.34	AF164370	DQ678086	
Morosphaeria ramunculicola 1	BCC 18404	GQ925838	GQ925853	
Morosphaeria ramunculicola 2	BCC 18405	GQ925839	GQ925854	
Morosphaeria ramunculicola 3	MAFF 239641	AB524454	AB524595	
Morosphaeria velatispora 1	JK 5304B	GU479760	GU479794	
Morosphaeria velatispora 2	BCC 17059	GQ925841	GQ925852	
Neottiosporina paspali	CBS 331.37	EU754073	EU754172	
Ophiosphaerella herpotricha	CBS 240.31	DQ678010	DQ678062	
Phaeosphaeria eustoma	CBS 573.86	DQ678011	DQ678063	
Phoma exigua	CBS 431.74	EU754084	EU754183	
Phoma radicina	CBS 111.79	EU754092	EU754191	
Pleomassaria siparia	CBS 279.74	DQ678027	DQ678078	
Pleospora herbarum	CBS 191.86	DQ247812	DQ247804	
Preussia terricola	DAOM 230091	AY544686	AY544726	
Prosthemium betulinum	CBS 127468	AB553644	AB553754	
Prosthemium canba	JCM 16966	AB553646	AB553760	
Pyrenophora phaeocomes	DAOM 222769	DQ499595	DQ499596	
Sporormiella (Preussia) minima	CBS 524.50	DQ678003	DQ678056	
Trematosphaeria pertusa	CBS 122371	GU348999	GU301876	

Index [RI], Relative Consistency Index [RC] and Homoplasy Index [HI]) were calculated for trees generated under different optimality criteria. Kishino-Hasegawa tests (KHT) (Kishino & Hasegawa 1989) were performed in order to determine whether trees were significantly different. Maximum parsimony bootstrap values (MPBP) equal or greater than 50% are indicated in each node (Fig. 1).

The model of evolution was performed by using MrModeltest 2.2 (Nylander 2004). Posterior probabilities (PP) (Rannala & Yang 1996) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes v. 3.0b4 (Huelsenbeck & Ronquist 2001). Six simultaneous Markov chains were run for 1000000 generations and trees were sampled every 100th generation and 10000 trees were obtained. The first 2000 trees, representing the burn-in phase of the analyses, were discarded while remaining 8000 trees used for calculating posterior probabilities in the majority rule consensus tree. Bayesian Posterior Probabilities (BYPP) with those equal or greater than 0.90 are indicated under each node (Fig. 1).

A maximum likelihood analysis was performed at the CIPRES webportal (Miller *et al.* 2010) using RAxML v.7.2.8 as part of the "RAxML-HPC2 on TG" tool (Stamatakis 2006, Stamatakis *et al.* 2008). A general time reversible model (GTR) was applied with a discrete gamma distribution and four rate classes. Fifty thorough maximum likelihood (ML) tree searches were produced in RAxML v. 7.2.7 under the same model, each one starting from a separate randomized tree. The best scoring tree was selected with a final likelihood value of -11521.442400. One thousand non parametric bootstrap iterations were run with the GTR model and a discrete gamma distribution. The resulting replicates were plotted on to the best scoring tree obtained previously. Maximum Likelihood bootstrap values (MLBP) equal or greater than 50 % are given in each node (Fig. 1). Phylogenetic trees were drawn using Treeview v. 1.6.6 (Page 1996).

Results

The combined SSU and LSU data set utilized 47 taxa with *Dothidea sambuci* as the outgroup taxon. The maximum parsimony dataset consists of 2367 total characters; where 1656 characters were constant, 369 variable characters were parsimony-uninformative and 342 characters were parsimony-informative. Kishino-Hasegawa (KH) test showed length = 1562 steps, CI = 0.578, RI = 0.727, RC = 0.420 and HI = 0.422. All trees were similar in topology and not significantly different (data not shown). A best scoring RAxML tree is shown in Fig. 1 with the value of -11521.442400. All *Helicascus* species, *Massarina thalassioideus* and *Morosphaeria elaterascus* form a monophyletic clade with strong support (Fig. 1). We therefore introduce two new species of *Helicascus* and two new combinations of *Massarina thalassioidea* and *Morosphaeria elaterascus* in *Helicascus* based on support in all three computational methods as well as morphological distinction.

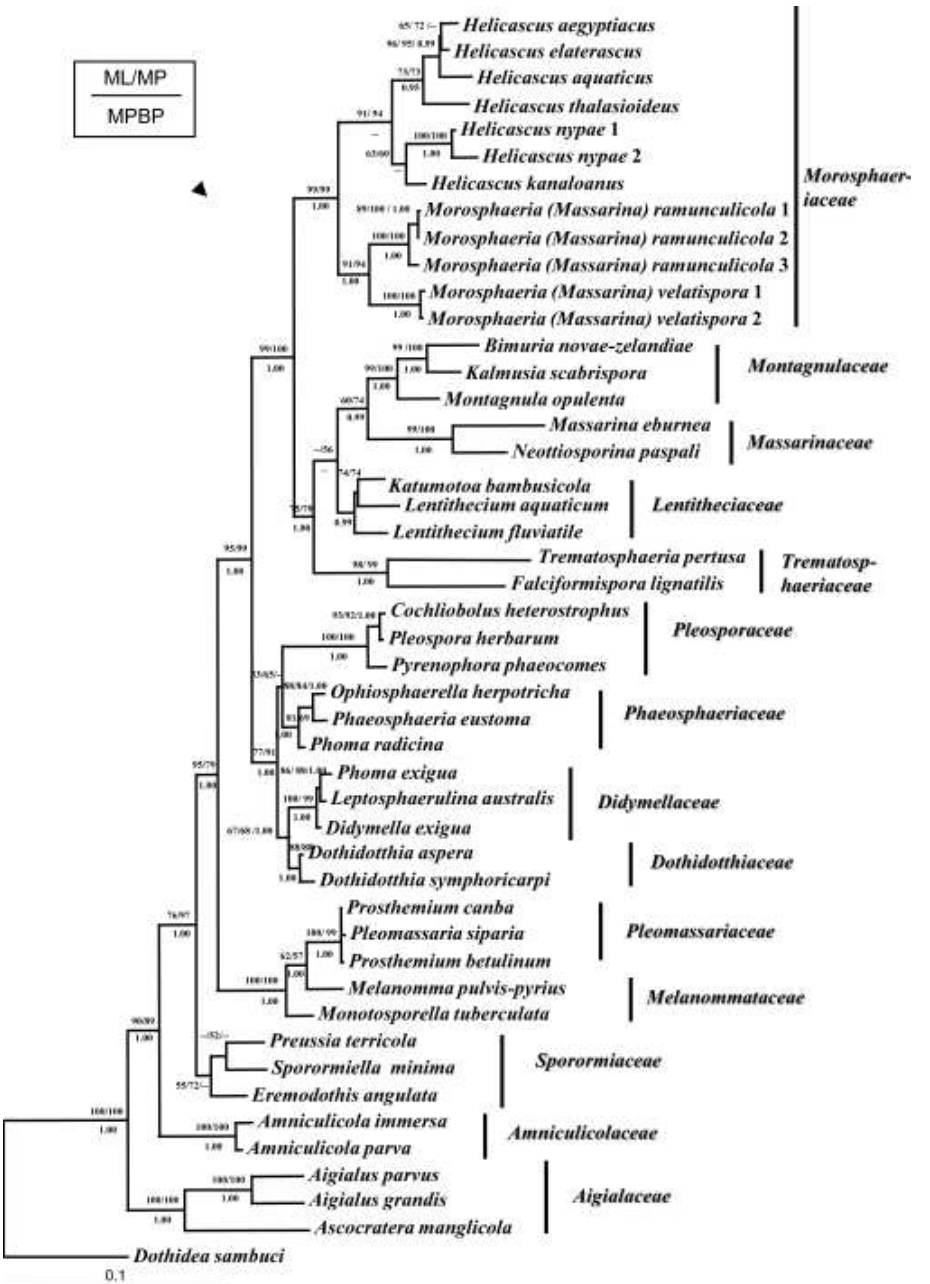

Fig. 1. RAxML tree based on LSU and SSU nrDNA sequences. Bootstrap support values for maximum likelihood (ML) and maximum parsimony (MP) greater than 50 % are above the nodes. The values below the nodes are Bayesian posterior probabilities (BS) above 0.95. Hyphen ("--") values lower than 50 % (MP) or 0.95 (BS). The tree was rooted to *Dothidea sambuci*.

Taxonomy

Helicascus aegyptiacus Abdel-Wahab & Abdel-Aziz, **sp. nov.** – Figs. 2,3.
Mycobank no.: MB 564270

Anamorph. – unknown.

Description. – Pseudostromata 450–950 µm wide, 500–650 µm high, comprising black fungal tissues growing in cortex of host, with 2–3(4) immersed, subglobose to triangular, dark locules, with a flattened base, and a single common ostiole; ostiole 200–260 high × 180–210 µm in diam., periphysate; periphyses 22–33 µm long, 1.2–2.5 µm in diam. Peridium of locules 46–60 µm thick in the upper region, consisting of 6–8 layers of cells of *textura angularis*; 20–30 µm thick in the basal region, comprising 4–7 layers of cells of *textura angularis*; and 180–220 µm thick at sides and angles of the base, comprising cells of *textura prismatica*. Pseudoparaphyses 1.2–2.5 µm wide, numerous, persistent, anastomosing above the asci, embedded in a gelatinous matrix. Asci 50–82 × 15–20 µm, 8-spored, bitunicate, fissitunicate, clavate, long pedicellate, base of the endoascus long, narrow and coiled within ectoascus, apically rounded without an obvious ocular chamber. Ascospores 27–35 × 8–14 µm, 2–3-seriate, subovoid to ellipsoidal, 1-septate, upper cell longer than lower cell, light-brown becoming brown when mature, thin-walled, rough-walled, surrounded by a gelatinous sheath.

Etymology. – In reference to the country where the fungus was first found.

Habitat. – On submerged decaying dicotyledonous wood.

Distribution. – Egypt.

Holotypus. – EGYPT, Sohag Governerate, River Nile, on submerged decaying dicotyledonous wood, 6 September 2004, *leg.* M. A. Abdel-Wahab, MFLU 12-0060 – ex-type living culture FWCC99

Notes. – *Helicascus aegyptiacus* is characterized by pseudostromata, with 2–3(4) immersed locules with a single common central ostiole, fissitunicate asci with a long, narrow and coiled endoascus, and unequally two-celled, verruculose ascospores surrounded by a gelatinous sheath. The morphological characters fit well with the current concept of *Helicascus*. *Helicascus aegyptiacus* is distinct in having thin-walled ascospores with a median to slightly sub-median septum vs. thick-walled ascospores with septa in the lower third of the ascospores in *H. kanaloanus* and *H. nypae* (Kohlmeyer 1969, Hyde 1991). Another freshwater fungus, *Morosphaeria elaterascus* (Shearer) S. Boonmee & K. D. Hyde, has characters in common with *H. aegyptiacus*, including one-septate, verruculose ascospores surrounded by a gelatinous sheath. However, *Morosphaeria elaterascus* has perithecial ascomata (Shearer 1993), while *H. aegyptiacus* has pseudostroma with 2–4 locules.

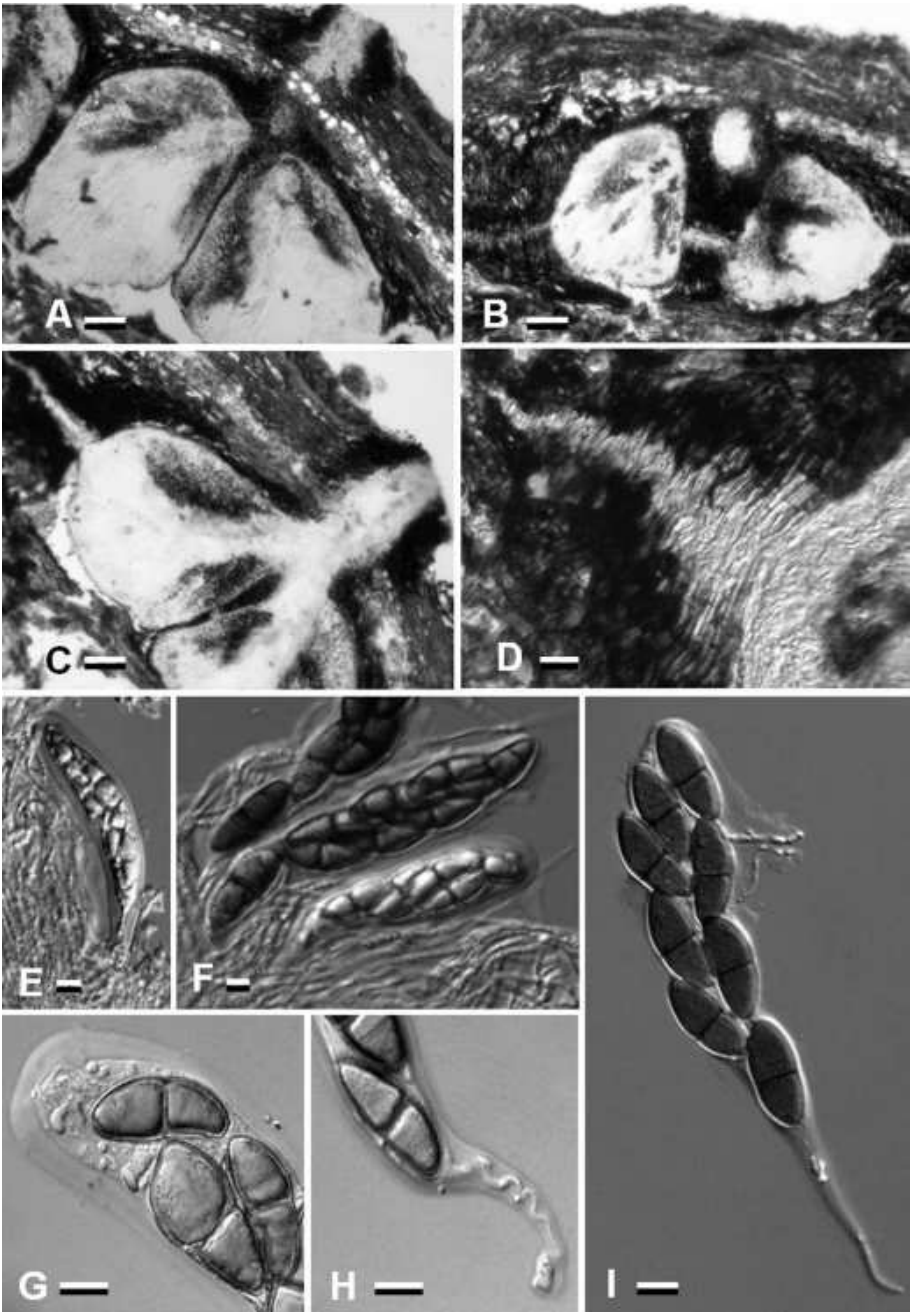

Fig. 2. *Helicascus aegyptiacus*, holotype. **A–C.** Vertical section through stroma showing several locules with one common ostiole. Note: the peridium at the top and the basal part of the stroma is thinner than at the sides of the stroma. **D.** Magnified part of the peridium at the sides showing the *textura prismatic* structure. **E–I.** Asci at different stages of maturation, **H** showing coiled endoascus. Bars: A–C 50 µm, D 25 µm, E–I 10 µm.

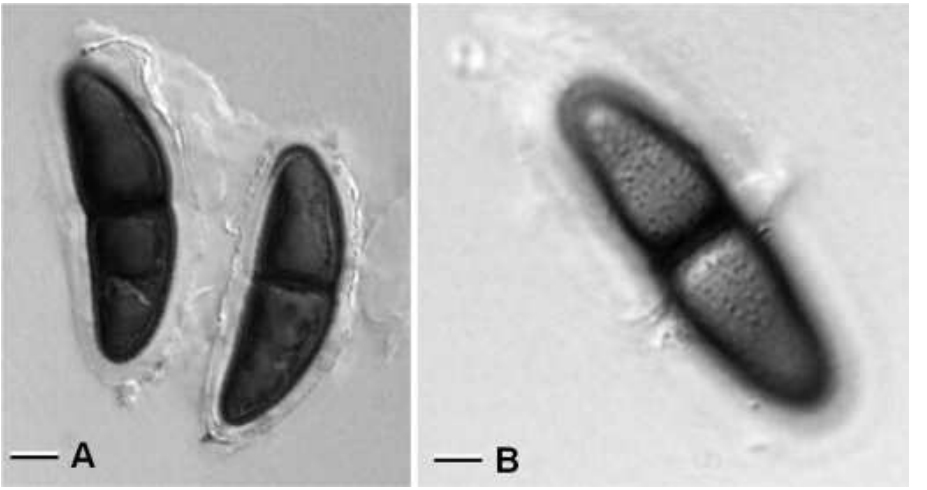

Fig. 3. *Helicascus aegyptiacus*, holotype. **A–B.** Rough ascospores surrounded by prominent gelatinous sheath, **B** showing rough wall. Bar 5 µm.

Helicascus aquaticus Huang Zhang & K. D. Hyde, **sp. nov.** – Figs. 4, 5.
Mycobank no.: MB 803926

Anamorph. – *Pleurophomopsis*-like.

Description. – Pseudostromata 800–1000 µm wide, 200–300 µm high, comprising brown to black fungal tissues growing in cortex of host, enclosing two locules, with flattened base, horizontally arranged under the pseudostroma. Locules 300–400 × 100–200 µm, immersed, lenticular to ampulliform, coriaceous, ostiolate. Ostiole converging at the centre, uniting into one common, central pore, papilla 130–200 × 60–100 µm, cylindrical, black, with periphyses. Peridium of locules, thin at the upper and basal regions, comprising brown-walled cells of *textura angularis*, fusing with the host cells, 20–30 µm wide at the sides, composed of thin-walled, *textura prismatica* cells. Pseudoparaphyses cellular, numerous, hypha-like, septate, embedded in a gelatinous matrix. Asci 90–140 (185) × 15–23 µm ($\bar{x}$ = 120.5 × 19, n = 10), 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 19–26 × 8–11 µm ($\bar{x}$ = 22.5 × 9.3, n = 20), mostly 2-seriate, ellipsoidal, round at the apices, 1-(2- or 3-) septate, slightly constricted at the midseptum, apical cell usually larger than basal cell (10–12 vs. 12–13 µm), brown, thin-walled, guttulate, with a deliquescing sheath.

Pycnidia (formed on water agar on sterilized pine needles within 38–45 days) uniloculate, superficial, with the base immersed, solitary, globose to subglobose, 90–120 µm in diam., wall 2–4 cell layers, covered by mycelium, composed of brown thin-walled cells of *textura angularis*. Conidi-

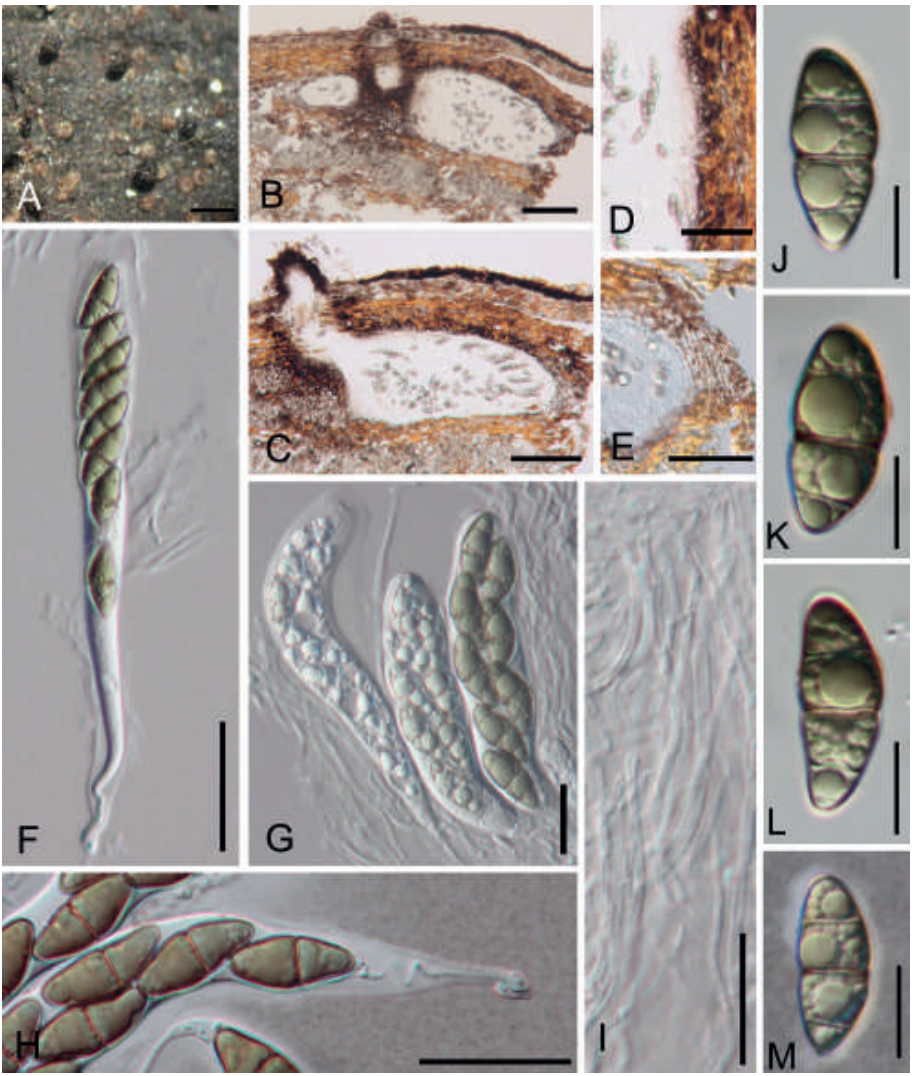

Fig. 4. *Helicascus aquaticus*, holotype. **A.** Ascomata on the host surface. **B.** Pseudostroma. **C.** Section of ascoma. **D.** Upper peridium. **E.** Peridium at the side. **F–H.** Asci. Note long, narrow and coiled base of endoascus. **I.** Pseudoparaphyses. **J–M.** Ascospores. L is in Indian ink. Note the deliquescing sheath in L. Bars: A 300 µm, B–C 100 µm, D–F 40 µm, G–I 20 µm, J–M 5 µm.

ophores reduced to conidiogenous cells. Conidiogenous cells 8–12 × 1–2 µm, holoblastic, cylindrical to subcylindrical, hyaline, hardly differentiated from the inner wall cells. Conidia 3.4–4.3(5.6) × 1.8–2.4 µm ($\bar{x}$ = 4 × 2 µm, n = 10), holoblastic, hyaline, unicellular, occasionally two-celled, ellipsoid to obovoid, thin-walled with 1 or 2 refractive globules, rounded at apex.

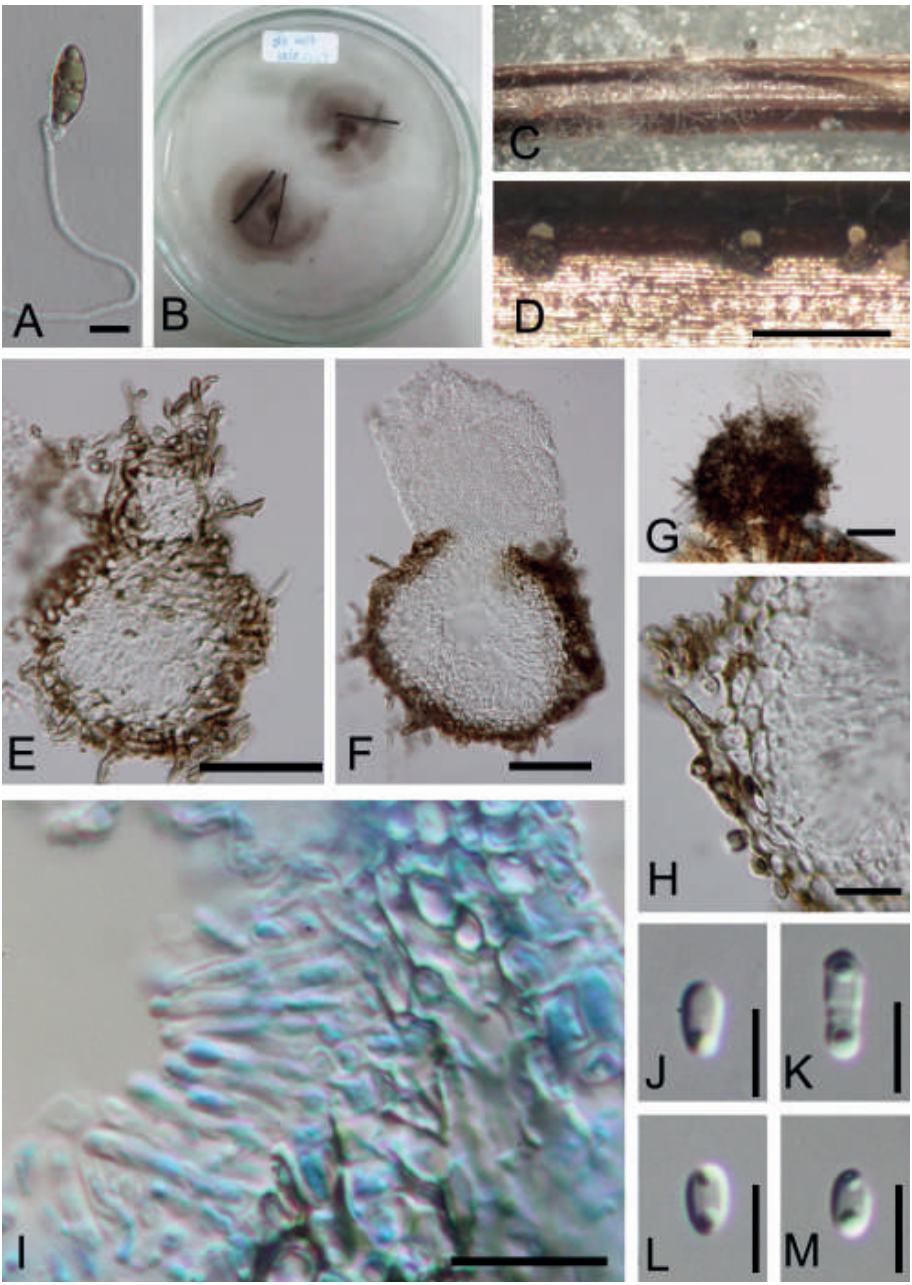

Fig. 5. *Helicascus aquaticus*, holotype. **A.** Ascospore germination. **B.** Colonies on water agar with pine needles. **C–D.** Conidiomata on pine needles. **E–G.** Section of conidiomata. H. Peridium of conidiomata. **I.** Conidiogenous cells. **J–M.** Conidia. Bars: A 10 µm, D 300 µm, E–G 40 µm, H–I 10 µm, J–M 5 µm.

Culture. – Ascospores germinating on WA within 12–24 hours. Colonies on WA, medium sparse, circular, brown to dark brown,1.5 cm diameter in 14 days at 25–28°C, flat, velvety, superficial mycelium, edge entire. Colonies on PDA (potato dextrose agar), dense, circular, brownish in the middle and white on the edge, reaching up to 2 cm diameter in 14 days at 25–28 °C, raised, fluffy, aerial mycelium, edge fimbriate.

Etymology. – From Latin *aquaticus*, in reference to the habitat, where the fungus was found.

Habitat. – On submerged wood.

Distribution. – Thailand.

Holotype. – THAILAND, Chiang Mai Prov., Doi Inthanon, on submerged wood, 16 November 2010, *leg.* Huang Zhang, MFLU11-0942 (holotype) – ex-type living culture MFLUCC10-0918 = IFRDCC2396-d3; ibid, MFLU11-1096 (isotype).

Notes. – *Helicascus aquaticus* is uniquely characterized by pseudostromata with two locules with ostioles converging at the centre, a thin peridium, and ellipsoidal, 1–3-septate, asymmetrical ascospores surrounded by a deliquescing sheath. *Helicascus aquaticus* is similar to *Morosphaeria elaterascus* in the features of asci and ascospores, but differs in having immersed pseudostromata with two locules. The most distinctive feature of *H. aquaticus* among *Helicascus* species is ascospores possessing three septa. Furthermore, *Helicascus kanaloanus* is distinguished from *H. aquaticus* as the former has a pseudostroma enclosing 3–5 loculi and unequally two-celled ascospores (Fig. 7. A–I). *Helicascus aegyptiacus* differs from *H. aquaticus* in having smaller asci, but larger ascospores.

The asexual state of *H. aquaticus* was obtained by using WA with pine needles. It is similar to *Pleurophomopsis nypae* (Hyde & Sutton 1992) in having superfical, globose to subglobose and thin-walled pycnidia, cylindrical and hyline conidiophores and holoblastic, hyaline, mostly unicellular, ellipsoid conidia. However, the pycnidia of *P. nypae* are larger (180–260 µm high, 150–245 µm diam.). *Pleurophomopsis nypae* was described from *Nypa fruticans* as was *Helicascus nypae* and is likely to be the asexual sate of *H. nypae*. Fresh collections and molecular data are required to confirm this.

Helicascus elaterascus (Shearer) Huang Zhang & K. D. Hyde, comb. nov.

Basionym. – *Kirschsteiniothelia elaterascus* Shearer, *Mycologia* 85: 963 (1993).

Synonym. – *Morosphaeria elaterascus* (Shearer) S. Boonmee & K. D. Hyde, *Mycologia* 103: 705 (2012)

MycoBank no.: MB 564329

Notes. – *Helicascus elaterascus* (≡ *Kirschsteiniothelia elaterascus* Shearer) clustered with other *Helicascus* species in a monophyletic clade with strong support (91/94 %, MP/ML), as also shown in Liu *et al.* (2011), Shearer *et al.* (2009), Suetrong *et al.* (2009) and Zhang *et al.* (2012 b). We used the same sequence data for *Kirschsteiniothelia elaterascus* (from strain HKUCC 7769) as Boonmee *et al.* (2012), who transferred the species to *Morosphaeria*, but no *Helicascus* sequence data were used in their paper. When

we removed all *Helicascus* sequences from our analysis (data not shown), *Helicascus elaterascus* clustered together with *Morosphaeria velatispora* with 90 % (MP) support (86 % in Boonmee *et al.* (2012)). When including all available *Helicascus* strains in our analysis *H. elaterascus* clustered with *Helicascus* with 91/94 % (MP/ML) support (Fig. 1). *Morosphaeria velatispora*, the type species of *Morosphaeria* has a pseudostroma similar to *Helicascus* species (Hyde & Borse 1986) and all species form a monophyletic clade with 99 % bootstrap support in *Morosphaeriaceae*, but the genera *Helicascus* and *Morosphaeria* are separated by high support.

Helicascus kanaloanus Kohlm. – Fig. 6.
MycoBank no.: MB 331762

Distribution. – Brunei (Hyde 1988, Hyde 1991), Hawaii (Hyde 1991), Philippine (Jones 1988), Malaysia (Jones & Tan 1987) Thailand (Hyde 1991), Sumatra (Hyde 1989)

Material examined: USA, Hawaii, Oahu, Kaneohe Bay, Heeia Swamp, on *Rhizophora mangle*, 4 June 1968 (Herb. J. Kohlmeyer No. 2566, holotype; No. 2565, 2567, paratype).

Helicascus thalassioideus (K. D. Hyde & Aptroot) Huang Zhang & K. D. Hyde, comb. nov. – Fig. 7

Basionym. – *Massarina thalassioidea* K. D. Hyde & Aptroot, *Nova Hedwigia* 66 (3–4): 498 (1998)
MycoBank no.: MB 803927

Anamorph. – unknown.

Description. – Pseudostromata 130–250 µm high, 100–150 µm wide, 520–780 µm long, comprising brown to black fungal material growing in cortex of host cells, visible on the host surface as blackened ostiolar dots, solitary or gregarious, carbonaceous. Ostiole central, opening rounded, periphysate. Peridium up to 70 µm wide, comprising several layers of thin-walled angular cells, hyaline inwardly and light-brown at the outside. Pseudoparaphyses 1.5–2.3 µm wide, hypha-like, numerous, septate, branched, embedded in a gelatinous matrix. Asci 74–120 × 14–22 µm, 8-spored, bitunicate, clavate to cylindrical, short pedicellate, apically rounded, with a cylindrical apical chamber. Ascospores 25–31 × 7–10 µm ($\bar{x}$ = 26.4 × 9 µm, n = 10), 2-3-seriate, ellipsoidal, 1-septate, symmetrical, slightly constricted at the septum, hyaline, becoming brown when old, lacking a mucilaginous sheath or appendages.

Habitat. – On submerged wood.

Distribution. – Australia (Hyde & Aptroot 1998, Vijaykrishna & Hyde 2006), Brunei (Hyde & Aptroot 1998), Hong Kong (Tsui *et al.* 2000, Ho *et al.* 2001, Tsui *et al.* 2001, Ho *et al.* 2002, Tsui & Hyde 2004), Philippines (Hyde & Aptroot 1998, Cai *et al.* 2003), Thailand (Kurniawati *et al.* 2010), Yunnan (China) (Cai *et al.* 2002, Luo *et al.* 2004).

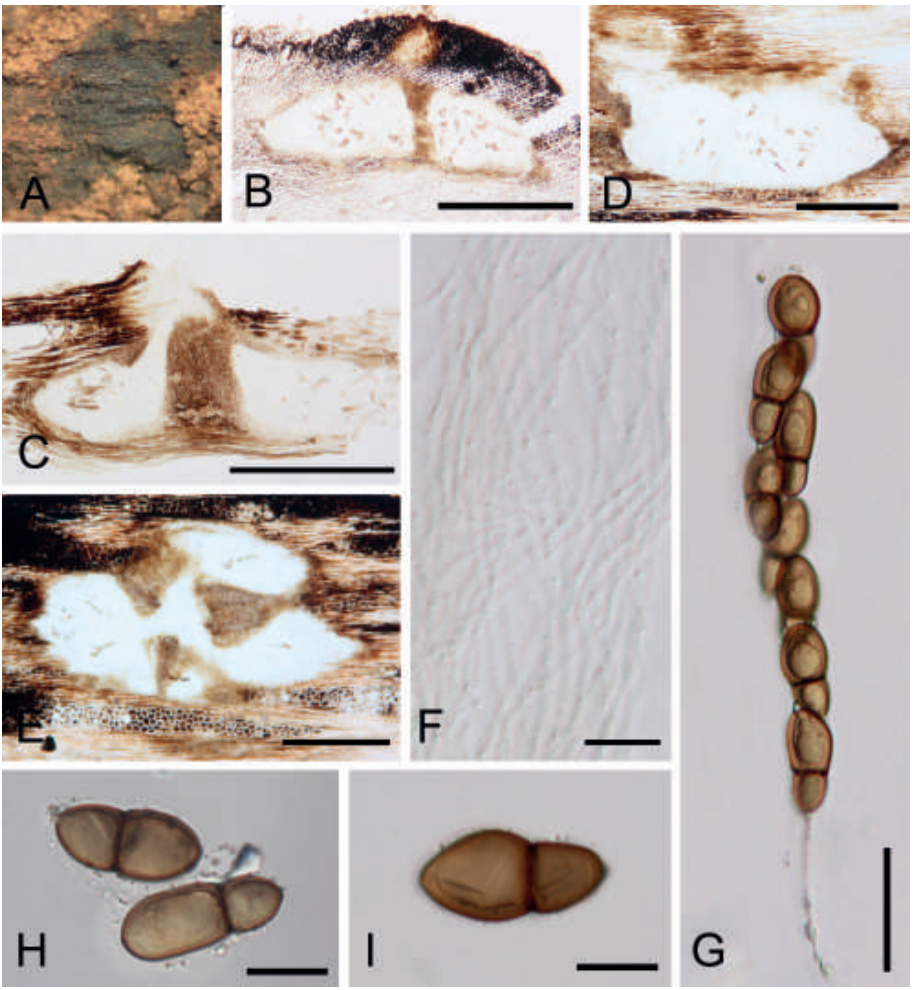

Fig. 6. *Helicascus kanaloanus* (A–C, F, I from holotype NY2566; D, E, G, H from paratype NY2565). **A.** Appearance of ascomata on host surface. **B–E.** Section of ascomata. Note the pseudostroma in E. **F.** Pseudoparaphyses. **G.** Ascus. **H–I.** Ascospores. Walls two layered, with germ pore at each end. Note the gelatinous, dissolving sheath in H. Bars: B–E 500 µm, F 10 µm, G 50 µm, H–I 20 µm.

Material examined: THAILAND, Chiang Rai, Hui Kang Pla Waterfall, on submerged wood, 18 January 2010, *leg.* Huang Zhang A43 (MFLU 10-0155) – ex-living culture MFLUCC10-0911 = IFRDCC 2427; ibid., on submerged wood, 18 January 2010, *leg.* Huang Zhang A45 (MFLU10-0156); ibid., on submerged wood, 18 January 2010, *leg.* Huang Zhang A47 (MFLU10-0157)

Notes. – *Helicascus thalassioideus* was first reported from freshwater habitats of Australia, Brunei and Philippines by Hyde & Aptroot (1998). It was assigned to the genus *Massarina sensu lato,* due to its wide pseudopara-

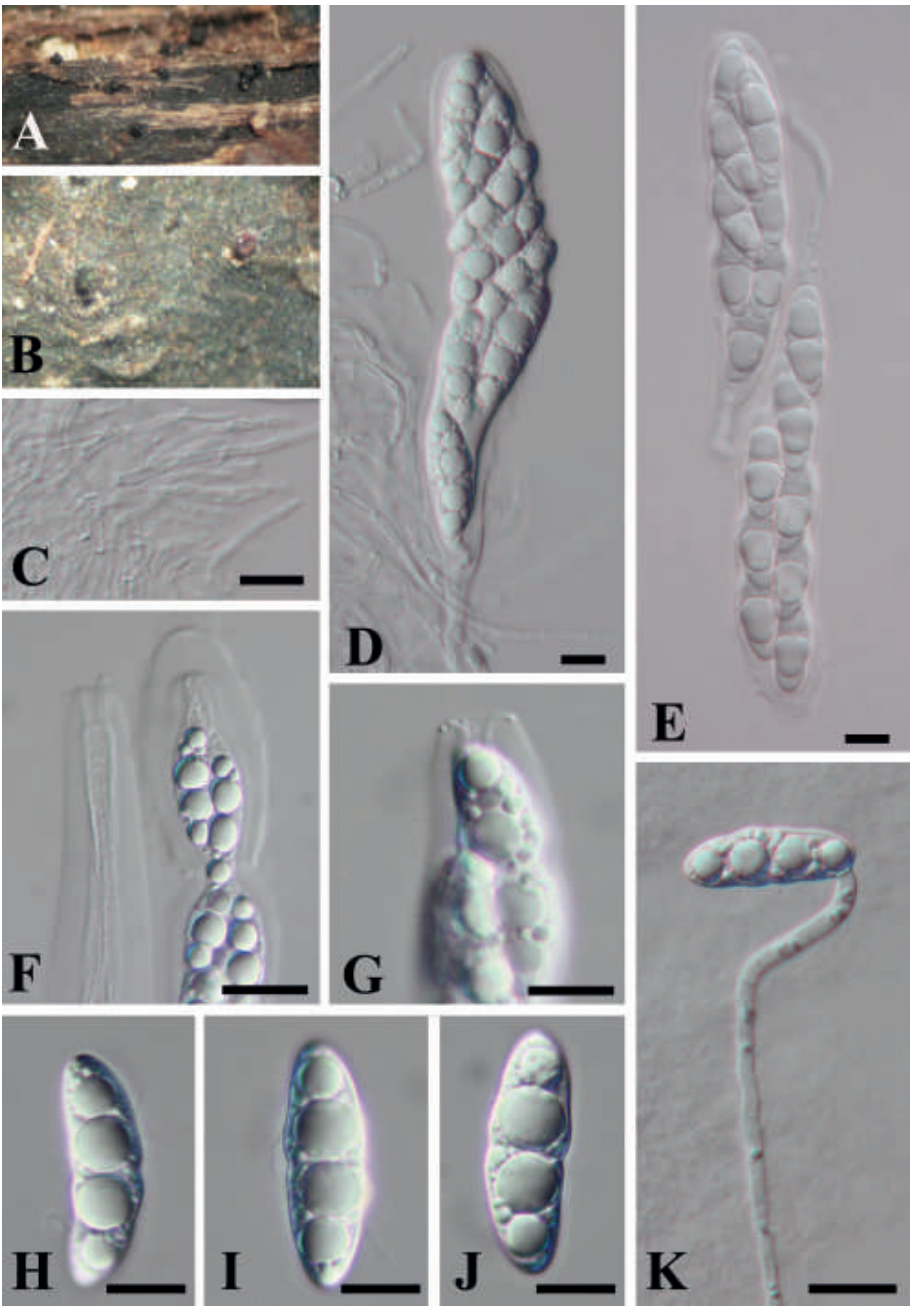

Fig. 7. *Helicascus thalassioideus*. **A–B.** Appearance of ascomata on wood. **C.** Pseudoparaphyses. **D–E.** Asci. **F–G.** Asci releasing ascospores. **H–J.** Ascospores. **K.** Germination of ascospore. Bars: C 15 µm, D–E 10 µm, F–G 15 µm, H–J 10 µm, K 15 µm.

physes, bitunicate asci and hyaline, 1-septate, ellipsoid ascospores. At that time, *Massarina* was poorly understood and comprised about 40 species with hyaline ascospores (Aptroot 1998). Liew *et al.* (2002) narrowed the concept of *Massarina* to include species similar to the type species *M. eburnea* (Tul. & C. Tul.) Sacc. and presently *Massarina* comprises *M. eburnea* and *M. cisti* S. K. Bose (Zhang *et al.* 2009 a, b). *Massarina thalassioidea* is not characteristic of *Massarina* and molecular data confirms its relationship with *Helicascus*.

Helicascus thalassioideus has characteristic lenticular locules under a pseudoclypeus and a single blackened ostiolar canal, and 1-septate ellipsoid ascospores. *H. thalassioideus* can be distinguished from other members of *Helicascus* by its symmetrical and mostly hyaline ascospores (brown only when old).

Key to the species of *Helicascus*

1. Ascospores hyaline when young ..... *H. thalassioideus*
1*. Ascospores coloured 2
2. Ascospores more than one septate..... *H. aquaticus*
2*. Ascospores with one septum 3
3. Ascospores smooth-walled..... *H. kanaloanus*
3*. Ascospores with verruculose walls..... 4
4. Ascospores 23–35 × 12–15 µm, from mangroves ..... *H. nypae*
4*. Ascospores 27–35 × 8–14 µm, from freshwater habitat..... *H. aegyptiacus*

Discussion

In the LSU and SSU phylogenetic tree, *Helicascus aquaticus*, *H. aegyptiacus* and *H. thalassioideus* grouped with other *Helicascus* species in a monophyletic clade with strong support (91/94 %, MP/ML). It is important to establish the sexual-asexual connections in ascomycetes as this characterises the different states in the life-cycle and provides a natural classification (Sivichai & Jones 2003, Shenoy *et al.* 2007a, 2010, Hyde *et al.* 2011, Wijayawardene *et al.* 2012a, b, c). The traditional method for determining the asexual state of ascomycetes is by culturing isolates on agar with substrates, i.e. rice straw or incubating small colony pieces in sterilized water (Tanaka *et al.* 2009, Liu *et al.* 2010) or more recently through molecular sequencing and phylogenetic analysis (Boonmee *et al.* 2011, Hyde *et al.* 2011, Liu *et al.* 2012, Wijayawardene *et al.* 2012 b). The asexual state of *H. aquaticus* was obtained by using pine needles in WA, but it also sporulated on PDA agar after 8 months incubation. It is also likely that *Pleurophomopsis nypae* is the asexual state of *Helicascus nypae* and thus *Pleurophomopsis* should become a synonym of *Helicascus*. We are presently trying to recollect *P. nypae* to confirm this.

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