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Annulatascus saprophyticus sp. nov. and *Pseudoannulatascus* gen. nov. to accommodate *Annulatascus biatriisporus* (Annulatascales, Sordariomycetes) from Thailand

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Abstract

A new *Annulatascus* species, *A. saprophyticus*, found on decaying wood in freshwater in northern Thailand is introduced in this paper. The new taxon is illustrated, described and compared with other species in the genus, as well as a key to genus is provided. It differs from other species in the genus in having straight up right necks at one end, paraphyses embedded in a gelatinous matrix, and 0–3-septate, fusoid to lunate ascospores, which are larger than other species in the genus. Phylogenetic analyses based on LSU gene data showed that *A. saprophyticus* belongs in *Annulatascus sensu strict* (Annulatascaceae, Annulatascales, Sordariomycetidae). Based on the molecular data and a reevaluation of morphology, a new genus *Pseudoannulatascus* in Annulatascaceae is introduced to accommodate *Annulatascus biatriisporus*.

Key words: Annulatascaceae, Aquatic fungi, LSU, Phylogeny, Taxonomy

Introduction

The genus *Annulatascus* was introduced by Hyde (1992) to accommodate two ascomycete species, with *A. velatisporus* K.D. Hyde as the type species, and *A. bipolaris* K.D. Hyde, which were collected from submerged decaying wood in Australia. *Annulatascus* is characterized by having immersed or superficial, black ascomata with long necks, unitunicate, cylindrical asci with relatively massive, refractive, apical rings and fusiform ascospores with appendages or sheaths (Hyde 1992, Boonyuen *et al*. 2012, Hu *et al*. 2012). Presently, 18 species are included in the genus (Barbosa *et al*.2008, Mohamed *et al*. 2011, Boonyuen *et al*. 2012, Hu *et al*. 2012) and most were reported from freshwater habitats in tropical areas (Barbosa *et al*.2008, Shearer *et al*. 2010, Boonyuen *et al*. 2012, Hu *et al*. 2012) and only two species (*A. citrisporus* J. Fröhl. & K.D. Hyde, *A. licualae* J. Fröhl. & K.D. Hyde) are known from terrestrial habitats on palm rachides (Fröhlich & Hyde 2000). Species in the genus *Annulatascus* could be recognized on the basis of morphology of ascospores and the presence or absence of a mucilaginous sheath (Tsui *et al*. 2002).

Abdel-Wahab *et al*. (2011) showed that *A. hongkongensis, A. nilensis*, and *A. velatisporus* clusters in the Annulatascaceae clade, but *A. biatriisporus* K.D. Hyde did not group with them based on the 28S rDNA sequence data, suggesting *Annulatascus* might be polyphyletic.

In this paper, we establish a new genus *Pseudoannulatascus* for the lineage of *Annulatascus biatriisporus* under a reevaluation of morphology and phylogenetic analyses, and also describe and illustrate a new *Annulatascus* species based on morphological characters and phylogenetic analyses of LSU sequence data.

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Materials and methods

Isolation and morphology

The specimens of decaying wood in freshwater were collected in November 2013from a stream in Mushroom Research Centre(N 19°07'13.7", E 98°43'52.9"), in Pha Deng Village, Pa Pae sub-district, Mae Taeng District, Chiang Mai 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 and Hyde (2003). The morphological observations were 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). Herbarium specimens were deposited at the herbarium of Mae Fah Luang University (MFLU) and the herbaria of Kunming Institute of Botany, Chinese Academy of Sciences (HKAS). The pure cultures were deposited in Mae Fah Luang University Culture Collection (MFLUCC) and International Collection of Microorganisms from Plants (ICMP).

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from fresh fungal mycelium grown on PDA at 25°C.The EZ gene™ Fungal gDNA Kit (GD2416) was used to extract DNA according to the manufacturer's instructions. The primer pair LROR and LR7was used to amplify partial large subunits nuclear ribosomal RNA gene (LSU) (Vilgalys & Hester 1990). The PCR thermal cycle program for LSU amplification were as follows: initially 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50 °C for 40seconds, elongation at 72 °C for 90 seconds, and final extension at 72°C for 10 mins.PCR products were purified using minicolumns, purification resin and buffer according to the manufacturer'sprotocols (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 out at Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, P.R. China).

Phylogenetic analysis

Sequences generated from this study were analyzed with other sequences obtained from the GenBank and those derived in Abdel-Wahab *et al*. (2011) and Boonyuen *et al*. (2012). The consensus sequences were then initially aligned using MAFFTv.7 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013)and further improved using Bioedit v.5.0.6 (Hall 2001) and ClustalX v. 1.83 (Thompson *et al*.1997) to allow maximum alignment and maximum sequence similarity.

A maximum likelihood analysis was performed using RAxMLGUI v. 1.3 (Silvestro & Michalak 2011). The optimal ML treesearch 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. Trees were viewed in Treeview (Page 1996).Sequences derived in this study were deposited in GenBank (Table 1).

TABLE 1. Isolates used in this study.

Taxon	Strain number	GenBank Accession number (LSU)
***Annulatascus saprophyticus* Z.L. Luo & K.D. Hyde**	MFLUCC 14-0035	KR868947
A. aquatorba Boonyuen & Sri-indr.	SS 2424	JN226107
A. biatriisporus K.D. Hyde	A 464-3	AY316352
A. hongkongensis W.H. Ho, Ranghoo, K.D. Hyde & Hodgkiss	HKUCC 3702	AF132319
A. nilensis Abdel-Wahab & Abdel-Aziz	IMI 397966	HQ616536
A. velatisporus K.D. Hyde	A 70-18	AY316354
A. velatisporus	HKUCC 3701	AF132320
A. velatisporus	R 047a	AY316355
Annulusmagnus triseptatus (S.W. Wong, K.D. Hyde & E.B.G. Jones) J. Campb. & Shearer	A 413-6B	AY590285
A. triseptatus	A 325-1D	AY590288
A. triseptatus	A 353–1F	AY590289
A. triseptatus	A 54–10A	AY590286
A. triseptatus	A 54-10E	AY590287

...continued on the next page

TABLE 1. (Continued)

Taxon	Strain number	GenBank Accession number (LSU)
Apiosordaria backusii (L.H. Huang) Guarro	ATCC 34568	AY780051
Aquaticola ellipsoidea W.H. Ho, K.M. Tsui, Hodgkiss & K.D. Hyde	A 411-3	AY316356
A. hongkongensis K.D. Hyde & E.C.Y. Liew	HKUCC 3703	AF132321
A. hyalomura W.H. Ho, K.M. Tsui, Hodgkiss & K.D. Hyde	R 038	AY590291
Ascitendus austriacus (Réblová, Winka & Jaklitsch) J. Campb. & Shearer	A 324-1F	AY590294
A.austriacus	A 44–28A	AY590292
A.austriacus	A 324–1B	AY590293
A.austriacus	CBS 102665	AF261067
A. triseptatus	A 413-6B	AY094186
Ascocollumdensa aquatic Ranghoo, K.D. Hyde & E.C.Y. Liew	HKUCC 3707	AF132325
Ascotaiwania mitriformis Ranghoo & K.D. Hyde	HKUCC 3706	AF132324
A. sawada H.S. Chang & S.Y. Hsieh	HKUCC 3705	AF132323
Bionectria ochroleuca (Schwein.) Schroers & Samuels	AFTOL-ID 187	DQ862027
Brunneosporella aquatic Ranghoo & K.D. Hyde	HKUCC 3708	AF132326
Cateractispora recepticuli Ranghoo, K.D. Hyde & E.C.Y. Liew	HKUCC 3710	AF132327
Cercophora appalachianensis O. Hilber & R. Hilber	HKUCC 3711	AF132328
Clohiesia corticola K.D. Hyde	HKUCC 3712	AF132329
Cyanoannulus petersenii Raja, J. Campb. & Shearer	R 044a	AY316358
Fluminicola coronata Ranghoo, K.D. Hyde & E.C.Y. Liew	HKUCC 3717	AF132332
Fusoidispora aquatica Vijaykr., Jeewon & K.D. Hyde	HKU(M) 17484	AY780365
Helvella compressa (Snyder) N.S. Weber	AFTOL-ID 66	AY544655
Hypoxylon subgilvum Berk. & Broome	CM AT-019	DQ840068
Mirannulata samuelsii Huhndorf, F.A. Fernández, A.N. Mill. & Lodge	SMH 1880	AY578353
Nais inornata Kohlm	ATCC 200453	AF539476
Nohea umiumi Kohlm. & Volkm.-Kohlm.	JK 5103F	U46893
Okeanomyces cucullatus (Kohlm.) K.L. Pang & E.B.G. Jones	LP-67	AY490787
Papulosa amerospora Kohlm. & Volkm.-Kohlm.	AFTOL-ID 748	DQ470950
Podospora comate Milovtz.	ATCC 36713	AY780072
Pseudoproboscispora caudae-suis (Ingold) J. Campb., Shearer, J.L. Crane & Fallah	A 40-1A	AY094191
P. caudae-suis	A 336–2D	AY094192
Sordaria fimicola (Roberge ex Desm.) Ces. & De Not.	HKUCC 3714	AF132330
Submersisphaeria aquatica K.D. Hyde	A 95-1B	AY094193
S. aquatica	A 354–1C	AY094194
Trichosphaeria pilosa (Pers.) Fuckel	089319b	AY590297
Vertexicola confuse Ranghoo, K.D. Hyde & E.C.Y. Liew	HKUCC 3715	AF132331
Xylaria hypoxylon (L.) Grev.	ATCC 42768	U47841

Results

Phylogeny

The partial LSU rDNA data set comprised 40 taxa with *Helvella compressa* (AFTOL-ID66) as the outgroup taxon and the manually adjusted dataset comprised942 characters including gaps.Most of the core genera in Annulatascaceae (Zelski *et al*. 2011) were included in our phylogenetic analysis and the best scoring RAxML tree is shown in Figure 1. The LSU rDNA sequence of noval *Annulatascus saprophyticus* aligned with representatives of the Annulatascaceae, along with representatives of Trichosphaeriales, Sordariales, Halosphaeriales and Xylariales. The phylogenetic trees generated by Maximum likelihood (ML) analysis showed that the new taxon clustered within the family Annulatascaceae *sensu strict* as sister taxon to species including *A. hongkongensis* and *A. velatisporus* (the type species of *Annulatascus*) (Fig. 1). *Annulatascus biatriisporus* grouped in a distinct clade apart from the type of the genus (*A. velatisporus*) and form a sister species to the *Cyanoannulus petersenii.* This would suggest that the genus *Annulatascus* could be polyphyletic. However, morphology of the *A.biatriisporus* is quite distinct from the *Annulatascus s. str.,* and therefore, new genus, *Pseudoannulatascus* is introduced.

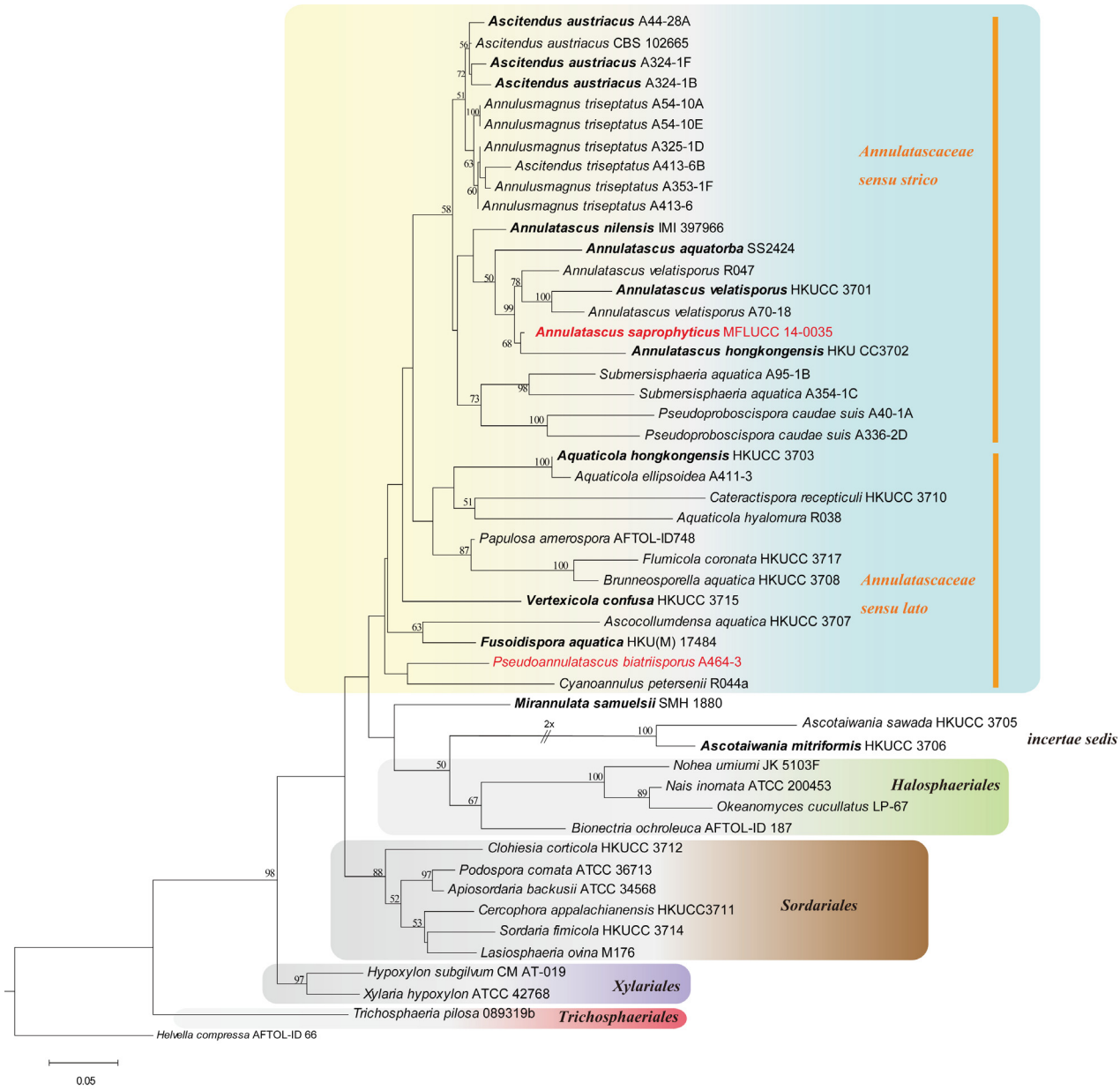

FIGURE 1.Consensus phylogram (50 % majority rule) of 1000 trees resulting from a RAxML analysis of the LSU sequence alignment of *Annulatascus*, *Annulusmagnus*, *Ascitendus* and other genera in family Annulatascaceae. RAxML bootstrap support values (MLB) and maximum parsimony bootstrap support values (MPB) are given at the nodes (MLB/MPB). The scale bar represents the expected number of changes per site. The tree is rooted to *Helvella compressa* (GenBank AY544655).

Taxonomy

Annulatascus saprophyticus Z.L. Luo & K.D. Hyde, *sp. nov.* **FIGURE 2.**

Index Fungorum: IF551104
Facesoffungi number: FoF00591
Etymology: With reference to saprobic life mode of the fungus.
Diagnosis: Differs from other taxa of Annulatascaceae in its larger ascospores
Holotype: MFLU 15–0070

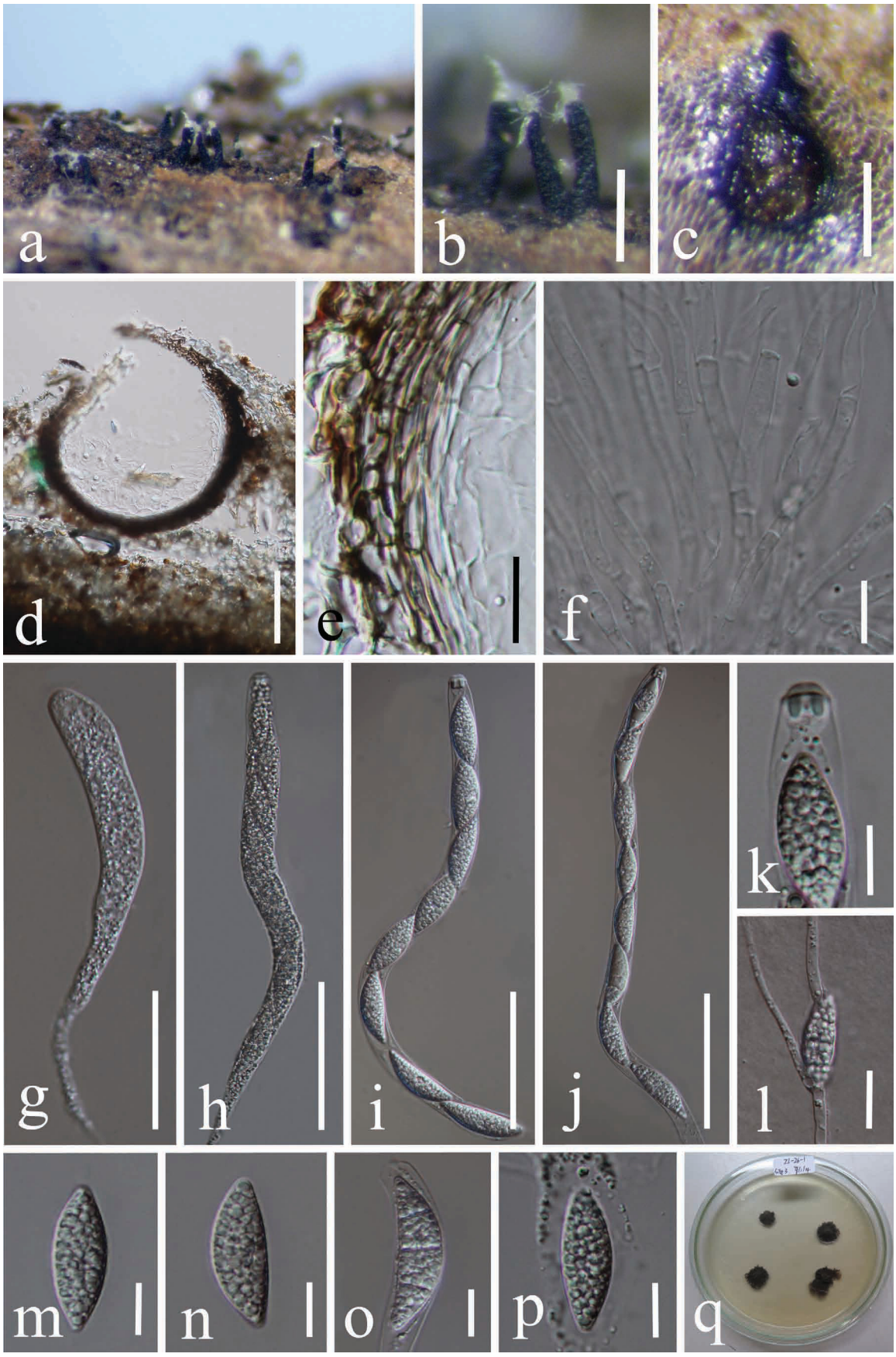

FIGURE 2. *Annulatascus saprophytica* (MFLU 15–0070, holotype). **a**. Ascomata on submerged wood. **b.** Ascomata necks on decaying wood. **c**–**d**. Sections of ascomata. **e**. Section through the peridium. **f**. Paraphyses. **g**–**j**. Asci. **k.** Ascal apical ring in side view. **l**. Germinating ascospore. **m**–**p**. Ascospores. **q**. Culture on MEA. Scale bars : b–c=200µm,d=80µm, h–j=50mm, g=30 µm, e–l =15 µm, f–k, m–p=10µm.

*Saprobic*on decaying wood submerged in freshwater. **Sexual state**: *Ascomata* 350–400 µm high, 230–260µmdiam,perithecioid, gregarious, partly immersed, globose to subglobose,coriaceous,papillate, black. *Ostiole* central, with straight upright necks at one end, black, periphysate.*Peridium* 20–30µm thick, comprising several layers of brown, thick-walled,compressed angularcells, which are hyaline inwardly. *Paraphyses* ca 4.5 µm, hypha-like, numerous, septate, unbranched and taperingdistally.*Asci*230.5–261×13.5–16.5 µm ($\bar{x}$=245.5 × 14.5µm, n=20) 8-spored, unitunicate, cylindrical, apically rounded, pedicellate, with a bilateral apical ring, 2.5–3.5 µm high × 4–5 µm wide. *Ascospores* 26.5–31×10–11 µm ($\bar{x}$= 28.5×10.5µm, n=20), uniseriate, 0–3-septate, fusiform,slightly constricted at the septa, hyaline, minutely verruculose, thick-walled, guttulate, with a thin mucilaginous sheath. **Asexual state**: Undetermined.

Material examined:—THAILAND, Chiang Mai Province, Mushroom Research Center (N 19°07'13.7", E 98°43'52.9", 850–905m), saprobic on decaying wood submerged in stream, November 2013, Zong-Long Luo, ZL-7 (MFLU15–0070, **holotype**), ex-type culture, MFLUCC14-0035, ICMP. *ibid.* (KUN, HKAS 86445, **isotype**).

Notes:—*Annulatascus saprophyticus* is characterized by its immersed, globose to subglobose, black, thick-walled ascomata, cylindrical, unitunicate asci with abilateral apical ring and 0–3-septate, fusiform, uniseriate ascospores. This fungus resembles *A. apiculatus* and *A. velatisporus* in having fusiform, uniseriate, hyaline ascospores with a mucilaginous sheath (Hyde 1992, Barbosa *et al*.2008). However, *A. velatisporus* differs with *A. saprophyticus* in having aseptate ascospores, larger ascomata, and a thicker peridium. *A. apiculatus* differs in the presence of an apiculus at both ends of the ascospores (Barbosa *et al*. 2008). Sequences of *A. saprophyticus* form a sister clade to *A. hongkongensis*, but *A. saprophyticus* differs with *A. hongkongensis* in having 0–3-septate and smaller ascospores (26.5–31×10–11 µm, versus 35–37.5 × 12.5–15µm).

Pseudoannulatascus Z.L. Luo, Maharach. & K.D. Hyde, *gen. nov*

Index Fungorum: IF551105
Facesoffungi number: FoF00603
Etymology: Named after its morphological similarity to *Annulatascus*.

Saprobic on decaying wood submerged in freshwater. **Sexual state**: *Ascomata*195–325µm high, 390–520µmdiam, immersed, ellipsoidal, solitary or gregarious,coriaceous, black. *Ostiole* central, with long, cylindrical necks, ca 300 µm long, 100 µm diam, black, periphysate. *Peridium*ca 50 µm thick, brown-black, coriaceous, comprising an inner layer of hyaline, thin–walled and an out layer of dark textura angularis. *Paraphyses* up to 4 µm thick at the base, hypha-like, numerous, septate and tapering distally. *Asci*210–260 ×12–17µm, 8-spored, unitunicate, cylindrical, apically rounded, with a short pedicel which tapers to a fine point, with a large apical ring, 3–4 µm high × 7–8 µm wide. *Ascospores*40–58 × 8–10µm, overlapping uniseriate, aseptate, long fusiform with weakly swollen ends, 1-celled, hyaline,with a thin mucilaginoussheath.**Asexual state**: Undetermined.

Notes:—*Pseudoannulatascus* is characterized by its immersed, ellipsoidal, black, coriaceous ascomata; cylindrical, unitunicate asci with a large apical ring and aseptate, long fusiform, overlapping uniseriate ascospores with weakly swollen ends. *Pseudoannulatascus biatriisporus* resembles all the species in the genus *Annulatascus* in having refractive, sphaerical ring at the ascus apex. But *Pseudoannulatascus biatriisporus* differs in having larger asci, larger, long fusiform ascospores with swollenends (Hyde 1995, Boonyuen *et al*. 2012). The *Pseudoannulatascus* is also similar to genus *Conlarium*, but the genus *Pseudoannulatascus* have a larger ascomata, paraphyses embedded in a matrix, larger asci, aseptate, long fusiform ascospores with swollenends (Hyde 1995, Liu *et al*. 2012). Abdel-Wahab *et al*. (2011) and Boonyuen *et al*. (2012) showed that *A. hongkongensis, A. nilensis*, and *A. velatisporu* clusters in the Annulatascaceae clade, but *Pseudoannulatascus biatriisporus* grouped in a separate clade sister to *Cyanoannulus petersenii* based on the 28S rDNA sequence data. Based on the sequence data and the distinct morphology, we treat this as a distinct genus in Annulatascaceae.

Type species: ***Pseudoannulatascus biatriisporus*** (K.D. Hyde) Z.L. Luo, Maharach. & K.D. Hyde, *comb.nov*

Index Fungorum: IF551106
Facesoffungi number: FoF00604
Basionym: *Annulatascus biatriisporus* K.D. Hyde, *Nova Hedwigia* 61(1–2): 119–140.

Key to the species of *Annulatascus*

1. Ascomatamilky, ascospres 24–28× 6–8µm, fusiform ..... *A. lacteus*
1. Ascomata black to dark brown 2
2. Ascospores aseptate 3
2. Ascospores septate 9
3. Ascospores small, 15–17.8 × 3.8–5µm, fusiform-rhomboid, found on plams ..... *A. licualae*
3. Ascospores larger, width often greater than 5µm 4
4. Ascospores ellipsoid to citriform 5
4. Ascospores mostly fusiform 6
5. Ascospores ellipsoid, 20–28 × 9–12µm ..... *A. joannae*
5. Ascospores citriform, 22.5–30.5 × 6.4–8.5µm, found on palms ..... *A. citriosporus*
6. Ascospores 26–42 × 9–12µm, fusiform, surrounded with thick, conspicuous mucilaginous sheath ..... *A. velatispora*
6. Ascospores smaller 7
7. Ascospores 21–30 × 6.5–8.5µm, surrounded with a mucilaginous sheath and a polar appendage ..... *A. bipolaris*
7. Ascospores with acute ends 8
8. Ascospores 21–25 × 8–10µm, with acute ends, without sheath or appendages ..... *A. menglensis*
8. Ascospores 19–24 × 6–7µm, with acute ends, with 2–4 large lipid globules ..... *A. aquaticus*
9. Ascospores 0–5-septate 10
9. Ascospores 32–52 × 7–10µm, 5–11-septate, constricted at the septate, surrounded by a large, irregular granular sheath ..... *A. nilensis*
10. Ascospores 16.5–25.5 × 6–9µm, fusiform, 1–5-septate, with bipolar pad-like appendages ..... *A. fusiformis*
10. Ascospores mostly 0–3-septate, lacking bipolar pad-like appendage, but may be surrounded by a mucilaginous sheath 11
11. Ascospores with mucilaginous sheath 12
11. Ascospores without mucilaginous sheath 6
12. Ascospores 35–37.5 × 12.5–15µm, 3-septate, ellipsoid with acute ends ..... *A. hongkongensis*
12. Ascospores without acute ends 13
13. Ascospores 23–36.5 × 8.8–10µm, 0–3-septate, fusiform, not constricted at septa ..... *A. apiculatus*
13. Ascospores obviously or slightly constricted at septa 14
14. Ascospores 18–33 × 6–12µm, 3-septate, with mucilaginous sheath verruculous at EM level ..... *A. triseptatus*
14. Ascospores with mucilaginous sheath but not verruculous at EM level 15
15. Ascospores 15–22.5 × 6.5–7.5µm, unicellular when young, becoming 2-septate at maturity ..... *A. liputii*
15. Ascospores 26.5–31 × 10–11µm, 0–3-septate, guttulate ..... *A. saprophyticus*
16. Ascospores relatively large, 42.5–52.5 × 7.5–10µm, 1–3-septate, fusiform ..... *A. tropicalis*
16. Ascospores smaller 17
17. Ascospores 15–22.5 × 5–7.5µm, 3-septate, slightly constricted at the septa, central cells brown, end cells with rounded apices, subhyaline ..... *A. aquatorba*
17. Ascospores 20–26 × 6–7µm, 3-septate, fusiform with blunt ends ..... *A. palmietensis*

Discussion

The application of molecular data can bring solid genetic information to define species boundaries in taxonomic studies. There are 18 *Annulatascus* names (Index Fungorum 2015) and only *A. aquatorba*, *A. hongkongensis*, *A. biatriiporus*, *A. nilensis*, and *A. velatisporus* have sequence data in GenBank. Therefore, further collections, isolation and sequence data are required to resolve the taxonomic assignment of individual species in this genus. Many of the genera in Annulatascaceae are not well resolved with LSU sequence data. Therefore further gene sequences are needed to determine genetic variation within the group.

Based on the morphological features, *Annulatascus saprophyticus* is referred to the genus *Annulatascus* based on its morphological characters of black ascomata with a long neck, unitunicate, cylindrical asci with relatively massive, refractive apical rings and fusiform ascospores with appendages or sheaths (Hyde 1992, Boonyuen *et al*.2012). Many authors used morphological characters such as dimensions of the ascomata, asci, ascospores and the substrata they naturally colonize to separate the taxa in *Annulatascus* (Tsui *et al*. 2002, Barbosa *et al*. 2008, Abdel-Wahab *et al*. 2011, Hu *et al*. 2012, Boonyuen *et al*. 2012). Out of them the most pronounced differences are in the size of the ascus and apical ring, ascospore septation and the presence of a mucilaginous sheath (Tsui *et al*. 2002, Boonyuen *et al*.2012). *A. saprophyticus* differs from other species in the genus by having a larger asci and ascospores. *A. saprophyticus* resembles other freshwater ascomycetes: *Annulusmagnus triseptatus* (Campbell & Shearer 2004). However, *A. triseptatus* differs from *A. saprophyticus* in having ascomata with short, setose-like hyphae, and ascospores which are 3-septate, broad-fusiform, slightly flattened on one side, becoming straw-coloured or brown with age.

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