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Morpho-molecular characterization and epitypification of *Annulatascus velatisporus*

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Abstract

The holotype of *Annulatascus velatisporus*, the type species of the genus *Annulatascus*, which is the core species of *Annulatascaceae* (*Annulatascales*) is in poor condition as herbarium material has few ascomata and molecular data could not be generated. Therefore, an epitype is herein designated for the precise delineation of this taxon. A morphological description and DNA characterization are provided for the epitype, obtained from the holotype location. Therefore, this specimen merits use as an interpretive type (epitype) for the holotype of *A. velatisporus* collected from submerged wood in a river, at Milaa Milaa Falls, north Queensland.

Keywords – aquatic fungi – phylogeny – submerged wood – taxonomy – type species

Introduction

The genus *Annulatascus* was introduced by Hyde (1992) to accommodate *A. bipolaris* K.D. Hyde, with *A. velatisporus* as the type species. Both species are associated with submerged decaying wood in Australia. Later, *A. bipolaris* was synonymized under *Cataractispora bipolaris* (K.D. Hyde) K.D. Hyde, S.W. Wong & E.B.G. Jones on the basis of its ascospore appendage ontogeny by Hyde et al. (1999). Morphologically *Annulatascus* species possess dark brown to black ascomata with long necks, unitunicate, long 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, Maharachchikumbura et al. 2016). Currently this genus comprises 19 species (Barbosa et al. 2008, Boonyuen et al. 2012, Hu et al. 2012, Luo et al. 2015), most of which are described from freshwater habitats in the tropics (Barbosa et al. 2008, Shearer et al. 2010, 2015). However, two species were discovered on palm rachides on land (Fröhlich & Hyde 2000).

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The holotype (BRIP 17373) of *A. velatisporus* that was deposited at the Plant Pathology Herbarium, Queensland is in poor condition with insufficient tissues (only a few of dried, empty ascomata are available on the holotype) from which the extraction of DNA is not possible and the ex-type culture is lost. As well, no previous molecular data from the type is available to clarify its phylogenetic placement. Three subsequent records of *A. velatisporus* are available in GenBank with LSU gene sequences (Raja et al. 2003) and recent phylogenetic analysis by Luo et al. (2015) showed their placements within the family *Annulatascaceae* as a sister taxon to *A. saprophyticus* Z.L. Luo & K.D. Hyde and *A. hongkongensis* W.H. Ho et al. However, no sequences are derived from a specimen collected from the location of the holotype herbarium material. The family *Annulatascaceae* based on *Annulatascus* as circumscribed is polyphyletic (Campbell and Shearer 2004). Furthermore, as *Annulatascaceae* is common and important on submerged wood in freshwater (Tsui et al. 2000, Ho et al, 2001, 2002, Cai et al. 2003), all genera need further study at the molecular level including *Annulatascus*.

In this study an epitype (*sensu* Ariyawansa et al. 2014) is designated, for *A. velatisporus* collected from the Millaa Millaa falls watershed, North Queensland, that is identical morphologically to the holotype material from same location. Descriptions, illustrations and combined LSU and SSU phylogenetic analysis are also provided.

Material and methodology

Sample collection, specimen examination

Pieces of decaying wood were collected from four sites in the Mulgrave river, North Queensland. The samples were collected by walking in the river and randomly picking up submerged, decaying woody debris. Pieces of wood less than 10-15 cm were picked up and put into a plastic zip-lock bags. The samples were then returned to the laboratory where they were placed into individual plastic containers and kept moist with sterile distilled water. Specimens were observed and examined with a Motic SMZ 168 stereomicroscope. Micro-morphological characters of the taxon were examined under a Nikon ECLIPSE 80i compound microscope and images were captured using a Nikon ECLIPSE 80i compound microscope with a Canon EOS 600D digital camera. Observations and photographs were made from material mounted in water and stained with Melzer's reagent. Measurements were made with the Tarosoft (R) Image Frame Work and images used for figures were processed with Adobe Photoshop CS3 Extended version 10.0 software. Isolates were derived by single spore isolation following the method of Chomnunti et al. (2014). Germinating ascospores were transferred to potato dextrose agar (PDA) after 12 h. Cultural characteristics, such as mycelium colour, shape, texture and growth rate, were recorded after incubating at 25 °C under normal light for a week. The herbarium specimens of the epitype (MFLU16-2204) was deposited at Mae Fah Luang University Herbarium and Adelaide Herbarium, while ex-epitype living cultures are deposited at Mae Fah Luang University culture collection (MFLUCC16-1441). Herbarium specimens of *A. velatisporus* PE0011-9a, PE0011-9b and PE0011-9c strains were deposited at the Fungarium of the University of Illinois (ILL 41204). *Facesoffungi* and Index Fungorum numbers are provided (Jayasiri et al. 2015, Index Fungorum 2016).

DNA extraction, PCR amplification and sequencing

The Biospin Fungus Genomic DNA Extraction Kit (BioFlux®, China), (Hangzhou, P. R. China) was used to extract DNA from herbarium material as well as fungal cultures grown on PDA for 14 days at 25 °C following manufacturer's instructions. The primer pair LROR and LR7 was used to amplify the partial large subunit nuclear ribosomal RNA gene (LSU) (Vilgalys & Hester 1990). The small subunit nuclear ribosomal RNA (SSU) gene was amplified by primer pairs NS1/NS4 (White et al. 1990). The amplification reactions were carried out in 25 µl PCR mixture contained 1.0µl of DNA template, 1 µl of each primers, 12.5 µl of 2× Easy Taq PCR SuperMix (mixture of EasyTaqTM DNA Polymerase, dNTPs, and optimized buffer, Beijing Trans Gen Biotech Co., Ltd., Chaoyang District, Beijing, PR China) and 9.5 µl of sterilized water.

Table 1 Taxa used in the phylogenetic analyses and their GenBank accession numbers. The newly generated sequences are indicated in blue and ex-type isolates are in bold.

Taxon	Voucher	GenBank Accessions	
		LSU	SSU
Amplistromataceae			
Amplistroma caroliniana	BEO9923	FJ532377	
A. erinaceum	**AH 43902**	**KC907374**	
A. longicollis	AH37870	HQ901790	
Annulatascaceae			
A. c.f. triseptatus	A413-6B	AY094186	-
A. hongkongensis	**HKUCC 3702**	**AF132319**	
A. nilensis	**IMI 397966**	**HQ616536**	-
A. saprophyticus	**MFLUCC 14-0035**	**KR868947**	-
***A. velatisporus* (herbarium)**	**MFLU16-2204**	**KX772397**	**KX772398**
***A. velatisporus* (culture)**	**MFLUCC16-1441**	**KY244031**	**KY244032**
A. velatisporus	R047	AY316355	-
A. velatisporus	HKUCC 3701	AF132320	-
A. velatisporus	A70-18	AY316354	-
A. velatisporus	PE0011 9a	KX977085	KX977088
A. velatisporus	PE0011 9b	KX977086	KX977089
A. velatisporus	PE0011 9c	KX977087	KX977090
A. triseptatus	A54-10E	AY590287	-
A. triseptatus	4325-1D	AY590288	-
A. triseptatus	A353-1F	AY590289	-
A. triseptatus	A413-6	AY590285	-
A. triseptatus	**CBS 128831**	**-**	**JQ429242**
A. triseptatus	MFLU 15-2681	KX998506	KX998507
Aquaticola ellipsoidea	A411-3	AY316356	-
A. hongkongensis	**HKUCC 3703**	**AF132321**	**-**
A. hyalomura	R038	AY590291	-
Ascitendus austriacus	A324-1F	AY590294	-
A. austriacus	A324-1B	AY590293	-
Ascobrunneispora aquatica	HKUCC 3708	AF132326	-
*Ascocollumdensa aquatica**	HKUCC 3707	AF132325	-
Ascolacicola austriaca	CBS 102665	AF261067	AF242263
Cataractispora recepticuli	**HKUCC 3710**	**AF132327**	**-**
Cyanoannulus petersenii	R044a	AY316358	-
Fluminicola coronata	HKUCC 3717	AF132332	-
Fusoidispora aquatica	HKU(M) 17484	AY780365	-
Pseudoproboscispora caudae-suis	A40-1A	AY094191	-
P. caudae-suis	A336-2D	AY094192	-
Submersisphaeria aquatica	A95-1B	AY094193	-
S. aquatica	A354-1C	AY094194	-
Vertexicola caudatus	HKUCC 3715	AF132331	-
Magnaporthaceae			
Bussabanomyces longisporus	CBS 125232	KM009154	KM009214
Magnaporthe salvinii	M21	JF414887	JF414862
Ophioceraceae			
Ophioceras dolichostomum	**CBS 114926**	**JX134689**	**JX134663**
O. dolichostomum	HKUCC 10113	DQ341507	DQ341485
Papulosaceae			
Papulosa amerospora	AFTOL ID 748	DQ470950	DQ470998
Pyriculariaceae			
Pyricularia borealis	CBS 461.65	-	KM009210
***Helvellaceae* (Out group)**			
Helvella compressa	AFTOL-ID 66	AY544655	AY544699

* = Undescribed ascomycete species belonging to the *Annu1atascaceae* which have black perithecia with long necks, cylindrical asci with a relative large refractive apical ring and ascospores with appendages.

Abbreviations – **AFTOL:** Assembling the Fungal Tree of Life, **ATCC:** The American Type Culture Collection, Manassas, **CBS:** Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, **HKUCC:** University of Hong Kong Culture Collection, Hong Kong, **MFLUCC:** Mae Fah Luang University Culture Collection, Thailand.

The PCR thermal cycle program for LSU and SSU amplification were as follows: initially denature 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for 40 seconds, elongation at 72 °C for 90 seconds, and final extension at 72 °C for 10 minutes. PCR products were purified using minicolumns, purification resin and buffer according to the manufacturer's protocols (Amersham product 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 used in this study were derived from GenBank and following the recent publications (Abdel-Wahab et al. 2011, Barbosa et al. 2008, Boonyuen et al. 2012, Luo et al. 2015, Maharachchikumbura et al. 2015, 2016) (Table 1). Only SSU and LSU sequences were incorporated for phylogenetic analyses due to the availability of sequenced data of available strains in GenBank. SSU and LSU gene sequences were deposited in GenBank (Table 1) together with one ITS barcode sequence (KX772399, KY320183) as an aid for future studies on this species. DNA sequences for each locus were initially aligned using MAFFTv.7 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013) and optimized manually when needed. Gaps were treated as missing data.

Phylogenetic analyses of the sequence data consisted of maximum likelihood (ML) and Bayesian inference (BI) analyses of the combined dataset. Maximum likelihood analysis was performed using RAxMLGUI v. 1.3 (Silvestro & Michalak 2011) and 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. Bayesian analysis was performed using MrBayes v. 3.0b4 (Ronquist & Huelsenbeck 2003). Nucleotide substitution models were determined with MrModeltest v. 2.2 (Nylander 2004). Posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were defined by Bayesian Markov Chain Monte Carlo (BMCMC) sampling method in MrBayes v. 3.0b4 (Huelsenbeck & Ronquist 2001). Six simultaneous Markov chains were run for 4000000 generations and trees were sampled every 100th generation resulting in 10000 total trees. 8000 trees were used for calculating posterior probabilities (PP) in the majority rule consensus tree, after discarding the first 2000 trees representing the burn-in phase (20 %) of the analysis. Resulting trees were visualized with TreeView v. 1.6.6 (Page 1996). Resulting trees were viewed in FigTree v. 1.4 (Rambaut 2009).

Results

Phylogeny

The combined SSU and LSU data set comprised 46 taxa with *Helvella compressa* (AFTOL-ID66) as the outgroup taxon. Most of the core genera in *Annulatascaceae* (Zelski et al. 2011) and strains from the families *Papulosaceae*, *Amplistromataceae*, *Magnaporthaceae* and *Pyriculariaceae* were included in the analysis and the best scoring RAxML tree (-9954.268237) is shown in Figure 1. Both ML and BI analyses generated trees with similar in topology.

Phylogenetic analyses showed that epitype of *Annulatascus velatisporus* grouped together with the other *A. velatisporus* isolates (PE0011-9a, PE0011-9b, PE0011-9c A70-18 and HKUCC 3701, R047) within family *Annulatascaceae sensu stricto* (Fig.1). *Aquaticola ellipsoidea* (A411-3), *Aq. hongkongensis* (HKUCC 3702), *Aq. hyalomura* (R038), *Cataractispora receptacularum* (HKUCC 3710), *Ascobrunneispora aquatica* (HKUCC 3708), *Fluminicola coronata* (HKUCC 3717), *Vertexicola confusa (cauadata)* (HKUCC 3715), *Ascocollumdensa aquatic* (HKUCC 3707), *Fusoidispora aquatica* (HKU(M) 17484), and *Cyanoannulus petersenii* (R044a), grouped in distinct clades apart from *Annulatascaceae sensu stricto*.

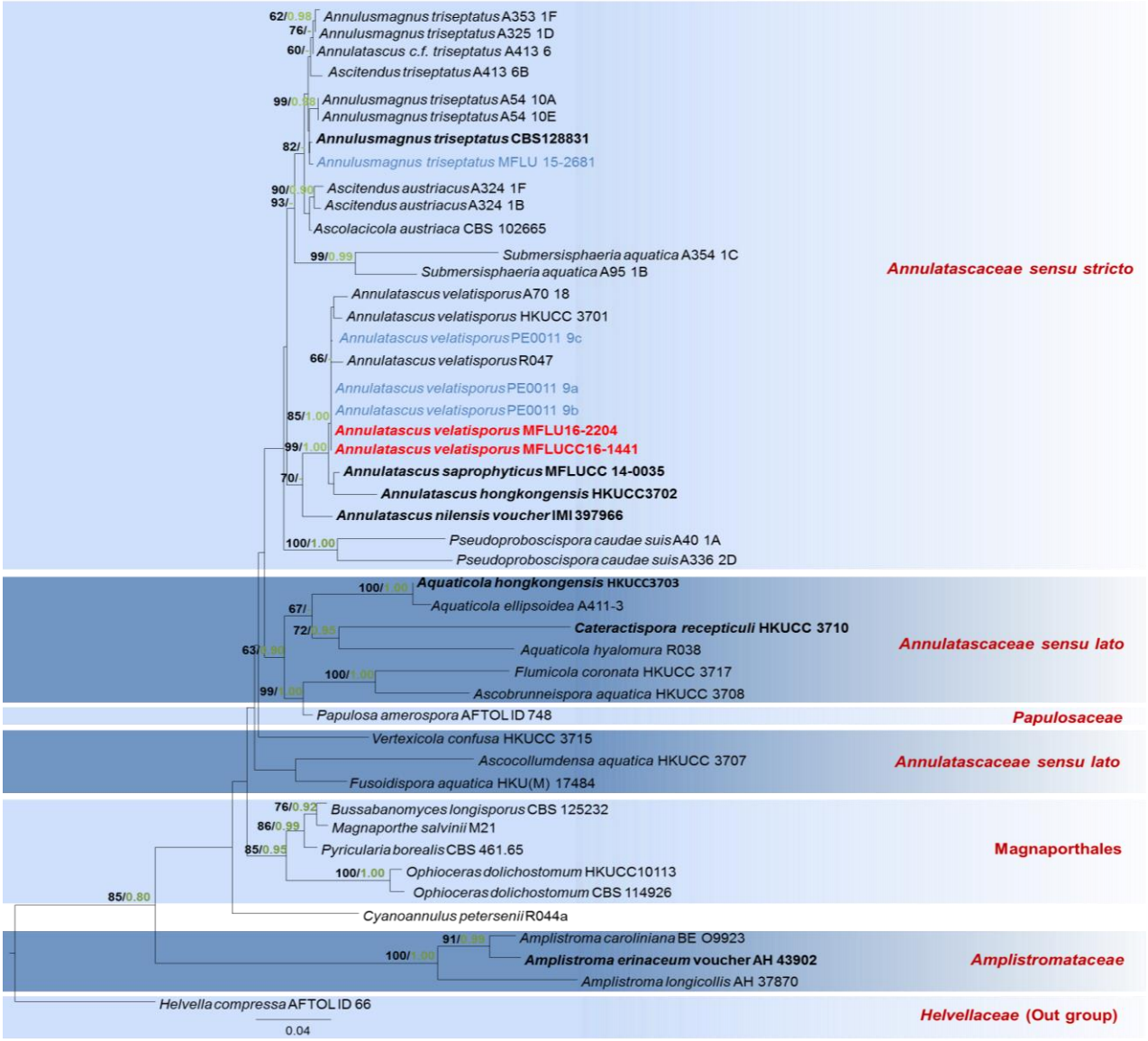

Fig. 1 – Best scoring RAxML tree based on a combined dataset of LSU and SSU sequence data. RAxML bootstrap support values (equal or greater than 60 %) and Bayesian posterior probabilities (equal or greater than 0.90) are given at the nodes (ML (black)/PP (green). The tree is rooted to *Helvella compressa* (AFTOL-ID 66). Sequences derived from ex-type strains are in bold, the ex-epitype strains generated from this study are in red and other stains generated from this study are in blue.

Taxonomy

Annulatascus velatisporus K.D. Hyde, *Australian Systematic Botany,* 5(1): 118 (1992) Figs 2,3

Epitypification identifier (Index Fungorum): IF552515

Saprobic on decaying wood submerged in freshwater. Sexual morph: *Ascomata* 445–500 µm high, 280–450 µm diameter, perithecioid, solitory, semi immersed to superficial, visible as small, black fruiting bodies on host surface, globose to subglobose, glabrous, carbonaceous, papillate, black. *Ostiole* central, with a neck, black, periphysate. *Peridium* 100–150 µm thick, of *textura angularis*, consisting of two layers, outer layer comprising several cell layers of brown to black cells, inner layer composed of hyaline elongated cells. *Paraphyses* 4 µm, numerous, septate, unbranched and tapering distally. *Asci* 224–300 × 10.5–14 µm ($\bar{x}$=225.5 × 12 µm, n=20) 8-spored, unitunicate, cylindrical, apically rounded, pedicellate, with a bilateral, non-amyloid apical ring, 6–8 µm high × 2–5 µm wide. *Ascospores* 19.5–28 × 8–12 µm ($\bar{x}$= 24 × 10 µm, n=30), overlapping uniseriate, aseptate, fusiform, hyaline, thick-walled, guttulate, with a thin mucilaginous sheath. Asexual morph: Undetermined.

Fig. 2 – *Annulatascus velatisporus* (BRIP 17373 holotype). a. Herbarium specimen of *Annulatascus velatisporus.* b. Host. c. Ascomata on substrate. d, e. Section through ascomata. f Section showing peridium. g, h. Asci. i. Apical ring. j. Apical ring in Melzer's reagent. k, l. Ascospores. Scale bars: d-e = 100 µm, f-h = 20 µm, i-l =10 µm.

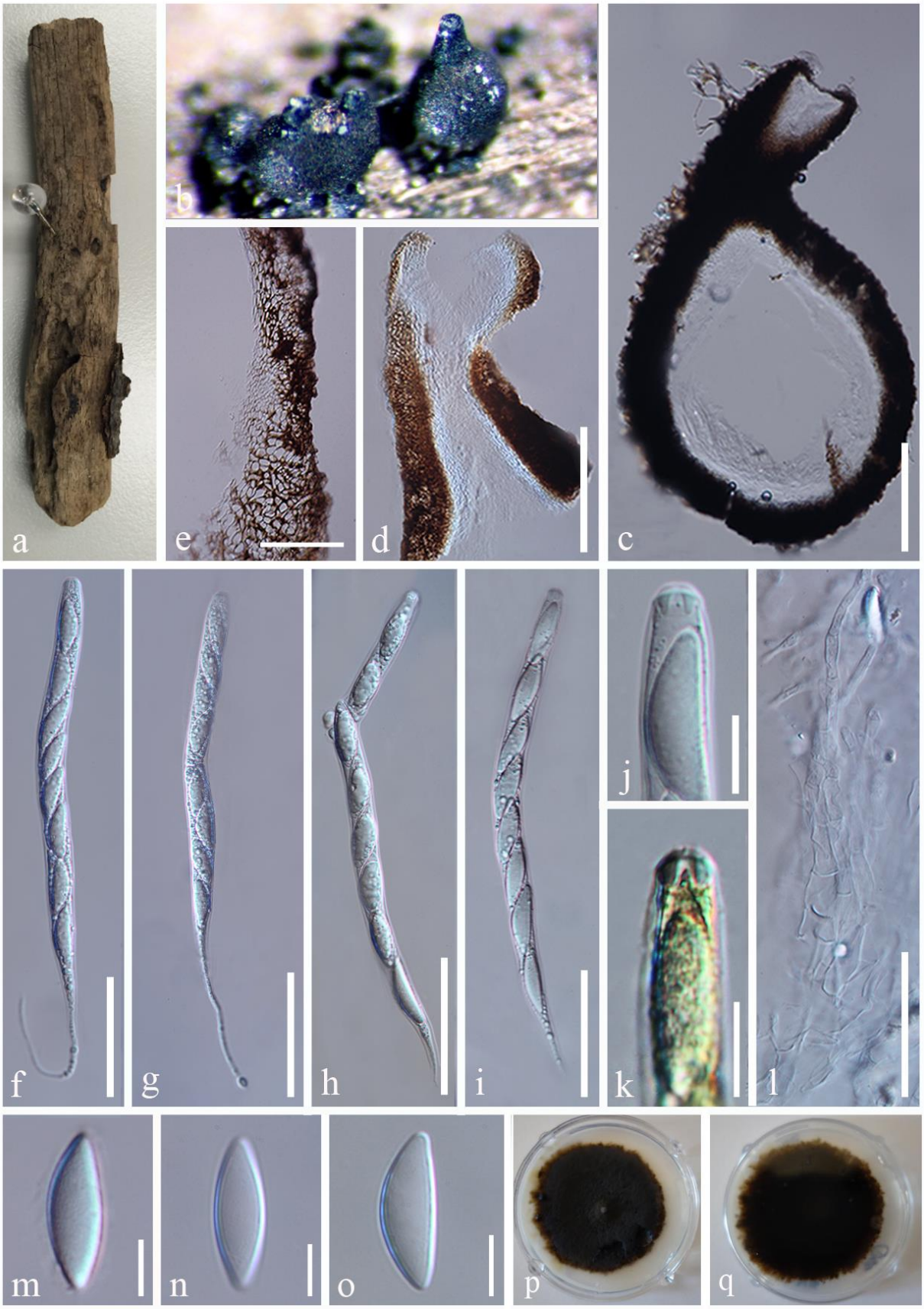

Fig. 3 – *Annulatascus velatisporus* (MFLU 16-2204 epitype). a. Host. b. Ascomata on submerged wood. c. Sections of ascomata. d. Section through neck region. e. Section through peridium. f–i. Asci. j. Apical ring. k. Apical ring in Melzer's reagent. l. Paraphyses. m–o. Ascospores. p,q. Culture on PDA (p = from above, q= from below). Scale bars: c = 100 µm, d,f–i = 50 µm, j-l = 10 µm, m–o = 5 µm.

Table 2 Synopsis of the characteristics of *Annulatascus velatisporus* K.D. Hyde and other collection.

Specimen	Ascomata (µm)	Asci (µm)	Apical ring (µm)	Ascospores (µm)	Host	Location	Reference
Annulatascus velatisporus BRIP 17373	445–460 × 326–345	224–260 × 11–13	5–8 × 2–5	22–25 × 7–9	Sub merged wood	Milaa Milaa Falls, North Queensland Australia	Hyde (1992). Holotype
Annulatascus velatisporus MFLU 16-2204	480–500 × 280–450	250–300 × 10.5–14 (n=20)	6–8 × 2–4	19.5–28 × 8–12 (n=30)	Sub merged wood	Queensland	This study Epitype

Culture characteristics – Colonies on PDA, reaching 8 cm diam. after 2 weeks at 20–25°C, medium dense, irregular, uneven edge, undulate margin, feathery, colony from above and below: dark brown, not produced pigmentation on PDA media.

Material examined – AUSTRALIA, North Queensland, Milaa Milaa Falls, on submerged wood in a river, July 1990, K.D. Hyde (BRIP 17373, **holotype**); AUSTRALIA, North Queensland, Mulgrave river, 17° 17724' S, 145° 72374 E, on decaying wood submerged in a river, 14 April 2015, Sally Fryar and Ben Cawson, MR102A (MFLU 16-2204**, epitype designated here**) ex-epitype culture, MFLUCC 16-1441 on decaying wood submerged in a river, 14 April 2015, Sally Fryar and Ben Cawson (AD-C 59642, **iso-epitype**).

Discussion

Taxa in *Annulatascaceae sensu stricto*; *Annulatascus*, *Annulusmagnus*, *Ascitendus*, *Pseudoproboscispora*, *Pseudoannulatascus* and *Submersisphaeria*, are freshwater ascomycetes growing on submerged wood. Based on molecular data, Campbell and Shearer (2004) opined that taxa assigned to *Annulatascus* were polyphyletic, and this was subsequently confirmed by various researchers (Abdel-Wahab et al. 2011, Boonyuen et al. 2012, Hu et al. 2012). Luo et al. (2015) introduced a new genus, *Pseudoannulatascus* to represent *A. biatriisporus* (Hyde 1995). *Annulatascus* species differ in their morphology, dimensions of the ascomata, asci and ascospores and the substrata they naturally colonize. The most pronounced differences are in size of the ascus and apical ring, ascospore septation and the presence of a mucilaginous sheath (Tsui et al. 2002).

Based on morphological characters such as black ascomata with a long neck, unitunicate, cylindrical asci with relatively massive, refractive apical ring and fusiform ascospores (Hyde 1992, Boonyuen et al. 2012), the newly obtained specimen is most similar to the holotype of *A. velatisporus*. According to Hyde's (1992) observations, ascospore sizes vary from 26–42 × 9–12 µm, while ranges of 22–25 × 7–9 µm were recorded from the dried holotype specimen. The relatively wide ascospore length ranges in holotype reported by Hyde (1992) might be a result of observing more than one collection. This ascospore size range (20–34 × 8–13) is also supported by measurements of 2000 ascospores of *A. velatisporus* during the dissertation studies (Zelski pers. comm.). The measurements of ascomata, asci and ascospores obtained in this study of both the holotype specimen and the epitype demonstrate that the two are unequivocally the same species (Table 2). The epitype designated herein can therefore be considered unambiguously *A. velatisporus*, and corresponds to the holotype description of the species. According to our phylogenetic analysis with combined LSU, and SSU data it is grouped with *A. velatisporus* isolates of PE0011-9a, PE0011-9b, PE0011-9c A70-18 and HKUCC 3701, and R047 are sister to two *A. velatisporus* strains, HKUCC 3701 and A70-18 (Raja et al. 2003) and collectively represent the *A. velatisporus*. Both holotype specimen and our new strain were obtained from the same geographical region of Queensland, Australia, where the species is common (Vijaykrishna & Hyde 2007). With strong morphological and molecular support, we designate this

specimen (MFLU16-2204) as the epitype specimen for *A. velatisporus* (*sensu* Ariyawansa et al. 2014) with the objective of providing the availability of a type specimen in good condition and corresponding molecular data for future studies.

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