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Helminthosporium velutinum and *H. aquaticum* sp. nov. from aquatic habitats in Yunnan Province, China

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Abstract

Helminthosporium species from submerged wood in streams in Yunnan Province, China were studied based on morphology and DNA sequence data. Descriptions and illustrations of *Helminthosporium velutinum* and a new species *H. aquaticum* are provided. A combined phylogenetic tree, based on SSU, ITS and LSU sequence data, place the species in Massarinaceae, Pleosporales. The polyphyletic nature of *Helminthosporium* species within Massarinaceae is shown based on ITS sequence data available in GenBank.

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

Introduction

The genus *Helminthosporium* Link was previously considered to be a widely distributed genus of dematiaceous hyphomycetes occurring on a wide range of hosts (Dreschler 1934, Ellis 1960, 1971, Manamgoda *et al*. 2011). With recent studies incorporating hyphomycetes in a natural classification, species considered to belong in the "*Helminthosporium*" complex were found to be polyphyletic (Shenoy *et al*. 2006, Bärlocher 2010, Baschien *et al*. 2013). Species in the graminicolous "*Helminthosporium*" complex (Dreschler 1923, 1934, Misra 1972, 1973), segregated in different genera *viz*. *Bipolaris* Shoemaker, *Curvularia* Boedijn, *Drechslera* Ito and *Exserohilum* Leonard and Suggs (Pleosporales), while some were accommodated in families of Corynesporaceae, Massarinaceae, Mycosphaerellaceae (Dothideomycetes) or other unrelated Ascomycetes (Shoemaker 2006, Zhang *et al*. 2012, Manamgoda *et al.* 2012, 2014, Woudenberg *et al*. 2013, Ariyawansa *et al*. 2015, Farr & Rossman 2015).

The type species of the genus, *Helminthosporium velutinum* is characterized by solitary, cylindrical, unbranched, brown conidiophores that produce obclavate, distoseptate conidia (Luttrell 1957, 1963, 1964, Hughes 1980). The conidiogenesis in *H. velutinum*is of the 'tretic'-type, i.e., a conidium primordium emerges through a pre-formed pore on the conidiogenous cell and develops into a full-grown conidium outside of the cell. Conidial secession is schizolytic. Conidia of *Helminthosporium* bear a conspicuously darkened hilum or "scar." (Alcorn 1988) Conidiogenous cells in *Helminthosporium* are polytretic and no corresponding scars on the conidiophore at the sites of conidium production. Most taxa in the *Helminthosporium* complex have later been placed in other genera, initially based on morphological characters and later based on molecular data, although some species remain unresolved. With recent advancement of taxonomy of other common genera previously classified in graminiscolous "*Helminthosporium*", it is essential to define the limits of *Helminthosporium sensu stricto*, based on precise phylogenetic placement with reference to its generic type.

In a recent survey of aquatic hyphomycetes of Yunnan, China, we encountered several isolates of *Helminthosporium* species associated with decaying wood in streams. The objectives of this study are to determine the phylogenetic

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placement of *Helminthosporium velutinum* based on these new collections and to introduce a new species *H. aquaticum*, with descriptions and illustrations.

Materials and methods

Isolation and morphology

Specimens of submerged decaying wood were collected from two streams in the Cangshan Mountain, Yunnan, China, during March to August 2014 and returned to the laboratory in plastic bags. The samples were incubated in plastic boxes lined with moistened paper towels at room temperature for one week. The samples were processed and examined following the methods described in Taylor and Hyde (2003). Morphological observations were made using a Motic SMZ 168 Series stereomicroscope and photographed by an OLYMPUS BX51 microscope-camera system. The fungal structures were measured using Image-Pro-Express software.

Single spore isolations were made to obtain pure cultures (Chomnunti *et al*. 2014). Dry culture specimens are deposited in the the Herbarium of Cryptogams Kunming Institute of Botany Academia Sinica (HKAS), Yunnan, China. Pure cultures are deposited in Mae Fah Luang University Culture Collection (MFLUCC) and duplicated in the culture collection of Kunming Institute of Botany Academia Sinica (KUMCC), Yunnan, China.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from fresh fungal mycelium grown on PDA at 25–27 °C. The EZ gene™ Fungal gDNA kit (GD2416) was used to extract DNA according to the manufacturer's instructions. The gene regions of the large subunit of the nuclear ribosomal DNA (LSU), the internal transcribed spacers (ITS) and small subunit of the nuclear ribosomal DNA (SSU) were amplified using the primer pairs LROR/LR7 (Vilgalys & Hester 1990), ITS5/ITS4, and NS1/ NS4 (White *et al*. 1990), respectively. The PCR mixture was including 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 mMMgCl2, stabilizer and enhancer), 1 µL of each primer (10 µM), 1 µL genomic DNA extract and 9.5 µL deionised water. 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'sprotocols (Amersham product code: 27–9602–01). The sequencing reactions were carried out by Shanghai Sangon Biological Engineering Technology and Services Co., Shanghai, P.R. China.

Phylogenetic analysis and species recognition

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. Two alignments were used based on the ex-type LSU, SSU and ITS sequences.

Phylogenetic reconstructions of individual gene-trees were performed using maximum likelihood (ML) and maximum parsimony (MP) methods. ML gene-trees were estimated using the software RAxML **7**.4.2 Black Box (Stamatakis *et al.* 2006) in the CIPRES Science Gateway (Miller *et al.* 2010), parsimony analysis in PAUP v. 4.0b10 (Swofford 2002), and substitution models were determined in MrModeltest v. 2.3 (Nylander 2004). PAUPv 4.0b10 was used to conduct maximum parsimony analyses. Trees were inferred using the heuristic search option with 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple parsimonious trees were saved.

Two phylogenetic analyses were carried out with a combined alignment of LSU, SSU and ITS to illustrate the relative placement of the isolates in Dothideomycetes and ITS phylogeny of *Helminthosporium* and closely related taxa retrieved from GenBank. Phylogenetic analyses and the comparison of each ITS sequence similarities coupled with the morphological characters were used to determine the species. All new sequence data generated in this study were deposited in GenBank (Table 1). Phylogenetic trees and data files were viewed in MEGA 5 (Tamura *et al.* 2011), Treeview (Page 1996) and Fig Tree v1.4 (Rambaut & Drummond 2008). The terminals of the trees (Figs. 1, 2) are labeled with GenBank accessions, species and the isolates/culture collection numbers.

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

Species	Collection/Isolate no.	GenBank accession no.		
		LSU	SSU	ITS
Alloconiothyrium aprootii	CBS 980.95	JX496234	-	JX496121
A. aprootii	CBS 981.95	JX496235	-	JX496122
Bimuria novae-zelandiae	CBS 107.79	AY016356	AY016338	-
Corynespora cassiicola	CBS 100822	GU301808	GU296144	-
C. leucadendri	CBS 135133	KF251654	-	KF251150
C. olivacea	CBS 114450	GU301809	-	-
C. smithi	CABI 5649b	GU323201	-	-
C. torulosa	CPC 15989	KF777207	-	KF777154
Deniquelata barringtoniae	MFLUCC 11–0257	KM213997	KM214000	KM214003
D. barringtoniae	MFLUCC 11–0422	JX254655	JX254656	JX254654
Didymosphaeria rubi-ulmifolii	MFLUCC 14–0023	KJ436585	KJ436587	KJ436585
Didymocrea sadasivanii	CBS 438.65	DQ384103	DQ384074	-
Helminthosporium aquaticum	**S-096**	**KU697306**	**KU697310**	**KU697302**
H. velutinum	**S-033**	**KU697304**	**KU697308**	**KU697300**
H. velutinum	**S-135**	**KU697303**	**KU697307**	**KU697299**
H. velutinum	**S-076**	**KU697305**	**KU697309**	**KU697301**
Karstenula rhodosoma	CBS 690.94	GU301821	GU296154	-
Kalmusia variisporum	CBS 121517	JX496143	-	JX496030
K. longisporum	CBS 582.83	JX496210	-	JX496097
Latendrea cordylinicola	MFLUCC 11–0148	KM213995	KM213998	KM214001
L. cordylinicola	MFLUCC 11–0150	KM213996	KM213999	KM214002
Massarina eburnea	CBS 473.64	GU301840	GU296170	-
Massarinaceae sp.	Isolate113/OG-2012	JQ780632	-	JQ780631
Montagnula aloes	CPC 19671	JX069847	-	JX069863
M. anthostomoides	CBS 615.86	GU205223	GU205246	-
M. spartii	CBS 183.58	GU205225	GU205250	-
Morosphaeria ramunculicola	BCC 18405	GQ925854	GQ925839	-
Neokalmusia scabrispora	CBS 120246	AB524453	AB524594	-
N. brevispora	CBS 120248	AB524600	AB524459	-
Paraconiothyrium africanum	CBS 121166	JX496142	EU295654	JX496029
P. cyclothyroides	CBS 972.95	JX496232	AY642524	JX496119
Paraphaeosphaeria angularis	CBS 167.70	JX496160	-	JX496047
P. viridescens	CBS 854.73	JX681076	-	JX496085
Phaeodothis winteri	CBS 182.58	GU301857	GU296183	-
Stagonospora paludosa	CBS 135088	KF25176	-	KF251257
S. pseudocaricis	CBS 135132	KF251762	-	KF251259

Results

Maximum Likelihood tree generated from RAxML analysis of LSU, SSU and ITS sequence data shows the phylogenetic position of the isolates within the class Dothideomycetes (Fig. 1). The combined alignment included 36 isolates including the out group taxon. The parsimony analysis revealed that the alignment comprised 2498 total characters, with 1933 characters being constant, 158 variable characters being parsimony un-informative and 407 (16%) characters being parsimony informative. The combined analysis of LSU, SSU and ITS sequence data placed the morphologically defined *Helminthosporium* isolates in the family Massarinaceae (Fig. 1).

The ITS phylogenetic tree (Fig. 2) of *Helminthosporium* and closely related taxa included 52 isolates with the out group taxon. The parsimony analysis revealed that of the 480 characters used for analysis, 301 characters are constant, 80 variable characters are parsimony informative and 99 (5%) characters are parsimony informative. Species were determined in accordance with the comparison of ITS sequence data and comparison of the morphological characters. The ITS phylogeny reveals the polyphyletic nature of *Helminthosporium* species. The generic type species of *Corynespora*, *C. cassiicola* is placed in a distinct family, Corynesporaceae. Therefore, the isolates grouped with *Helmonthosporium* species in Massarinaceae may represent different unidentified genera of hyphomycetes.

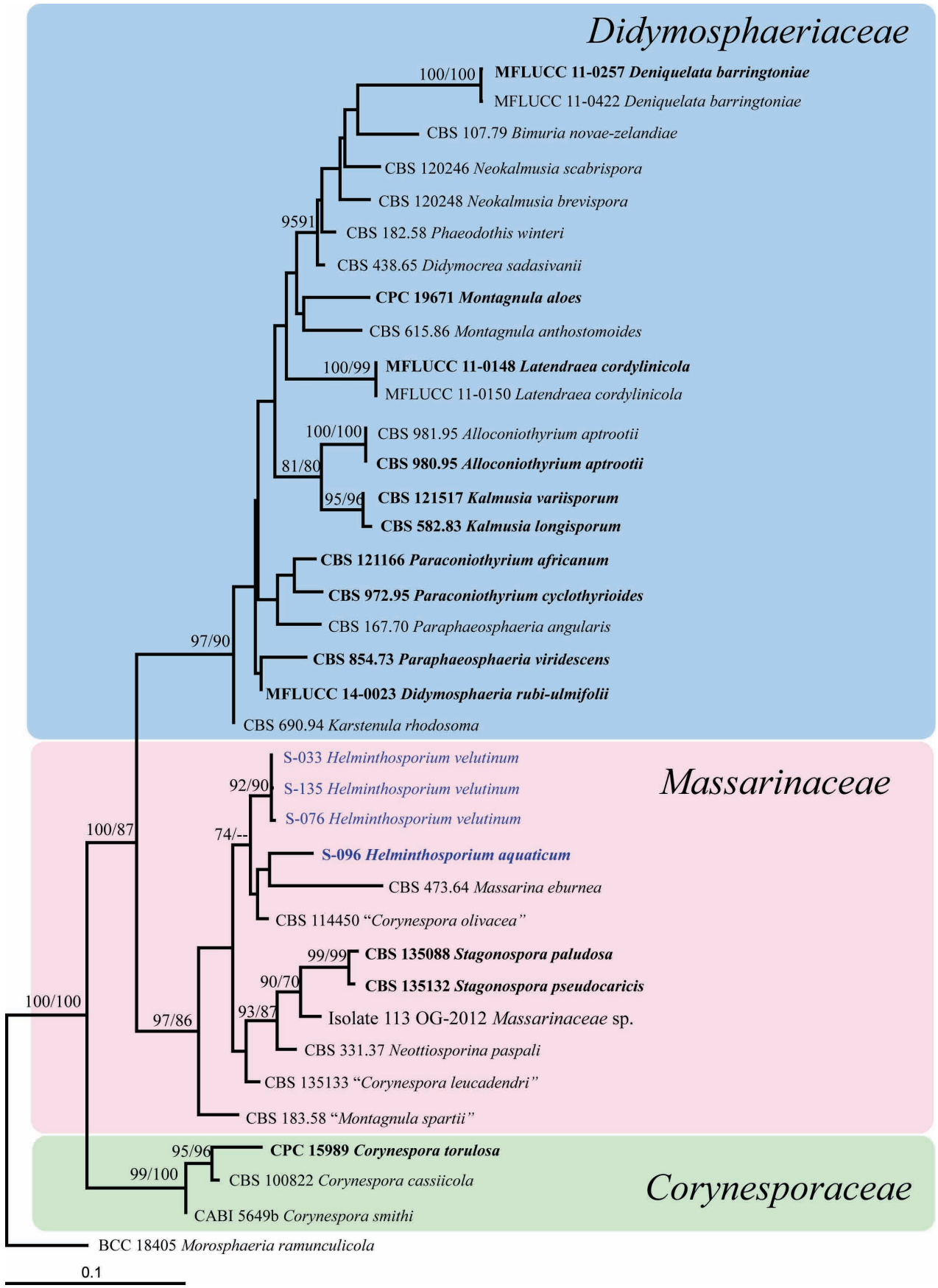

FIGURE 1. Maximum likelihood tree generated from analysis of combined LSU, SSU and ITS sequence data of taxa from Corynesporaceae, Didymosphaeriaceae and Massarinaceae. ML bootstrap and parsimony bootstrap values ≥70 % are displayed above or below each branch. Ex-type isolates are in bold. Isolates newly added in this study are in blue. The tree is rooted with *Morosphaeria ramunculicola* (Morosphaeriaceae).

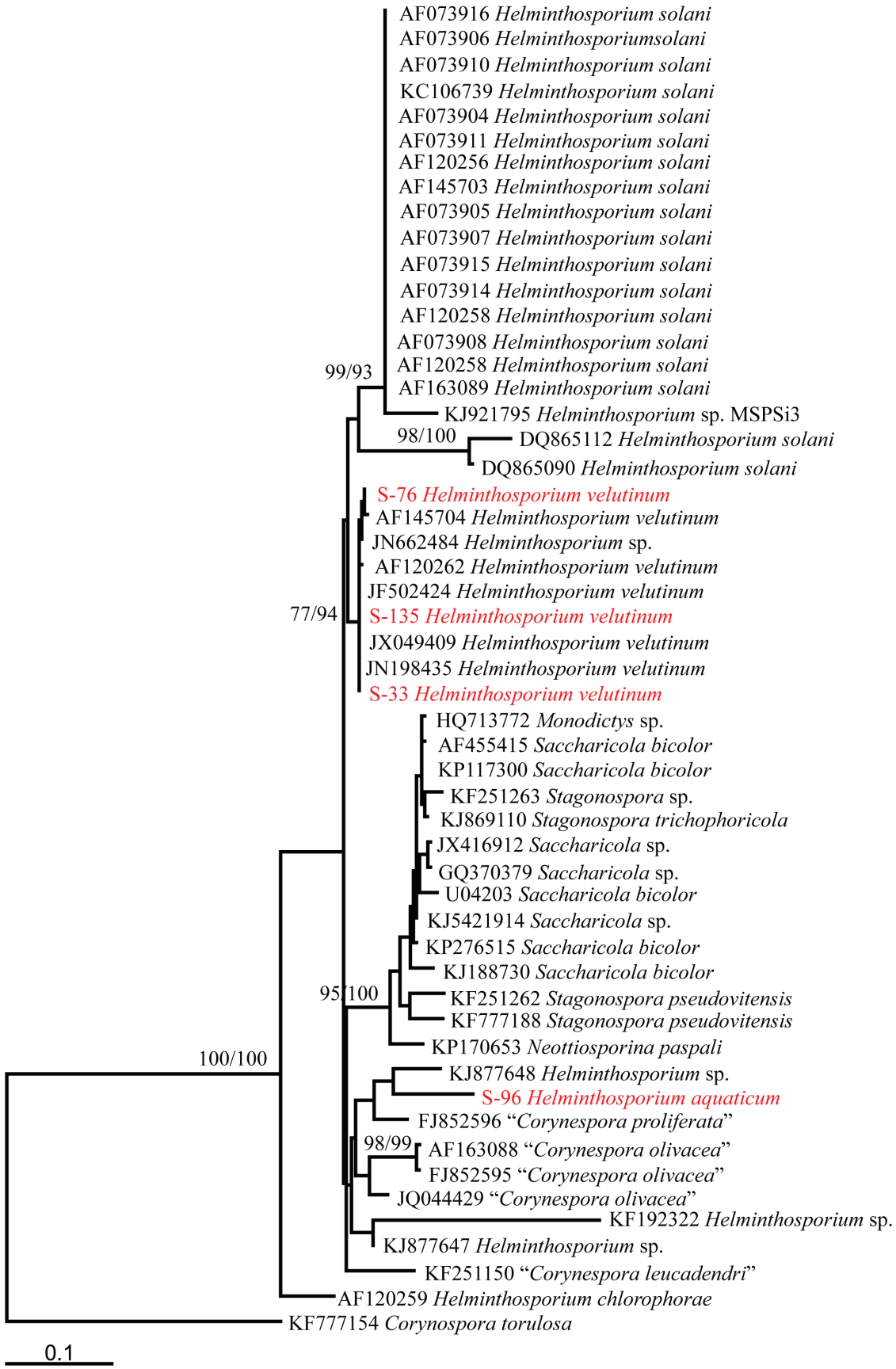

FIGURE 2. Maximum likelihood tree generated from analysis of ITS sequence data from *Helminthosporium* species and closely related taxa available in GenBank which shows the polyphyletic nature of the genus. ML bootstrap and parsimony bootstrap values ≥70% are displayed above or below each branch. New isolates added in this study are in red. The tree is rooted with *Corynespora torulosa* (Corynesporaceae).

Taxonomy

Helminthosporium velutinum Link [as 'Helmisporium'], Mag. Gesell. naturf. Freunde, Berlin 3(1–2): 10, tab. 1:9 (1809) **FIGURE 3.**

Facesoffungi number: FoF 01053
Reported synonyms (see Index Fungorum 2016)

Colonies saprobic, dark brown, effuse, velvety. *Mycelium* immersed, composed of branched, septate, thick-walled hyphae. *Conidiophore*s mononematous, macronematous, mostly unbranched, proliferating, dark brown, 530–655 µm long ($\bar{x}$=594 µm, SD=62, n=10), 16–18 µm wide ($\bar{x}$=17 µm, SD=1, n=10), 17–23-septate, erect or flexuous, tapering towards apex, bulbous at base with cells near apex of conidiophore guttulate and fertile. *Conidiogenous cells* polytretic integrated, intercalary and terminal. *Conidia* 67–79 µm long ($\bar{x}$=73 µm, SD=6, n=20), 15–19 µm wide ($\bar{x}$=17 µm, SD=2, n=20), single, obclavate, pale brown to brown, 7–9-distoseptate, smooth, straight or curved, base slightly truncate, cicatrized and wider than apex, dark brown, apical cell paler than other cells, rounded at apex, guttulate when young, non-guttulate at maturity. Conidial secession schizolytic.

Material examined: CHINA, Yunnan Province, Dali, WanHua stream, saprobic on decaying wood submerged in stream, July 2014, Qin-yan Li, 2WHXM H 1–1 (S-135), (HKAS 84015, **reference specimen designated here**), living culture MFLUCC 15–0428 =KUMCC; *ibid.,* Linquan stream, saprobic on decaying wood submerged in stream, March 2014, Hong-yan Su, LQXM 19–4 (S-033), HKAS 83990, culture MFLUCC 15–0423 = KUMCC; *ibid.* Heilong stream, saprobic on decaying wood submerged in stream, March 2014, Xiao-ying Liu, HLXM 45–1 (S-076), HKAS 84000, living culture MFLUCC 15–0243 =KUMCC.

Notes:—*Helminthosporium velutinum* is found on a wide range of dead plant material and it is considered to be a cosmopolitan taxon. Based on a megablast search of NCBIs GenBank nucleotide database the closest hits using the ITS sequence data of the isolate S-135 are *Helminthosporium velutinum* strain 1-05 (GenBank JF502424; identities = 455/455(100%), gaps = 0/455(0%), *H. velutinum* (GenBank JN19843; identities = 455/456(99%), gaps = 1/456(0%)) and *H. velutinum* strain ATCC38969 (GenBankAF120262; identities = 454/455(99%), gaps = 0/455(0%)) and *H. velutinum* strain CBS 360.75 (GenBankAF145704; identities = 452/455(99%), gaps = 0/455(0%)). Based on morphology, and the similarity of the ITS sequences available from various collections worldwide, we recognize our isolates as being *H. velutinum*. Being primarily associated with dead plant material in terrestrial ecosystems, the fungus might have been introduced to the streams from the neighboring riparian terrestrial environment.

Helminthosporium aquaticum H.Y. Su, Z.L. Luo & K.D. Hyde, sp. nov. **FIGURE 4.**

Etymology: Referring to the occurrence of the fungus in aquatic environments
Index Fungorum number: IF 551974
Facesoffungi number: FOF 01054,
Holotype: HKAS89692

Colonies saprobic, effuse, velvety, dark brown or black. Mycelium immersed, consisting of branched, septate, smooth, thick-walled hyphae. *Conidiophore*s mononematous, macronematous, unbranched, dark brown, 410–580µm long ($\bar{x}$=494 µm, SD=84, n=10), 13–17 µm wide ($\bar{x}$=15 µm, SD=2, n=10), 14–23-septate, erect, flexuous, paler towards the apex, bulbous at base, smooth, solitary or in groups of 2–4. *Conidiogenous cells* polytretic, cylindrical, integrated, intercalary and terminal. *Conidia* 70–80 µm long ($\bar{x}$=75 µm, SD=5, n=20), 16–18 µm wide ($\bar{x}$=17 µm, SD=1, n=20), single, obclavate, acropleurogenous, 8–10-distoseptate, dry, pale brown to brown, straight or curved, truncate and cicatrized at base, wider than apex, dark brown, apical cell paler than other cells, guttulate. Hilum was flat ring bounded by the ruptured lateral walls. *Conidial secession* schizolytic.

Material examined: CHINA, Yunnan Province, Dali, Heilong stream, saprobic on decaying wood submerged in stream, March 2014, Hong-Yan Su, HLX 22–5(S-096), (HKAS89692, **holotype**); ex-type culture, MFLUCC 15–0357 =KUMCC; *ibid.* (DLU14–096, **isotype**).

Notes:—Based on a megablast search of NCBIs GenBank nucleotide database the closest hits using the ITS sequence of isolate S-96 are *Corynespora proliferata* CBS 112393 (GenBank = FJ852596, identities = 476/522 (91%), gaps = 11/522 (2%)), *Helminthosporium* sp. XXJW-2014a (GenBank = KJ877648, identities = 432/465 (93%),

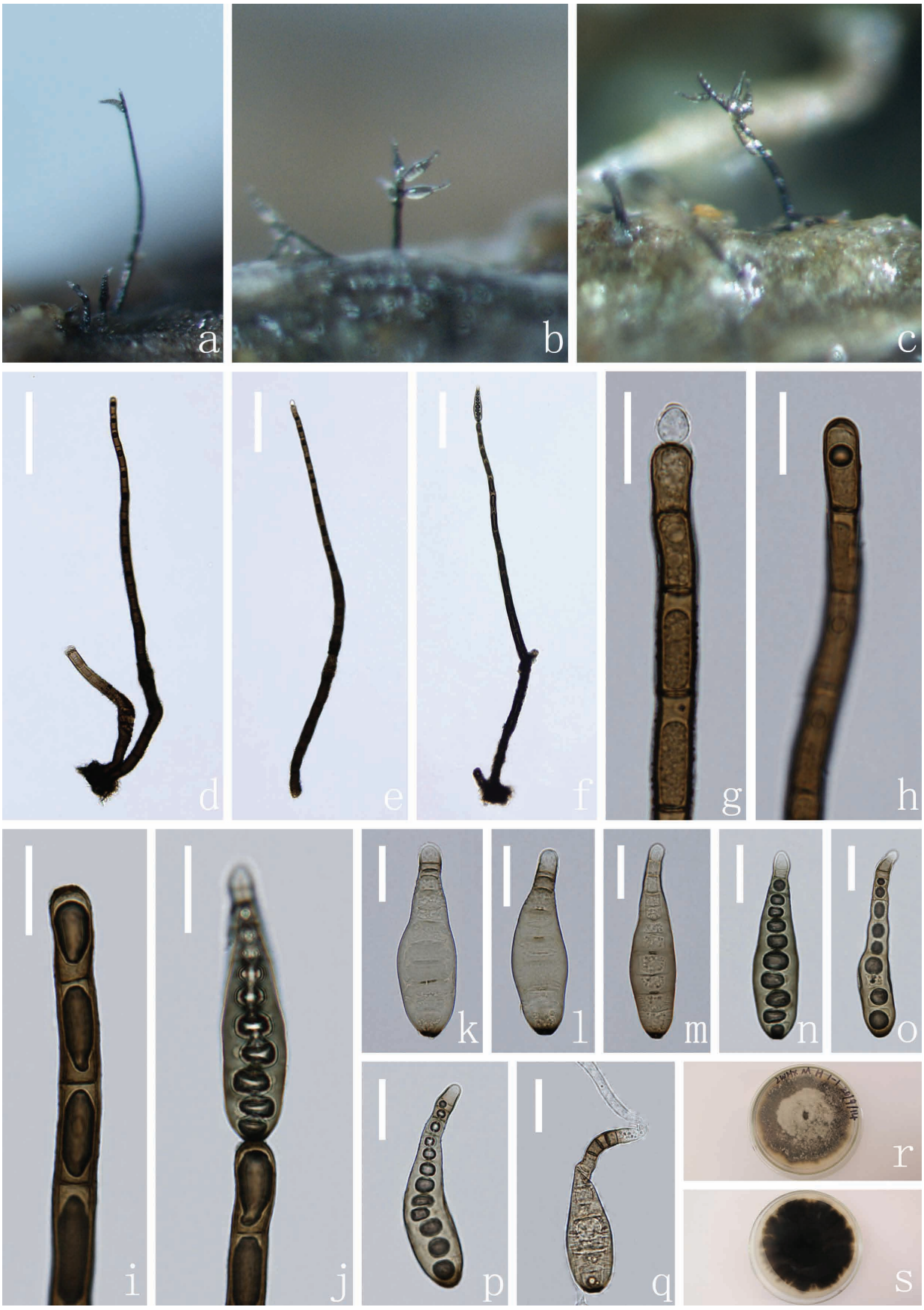

FIGURE 3. *Helminthosporium velutinum* (**reference specimen**) a–c. Colonies on the substrate; d, e. Conidiophores; f. Conidiophore and conidia; g, j. Conidiogenesis; h–i. Apical portion of conidiophores; k–p. Conidia; q. Germinating conidium; r–s. Culture on PDA after 30 days. Scale bars: d–f = 100 µm, g–q = 20 µm.

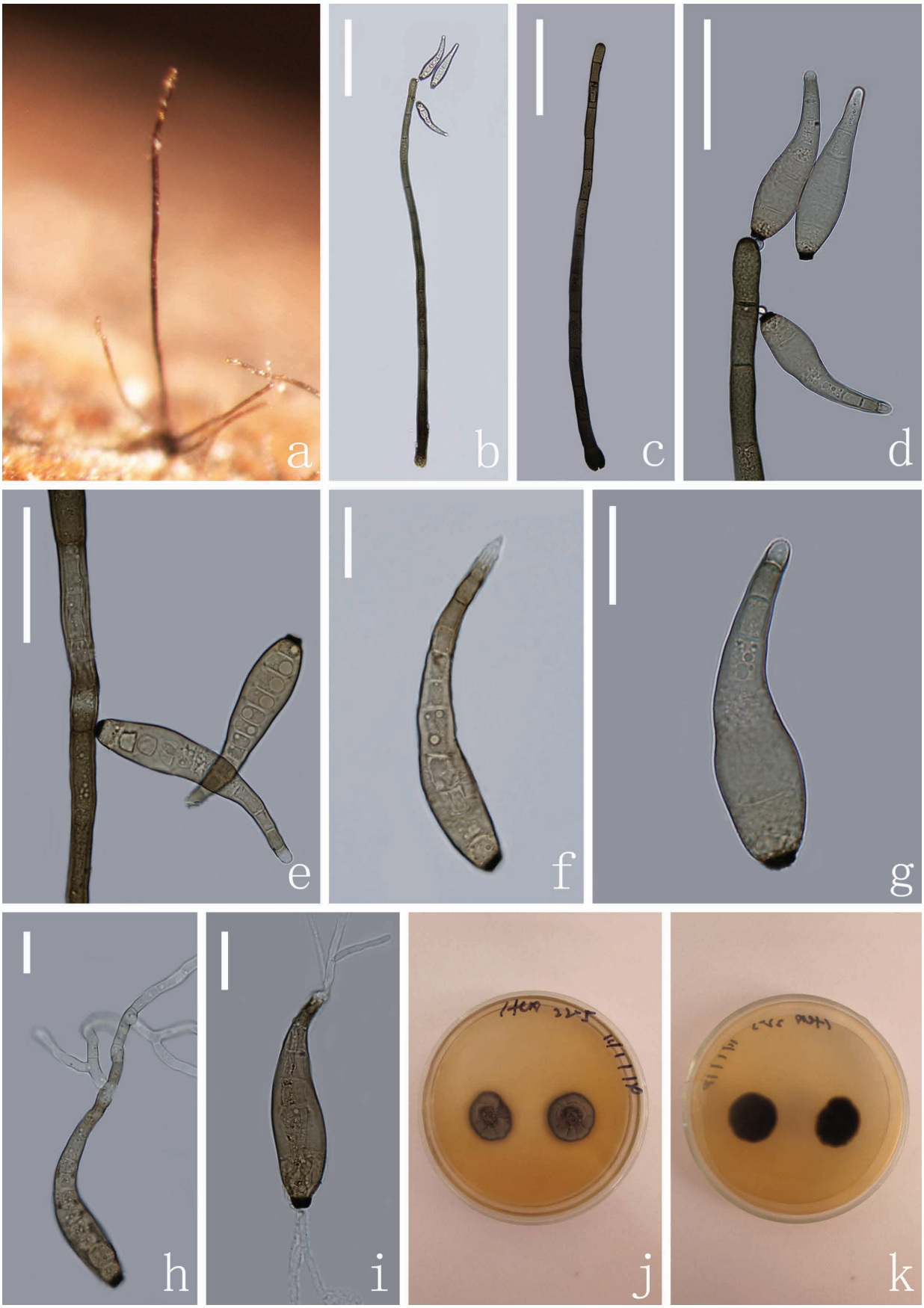

FIGURE 4. *Helminthosporium aquaticum* (**holotype**) a. Colony on the substrate; b. Conidiophores and conidia; c. Conidiophore; d–e. Conidiogenesis; f–g. Conidia; h–i. Germinating conidia; j–k. Cultures on MEA. Scale bars: b= 100 µm, d–e=50 µm, f–I= 20 µm.

gaps = 8/465 (1%)), *Corynespora olivacea* CBS 291.74 (GenBank = FJ852595, identities = 475/531 (89%), gaps = 15/531 (2%)) and *Helminthosporium* sp. XXJW-2014b (GenBank = KJ877647; identities = 438/478 (92%), gaps = 10/478 (2%)). The *Corynospora* species retrieved in the megablast search grouped apart from Corynosporaceae and are likely to be represent tentatively named taxa otherwise different unidentified genera. The conidia could easily be confused with species of *Corynespora*. However, the conidiophores of *Helminthosporium* are polytretic and proliferate sympodially, while those of *Corynespora* are monotretic and proliferate percurrently. The new species, *H. aquaticum*, is morphologically very similar to *H. velutinum*, but can be distinguished based on culture characteristics and molecular data.

Discussion

Studies of lignicolous aquatic hyphomycetes in China and elsewhere in the world have re-defined poorly studied genera of fungi. Many novel fungal taxa have been introduced from aquatic habitats (Belliveau & Bärlocher 2005, Pascoal *et al.* 2005, Cai *et al.* 2007, Shearer *et al.* 2007). Testing the polyphyletic nature of asexual fungi found in aquatic ecosystems and incorporating them in a natural classification is important to resolve taxonomic and nomenclatural issues of little known genera (Campbell *et al.* 2006, Jones *et al.* 2008, Seena *et al.* 2010).

The previous classification of helminthosporoid fungi is dated, and was essentially an artificial system of classification that can now be improved using molecular data and establishing informative taxonomic characters. The application of the name "*Helminthosporium*" in much of the plant pathology and taxonomic literature, referring to helminthosporoid genera, may be largely redundant. Many of the plant pathogenic taxa conventionally named as "*Helminthosporium*" are species of *Bipolaris*, *Curvularia*, *Drechslera* and *Exerohilum*, which are unrelated to *Helminthosporium velutinum* (Manamgoda *et al.* 2014). However, resurrection of the genus *Helminthosporium* and studying the diversity and biology is needed as this may resolve the taxonomy of many doubtful species and some important plant pathogens. In addition to *Helminthosporium velutinum* and *H. aquaticum* in *Helminthosporium sensu stricto*, we included the isolates of *H. solani* with the sequence data available in GenBank. *Helminthosporium solani* is an important plant pathogen that causes silver scurf disease on potato, resulting in a cosmetic surface blemish to the tubers, and weight loss in storage (Firman & Allen 1995, Olivier *et al.* 1998, Hervieux *et al.* 2002). *Helminthosporium aquaticum* is close to *H. velutinum* in morphology and DNA sequence data (Fig. 1), although ITS data show strong polyphyly (Fig. 2). Future research with many more isolates, multi-gene sequence data and voucher specimens and careful sampling of other closely related genera including *Massarina*, *Stagonospora*, *Saccharicola* and *Neottiosporina* is needed to circumscribe the limits of *Helminthosporium*.

We have compiled and documented numerous hyphomycetes from aquatic habitats from Yunnan Province in recent parallel studies (Cai *et al.* 2007, Liu *et al.* 2015, Su *et al.* 2015). We have collected and studied several genera in Dothideomycetes (e.g. *Curvularia*, *Phragmocephala* (Su *et al.* 2015) and Sordariomycetes *Minimelanolocus* (Liu *et al.* 2015)). These studies of freshwater fungi will also eventually contribute to an updated taxonomy and phylogeny of poorly studied genera of fungi in Dothideomycetes and Sordariomycetes (Hyde *et al.* 2013, Wijayawardene *et al.* 2014, Maharachchikumbura *et al*. 2015, Senanayake *et al.* 2015). An extended sampling of lignicolous fungi from diverse aquatic ecosystems may reveal the diversity and dynamics of composition of these ecosystems. Habitat destruction and anthropogenic activities as well as global warming may impact on the diversity of fungal species and, therefore, aquatic fungi also are useful as ecological indicators (Hyde *et al.* 2016). An understanding of the taxonomy of poorly studied taxa in aquatic environments *will* contribute to the knowledge of global biodiversity estimates and to updating regional checklists.

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