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Phylogenetic and morphological appraisal of *Diatrype lijiangensis* sp. nov. (*Diatrypaceae*, Xylariales) from China

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Abstract

The majority of *Diatrype* species are saprobes, while a few species are pathogens which form cankers on forest trees. The placement of several species in the genus *Diatrype* is uncertain, as the phylogeny has yet to be well-resolved with extensive taxon sampling and authentic ex-type cultures. In this study, a diatrype-like taxon was found on decaying wood in Lijiang, Yunnan Province, China. Morphological characteristics and phylogenetic analyses of combined ITS and β-tubulin sequences, supported the conclusion that the taxon is a new species, which is named as *Diatrype lijiangensis*. The new species differs from other species of *Diatrype* in asci and ascospore characteristics. The morphological similarities and dissimilarities of the new species with phylogenetically close alliances are discussed. Micro-morphological characteristics of this novel taxon are illustrated with descriptions.

Key words – 1 new species – Phylogeny – Sordariomycetes – Taxonomy

Introduction

Diatrypaceae Nitschke, a family in Xylariales, comprises 17 genera (Index Fungorum 2019) with *Diatrype* Fr. as the type genus (Maharachchikumbura et al. 2015, 2016, Senanayake et al. 2015, de Almeida et al. 2016, Dayarathne et al. 2016, Mehrabi et al. 2016, Senwanna et al. 2017, Shang et al. 2017, Wijayawardene et al. 2018). Species of *Diatrypaceae* are mostly saprobes on decaying wood (Carter 1991, Acero et al. 2004, Trouillas & Gubler. 2004, Mehrabi et al 2015, de Almeida et al. 2016, Shang et al. 2017), however several species are pathogens and endophytes (Acero et al. 2004, de Errasti et al. 2014, Shang et al. 2017). The taxa of *Diatrypaceae* have perithecial ascomata, a poor or well-developed stroma with an ostiole, short to long neck, clavate or spindle-shaped asci and allantoid ascospores (Trouillas et al. 2010, Mehrabi et al. 2015, Dayarathne et al. 2016, de Almeida et al. 2016, Senwanna et al. 2017, Shang et al. 2017). However, the intraspecific variations in stromatal characteristics make the delineation of the genera in

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Diatrypaceae challenging (Vasilyeva & Stephenson 2004, Dayarathne et al. 2016). Asexual morphs have been reported to be either coelomycetous, ex: *Cytosporina* Sacc and *Libertella* Desm. (de Almeida et al. 2016, Senwanna et al. 2017, Shang et al. 2017) or hypomycetous, ex: genus *Phaeoisaria* which has recently been linked to the family *Pleurotheciaceae* (Luo et al. 2018). The asexual morph has not been used to identify species in *Diatrypaceae* (de Almeida et al. 2016, Senwanna et al. 2017).

Recent studies have provided updated phylogenetic analyses of *Diatrypaceae* (Mehrabi et al. 2015, Dayarathne et al. 2016, de Almeida et al. 2016, Mehrabi et al. 2016, Senwanna et al. 2017, Shang et al. 2017). However, phylogenetic placement of *Eutypa*, *Diatrype* and *Diatrypella* in this family remains unresolved (de Almeida et al. 2016, Senwanna et al. 2017, Shang et al. 2017). Therefore, more taxon sampling and extended molecular data are needed to elucidate their natural placements and close alliances (Shang et al. 2017).

In this study, morphological characteristics and molecular phylogenetic analyses showed that the new fungus groups within *Diatrype* and forms a strongly supported clade. The objectives of this study are to 1) introduce a new species of *Diatrype* and 2) strengthen its taxonomic placement using both morphological characteristics and phylogenetic analyses results of maximum likelihood and Bayesian analyses, based on combined ITS and β-tubulin sequences.

Material and Methods

Decaying woody material were collected from Lijiang, Yunnan Province, China, N 27º 00' 30.8", E 100º 11' 26.1", 3234 m in September 2018 and brought to the laboratory in a Zip-lock plastic bag. Samples were examined under a Motic SMZ 168 Series microscope and photographed using a Carl Zeiss Discovery V8 stereo-microscope fitted with Axiocam. Hand sections of the ascomata, were mounted on 5% KOH. Sections of ascomata and other micro-morphological characteristics were photographed using a Nikon ECLIPSE 80i compound microscope fitted with a Canon 550D digital camera. All microscopic measurements were made with Tarosoft Image Frame Work (v.0.9.0.7).

Images used for figures were processed with Adobe Photoshop CS3 Extended version 10.0 software (Adobe Systems, USA). The holotype specimen was deposited in the Mae Fah Luang University (MFLU) Herbarium, Chiang Rai, Thailand. Faces of Fungi and Index Fungorum numbers were provided as outlined in Jayasiri et al. (2015) and Index Fungorum (2019) respectively. The new species was established based on the recommendations of Jeewon & Hyde (2016). We could not obtain a pure culture of this fungus and all the morphological characteristics and phylogenetic data were obtained from fresh fruiting structures.

DNA extraction, PCR amplification and gene sequencing

DNA was extracted directly from fruiting bodies of the fungus as outlined by Wanasinghe et al. (2018). An E.Z.N.A. ® Forensic DAT (D3591 – 01, Omega Bio – Tek) DNA extraction kit was used to extract DNA by following the manufacturer's instructions. DNA samples that were intended for use as a template for PCR were stored at 4ºC for use in regular work and duplicated at -20 °C for long-term storage. DNA sequence data were obtained from internal transcribed spacers (ITS) and β-tubulin. The internal transcribed spacers (ITS) were amplified with primers ITS4 and ITS5 (White et al. 1990) while the β-tubulin was amplified with primers Bt2a and Bt2b (Glass & Donaldson 1995).

The components for the PCR amplification are described below. The final volume of the PCR mixture was 25 µl with 2.0 µl of DNA template, 1 µl of each forward and reverse primers, 12.5 µl of 2 × Easy Taq PCR Super Mix (mixture of Easy Taq TM DNA Polymerase, dNTPs, obtained buffer (Beijing Trans Gen Biotech Co., Chaoyang District, Beijing, PR China) and 8.5 µl sterilized water. The PCR thermal cycle program for ITS gene was used as: initial denaturation at 94ºC for 3 mins, followed by 35 cycles of 30 sec at 94ºC, 50 sec at 55ºC, and 1 min at 72ºC, with a final extension of 10 mins at 72ºC. Amplification of β-tubulin was accomplished by an initial denaturation at 94ºC for 3 mins, followed by 35 cycles consisted of denaturation at 94ºC for 30 sec,

annealing at 55ºC for 50 sec, elongation at 72ºC for 1 min with a final extension for 10 mins at 72ºC.

PCR products were observed on 1% agarose electrophoresis gels stained with ethidium bromide. Purification and DNA sequencing were performed by Shanghai Sangon Biological Engineering Technology & Services Co. (Shanghai, P.R. China). The nucleotide sequence data acquired were deposited in GenBank and alignments and the trees were submitted to TreeBASE under submission ID 24320.

Phylogenetic analyses and species recognition

Sequence homologies were searched by using the NCBI BLAST search engine for the preliminary identification (https://www.ncbi.nlm.nih.gov). Phylogenetic analyses were constructed based on ITS and β-tubulin sequence data. Sequences of available closely related taxa from the family *Diatrypaceae* were obtained from GenBank and following Senwanna et al. (2017) (Table 1). *Kretzschmaria deusta* (CBS 826.72) and *Xylaria hypoxylon* (CBS 122620) were selected as the outgroup taxa. Multiple sequence alignments were generated with MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/index.html; Katoh et al. 2017), and where necessary were manually adjusted using Bioedit v. 7.0.5.2 (Hall 1999).

Table 1 Taxa used in the phylogenetic analyses and their GenBank accession numbers. The newly generated sequence from this study, and the ex-type isolates are given in bold.

Taxon	**Strain**	**GenBank Accessions**	
		ITS	**β -tubulin**
Allocryptovalsa polyspora	MFLU:17-1218	NR153588	–
Allocryptovalsa rabenhorstii	WA07CO	HQ692620	HQ692522
Allocryptovalsa rabenhorstii	WA08CB	HQ692619	HQ692523
Anthostoma decipiens	IPVFW349	AM399021	AM920693
Anthostoma decipiens	JL567	JN975370	JN975407
Cryptosphaeria ligniota	**CBS 273.87**	**KT425233**	**MF359667**
Cryptosphaeria pullmanensis	HBPF24	KT425202	GQ294014
Cryptosphaeria pullmanensis	ATCC 52655	KT425235	KT425170
Cryptosphaeria subcutanea	DSUB100A	KT425189	KT425124
Cryptosphaeria subcutanea	**CBS 240.87**	**KT425232**	**KT425167**
Cryptovalsa ampelina	A001	GQ293901	GQ293972
Cryptovalsa ampelina	DRO101	GQ293902	GQ293982
Diatrype bullata	UCDDCh400	DQ006946	DQ007002
Diatrype disciformis	MFLUCC 15-0538	KRO92795	–
Diatrype disciformis	CBS 205.87	AJ302437	–
Diatrype lijiangensis	**MFLU:19-0717**	**MK852582**	**MK852583**
Diatrype spilomea	D17C	AJ302433	–
Diatrype stigma	DCASH200	GQ293947	GQ294003
Diatrype undulata	D20C	AJ302436	–
Diatrype virescens	1057	KU320619	–
Diatrypella frostii	UFMGCB 1917	HQ377280	–
Diatrypella heveae	**MFLU:17-1216**	NR154046	**HQ692502**
Diatrypella major	Isolate 1058	KU320613	–
Diatrypella tectonae	MFLUCC 12-0172a	KY283084	–
Diatrypella tectonae	**MFLUCC 12-0172b**	**KY283085**	**KY421043**
Diatrypella verruciformis	UCROK1467	JX144793	JX174093
Diatrypella verruciformis	UCROK754	JX144783	JX174083
Diatrypella vulgaris	**HVGRF03**	**HQ692590**	**HQ692502**
Eutypa armeniacae	ATCC 28120	DQ006948	DQ006975
Eutypa guttulata	HUEFS 192075	AJ302450	–
Eutypa lata	EP18	HQ692611	HQ692501
Eutypa lata	RGA01	HQ692614	HQ692497
Eutypella citricola	HVVIT07	HQ692579	HQ692512
Eutypella citricola	HVGRF01	HQ692589	HQ692521
Eutypella vitis	UCD2428TX	FJ790851	GU294726

Table 1 Continued.

Taxon	Strain	GenBank Accessions	
		ITS	β -tubulin
Eutypella vitis	UCD2291AR	HQ288224	HQ288303
Halodiatrype avcenniae	**MFLUCC 15-0953**	**KX573916**	**KX573931**
Halodiatrype salinicola	**MFLUCC 15-1277**	**KX573915**	**KX573932**
Kretzschmaria deusta	**CBS 826.72**	**KU683767**	**KU684190**
Monosporascus cannonballus	CMM3646	JX971617	–
Monosporascus cannonballus	**ATCC 26931**	**NR111370**	–
Peroneutypa alsophila	EL58C	AJ302467	–
Peroneutypa comosa	BAFC 393	KF964568	
Peroneutypa diminutispora	**MFLUCC 17-2144**	MG873479	**MH316765**
Peroneutypa kochiana	EL53M	AJ302462	–
Peroneutypa rubiformis	MFLUCC 17-2142	MG873477	–
Peroneutypa scoparia	MFLUCC 11-0478	KU940151	–
Quaternaria quaternata	GNF13	KR605645	–
Quaternaria quaternata	CBS 278.87	AJ302469	–
Xylaria hypoxylon	**CBS 122620**	**AM993141**	–

Phylogenetic analyses of both individual and combined aligned data were performed under maximum-likelihood (ML) and Bayesian (BI) criteria. Terminal ends of sequences and ambiguous regions were deleted manually and excluded from the dataset. The phylogeny web tool "ALTER" (Glez-Peña et al. 2010) was used to convert sequence alignment from FASTA to PHYLIP for RAxML analysis and FASTA to NEXUS format for Bayesian analysis. The estimated model of maximum likelihood and Bayesian analyses were performed independently for each locus using MrModeltest v.2.2 (Nylander 2004). Maximum likelihood trees were generated using RAxML-HPC2 on XSEDE (8.2.8) (Stamatakis 2006, Stamatakis et al. 2008) in the CIPRES Science Gateway platform (Miller et al. 2010).

MrBayes v. 3.0b4 was used to perform Bayesian analysis (Ronquist & Huelsenbeck 2003). GTR + GAMMA + I nucleotide substitution best-fit model is determined with MrModeltest v. 2.2 (Nylander 2004). The Bayesian Markov Chain Monte Carlo (BMCMC) sampling method in MrBayes v. 3.0b4 (Huelsenbeck & Ronguist 2001) was used to define Bayesian Posterior Probabilities (BP) (Rannala et al. 1998, Zhaxybayeva & Gogarten 2002). Six simultaneous Markov Chains were run for 5 million generations and the trees were sampled every 100th generation. The first 10% of trees that represented the burn-in phase were discarded, and only the remaining 90% of trees were used for calculating posterior probabilities (PP) in the majority rule consensus tree. The resulting trees are displayed using FigTree v1.4.0 (Rambaut 2012) and copied to Microsoft PowerPoint 2013 and converted to jpeg file in Adobe Photoshop CS6 version 13.0. (Adobe Systems. U.S.A.).

Results

Phylogenetic analyses

Our preliminary dataset comprised representatives of the family *Diatrypaceae* and our strain nested together with *Diatrype sensu stricto* based on combined ITS and β-tubulin data. We excluded other *Diatrype* species which were not cladded in *Diatrype sensu stricto* from the final analysis (Fig. 1). Based on megablast search of the NCBI nucleotide database using the ITS sequence, the highest similarities were found with *D. spilomea* [GenBank AJ302433; Identities = 587/600 (97%), Gaps = 75/600 (12%)], *D. stigma* [GenBank KX828152; Identities = 517/600 (97%), Gaps = 87/600(14%)] and *D. virescens* [GenBank MH864890; Identities = 584/607 (96%), (Gaps =7/607 (2%)]. Based on megablast search of the NCBI nucleotide database using the β-tubulin sequence, the highest similarities were found with undefined *Diatrype* species. The results obtained by both ML and BI analyses of the combined ITS and β-tubulin dataset comprised selected 50 taxa including the new strain.

Phylogenetic analyses obtained from maximum likelihood and Bayesian inference analysis showed similar topologies and were not significantly different. The best scoring RAxML tree was selected to represent the relationship analyses obtained among taxa, with the final ML optimization likelihood value of -8747.484409 and is shown in Fig. 1. Bayesian posterior probabilities from MCMC were evaluated with final average standard deviation of split frequencies = 0.006514. The new strain, MFLU 19-0717, was grouped in *Diatrype sensu sticto* within *Diatrypaceae* and formed a distinct clade (ML/BP=97/1.00) with high bootstrap support (Fig. 1). *Diatrype sensu sticto* comprises *D. stigma*, *D. undulate* and *D. bullata* and *D. spilomea* species with the type *D. disciformis* and this concur the phylogenetic studies of Dayarathne et al. 2016, de Almeida et al. 2016, Senwanna et al. 2017, Shang et al. 2017. We have defined *Diatrype sensu sticto* based on the published sequence data from reference stain of the type species *D. disciformis* (Senanayake et al. 2015).

Taxonomy

Diatrype lijiangensis Thiyagaraja & Wanas., sp. nov.

Index Fungorum Number: IF556377; Facesoffungi number: FoF06032

Etymology – The specific epithet "*lijiangensis*" refers to the name of the place, from which the type specimen of the species was collected.

Holotype – MFLU 19-0717

Saprobic on decaying woody bark. Sexual morph: *Ascostromata* 1 mm diam., black, superficial, solitary to gregarious, subglobose or ellipsoidal, carbonaceous. *Ascomata* 170–460 × 200–300 μm ($\overline{x}$ = 300 × 250 μm, n = 10), perithecial, black, subglobose to ovoid, clustered, immersed in ascostroma, glabrous, 2–5 loculate, ostiolate. *Ostiole* papillate or apapillate, central, ostiolar canal filled with periphyses. *Peridium* 15–25 μm wide, composed of two layers, outer layer comprising several layers of thick-walled, dark brown to black cells of *textura angularis*, inner layer comprising 3–5 layers of thin-walled, hyaline cells of *textura angularis*. *Hamathecium* 140–165 μm wide, hyaline. *Paraphyses* 2–4 μm wide, arising from base of perithecia, composed of long, narrow, unbranched, septate, guttulate, narrowing and tapering towards the apex, with apex blunt. *Asci* 50–90 × 6–9 μm ($\overline{x}$ = 65 × 8 μm, n = 20), 8-spored, unitunicate, thin-walled, clavate to cylindric clavate, long pedicillate, apically truncate. *Ascospores* 6–8 × 1–2 μm ($\overline{x}$ = 7 × 1.5 μm, n = 30), overlapping bi-seriate, allantoid, aseptate, hyaline to pale-brown, with one to few small guttules, slightly to moderately curved and smooth-walled. Asexual morph: Undetermined.

Material examined – China, Yunnan Province, Lijiang, on dead wood of unidentified host, N 27º 00' 30.8", E 100º 11' 26.1", 3234m, 7 September 2018, V. Thiyagaraja (MFLU 19-0717, holotype).

Addition GenBank number – LSU (MK810546)

Discussion

The genus *Diatrype* was introduced by Fries (1849) with the type species *D. disciformis* (Hoffm.) (Tilak 1964). It comprises 60 species (Wijayawardene et al. 2017), of which the majority are saprobes, with a few species that are pathogens which form cankers on forest trees. Its asexual morph is reported as libertella-like (Senanayake et al. 2015, Dayarathne et al. 2016, Wijayawardene et al. 2017). The species of *Diatrype* possess characteristics that include perithecia embedded in discoid or widely effuse stromata that are erumpent from the bark (Vasilyeva & Stephenson. 2009). The young stromata are sometimes covered with a layer of sterile tissue that eventually peels off to expose a fertile surface extruded with papillate or stellate ostioles (Vasilyeva & Stephenson. 2009, Dayarathne et al. 2016). The polysporous ascus feature has been traditionally used to distinguish the species of *Diatrype* from those of *Diatrypella* (Liu et al. 2015).

The most recent phylogenetic analysis for the genera of *Diatrypaceae* is provided by Senwanna et al. (2017) with twelve genera. The genera *Diatrype*, *Diatrypella* and *Eutypa* are polyphyletic within the family as found in several previous studies (Acero et al. 2004, Trouillas et

al. 2011, Mehrabi et al. 2015, 2016, de Almeida et al. 2016, Senwanna et al. 2017, Shang et al. 2017). In this study, we introduce a new species to the family *Diatrypaceae* based on molecular phylogenetic analyses of combined ITS and β-tubulin sequence data and morphological characteristics. According to our phylogenetic analyses, the genus *Diatrype sensu stricto* formed a district clade (ML/BP=97/1.00) with selected taxa including *D. disciformis*, *D. stigma*, *D. undulate*, *D. virescens*, *D. bullata*, *D. spilomea*, and our new species *D. lijiangensis*. However, further studies need to clarify the taxonomic placement of these taxa.

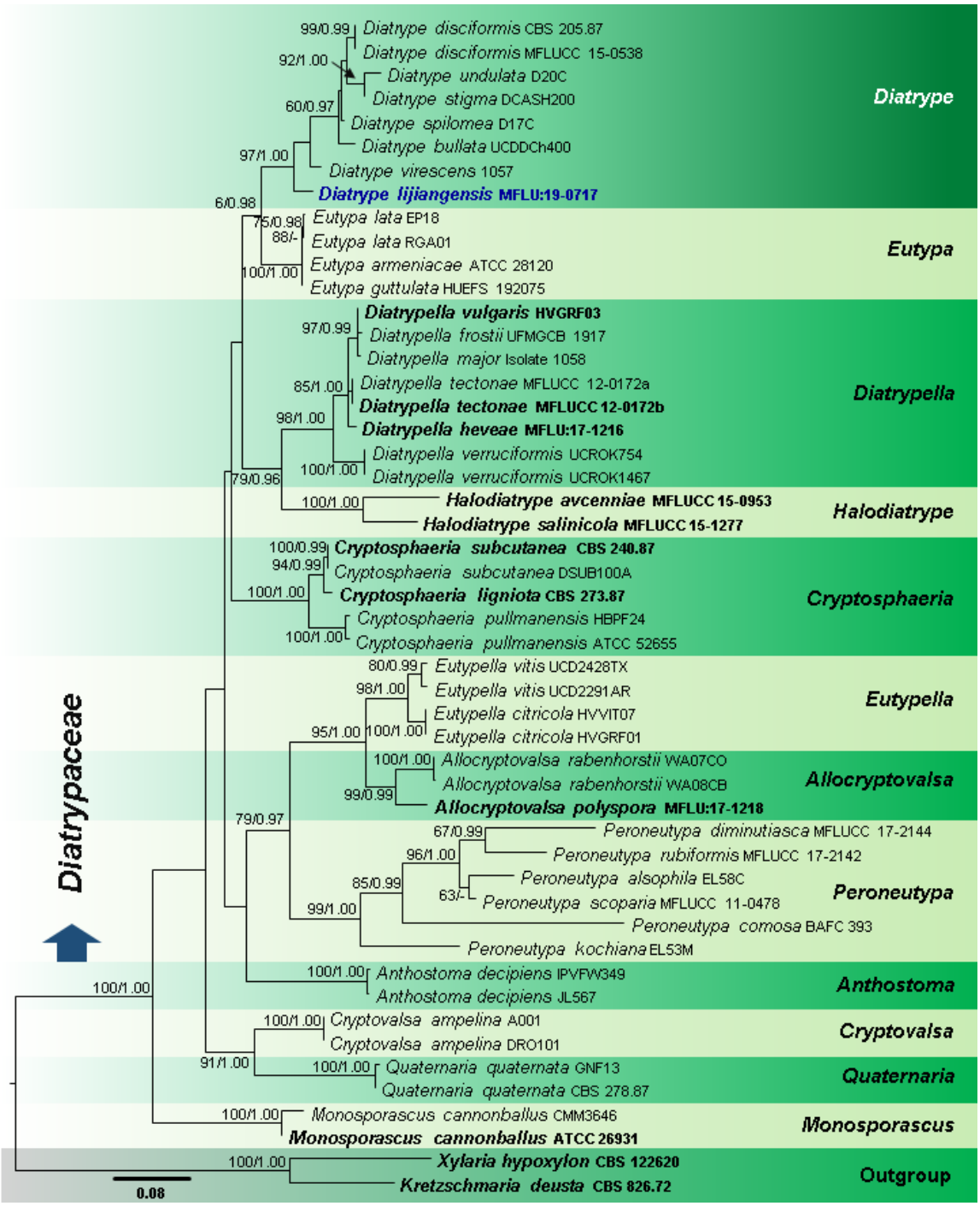

Fig. 1 – RAxML tree based on analysis of combined ITS and β-tubulin partial sequence data. Bootstrap support values for ML equal or greater than 60%, and Bayesian posterior probabilities (BP) equal or greater than 0.90 are given as ML/BP above the nodes. The tree is rooted to *Kretzschmaria deusta* (CBS 826.72) and *Xylaria hypoxylon* (CBS 122620). All ex-type strains are displayed in bold and the new species that was found in this study is displayed in blue bold.

Our novel taxon *D. lijiangensis* exhibits distinct morphological characteristics: viz. long pedicellate asci (50–90 µm) with superficial stromata (Fig. 2) which contrast to those recorded for *D. acericola* (23–27 µm), *D. albopruinosa* (40–60 µm), *D. bullata* (25–30 µm), *D. hypoxyloides* (50–90 µm), *D. macounii* (25–30 µm) *D. stigma* (25–30 µm) and *D. subundulata* (35–40 µm) which were previously collected from China (Vasilyeva & Ma 2014) (Table 2).

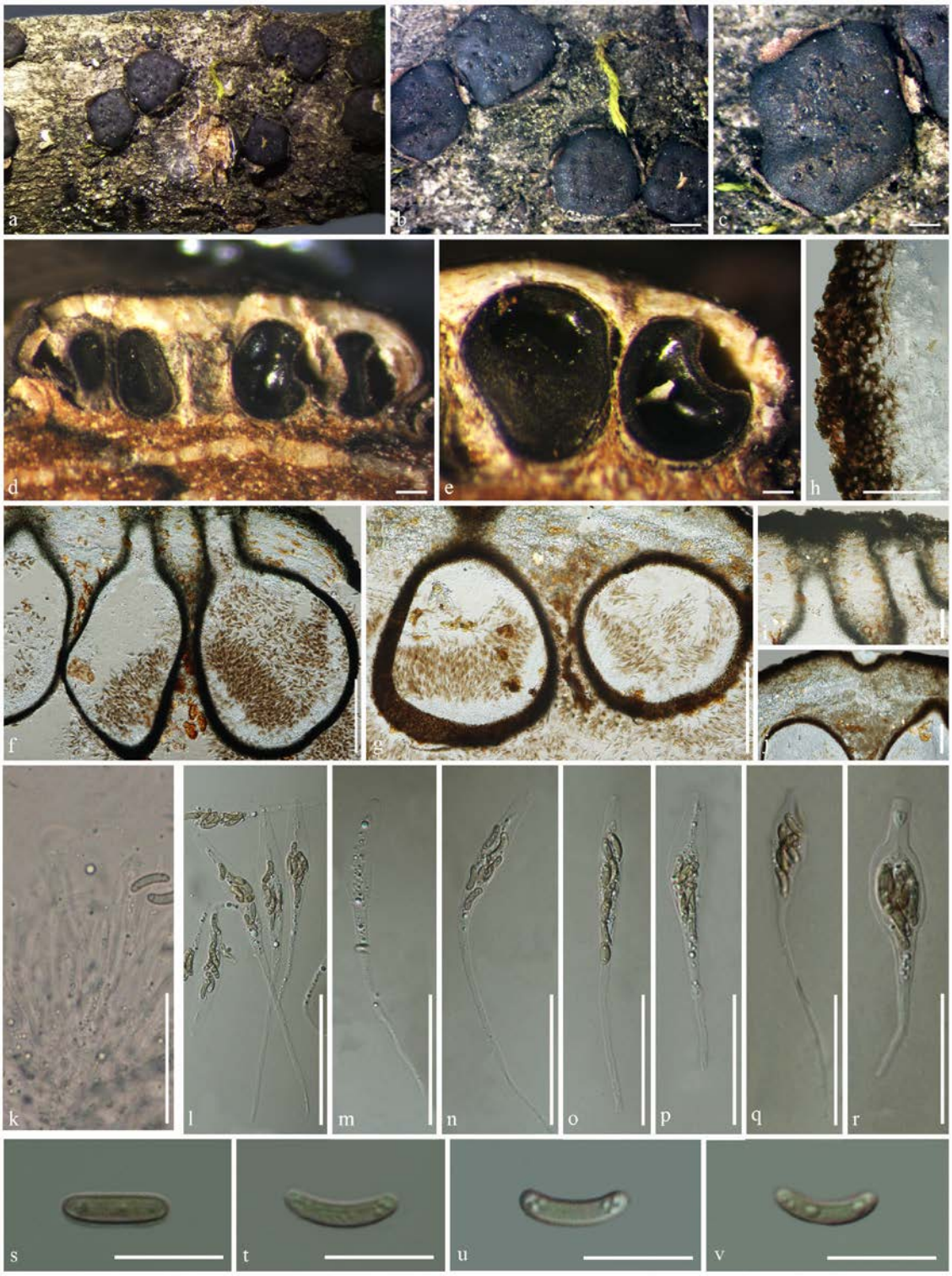

Fig. 2 – *Diatrype lijiangensis* (MFLU 19-0717, holotype). a-c Stromata on substrate. d, e Cross-section of stroma. f, g Vertical section through stroma showing ostioles and perithecia. h Peridium. i, j Ostiolar canals. k Paraphyses. l-r Asci. r-u Ascospores. Scale bars: b-e = 200 µm, f, g = 100 µm, h = 30 µm, k-r = 20 µm, s-v = 5 µm

Table 2 Synopsis of *D. lijiangensis* and related species discussed in this study

Species name	Colour of entostroma	Ascus length (µm)	Ascus width (µm)	Ascospore color	Ascospore length (µm)	Ascospore width (µm)	Reference
D. lijiangensis	Dark brown	50–90	6–9	Hyaline to pale-brown	6–8	1–2	This study
D. acericola	Brownish or almost black	23–27	5–7	Very slightly yellowish	7.5–9	0.9–1.1	Vasilyeva & Ma 2014
D. albopruinosa	Brownish or almost black	40–60	10–15	Brownish	12–15(–18)	3.5–4	Vasilyeva & Ma 2014
D. atlantica	Chocolate colored	30–40	4–6	Hyaline	(6–)7–9(–10)	–	Vasiljeva & Stephenson. 2009
D. bullata	Light to dark brown	25–30	5–7	Slightly yellowish	7.5–9	–	Vasilyeva & Ma 2014
D. caryae	Light brown	28–33	4–5	Hyaline	5–7	–	Vasiljeva & Stephenson. 2009
D. decorticata	Pallid-brown or brown	30–40	4–6	Hyaline	6–8	–	Vasiljeva & Stephenson. 2009
D. enteroxantha	Brown or black	18–28.5	5–9	Subhyaline	7–10	1.5–2.5	Almeida et al. 2014
D. hypoxyloides	Chocolate-brown	(15–)20–25	4–6	Hyaline	4–6	–	Vasilyeva & Ma 2014
D. ilicina	Dark brown	25–35	4–6	Hyaline	5–7	–	Vasiljeva & Stephenson. 2009
D. macounii	Dark brown	25–30	4–6	Slightly yellowish	4–6	0.7-1	Vasilyeva & Ma 2014
D. spilomea	Black	25–30	4–6	–	5–7	–	Vasilyeva & Ma 2004
D. stigma	Brownish	25–30	5–7	Hyaline	6–8	1.5-2	Vasilyeva & Ma 2014
D. stigmaoides	Grey or dark-grey	20–30	5–6	Hyaline	4–6	–	Vasiljeva & Stephenson. 2009
D. subundulata	Dark brown	35–40	5–7	Yellowish	7–9	1.7-1.9	Vasilyeva & Ma 2014

The comparatively longer asci (50–90 µm) of *D. lijiangensis* were observed to delineate from extant species such as *D. decorticata* (30–40 µm), *D. caryae* (28–33 µm), *D. atlantica* (30–40 µm), *D. ilicina* (25–35µm), *D. stigmaoides* (20–30 µm) (Vasiljeva & Stephenson 2009), *D. aceris-rubrae* (35–50 µm) (Vasilyeva & Stephenson 2014), *D. Montana* (30–35 µm), *D. rappazii* (26–30 µm) and *D. subaffixa* (30–40 µm) (Vasilyeva & Stephenson 2004). Our taxon shares similar morphological characteristics with the type species *D. disciformis,* but differs in having aseptate paraphyses whereas *D. disciformis* has septate paraphyses (Senanayake et al. 2015). *D. disciformis* phylogenetically differs from our new taxon in 2.47 % (14/565) base pair differences present in ITS gene but β-tubulin sequence data were not available in GenBank for comparisons. *D. virescens* phylogenetically closely related to our taxon but, differs in 4.77 % (27/565) base pair differences in ITS gene region and β-tubulin sequence data were not available in GenBank for comparisons. These two species exhibit distinct morphological differences in asci and ascospore characteristics whereas, asci and ascospores of *D. virescens* are 35–40 µm long and (10)12–14 µm long respectively (Vasiljeva & Stephenson 2004).

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