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Sexual morph of *Lasiodiplodia pseudotheobromae* (*Botryosphaeriaceae*, Botryosphaeriales, Dothideomycetes) from China

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Abstract

The sexual morph recorded here for *Lasiodiplodia pseudotheobromae*, was collected from dead leaves of *Plukenetia volubilis* L. (*Euphorbiaceae*) in Yunnan Province, China. The sexual-asexual connection in *Lasiodiplodia pseudotheobromae* was confirmed by phylogenetic analyses of combined ITS and *tef1-α* sequence data. This is the first report of a sexual morph with molecular evidence for this species. The important characteristics of this species are immersed to erumpent ascomata, papillate ostioles, cylindric-clavate asci with a short pedicel, well-developed ocular chamber and ellipsoidal to fusiform, golden to dark brown, aseptate ascospores. The sexual morph of this species is compared with other *Lasiodiplodia* sexual morphs and a comprehensive description and micrographs are provided.

Key words – Morphology – phylogeny – taxonomy

Introduction

Botryosphaeriaceae is a well-established and diverse family in the order Botryosphaeriales of Dothideomycetes. The family was introduced by Theissen & Sydow (1918) as a sub-family of *Pseudosphaeriaceae* (Liu et al. 2012, Abdollahzadeh et al. 2013, Phillips et al. 2013, Netto et al. 2014, Coutinho et al. 2016). Taxa in *Botryosphaeriaceae* are morphologically diverse and can be characterized by uni- to multilocular ascomata with multi-layered walls, hyaline, hypha-like pseudoparaphyses, bitunicate asci and hyaline, pale or dark brown ascospores (Liu et al. 2012, Phillips et al. 2013). Species of *Botryosphaeriaceae* are cosmopolitan in distribution occurring on a wide range of hosts from tropical and temperate regions, as pathogens, endophytes or saprobes (von Arx & Müller 1954, Barr 1987, Abdollahzadeh et al. 2013, Phillips et al. 2013, Slippers et al. 2013, Machado et al. 2014). Many sexual and asexual genera have been introduced in *Botryosphaeriaceae* (Phillips et al. 2005, Crous et al. 2006, Phillips et al. 2008, Schoch et al. 2009, Liu et al. 2012, Wijayawardene et al. 2012, Hyde et al. 2013, 2014).

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Lasiodiplodia was introduced by Ellis and Everhart (1894) with *L. tubericola* as the type species, although the genus was formally described by Clendenin (1896). Species of *Lasiodiplodia* commonly occur in tropical and subtropical regions and some cause severe damage especially to cultivated plants (Punithalingam 1980, Netto et al. 2014, Correia et al. 2016, Rosado et al. 2016). *Lasiodiplodia* species can be distinguished from morphologically closely related genera in having pycnidial paraphyses and longitudinal striations on the mature conidia (Sutton 1980, Phillips et al. 2008, Abdollahzadeh et al. 2010, Netto et al. 2014). According to Index Fungorum (2016) there are 42 *Lasiodiplodia* species. However, the sexual morphs of *Lasiodiplodia* species have rarely been recorded and are known for only three *Lasiodiplodia* species, namely, *L. gonubiensis* Pavlic, Slippers & M.J. Wingf., *L. lignicola* (Ariyaw., J.K. Liu & K.D Hyde) A.J.L. Phillips, A. Alves & Abdollahz and *L. theobromae* (Pat.) Griffon & Maubl. (Liu et al. 2012, Phillips et al. 2013, Trakunyingcharoen et al. 2015).

The aim of the present paper is to report for the first time the sexual morph of *Lasiodiplodia pseudotheobromae*, which was collected from dead leaves of *Plukenetia volubilis* in Xishuangbanna (Yunnan Province), China. The identity of our strain (MFLUCC 16-0805) as *Lasiodiplodia pseudotheobromae* was confirmed by phylogenetic analyses of combined ITS and *tef1-α* sequence data by maximum-likelihood (ML), maximum-parsimony (MP) and Bayesian Inference (BI).

Materials & Methods

Sample collection, morphological studies and isolation

Fresh specimens were collected from Xishuangbanna in Yunnan Province, China. Specimens were taken to the laboratory in zip lock bags and examined with a JNOEC series JSZ4 stereomicroscope. Micro-morphological characters were determined with an Olympus series SZ61 compound microscope. Hand cut sections of ascomata were mounted in sterile water for microscopic studies and photomicrography. Images were taken with a Nikon Eclipse 80i compound microscope with a Canon EOS 600D digital camera. Permanent slides were prepared by mounting fungal material in lactoglycerol and sealed by applying nail-polish around the margins of cover slips. Measurements were made with the Tarosoft (R) Image Frame Work program and images used for figures were processed with Adobe Photoshop CS3 extended version 10.0 (Adobe Systems, USA).

Isolates were obtained from single ascospores following the methods described in Chomnunti et al. (2014). Germinated ascospores were transferred to potato dextrose agar (PDA) and incubated at 25°C in normal light. Culture characteristics were observed after two weeks. Specimens were deposited in the Mae Fah Luang University Herbarium (MFLU), Chiang Rai, Thailand and Kunming Institute of Botany herbarium (HKAS). Living cultures were deposited in Mae Fah Luang University Culture Collection (MFLUCC) and Kunming Institute of Botany Culture Collection (KUMCC). Faces of Fungi numbers are registered as described in Jayasiri et al. (2015).

DNA extraction and PCR amplification

Genomic DNA was extracted from mycelium with Biospin fungus Genomic DNA extraction kit (BioFlux®, Hangzhou, P. R. China) following the manufacturer's protocol. DNA was kept at 4°C for the DNA amplification and maintained at -20°C for long term storage. The ITS region was amplified with primers ITS1 and ITS4 (White et al. 1990) as described in Alves et al. (2004). The primers TEF1-728F and TEF1-986R (Carbone and Kohn, 1999) were used to amplify translation elongation factor (*tef1- α*) as described in Phillips et al. (2005). Quality of PCR products were checked on 1 % agarose electrophoresis gels stained with ethidium bromide. The amplified PCR fragments were sequenced by Sangon Biotech (Shanghai) Co., Ltd, P.R. China. Nucleotide sequences were deposited in GenBank (Table 1).

Sequencing and sequence alignment

Sequence homologies for the assembled consensus sequences were analyzed by BLAST searches of the National Center for Biotechnology Information (NCBI) and for the preliminary identification of the isolates used in the analyses. Other sequences used in the analyses (Table 1) were obtained from GenBank based on recently published data (Abdollahzadeh et al. 2013, Coutinho et al. 2016).

Phylogenetic analysis

The multiple alignments were made with MAFFT v. 7 at the web server (http://mafft.cbrc.jp/alignment/server), using default settings (Katoh & Standley 2013). The alignment was refined manually with BioEdit v. 7.0.5.2 (Hall 1999) where necessary. Maximum likelihood analysis was performed by RAxML (Stamatakis 2010) implemented in RAxMLGUI 1.3 (Silvestro & Michalak 2012). Maximum parsimony analysis (MP) was performed in PAUP v. 4.0b10 (Swofford 2002), with the heuristic search option and 1,000 random replicates. Maxtrees was set to 1000 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 Index [RI], Relative Consistency Index [RC] and Homoplasy Index [HI] were calculated for trees generated under different optimality criteria. The Kishino-Hasegawa tests (Kishino & Hasegawa 1989) were performed to determine whether the trees inferred under different optimality criteria were meaningfully different.

Evolutionary models for phylogenetic analyses were selected independently for each locus using MrModeltest v. 3.7 (Posada & Crandall 1998) under the Akaike Information Criterion (AIC). A Bayesian analysis was conducted with MrBayes v. 3.1.2 (Huelsenbeck & Ronqvist 2001) to evaluate posterior probabilities (PP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) by Markov Chain Monte Carlo sampling (BMCMC). Phylograms were visualized with FigTree v1.4.0 program (Rambaut, 2012) and annotated in Microsoft Power Point (2010). The final alignment and tree were deposited in TreeBASE, submission ID: 20127.

Table 1 GenBank and culture collection accession numbers of species included in the phylogenetic study. The newly generated sequence is shown in blue. The ex-type strains are shown as bold.

Taxon	**Culture accession no.***	**GenBank Accession no.**	
		ITS	***tef1-α***
Diplodia corticola	CBS 112547	AY259110	DQ458872
Diplodia corticola	CBS 112549	KF766156	KF766398
Diplodia cupressi	CBS 168.87	KF766157	DQ458878
Diplodia cupressi	CBS 261.85	DQ458894	DQ458879
Diplodia mutila	CBS 112553	AY259093	AY573219
Diplodia mutila	CBS 230.30	DQ458886	DQ458869
Diplodia pinea	CBS 109725	DQ458896	DQ458881
Diplodia pinea	CBS 109943	DQ458898	DQ458883
Diplodia scrobiculata	CBS 109944	DQ458899	DQ458884
Diplodia scrobiculata	CBS 113423	DQ458900	DQ458885
Diplodia seriata	CBS 112555	AY25909	AY573220
Diplodia seriata	CBS 119049	DQ458889	DQ458874
Diplodia tsugae	CBS 418.64	DQ458888	DQ458873
Lasiodiplodia citricola	IRAN 1521	GU945353	GU945339
Lasiodiplodia citricola	IRAN 1522	GU945354	GU945340
Lasiodiplodia crassispora	CMW 13488	DQ103552	DQ103559

Taxon	Culture accession no.*	GenBank Accession no.	
		ITS	*tef1-α*
Lasiodiplodia crassispora	WAC 12533	DQ103550	DQ103557
Lasiodiplodia gilanensis	IRAN 1501	GU945352	GU945341
Lasiodiplodia gilanensis	IRAN 1523	GU945351	GU945342
Lasiodiplodia gonubiensis	CBS 116355	AY639594	DQ103567
Lasiodiplodia gonubiensis	CBS 115812	DQ458892	DQ458877
Lasiodiplodia hormozganensis	IRAN 1498	GU945356	GU945344
Lasiodiplodia hormozganensis	IRAN 1500	GU945355	GU945343
Lasiodiplodia iraniensis	IRAN921	GU945346	GU945334
Lasiodiplodia iraniensis	IRAN 1502	GU945347	GU945335
Lasiodiplodia lignicola	MFLUCC 11-0435	JX646797	JX646862
Neofusicoccum luteum	CBS 110299	AY259091	AY573217
Lasiodiplodia parva	CBS494.78	EF622084	EF622064
Lasiodiplodia parva	CBS 495.78	EF622085	EF622065
Lasiodiplodia pseudotheobromae	CBS 304.79	EF622079	EF622061
Lasiodiplodia pseudotheobromae	CBS 374.54	EF622080	EF622059
Lasiodiplodia pseudotheobromae	CBS 116460	EF622078	EF622058
Lasiodiplodia pseudotheobromae	CBS 116459	EF622077	EF622057
Lasiodiplodia pseudotheobromae	IBL 241	KT247479	KT247483
Lasiodiplodia pseudotheobromae	CJA 36	GU973875	GU973867
Lasiodiplodia pseudotheobromae	**MFLUCC 16-0805**	**KY056126**	**KY056125**
Lasiodiplodia pseudotheobromae	IBL 266	KT247480	KT247484
Lasiodiplodia rubropurpurea	WAC 12535	DQ103553	DQ103571
Lasiodiplodia rubropurpurea	WAC 12536	DQ103554	DQ103572
Lasiodiplodia theobromae	CAA 006	DQ458891	DQ458876
Lasiodiplodia theobromae	CBS 112874	EF622075	EF622055
Lasiodiplodia theobromae	CBS 113520	EF622074	EF622054
Lasiodiplodia theobromae	CBS 289.56	EF622070	EF622050
Lasiodiplodia venezuelensis	WAC 12539	DQ103547	DQ103568
Lasiodiplodia venezuelensis	WAC 12540	DQ103548	DQ103569

Abbreviations of culture collections: CAA A. Alves, Universidade de Aveiro, Portugal; CBS Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CJA Kurdistan University, Sanandaj, Kurdistan Province, Iran; CMW Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; IBL Independent Biological Laboratories, Israel; IRAN Iranian Research Institute of Plant Protection, Iran, Tehran; MFLUCC Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; WAC Department of Agriculture Western Australia Plant Pathogen Collection, Perth, Australia.

Results

Phylogenetic analysis

The combined ITS and *tef1-α* dataset consisted of 45 taxa including our strain (*Lasiodiplodia pseudotheobromae*; MFLUCC16-0805), with *Neofusicoccum luteum* (CBS 110299) as the outgroup taxon. The combined dataset consisted of 847 constant characters and 44 parsimony-uninformative characters. Maximum parsimony analysis of the remaining 165 parsimony-informative characters resulted in 1000 trees with TL = 405, CI = 0.704, RI = 0.896, RC = 0.630, HI = 0.296. Maximum likelihood (ML), maximum parsimony (MP) and Bayesian

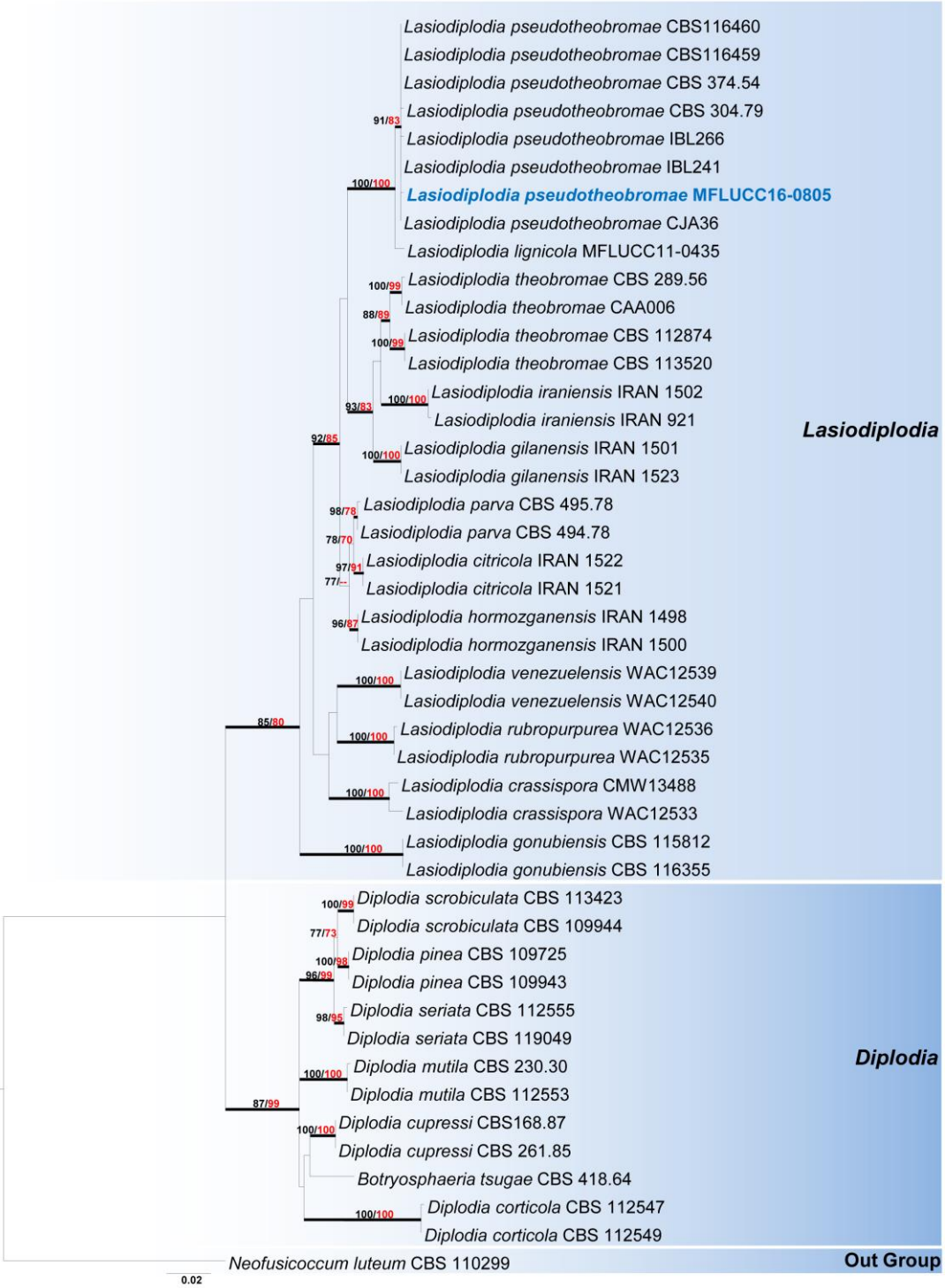

Fig. 1 RAxML tree based on analysis of a combined ITS and *tef1-α* partial sequences. Bootstrap support values for maximum parsimony (MP, red) and maximum likelihood (ML, black) greater than 70 % are defined above the nodes. Bayesian posterior probabilities (PP) greater than 0.90 are shown as bold branches. The tree is rooted to *Neofusicoccum luteum* (CBS 110299). The new strain is shown in blue. Ex-type strains are shown in bold.

posterior probability analyses (PP) resulted in trees with similar topologies that did not differ significantly from one another (data not shown). The final RAxML tree is shown in Fig. 1, with the final ML optimization likelihood value of -3295.519404 (ln). The sexual morph of *Lasiodiplodia pseudotheobromae* strain (MFLUCC 16-0805) clustered with other strains of *Lasiodiplodia pseudotheobromae* in a well-supported clade (91% ML, 83% MP, 0.91 PP, Fig. 1).

Taxonomy

An emended description of *Lasiodiplodia pseudotheobromae* is provided and discussed below.

Lasiodiplodia pseudotheobromae A.J.L. Phillips, A. Alves & Crous, Fungal Diversity 28: 8 (2007). Fig. 2

Facesoffungi number: FoF0264

Saprobic on dead leaves of *Plukenetia volubilis* L. **Sexual morph**: Ascomata apearing as black spots on host surface, globose to subglobose, 270–320 µm diam., 265–275 µm high, gregarious, scattered to clustered, immersed or erumpent through host surface, slightly raised, glabrous, uni-loculate. *Ostioles* up to 59–63 µm diam., 68–73 µm high, setae- like periphyses, central circular, papillate. *Peridium* 35–45 µm wide, thin to thick-walled with equal thickness, composed of several layers of dark brown to black pseudoparenchymatous cells, arranged in a *textura angularis* to *textura prismatica. Hamathecium* composed of dense, broad cellular pseudoparaphyses, 3.2–3.9 µm wide, filamentous, septate, constricted at the septum, anastomosing at the apex, embedded in a hyaline gelatinous matrix. *Asci* (159–)168–182(–219) × (24–)28–31(–40) µm ($\bar{x}$ = 178 × 29.9 µm, n = 20), 8-spored, bitunicate, fissitunicate, cylindric-clavate, short furcate or obtuse pedicel, apically rounded, with well-developed ocular chamber. *Ascospores* (31.5–)35–38(–41) × 14–16µm ($\bar{x}$ = 36.1 × 15.5 µm, n = 20), overlapping uni- to bi-seriate, ellipsoidal to fusiform, with rounded ends, initially hyaline to pale yellowish, becoming golden to dark brown at maturity, aseptate, straight to curved, smooth-walled. **Asexual morph**: see Alves et al. (2008).

Culture characteristics: Colonies on PDA reaching 37 mm diameter after 2 weeks at 20–25 °C, colonies medium sparse, circular, flat, surface slightly rough with edge entire, margin well-defined, cottony to fairly fluffy with sparse aspects, colony from above: brown to black at the margin, white to grey at the centre; reverse, brown to black at the margin, light brown to yellowish at the centre; mycelium light brown to whitish grey with tufting; not producing pigments in PDA.

Material examined: China, Yunnan Province, Xishuangbanna, Nabanhe, dead leaves of *Plukenetia volubilis* L. (*Euphorbiaceae*), 20 November 2015, D.S. Tennakoon, DXH 024 (MFLU 16-1386, *ibid*. HKAS 93716), living culture, MFLUCC 16-0805, KUMCC 15-0571.

Notes: The sexual morphs of *Lasiodiplodia* species have rarely been recorded and are known only for *L. gonubiensis*, *L. lignicola* and *L. theobromae*. Morphologically, *Lasiodiplodia pseudotheobromae* is distinct from the sexual morphs of these other species. *Lasiodiplodia pseudotheobromae* resembles *L. gonubiensis* in having dark brown to black, unilocular, globose ascomata with central ostiole but, *Lasiodiplodia gonubiensis* has ascospores with a terminal apiculus, which is not found in *L. pseudotheobromae*. Ascospores of *L. gonubiensis* are normally hyaline, becoming pigmented and septate when the ascospores are released, but in *L. pseudotheobromae* ascospores are golden to dark brown and aseptate. *Lasiodiplodia lignicola* differs morphologically from *L. pseudotheobromae* in having multi-loculate ascomata, size range of ascomata, asci and host occurrence (Table 2.), although, the size range and colour of ascospores is quite similar to *L. pseudotheobromae*. The size ranges of *Lasiodiplodia* species sexual morphs are shown in Table 2.

Key to recorded sexual morphs of *Lasiodiplodia* species

1. Ascomata uni-loculate…… **2**
1. Ascospores multi-loculate …… ***L. lignicola***
2. Ascospores without a terminal apiculus, aseptate…… **3**
2. Ascospores with a terminal apiculus, 1–2-septate within ascoma or shortly after discharge …… ***L. gonubiensis***
3. Ascospores hyaline, associated with various hosts …… ***L. theobromae***
3. Ascospores golden brown, associated with *Plukenetia volubilis* L ***L. pseudotheobromae***

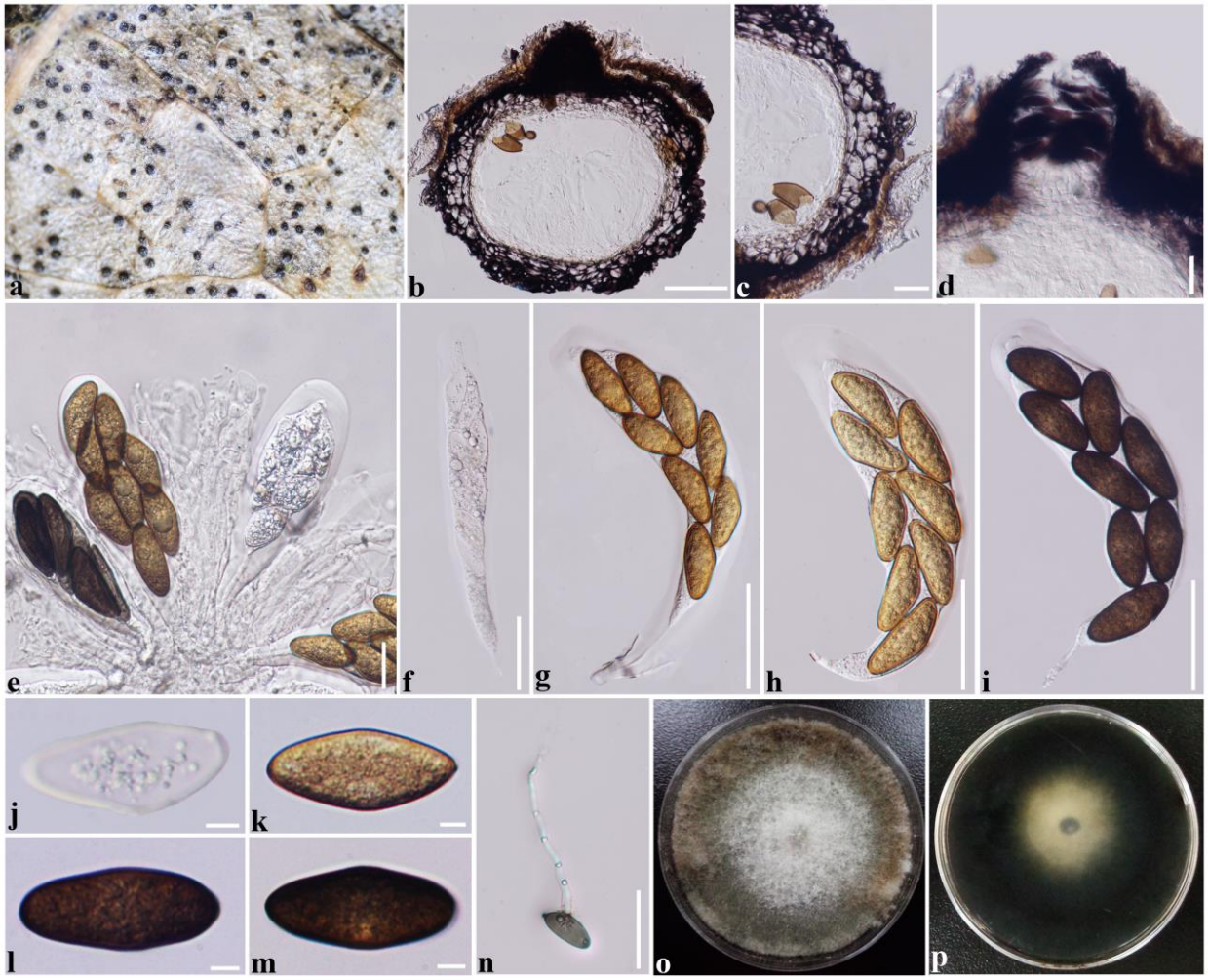

Fig. 2 – *Lasiodiplodia pseudotheobromae* (MFLUCC 16-0805) **a** Appearance of ascomata on the host. **b** Vertical section of ascoma. **c** Section of peridium. **d** Section through neck. **e** Asci embedded in pseudoparaphyses. **f** Immature ascus. **g–i** Asci. **j–m** Ascospores. **n** Germinated ascospore. **o** Colony from above. **p** Colony from below. Scale bars: b = 50 µm, c–e = 20 µm, f–i = 50 µm, j–m = 5 µm, n = 50 µm.

Discussion

In this study, we describe the sexual morph of a strain of *Lasiodiplodia pseudotheobromae* that was identified based on analysis of combined ITS and TEF-α genes. This is the first time a sexual morph has been reported for this species.

The sexual morphs of *Lasiodiplodia* are known only in *Lasiodiplodia theobromae*, *L. lignicola* and *L. gonubiensis*. *Lasiodiplodia pseudotheobromae* can be distinguished from *L. theobromae* in having golden brown to dark brown ascospores and setae-like periphyses in the ostiole. In *L. theobromae*, ascospores are hyaline and periphyses are lacking. The size range of asci and hosts also differ in *L. theobromae*. However, the sexual-asexual morph connection of *L. theobromae* has not yet been conclusively proven (Phillips et al. 2013). Liu et al. (2012) introduced *Auerswaldia lignicola* as a new sexual morph species. Phillips et al. (2013) transferred this species to *Lasiodiplodia* as *L. lignicola*. *Lasiodiplodia lignicola* was characterized by multi-loculate ascomata and ascospores that are reddish-brown to dark brown, aseptate, fusiform to ellipsoid and with narrowly rounded ends. The sexual morph of *Lasiodiplodia gonubiensis* was described by Trakunyingcharoen et al. (2015) and is characterized by having hyaline, aseptate ascospores, that rarely turn pale brown, that are 1–2 septate within the ascoma or shortly after discharge and have hyaline apiculi at both or either ends.

As a result of this study the number of species of *Lasiodiplodia* with known sexual morphs increases to four. Our observations confirm that ascospores of *Lasiodiplodia* species are coloured and can be 1- or 2-septate. Further collections are required to resolve the sexual and asexual linkage of other *Lasiodiplodia* species.

Table 2 Synopsis of recorded sexual morphs of *Lasiodiplodia* species discussed in this study

***Lasiodiplodia* species**	**Size (µm)**			**Ascospores**		**References**
	Ascomata	**Asci**	**Ascospores**	**septation**	**Colour**	
L. gonubiensis	530–600 × 400–500	150–180 × 27.5–30	35–37.5 × 17.5–20	Rarely 1–2 septate	Rarely pale brown	Trakunyingcharoen et al. 2015
L. lignicola	500–750 × 500–1000	85 × 15–20	15–20 × 8–10	Aseptate	Reddish brown to dark brown	Phillips et al. 2013
L. pseudotheobromae	270–320 × 265–275	168–182 × 28–31	35–38 × 14–16	Aseptate	Golden to dark brown	This study
L. theobromae	250–400	90–120	30–35 × 11–14	Aseptate	Hyaline	Phillips et al. 2013

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