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RESEARCH ARTICLE

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Phylogeny and morphology of four new species of *Lasiodiplodia* from Iran

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Key words

Botryosphaeriaceae
EF-1α
ITS
Lasiodiplodia
phylogeny
taxonomy

Abstract Four new species of *Lasiodiplodia*; *L. citricola*, *L. gilanensis*, *L. hormozganensis* and *L. iraniensis* from various tree species in Iran are described and illustrated. The ITS and partial translation elongation factor-1α sequence data were analysed to investigate their phylogenetic relationships with other closely related species and genera. The four new species formed well-supported clades within *Lasiodiplodia* and were morphologically distinct from all other known species.

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INTRODUCTION

Members of the *Botryosphaeriaceae* (*Botryosphaeriales*, *Dothideomycetes*, *Ascomycota*) are cosmopolitan and occur on a wide range of monocotyledonous, dicotyledonous and gymnosperm hosts (von Arx & Müller 1954, Barr 1987). They are associated with various symptoms such as shoot blights, stem cankers, fruit rots, dieback and gummosis (von Arx 1987) and are also known as endophytes (Slippers & Wingfield 2007). Based on 28S rDNA sequence data Crous et al. (2006) showed that *Botryosphaeria* is polyphyletic and they divided it into several genera distinguishable by conidial morphology and phylogenetic data. *Botryosphaeria* was thus restricted to species with *Fusicoccum* anamorphs. However, the clade containing *Diplodia*/*Lasiodiplodia* could not be fully resolved. In a multigene genealogy Phillips et al. (2008) resolved and separated this clade into six genera including *Diplodia*, *Lasiodiplodia*, *Neodeightonia*, *Barriopsis*, *Phaeobotryon* and *Phaeobotryosphaeria*. Morphological characters of the anamorphic and teleomorphic states also supported the separation of these genera.

Lasiodiplodia species are common, especially in tropical and subtropical regions where they cause a variety of diseases (Punithalingam 1980). According to Sutton (1980) the genus is based on *Lasiodiplodia theobromae*. The main features that distinguish this genus from other closely related genera are the presence of pycnidial paraphyses and longitudinal striations on mature conidia. Thus far 20 species have been described and they are differentiated on the basis of conidial and paraphyses morphology. The more recently described species (described since 2004) have been separated not only on morphology, but also on the basis of ITS and EF-1α sequence data. Punithalingam (1976) included several of the species known at that time as synonyms of *L. theobromae* since he could not separate them on morphological characters. However, on account of its morphological variability and wide host range it seems likely that *L. theobromae* is a species complex. Recent studies based on sequence data have confirmed this and eight new species have been described since 2004 (Pavlic et al. 2004, 2008, Burgess et al. 2006, Damm et al. 2007, Alves et al. 2008).

There have been no studies on the *Lasiodiplodia* species in Iran apart from a few reports of *L. theobromae*. In a survey of *Botryosphaeriaceae* in Iran some *Lasiodiplodia* isolates that differed from *L. theobromae* in terms of morphology and ISSR fingerprinting profile were found. The aim of this study was to characterise these isolates in terms of anamorph morphology and phylogenetic analysis.

MATERIALS AND METHODS

Fungal isolation

During a survey of *Botryosphaeriaceae* in different regions of Iran in 2005–2007 some 30 *Lasiodiplodia*-like isolates were collected from various tree species showing symptoms of branch dieback, cankers and fruit rot. Isolations were made from single conidia or by directly plating out pieces of diseased tissue after surface sterilization (1–4 min in 70 % ethanol). Representative isolates were deposited in the culture collection of the Iranian Research Institute of Plant Protection (IRAN, Tehran, Iran) and the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). Isolates included in the morphological and phylogenetic analyses are listed in Table 1.

Morphology and culture characteristics

To induce sporulation, isolates were transferred to 2 % water agar with sterilised pine needles on the agar surface and incubated under mixed near-UV and cool-white fluorescent light in a 12 h light-dark regime for 2–6 wk at 25 °C. Vertical sections through conidiomata were made for some isolates with a Leica CM1100 cryostat microtome. Structures were mounted in 100 % lactic acid and digital images were recorded with a Leica DFC 320 camera on a Leica DMR HC microscope. Measurements were made with the Leica IM500 measurement module. From measurements of 50 conidia the mean, standard deviation

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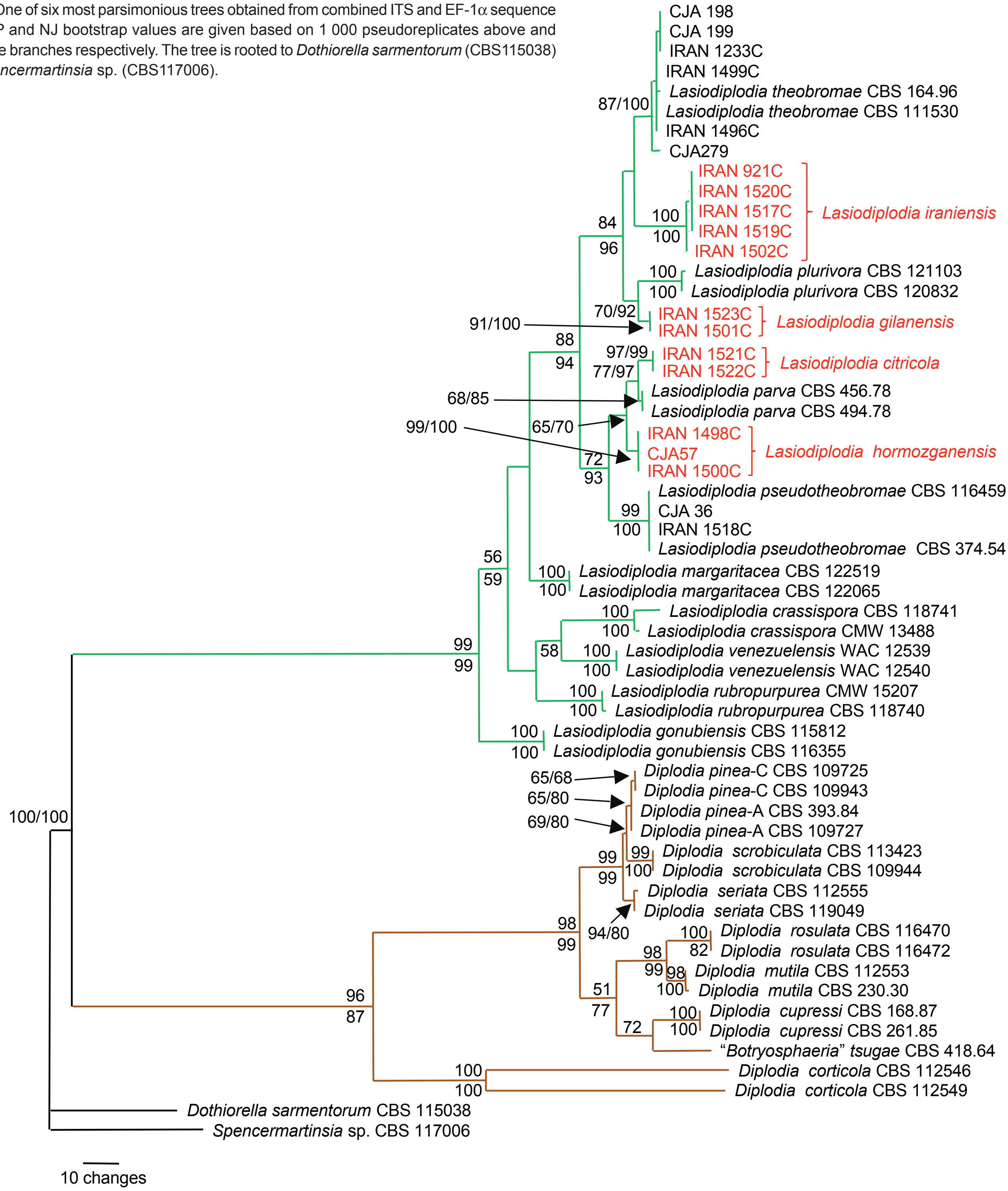
Table 1 Isolates included in the phylogenetic study.

Species	Culture no.	Substrate	Locality	Collector	GenBank	
					ITS	EF-1α
"*Botryosphaeria*" *tsugae*	CBS 418.64	*Tsuga heterophylla*	Canada	A. Funk	DQ458888	DQ458873
Diplodia corticola	CBS 112549	*Quercus suber*	Portugal	A. Alves	AY259100	AY573227
	CBS 112546	*Quercus ilex*	Spain	M. E. Sánchez/A. Trapero	AY259090	EU673310
D. cupressi	CBS 168.87	*Cupresuss sempervirens*	Bet Dagan, Israel	Z. Solel	DQ458893	DQ458878
	CBS 261.85	*Cupresuss sempervirens*	Bet Dagan, Israel	Z. Solel	DQ458894	DQ458879
D. mutila	CBS 112553	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY259093	AY573219
	CBS 230.30	*Phoenix dactylifera*	USA	L.L. Huillier	DQ458886	DQ458869
D. pinea	CBS 393.84	*Pinus nigra*	Netherlands	H.A. van der Aa	DQ458895	DQ458880
	CBS 109727	*Pinus radiata*	South Africa	W.J. Swart	DQ458897	DQ458882
	CBS 109725	*Pinus patula*	Indonesia	M.J. Wingfield	DQ458896	DQ458881
	CBS 109943	*Pinus patula*	Indonesia	M.J. Wingfield	DQ458898	DQ458883
D. rosulata	CBS 116470	*Pinus africana*	Ethiopia	A. Gure	AY210344	EU430267
	CBS 116472	*Pinus africana*	Ethiopia	A. Gure	AY210345	EU430268
D. scrobiculata	CBS 113423	*Pinus greggii*	Mexico	M.J. Wingfield	DQ458900	DQ458885
	CBS 109944	*Pinus greggii*	Mexico	M.J. Wingfield	DQ458899	DQ458884
D. seriata	CBS 112555	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY25909	AY573220
	CBS 119049	*Vitis* sp.	Italy	L. Mugnai	DQ458889	DQ458874
Dothiorella sarmentorum	CBS 115038	*Malus pumila*	Netherlands	A.J.L. Phillips	AY573206	AY573223
Lasiodiplodia citricola	IRAN 1521C	*Citrus* sp.	Iran	A. Shekari	GU945353	GU945339
	IRAN 1522C	*Citrus* sp.	Iran	J. Abdollahzadeh/A. Javadi	GU945354	GU945340
L. crassispora	CMW 13488	*Eucalyptus urophylla*	Venezuela	S. Mohali	DQ103552	DQ103559
	CBS 118741	*Santalum album*	Australia	T.I. Burgess/B. Dell	DQ103550	DQ103557
	IRAN 1501C	Unknown	Iran	J. Abdollahzadeh/A. Javadi	GU945352	GU945341
L. gilanensis	IRAN 1523C	Unknown	Iran	J. Abdollahzadeh/A. Javadi	GU945351	GU945342
L. gonubiensis	CBS 115812	*Syzygium cordatum*	South Africa	D. Pavlic	DQ458892	DQ458877
	CBS 116355	*Syzygium cordatum*	South Africa	D. Pavlic	AY639594	DQ103567
L. hormozganensis	IRAN 1498C	*Mangifera indica*	Iran	J. Abdollahzadeh/A. Javadi	GU945356	GU945344
	IRAN 1500C	*Olea* sp.	Iran	J. Abdollahzadeh/A. Javadi	GU945355	GU945343
	CJA57	*Mangifera indica*	Iran	J. Abdollahzadeh/A. Javadi	GU945357	GU945345
L. iraniensis	IRAN 921C	*Mangifera indica*	Iran	N. Khezrinejad	GU945346	GU945334
	IRAN 1502C	*Juglans* sp.	Iran	A. Javadi	GU945347	GU945335
	IRAN 1517C	*Citrus* sp.	Iran	J. Abdollahzadeh/A. Javadi	GU945349	GU945337
	IRAN 1519C	*Mangifera indica*	Iran	J. Abdollahzadeh/A. Javadi	GU945350	GU945338
	IRAN 1520C	*Salvadora persica*	Iran	J. Abdollahzadeh/A. Javadi	GU945348	GU945336
L. margaritacea	CBS 122519	*Adansonia gibbosa*	Western Australia	T.I. Burgess	EU144050	EU144065
	CBS 122065	*Adansonia gibbosa*	Western Australia	T.I. Burgess	EU144051	EU144066
L. parva	CBS 494.78	*Cassava-field soil*	Colombia	O. Rangel	EF622084	EF622064
	CBS 456.78	*Cassava-field soil*	Colombia	O. Rangel	EF622083	EF622063
L. plurivora	CBS 121103	*Vitis vinifera*	South Africa	F. Halleen	AY343482	EF445396
	CBS 120832	*Prunus salicina*	South Africa	U. Damm	EF445362	EF445395
L. pseudotheobromae	CBS 116459	*Gmelina arborea*	Costa Rica	J. Carranza-Velásquez	EF622077	EF622057
	CBS 374.54	*Coffea* sp.	Zaire	Unknown	EF622080	EF622059
	IRAN 1518C	*Citrus* sp.	Iran	J. Abdollahzadeh/A. Javadi	GU973874	GU973866
	CJA36	*Citrus* sp.	Iran	J. Abdollahzadeh/A. Javadi	GU973875	GU973867
L. theobromae	CBS 164.96	Fruit on coral reef coast	New Guinea	A.Aptroot	AY640255	AY640258
	CBS 111530	Unknown	Unknown	Unknown	AY622074	AY622054
	IRAN 1233C	Unknown	Iran	Unknown	GU973868	GU973860
	IRAN 1496C	*Mangifera indica*	Iran	J. Abdollahzadeh/A. Javadi	GU973869	GU973861
	IRAN 1499C	*Mangifera indica*	Iran	J. Abdollahzadeh/A. Javad	GU973870	GU973862
	CJA198	Unknown	Iran	Unknown	GU973871	GU973863
	CJA199	Unknown	Iran	Unknown	GU973872	GU973864
	CJA279	*Coccos* sp.	Unknown	J. Abdollahzadeh	GU973873	GU973865
L. venezuelensis	WAC 12539	*Acacia mangium*	Venezuela	S. Mohali	DQ103547	DQ103568
	WAC 12540	*Acacia mangium*	Venezuela	S. Mohali	DQ103548	DQ103569
Spencermartinsia sp.	CBS 117006	*Vitis vinifera*	Spain	J. Luque & S. Martos	AY905555	AY905562

Fig. 1 One of six most parsimonious trees obtained from combined ITS and EF-1α sequence data. MP and NJ bootstrap values are given based on 1 000 pseudoreplicates above and below the branches respectively. The tree is rooted to *Dothiorella sarmentorum* (CBS115038) and *Spencermartinsia* sp. (CBS117006).



and 95 % confidence intervals were calculated. Dimensions are given as the range of measurements with extremes in parentheses followed by 95 % confidence limits and mean ± standard deviation. Dimensions of other structures are given as the range of at least 20 measurements. Colony morphology, colour (Rayner 1970), and growth rates between 5 and 35 °C in 5 °C intervals, were determined on 2 % malt extract agar (MEA, Difco laboratories) in the dark. Nomenclatural novelties and descriptions were deposited in MycoBank (www.MycoBank.org; Crous et al. 2004).

Phylogenetic analysis

Isolates were grown in 2 % malt extract broth (MEB) incubated at room temperature for 4–7 d. Mycelial mats were collected by filtration and washed with sterile distilled water and freeze dried with an Edward MicroModulyo 1.5K System (England) freeze drier. DNA was extracted using the method of Raeder & Broda (1985) with modifications as described by Abdollahzadeh et al. (2009). PCR reaction mixtures were prepared according to Alves et al. (2004), with the addition of 5 % DMSO to improve the amplification of some difficult DNA templates. The ITS1-5.8S-ITS2 plus D1/D2 domain of the 28S rDNA gene, and the translation elongation factor-1alpha (EF-1α) were amplified with the primer pairs ITS1 (White et al. 1990) / NL4 (O'Donnell 1993) and EF1-688F/EF1-1251R (Alves et al. 2008), respectively. PCR conditions, purification and sequencing were as described in Abdollahzadeh et al. (2009). The nucleotide sequences were read and edited with Bioedit Sequence Alignment Editor v7.0.9.0 (© 1997–2007, Tom Hall). Sequences of both DNA regions of additional isolates were retrieved from GenBank (Table 1).

Table 2 Conidial and paraphyses dimension of *Lasiodiplodia* spp. examined in this study and previous studies.

Species	Conidial dimensions (µm)	L/W ratio	Paraphyses (µm)		Reference
			Length	Width	
L. abnormis	25–28 × 13–15	–	–	–	Saccardo (1913)
L. citricola	22.5–26.6 × 13.6–17.2	1.6	125	4	This study
L. crassispora	27–30 × 14–17	1.8	70	4	Burgess et al. 2006
L. fiorii	24–26 × 12–15	–	–	–	Saccardo (1913)
L. gilanensis	28.6–33.4 × 15.6–17.6	1.9	95	4	This study
L. gonubiensis	32–36 × 16–18.5	1.9	70	4	Pavlic et al. 2004
L. hormozganensis	19.6–23.4 × 11.7–13.3	1.7	83	4	This study
L. iraniensis	18.7–22.7 × 12.1–13.9	1.6	127	4	This study
L. margaritacea	14–17 × 11–12	1.3	50	4	Pavlic et al. 2008
L. paraphysaria	30–32 × 15–16	–	90–100	3	Saccardo (1913)
L. parva	18.3–22.1 × 10.7–12.3	1.8	105	4	Alves et al. 2008
L. plurivora	26.7–32.5 × 14.4–16.7	1.9	130	10	Damm et al. 2007
L. pseudotheobromae	25.5–30.5 × 14.8–17.2	1.7	58	4	Alves et al. 2008
	21.7–26.3 × 13.4–14.8	1.7	60	3–4	This study
L. ricinii	16–19 × 10–11	–	25–35	2	Saccardo (1913)
L. rubropurpurea	24–33 × 13–17	1.9	70	4	Burgess et al. 2006
L. theobromae	23.6–28.8 × 13–15.4	1.9	55	4	Alves et al. 2008
	22.4–24.2 × 12.9–14.3	1.8	58	2–3	This study
L. thomasiana	28–30 × 11–12	–	89–90	1.5	Saccardo (1913)
L. undulata	20–32 × 13.5–19.2	–	–	–	Abbas et al. (2004)
L. venezuelensis	26–33 × 12–15	2.1	70	4	Burgess et al. 2006

The nucleotide sequences were aligned with ClustalX v1.83 (Thompson et al. 1997) and manually adjusted when necessary. Phylogenetic information contained in indels (insertions/deletions) was incorporated into the phylogenetic analyses using simple indel coding as implemented by GapCoder (Young & Healy 2003). Trees were rooted to *Dothiorella sarmentorum* and *Spencermartinsia* sp. Phylogenetic analyses were performed using PAUP v4.0b10 (Swofford 2003) for neighbour-joining (NJ) and maximum-parsimony (MP) analyses. The neighbour-joining analysis was performed using Kimura-2-parameter nucleotide substitution model (Kimura 1980). All characters were unordered and of equal weight. Bootstrap values were obtained from 1 000 NJ bootstrap replicates. Maximum-parsimony analysis was performed using the heuristic search option with 1 000 random taxon additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated by 1 000 bootstrap replications (Hillis & Bull 1993). Other measures used were consistency index (CI), retention index (RI) and homoplasy index (HI). A partition homogeneity test was done to determine the possibility of combining the ITS and EF-1α datasets (Farris et al. 1995, Huelsenbeck et al. 1996). New sequences were deposited in GenBank (Table 1) and the alignment in TreeBASE (S10302).

RESULTS

Phylogenetic analysis

The partition homogeneity test in PAUP was not significant (P = 0.08) indicating that the ITS (566 characters) and EF-1α (330 characters) datasets were congruent. Therefore the two datasets were combined in a single analysis. ITS and EF-1α sequences for the 20 isolates studied were combined and aligned with 37 sequences of 19 taxa, including the outgroup, retrieved from GenBank. Incomplete portions at the ends of the sequences were excluded from the analyses. The combined dataset after alignment contained 987 characters including alignment gaps, of which 74 were excluded, 552 were constant, 62 were variable and parsimony-uninformative and 299 were parsimony-informative. A heuristic search of the remaining 299 parsimony-informative characters resulted in six most parsimonious trees of 645 steps (CI = 0.73, HI = 0.27, RI = 0.914), each with the same topology. NJ analysis produced a tree with the same topology as the MP trees. One of the MP trees is shown in Fig. 1 with bootstrap support values for MP above and NJ below the branches.

Taxonomy

All isolates obtained in this study (Table 1) produced pycnidia on pine needles on 2 % WA within 3–4 wk. No sexual structures were observed in this study. Based on ITS and EF-1α sequence data and anamorph morphology (Table 2) six species were identified. Of these, *L. theobromae* and *L. pseudotheobromae* are known species. The remaining four species are described here as new.

Lasiodiplodia citricola Abdollahzadeh, Javadi & A.J.L. Phillips, *sp. nov.* — MycoBank MB516777; Fig. 2

Teleomorph. Unknown.

Lasiodiplodia parva similis sed conidiis majoribus, (20–)22–27(–31) × (10.9–)12–17(–19) µm.

Etymology. Named for the host it was first isolated from, namely *Citrus*.

Conidiomata stromatic, pycnidial, produced on pine needles on WA within 2–4 wk, superficial, dark brown to black, covered with dense mycelium, mostly uniloculate, up to 2 mm diam, solitary, globose, thick-walled, non-papillate with a central ostiole. *Paraphyses* hyaline, cylindrical, thin-walled, initially aseptate, becoming up to 1–5 septate when mature, occasionally branched, rounded at apex, occasionally basal, middle or apical cells swollen, up to 125 µm long, 3–4 µm wide. *Conidiophores* absent. *Conidiogenous cells* holoblastic, discrete, hyaline, smooth, thin-walled, cylindrical, proliferating percurrently with 1–2 annellations, 11–16 × 3–5 µm. *Conidia* initially hyaline, aseptate, ellipsoid to ovoid, with granular content, both ends broadly rounded, wall < 2 µm, becoming pigmented, verruculose, ovoid, 1-septate with longitudinal striations, (20–)22–27(–31) × (10.9–)12–17(–19) µm, 95 % confidence limits = 24.1–24.9 × 15–15.7 µm (av. ± S.D. = 24.5 ± 0.2 × 15.4 ± 1.8 µm, l/w ratio = 1.6 ± 0.2).

Culture characteristics — *Colonies* with abundant aerial mycelium reaching to the lid of Petri plate, aerial mycelium becoming smoke-grey (21''''f) to olivaceous-grey (21'''''i) or iron-grey (23'''''k) at the surface and greenish grey (33''''i) to

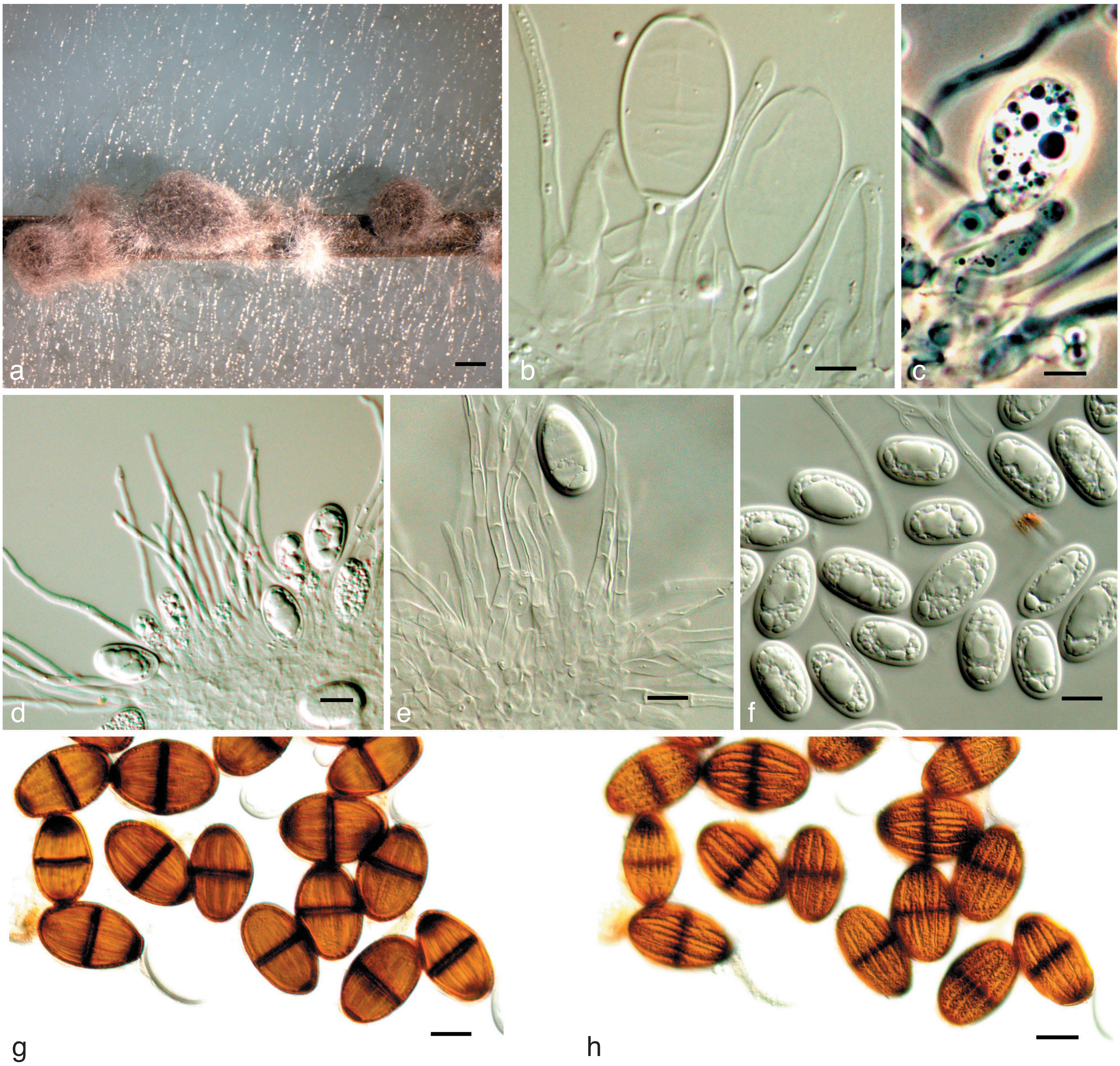


Fig. 2 *Lasiodiplodia citricola* holotype. a. Conidiomata on pine needles in culture; b. conidia developing on conidiogenous cells between paraphyses; c. annellations; d, e. paraphyses; f. hyaline, immature conidia; g, h. mature conidia in two different focal planes to show the longitudinal striations. — Scale bars: a = 1 000 µm; b, c = 5 µm; d–h = 10 µm.

dark slate blue (39''''k) at the reverse after 2 wk in the dark at 25 °C. Colonies reaching 85 mm on MEA after 2 d in the dark at 25 °C. Cardinal temperatures for growth; min ≤ 10 °C, max ≥ 35 °C, opt 25–30 °C.

Substrate — *Citrus* sp.

Distribution — Chaboksar (Gilan Province), Sari (Mazandaran Province), Northern Iran.

Specimens examined. Iran, Gilan Province, Chaboksar, on twigs of *Citrus* sp., June 2007, *J. Abdollahzadeh* and *A. Javadi*, holotype IRAN 14270F, culture ex-type IRAN 1522C = CBS 124707; Mazandaran Province, Sari, on twigs of *Citrus* sp., June 2007, *A. Shekari*, IRAN 1521C = CBS 124706.

Notes — Phylogenetically *Lasiodiplodia citricola* is closely related to *L. parva*, but conidia of *L. citricola*, (20–)22–27(–31) × (10.9–)12–17(–19) µm, are longer and wider than those of *L. parva*, (15.5–)16–23.5(–24.5) × (10–)10.5–13(–14.5) µm. This species produces a pink pigment in PDA cultures at 35 °C.

Lasiodiplodia gilanensis Abdollahzadeh, Javadi & A.J.L. Phillips, *sp. nov.* — MycoBank MB516778; Fig. 3

Teleomorph. Unknown.

Lasiodiplodia plurivora similis sed paraphyses brevoribus et angustioribus.

Etymology. Named after Gilan Province in Iran where it was first found.

Conidiomata stromatic, pycnidial, produced on pine needles on WA within 2–4 wk, superficial, dark brown to black, covered with dense mycelium, mostly uniloculate, up to 940 µm, solitary, globose, thick-walled, non-papillate with a central ostiole. *Paraphyses* hyaline, cylindrical, thin-walled, initially aseptate, becoming up to 1–3 septate when mature, rarely branched, rounded at apex, up to 95 µm long, 2–4 µm wide. *Conidiophores* absent. *Conidiogenous cells* holoblastic, discrete, hyaline, smooth, thin-walled, cylindrical, 11–18 × 3–5 µm. *Conidia* initially hyaline, aseptate, ellipsoid to ovoid, with granular content, rounded at apex, base mostly truncate, wall < 2 µm, becoming pigmented, verruculose, ellipsoid to ovoid, 1-septate with longitudinal striations, (25.2–)28–35(–38.8) × (14.4–)15–18(–19) µm, 95 % confidence limits = 30.6–31.4 ×

16.5–16.7 µm (av. ± S.D. = 31 ± 2.4 × 16.6 ± 1 µm, l/w ratio = 1.9 ± 0.2).

Culture characteristics — *Colonies* with abundant aerial mycelia reaching to the lid of Petri plate, aerial mycelia becoming smoke-grey (21''''f) to olivaceous-grey (21'''''i) at the surface and greenish grey (33''''i) to dark slate blue (39''''k) at the reverse after 2 wk in the dark at 25 °C. Colonies reaching 80 mm on MEA after 2 d in the dark at 25 °C. Cardinal temperatures for growth; min ≤ 10 °C, max ≥ 35 °C, opt 25–30 °C.

Substrate — Unknown.

Distribution — Rahimabad-Garmabdost (Gilan Province), Northern Iran.

Specimens examined. Iran, Gilan Province, Rahimabad-Garmabdost, on twigs of unknown woody plant, June 2007, *J. Abdollahzadeh* and *A. Javadi*, holotype IRAN 14272F, culture ex-type IRAN 1523C = CBS 124704; Gilan Province, Rahimabad-Garmabdost, on twigs of unknown woody plant, June 2007, *J. Abdollahzadeh* and *A. Javadi*, IRAN 1501C = CBS 124705.

Notes — Phylogenetically *L. gilanensis* is closely related to *L. plurivora*, but can be distinguished on average conidial dimensions. Moreover, the paraphyses of *L. gilanensis* are up to 95 µm long and 4 µm wide, whereas paraphyses of *L. plurivora* are up to 130 µm long and 10 µm wide (Damm et al. 2007). Also, the 1–3 basal cells of *L. plurivora* paraphyses are often broader than the apical cells whereas, in *L. gilanensis*, they are the same as the apical cells. This species produces a pink pigment in PDA cultures at 35 °C.

Lasiodiplodia hormozganensis Abdollahzadeh, Zare & A.J.L. Phillips, *sp. nov.* — MycoBank MB516779; Fig. 4

Teleomorph. Unknown.

Lasiodiplodia citricola et *L. parva* phylogenetice simile. Differt a *L. parva* conidiis majoribus (20.2 ± 1.9 × 11.5 ± 0.8 µm) et *L. citricola* minoribus (24.5 ± 0.2 × 15.4 ± 1.8 µm), et paraphyses minoribus.

Etymology. Named after Hormozgan Province in Iran where it was first found.

Conidiomata stromatic, pycnidial, produced on pine needles on WA within 2–4 wk, superficial, dark-brown to black, covered

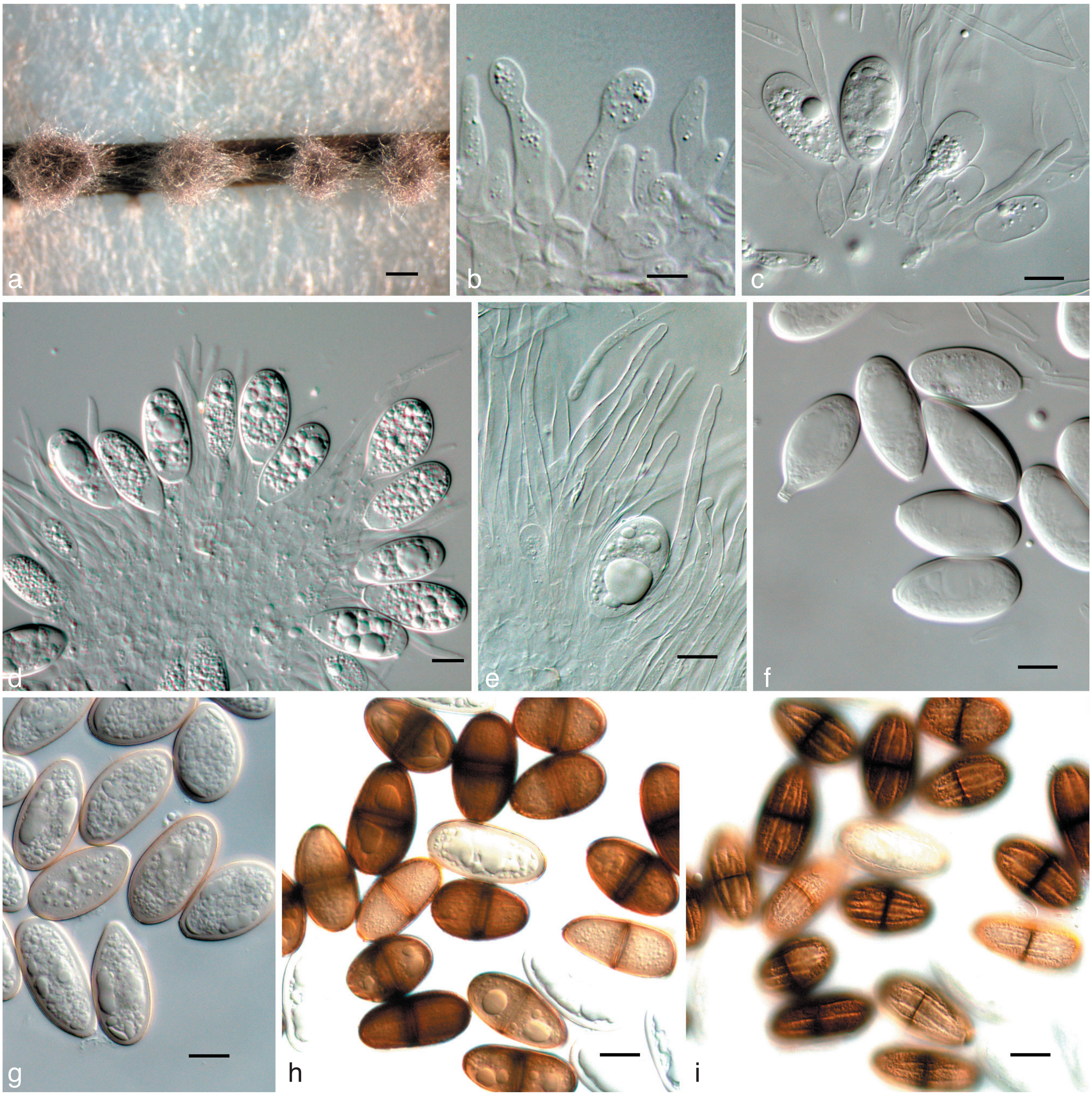


Fig. 3 *Lasiodiplodia gilanensis* holotype. a. Conidiomata on pine needles in culture; b–d. conidia developing on conidiogenous cells between paraphyses; e. paraphyses; f, g. hyaline, immature conidia; h, i. mature conidia in two different focal planes to show the longitudinal striations. — Scale bars: a = 1 000 µm; b, c = 5 µm; d–i = 10 µm.

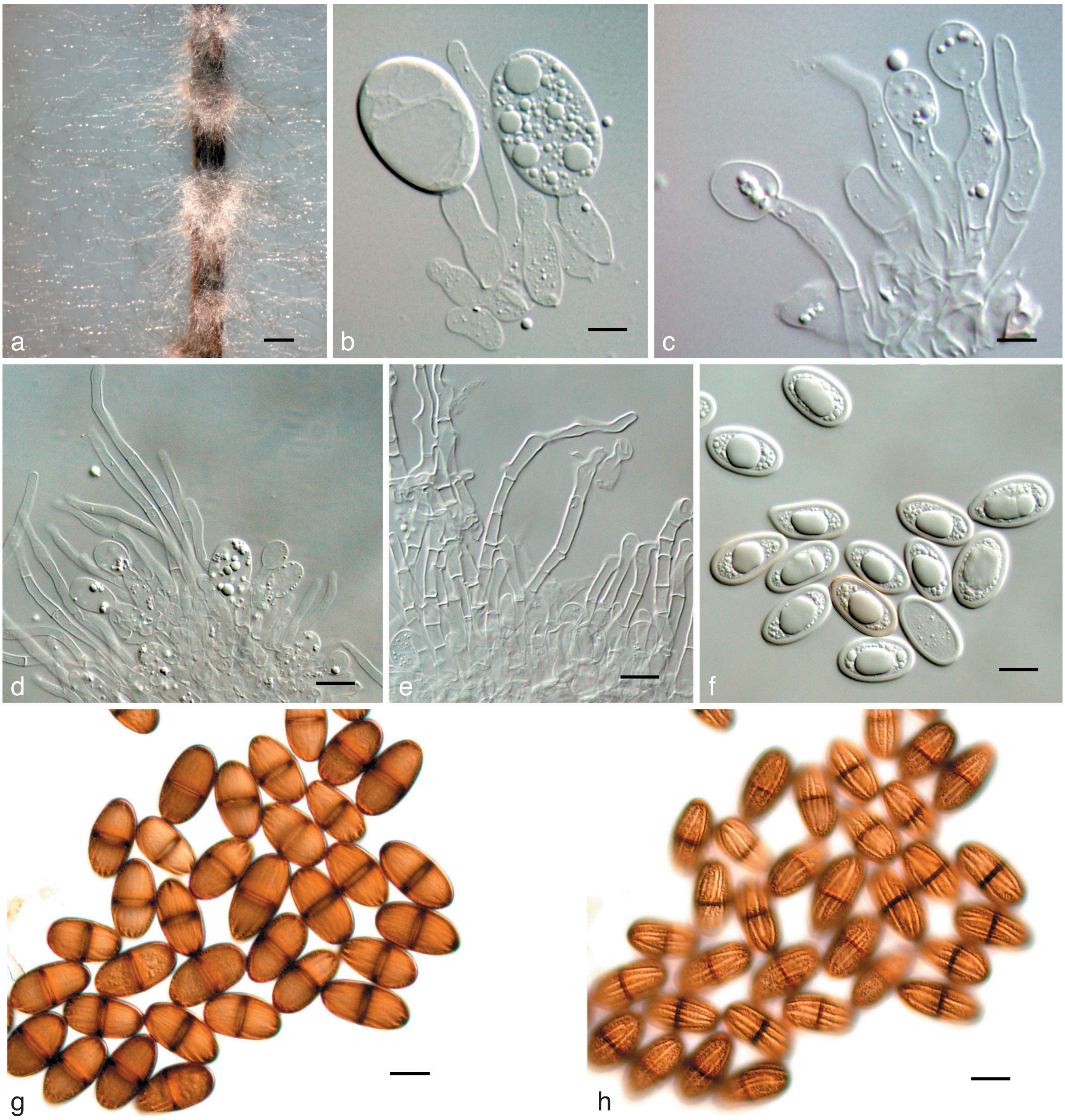


Fig. 4 *Lasiodiplodia hormozganensis* holotype. a. Conidiomata on pine needles in culture; b, c. conidia developing on conidiogenous cells between paraphyses; d, e. paraphyses; f. hyaline immature conidia; g, h. mature conidia in two different focal planes to show the longitudinal striations. — Scale bars: a = 1 000 µm; b, c = 5 µm; d–h = 10 µm.

with dense mycelium, mostly uniloculate, up to 950 µm, solitary, globose, thick-walled, non-papillate with a central ostiole. *Paraphyses*, hyaline, cylindrical, thin-walled, initially aseptate, becoming up to 1–7 septate when mature, rarely branched, occasionally basal, middle or apical cells swollen, rounded at apex, up to 83 µm long, 2–4 µm wide. *Conidiophores* absent. *Conidiogenous cells* holoblastic, discrete, hyaline, smooth, thin-walled, cylindrical, 9–15 × 3–5 µm. *Conidia* initially hyaline, aseptate, ellipsoid to cylindrical, with granular contents, rounded at apex, base round or truncate, wall < 2 µm, becoming pigmented, verruculose, ellipsoid to ovoid, 1-septate with longitudinal striations, (15.3–)18–24(–25.2) × 11–14 µm, 95 % confidence limits = 21.2–21.7 × 12.4–12.6 µm (av. ± S.D. = 21.5 ± 1.9 × 12.5 ± 0.8 µm, l/w ratio = 1.7 ± 0.2).

Culture characteristics — *Colonies* with abundant aerial mycelium reaching to the lid of Petri plate, aerial mycelium becoming smoke-grey (21''''f) to olivaceous-grey (21'''''i) at the surface and greenish grey (33''''i) to dark slate blue (39''''k) at the reverse after 2 wk in the dark at 25 °C. Colonies reaching 83 mm on MEA after 2 d in the dark at 25 °C. Cardinal temperatures for growth; min ≤ 10 °C, max ≥ 35 °C, opt 25–30 °C.

Substrates — *Mangifera indica*, *Olea* sp.

Distribution — Rudan-Kheirabad (Hormozgan Province), Southern Iran.

Specimens examined. Iran, Hormozgan Province, Rudan, on twigs of *Olea* sp., June 2007, *J. Abdollahzadeh* and *A. Javadi*, holotype IRAN 14271F, culture ex-type IRAN 1500C = CBS 124709; Hormozgan Province, Rudan-Kheirabad, on twigs of *Mangifera indica*, June 2007, *J. Abdollahzadeh* and *A. Javadi*, IRAN 1498C = CBS 124708; Hormozgan Province, Rudan-Kheirabad, on twigs of *Mangifera indica*, Mar. 2007, *J. Abdollahzadeh* and *A. Javadi*, CJA 57.

Notes — Phylogenetically this species is closely related to *L. citricola* and *L. parva* but can be distinguished on average conidial dimensions and length of its paraphyses. Conidia of

L. hormozganensis are larger (21.5 ± 1.9 × 12.5 ± 0.8 μm) than those of *L. parva* (20.2 ± 1.9 × 11.5 ± 0.8 μm), but smaller than those of *L. citricola* (24.5 ± 0.2 × 15.4 ± 1.8 μm). Paraphyses of *L. hormozganensis* are shorter (up to 83 μm) than those of *L. parva* (up to 105 μm), and *L. citricola* (up to 125 μm). This species did not produce a pink pigment in PDA cultures at 35 °C.

Lasiodiplodia iraniensis Abdollahzadeh, Zare & A.J.L. Phillips, *sp. nov.* — MycoBank MB516780; Fig. 5

Teleomorph. Unknown.

Lasiodiplodia theobromae phylogenetice simile, sed conidiis minoribus.

Etymology. Named after Iran where it was first found.

Conidiomata stromatic, pycnidial, produced on pine needles on WA within 2–4 wk, superficial, dark brown to black, covered with dense mycelium, mostly uniloculate, up to 980 μm, solitary, globose, thick-walled, non-papillate with a central ostiole. *Paraphyses*, hyaline, cylindrical, thin-walled, initially aseptate, becoming up to 1–6 septate when mature, rarely branched, occasionally basal, middle or apical cells swollen, rounded at apex, up to 127 μm long, 2–4 μm wide. *Conidiophores* absent. *Conidiogenous cells* holoblastic, discrete, hyaline, smooth, thin-walled, cylindrical, 9–16 × 3–5 μm. *Conidia* initially hyaline, aseptate, subglobose to subcylindrical, with granular content, both ends rounded, wall < 2 μm, becoming pigmented, verruculose, ellipsoid to ovoid, 1-septate with longitudinal striations, (15.3–)17–23(–29.7) × 11–14 μm, 95 % confidence limits = 20.6–20.8 × 13–13.1 μm (av. ± S.D. = 20.7 ± 2 × 13 ± 0.9 μm, l/w ratio = 1.6 ± 0.2).

Culture characteristics — *Colonies* with abundant aerial mycelium reaching to the lid of Petri plate, aerial mycelium becoming smoke-grey (21''''f) to olivaceous-grey (21'''''i) at the surface and greenish grey (33''''i) to dark slate blue (39''''k) at the reverse after 2 wk in the dark at 25 °C. Colonies reaching 80 mm on MEA after 2 d in the dark at 25 °C. Cardinal temperatures for growth; min ≤ 10 °C, max ≥ 35 °C, opt 25–30 °C.

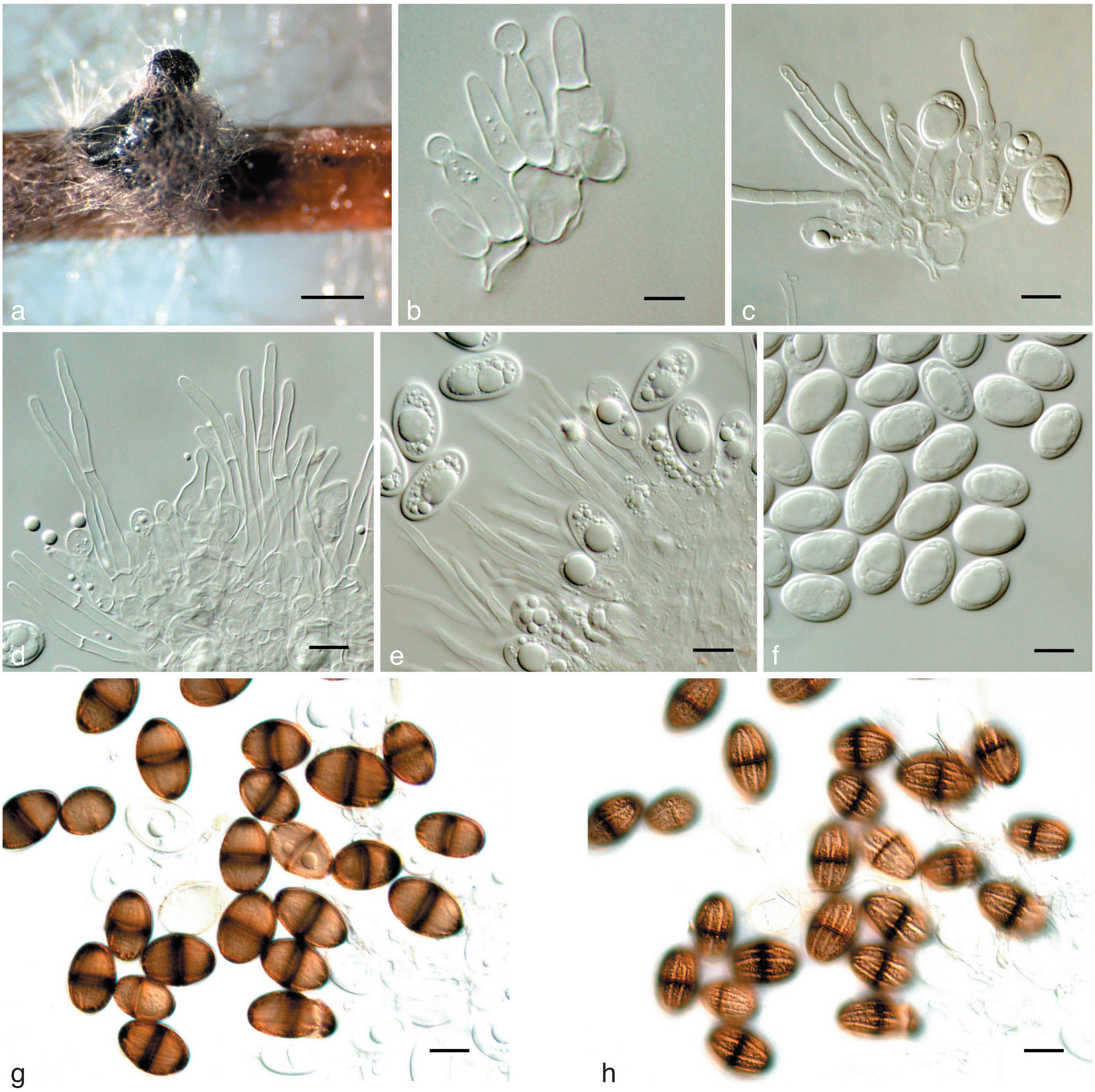


Fig. 5 *Lasiodiplodia iraniensis* holotype. a. Conidiomata on pine needles in culture; b, c. conidia developing on conidiogenous cells between paraphyses; d, e. paraphyses; f. hyaline, immature conidia; g, h. mature conidia in two different focal planes to show the longitudinal striations. — Scale bars: a = 500 μm; b, c = 5 μm; d–h = 10 μm.

Substrates — *Mangifera indica*, *Eucalyptus* sp., *Citrus* sp., *Salvadora persica*, *Juglans* sp., *Terminalia catapa*.

Distribution — Hormozgan & Golestan Provinces, Southern and Northern Iran.

Specimens examined. Iran, Hormozgan Province, Bandar Abbas, Geno mountain, on twigs of *Salvadora persica*, Mar. 2007, *J. Abdollahzadeh* and *A. Javadi*, holotype IRAN 14268F, culture ex-type IRAN 1520C = CBS 124710; Golestan Province, Gorgan-Toshan, on twigs of *Juglans* sp., June 2007, *A. Javadi*, IRAN 1502C = CBS 124711; Additional isolates are listed in Table 1.

Notes — Phylogenetically *L. iraniensis* is clearly distinct from other species, but is most closely related to *L. theobromae*. Conidia of *L. iraniensis* are smaller ((15.3–)17–23(–29.7) × 11–14 µm) than *L. theobromae* ((19–)21–31(–32.5) × (12–)13–15.5 (–18.5) µm). Conidial dimensions of *L. iraniensis* are similar to those of *L. parva*, but the subglobose to subcylindrical conidia with both ends rounded distinguish this species from *L. parva*, in which the conidia are ovoid with apex broadly rounded and the base rounded or truncate. This species produces a pink pigment in PDA cultures at 35 °C.

DISCUSSION

In this study six species of *Lasiodiplodia* were associated with a variety of symptoms on a range of woody hosts in Iran. Four of these (*L. citricola*, *L. gilanensis*, *L. hormozganensis* and *L. iraniensis*) are recognised as new. All four species can be distinguished morphologically and phylogenetically from one another and from previously described species.

Although 24 species are currently known in *Lasiodiplodia* (including those described here), cultures of only 12 are available, and all of these are of species described since 2004. For this reason it was possible to include only the more recently described species in the phylogenetic analysis. Thus, it is possible that some of the species described before 2004 are the same as those included in the phylogenetic tree in this paper. To complicate matters, none of the currently extant isolates of *L. theobromae* can be linked to the type. Pavlic et al. (2004) were unable to locate the holotype of *L. theobromae* and relied on the original description of this species, and its various synonyms, to differentiate *L. gonubiensis* from *L. theobromae*. Burgess et al. (2006), Damm et al. (2007) and Alves et al. (2008) followed the example of Pavlic et al. (2004) and included strains that have previously been recognised as representative of *L. theobromae* in their phylogenies. This is not wholly satisfactory, but until the species is recollected and an epitype is proposed there is no alternative. However, that does not resolve the possibility that new species names are applied to existing species. To differentiate species in the absence of cultures or sequence data it is necessary to rely on morphological characters and the original descriptions of the older species.

Species in *Lasiodiplodia* have been distinguished based on their DNA phylogeny in association with conidial morphology and dimensions, and morphology and size of paraphyses. Burgess et al. (2006) used septation of pycnidial paraphyses to differentiate *Lasiodiplodia* species including *L. crassispora*, *L. gonubiensis*, *L. rubropurpurea*, *L. theobromae* and *L. venezuelensis*. However, this character needs to be interpreted carefully since paraphyses are aseptate when they are young but later they become septate. For example, Burgess et al. (2006) could not find septate paraphyses in the isolates of *L. theobromae* that they studied. Nevertheless, septa have been reported in this species by Punithalingam (1976) and Alves et al. (2008). Damm et al. (2007) distinguished *L. plurivora* from *L. crassispora* and *L. venezuelensis* on the length and shape of the paraphyses. In a similar way in the present study maximum length of paraphyses differentiated *L. gilanensis* from *L. plurivora*, and *L. hormozganensis* from *L. parva* and *L. citricola*. Burgess et al. (2006) used conidial dimensions to differentiate *L. crassispora*, *L. rubropurpurea* and *L. venezuelensis* from *L. gonubiensis* and *L. theobromae*. Furthermore, Alves et al. (2008) distinguished *L. parva*, and Pavlic et al. (2008) distinguished *L. margaritacea* from all other species on account of their small conidia.

Culture morphology has rarely been used as a character for species separation in *Lasiodiplodia*. However, Alves et al. (2008) distinguished *L. parva* and *L. pseudotheobromae* from *L. theobromae* based on the ability of the first two species to produce a pink pigment in PDA cultures at 35 °C. However, in this study all species except *L. hormozganensis* produced a pink pigment in PDA cultures at 35 °C and the *L. theobromae* isolates produced a very strong pigment. Furthermore, all isolates studied in the present work could grow at 10 °C, which is in contrast to the report of Alves et al. (2008) who found that only *L. pseudotheobromae* was capable of growing at this temperature. Thus, cultural characters can vary widely between isolates of any given species, and thus are of limited value in species determination.

Punithalingam (1976) regarded *L. nigra*, *L. triflorae* and *L. tubericola* as synonyms of *L. theobromae* and this was confirmed from the morphological data presented by Pavlic et al. (2004) for these four species. According to descriptions of *L. abnormis*, *L. fiorii* and *L. thomasianae* given by Saccardo (1913), these are also likely to be synonyms of *L. theobromae*, but this would have to be confirmed from a study of type material. From Saccardo's (1899) description of *L. paraphysaria* (under *Diplodia paraphysaria*) this species is similar to *L. gonubiensis* except that the conidia are smaller (30–32 × 15–16 µm) and the paraphyses are longer (90–100 µm). Nevertheless, conidia of *L. paraphysaria* are substantially longer than any other known species of *Lasiodiplodia*, apart from *L. gonubiensis*. On the other hand, conidia of *L. ricinii* have similar dimensions to *L. parva* (16–19 × 10–11 µm), but the paraphyses are much shorter (25–35 µm). Little information is available on *L. undulata*. Abbas et al. (2004) regarded this as a synonym of *L. theobromae* and report the conidia as 20–32 × 13.5–19.2 µm. In the original description, Berkeley (1868) gives the conidia as 33 µm long, and this was confirmed by Saccardo (1884) who reported them as 30–33 µm long. Since conidia of *L. theobromae* rarely exceed 30 µm (Punithalingam 1976, Pavlic et al. 2004, Alves et al. 2008) it seems unlikely that *L. undulata* is a synonym of *L. theobromae*.

Since 2004, 12 new species have been described in *Lasiodiplodia*, while in the preceding 108 years only 13 species were introduced. The recent increase in the number of species recognised is largely due to the use of phylogenetic data, but is also due to sampling in relatively unexplored regions including Venezuela (Burgess et al. 2006), Western Australia (Pavlic et al. 2008) and Iran (this paper).

Since 2004 phylogenetics has played a significant role in distinguishing species in *Lasiodiplodia*. Pavlic et al. (2004) used ITS sequence data to distinguish *L. gonubiensis* from *L. theobromae*. Burgess et al. (2006) described a further three new *Lasiodiplodia* species clearly separated from *L. theobromae* based on ITS sequences. Inclusion of EF-1α sequences in the phylogenetic analysis gave stronger support for these species (Burgess et al. 2006). In a study of *Botryosphaeriaceae* on *Prunus* species in South Africa, Damm et al. (2007) described *L. purivora* as a new species. This species is closely related to *L. theobromae* and the two species could not be distinguished solely on the basis of ITS sequence data but they were clearly separated when EF-1α data was included. Alves et al. (2008)

used ITS and EF-1α together with morphological data to characterise a collection of isolates originally identified as *L. theobromae*. In this way they showed that *L. theobromae* is a complex of cryptic species and described *L. pseudotheobromae* and *L. parva* as new. In the present paper we reveal a further four species in this complex. The eight species currently recognised in the complex cannot be distinguished solely on their ITS sequences, and phylogenetic separation is effectively based on a single gene region, namely EF-1α. However, the differences in EF-1α are fixed within the isolates studied thus far and the species can be separated on morphological features. Nevertheless, if further species appear in this complex in the future it would seem prudent to include further gene regions in the analyses to strengthen the support for them and to separate the existing ones.

All the new species described in this study were isolated from dead twigs of various hosts, but it is not known if they are primary pathogens or saprobes that developed on diseased wood. While *L. citricola* was isolated only from *Citrus* sp., it is not possible to determine any degree of host specificity. Indeed, the other three new species were each isolated from several different hosts thus suggesting a plurivorous nature. Although *L. theobromae* has been reported from more than 500 hosts (Punithalingam 1976), host ranges of species described in recent years have been reportedly restricted (Pavlic et al. 2004, Burgess et al. 2006, Damm et al. 2007). However, it is not clear if the narrow host range of the more recently described species is a reflection of sampling rather than a real representation of host range. Thus it is highly possible that there is a variation in the breadth of host range between species as seen in other genera in the *Botryosphaeriaceae*. For example, *D. seriata* has a very broad host range while *D. pinea* is restricted to pines and *D. corticola* is restricted to *Quercus* species.

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