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Phylogenetic and morphological appraisal of *Leptosphaeria italica* sp. nov. (*Leptosphaeriaceae*, Pleosporales) from Italy

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Abstract

A fungal species with bitunicate asci and ellipsoid to fusiform ascospores was collected from a dead branch of *Rhamnus alpinus* in Italy. The new taxon morphologically resembles *Leptosphaeria*. Maximum likelihood (ML) and Bayesian analyses of a combined LSU and ITS sequence dataset confirm its placement in *Leptosphaeria sensu stricto*. The new taxon is distinct from other species based on morphology and phylogeny and is thus introduced as a new species, *viz*. *L. italica.* The new species is compared with other *Leptosphaeria* species and a comprehensive description and micrographs are provided.

Key words – ITS – LSU – molecular phylogeny – morphology – taxonomy

Introduction

Leptosphaeriaceae is an important group of fungi in the order Pleosporales (Zhang et al. 2012, Hyde et al. 2013), including economically important pathogens on a number of hosts (De Gruyter et al. 2013, Hyde et al. 2014). Barr (1987) introduced the family *Leptosphaeriaceae* and typified it with the genus *Leptosphaeria*, with *L. doliolum* (Pers.) Ces. & De Not. as the type species. The genus *Leptosphaeria* is saprobic or pathogenic on stems and leaves of herbaceous or woody plants in terrestrial and possibly aquatic habitats (Ariyawansa et al. 2015, Jones et al. 2015). Eriksson & Hawksworth (1986, 1990) accepted Barr's separation of *Leptosphaeriaceae* from *Pleosporaceae*. *Leptosphaeriaceae* is phylogenetically well-established in the suborder *Pleosporineae* (Schoch et al. 2009, Zhang et al. 2012, Wijayawardene et al. 2014, Ariyawansa et al. 2015). Even though the family shares some similarities with *Phaeosphaeriaceae*, it can clearly be distinguished by its peridium structure, hosts and asexual morphs (Câmara et al. 2002). The members of

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Leptosphaeriaceae occur abundantly on dicotyledons and have a relatively wide peridium comprising scleroplectenchymatous cells with coniothyrium-like and phoma-like asexual morphs (Câmara et al. 2002, Kirk et al. 2008, Zhang et al. 2009, Wijayawardene et al. 2012, Hyde et al. 2013, Phookamsak et al. 2014). *Phaeosphaeriaceae* species are commonly found on monocotyledons and the peridium is relatively thin, comprising pseudoparenchymatous cells (Câmara et al. 2002, Kirk et al. 2008, Zhang et al. 2009, 2012, Hyde et al. 2013, Phookamsak et al. 2014).

In the wider sense, the genus *Leptosphaeria* is characterized by superficial ascomata with thick-walled, scleroplectenchymatous cells, arranged in a *textura angularis*, with an outer black amorphous layer not fusing with the host tissues and pale to dark brown, septate ascospores in bitunicate asci (Camara et al. 2002, Ariyawansa et al. 2015). The asexual morphs of *Leptosphaeria* are coniothyrium-like and phoma-like, comprising depressed, globose conidiomata, with a flattened base and cylindrical necks. The conidiomata wall is scleroplectenchymatous and conidia ellipsoidal to subcylindrical (Boerema et al. 1994, Hyde et al. 2011, Wijayawardene et al. 2012).

The objectives of this study are to 1) introduce a new species in *Leptosphaeria*, and 2) investigate its taxonomic placement using maximum likelihood and Bayesian analyses of combined LSU and ITS sequence data, based on the backbone tree provided by Ariyawansa et al. (2015).

Material and methods

A Dothideomycete species was collected from a dead branch of a deciduous tree in Italy, and dried herbarium material is deposited at the Mae Fah Luang University herbarium, Thailand (MFLU) and the Herbarium of Kunming Institute of Botany (HKAS), China. Slides were prepared by hand-sectioning of dried material and examined under the Olympus SZH10 stereo microscope. Morphological structures were examined by compound light microscope (Nikon Eclipse 80i compound microscope) and photomicrography carried out using a Canon 550D digital camera fitted to the microscope. Measurements of characters were made with Tarosoft (R) Image Frame Work version 0.9.7, measurements are based on up to 30 units and are reported as the extremes (maximum and minimum).

Faces of fungi numbers were obtained as explained in Jayasiri et al. (2015) and IF numbers in Index Fungorum (2015).

DNA extraction, PCR amplification and gene sequencing

DNA was extracted directly from ascomata using a DNA extraction kit (E.Z.N.A.® Forensic DNA kit, D3591- 01,Omega Bio-Tek) following the manufacturer's instructions.

The partial large subunit nuclear rDNA (28S, LSU) was amplified with primers LROR and LR5 (Vilgalys and Hester 1990). The internal transcribed spacers (5.8S, ITS) were amplified by ITS4 and ITS5 primer pairs as described by White et al. (1990). The components for the PCR were used as follows. The final volume of the PCR mixture was 25 µl with 1.0 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, and optimized buffer, Beijing Trans Gen Biotech Co., Chaoyang District, Beijing, PR China) and 9.5 µl sterilized water.

The amplification was performed with an initial denaturing step of 94 °C for 3 min, followed by 30 amplification cycles of 94 °C for 30 s and 55 °C for 50 s for annealing and 72 °C for 1 min and a final extension step of 72 °C for 10 min (Phillips et al. 2008). The PCR products were observed on 1% agarose electrophoresis gels stained with ethidium bromide. Purification and DNA sequencing was performed by Shanghai Sangon Biological Engineering Technology & Services Co. (Shanghai, P.R. China). The nucleotide sequence data acquired were deposited in GenBank (Table 1).

Phylogenetic analyses

Phylogenetic analyses was based on ITS and LSU sequence data and sequence homologies using the BLAST search engine of the National Centre for Biotechnology Information (NCBI) for

the preliminary identification of the new isolate (Udayanga et al. 2012). Sequences of the available closely related taxa from the family *Leptosphaeriaceae* were obtained from GenBank following Ariyawansa et al. (2015) (Table 1). Members of the genus *Leptosphaeria* was included in this analyses, with *Alternariaster helianthi* as an outgroup taxon.

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

Taxon	*Culture Accession No*	GenBank Accession	
		ITS	LSU
Alloleptosphaeria italica	**MFLUCC 14-0934**	KT454722	KT454714
Alternariaster helianthi	**CBS 327.69**	**KC609335**	**KC584369**
Leptospharia ebuli	**MFLU 14-0828**	**KP744446**	**KP744488**
Leptosphaeria errabunda	CBS 129998	JF740219	-
	CBS 129999	JF740218	-
	CBS 129997	JF740220	-
	CBS 617.75	JF740216	JF740289
	CBS 125978	JF740217	JF740290.
Leptosphaeria macrocapsa	CBS 640.93	JF740237	JF740304
Leptospharia doliolum	**CBS 541.66**	**JF740206**	**JF740284**
	CBS 504.75	JF740209	JX681095
	CBS 130000	JF740210	-
	CBS 155.94	JF740207	JF740282
	CBS 505.75	JF740205	GQ387576
	CBS 125979	JF740208	JF740283
	MFLUCC 15-1875	KP729444	KP729445
Leptosphaeria veronicae	CBS 145.84	JF740254	JF740320
	CBS 126583	JF740255	JF740321
Leptosphaeria conoidea	**CBS 616.75**	**JF740201**	**JF740279**
	CBS 125977	JF740202	JF740280
Leptosphaeria sclerotioides	**CBS 144.84**	**JF740192**	**JF740269**
	CBS 148.84	JF740193	JF740270
Leptosphaeria slovacica	CBS 389.80	JF740247	JF740315
	CBS 125975	JF740248	JF740316
Leptosphaeria sydowii	CBS 385.80	JF740244	JF740313
	CBS 125976	JF740245	JF740314
Leptosphaeria italica	**MFLU 15-0174**	**KT443984**	**KT783670**
Leptosphaeria pedicularis	CBS 390.80	JF740224	JF740294
	CBS 126582	JF740223	JF740293
Leptosphaeria cichorium	**MFLUCC 14-1063**	**KT454720**	**KT454712**
Neoleptosphaeria rubefaciens	CBS 223.77	JF740243	JF740312
	CBS 387.80	JF740242	JF740311
Pseudoleptosphaeria etheridgei	CBS 125980	NR111620	JF740291

Abbreviations – **CBS:** Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; **MFLUCC:** Mae Fah Luang University Culture Collection, Chiang Rai, Thailand.

Multiple sequence alignments were generated with MAFFT v. 6.864b (http://mafft.cbrc.jp/alignment/server/index.html). The alignments were checked visually and improved where necessary and manually optimized to allow maximum alignment and maximum

sequence similarity as detailed in Hall (1999) and ClustalX (Kohli & Bachhawat 2013). Best-fit model of evolution for each data set for Bayesian and Maximum Likelihood analyses was determined by MrModeltest 2.2 (Nylander 2004).

Maximum likelihood trees were generated using the RAxML software (Stamatakis et al. 2008) implemented in raxmlGUI v.0.9b2 (Silvestro & Michalak 2010), Rapid bootstrap with nonparametric bootstrap iterations (Stamatakis et al. 2008) was run in 1,000 replicates with the GTR model and a discrete gamma distribution (Liu et al. 2011). The best scoring tree from a separate randomized tree under the same model was selected with a final likelihood value of -4057.667790 and data files were viewed in MEGA v. 5 (Tamura et al. 2011).

The model of nucleotide substitution was estimated using Topali v. 2.5 and Bayesian inference (BI) analyses computed by Topali 2.5 (Milne et al. 2004). The likelihood parameters for BI were based on the GTR+G model. Posterior probabilities (PP) were estimated over 5 000 000 generations via four independent runs of four simultaneous MCMCMC chains with every 100th tree saved. The first 25% of the sampled trees were discarded as 'burn in'. Bayesian Posterior Probabilities (PP) equal or greater than 0.90 is given below or above each node (Fig. 1). Phylogenetic trees and data files were viewed in MEGA v. 5 (Tamura et al. 2011), TreeView v. 1.6.6 (Page 1996) and FigTree v. 1.4 (Rambaut & Drummond 2008).

RESULTS

Phylogenetic analyses

The results obtained by both maximum likelihood (ML) and Bayesian inference (BI) analyses of the combined LSU and ITS dataset comprised 33 taxa including the new strain were identical. The new strain, *Leptosphaeria italica* (MFLU 15-0174), grouped in *Leptosphaeria sensu stricto* in *Leptosphaeriaceae* and formed a distinct clade with low bootstrap support (Fig. 1).

TAXONOMY

Leptosphaeria italica M.C. Dayarathne, Camporesi & K.D. Hyde, **sp. nov**

Index Fungorum number: IF551552; Facesoffungi Number: FoF01040 Fig. 2

Etymology – Name reflects the country Italy, from where the species was collected.

Holotype – MFLU 15-2251

Saprobic on dead branch of *Rhamnus alpinus*. **Sexual morph**: *Ascomata* 285–294 µm high, 248–260 µm diam., solitary to gregarious, superficial or semi-immersed on host tissue, visible as black spots on host surface, sometimes bi-loculate, globose to subglobose, brown to dark brown, *Ostiole* apex dark brown to black, ostiolar canal filled with periphyses, papilla not conspicuous *Peridium* 38–40 µm wide, comprising two cell types, outer layer composed of large, heavily pigmented, thick-walled cells of *textura angularis*, inner layer composed of scleroplectenchymatous cells of *textura angularis*. *Hamathecium* comprising numerous, 1–2 µm diam., slime coated, branched, cellular pseudoparaphyses. *Asci* 60–112 × 7–12 µm ($\bar{x}$ = 86 × 9.5 µm, n = 30), 8-spored, bitunicate, numerous, cylindrical to cylindric-clavate, short pedicellate, apically rounded, with indistinct ocular chamber. *Ascospores* 12–18 × 4–6 µm ($\bar{x}$ = 15 × 5 µm, n = 30), uni to bi-seriate, pale brown when immature, becoming yellowish brown to brown at maturity, ellipsoid to broadly fusiform, with rounded to acute ends, slightly clavate with narrow towards the base, 3-septate, rarely 1–2 septate, strongly constricted at septum, widest above the central septum, smooth-walled. **Asexual morph**: Undetermined.

Known distribution – On dead and hanging branches of Rhamnus alpinus, Italy.

Material examined – ITALY. Province of Forlì-Cesena [FC], Monte Fumaiolo, on dead and hanging branches of *Rhamnus alpinus* L. ssp. Fallax (Boiss.) Marie & Petitmangin (*Rhamnaceae*), 17 March 2014, E. Camporesi, IT-1766 (MFLU 15-0174, **holotype**), **(isotype** in HKAS, under the code of HKAS 86440).

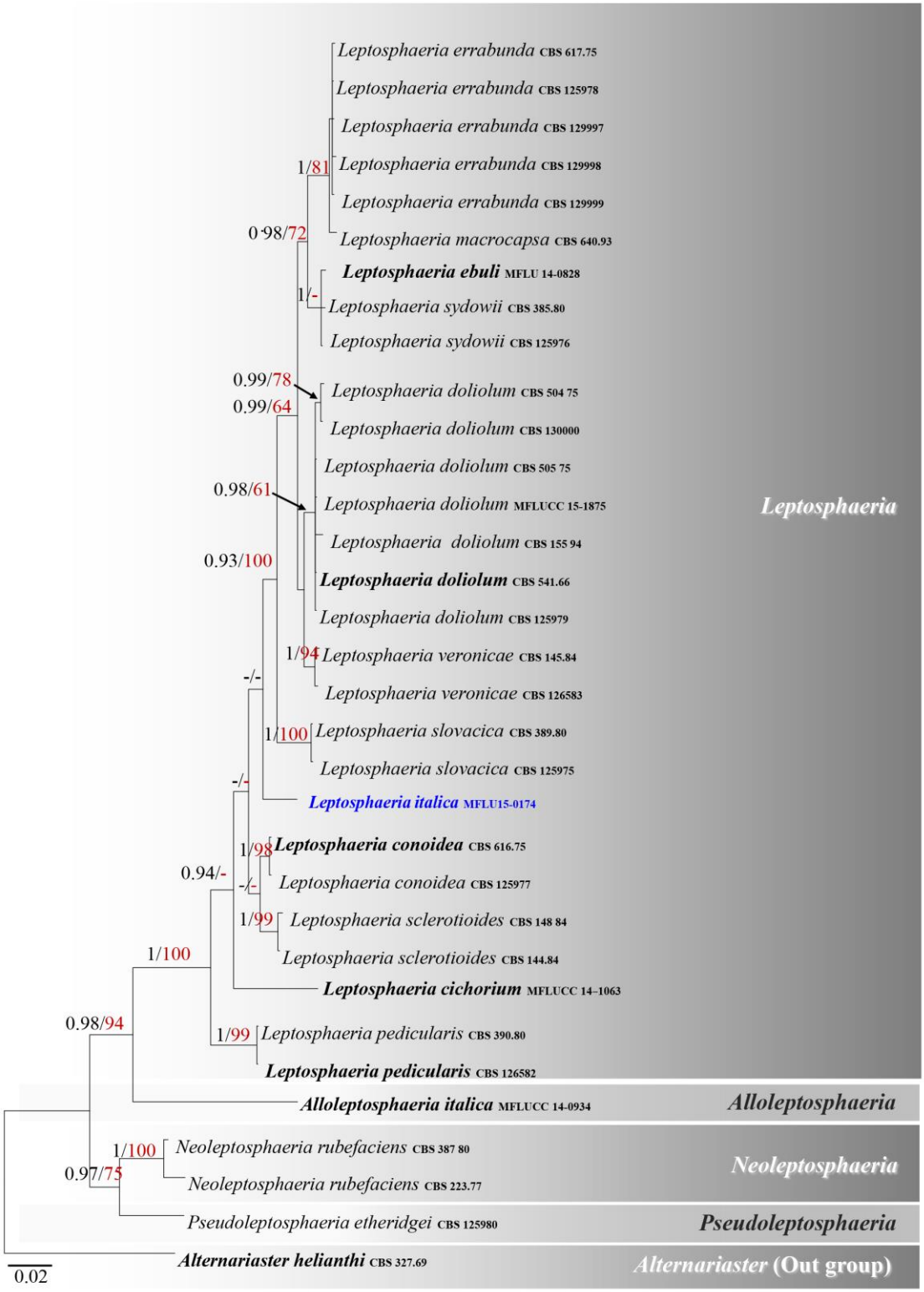

Fig. 1 – Bayesian 50% majority rule consensus tree based on analyses of a combined dataset of LSU and ITS partial sequence data. Bootstrap values for maximum likelihood (ML, blue) higher than 60% and Bayesian posterior probabilities (PP, black) greater than 0.90 are given above the nodes. The tree is rooted to *Alternariaster helianthi* (CBS 327.69). All ex-type strains are in bold.

Notes – Single spore isolation was not successful and DNA was extracted directly from the fresh fruiting bodies. Therefore, living cultures are not available.

Gene sequence data – ITS (KT443984), LSU (KT783670).

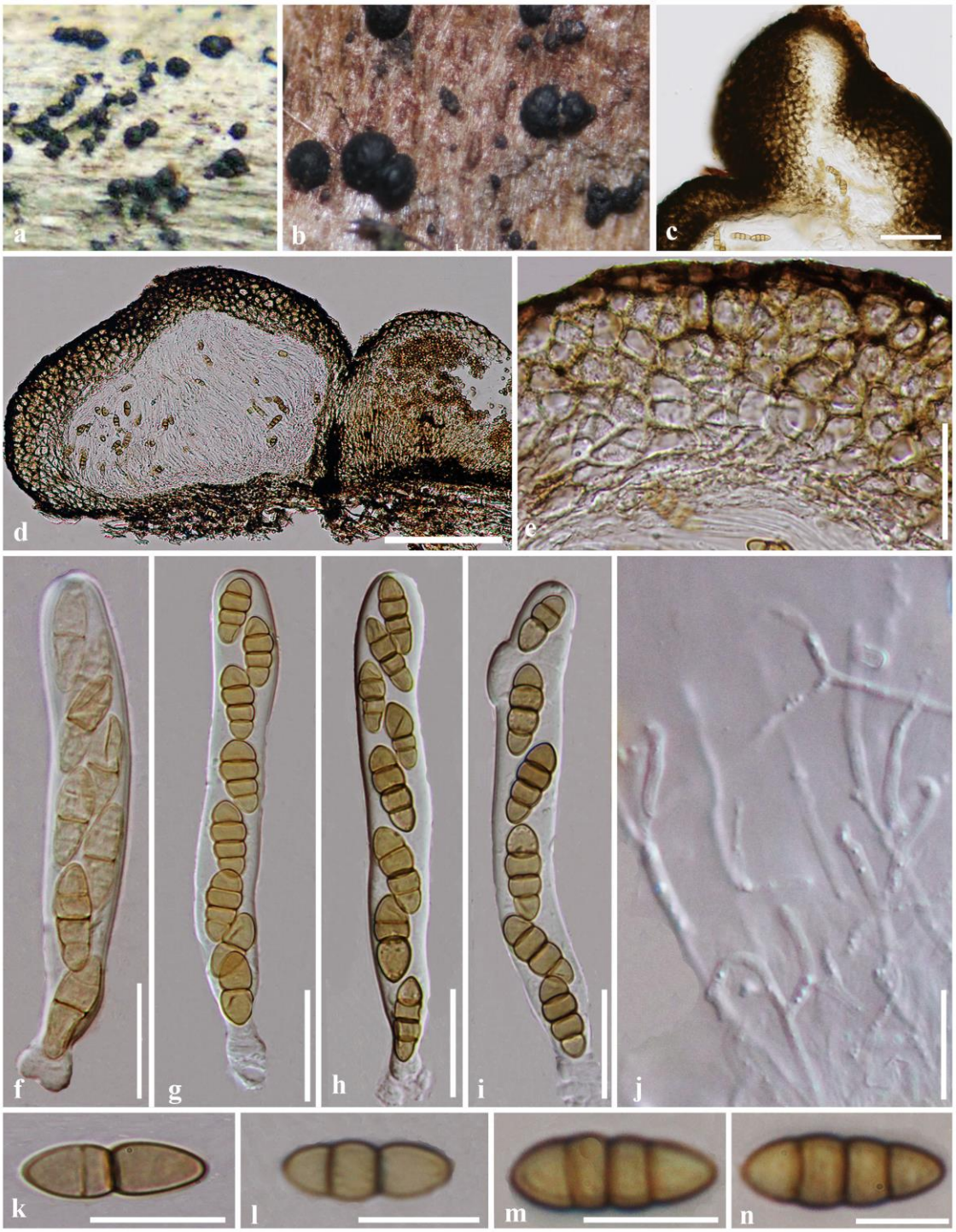

Fig. 2 – *Leptosphaeria italica* (MFLU15-0174, **holotype**). a, b. Ascomata on host. c. Section through ostiolar region. d. Section through ascoma. e. Peridium. f–i. Asci. j. Pseudoparaphyses. k–n. Ascospores. Scale bars: d = 100 µm, c, e = 50 µm, f–j = 20 µm, k–n = 10 µm.

Discussion

The genus *Leptosphaeria* was introduced by Cesati & de Notaris (1863) comprising 26 species, with *L. doliolum* as the lectotype species (Shearer et al. 1990). Material used by Persoon (1800) in the National Herbarium Netherlands, Leiden University (L), was selected to lectotypify *S. doliolum* the basionym of *Leptosphaeria doliolum* (Shearer et al. 1990). *Leptosphaeria sensu stricto* species are characterized by a peridium of scleroplectenchymatous cells and 3-septate, reddish to yellowish brown ascospores, which are narrowly fusoid with sharp to narrowly rounded ends, and with the cell above the central septum widest (Ariyawansa et al. 2015).

Leptosphaeria italica is saprobic on dead stems of *Rhamnus alpines* and has (1–2-) 3-septate, yellowish brown to brown, ellipsoid to fusiform, somewhat clavate ascospores (12–18 × 4–6 µm), strongly constricted at the septa, with the cell above central septum widest and thus differs from *L. doliolum*. As far as we are aware, no *Leptosphaeria* are known from *Rhamnus alpinus.* We compared the new strain with *Melanomma fuscidulum* Sacc. from *Rhamnus* sp. and they share some similar characters, but *M. fuscidulum* differs from *L. italica* with ascospore size (12–18 × 3.5–4 µm) and being 2-celled (Saccardo 1878). In our combined analyses of ITS and LSU sequence data of members of the family *Leptosphaeriaceae*, taxa from the genus *Leptosphaeria sensu stricto* formed a distinct clade including *L. errabunda* (Desm.) Gruyter, Aveskamp & Verkley, *L. macrocapsa* (Trail) Gruyter, Aveskamp & Verkley, *L. sydowii* (Boerema, Kesteren & Loer.) Gruyter, Aveskamp & Verkley, *L. ebuli* Jayasiri, Camporesi & K.D. Hyde, *L. veronicae* (Hollós) Gruyter, Aveskamp & Verkley, *L. doliolum*, *L. conoidea* (De Not.) Sacc., *L. sclerotioides* (Preuss ex Sacc.) Gruyter, Aveskamp & Verkley, *L. slovacica* Picb., *L. pedicularis* (Fuckel) Gruyter, Aveskamp & Verkley, *L. cichorium* Phukhamsakda, Camporesi, Ariyawansa & K.D. Hyde and our new species *L. italica*, the latter forming a distinct lineage with comparatively low statistical support (Figure 1). *Leptosphaeria italica* has similar features to *L. solani* Romell ex Berl. and *L. slovacica. Leptosphaeria solani* differs from *L. italica* in having narrowly clavate to elliptical asci, and ascospores with globose terminal appendages (Shoemaker 1984). *Leptosphaeria slovacica* is phylogenetically distantly related to *L. italica*, and ascospores of *L. slovacica* differ in being broadly fusiform, 3-septate, and olivaceous yellow with acute end cells.

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