Mycosphere 7 (9): 1368–1377 (2016) www.mycosphere.org ISSN 2077 7019

Article – special issue
Doi 10.5943/mycosphere/7/9/10

Neoleptosphaeria jonesii sp. nov., a novel saprobic sexual species, in Leptosphaeriaceae

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Wanasinghe DN, Camporesi E, Hu DM 2016 – *Neoleptosphaeria jonesii* sp. nov., a novel saprobic sexual species, in Leptosphaeriaceae. Mycosphere 7 (9), 1368–1377, Doi 10.5943/mycosphere/7/9/10

Abstract

Neoleptosphaeria is a genus of ascomycetes known only from its asexual morphs (coelomycetous) and its species have saprobic and / or endophytic life modes. We obtained LSU, SSU and ITS sequence data from a single spore isolation of a freshly collected specimen. A phylogeny of representative strains of the genus and other taxa in Leptosphaeriaceae was obtained. *Neoleptosphaeria* proved to be strongly monophyletic but related to other genera in Leptosphaeriaceae. Phylogenetic analyses place our new isolate in a strongly supported clade with the generic type of *Neoleptosphaeria* (*N. rubefaciens*). The sexual morph of *Neoleptosphaeria* is therefore established and includes the first genus with muriform ascospores in Leptosphaeriaceae.

Keywords – asexual morph – dictyospores – Italy – phylogeny – taxonomy

Introduction

Barr (1987) established the family Leptosphaeriaceae species with having a conical or globose ascomata, narrow asci with thin walls and coelomycetous asexual morphs in the order Pleosporales. Leptosphaeriaceae is typified by the genus *Leptosphaeria* and taxa in the family can be saprobic, hemibiotrophic or parasitic on stems and leaves of herbaceous or woody plants in terrestrial habitats (Hyde et al. 2013, Ariyawansa et al. 2015, Liu et al. 2015, Hyde et al. 2016). The classification of genera and species in Leptosphaeriaceae has been challenging due to the lack of understanding of the significance of morphological characters used to differentiate taxa, as well as the lack of DNA based molecular data from ex-type strains (Ariyawansa et al. 2015). Hyde et al. (2013) provided an inclusive view of Leptosphaeriaceae and accepted *Leptosphaeria*, *Neophaeosphaeria*, *Paraleptosphaeria* (sexual genera) *Heterospora*, *Plenodomus* and *Subplenodomus* (asexual genera) in the family. Alves et al. (2013) introduced *Alternariaster* to accommodate *Alternaria helianthi* as the first hyphomycetous record for Leptosphaeriaceae based on morphology coupled with DNA sequence data, while

Submitted 23 November 2016, Accepted 10 December, Published online *
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Trakunyingcharoen et al. (2014) placed *Sphaerellopsis* in the family. Ariyawansa et al. (2015) provided comprehensive descriptions for all genera in Leptosphaeriaceae along with illustrations and a well-resolved backbone tree. Ariyawansa et al. (2015) excluded *Neophaeosphaeria* from Leptosphaeriaceae and introduced *Alloleptosphaeria*, *Neoleptosphaeria* and *Pseudoleptosphaeria* in the family based on evidence from molecular phylogeny, as well as morphological characters. Liu et al. (2015) introduced *Leptosphaeria ebuli* as a new species, *Paraleptosphaeria nitschkei* and *Plenodomus agnitus* as reference specimens to Leptosphaeriaceae based on both molecular data coupled with morphology. Hyde et al. (2016) updated the phylogeny of Leptosphaeriaceae by introducing *Leptosphaeria cirsii* and *L. irregularis* as new species to *Leptosphaeria*.

This paper reports on a saprobic Leptosphaeriaceae species which was collected on dead twigs of *Clematis vitalba* in Italy and identified as a new species of *Neoleptosphaeria*. Combined analyses of LSU, SSU and ITS sequence data, using maximum-likelihood (ML), maximum-parsimony (MP) and Bayesian analyses (BYPP), clearly show that *Neoleptosphaeria* is a well-supported genus (90% ML / 99% MP / 1.00 BYPP, Fig. 1) in the family.

Materials and methods

Sample collection, morphological studies and isolation

Fresh material was collected from Forlì-Cesena Province in Italy and brought to the laboratory in Zip lock plastic bags. Samples were examined with a Motic SMZ 168 Series microscope. Hand sections of the fruiting structures were mounted in water for microscopic studies and photomicrography. The taxa were examined using a Nikon ECLIPSE 80i compound microscope and photographed with a Canon 550D digital camera fitted to the microscope. India ink was added to water mounts to show the presence of a gelatinous sheath around the ascospores. Measurements were made with the Tarosoft (R) Image Frame Work program and images used for figures processed with Adobe Photoshop CS3 Extended version 10.0 software (Adobe Systems, USA).

Single ascospore isolation was carried out following the method described in Chomnunti et al. (2014). Germinated spores were individually transferred to Potato dextrose agar (PDA) plates and grown at 16°C in the daylight. Colony colour and other characters were observed and measured after three weeks. The specimens are deposited at the Mae Fah Luang University (MFLU) Herbarium, Chiang Rai, Thailand. Living cultures are deposited at the Culture Collection of Mae Fah Luang University (MFLUCC). Faces of Fungi number is provided in Jayasiri et al. (2015) and Index Fungorum numbers as in Index Fungorum (2016).

DNA extraction and PCR amplification

Fungal isolates were grown on potato-dextrose agar (PDA) for 3–4 weeks at 16 °C and total genomic DNA was extracted from fresh mycelium using the Biospin Fungus Genomic DNA Extraction Kit-BSC14S1 (BioFlux, P.R. China) following the instructions of the manufacturer. The DNA extractions were stored at 4 °C for regular use and duplicated at -20 °C for long term storage.

DNA amplification was performed by polymerase chain reaction (PCR). Three partial gene portions were used in this study: the internal transcribed spacers (ITS), the large subunits of the nuclear ribosomal RNA genes (LSU) and small subunits of the nuclear ribosomal RNA genes (SSU). ITS was amplified using the primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). LSU was amplified using the primers LROR (5'-TCCTGAGGGAAACTTCG-3') and LR5 (5'-ACCCGCTGAACTTAAGC-3') (Vilgalys & Hester 1990, Rehner & Samuels 1994). SSU was amplified using the primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3') (White et al. 1990). The PCR thermal cycle program for ITS, LSU and SSU amplification was as follows: initially denaturing step of 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing

at 56 °C for 45 s, elongation at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The amplified PCR fragments were sent to a commercial sequencing provider (BGI, Ltd Shenzhen, P.R. China). The nucleotide sequence data acquired were deposited in GenBank (Table 1). The finalized alignment and tree were deposited in TreeBASE, submission ID: 20235 (http://www.treebase.org/).

Sequencing and sequence alignment

Sequences generated from different primers were analyzed with other sequences from GenBank. The related sequences were determined using a BLAST search to recognize closest matches with taxa in Leptosphaeriaceae and recently published data (Ariyawansa et al. 2015, Liu et al. 2015, Hyde et al. 2016). Sequences were automatically aligned with MAFFT v. 7 (http://mafft.cbrc.jp/alignment/ server/index.html; Katoh & Standley 2013), and improved manually when necessary using BioEdit v. 7.0.5.2 (Hall 1999).

Phylogenetic analysis

Phylogenetic analyses of both individual and combined aligned data consisted of maximum-likelihood, maximum parsimony and Bayesian analyses. The sequence alignments were converted to NEXUS file (.nex) for maximum parsimony and Bayesian analyses using ClustalX2 v. 1.83 (Thompson et al. 1997). The NEXUS file was prepared for MrModeltest v. 2.2 after deleting the symbols ="ABCDEFGHIKLMNOPQRSTUVWXYZ" (Nylander 2004) in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2002). For the Randomized Accelerated Maximum Likelihood (RAxML) analysis, sequence alignments were converted to PHYLIP file (.phy) using ALTER (alignment transformation environment: http://sing.ei.uvigo.es/ALTER/; 2016). Parsimony analysis was performed in PAUP using the heuristic search option with 1000 random sequence additions and tree bisection-reconnection (TBR) via branch swapping algorithm. All molecular characters were unordered and given equal weight, analyses were performed with gaps treated as missing data; the COLLAPSE command was set to minbrlen. Maxtrees were set at 5000, branches of zero length were collapsed and all multiple, equally parsimonious trees saved. Clade constancy was measured using bootstrap (BT) analysis with 1000 replicates, with 10 replicates of each random stepwise addition of sequences. 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 trees were significantly different. Maximum parsimony bootstrap values equal or greater than 70 % are given above each node (Fig. 1). The evolutionary models for Bayesian analysis and maximum-likelihood were selected independently for each locus using MrModeltest v. 2.3 (Nylander 2004) under the Akaike Information Criterion (AIC) implemented in both PAUP v. 4.0b10. The GTR+I+G model resulted in each locus for Bayesian and maximum-likelihood analyses by AIC in MrModeltest as a best-fit model.

Bayesian analysis was performed in 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). Six simultaneous Markov chains were run for 50,000,000 generations and trees were sampled every 5000th generation. The distribution of log-likelihood scores was examined to determine stationary phase for each search and to decide if extra runs were required to achieve convergence, using the program Tracer 1.5 (Rambaut & Drummond 2007). All sampled topologies beneath the asymptote (10 %) were discarded as part of a burn-in procedure; the remaining trees were used for calculating posterior probabilities (PP) in the majority rule consensus tree. BYPP greater than 0.95 are given above each node (Fig. 1).

Maximum likelihood trees were generated using the RAxML-HPC2 on XSEDE (8.2.8) (Stamatakis 2008, 2014) in the CIPRES Science Gateway platform (Miller et al. 2010) using GTR+I+G model of evolution. Maximum likelihood bootstrap values (ML) equal or greater than 70 %

Table 1 Taxa used in the phylogenetic analysis and their corresponding GenBank numbers. The newly generated sequence is indicated in bold.

Taxon	Culture accession no.	GenBank accession no.*		
		ITS	LSU	SSU
Alloleptosphaeria italica	MFLUCC 14-0934	KT454722	KT454714	NA
Alternariaster bidentis	CBS 134021	KC609333	KC609341	KC609347
Alternariaster centaureae-diffusae	MFLUCC 14-0992	KT454723	KT454715	KT454730
Alternariaster centaureae-diffusae	MFLUCC 15-0009	KT454724	KT454716	KT454731
Alternariaster helianthi	CBS 327.69	KC609335	KC584369	KC584627
Camarosporium aborescentis	MFLUCC 14-0604	KP711377	KP711378	KP711379
Camarosporium arezzoensis	MFLUCC 14-0238	KP120926	KP120927	KP120928
Camarosporium aureum	MFLUCC 14-0620	NR_137970	KP744478	KP753948
Camarosporium caraganicola	MFLUCC 14-0605	KP711380	KP711381	KP711382
Coniothyrium palmarum	CBS 400.71	AY720708	EU754153	AY642513
Coniothyrium palmarum	CBS 758.73	NA	JX681085	EU754055
Cucurbitaria berberidis	CBS 394.84	NA	JX681088	GQ387544
Cucurbitaria berberidis	MFLUCC 11-0386	NA	KC506796	KC506800
Didymella exigua	CBS 183.55	GU237794	EU754155	EU754056
Heterospora chenopodii	CBS 115.96	JF740227	EU754188	EU754089
Heterospora chenopodii	CBS 448.68	FJ427023	EU754187	EU754088
Heterospora dimorphospora	CBS 165.78	JF740204	JF740281	JF740098
Heterospora dimorphospora	CBS 345.78	NR_111618	GU238069	GU238213
Leptosphaeria cichorium	MFLUCC 14-1063	KT454720	KT454712	KT454728
Leptosphaeria doliolum	MFLU 15-1875	KT454727	KT454719	KT454734
Leptosphaeria doliolum	CBS 541.66	JF740206	JF740284	NA
Leptosphaeria slovacica	CBS 389.80	JF740247	JF740315	JF740101
Leptosphaeria slovacica	CBS 125975	JF740248	JF740316	NA
Neoleptosphaeria jonesii	**MFLUCC 16-1442**	**KY211869**	**KY211870**	**KY211871**
Neoleptosphaeria rubefaciens	CBS 223.77	JF740242	JF740312	NA
Neoleptosphaeria rubefaciens	CBS 387.80	JF740243	JF740311	NA
Ophiosphaerella herpotricha	CBS 620.86	KF498728	DQ678062	DQ678010
Paraleptosphaeria dryadis	CBS 643.86	JF740213	GU301828	KC584632
Paraleptosphaeria nitschkei	MFLU 13-0688	KR025860	KR025864	NA
Paraleptosphaeria orobanches	CBS 101638	JF740230	JF740299	NA
Paraleptosphaeria praetermissa	CBS 114591	JF740241	JF740310	NA
Paraleptosphaeria rubi	MFLUCC 14-0211	KT454726	KT454718	KT454733
Paraphoma radicina	CBS 111.79	FJ427058	EU754191	EU754092
Phaeosphaeria oryzae	CBS 110110	KF251186	GQ387591	GQ387530
Plenodomus chrysanthemi	CBS 539.63	NR_111622	GU238151	GU238230
Plenodomus guttulatus	MFLU 15 1876	KT454721	KT454713	KT454729
Plenodomus lingam	CBS 260.94	JF740235	JF740307	NA
Plenodomus salviae	MFLUCC 13-0219	KT454725	KT454717	KT454732
Plenodomus visci	CBS 122783	NR119957	EU754195	EU754096
Pyrenochaeta nobilis	CBS 407.76	NR_103598	EU754206	DQ898287
Sphaerellopsis filum	CBS 234.51	KP170655	KP170723	NA
Sphaerellopsis macroconidialis	CBS 233.51	KP170658	KP170726	NA
Sphaerellopsis macroconidialis	CBS 658.78	KP170659	KP170727	NA
Sphaerellopsis paraphysata	CPC 21841	KP170662	KP170729	NA
Subplenodomus apiicola	CBS 285.72	JF740196	GU238040	GU238211
Subplenodomus drobnjacensis	CBS 270.92	JF740212	JF740286	JF740100
Subplenodomus valerianae	CBS 499.91	JF740252	JF740319	GU238229
Subplenodomus valerianae	CBS 630.68	JF740251	GU238150	GU238229
Subplenodomus violicola	CBS 306.68	FJ427083	GU238156	GU238231

*NA: No sequence available in GenBank.

Abbreviations: CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC Collection of Pedro Crous housed at CBS; IMI International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand.

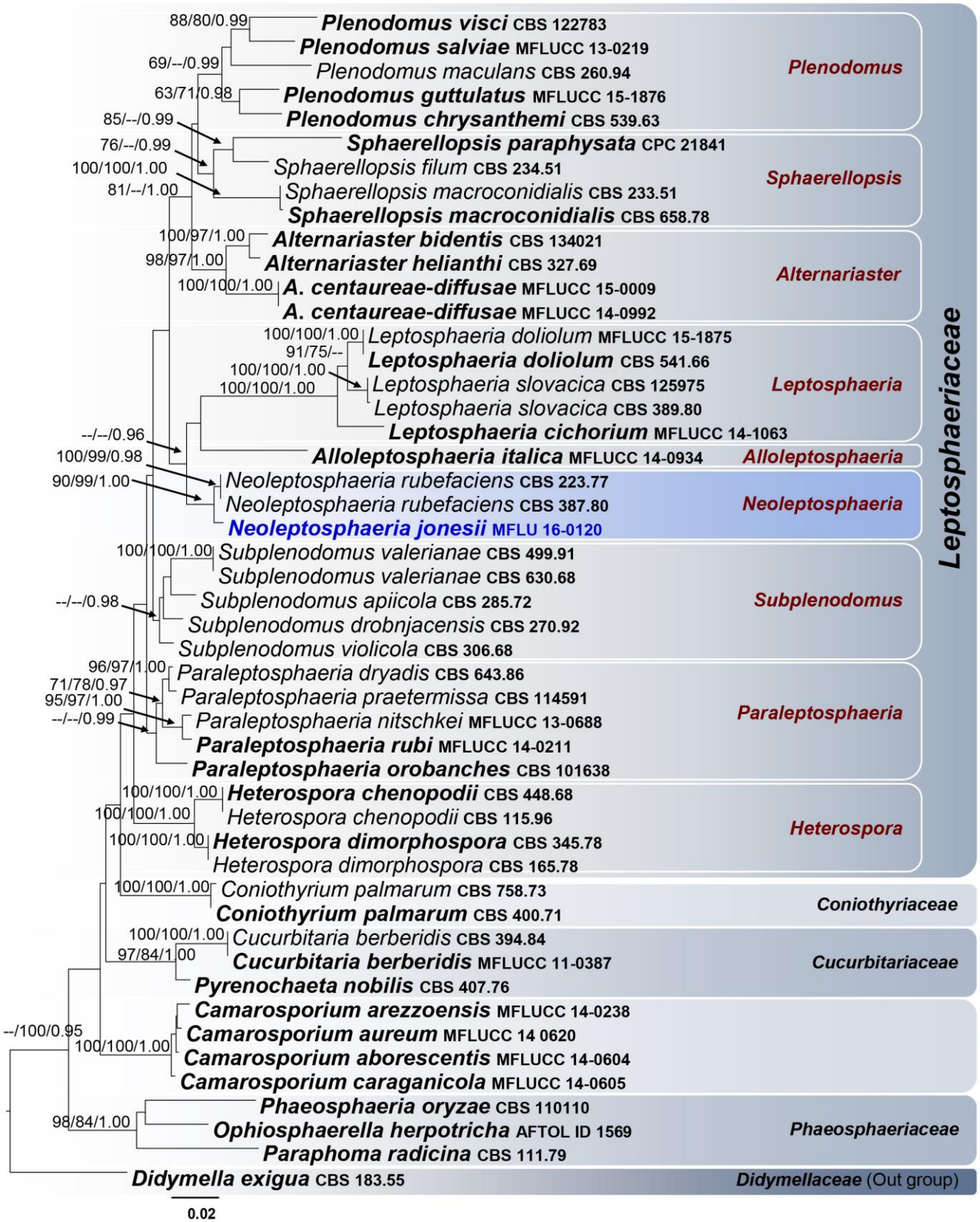

Fig. 1 – RAxML tree based on a combined dataset of LSU, SSU and ITS partial sequences. Bootstrap support values for maximum likelihood (ML), maximum parsimony (MP) higher than 70 % and Bayesian posterior probabilities (BYPP) greater than 0.95 are given above the each branch respectively. The new isolate is in blue. Ex-type strains are in bold. The tree is rooted to *Didymella exigua* in the *Didymellaceae*.

are given above each node (Fig. 1). Phylograms were visualized with FigTree v1.4.0 program (Rambaut, 2012) and reorganized in Microsoft power point (2007) and Adobe Illustrator®.

Results and Discussion

Phylogenetic analysis

The combined LSU, SSU and ITS gene dataset comprised 48 sequences with strains from Leptosphaeriaceae and our new strains. RAxML analysis yielded a best scoring tree (Fig. 1) with a final ML optimization likelihood value of -11115.225636. The matrix had 549 distinct alignment patterns, with 20.37% of undetermined characters or gaps. Estimated base frequencies were as follows; A = 0.249926, C = 0.215820, G= 0.270487, T = 0.263767; substitution rates AC = 1.565898, AG = 3.058216, AT = 2.269071, CG = 0.645547, CT = 7.129065, GT = 1.000; proportion of invariable sites I = 0.805429; gamma distribution shape parameter α = 0.572702. The maximum parsimonious dataset consists of 2698 characters, of which 2319 were constant, 298 parsimony-informative and 81 parsimony-uninformative. The parsimony analysis of the data matrix resulted in five equally parsimonious trees with a length of 1584 steps (CI=0.399, RI=0.638, RC=0.255, HI: 0.601) in the first tree.

The topology of the tree is in accordance with Ariyawansa et al. (2015), Liu et al. (2015), Hyde et al. (2016) based on maximum likelihood analysis. The species in each genus are also spread throughout the tree with significant support (except *Subplenodomus*). Our strain of *Neoleptosphaeria jonesii* (MFLUCC 16-1442) grouped in an isolated clade sister to *Neoleptosphaeria rubefaciens* (CBS 223.77 and 367.80) with 90 % ML, 99% MP and 1.00 PP support (Fig. 1).

Taxonomy

Neoleptosphaeria Ariyawansa & K.D. Hyde, Fungal Divers. 74: 36 (2015) **emended.**
Index Fungorum Number: IF551464
Facesoffungi Number: FoF 01157

Pathogenic or *saprobic* on wood, bark and fruits of herbaceous or woody plants in terrestrial habitats. S**exual morph**: *Ascomata* black, superficial to semi-immersed, fully or partly erumpent, solitary, globose, black, ostiolate. *Ostiole* central, short, filled with hyaline cells. *Peridium* composed of blackish to dark brown cells of *textura angularis*, cells towards the inside lighter, composed of thin-walled cells of *textura angularis*. *Hamathecium* comprising numerous, branched septate, pseudoparaphyses. *Asci* 8-spored, bitunicate, fissitunicate, cylindrical, short-pedicellate. *Ascospores* overlapping uniseriate, muriform, mostly ellipsoidal, 4−5-transversely septate, with 1 vertical septum, constricted at central septum, initially hyaline, becoming brown at maturity, slightly paler, conical and narrow at the ends, guttulate, surrounded by a mucilaginous sheath. **Asexual morph**: see Ariyawansa et al. (2015).

Type species − *Neoleptosphaeria rubefaciens* (Togliani) Ariyawansa & K.D. Hyde, Fungal Diversity 74: 37 (2015)

Neoleptosphaeria jonesii Wanasinghe, Camporesi & K.D. Hyde, **sp. nov.**
Index Fungorum Number: IF552569
Facesoffungi Number: FoF 02716 Fig. 2, 3

Etymology − In honour of Prof. E.B. Gareth Jones for his immense contribution to mycology

Holotype − MFLU 16-0120

Saprobic on dead branches of *Clematis vitalba* L. **Sexual morph**: *Ascomata* 400–500 µm high, 420–470 µm diam. ($\overline{x}$ = 434.4 × 462.1 µm, n = 10), black, superficial to semi-immersed, fully or partly erumpent, solitary, globose, rough or hairy, ostiolate. *Ostiole* 110–150 µm long, 50–100 µm diam. ($\overline{x}$ =

135 × 70 µm, n = 10), central, smooth, with ostiolar canal filled with hyaline cells. *Peridium* 50–80 µm wide at the base, 30–50 µm wide at the sides, comprising 8–10 layers, with outer layer heavily pigmented, thick-walled, comprising blackish to dark brown cells of *textura angularis*, cells towards the inside lighter, with inner layer composed 2–3 layers, hyaline, flattened, thin-walled cells of *textura angularis*. *Hamathecium* comprising numerous, 2–3 µm (n = 40) wide, filamentous, branched septate, pseudoparaphyses. *Asci* 120–130 × 10–13 µm ($\bar{x}$ = 124.4 × 11.5 µm, n = 40), 8-spored, bitunicate, fissitunicate, cylindrical, short-pedicellate, rounded at apex with a minute ocular chamber. *Ascospores* 19–23 × 6–8 µm ($\bar{x}$ = 21 × 7.5 µm, n = 50), overlapping uniseriate, muriform, mostly ellipsoidal, 4−5-transversely septate, with 1 vertical septum, constricted at middle septum, initially hyaline, becoming brown at maturity, slightly paler, conical and narrow at the ends, surrounded by a mucilaginous sheath. **Asexual morph**: Coelomycetous phoma-like. *Pycnidia* solitary to confluent, on upper surface or submerged in agar, globose to subglobose, setose, with apapillate or papillate ostiole, olivaceous to olivaceous-black, the wall with *pseudoparenchymatal* cells. *Conidiophores* hyaline, cylindrical to sub cylindrical, arising from the inner layers of conidioma. *Conidiogenous cells* hyaline, enteroblastic, phialidic, discrete, or integrated in septate. *Conidia* 3–4 × 2–2.5 µm ($\bar{x}$ = 4.3 × 2.3 µm, n = 50) aseptate, cylindrical/ellipsoidal, eguttulate or with 1–2 min guttulate.

Culture characteristics − Colonies on PDA reaching 3 cm diam. after 30 days at 16 °C, circular, smooth margin white at first, dirty white to iron after 4 weeks, flat on the surface, without aerial mycelium, reverse iron (Fig. 3). Hyphae septate branched, hyaline, thin-walled.

Known distribution − Italy, on dead twigs of *Clematis vitalba*.

Material examined − ITALY, Forlì-Cesena, Bagno di Romagna, Pietrapazza, on dead stem of *Clematis vitalba* (Ranunculaceae), 20 January 2013, Erio Camporesi, IT 1021 (MFLU 16-0120, **holotype) isotype** in BBH, ex-type living culture, MFLUCC 16-1442.

Notes − *Neoleptosphaeria* was described by Ariyawansa et al. (2015) as a monotypic genus to accommodate *N. rubefaciens*. Two strains of *Neoleptosphaeria rubefaciens* were included in the phylogeny of Ariyawansa et al. (2015) *viz.* CBS 223.77, isolated from twig of *Quercus* sp. (Fagaceae) in Switzerland and CBS 223.77, isolated from wood of *Tilia europaea* (Tiliaceae) in the Netherlands, and no sexual morph was reproted (De Gruyter et al. 2013). Here we add the asexual and sexual morphs of *Neoleptosphaeria jonesii* from *Clematis vitalba* in Italy. *Neoleptosphaeria jonesii* resembles *N. rubefaciens* in having cylindrical/ellipsoidal, hyaline conidia with 1–2 guttules. The morphology of the sexual morph of *Neoleptosphaeria jonesii* is more close to *Leptosphaeria doliolum*, *Cucurbitaria berberidis* and *Camarosporium arezzoensis* in having globose ascomata, a central ostiole filled with hyaline cells, sides of peridium wider than at the base, cylindrical, short-pedicellate asci which are rounded at apex and with a minute ocular chamber, and overlapping uniseriate, ellipsoidal, muriform ascospores which are mostly, conical and narrow at the ends (Hyde et al. 2013, Ariyawansa et al. 2015, Tibpromma et al. 2015). However they are not closely related in multi-gene analyses (Fig. 1).

Consequently, based on the morphology of asexual morph and the phylogeny we introduce our new taxon, *Neoleptosphaeria jonesii* as the second species of *Neoleptosphaeria*. In Leptosphaeriaceae, the sexual morphs of *Pseudoleptosphaeria*, *Sphaerellopsis* and *Subplenodomus* are still undetermined. Further collections with fresh specimens are needed to link the asexual-sexual morphs.

Acknowledgments

Dhanushka Wanasinghe extend his sincere appreciations to Prof. E.B.G. Jones for funding his research. Dian-Ming Hu thanks the grant number NSFC 31460009 and NSFC 31500021 for funding this research.

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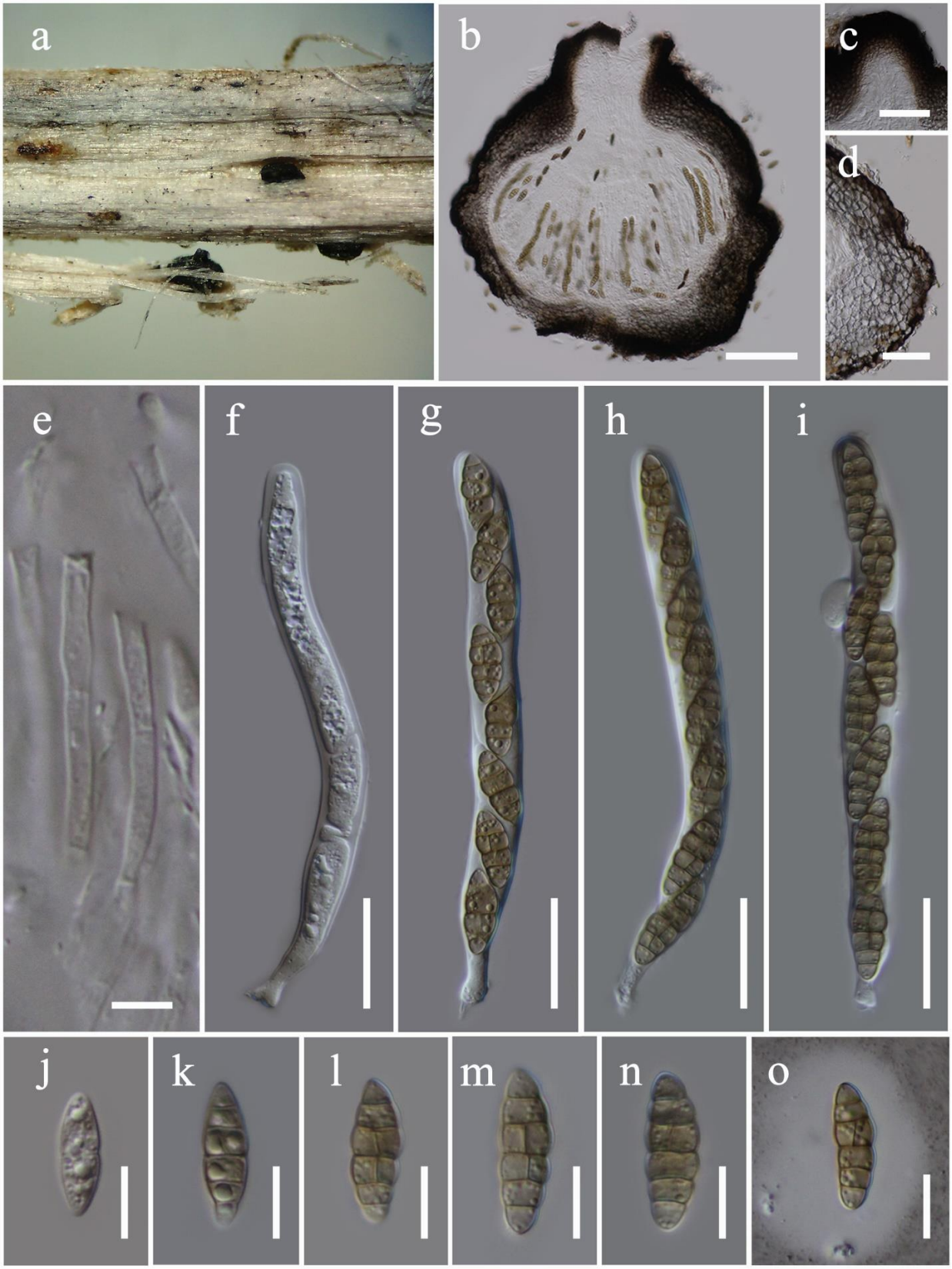

Fig. 2 – *Neoleptosphaeria jonesii* (holotype). **a**. Appearance of ascomata on host substrate. **b.** Section of ascoma. **c.** Close up of ostiole. **d.** Peridium. **e**. Pseudoparaphyses. **f-i**. Asci. **j-o**. Ascospores (note the ascospore stained in Indian ink to show the mucilaginous sheath in o). Scale bars: b = 100 µm, c,d = 50 µm, e = 5 µm, f–i = 20 µm, j–o = 10 µm.

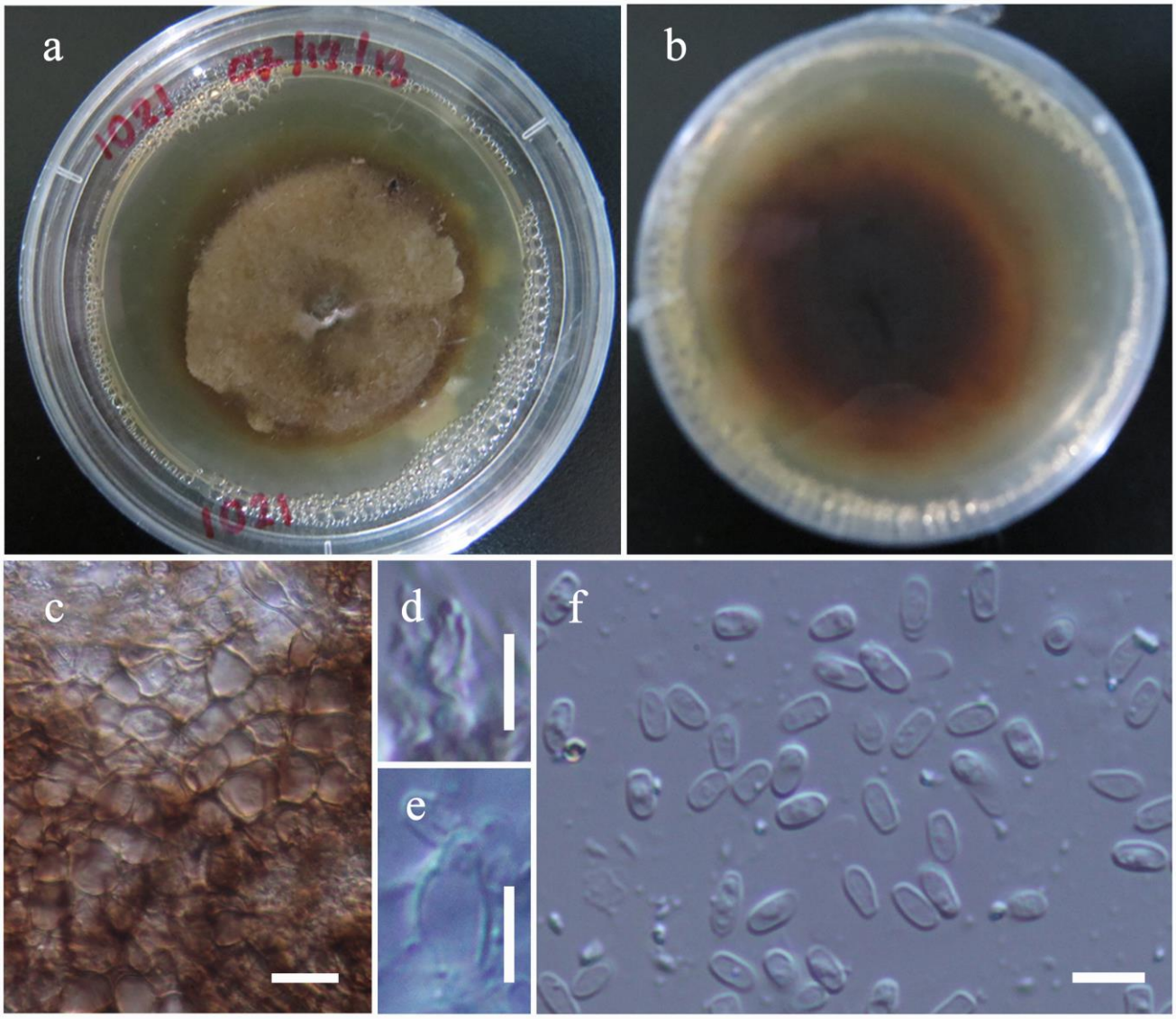

Fig. 3 – *Neoleptosphaeria jonesii* (ex-type culture). a,b Culture on PDA (note b reverse). c Peridium cells of squashed conidiomata. d,e Conidiogenous cells. f Mature and immature conidia. Scale bars: c = 10 µm, d–f = 5 µm.

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