Cryptogamie, Mycologie, 2013, 34 (4): 303-319

Phylogeny and morphology of *Leptosphaerulina saccharicola* sp. nov. and *Pleosphaerulina oryzae* and relationships with *Pithomyces*

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Abstract – A Dothideomycete species, associated with leaf spots of sugarcane (*Saccharum officinarum*), was collected from Nakhonratchasima Province, Thailand. A single ascospore isolate was obtained and formed the asexual morph in culture. ITS, LSU, *RPB2* and *TEF1α* gene regions were sequenced and analyzed with molecular data from related taxa. In a phylogenetic analysis the new isolate clustered with *Leptosphaerulina americana*, *L. arachidicola*, *L. australis* and *L. trifolii* (*Didymellaceae*) and the morphology was also comparable with *Leptosphaerulina* species. *Leptosphaerulina saccharicola* is introduced to accommodate this new collection which is morphologically and phylogenetically distinct from other species of *Leptosphaerulina*. A detailed description and illustration is provided for the new species, which is compared with similar taxa. The type specimen of *Pleosphaerulina oryzae*, is transferred to *Leptosphaerulina*. It is redescribed and is a distinct species from *L. australis*, with which it was formerly synonymized. *Leptosphaerulina* species have been linked to *Pithomyces* but the lack of phylogenetic support for this link is discussed. The character of the asexual morph of *Leptosphaerulina*, which is similar to *Pithomyces*, may to have evolved on separate occasions.

Asexual morph / *Didymellaceae* / Phylogeny / Plant disease / Taxonomy

INTRODUCTION

Dothideomycetes are the largest and most varied class of Ascomycota comprising 22 orders, 105 families, 678 genera and more than 19,000 species (Kirk *et al.*, 2008; Schoch *et al.*, 2006, 2009; Lumbsch & Huhndorf, 2010; Hyde *et al.*, 2013). Species of Dothideomycetes and their asexual morphs are found as endophytes, epiphytes or pathogens on living plants and as saprobes on decaying organic matter including dicotyledons, grasses and other monocotyledons (Schoch

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doi/10.7872/crym.v34.iss4.2013.303

et al., 2006; Zhang *et al.*, 2009). Some genera of Dothideomycetes (e.g. *Botryosphaeria*, *Cochliobolus*, *Didymella*, *Mycosphaerella*, *Leptosphaeria*, *Leptosphaerulina*, *Phaeosphaeria*) and their asexual morphs (e.g. *Alternaria*, *Stemphylium*) may cause serious disease of economic crops (*e.g.* corn, rice, banana, wheat, sugarcane) worldwide (Shoemaker & Babcock, 1989; Schoch *et al.*, 2006, 2009; Zhang *et al.*, 2009; Liu *et al.,* 2012). In this study we collected a Dothideomycete from diseased sugarcane (*Saccharum officinarum*), and it was subsequently identified as a *Leptosphaerulina* species.

Leptosphaerulina was introduced by McAlpine (1902) with *L. australis* on apricot leaves (*Prunus armeniaca* L.) being the type and has also been recorded as associated with *Dolichos*, *Poa, Lolium* and *Vitis* (McAlpine, 1902; Graham & Luttrell, 1961). The asexual state has been reported as *Pithomyces* (Ellis, 1971; Morgan-Jones, 1987; Zhang & Zhang, 2003; Hyde *et al*., 2011; Wijayawardene *et al*., 2012). A linkage with *P. flavus* Berk. & Broome (1873), the morphologically distinct type species of *Pithomyces,* however, has not been established with molecular data, and this relationship must be considered questionable. *Leptosphaerulina* species have ascomata that are ostiolate, papillate and immersed to erumpent, and the bitunicate asci are distinctly saccate. Ascospores are oblong to cylindrical, generally muriform, and mostly hyaline, but become brown at maturity (Graham & Luttrell, 1961; Crivelli, 1983; Abler, 2003; Zhang *et al*., 2012). The genus *Leptosphaerulina* was placed in the family *Pseudosphaeriaceae* based on its morphological characters (Höhnel, 1907; Luttrell, 1955; Graham & Luttrell, 1961; Barr 1982). Subsequently, it was accommodated in *Pleosporaceae* (Eriksson & Hawksworth, 1998; Kirk *et al*., 2001; Eriksson 2005). However Kodsueb *et al*. (2006) studied this genus based on phylogenetic analysis and were unable to resolve the placement of *Leptosphaerulina* in either of these families. Recent molecular data have confirmed the placement of *Leptosphaerulina* in *Didymellaceae* (Aveskamp *et al*., 2010; Zhang *et al*., 2012; Hyde *et al*., 2013).

Species of *Leptosphaerulina* and their asexual morphs are reported as saprobic or parasitic on leaves or stems of various plants including important crop plants. For example, *L. trifolii* (Rostr.) Petr. causes pepper spot on *Trifolium*, *Medicago* and various other hosts, *L. arachidicola* W.Y. Yen, M.J. Chen & K.T. Huang is pathogenic on leaves of *Arachis* and *L. brassicae* Karan causes leaf spots on *Brassica* (Yen *et al.*, 1956; Karan, 1964; Roux, 1986; Abler, 2003; Ahonsi *et al*., 2010). *Leptosphaerulina americana* (Ellis & Everh.) J.H. Graham & Luttr., and *L. australis* McAlpine have been reported as saprobes on dead plant materials (Graham & Luttrell, 1961; Irwin & Davis, 1985; Zhang *et al*., 2012). The genus has morphologically been relatively well-studied. Graham & Luttrell (1961) recognized six species (*Leptosphaerulina australis*, *L. americana*, *L. arachidicola*, *L. argentinensis* (Speg.) J.H. Graham & Luttr., *L. Briosiana* (Pollacci) Graham & Luttr. and *L. trifolii* from forage plants. These species were differentiated based on the size and septation of ascospores, the host and characteristics in culture. Irwin & Davis (1985) accepted only four species (*L. americana, L. arachidicola, L. argentinensis* and *L. trifolii*), and placed *L. australis* and *L. briosiana* in synonymy with *L. trifolii*, following Booth & Pirozynski (1967). Olanya & Campbell (1990) found that ascospores were not a good character to distinguish species of *Leptosphaerulina*. It appears that morphological characters in *Leptosphaerulina* overlap and may comprise species complexes as has been shown in several other recently evolving plant pathogenic genera (e.g. *Bipolaris,* Manamgoda *et al*., 2012; *Diaporthe*, Udayanga *et al*., 2012; *Phyllosticta,* Wikee *et al*., 2011; Wulandari *et al*., 2009).

We are carrying out research of Dothideomycetes on monocotyledons in Thailand, and the present study details one pathogenic and one saprobic taxa on

leaves of *Saccharum officinarum* and *Oryza sativa,* respectively. One is a new species, *Leptosphaerulina saccharicola* which is introduced in this paper based on both of morphology and molecular study. A *Pithomyces*-like asexual state of this species was obtained in culture and thus these genera are discussed and compared using molecular and morphological data. The second taxon is a new combination for *Leptosphaerulina oryzae* (basionym: *Pleosphaerulina oryzae* I. Miyake), which we carry out based on the morphology of the re-examined type species.

MATERIAL AND METHODS

Isolation and identification

The specimen was collected from Nakhonratchasima Province in Thailand and returned to the laboratory for examination following the methods described by Taylor & Hyde (2003), Chomnunti *et al.* (2011) and Liu *et al.* (2011). The specimen was observed and examined under a Motic SMZ 168 Series stereomicroscope. Micromorphological images were captured using a Nikon ECLIPSE 80i compound microscope with a Canon EOS 550D digital camera. Indian ink was used to reveal any mucilaginous sheath surrounding the ascospores. Measurements were made with Tarosoft (R) Image Frame Work version 0.9.7 (Liu *et al.*, 2010; Chomnunti *et al.*, 2011).

A culture was derived via single spore isolation following the methods described in Chomnunti *et al.* (2011). Ascomata were cut with a razor blade, the centrum tissue containing ascospores were removed using a sterile needle and placed in sterile water. The spore drop which contained the ascospores suspension were placed on quarter strength Sabouraud Dextrose Agar (SDA 3.9 g / 0.6 g yeast extract / 9.0 g bacteriological agar in 600 ml sterile distilled water) and incubated overnight at room temperature. The germinated spores were removed to Malt Extract Agar (MEA; 33.6 g/L sterile distilled water, Difco malt extract) and Potato Dextrose Agar (PDA; 39 g/L distilled water, Difco potato dextrose). The culture is deposited at Mae Fah Luang University Culture Collection (MFLUCC). The specimens are deposited at the herbarium of Mae Fah Luang University (MFLU), Chiang Rai, Thailand with duplicates culture in International Collection of Microorganisms from Plants (ICMP), Landcare Research, New Zealand.

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from fresh fungal mycelium grown on MEA/PDA media agar at 25-27°C. The Biospin Fungus Genomic DNA Extraction Kit (BioFlux®, Hangzhou, and P.R. China) was used to extract DNA according to the manufacturer's instructions.

DNA amplification was performed by Polymerase Chain Reaction (PCR). Four partial gene portions were used in this study: the large subunits of the nuclear ribosomal RNA genes (LSU), the internal transcribed spacers (ITS) and two protein coding genes, namely the translation elongation factor 1-alpha gene (*TEF1α*) and the partial RNA polymerase second largest subunit (*RPB2*). The primers used were LROR and LR5 (Vilgalys & Hester, 1990) for LSU, ITS5 and ITS4 (White *et al.*, 1990) for ITS, EF1-983F and EF1-2218R (Rehner, 2001) for *TEF1α* and fRPB2-5F and fRPB2-7cR (Liu *et al.*, 1999) for *RPB2*. The PCR

thermal cycle program for ITS, LSU and *TEF1α* amplification were as follows: initially 94°C for 3 mins, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 50 seconds, elongation at 72°C for 1 mins, and final extension at 72°C for 10 mins. The PCR thermal cycle program for the partial RNA polymerase second largest subunit (*RPB2*) was followed as initially 95°C for 5 mins, followed by 40 cycle of denaturation at 95°C for 1 mins, annealing at 52°C for 2 mins, elongation at 72°C for 90 seconds, and final extension at 72°C for 10 mins. The PCR products were purified by using the PCR Purification Kit according to manufacturer's protocol. DNA sequencing was performed by Shanghai Sangon Biological Engineering Technology & Services Co. (Shanghai, P.R. China).

Phylogenetic analysis

The generated ITS, LSU and TEF1 were analyzed with other GenBank sequences (Table 1). The sequences were performed to indicate the closest matches with taxa in *Didymellaceae* by Blast search. In addition, the fungal members of *Cucurbitariaceae*, *Dothidotthiaceae*, *Melanommaceae*, *Leptosphaeriaceae*, *Phaeosphaeriaceae*, *Pithomyces* and *Pleosporaceae* were included in this analysis. *Botryosphaeria dothidea* was selected as outgroup. The fungal sequence strains were combined and aligned with SATé (Liu *et al.*, 2009) using MAFFT v. 7.036 (Katoh & Standley, 2013) and improved in MEGA5 (Tamura *et al.*, 2011). The alignments were checked and improved manually where necessary. The Phylogenetic relationships were inferred using Maximum-parsimony (MP) in PAUP* 4.0b10 (Swoord, 2002), and MrBayes v. 3.0b4 (Ronquist & Huelsenbeck, 2003) for Bayesian analysis. Maximun likelihood analysis used RAxML v.7.2.8 as part of the RAxML-HPC2 on TG" tool (Stamatakis, 2006; Stamatakis *et al.*, 2008) and was performed at the CIPRES webportal (Miller *et al.*, 2010).

Maximum-parsimony (MP) search was performed using the tree heuristic search option with TBR branch swapping and 1,000 random sequence additions. Maxtrees were set up to 500, branches of zero length were collapsed and all multiple parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated by 1,000 bootstrap replications resulting from maximum parsimony analysis, each with 10 replicates of random stemwise addition of taxa ((Hillis & Bull, 1993). Other measures used were consistency index (CI), retention index (RI), rescaled consistency index [RC] and homoplasy index (HI). Bayesian analysis was performed by MrBayes v. 3.0b4 (Ronquist & Huelsenbeck, 2003) with the best-fit model of sequences evolution estimated with MrModeltest 2.2 (Nylander, 2004). Markov Chain Monte Carlo sampling (BMCMC) was used to determine the posterior probabilities (PP) (Rannala & Yang, 1996; Zhaxybayeva & Gogarten, 2002) in MrBayes v. 3.0b4 (Huelsenbeck & Ronquist, 2001). Six simultaneous Markov chains were run for 1000000 generations sampling one tree every 100th generations of trees (resulting 10001 total trees). The burn-in (first 2000 trees) which represented the phase of the analysis were discarded and the remaining 8000 trees were used to built a majority rule consensus tree (Cai *et al.*, 2006; Cai *et al.*, 2008; Liu *et al.*, 2011) with posterior probabilities (PP). A consensus phylogram (50% majority rule) was presented in Fig. 2. Maximum likelihood analysis was performed by RAxML v.7.2.8 as part of the RAxML-HPC2 on TG" tool (Stamatakis, 2006; Stamatakis *et al.*, 2008) at the CIPRES webportal (Miller *et al.*, 2010). A discrete gamma and the four rate classes were relevant with a general time revisable model (GTR) and fifty through

Table 1. Isolates used in this study and their GenBank accession numbers. The newly generated sequences are indicated in bold

Taxon	*Culture Accession No*[1]	*GenBank Accession No*[2]		
		LSU	*ITS*	*TEF1α*
Boeremia exigua var. *exigua*	CBS 431.74	EU754183	FJ427001	GU349080
Botryosphaeria dothidae	CMW 8000	AY928047	AY236949	AY236898
Cochliobolus sativus	DAOM 226212	DQ678045	–	–
Cucurbitaria berberidis	CBS 394.84	GQ387605	–	–
Cucurbitaria berberidis	CBS 363.93	GQ387606	JF740191	–
Didymella cucurbitacearum	IMI 373225	AY293792	AY293804	–
Didymella exigua	CBS 183.55^{T}	EU754155	GU237794	–
Didymella fabae	CBS 524.77	EU754133	GU237880	–
Dothidotthia aspera	CPC 12933	EU673276	–	–
Dothidotthia symphoricarpi	CPC 12929	EU673273	–	–
Entodesmium rude	CBS 650.86	GU301812	–	GU349012
Herpotrichia juniperi	CBS 468.64	DQ384093	GQ203759	–
Leptosphaeria doliolum	CBS 505.75^{T}	GU301827	JF740205	GU349069
Leptosphaeria maculans	DAOM 229267	DQ470946	–	DQ471062
Leptosphaeria maculans	CBS 275.63	JF740306	JF740234	–
Leptosphaerulina americana	CBS 213.55	GU237981	GU237799	–
Leptosphaerulina arachidicola	CBS 275.59	GU237983	GU237820	–
Leptosphaerulina australis	CBS 311.51^{T}	FJ795500	–	GU456272
Leptosphaerulina australis	CBS 317.83	EU754166	GU237829	GU349070
Leptosphaerulina saccharicola	**MFLUCC 11-0169**	**KF670716**	**KF670717**	**KF670715**
Leptosphaerulina trifolii	CBS 235.58	GU237982	GU237806	–
Melanomma pulvis-pyrius	CBS 371.75	GU301845	–	GU349019
Neosetophoma samarorum	CBS 138.96^{T}	GQ387578	FJ427061	–
Ophiosphaerella herpotricha	CBS 240.31	DQ767656	–	DQ767639
Paraphoma radicina	CBS 111.79^{T}	EU754191	FJ427058	GU349076
Phaeosphaeria oryzae	CBS 110110	GQ387591	–	–
Phaeosphaeriopsis musae	CBS 120026^{T}	GU301862	–	GU349037
Phoma herbarum	CBS 276.37^{T}	DQ678066	JF810524	DQ677909
Pithomyces chartarum	DS1bioJ1b	HM216194	HM216213	–
Pithomyces valparadisiacus	CBS 113339	EU552152	EU552152	–
Plenodomus biglobosus	CBS 298.36	GU237980	–	–
Plenodomus chrysanthemi	CBS 539.63^{T}	GU238151	JF740253	–
Pleospora herbarum	CBS 191.86^{T}	DQ247804	–	DQ471090
Pyrenochaeta nobilis	CBS 407.76^{T}	EU754206	EU930011	–
Pyrenochaeta nobilis	CBS 566.75	GQ387616	–	–
Pyrenophora phaeocomes	DAOM 222769	DQ499596	DQ491507	DQ497607
Pyrenophora tritici-repentis	AFTOL-ID 173	AY544672	–	DQ677882
Setomelanomma holmii	CBS 110217	GU301871	–	GU349028
Setosphaeria monoceras	CBS 154.26	AY016368	–	–

Abbreviations: **AFTOL:** Assembling the Fungal Tree of Life; **CBS:** Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; **CMW:** Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; **CPC:** Collection of Pedro Crous housed at CBS; **DAOM:** Plant Research Institute, Department of Agriculture (Mycology), Ottawa, Canada; **ICMP:** International Collection of Micro-organisms from Plants, Landcare Research, New Zealand; **IMI:** International Mycological Institute, CABI-Bioscience, Egham, Bake-ham Lane, U.K.; **MFLUCC:** Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; T ex-type/ex-epitype isolates.

maximum likelihood (ML) tree were performed at the same model in RAxML v.7.2.7 with each one starting from the separate randomized trees. One thousand non parametric bootstrap iterations were run with the GTR model and a discrete gamma distribution; the resulting replicates were plotted on to the best scoring tree obtained previously. The phylogram was visualized in Treeview (Page, 1996). The sequences in this study are deposited in GenBank (KF670716, KF670717, KF670715).

RESULTS AND DISCUSSION

Phylogenetic analysis

The combined LSU, ITS and *TEF1α* gene data set consists of 39 taxa, with *Botryosphaeria dothidea* as the outgroup taxon. The dataset consists of 2512 characters after alignment, of which 1854 characters are constant and 418 sites (18%) are parsimony informative. A heuristic search with random addition sequences (1000 replicates) and treating gaps as missing characters generated six equally parsimonious trees. All trees were similar in topology and not significantly different (data not shown). The best scoring RAxML tree is shown in Fig. 1. Bootstrap support (BS) values of ML and MP (equal to or greater 50% based on 1,000 replicates) are shown above the nodes and values of the Bayesian posterior probabilities (PP) (equal to or above 95% based on 1,000 replicates) from MCMC analyses are indicated below nodes.

The phylogenetic trees obtained from maximum likelihood, maximum parsimony and Bayesian analyses gave similar results relating to family. The families *Cucurbitariaceae*, *Didymellaceae*, *Dothidotthiaceae*, *Leptosphaeriaceae*, *Phaeosphaeriaceae* and *Pleosporaceae* clustered into the suborder *Pleosporineae*. The strains in *Leptosphaeriaceae* did not form a good clade none of the phylogenetic models (ML, MP and Bayesian), however once more genes (*RPB2*) were added this could be improved. In this study, because of the limitation of multi-loci combination, we chose three genes (LSU, ITS and *TEF1α*) for our analysis, the strain of *Leptosphaeria doliolum* (CBS 505.75) did not cluster with the other four selected strains of *Leptosphaeriaceae*. *Leptosphaerulina saccharicola* (MFLUCC11-0169) clustered together with four other *Leptosphaerulina* species and formed a well supported clade (99% ML, 98% MP and 1.00 PP) with *L. arachidicola* (CBS 275.59) in *Didymellaceae*. The phylogenetic tree resulting from the combined genes showed that *L. saccharicola* is distinct from *L. americana*, *L. arachidicola*, *L. australis* and *L. trifolii*, with the latter three species clades probably being species complexes. Two strains of *Pithomyces* did not cluster in the suborder *Pleosporineae* and were included in *Didymellaceae* with *Leptosphaerulina*.

Based on the phylogenetic analysis and morphology, therefore we introduce a new species, *Leptosphaerulina saccharicola* R. Phookamsak, J.K. Liu & K.D. Hyde, sp. nov. The description and illustrations of this new species are provided as below, as well as the culture characters and details of asexual morphs.

Taxonomy

Leptosphaerulina saccharicola Phookamsak, J.K. Liu & K.D. Hyde., **sp. nov.** **Fig. 2**

MycoBank: MB 805600

Etymology: Referring to the host on which the fungus was found.

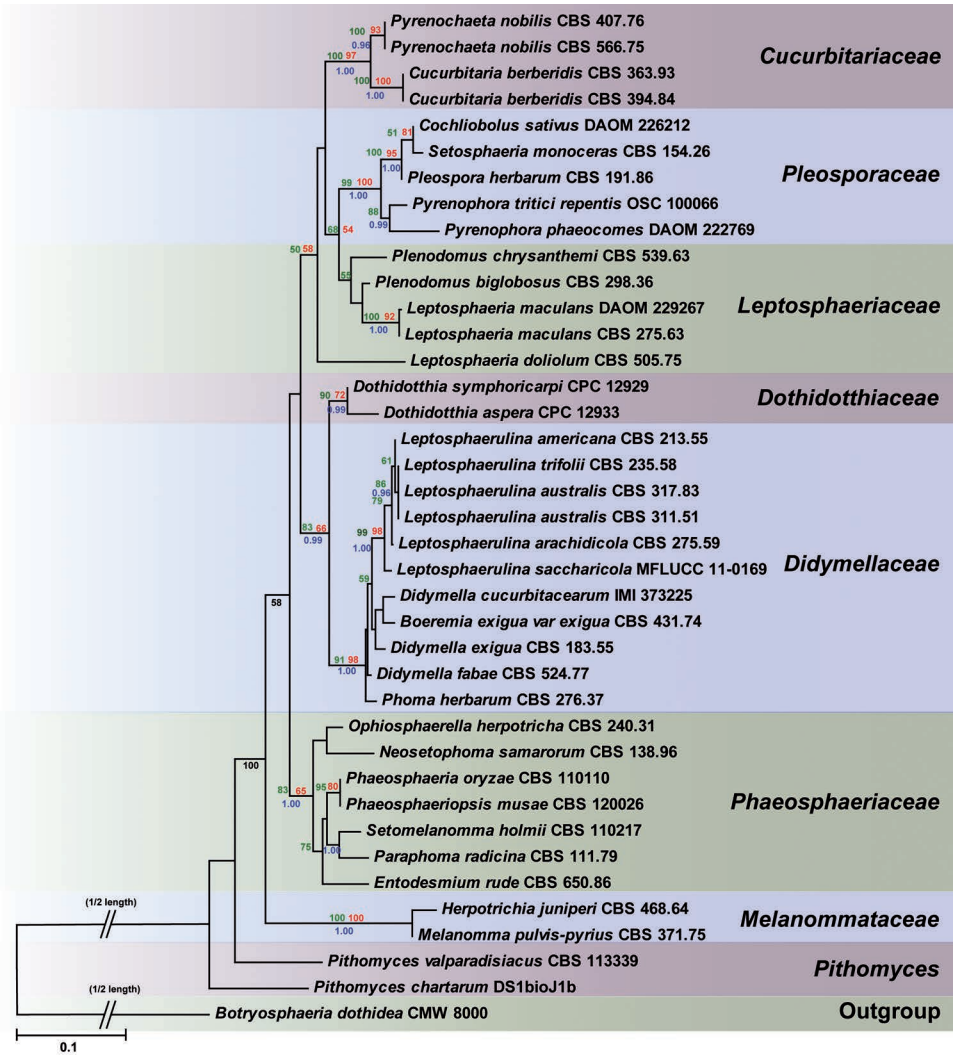

Fig. 1. RAxML tree based on a combined dataset of LSU, ITS and *TEF1* sequences. Bootstrap support values for maximum likelihood (ML, green) and maximum parsimony (MP, red) greater than 50% are given above the nodes. Bayesian posterior probabilities (BYPP, blue) above 0.95 are given below the nodes. The tree was rooted to *Botryosphaeria dothidea*.

HOLOTYPUS: MFLU11-0205

Pathogenic, associated with leaf spots, about 8-40 mm diam., visible as pale brown to brown regions, separated from healthy part of leaf by dark brown margins. Sexual state: *Ascomata* 70-110 µm high, 100-140 µm diam, immersed under epidermis, uniloculate, globose to subglobose, membranous, brown, gregarious, scattered to clustered. *Ostioles* circular, central, papillate. *Peridium* 8-16 µm wide, comprising a few layers, the outer layers comprised of brown walled cells of *textura angularis*, the inner layer comprised of pale brown to hyaline,

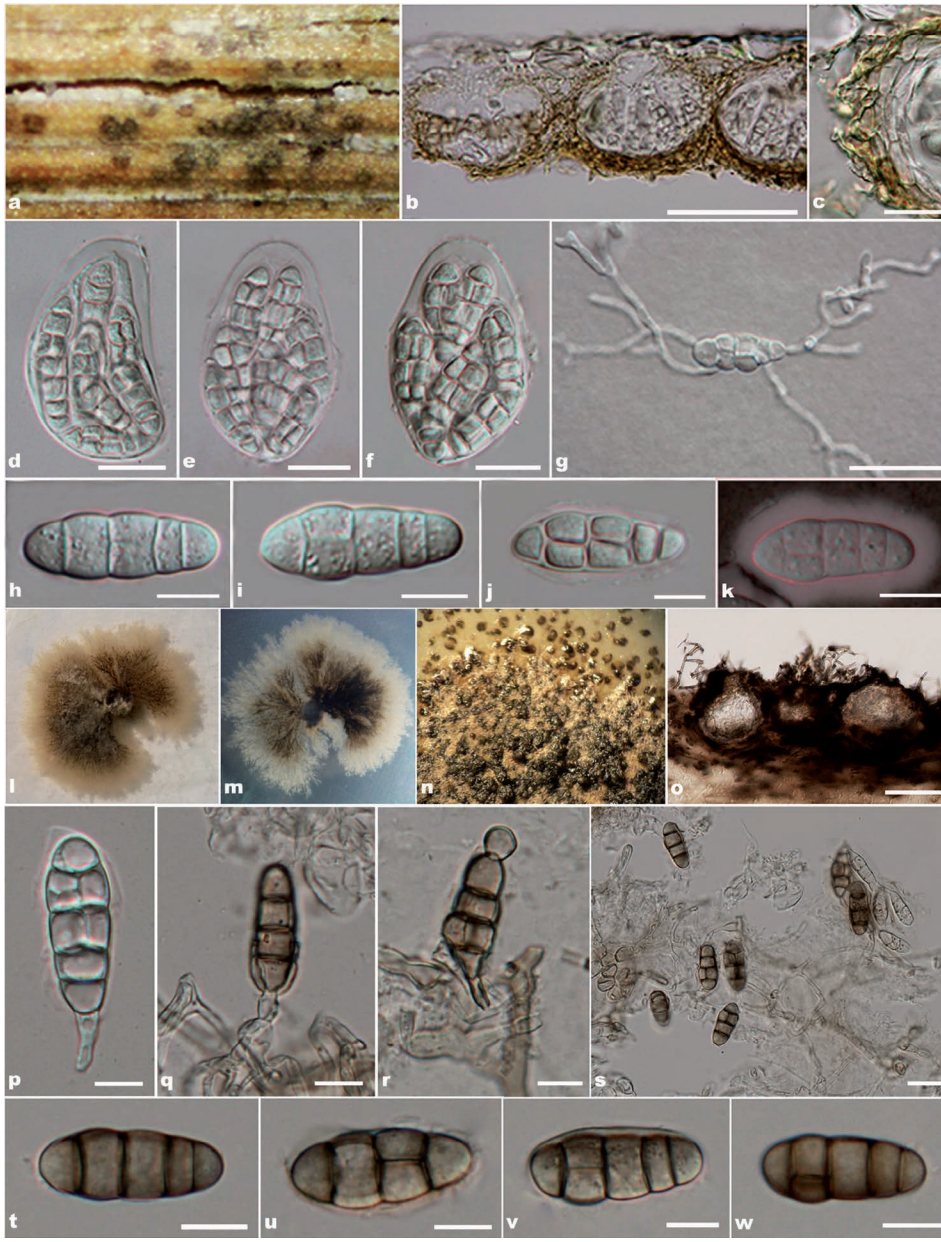

Fig. 2. ***Leptosphaerulina saccharicola*** (Holotype: MFLU11-0205). **a.** Fruiting bodies on host tissue. **b.** Section through ascomata. **c.** Section of peridium. **d-f.** Asci. **g.** Germinating ascospore. **h-j.** Ascospores. **k.** Ascospore stained in Indian ink showing sheath. **l-m.** Living culture on PDA. **n.** Ascomata developing in living culture. **o.** Section through ascomata forming on culture. **p-r.** Conidiogenous cells. **s-w.** Conidia. **Scale bars:** b, o = 100 µm, g = 50 µm, c, d, e, f, s = 20 µm. h, i, j, k, p, q, r, t, uv, w = 10 µm.

elongate thick-walled cells, of *textura angularis*. *Hamathecium* lacking pseudoparaphyses. *Asci* (49-)60-80(-86) × (32-)35-45(-49) µm ($\bar{x}$ = 67.9 × 39.4 µm, n = 25), 8-spored, bitunicate, fissitunicate, saccate, obpyriform, ovoid or amygdaliform, apedicellate, apically rounded with well-developed ocular chamber, thick-walled at the apex, arising from the base of the ascoma. *Ascospores* (25-)27-32(-35.5) × (9-)10-11.5(-12) µm ($\bar{x}$ = 29.6 × 11 µm, n = 30), overlapping or irregularly triseriate, oblong to cylindrical or ellipsoidal, muriform or phragmosporous, hyaline, with 4 transverse septa, and 0-2 longitudinal septa, usually widest in the second cell, smooth-walled, with small guttules, surrounded by distinctive structured mucilaginous sheath. Spore germinating initially at both ends and later in all cells, germinating within 12 hours on WA, newly emerging hyphae broadly branching. Asexual state produced on PDA and CMA, hyphae usually hyaline when young, becoming brown to dark brown when mature. *Conidiophores* 6-11.5 × 2.5-5 µm, mononematous, solitary, narrower than vegetative hyphae, with one apical pore, mostly unbranched, septate, initially hyaline, becoming light brown, walls smooth. *Conidiogenous cells* 3-8 × 2-5 µm, holoblastic, integrated, terminal, cylindrical, hyaline to brown. *Conidia* (23-)(24-)25-30(-32)(-33) × (9-)10-12(-13) µm ($\bar{x}$ = 29.2 × 11.5 µm, n = 30), solitary thalloconidia, oblong to cylindrical or ellipsoidal, muriform or phragmosporous, initially hyaline, becoming brown to dark brown, mostly with 3-4 transverse septa and 0-1 longitudinal septa.

Culture characters: Colonies on PDA 35-45 mm diameter after 1 week at 25-30°C; circular, brown to dark brown, white to pale yellow at the edge, sometimes dark grey, covered by white fluffy hyphae, flattened; reverse white to pale yellow at the edge, brown to dark brown in the middle with fimbriate edge, opaque, floccose to fluffy, no pigments produced, after 4 weeks black ascomata produced on colony and submerged in agar.

Material examined: THAILAND, Nakhonratchasima, Huaithalaeng District, Loongpradoo Village, on living leaves of *Saccharum officinarum* L., 1 November 2010, R. Phookamsak, RP0085 (MFLU11-0205, holotype); extype living culture = MFLUCC 11-0169 = ICMP 19875.

Gene sequence data: ITS (KF670717), LSU (KF670716), *TEF1α* (KF670715) and *RPB2* (KF670714).

Commentary: Leptosphaerulina saccharicola shares similar morphological characters with *L. arachidicola*, *L. australis* and *L. trifolii* in having ascospores of similar size and septation and in culture characteristic and differs from *L. americana* by size and septation of ascospores and host preferences (Graham & Luttrell, 1961). *Leptosphaerulina saccharicola* is most similar to *L. Arachidicola*, although the latter was reported as causing leaf disease of only *Arachis* spp. while *L. saccharicola* is associated with leaf disease of *Saccharum officinarum*. Our phylogenetic analysis also showed that *L. saccharicola* is most similar to *L. arachidicola*, but was in a different clade. *Leptosphaerulina saccharicola* differs from *L. australis* in having ascospores with transverse septa and in the pigment production on media. In *L. australis* ascospores have 5 transverse septa and produce a pink pigment when grown in V-8 juice agar, while *L. saccharicola* has ascospores with 4 transverse septa and does not produce pigment in V-8 juice agar. *Leptosphaerulina arachidicola*, *L. australis* and *L. saccharicola* are morphologically similar to *L. trifolii*, and Booth & Pirozynski (1967) synonymised *L. australis* under *L. trifolii*. However, these species differ in the size of ascospores, growth on media and host preferences. *Leptosphaerulina trifolii* has larger ascospores and asci; it is associated with leaf disease of *Medicago* and *Trifolium*, grows slowly on V-8 juice agar, while other species are fast growing (Graham & Luttrell, 1961; Miles, 1925; Miller, 1925; Abler, 2003). We have compared the

morphological characters of species of *Leptosphaerulina* on other monocotyledonous plants with *Leptosphaerulina saccharicola*; our species differs in ascospore septation and host (Table 2).

The asexual morph of *Leptosphaerulina saccharicola* formed in culture, and has similar characters with some putative *Pithomyces* species. The type species of *Pithomyces* is *P. flavus* which was collected from a monocotyledonous plant in Sri Lanka (Peradeniya) by Berkeley and Broome in December 1868 (Berkeley & Broome, 1873). *P. flavus* produces yellow or olive-yellow, soft, spreading colonies on the host and has 15×10 µm, 4-5-septate, straw-colored to dark brown, echinulate, doliiform conidia, with darkened septal bands, which are widest in the centre (Berkeley & Broome, 1873; Ellis, 1971). The asexual state of *L. saccharicola* differs from *P. flavus* in colony appearance, conidia shape (generally being wider at one end), a part of the conidiophore not remaining attached to the base of the conidia, lack of longitudinal septa and septal bands. Thus, we do not consider *L. saccharicola* to be related to the type species of *Pithomyces*. Several species with somewhat similar characters to *Pithomyces* such as *P. chartarum* (Berk. & M.A. Curtis) M.B. Ellis have also been placed in *Pithomyces*. The link between *P. chartarum* and *L. chartarum* was reported by Roux (1986), based on cultural studies. The conidia of *P. chartarum* are morphologically similar to the ascospores of *L. chartarum*, although the latter are smooth and hyaline to light brown. *P. saccharicola* was reported from China by Zhang & Zhang (2003), also from *Saccharum officinarum*; however the asexual morph of *L. saccharicola* differs from *P. saccharicola* in septation of conidia. The asexual morph of *L. saccharicola* has 3-4 transverse septa and 0-1 longitudinal septum, while *P. saccharicola* has 1-2 transverse septa with longitudinal septa being absent or rare (Zhang & Zhang, 2003). These *Pithomyces* species have not been linked to the type species, *P. flavus*. Thus, there is considerable confusion surrounded the genus *Pithomyces* which requires further work at the molecular level using fresh collections.

Pithomyces chartarum (strain DS1bioJ1b) and *P. valparadisiacus* (strain CBS113339) were clearly unrelated to *Leptosphaerulina* in our phylogenetic analysis. We therefore treat *Pithomyces* in a strict sense based on *P. flavus* as having yellow or olive-yellow, soft, spreading colonies on the host and straw-colored to dark brown, echinulate, doliiform, trans-septate conidia, with darkened septal bands, and widest in the centre, while *Pithomyces*-like species related to *Leptosphaerulina* have conidia that are brown to dark brown, smooth-walled, cylindrical, with 3-4 transverse septa and 0-1 longitudinal septa and without a part of the conidiophore remaining attached to the base of the conidia. A third group which may include *P. chartarum*, does not cluster with *Leptosphaerulina* and may need to be accommodated in a separate genus. However, further studies are needed.

It is likely that the genus *Leptosphaerulina* comprises several species complexes with each complex having several morphologically similar taxa, but which are phylogenetically distinct and infect different hosts. Detailed studies of species in this genus should be carried out using multigene phylogeny to establish if this is the case. Recent studies have shown that pathogenic genera on economic crops usually comprise species complexes with each complex comprising morphologically similar, but phylogenetically well differentiated species. For example, *Colletotrichum* contains nine species complexes, with *C. Gloeosporioides sensu lato* comprising 22 cryptic species plus one subspecies (Cannon *et al*., 2012; Weir *et al*., 2012). Similar scenarios have been found in the pathogenic genera *Alternaria, Diaporthe, Fusarium, Pestalotiopsis* and *Phyllosticta* (Summerell *et al*.,

Table 2. Synopsis of *Leptosphaerulina* species discussed in this study

Species	*Size (µm)*			*Septation*		*Hosts*	*Source references*
	Ascospores	*Asci*	*Ascomata (diam)*	*Transverse septa*	*Longitudinal septa*		
L. americana	34-49 × 13-18	101-106 × 45-48	126-140	5-6	2-5	*Trifolium pretense*, *Phleum pratense*	Graham & Luttrell (1961)
L. arachidicola	23-40 × 11-17	53-87 × 28-42	64-140	3.5	0-2	*Arachis* spp.	Graham & Luttrell (1961)
L. australis	30-32 × 11	75-80 × 28-50	150	5	2	Various hosts	McAlpine (1902), http://nt.ars- grin.gov/fungaldatabases/fungushost/new_frameFungusHostReport.cfm
L. calamagrostidis	19-23.1 × 6.3-7.3	63-81.9 × 14.7-16.8	126-175	3-5	1-3	*Calamagrostis epigeios*	Pisareva (1964)
L. chartarum	23(25-)27 × 7(-8)-12	100-150 × 60-100		3	1	*Galenia procumbens*	Roux (1986)
L. olivaceogrisea	14-20 × 5-9(12)	45-65 × 15-20(-29)	60-170	3-6	1-2	*Carex firma*, *Dryas octopetala*	Nograsek (1990)
L. oryzae	28-30 × 10-11	60-78 × 38-51	120-170	4-5	2-3	*Oryza sativa*	This study
L. saccharicola	27-32 × 10-11.5	60-80 × 35-45	100-140	4	0-2	*Saccharum officinarum*	This study
L. trifolii	25-49 × 11-21	62-95 × 42-59	124-207	3-4	0-2	Various hosts	Graham & Luttrell (1961), http://nt.ars- grin.gov/fungaldatabases/fungushost/new_frameFungusHostReport.cfm

2010; Glienke *et al*., 2011; Summerell & Leslie, 2011; Maharachchikumbura *et al*., 2011, 2012; Udayanga *et al*., 2011, 2012; Wang *et al*., 2011; Wikee *et al*., 2011; Weir *et al*., 2012; Woudenberg *et al*., 2013). The molecular data presented here, indicates that *Leptosphaerulina* comprises species complexes. Analysis of LSU gene sequence data alone cannot separate the species, although they have different morphological characters, and different asexual morphs. Combined gene analysis of five genes, which included two protein genes (*TEF1α* and *RPB2*), indicated that the *Leptosphaerulina* species used in the analysis are distinct species. The species appear to be host or family specific, but further taxa sampling and gene data is required to address the diversity in this genus.

Leptosphaerulina oryzae (I. Miyake) Phookamsak, J.K. Liu & K.D. Hyde, **comb. nov.** **Fig. 3**

MycoBank: MB 805608

≡ *Pleosphaerulina oryzae* I. Miyake, J. Coll. Agric. imp. Univ. Tokyo2: 250 (1910)

≡ *Pringsheimia oryzae* (T. Miyake) Hara, A Monograph of Rice Diseases: 78 (1959)

Saprobic on *Oryza sativa*. Sexual state: *Ascomata* 100-120 μm high, 120-170 μm diam, immersed below epidermis, solitary, scattered, uniloculate, globose to subglobose, membranous, pale brown to brown. *Ostioles* circular, central, papillate. *Peridium* 2.5-8 μm wide, comprising 1-2 layers of brown to dark brown, thin-walled cells, of *textura angularis*. *Hamathecium* lacking pseudoparaphyses. *Asci* (58-)60-78(-85) × (36-)38-51(-54) μm ($\bar{x}$ = 70.8 × 45.4 μm, n = 25), 8-spored, bitunicate, fissitunicate, saccate, obpyriform or ovoid, sessile, apically rounded with well-developed ocular chamber, thick-walled, arising from the base of ascoma. *Ascospores* (26.5-)28-30(-33) × (9.5-)10-11(-12) μm ($\bar{x}$ = 30.1 × 10.9 μm, n = 30), irregularly tri-seriate, oblong to cylindrical or ellipsoidal, hyaline to brown, muriform, with 4-5 transverse septa, and 2-3 longitudinal septa, usually widest at the second cell, smooth-walled, surrounded by distinct mucilaginous sheath.

Material examined: Japan, on dead leaves of *Oryza sativa*, September 1907 (Hara, F8478-type, as *Pleosphaerulina oryzae*).

Commentary: Pleosphaerulina oryzae was introduced by Miyake (1910) as saprobic on *Oryza sativa* in Japan. This species was placed as a synonym of *Leptosphaerulina australis* (Graham & Luttrell 1961). We observed the type specimens of *Pleosphaerulina oryzae* and compared the morphological characters with the original description of *L. australis* (McAlpine 1902). *Pleosphaerulina oryzae* is similar to *L. australis*, but they differ in the septation of ascospores, with *P. oryzae* mostly having 4 (rarely 5) transverse septa, while *L. australis* has 5 transverse septa (McAlpine 1902). The hosts also differ — rice (*Oryza sativa*, *Poaceae*), for *P. oryzae* and apricots (*Prunus armeniaca* L., *Rosaceae*) for *L. australis*. The new combination is supported by morphology but, unfortunately, type specimens of *Leptosphaerulina australis* could not be located. Epitypification and molecular data is required to resolve this species. *Leptosphaerulina saccharicola* has similar characters to those of *L. oryzae* in the size and shape of ascomata, asci and ascospores. However, *L. oryzae* has more longitudinal septa (2-3) than *L. saccharicola* (0-2).

Fig. 3. ***Leptosphaerulina oryzae*** (S, F8478_type of *Pleosphaerulina oryzae*). **a.** Herbarium specimens and label of *Pleosphaerulina oryzae*. **b.** Ascoma on substrate. **c.** Section through ascoma. **d.** Section through peridium. **e.** Asci. **f-i.** Ascus. **j-l.** Immature ascospore. **m-n.** Mature ascospore. **Scale bars:** c, d, e, f, g, h, i = 20 µm, j, k, l, m, n = 10 µm.

Acknowledgements. This study is supported by Royal Golden Jubilee Ph. D. Program (PHD/0090/2551) under Thailand Research Fund and Mae Fah Luang University (56 1 01 02 00 32), both of which are gratefully acknowledged for scholarship and laboratory support. Wen Jing Li and the International Fungal Research & Development Centre, Research Institute of Resource Insects, Chinese Academy of Forestry are grateful for use of molecular data. The curator of the herbarium S is thanked for lending the herbarium specimen.

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