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Leucaenicola osmanthi sp. nov. (Bambusicolaceae, Pleosporales), causing leaf spot of *Osmanthus fragrans* in Taiwan

HIRAN A. ARIYAWANSA[1], ICHEN TSAI[1], WAEL N. HOZZEIN[2, 3] & KASUN M. THAMBUGALA[4]
[1]*Department of Plant Pathology and Microbiology, College of Bio-Resources and Agriculture, National Taiwan University, Taiwan*
[2]*Bioproducts Research Chair, Zoology Department, College of Science, King Saud University, Riyadh, Saudi Arabia*
[3]*Botany and Microbiology Department, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt*
[4] *Genetics and Molecular Biology Unit, Faculty of Applied Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka.*
**Corresponding authors' emails: ariyawansa44@ntu.edu.tw; kasun@sci.sjp.ac.lk*

Abstract

Osmanthus fragrans naturally occurs in Taiwan, and is now widely cultivated as an ornamental plant. Signs of leaf spots caused by unknown species has been detected on *O. fragrans* saplings in Nangang District, Taipei City, Taiwan. This investigation aimed to illustrate the fungal species by engaging morphological features, pathogenicity tests, and DNA sequence comparisons for the ITS, LSU, SSU, *rpb*2 and *tef*1 gene sequences. A new species is proposed and identified here as *Leucaenicola osmanthi*. *Leucaenicola osmanthi* can be differentiated from the phylogenetically close taxa, *L. aseptata* and *L. phraeana*, by much larger conidiomata, conidiogenous cells and conidia. Moreover, this is the first report of a species belonging to *Leucaenicola* on *Osmanthus fragrans* in Taiwan.

Keywords: 1 new species, diversity, pathogen, phylogeny, taxonomy

Introduction

Osmanthus fragrans Lour. is a plant that belongs to the family Oleaceae (Ômura *et al*. 2000), usually native to warm temperature zones of Asia such as, southern China, Taiwan and southern Japan (Hung *et al*. 2012). *Osmanthus fragrans* is known to be an evergreen dioecious bush or small tree (Hung *et al*. 2012). The flowers come in a variety of white, pale yellow, golden yellow, or orange yellow coloration with a four-lobed corolla, and have a powerful fragrance. It is used not only as an ornamental plant, but also as an additive in food, tea, and other brews because of its powerful fragrance (Hung *et al*. 2012). Several fungi are known to cause foliar diseases of *O. fragrans*. According to the USDA database, 74 fungal species have been known affect *Osmanthus fragrans* (Farr & Rossman 2020). Anthracnose caused by *Colletotrichum gloeosporioides*, leaf spots caused by *Pseudocercospora osmanthicola*, leaf blight caused by *Phyllosticta osmanthicola*, and sooty mold caused by *Aithaloderma clavatisporum* are common fungal diseases affecting *O. fragrans* in Taiwan (Tzean *et al*. 2019).

Leucaenicola has recently been introduced by Jayasiri *et al*. (2019) to accommodate coelomycetous species isolated from the decaying pod of *Leucaena* sp. and classified under Bambusicolaceae, Pleosporales, on the basis of morphology and phylogeny. Currently, *Leucaenicola* contains two species namely *Leucaenicola aseptata* and *L. phraeana* (Jayasiri *et al*. 2019). The genus is characterized by globose to subglobose, ostiolate conidiomata, enteroblastic, phialidic, globose to flask-shaped conidiogenous cells and one-celled, initially hyaline, but turns brown at maturity, oblong to ellipsoidal, aseptate conidia (Jayasiri *et al*. 2019).

Taiwan is known for its excellent biodiversity due to its warm and humid weather (Hsieh & Li 1991, Sivanesan & Hsieh 1989, Tzean *et al*. 1997). A number of surveys conducted within the last few years have broadened our understanding of the dothideomycetous fungal flora in Taiwan (Ariyawansa *et al*. 2018a, b, 2019, Chang & Wang 2009, Tennakoon *et al*. 2018, Yang *et al*. 2016). During a survey conducted in Taiwan on fungal diseases associated with *Osmanthus fragrans*, an unidentified coelomycetous fungus was discovered. Therefore, this study aims to describe unidentified coelomycetous species causing necrotic lesions on leaves of *O. fragrans* and establish its taxonomy based on morphological and molecular studies.

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Material and Methods

Sample collection and fungal isolation

Infected *O. fragrans* leaves were gathered from Nangang Tea Processing Demonstration Centre, Nangang District, Taipei City, Taiwan, during March to April 2018. Symptomatic leaves were taken to the laboratory in Ziplock plastic bags. The samples were treated and inspected according to the method described in Ariyawansa *et al*. (2018 a, b). Observation of fresh materials was done under a Motic SMZ 168 dissecting microscope for identification and isolation of fruiting bodies. Hand sections of the fruiting structures were mounted in water for microscopic studies and photomicrography. Isolations were made from single conidia, following a modified method of Ariyawansa *et al*. (2018 a, b). Morphological descriptions were made for strains cultured on 2% potato dextrose agar (PDA; HiMedia®). Conidiomatal development was observed on water agar (WA; BioShop®) with double-autoclaved pine needles placed onto the agar surface (PNA). Incubation of cultures was done at room temperature (25 °C) under blue light condition for 7 days. Microscopic measures were made in distilled water with at least 30 measurements per structure being observed with an Olympus BX51 microscope using differential interference contrast (DIC) illumination. Voucher specimens were deposited in the herbarium of Department of Plant Pathology and Microbiology, National Taiwan University (NTUH). Living cultures were deposited at the Department of Plant Pathology and Microbiology, National Taiwan University Culture Collection (NTUCC). Nomenclatural novelties were deposited in MycoBank.

DNA extraction, PCR amplification and sequencing

Single spore fungal isolates were grown on PDA for 28 days at 25 °C in the dark. Genomic DNA was extracted from the growing mycelium using the EasyPure Genomic DNA Spin Kit (Bioman®) following the manufacturer's protocol (BIOMAN SCIENTIFIC CO., LTD).

The amplification procedure was performed in a 25 µl reaction volume containing 5–10 ng DNA, 0.8 units Taq polymerase, 1X PCR buffer, 0.2 mM d'NTP, 0.3 µM of each primer with 1.5 mM $MgCl_2$ (Ariyawansa *et al*. 2019). PCR conditions for ITS (internal transcribe spacer), SSU (small subunit of the nrRNA gene) and LSU (large subunit of the nrRNA gene) adopted the method of Ariyawansa *et al*. (2019). Amplification of partial *rpb*2 (RNA polymerase II second largest subunit gene) and partial *tef*1 (translation elongation factor 1-α gene) adopted the method of Ariyawansa *et al*. (2018a, b). The following primer sets were used for these genes: ITS: ITS4/ITS5; LSU: LR0R/LR5; SSU: NS1/NS4 (Liu *et al*. 1999, Sung *et al*. 2007, White *et al*. 1990); *tef*1: EF1-983F/-2218R (Carbone & Kohn, 1999) and *rpb*2: fRPB2-5F/ fRPB2-7cR (Ariyawansa *et al*. 2018a, b). The PCR products were tested on 1.5% agarose gels stained with SYBR safe DNA gel stain. PCR products were cleaned and sequenced at the Genomics Company, New Taipei, Taiwan using Sanger sequencing method. To acquire consensus sequences from sequences produced from forward and reverse primers, DNASTAR Lasergene SeqMan Pro v.8.1.3 was used. Data of newly obtained sequences were kept in the NCBI GenBank under the accession numbers provided in TABLE 1.

Sequence alignment and phylogenetic analysis

ITS, LSU, SSU, *rpb*2 and *tef*1 gene sequences were used in the phylogenetic analyses. NCBI BLAST searches were conducted to identify the closest matches in GenBank. All sequences obtained from GenBank and used by Ariyawansa *et al*. (2018a, b), Chen *et al*. (2015), Hernandez-Restrepo *et al*. (2017), Hyde *et al*. (2013), Jayasiri *et al*. (2019), Tanaka *et al*. (2015), Valenzuela-Lopez *et al*. (2018), Wanasinghe *et al*. (2017) are listed in TABLE 1. Multiple sequence alignments were generated with MAFFT v. 6.864b (http://mafft.cbrc.jp/alignment/server/index.html). The alignments were visually inspected and improved manually where necessary. All introns and exons were discretely arranged. Single gene phylogenies were inferred for ITS, LSU, SSU, *rpb*2 and *tef*1, and finally subjected to a concatenated gene analysis. Manual comparison of the topologies of the tress obtained from each gene was made to confirm the similarity between the overall tree topology of the individual datasets and that of the tree obtained from the combined alignment.

Individual selection of evolutionary models for phylogenetic analyses of each gene was done using MrModeltest v. 2.3 (Nylander 2004) under the Akaike Information Criterion (AIC) implemented in both PAUP v. 4.0b10 and MrBayes v. 3. A maximum likelihood analysis was performed at the CIPRES webportal (Miller *et al*. 2010) using RAxML-HPC2 on XSEDE (v 8.2.8) with default parameters and bootstrapping with 1000 replicates (Stamatakis 2014). The resulting

replicates were assigned to the best scoring tree obtained earlier. Maximum Likelihood bootstrap values (ML) equal or greater than 70 % are presented below or above each node (FIGURE 1). Posterior probabilities (BP) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v. 3.0b4 (Huelsenbeck & Ronquist 2001). Six simultaneous Markov chains were initially run for 10×10^6 generations, and for every 1000th generation a tree was sampled (critical value for the topological convergence diagnostic set to 0.01, options of "stoprule = yes" and "stopval = 0.01"). MCMC heated chain was set with a "temperature" value of 0.15. Log-likelihood scores distribution was examined to determine the stationary phase for each search and determine the need or not for extra runs to achieve convergence, using the program Tracer 1.5 (Rambaut & Drummond 2007). All sampled topologies beneath the asymptote (20%) were discarded as part of a burn-in procedure, the remaining trees were used for calculating posterior probabilities in the majority rule consensus tree. BP equal or greater than 0.95 are given below or above each node (FIGURE 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).

TABLE 1. GenBank accession numbers of isolates included in this study. New sequences are shown in bold. An en dash indicates missing data

Taxon	Strain ID	LSU	ITS	SSU	*rpb2*	*tef1*
Bambusicola splendida	MFLUCC 11-0439	KU863110	NR_121549	JX442042	KU940168	KP761726
Bambusicola irregulispora	MFLUCC 11-0437	JX442036	NR_121547	JX442040	KP761719	KP761723
Bambusicola bambusae	MFLUCC 11-614	JX442035	NR_121546	JX442039	KP761718	KP761722
Bambusicola didymospora	MFLUCC 10–0557	KU863105	KU940117	KU872111	KU940164	KU940188
Bambusicola loculata	MFLUCC 13-0856	KP761729	KP761732	KP761735	KP761715	KP761724
Bambusicola triseptatispora	MFLUCC 11-0166	KU863109	NR_153624	-	KU940167	-
Bambusicola massarinia	MFLUCC 11-0389	KU863111	NR_121548	KU872115	KU940169	KU940192
Bambusicola thailandica	MFLUCC 11-0147	KU863108	KU940119	KU872113	KU940166	KU940191
Palmiascoma gregariascomum	MFLUCC 11-0175	KP744495	NR_154316	KP753958	KP998466	-
Leucaenicola phraeana	MFLUCC 18-0472	NG_066317	MK347785	NG_065784	MK434867	MK360060
Leucaenicola aseptata	MFLUCC 17-2423	NG_066309	NR_163332	NG_065776	MK434891	MK360059
Leucaenicola osmanthi	**NTUCC 18-101-1**	**MN908612**	**MN908565**	**MN908609**	**MN915020**	**MN918596**
Leucaenicola osmanthi	**NTUCC 18-101-2**	**MN908611**	**MN908566**	**MN908608**	**MN915018**	**MN918597**
Leucaenicola osmanthi	**NTUCC 18-101-3**	**MN908610**	**MN908564**	**MN908607**	**MN915019**	**MN918598**
Sulcatispora acerina	CBS 139703	AB807534	AB809635	AB797244	-	AB808509
Sulcatispora berchemiae	CBS 139704	LC014610	LC014597	LC014605	-	LC014615

Pathogenicity test

The pathogenicity of the strain was tested on healthy leaves of *Osmanthus fragrans* obtained from the original collection sites at Tea Processing Demonstration Centre, Taipei City, Nangang District, Taiwan; the top and bottom surfaces of leaves were sterilized with 70% ethanol. For each isolate, 15 *Osmanthus fragrans* leaves were inoculated. An agar plug (1 cm diam) with mycelium was cut from the periphery of five days-old culture grown on Malt extract agar (MEA) medium (25 °C). The leaves were divided into three sets, each with five leaves. The first set of leaves was injured by pin-pricking and inoculated by agar plugs (1 cm diam) with fungal mycelium. The second set which comprised

unwounded leaves had Agar plugs with fungal mycelium placed on the leaf surface. The third set of leaves, not pin-pricked, was inoculated with MEA agar plugs without fungal mycelium (control). The inoculated test leaves were preserved in sterile, moist plastic boxes for a period of 14 days. Daily observations were made on the development of disease symptoms. All fungal isolates included in pathogenicity tests were re-isolated through single spore isolation from the diseased *Osmanthus fragrans* leaves to confirm their identity with both molecular and morphological methods, as earlier above.

Results

Phylogeny

The tree topology in the ML analyses was similar and corresponded to those obtained in the Bayesian analyses. FIGURE 1 presents the results of the molecular phylogenetic analyses.

The dataset consists of 4379 characters ITS 422, LSU 945, SSU 1034, *rpb*2 1080, and *tef*1 938. The result of the Bayesian analysis was 5000 trees after 5000000 generations from the topological convergence. The first 1000 trees, which represented the burn-in phase of the analyses, were discarded, while the remaining trees were used for calculating posterior probabilities in the majority rule consensus tree.

The best scoring RAxML tree had the Likelihood value of: -4812.054120. Phylogenetic trees obtained from ML and Bayesian analysis yielded trees with similar overall topology at the species level which corresponds with previous studies based on ML and Bayesian analysis (Ariyawansa *et al*. 2015, 2018a, b, Chen *et al*. 2015, Hernandez-Restrepo *et al*. 2017, Hyde *et al*. 2013, Jayasiri *et al*. 2019, Valenzuela-Lopez *et al*. 2018, Wanasinghe *et al*. 2017).

The final alignment included 16 strains, representing 12 distinct clades in Bambusicolaceae. There is variation between the support values for the different phylogenetic methods; the Bayesian posterior probabilities being higher than the RAxML bootstrap support values (FIGURE 1). Outcomes from ML and Bayesian analysis indicated that the clade including the three strains of *Leucaenicola* NTUCC 18-101-1, 18-101-2 and 18-101-3 introduced in this study, form a monophyletic clade with high statistical support and sister clade to the strains *Leucaenicola phraeana* MFLUCC 18-0472 and *Leucaenicola aseptata* MFLUCC 17-2423 in both single gene and concatenated datasets analysis. Therefore as a recommendation, the new lineage becomes the novel taxon *Leucaenicola osmanthi*.

Alignments were investigated parallel to single gene study of ITS, LSU, SSU, *rpb*2 and *tef*1 alignments of the four phylogenies. TABLE 2 presents a detailed valuation of the alignment properties and nucleotide substitution models.

TABLE 2. Evaluation of alignment properties of genes and nucleotide substitution models used in the phylogenetic analyses.

Genes/loci	LSU	ITS	SSU	*rpb*2	*tef*1
Alignment strategy (MAFFT v6)	G-INS-1	G-INS-1	G-INS-1	G-INS-1 +manual	G-INS-1 +manual
Nucleotide substitution models for Bayesian analysis (determined by MrModeltest)	GTR+I+G	HKY+I+G	GTR+I+G	GTR+I+G	GTR+I+G

TABLE 3. Summary of *Leucaenicola* species discussed in this study.

Taxon	**Conidiomata (µm)**	**Conidiogenous cells (µm)**	**Conidia (µm)**	**Locality**	**Host**	**Reference**
Leucaenicola phraeana	90–115 × 130–150	3–4 × 1.5–2	3–4 × 1.5–2	Thailand	*Leucaena* sp.	Jayasiri *et al*. (2019)
Leucaenicola aseptata	80–100 × 100–125	2.5–3 × 1.5–2	3–4 × 1.5–2	Thailand	*Leucaena* sp.	Jayasiri *et al*. (2019)
Leucaenicola osmanthi	125–215 × 280–390	5–11 × 2–4	3–4 × 2–3	Taiwan	*Osmanthus fragrans*	This study

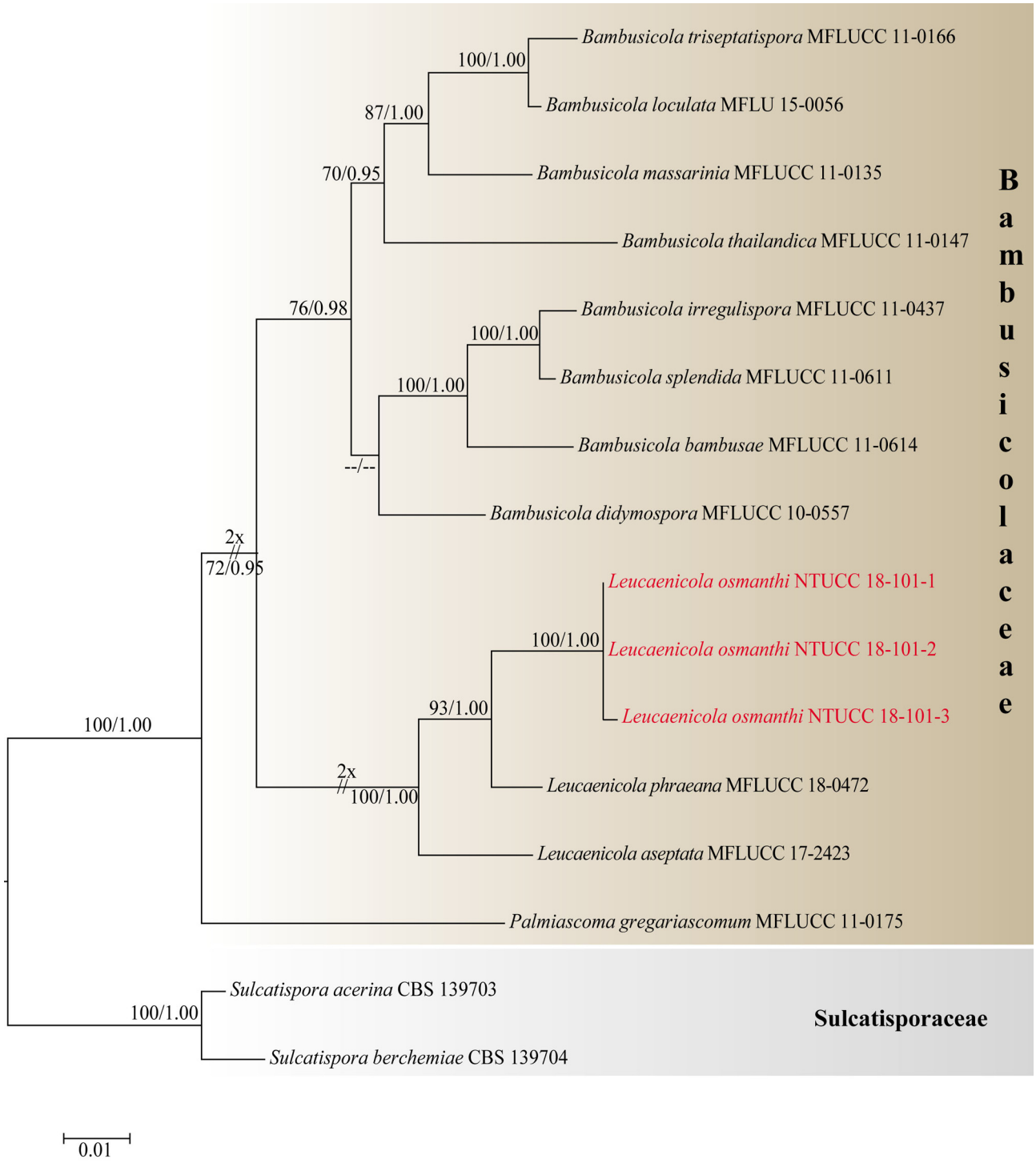

FIGURE 1. RAxML tree obtained from the concatenated DNA sequence data of ITS, LSU, SSU, *rpb*2 and *tef*1 genes. The new isolates are shown in red. ML bootstrap values (BS) ≥70 % and Bayesian posterior probabilities (PP) ≥ 0.95 are presented at the nodes. The branches *Leucaenicola* clades were scaled to half to allow an enhanced arrangement of the tree. The scale bar presents the number of estimated substitutions per site. *Sulcatispora acerina* (CBS 139703) and *Sulcatispora berchemiae* (CBS 139704) (Sulcatisporaceae, Pleosporales) were used as outgroups for rooting the tree.

Taxonomy

Leucaenicola osmanthi Ariyawansa, I. Tsai & Thambugala *sp. nov.* FIGURE 2.

MycoBank number: MB833892

Type:—TAIWAN. Taipei City, Nangang District, Nangang Tea Processing Demonstration Center (N: 25°07′10″, E: 121°61′21″), on leaves of *Osmanthus fragrans* (Oleaceae), 24 March 2018, Tsai Ichen, **holotype** NTUH 18-101-1, ex-holotype NTUCC 18-101-1.

Associated with leaf lesions of *Osmanthus fragrans*. *Leaf lesions* expanded, developing from apex to middle of leaves. *Sexual morph*: undetermined. *Asexual morph*: *Conidiomata* 125–215 × 280–390 µm ($\bar{x}$ = 172.7 × 331.3 µm), pycnidial, solitary, scattered, immersed to slightly erumpent through the host tissues, uni-loculate, globose to subglobose. *Conidiomatal wall* comprising few layers of brown to lightly-pigmented, thick-walled, cells of *textura angularis*. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* 5–11 × 2–4 µm ($\bar{x}$ ± SD = 8.5 ± 1.6 × 3.4 ± 0.5 µm), holoblastic, hyaline, smooth, ampulliform to doliiform or cylindrical, lining the conidiomatal cavity. *Conidia* 3–4 × 2–3 µm ($\bar{x}$ ± SD = 3.5 ± 0.2 × 2.3 ± 0.1 µm), ellipsoidal to cylindrical, initially hyaline, becoming pale brown, thin-walled, smooth, aseptate, with 1–2 guttules.

Etymology:—The species epithet *"osmanthi*" refers to the host genus on which the fungus was collected.

Additional material examined:—TAIWAN. Taipei City, Nangang District, Nangang Tea Processing Demonstration Center (N: 25°07′10″, E: 121°61′21″), on leaves of *Osmanthus fragrans* (Oleaceae), 24 March 2018, Ariyawansa H. A., NG14-2 (NTUH 18-101-2), ex-type culture (NTUCC 18-101-2); *ibid*. 25 April 2018, Ariyawansa H. A., NG14-3 (NTUH 18-101-3), ex-type culture (NTUCC 18-101-3) .

Notes: Morphologically and phylogenically, *Leucaenicola osmanthi* is a unique taxon (FIGURES 1 and 2). *Leucaenicola osmanthi* varies from the generic type; *Leucaenicola aseptata* by relatively larger conidiomata (125–215 × 280–390 µm versus 80–100 × 100–125 µm), larger conidiogenous cells (5–11 × 2–4 µm versus 2.5–3 × 1.5–2 µm), larger conidia (3–4 × 2–3 µm versus 3–4 × 1.5–2 µm), host (*Osmanthus* versus *Leucaena*), pathogenicity (pathogenic versus saprobic) and distribution (Taiwan versus Thailand). Our new species diverges from its phylogenetically closely related species, *Leucaenicola phraeana* by larger conidiomata (125–215 × 280–390 µm versus 90–115 × 130–150 µm), larger conidiogenous cells (5–11 × 2–4 µm versus 3–4 × 1.5–2 µm), relatively larger conidia (3–4 × 2–3 µm versus 3–4 × 1.5–2 µm), host (*Osmanthus* versus *Leucaena*), pathogenicity (pathogenic versus saprobic) and distribution (Taiwan versus Thailand).

Pathogenicity test

From the results of pathogenicity evaluation, it was revealed that with wound inoculation, *Leucaenicola osmanthi* were pathogenic on leaves of *Osmanthus fragrans*, with comparable symptoms to those under natural conditions in the field. The wounded *Osmanthus fragrans* leaves initially developed small, circular, ash-coloured spots, which subsequently transformed into brown to black spots. Following 10 days of incubation, the spots increased in diameter to 5 mm, which further increased and became sunken, causing soft decay of the leaf tissues with white mycelia coverings. On the contrary, there were no observed symptoms on non-wounded leaves, suggesting the dependence of symptom development on wounding. Four replicates with three times repetitions were used for this experiment. In every instance, similar results were obtained. All of the isolates of *Leucaenicola osmanthi* were greatly pathogenic to the artificially infected *Osmanthus fragrans* leaves. The fungi were re-isolated from wounds of the diseased leaves with 100% frequency, and their morphological characteristics and gene sequences were identical to the original ones, which confirmed that *Leucaenicola osmanthi* is a causal agent for *Osmanthus fragrans* leaf spot disease.

Discussion

Jayasiri *et al*. (2019) introduced *Leucaenicola* to accommodate two new fungal species namely, *L. aseptata* and *L. phraeana* isolated from decaying pod of *Leucaena* sp. During our survey on pleosporalean taxa associated with *Osmanthus fragrans*, one coelomycetous species causing leaf spots were recognized by DNA sequences data and morphological comparison. The phylogenetic analyses based on the nucleotide sequences of single and combined ITS, LSU, SSU, *rpb*2 and *tef*1 delivered strong confirmation that *Leucaenicola osmanthi* belong to *Leucaenicola*,

and it forms separate clades, thus demonstrating a separation of the new species from other taxa of the genus with high bootstrap support (FIGURE 1). In addition, the summary of the characters of *Leucaenicola* taxa, including major morphological characters and related data is presented in TABLE 3. To the best of our knowledge, this is the first record of *Leucaenicola* taxon as a pathogen of *Osmanthus fragrans* in Taiwan.

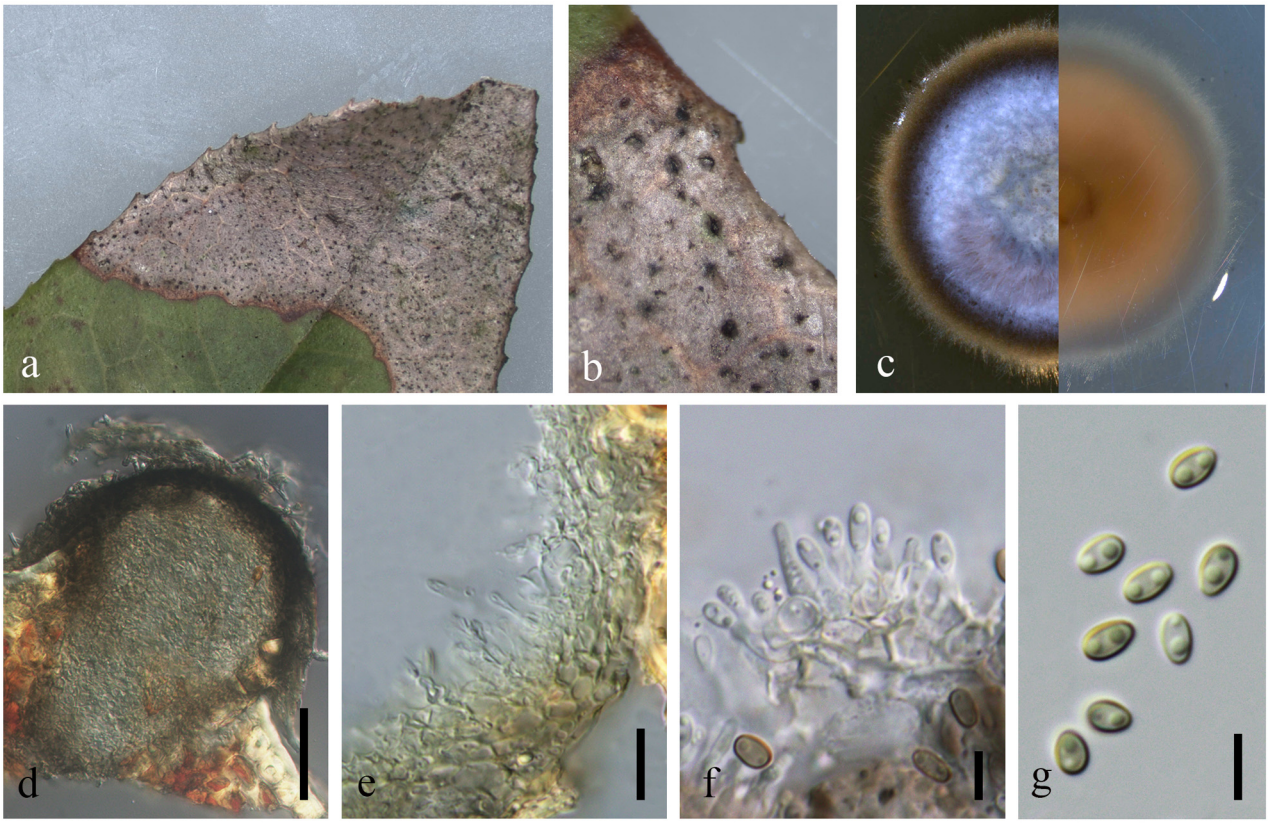

FIGURE. 2. ***Leucaenicola osmanthi*** (NTUH 18-101-1, **holotype**). a, b. Appearance of Conidiomata on host surface. c. Cultures on PDA, from above and below. d. Vertical section through conidioma. e. Conidiomatal wall. f. Conidiogenous cells and developing conidia. g. Conidia. Scale bars: d = 40 µm, e = 10 µm f–g = 5 µm.

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