RESEARCH ARTICLE

First Report of *Leptosphaerulina saccharicola* Isolated from Persimmon (*Diospyros kaki*) Tree Bark in Korea

Okouma Nguia Fulbert[1,†], Benjamin Yaw Ayim[1,†], Kallol Das[1], Yang-Sook Lim[2], Seung-Yeol Lee[1,3], Hee-Young Jung[1,3*]

[1]School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, Daegu 41566, Korea

[2]Agricultural Environment Research Department, Gyeongsangbuk-do Agricultural Research & Extension Services, Daegu 41404, Korea

[3]Institute of Plant Medicine, Kyungpook National University, Daegu 41566, Korea

[*]Corresponding author:heeyoung@knu.ac.kr

[†]This authors contributed equally to this work.

ABSTRACT

A fungal strain, designated PTT-2, was isolated from the bark of the trunk of a persimmon (*Diospyros kaki*) tree in Cheongdo, Korea. The isolate showed morphological similarities with *Leptosphaerulina saccharicola*. Strain PTT-2 had more rapid growth on potato dextrose agar medium than on oatmeal agar, malt extract agar, and synthetic nutrient poor agar media, with colony sizes of 53.8 mm, 49.8 mm, 48.4 mm, and 28.1 mm after 7 days at 25°C temperature, respectively. Strain PTT-2 produced ascospores, which had irregular wavy edges, oblong to ellipsoidal shape, hyaline appearance and 23.6 × 10 µm size. The black ascomata were developed on PDA medium, and asci were recorded. A BLAST search of the internal transcribed spacer (ITS) region, *TEF1-α* and *RPB2* gene sequences revealed that strain PTT-2 showed more than 99% nucleotide similarity with a strain of *Leptosphaerulina saccharicola* previously reported from Thailand. A neighbor-joining phylogenetic tree was constructed by concatenating the above-mentioned sequences, and showed that strain PTT-2 clustered in the same clade with *L. saccharicola.* Based on these findings, this is the first record of *Leptosphaerulina saccharicola* occurring in Korea.

Keywords: Bark, Dothideomycetes, *Leptosphaerulina saccharicola*, Persimmon

pISSN : 0253-651X
eISSN : 2383-5249

Kor. J. Mycol. 2019 March, 47(1): 13-8
https://doi.org/10.4489/KJM.20190002

Received: January 30, 2019
Revised: March 6, 2019
Accepted: March 8, 2019

INTRODUCTION

Dothideomycetes, the largest and most varied class of Ascomycota, consist of 22 orders, 105 families, 178 genera, and over 19,000 species [1], and the genus *Leptosphaerulina* is classified under this class. The *Leptosphaerulina* is endemic to North America, South America, South Africa, Asia, Australia and Europe and comprises around 25 described species [2-6]. The anamorphic stage of *Leptosphaerulina* was reported for the first time in a study in the Karoo region of South Africa

by Daniel McAlpine in the year 1902, and *Leptosphaerulina australis* was designated as the type specimen [6]. It is reported that *Leptosphaerulina* species are common fungi that can colonize several turf grass species under humid conditions and peanuts [7-8]. The fungus causes damage to 10 different angiosperm host genera belonging to 7 families of dicotyledons [9]. Species of *Leptosphaerulina* have been classified as saprophytes, senectophyte, has weak pathogenicity in turfgrasses [10]. Recently, *L. australis* has been reported for the first time in Korea [11].

In this study, a fungal isolate PTT-2 was obtained from the bark of the trunk of a persimmon (*Diospyros kaki*) tree in Cheongdo, and it was identified as a member of the genus *Leptosphaerulina*. Based on the morphological and molecular characteristics, the isolate was identified as *L. saccharicola* which has not been reported in Korea. This is the first report of its isolation and identification in Korea.

MATERIALS AND METHODS

Collection of bark sample and fungal strain isolation

During screening of fungal species in 2018, a fungal strain PTT-2 was isolated from the bark of a persimmon tree in Cheongdo, Korea (35°40′12.6″N, 128°35′52.5″E). A small portion of the bark of the tree was scraped onto potato dextrose agar (PDA) media and then incubated at 25°C. The growth of the colonies was observed for 2~3 days before the colonies were transferred to new PDA media and incubated again at 25°C. Strain PTT-2 was selected from numerous other fungal strains for further morphological and molecular phylogenetic analyses.

Morphological observation

To study growth and morphological characteristics, strain PTT-2 was cultured on four different media: PDA, oatmeal agar (OA), malt extract agar (MEA) and synthetic nutrient poor agar (SNA) [1,11]. After 7 days, colony characteristics such as color, shape, and size were recorded. Colonies on PDA, OA, MEA and SNA media were illuminated with ultraviolet light on a 12 hrs diurnal cycle for 10 days at 25°C to induce sporulation. Morphological characteristics were observed under a light microscope (BX-50; Olympus, Tokyo, Japan).

Table 1. GenBank numbers of fungal strains used for phylogenetic analyses in this study

Species	Strains Numbers	GenBank Accession numbers			
		ITS	LSU	*TEF1-α*	*RPB2*
Boeremia exigua var. *exigua*	CBS 431.74	FJ427001	JX681074	KY484687	KT389569
Botryosphaeria qingyuanensis	CERC 2946	KX278000	MF410042	MF410151	KX278105
Leptosphaerulina argentinensis	CBS 569.94	MH862490	MH874133	GU349008	GU357759
L. australis	CBS 317.83	GU296160	MH873322	GU349070	KT389640
L. doliolum var. *doliolum*	CBS 505.75	MH860947	MH872714	GU349069	KT389640
L. maculans	ICMP:19875	KF670717	KF670716	KF670715	KF670714
L. saccharicola	AFTOL-ID 277	KT225526	DQ470946	DQ471062	DQ470894
L. saccharicola	PTT-2	LC465237	LC465238	LC465240	LC465239

DNA extraction, PCR amplification, and sequencing analysis

For phylogenetic analysis, DNA from strain PTT-2 was extracted from mycelia on PDA using the HiGene Genomic DNA Prep Kit (BIOFACT, Daejeon, Korea) following manufacturer's instructions. PCR amplification was performed to amplify the internal transcribed spacer (ITS) rDNA region using primers ITS1F/ITS4, the partial sequence of the translation elongation factor 1-α (*TEF1-α*) gene region using primers EF1-983F/EF1-2218R, the large subunit (LSU) gene region using primers LROR/LR7 and the RNA polymerase II largest subunit gene (*RPB2*) using primers fRPB2-5F/fRPB2-7CR [12-16]. Amplified PCR products were purified with ExoSAP-IT (Thermo FisherScientific, Waltham, MA, USA) and sequenced by Solgent (Daejeon, Korea). The obtained sequences of strain PTT-2 were deposited in NCBI GenBank, with accession number of LC465237 for ITS, LC465238 for LSU, LC465240 for *TEF1-α* and LC465239 for *RPB2* gene sequences.

Phylogenetic analysis

Sequences of allied species were retrieved from NCBI GenBank. Phylogenetic trees were constructed based on a concatenated dataset of ITS regions, partial of *TEF1-α*, LSU and *RPB2* gene sequences using the neighbor-joining (NJ) method in the MEGA 6 software program with bootstrap analysis of 1,000 replications [17].

RESULTS AND DISCUSSION

Morphology of the strain PTT-2

Colonies of PTT-2 grew reaching 53.8 mm, 49.8 mm, 48.4 mm, and 28.1 mm in diameter, after 7 days of incubation at 25ºC on PDA, OA, MEA and SNA media, respectively (Fig. 1). The mycelial colonies were circular on PDA, OA and MEA media, but wavy and circular on SNA medium. The morphological characteristics of the colonies were compared with previous description of *L.*

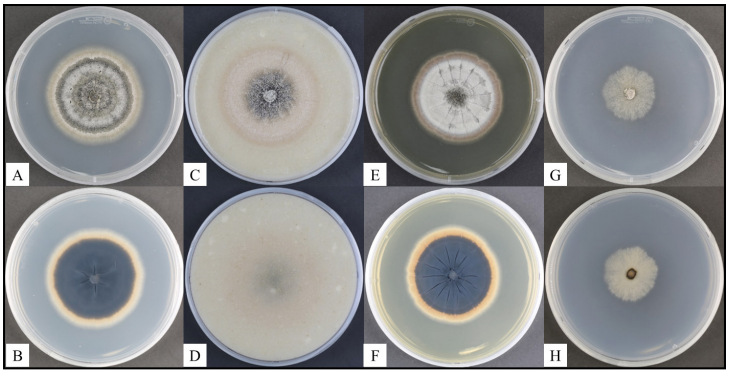

Fig. 1. Cultural characteristics of *Leptosphaerulina saccharicola* PTT-2. A, B, colonies on potato dextrose agar; C, D, colonies on oatmeal agar; E, F colonies on malt extract agar; G, H colonies on synthetic nutrient poor agar (scale bar = 10 μm).

saccharicola (Table 2) [1]. Ascospores of strain PTT-2 were initially hyaline, becoming brown to dark brown on PDA, and reached 29.6 × 11 μm in size. Transverse and longitudinal septa were also observed. The number of longitudinal septa ranged from 0~2, while the transverse septa from 0~1 [1]. Strain PTT-2 produced abundant hyaline ascospores, which had irregular wavy edge, triseriate oblong to ellipsoidal shape and 23.6 × 10 μm diameter (Table 2). The number of longitudinal septa of ascospores ranged from 0~1, while transverse septa numbered 3~4. The asci and ascospores were observed (Fig. 2). The morphological characteristics observed for strain PTT-2 were very similar to the previously described characteristics of *L. saccharicola*.

Table 2. Comparison of morphological characteristics of isolate PTT-2 with reference to strains of *Leptosphaerulina saccharicola*

Morphology		PTT-2 strain[a]	*Leptosphaerulina saccharicola*[b]
Colony	Characteristics	Brown to dark brown on PDA, covered with white fluffy hyphae with circular wavy edge, white to pale yellow at the edge. Dark brown to blackish color in the middle at 7 d post-inoculation at 25℃	Brown to dark brown developed on PDA, 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 at 7 d post-inoculation at 25~30℃
	Diameter	PDA: 53.8 mm; MEA: 48.4 mm; OA: 49.9 mm; SNA: 28.1 mm	PDA: 35~45 mm; MEA: N/A; OA: N/A
	Shape	Circular on PDA, OA, MEA but wavy circular on SNA	Circular on PDA
Asci	Color	Hyaline to brown	Initially hyaline, becoming brown to dark brown on PDA in 7 days at 25~30 ℃
	Diameter	23.6 × 10 μm, n=22	29.6 × 11 μm, n=30
Ascospore	Shape	Irregular, wavy edge, triseriate, oblong to ellipsoidal	Irregular triseriate, oblong to cylindrical or ellipsoidal
	Number of transverse septa	3~4	4
	Number of longitudinal septa	0~1	0~2

[a]Fungal strain studied in this study; N/A, characteristic was not available
[b]Source of description [1]

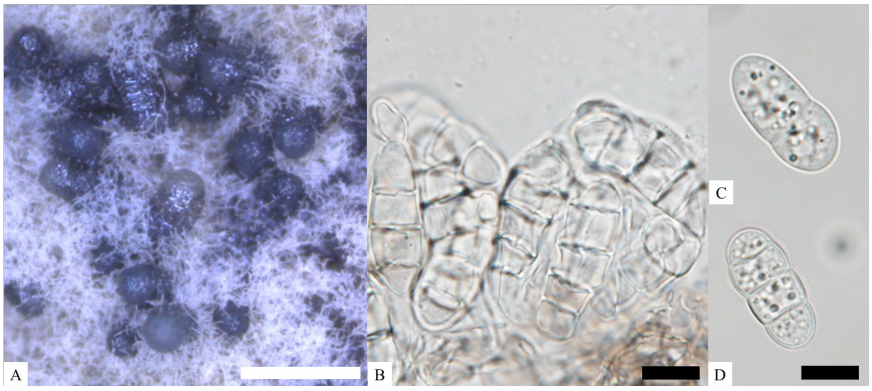

Fig. 2. Morphological characteristics of *Leptosphaerulina saccharicola* PTT-2. A, asci; B, C, D, ascospores of PTT-2 (scale bar: A = 250 μm, B~D = 10 μm).

Molecular phylogeny of strain PTT-2

After sequencing analysis, sequences of 501 bp, 1,271 bp, 930 bp and 1,048 bp were obtained from ITS regions, LSU, *TEF1-α* and *RPB2* gene, respectively. BLAST search results revealed that strain PTT-2 shared 99% identities with the ITS regions, *TEF1-α* and *RPB2* gene sequences with three other strains of *L. saccharicola* (MG583749, KF670715, KF670714). For LSU, blast search results showed the 100% similarities with a strain of *L. australis* (MH871269). A concatenated dataset of ITS, LSU, *TEF1-α* and *RPB2* gene sequences was used to determine the molecular relationship between the present Korean isolate and *Leptosphaerulina* species retrieved from GenBank. A NJ tree showed that PTT-2 strain clustered in the same clade with the other *L. saccharicola* strains, indicating that PTT-2 is a strain of *L. saccharicola* (Fig. 3). Although *L. saccharicola* associated with leaf disease of *Saccharum officinarum*, but also as an important pathogen of turf grass [1]. In view of this, the pathogen is very relevant to agricultural production and further studies need to investigate its potential pathogenicity to aid identifying relative cultivars that are susceptible to the pathogen. To our knowledge, this is the first report of *Leptosphaerulina saccharicola* in Korea and its identity is strongly supported by the morphological and molecular evidence presented here.

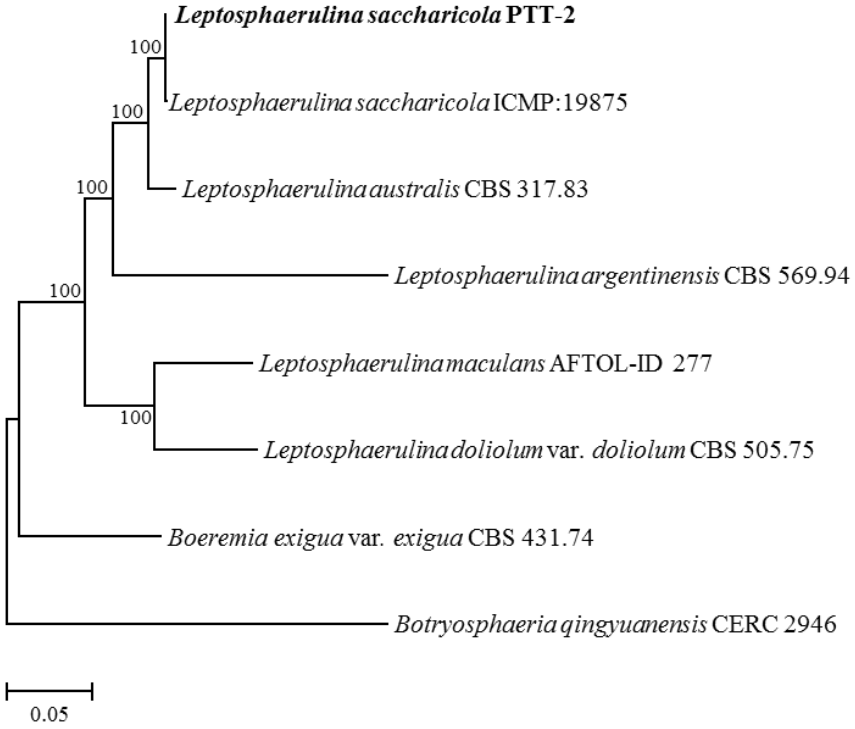

Fig. 3. Neighbor-joining phylogenetic tree, based on the concatenated ITS, *TEF1-α*, LSU and *RPB2* gene sequences shows the phylogenetic position of *Leptosphaerulina saccharicola* PTT-2 among members of the genus *Leptosphaerulina*. The strain isolated in this study was shown in boldface. Bootstrap values (based on 1,000 replications) are shown at the branch points. *Botryosphaeria qingyuanensis* CERC 2946 was used an outgroup.

ACKNOWLEDGEMENTS

This research was supported by a project on the survey and excavation of Korean indigenous species of the National Institute of Biological Resources (NIBR 201801105).

REFERENCES

1. Rungtiwa P, Jian KL, Ekachai C, Eric HCM, Kevin DH. Phylogeny and morphology of *Leptosphaerulina saccharicola* sp. nov. and *Pleosphaerulina oryzae* and relationships with Pithomyces. Cryptogamie Mycol 2013;34:303-19.
2. Inderbitzin P, Gareth JEB, Vrijmoed LLP. A new species of *Leptosphaerulina* from decaying mangrove wood from Hong Kong. Mycoscience 2000;41:233-7.
3. Roux C. *Leptosphaerulina chartarum* sp. nov. the teleomorph of *Pithomyces chartarum*. Trans Br Mycol Soc 1986;86:319-23.
4. Irwin JAG, Davis RD. Taxonomy of some *Leptosphaerulina* spp. on legumes in Eastern Australia. Aust J Bot 1985;33:233-7.
5. Graham JH. Species of *Leptosphaerulina* on forage plants. Phytopathology 1961;51:680-93.
6. McAlpine D. Fungus diseases of stone-fruit trees in Australia and their treatment, Melbourne: Government Printer;1902.
7. Mitkowski NA, Browning M. *Leptosphaerulina australis* associated with intensively managed stands of *Poa annua* and *Agrostis palustris*. Can J Plant Pathol 2004;26:193-8.
8. Rao PN, Karan D. Some new hosts to *Leptosphaerulina* McAlp. from India. Mycopathol Mycol Appl 1964;22:91-5.
9. Kim JH, Shim GY, Kim YH. Occurrence of *Leptosphaerulina* leaf blight on Kentucky bluegrass caused by *Leptosphaerulina*. Res Plant Dis 2010;16:94-6.
10. The Korean Society of Plant Pathology. List of plant disease in Korea, 5th ed. Suwon: Korean Society of Plant Pathology; 2009.
11. Li W, Back CG, Lee SY, Ten LN, Jung HY. First report of *Leptosphaerulina australis* isolated from soil in Korea. Kor J Mycol 2018;46:369-74.
12. White T, Bruns T, Lee S, Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR Protocols: a guide to methods and applications. San Diego: Academic Press; 1990. p. 315-22.
13. Gardes M, Bruns TD. ITS primers with enhanced specificity for basidiomycetes- application to the identification of mycorrhizae and rusts. Mol Ecol 1993;2:113-8.
14. Rehner SA, Buckley E. A *Beauveria* phylogeny inferred from nuclear ITS and EF1-alpha sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. Mycologia 2005;97:84-8.
15. Liu YJ, Whelen S, Hall BD. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. Mol Biol Evol 1999;16:1799-808.
16. Vilgalys R, Hester M. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol 1990;172:4238-46.
17. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA 6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013;30:2725-9.