

## RESEARCH NOTE

# Morphology and Molecular Phylogeny of *Pseudocercospora fatouae* Associated with Hypophyllous Leaf Mold on *Fatoua villosa* in Korea

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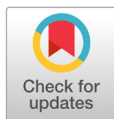
## ABSTRACT

Dark brown leaf molds growing in a vein-limited pattern have been continuously observed on the lower leaf surface of *Fatoua villosa* in Korea. Fifteen samples deposited in the Korea University herbarium were examined for fungal morphological characterization. Two monoconidial isolates were obtained in 2022, and molecular analysis was performed based on a combined multigene dataset of the internal transcribed spacer (ITS) region and partial actin (*actA*), translation elongation factor 1-alpha (*tef1*), and DNA-directed RNA polymerase II second-largest subunit (*rpb2*). Based on morphological features and molecular phylogenetic analysis, the fungus *Pseudocercospora fatouae* was identified. To the best of our knowledge, it is the first study reporting the presence of *P. fatouae* on *F. villosa* in Korea.

**Keywords:** Biocontrol, Hairy crabweed, Mulberryweed, Multi-locus DNA dataset

*Fatoua villosa* (Thunb.) Nakai, commonly known as mulberryweed or hairy crabweed, belongs to the family Moraceae. It is native to Eastern Asia and was introduced to Louisiana, USA, in the 1950s. Given its ability to reproduce and spread rapidly, it has been naturalized throughout much of the eastern United States and is found from Texas to Florida and north of Michigan and Delaware [1-3]. This species was recently reported in Australia (Queensland) and Europe (France) [<https://gd.eppo.int/taxon/FTOVI>].

To eradicate this troublesome weed, multiple attempts have been made using chemicals but not effective biological tools [4,5]. Some phytopathogenic fungi, such as *Pseudocercospora fatouae* Goh & W.H. Hsieh in China and Taiwan, *Podospaera pseudofusca* (U. Braun) U. Braun & S. Takam. in China and Japan, and *Podospaera xanthii* (Castagne) U. Braun & Shishkoff in Korea, have been identified on this weed [6,7]. Given that *P. fatouae* is a culturable fungus, it can be considered a potential biocontrol agent. However, its distribution and biology are poorly understood. The purpose of this study was the morphological and molecular characterization of the fungus on *F. villosa* in Korea.



## OPEN ACCESS

pISSN : 0253-651X  
eISSN : 2383-5249

Kor. J. Mycol. 2023 June, 51(2): 147-153  
<https://doi.org/10.4489/KJM.20230017>

**Received:** March 30, 2023

**Revised:** June 29, 2023

**Accepted:** June 29, 2023

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During our field forays in Korea, fifteen samples of fungus-infected *F. villosa* were collected and preserved in the Korea University herbarium (KUS) (Table 1). Preliminary microscopy confirmed the fungus on these samples as *P. fatouae* based on the characteristic growth pattern on the lower leaf surface and the morphology of conidiophores and long conidia. Detailed morphological characterization and molecular analysis were performed using two samples collected in 2022.

**Table 1.** Sources of fungal materials and sequence database accession numbers.

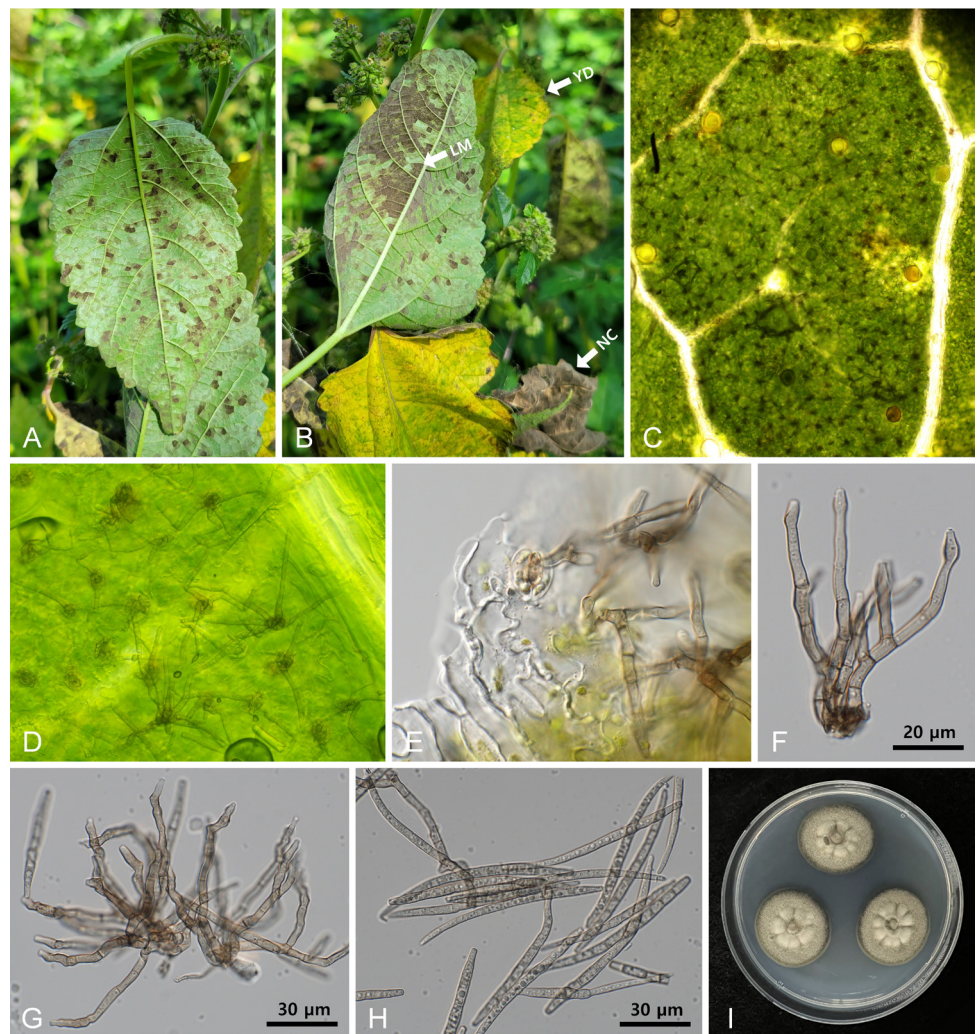
Voucher specimen number (KUS)	Collection date	Collection place	KACC number	GenBank accession numbers			
				ITS	<i>actA</i>	<i>tef1</i>	<i>rpb2</i>
F17462	Aug 30, 2000	Pocheon	-	-	-	-	-
F17797	Oct 9, 2000	Dongducheon	-	-	-	-	-
F18341	Aug 31, 2001	Pocheon	-	-	-	-	-
F19028	Sep 18, 2002	Pocheon	-	-	-	-	-
F20804	Oct 11, 2004	Hoengseong	-	-	-	-	-
F21313	Sep 4, 2005	Hoengseong	-	-	-	-	-
F22114	Sept 8, 2006	Hoengseong	-	-	-	-	-
F22203	Sep 24, 2006	Hongcheon	-	-	-	-	-
F22750	Aug 3, 2007	Hoengseong	-	-	-	-	-
F22919	Sep 21, 2007	Hoengseong	-	-	-	-	-
F23078	Oct 19, 2007	Jecheon	-	-	-	-	-
F24526	Sep 7, 2009	Youngwol	-	-	-	-	-
F27124	Oct 15, 2012	Jeju	-	-	-	-	-
F33248	Sep 20, 2022	Jinan	410294	OQ569347	OQ606969	OQ606973	OQ606971
F33291	Sep 27, 2022	Wanju	410295	OQ569348	OQ606970	OQ606974	OQ606972

KACC: Korean Agricultural Culture Collection, Rural Development Administration; ITS: internal transcribed spacer; *actA*: actin; *tef1*: translation elongation factor 1-alpha; *rpb2*: DNA-directed RNA polymerase II second-largest subunit.

For morphological investigations, a small amount of fungus was scraped from fresh samples, mounted in a drop of sterile water, and examined under an optical microscope (Equipped with a KCS-3.1C Imaging System, Carl Zeiss AX10, Göttingen, Germany). A minimum of 30 measurements were performed for each diagnostic structure. To obtain a pure conidial culture, conidia were collected from lesions using a sterile scalpel and placed in Eppendorf tubes containing sterile water. The conidial suspension was streaked onto 2% water agar (WA, Junsei, Tokyo, Japan) plates supplemented with 100 mg/L of streptomycin sulfate and incubated at 25°C. After two days, colonies were transferred onto potato dextrose agar (PDA, Difco, France) plates. The isolates were obtained from KUS-F33248 and F33291 and submitted to the Korean Agricultural Culture Collection of the Rural Development Administration.

Symptoms initially appeared as pale greenish or yellowish foliar spots on the upper leaf surface, corresponding to dark-gray fuliginous growth on the lower surface. With disease progression, the leaf lesions became vein-limited and angular (Fig. 1A). Ultimately, by coalescing the leaf lesions, more than half of the leaf surface was covered with chocolate-colored fungal growth (Fig. 1B). Stromata were nearly absent or contained only aggregates of several swollen cells in the stomatal openings (Figs. 1C and D). Conidiophores were emerging from stomatal openings, 2-10 in loosely divergent fascicles, olivaceous

brown, darker than conidia, 0-3 times branching, tortuous and geniculate, sinuous, 3-6-septate, 30-70  $\mu\text{m}$  long, 3-5  $\mu\text{m}$  wide, and conidial scars inconspicuous (Figs. 1E and F). Conidia were solitary, filiform to cylindrical, pale olivaceous, substraight or slightly curved, 4-10-septate, apex subobtuse, base subtruncate, 50-110  $\mu\text{m}$  long, 3.5-5.0  $\mu\text{m}$  wide, and hilum unthickened and inconspicuous (Figs. 1G and H). Two-week-old fungal colonies grown on PDA at 25°C measured 23-25 mm in diameter and were olivaceous gray to khaki with smooth undulate margins (Fig. 1I). The reverse culture was green-black. These characteristics are similar to those of *P. fatouae* Goh & W. H. Hsieh [8].



**Fig. 1.** *Pseudocercospora fatouae* hypophyllous leaf mold on *Fatoua villosa*. (A) Early symptoms of angular leaf mold on the lower leaf surface. (B) Leaf symptoms in the later stage of disease development. Note the leaf mold on the lower leaf surface (LM), yellow discoloration on the upper leaf surface (YD), and necrosis of the affected leaves (NC). (C) A close-up view of the lower leaf surface with numerous conidiophores. (D, E) Conidiophores emerging from the stomatal openings. (F) Conidiophore showing branched character. (G) Conidiophores with small stomata and a young conidium on the tip (arrow). (H) Conidia. (I) Three-week-old colonies of *P. fatouae* growing on potato dextrose agar at 25°C.

For molecular analysis, genomic DNA was extracted from 2-week-old cultures grown on PDA at 25°C using Maglisto™ 5M kits (Bioneer, Daejeon, Korea) according to the manufacturer's protocol. The internal transcribed spacer (ITS) region, including protein coding genes such as, actin (*actA*), translation elongation factor 1-alpha (*tef1*), and DNA-directed RNA polymerase II second largest subunit (*rpb2*), was amplified and sequenced using primer pairs V9G/ITS4, ACT-512F/ACT-783R and EF1-728F/EF1-986R through the polymerase chain reaction (PCR) steps described previously by Nakashima et al. [9]. Amplification of *rpb2* gene was not successful with RPB2-5f2/fRPB2-7cR or fRPB2-5F/fRPB2-7cR. As a result, we developed the following primers for amplification of this gene: forward P-RPB2-F (5'-ACC ACT CCC ATC GGT CGA GA-3') and reverse P-RPB2-R (5'-GGT GTC ATG CTG ATC ATA GC-3'); the PCR procedure was as follows: initial denaturation (94°C for 2 min), five amplification cycles at 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s, followed by 30 amplification cycles at 95°C for 45 s, 54°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 5 min). The PCR products were sequenced in both directions using the same primers by a commercial sequencing company (Bioneer, Daejeon, Korea). Forward and reverse sequences were assembled, and the consensus sequences were deposited in GenBank (Table).

The alignment for each gene was prepared separately in MEGA 11 [10] and then combined into one multigene dataset of ITS+*act*+*tef1*+*rpb2* using SequenceMatrix 1.8 software [11]. *Trochophora simplex* (CBS 214744) and *Pallidocercospora heimioides* (CBS 111190) were selected as outgroups. Maximum parsimony analysis was conducted in PAUP\* 4.0a using a heuristic search option [12]. The robustness of the tree was evaluated using bootstrap analysis with 1,000 replicates.

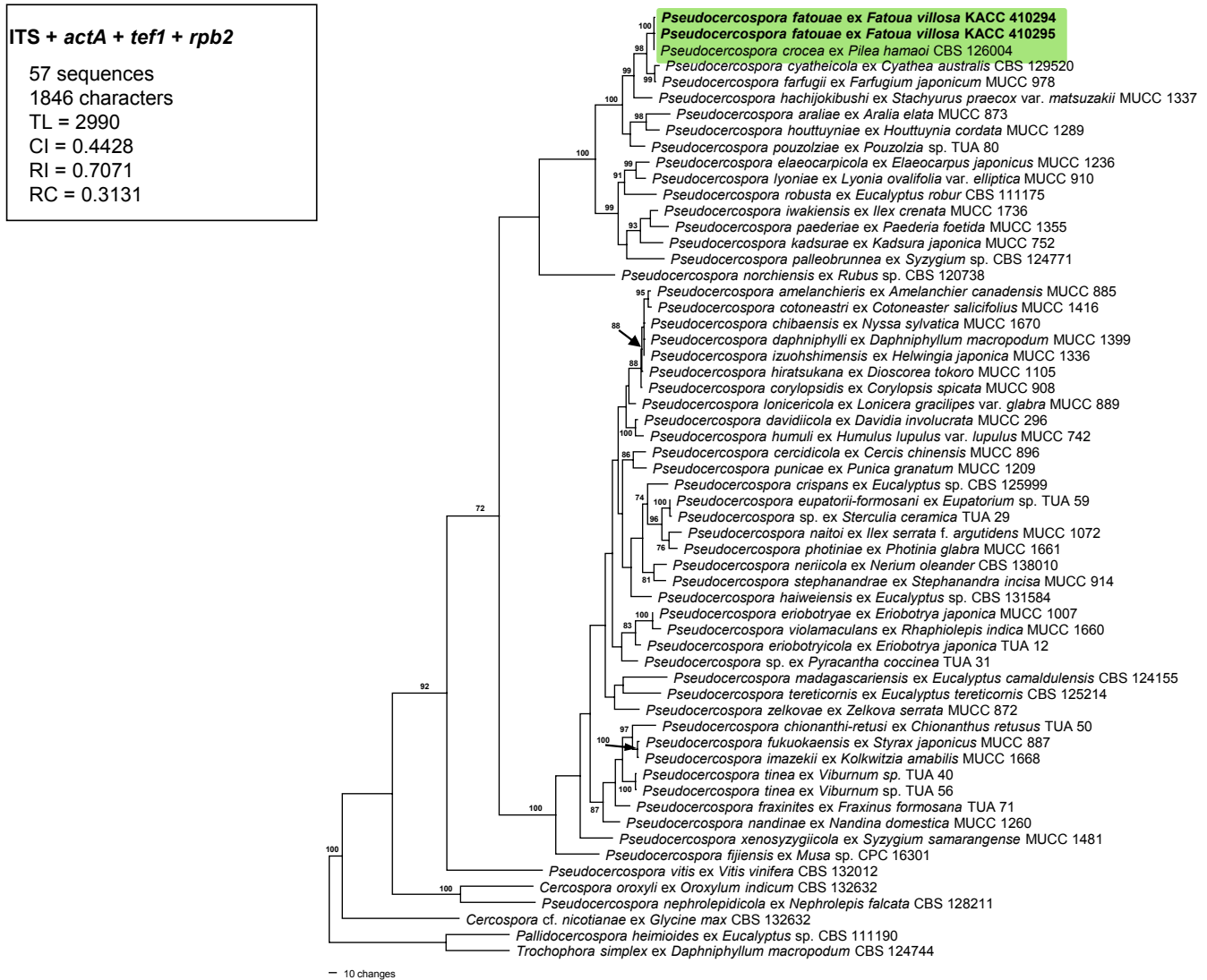
The resulting sequences obtained from the two samples were almost identical; only a one-bp difference was found in *actA*. A BLASTn search was performed for newly obtained sequences to compare their similarity with other reference sequences in the GenBank. The findings revealed 100% identity to *Pseudocercospora crocea* (MH863878, GU384502) for the ITS and *tef1* genes, whereas for *actA*, 99.5% similarity to *P. balsaminae* (GU320367) and *P. farfugii* (LC599423) and for *rpb2*, 99.84% similarity to *P. farfugii* (LC599603) was observed.

The final alignment consisted of 57 sequences and 1,846 characters, of which 187 (10.13%) were variable and parsimony uninformative, and 605 (32.77%) were informative for parsimony analysis. As demonstrated in the resulting tree, the sequences of *P. fatouae* found on *F. villosa* were placed in a distinct clade with those of *P. crocea*; this was supported by a 100% bootstrap value (Fig. 2). Given the lack of *rpb2* sequences for *P. crocea* (CBS126004) in GenBank, only the sequences of ITS (GU269792), *actA* (GU320493), and *tef1* (GU320493) obtained from that isolate were included in the current analysis. However, *P. crocea* described on *Pilea hamaoi* (Urticaceae) in 2013 [13] differs from the current species by having amphigenous caespituli (vs. hypogenous caespituli), a well-developed stroma of 40-100 µm diam. (vs. lacking or only an aggregate of several swollen cells), and shorter conidiophores, 17-24 µm long (vs. 30-70 µm long). As a result, *P. crocea* is not close to, or synonymous with, *P. fatouae*.

*P. fatouae* was first described by Hsieh & Goh [8] as a new species in Taiwan in 1990. Previously, *Cercospora fatouae* (originally known as *C. fatuae*) was described as a new species in Japan in 1904 [14].



The morphological description of *C. fatouae* is extremely close to that of *P. fatouae* in having hypogenous fructification with dark fuliginous fungal growth, branched conidiophores, and long conidia (90-120×3-4 μm) in the same genus, *Fatoua spinosa* var. *subcordata*, indicating that these two species are synonymous. Therefore, we propose the correct name for this fungus as *Pseudocercospora fatouae* (Henn.) Goh & W. H. Hsieh.



**Fig. 2.** A phylogenetic tree of *Pseudocercospora fatouae* was constructed using the maximum parsimony method based on a combined multigene dataset of ITS+actA+tef1+rpb2 of 59 sequence samples. The isolates obtained in this study are shown in boldface. Bootstrap values (>70%) were provided on the relevant branches. Tree scores, such as tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC), are presented in the box given on the left side of the Figure. ITS, internal transcribed spacer; actA, actin; tef1, translation elongation factor 1-alpha; rpb2, DNA-directed RNA polymerase II second-largest subunit.

Alien-invasive plants are of great concern worldwide. Mulberryweed (*F. villosa*) is an Asian plant that is invasive in North America and, recently, in France. Understanding the diversity of phytopathogenic fungi on mulberryweed in its indigenous regions is critical for understanding this weed and developing effective control measures. Leaf mold (*P. fatouae*) on mulberry weeds has been reported in China and Taiwan [7]. To the best of our knowledge, this is the first report of *P. fatouae* infection in *F. villosa* in Korea. This study contributes to the understanding of the biology of *P. fatouae* by providing morphological characteristics and molecular phylogenetic data, as well as depositing two monoconidial isolates from Korea in the culture collection.

## DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

## ACKNOWLEDGEMENTS

This study was carried out with the support of the Cooperative Research Program for Agricultural Science and Technology Development (Project Nos. PJ014976), Rural Development Administration, Republic of Korea. H. D. Shin was supported by a grant (K2123391) from Korea University, Korea.

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