RESEARCH NOTE

Morphology and Molecular Phylogeny of *Pseudocercospora securinegae* on *Flueggea suffruticosa* Based on Korean Isolates

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ABSTRACT

This study provides a detailed morphological description of *Pseudocercospora securinegae*, a species that has long been overlooked, supplemented by comprehensive illustrations of its diagnostic features. The taxonomic value of this study is further supported by molecular phylogenetic analyses based on a multigene dataset, including the internal transcribed spacer (ITS) region, actin (*actA*), translation elongation factor 1-alpha *(tef1*), and DNA-directed RNA polymerase II second largest subunit (*rpb2*). Notably, a sequence of the *rpb2* gene for this fungus is presented here for the first time.

Keywords: Arching bushweed, Leaf spot, Multi-locus DNA dataset, Phytopathogen, *Securinega suffruticosa*

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Pseudocercospora securinegae (Togashi & Katsuki) Deighton is a fungal pathogen that causes leaf spot disease. It was originally described as *Cercospora securinegae* Togashi & Katsuki from *Securinega suffruticosa* (Pall.) Rehder (currently *Flueggea suffruticosa* (Pall.) Baill.) in Japan [1]. Its current taxonomic placement within the genus *Pseudocercospora* was established by Deighton in 1976 [2].

In Korea, the occurrence of *Ps. securinegae* on *F. suffruticosa* was first observed during a survey of phytopathogenic fungi in 2003. A relevant specimen collected in Yangpyeong was deposited at the Westerdijk Fungal Biodiversity Institute, and the corresponding isolate was registered as CBS 131930 (Centraalbureau voor Schimmelcultures; CBS). Although this strain was later included in a taxonomic study of the genus *Pseudocercospora* by Crous et al. [3], its detailed morphological description was not provided. Our field observations show that the disease can be severe in Korea, with extensive leaf spots leading to premature defoliation of up to 80% before the expected leaf fall in autumn.

Subsequent comprehensive studies on cercosporoid fungi—i.e., Shin and Kim [4], Nakashima et al. [5], and Groenewald et al. [6]—overlooked this fungus. Moreover, nucleotide sequence data obtained from one aforementioned strain of *Ps. securinegae* are available in GenBank, which restricts conducting reliable molecular comparisons and phylogenetic placement.

Despite its early report, subsequent taxonomic studies have largely overlooked *Ps. securinegae*; hence, its morphological features have yet to be sufficiently characterized. To address this gap, this study provides a detailed morphological description supported by photomicrographs and supplemented with additional sequence data, including sequences of the DNA-directed RNA polymerase II second-largest subunit (*rpb2*) gene for the first time.

During our field forays in Korea, 14 samples of *F. suffruticosa* with leaf spot symptoms of *Pseudocercospora* infection were collected and preserved in the herbaria of Korea University (KUS; Seoul, Korea) and Jeonbuk National University (JBNU; Jeonju, Korea). Detailed information regarding the herbarium number, location, and collection dates are listed in Table 1.

Table 1. Korean samples of *Pseudocercospora securinegae* from *Flueggea suffruticosa* used in this study

Voucher specimen number	Collection date	Collection place	KACC/CBS number	GenBank accession numbers			
				ITS	*actA*	*tef1*	*rpb2*
KUS-F19756	30 Sep 2003	Yangpyeong	CBS 131930	GU269776	GU320479	GU384487	-
KUS-F20561	4 Aug 2004	Hoengseong	-	-	-	-	-
KUS-F21419	23 Sep 2005	Hoengseong	-	-	-	-	-
KUS-F21967	11 Aug 2006	Yangpyeong	-	-	-	-	-
KUS-F22039	3 Sep 2006	Hoengseong	KACC 42527	PX376255	PX413061	PX423942	PX436606
KUS-F22749	3 Aug 2007	Hoengseong	KACC 43030	PX376256	PX413062	PX423939	PX436607
KUS-F25219	4 Sep 2010	Chuncheon	KACC 46414	PX376257	PX413063	PX423940	PX436608
KUS-F25347	27 Sep 2010	Yeongwol	-	-	-	-	-
KUS-F26101	5 Sep 2011	Hongcheon	-	-	-	-	-
KUS-F26738	23 Jul 2012	Hongcheon	-	-	-	-	-
KUS-F32608	3 Nov 2021	Jeonju	-	-	-	-	-
KUS-F34197	2 Sep 2024	Gapyeong	KACC 411232	PX376260	PX413066	PX423943	PX436611
KUS-F34239	19 Sep 2024	Hongcheon	KACC 411233	PX376258	PX413064	PX423938	PX436609
JBNU-F0519	4 Oct 2024	Wanju	KACC 411234	PX376259	PX413065	PX423941	PX436610

CBS: Centraalbureau voor Schimmelcultures culture collection; 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.

Fresh specimens were used for conducting morphological observations of the fungus using an Olympus BX50 microscope (Olympus, Tokyo, Japan). Stromata-bearing conidiophores and conidia were picked with a sterile needle, mounted in a drop of sterile water, and examined. Photomicrographs of taxonomic features were captured using a Zeiss AX10 microscope equipped with an AxioCam MRc5 digital camera (Carl Zeiss, Oberkochen, Germany). A total of 30 measurements were recorded for each diagnostic structure. To obtain pure conidial cultures, conidia were isolated from symptomatic tissues using sterile forceps and streaked onto 2% potato dextrose agar (PDA; Difco, France) supplemented with 200 mg/L streptomycin sulfate, followed by incubation at 25°C. After four days, the germinated conidia were transferred to a new PDA plate without antibiotics. A total of six isolates were obtained and deposited in the Korean Agricultural

Culture Collection (KACC; Rural Development Administration, Korea). The accession numbers are listed in Table 1.

Observed morphological characteristics of the fungus include the following: Leaf spots were amphigenous, irregular to angular, 5–15 mm in diameter (or larger by coalescing discrete lesions), with greyish lesion with dark brown margin (Fig. 1A–D). Caespituli were amphigenous, albeit primarily hypophyllous, fasciculate to solitary (Fig. 1E). Mycelium was internal and external, with septate and branched hyphae. Stroma were well-developed, sub-globular, erumpent, light to dark brown, up to 30–60

Fig. 1. Symptoms and morphological characteristics of *Pseudocercospora securinegae* on *Flueggea suffruticosa*. (A) Leaf spots with a greyish lesion on infected leaves. (B) Close-up view of individual leaf spots showing a well-defined greyish lesion. (C, D) Overall view of infected plants showing severe disease symptoms in early September; nearly all leaves had fallen, and only several new leaves remained (arrows). (E) Abundant greyish fungal fructification on the upper leaf surface. (F) Stromata. (G) Conidiophores and conidiogenous cells. (H, I) Conidia. (J) Four-week-old colonies of *Ps. securinegae* growing on a potato dextrose agar at 25°C.

μm in diameter (Fig. 1F). Conidiophores were numerous, in dense fascicles, smooth-walled, 2–4-septate, cylindrical to sub-cylindrical, straight, 14–37(–40) × (2.5–)3–5 μm. Conidiogenous cells were monoblastic, hyaline, unbranched, 3–6 μm, with inconspicuous scars (Fig. 1G). Conidia were solitary, narrowly obclavate to cylindrical, smooth, hyaline, straight to slightly curved, 6–11-septate, apex obtuse, base truncate, 25–85(–92) × (2.9–)3.3–5.4 μm, with an unthickened and not darkened hila (Fig. 1H–I). Four-week-old fungal colonies grown on PDA at 25°C were slow-growing, 10 mm in diameter, olivaceous to greyish towards the center, with an erose or dentate margin, developed moderate aerial mycelium, and dark olivaceous to black on the reverse surface (Fig. 1J).

Due to the absence of a detailed description of *Ps. securinegae* in previous studies, the morphological characteristics of the Korean isolates were compared with an original description of *C. securinegae* [1], from which the species was transferred. The Korean isolates correspond well with the original description of *C. securinegae* in stromata size (35–60 μm vs. 35–50 μm), conidiophore morphology and dimensions (14–37(–40) × (2.5–)3–5 μm vs. 25–40 × 3–4 μm), and conidial shape and size (25–85(–92) × (2.9–)3.3–5.4 μm vs. 25–125 × 3–4 μm). Although our specimens exhibited slightly larger stromata and more septate conidia, these differences correspond to the expected intraspecific variation of *Pseudocercospora*. In addition, among *Flueggea* hosts, *Ps. aberrans* has been reported on *F. virosa* from China [7] and was therefore considered in our comparative assessment. The species shows clear distinguishing features, including smaller stromata (15–35 μm), very long conidiophores (up to 205 μm), and pale olivaceous, 1–3-septate conidia. These distinct characteristics clearly separate *Ps. aberrans* from Korean isolates.

To conduct molecular analysis, genomic DNA was extracted from six isolates cultured for two weeks on PDA using Maglisto™ 5M kits (Bioneer, Daejeon, Korea) following the manufacturer's protocol. The primer pairs V9G/ITS4, ACT-512F/ACT-783R, EF1-728F/EF-986R, and P-RPB2-F/P-RPB2-R were used to amplify and sequence the nucleotide sequences of the internal transcribed spacer (ITS) region, actin (*actA*), translation elongation factor 1-alpha (*tef1*), and *rpb2* genes, respectively [5,8,9]. The polymerase chain reaction products were purified and sequenced by a commercial sequencing company (Bionics, Seoul, Korea). The obtained forward and reverse sequences were assembled in MEGA 11 [10], and concatenated data were deposited in the NCBI database (Table 1).

The dataset of each gene was created and aligned separately, and each alignment was combined into a single multigene dataset of ITS + *actA* + *tef1* + *rpb2* using the SequenceMatrix software [11]. *Trochophora simplex* (CBS 214744) was selected as an outgroup [5]. A phylogenetic tree was constructed using three different methods: maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) analyses. For the MP analysis, a heuristic search with the tree-bisection algorithm was used in PAUP* 4.0a [12]. The final dataset contained 34 taxa with a total of 1,853 characters, of which 225 (12.14%) were variable and parsimony-uninformative, and 541 (29.2%) were informative for the parsimony analysis. The ML analysis was conducted using raxmlGUI 2.0.13, with the GTR substitution model and a GAMMA distribution [13]. The reliability of the trees was tested using 1,000 bootstrap replications. To perform the BI analysis, the optimal nucleotide substitution model was first determined using MrModelTest version 2.4 [14], which identified GTR + I + G as the best model. This model was then used for the BI analysis in MrBayes version

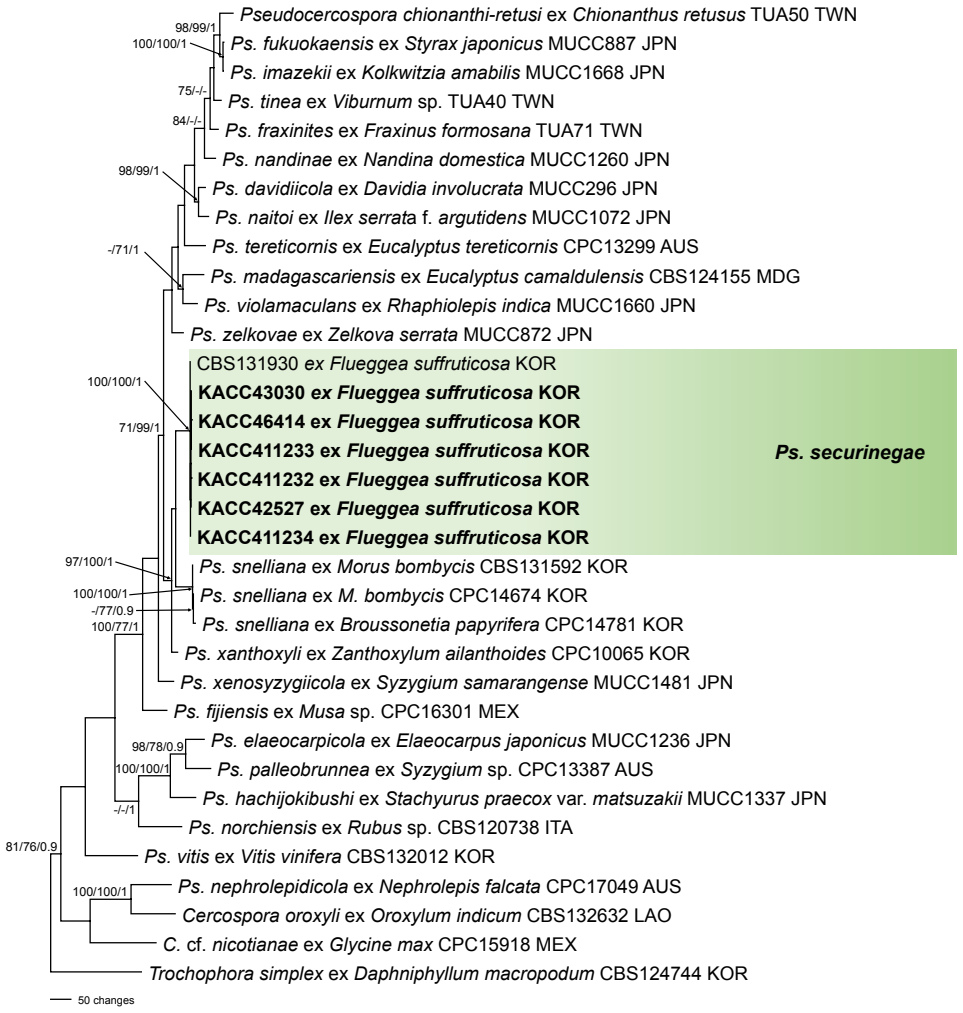

Fig. 2. A maximum parsimony tree of *Pseudocercospora securinegae* constructed using a multigene dataset of ITS + *actA* + *tef1* + *rpb2*. Isolates obtained in this study are shown in bold. Bootstrap values (>70%) obtained from maximum parsimony and maximum likelihood as well as posterior probabilities (>90%) from Bayesian inference are shown on the respective related branches. ITS: internal transcribed spacer; *actA*: actin; *tef1*: translation elongation factor 1-alpha; *rpb2*: DNA-directed RNA polymerase II second-largest subunit.

3.2.7 [15]. The analysis utilized a Markov Chain Monte Carlo approach with four chains, running for 1,000,000 generations and sampling every 1,000 generations. Bayesian consensus trees were generated after a burn-in period of 25%. The resulting tree was visualized using FigTree version 1.4.4 [16]. Bootstrap support values greater than 70% from the MP and ML analyses, along with posterior probabilities of 0.90 or higher from the BI analysis, were added to the corresponding branches and nodes of the tree to indicate the robustness of the branches. The six newly obtained isolates clustered together with *Ps. securinegae* (CBS 131930) and formed a distinct clade, separated from other *Pseudocercospora* species. This branch was strongly supported by high bootstrap values and posterior probabilities from the MP, ML, and BI analyses (100/100/1) (Fig. 2).

The resulting sequences were compared with reference sequences in GenBank using the BLASTn search. The ITS region sequences of all six isolates were identical and shared 99.56% similarity with *Ps. fuligena* (GU214675) and *Ps. chengtuensis* (GU214672). For the *actA* gene, the sequences from all six isolates were also identical and showed 100% identity with *Ps. securinegae* (GU320479). For the *tef1* gene, three isolates (KACC411234, KACC42527, and KACC411232) were identical and exhibited 99.68% similarity with *Ps. securinegae* (GU384487). The remaining three isolates (KACC411233, KACC43030, and KACC46414) differed by a single nucleotide substitution (adenine to thymine), resulting in 99.36% similarity with the same reference sequence. Due to the unavailability of *Ps. securinegae rpb2* sequence data in GenBank, one isolate (KACC411234) showed 98.06% similarity with *Ps. halleriae* (PP404492), whereas the remaining five isolates differed by one nucleotide (cytosine to thymine) and showed 97.89% similarity with *Ps. halleriae* (PP404492).

F. suffruticosa (Euphorbiaceae) is a shrub or small tree species native to Russia and East Asia, including China, Japan, Korea, and Mongolia [17]. In Korea, it commonly grows across the country along forest edges, valleys, and mountainous areas, and its young sprouts are edible, having been widely used as an indigenous vegetable [18–20]. Historically, *F. suffruticosa* has also been used in traditional medicine in East Asia, primarily for the treatment of infantile paralysis, neurasthenia, and facial paralysis [21]. Its chemical constituents include a variety of bioactive compounds, such as alkaloids, terpenoids, flavonoids, and phenols. Among these, Securinega alkaloids are particularly important natural products because of their pharmaceutical potential [22]. Leaf spot disease caused by *Ps. securinegae* can significantly affect plant growth, as premature defoliation reduces the photosynthetic capacity of infected plants, which, in turn, weakens the overall vigor and decreases the amount of harvestable plant material. Premature defoliation may also influence the accumulation of key bioactive compounds that are important for the medicinal use of this species.

Although plants belonging to the genus *Flueggea* are widely used in traditional medicine, little is known about the phytopathogenic fungi associated with this genus [23]. To date, only a few fungi have been reported on *F. suffruticosa*, including *Erysiphe securinegae* (powdery mildew) in China, Japan, Korea, and Russia; *Nothoravenelia japonica* (rust) in China and Korea; and *Ps. securinegae* (leaf spot) in China, Japan, and Korea [3,24–26]. The distribution of *F. suffruticosa*, which is native to East Asia, aligns with the reports of *Ps. securinegae*, which has been documented only in China, Japan, and Korea.

This study provides the first comprehensive description of the morphological characteristics of *Ps. securinegae* from Korean isolates and contributes additional molecular data for the species—*rpb2* gene sequences in particular, which were previously unavailable for this species. Given the medicinal importance of *F. suffruticosa* and its widespread distribution across the Korean Peninsula, our findings provide valuable insights into the identity and biology of this fungal pathogen.

CONFLICT OF INTEREST

No conflict of interest was reported by the authors.

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