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RESEARCH NOTE

# Morphology and Molecular Phylogeny of *Pseudocercospora rubigena* Associated with Leaf Spot on *Rubus corchorifolius* in Korea

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## ABSTRACT

*Pseudocercospora rubigena* is a recently introduced leaf spot pathogen on *Rubus* sp. based on single-strain identification. This study addressed the limitations of the initial description of *Ps. rubigena* by examining multiple isolates of *Rubus corchorifolius* in Korea, emphasizing its first confirmed occurrence on this host. Notably, shorter conidia with a narrow septation range are considered key characteristics that distinguish *Ps. rubigena* from related species. Sequences of the internal transcribed spacer (ITS) region, along with protein-coding genes such as partial actin (*actA*), partial translation elongation factor 1-alpha (*tef1*), and partial DNA-directed RNA polymerase II second-largest subunit (*rpb2*), were obtained from three Korean isolates. Later, these sequences were included in phylogenetic analyses using a multigene dataset of ITS, *act*, *tef1* and *rpb2*. The results showed that *Pseudocercospora* isolates from *R. corchorifolius* clustered in a single clade with *Ps. rubigena*, thus confirming the consistency between their morphological characteristics and those of the latter. This study clarifies the morphological and molecular phylogeny of *Ps. rubigena*, contributing to the taxonomy of *Pseudocercospora* species and providing insights into its host specificity and distribution.

**Keywords:** cercosporoid fungi, Mycosphaerellaceae, phytopathogen, *Pseudocercospora rubi*, Rosaceae

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The genus *Pseudocercospora* Speg. is a diverse group of fungi known to cause leaf spot diseases on a wide range of plants globally. Using new molecular phylogenetic approaches, this genus has undergone significant taxonomic reclassification, enhancing our understanding of its biodiversity. New species continue to be discovered, and previously uncertain species classifications are being resolved [1,2]. Among these, *Pseudocercospora rubi* (Sacc.) Deighton was first reported on *Rubus* sp. in the United States in 1984 [3] and has since been recognized as the major leaf spot pathogen affecting various *Rubus* L. species. The genus encompasses more than 30 plants species, of them *R. corchorifolius* L. f., *R. coreanus* Miq., and *R. pungens* var. *oldhamii* (Miq.) Maxim. (= *R. oldhamii* Miq.) have been listed as potential hosts for this fungus in Korea [4,5]. Its classification has long been based on morphological traits, such as branched mycelia, short conidiophores, and a high range of septation in conidia.

In addition to *Ps. rubi*, several other *Pseudocercospora* species infect *Rubus* plants, such as *Ps. heteromalla* (Syd.) Deighton in India, South Africa, China, and Iran [6–9]; *Ps. norchiensis* Crous in Iran and New Zealand [1,10]; *Ps. rubicola* (Thüm.) X.J. Liu & Y.L. Guo in China [11]; and *Ps. pancratii* (Ellis & Everh.) U. Braun & R.F. Castañeda in the United States [12]. Despite these findings, detailed morphological descriptions and molecular phylogenetic characterizations of some of the above-mentioned species remain incomplete, leaving gaps in our understanding of their classification and host ranges.

Recently, *Ps. rubigena* Crous, Y. Zhang ter. & Yuan Yuan Chen was described as a new pathogen on *Rubus* sp. from China [2]. Although it was phylogenetically distinct from the recorded sequences of other *Pseudocercospora* species, its morphological traits closely matched those of *Ps. rubi*, as both species share similar features. Furthermore, the conclusions in *Ps. rubigena* are constrained by the reliance on molecular phylogenetic results for a single strain. To address these gaps and clarify the species concept of *Pseudocercospora* on *Rubus corchorifolius* in Korea, multiple *Pseudocercospora* isolates from selected host plants collected across various regions were included in this study to confirm their identity and provide further insights into their morphological variability and distribution.

In total, eight samples of *R. corchorifolius* infected by *Pseudocercospora* leaf spots collected during our field forays and preserved in the Herbaria of Korea University (KUS) and Jeonbuk National University (JBNU) were involved in the present study (Table 1). Culture isolation and molecular analysis were performed using fresh specimens, which were later deposited in relevant databases.

The morphological characteristics of the fungus were observed using an Olympus BX50 microscope (Olympus, Tokyo, Japan). Photomicrographs were captured using a Zeiss AX10 microscope equipped with an AxioCam MRc5 camera (Carl Zeiss, Oberkochen, Germany). At least thirty measurements were performed for each diagnostic structure. To obtain monoconidial isolates, conidia collected from young lesions were placed in Eppendorf tubes containing sterile water. The prepared conidial suspension was then streaked onto 2% water agar (WA; Junsei, Tokyo, Japan) plates supplemented with 100 mg/L of streptomycin sulfate and incubated at 25℃. After 2 days, germinating conidia were transferred onto 2% potato dextrose agar (PDA; Difco, France) plates. The obtained colonies were deposited at the Korean Agricultural Culture Collection (KACC), Rural Development Administration, Korea (Table 1).

**Table 1.** Sources of Korean fungal specimens used in this study, with addition of accession numbers of obtained strains, and sequences deposited in the NCBI and KACC, respectively

| Voucher specimen number | Collection date | Collection place | KACC number | GenBank accession numbers | | | |
|---|---|---|---|---|---|---|---|
| | | | | ITS | *actA* | *tef1* | *rpb2* |
| KUS-F18082 | Nov 11, 2000 | Wando | - | - | - | - | - |
| KUS-F25065 | Jul 13, 2010 | Gimje | - | - | - | - | - |
| KUS-F28924 | Oct 13, 2015 | Naju | - | - | - | - | - |
| KUS-F28933 | Oct 13, 2015 | Naju | - | - | - | - | - |
| KUS-F30859 | Nov 15, 2018 | Mokpo | - | - | - | - | - |
| KUS-F32498 | Oct 4, 2021 | Gunsan | 411022 | PQ596155 | PV131708 | PV156644 | PV156647 |
| KUS-F32638 | Nov 15, 2021 | Buan | - | - | - | - | - |
| KUS-F33151 | Sep 1, 2022 | Wanju | 410454 | PQ596154 | PV131709 | PV156645 | PV156648 |
| JBNU-F0274 | Nov 2, 2023 | Wanju | 411010 | PQ596167 | PV126031 | PV156643 | PV156646 |

NCBI: National Center for Biotechnology Information; 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.

Leaf spots were amphigenous, irregular to angular, 3–6 mm in diameter, vein-limited, with a light creamy to khaki center, surrounded by a dark brown margin (Fig. 1A–B). Caespituli were amphigenous but primarily epiphyllous, fasciculate to solitary. Stromata were well-developed, composed of several swollen hyphal cells, pale to light-brown, erumpent, and up to 50 μm in diameter (Fig. 1C–D). Mycelium was internal, consisting of branched, septate, brownish hyphae. Conidiophores were numerous, forming dense fascicles, 25–57 × 3–6 μm, brown to pale brown, smooth-walled, subcylindrical, straight or slightly curved, and 1–3-septate. Conidiogenous cells were unbranched, pale to medium brown, 10–21 × 3–4 μm, with inconspicuous scars (Fig. 1E). Conidia were solitary, hyaline, narrowly obclavate to subcylindrical, straight or slightly flexuous, 1–6-septate, with an obtuse apex and truncate base, 47–80 × 3–4 μm, and featuring unthickened, non-darkened hila (Fig. 1F–G). One-week-old colonies grown on PDA at 25℃ were 7–10 mm in diameter, khaki in appearance, undulate, with a clear margin, and developed moderate aerial mycelium (Fig. 1H). The morphological characteristics of our isolates were consistent with those of *Ps. rubigena* [2].

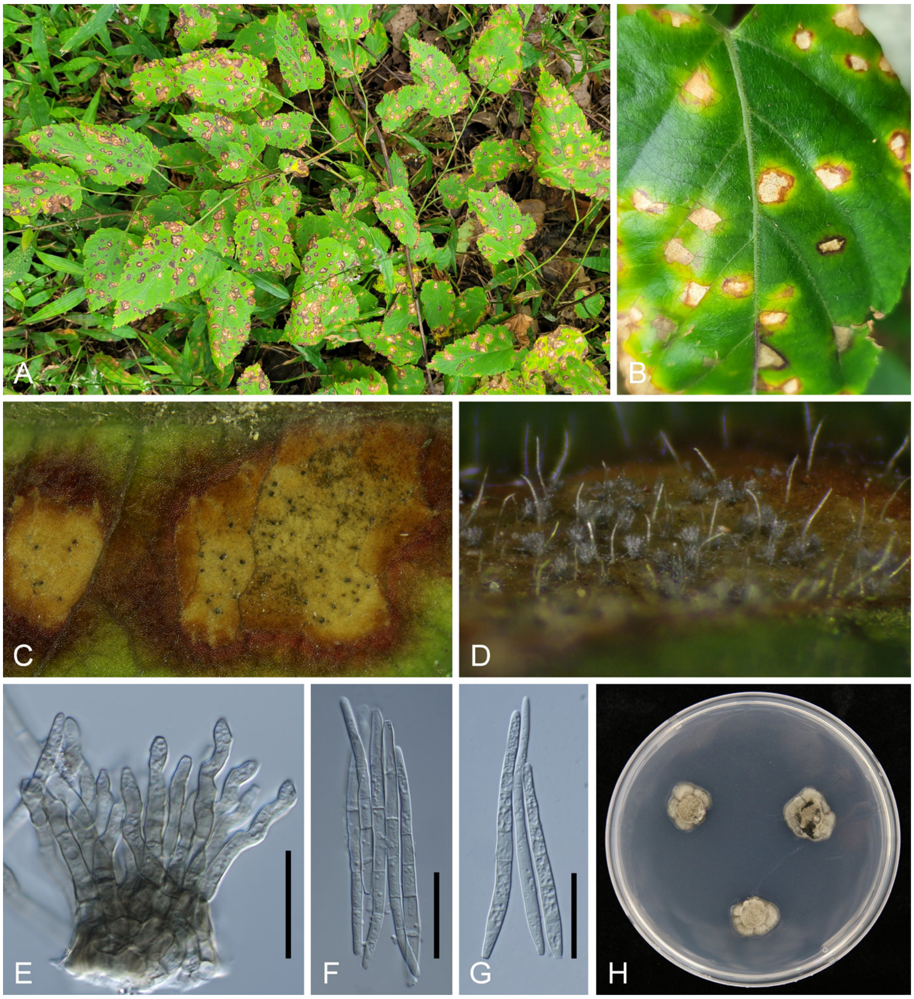

**Fig. 1.** Leaf spot disease caused by *Pseudocercospora rubigena* on *Rubus corchorifolius*. (A) Symptoms on infected plants in the field. (B) Close-up of leaf spots. (C) Lesions with distinct brown margins and abundant caespituli in the creamy center. (D) Heavy fructification showing greyish conidial masses. (E) Conidiophores. (F, G) Conidia. (H) One-week-old colony of *Ps. rubigena* grown on potato dextrose agar at 25℃. Scale bar indicates 20 μm.

To confirm the morphology-based identification, DNA was extracted from three two-week-old colonies grown on PDA to perform molecular phylogenetic analyses. Nucleotide sequences of the internal transcribed spacer (ITS) region, along with protein-coding genes such as partial actin (*actA*), partial translation elongation factor 1-alpha (*tef1*), and partial DNA-directed RNA polymerase II second-largest subunit (*rpb2*), were determined using the primer pairs V9G/ITS4, ACT-512F/ACT-783R, EF1-728F/EF1-986R, and P_RPB2-F/P_RPB2-R, respectively [13,14]. Assembled sequences were submitted to GenBank (Table 1). For phylogenetic analyses, a data matrix was generated for each gene individually and aligned using the MUSCLE algorithm in MEGA 11 [15]. All four datasets were then concatenated into a single multigene dataset in the order ITS, *act*, *tef1* and *rpb2* using SequenceMatrix [16]. *Trochophora simplex* (CBS 214744) and *Pallidocercospora heimioides* (CBS 111190) were selected as outgroups [13]. Maximum parsimony (MP) analysis was performed in PAUP* 4.0a using a heuristic search with the tree-bisection algorithm [17], while maximum likelihood (ML) analysis was conducted in raxmlGUI 2.0.13 based on the GTR substitution model with GAMMA distribution [18]. Tree robustness was assessed via 1,000 bootstrap replications. The final alignment included 31 sequences and 1,912 characters, of which 206 (10.77%) were variable and parsimony-uninformative, while 535 (29.55%) were informative for parsimony analysis.

BLASTn search results showed 99.85% similarity in the ITS region with *Pseudocercospora* sp. on *Syzygium cordatum* (KX287305), differing by a single base (adenine in all three Korean isolates *vs*. thymine in the reference strain). The *actA* and *rpb2* sequences were 100% identical to *Ps. rubigena* (PP404605), while *tef1* showed 99% identity with *Ps. rubigena* (PP404923). In the phylogenetic tree, all three Korean isolates clustered in a distinct, highly supported branch (100/100 BS values) with *Ps. rubigena* (Fig. 2). The phylogenetic separation of *Ps. rubi*, *Ps. rubigena*, *Ps. norchiensis*, and *Ps. pancratii* into distinct clades indicates genetic divergence among *Rubus* leaf spot pathogens, suggesting adaptation to specific environments and hosts.

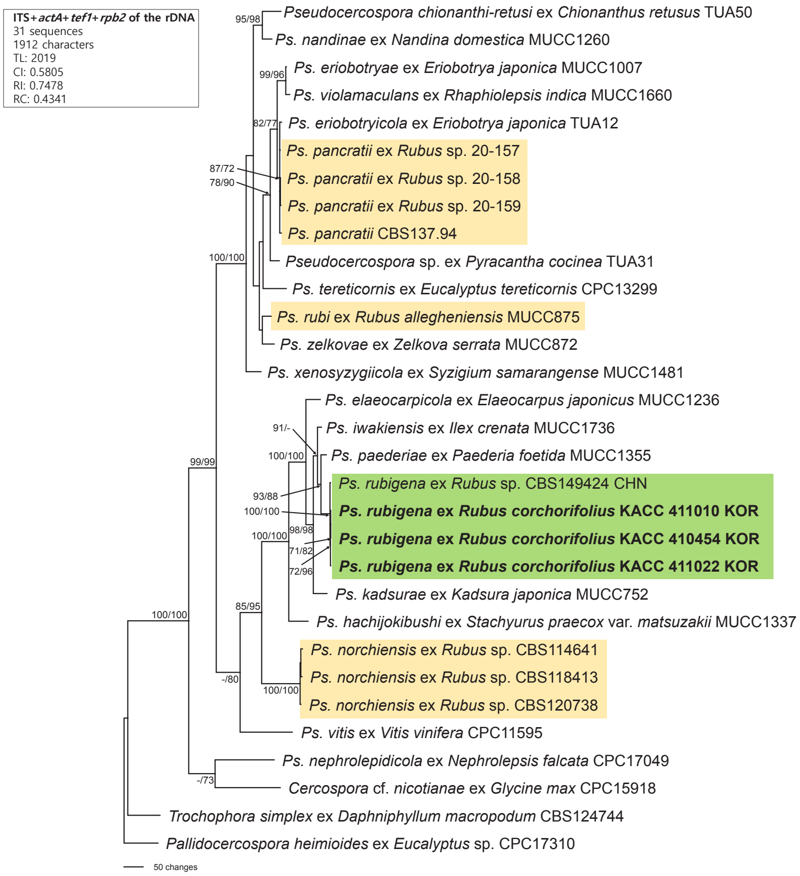

**Fig. 2.** Phylogenetic tree of *Pseudocercospora rubigena* on *Rubus corchorifolius* constructed using maximum parsimony (MP) based on a combined multigene dataset (ITS + *actA* + *tef1* + *rpb2*) comprising 31 sequences. Isolates obtained in this study are shown in bold. Bootstrap values (> 70%) from MP and maximum likelihood (ML) analyses are indicated on corresponding branches, respectively. Tree scores, such as tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC), are provided in the box on the left. ITS: internal transcribed spacer; *actA*: actin; *tef1*: translation elongation factor 1-alpha; *rpb2*: DNA-directed RNA polymerase II second-largest subunit.

The morphology of Korean specimens on *R. corchorifolius* was compared with *Ps. rubigena* and *Ps. rubi* previously reported in Korea (Table 2). Conidia of *Ps. rubi* are longer (up to 132 μm), with more septa (3–11), and conidiophores are narrower (up to 4 μm) than those of *Ps. rubigena* and the Korean isolates. However, under *in vitro* conditions, conidia of *Ps. rubigena* have been measured up to 123.5 μm in length, with (3–)5–10 septa [2], closely resembling the characteristics of *Ps. rubi* reported in Korea. Owing to the lack of reliable data, colony morphology comparisons between *Ps. rubigena* and *Ps. rubi* were not provided. Nevertheless, these findings support the conclusion that the previously reported *Ps. rubi* on *R. corchorifolius* in Korea is actually *Ps. rubigena*. Separately, ITS, LSU, *rpb2*, *actA*, and *tef1* sequences obtained from MUCC 875 on *Rubus allegheniensis* in Japan have been deposited in GenBank. However, owing to the absence of molecular-phylogenetic confirmation of *Ps. rubi* in Korea, further studies using updated molecular-phylogenetic approaches are required to verify its presence.

**Table 2.** Morphological comparison of Korean isolates with *Pseudocercospora rubigena* and *Ps. rubi*

| Fungus | Leaf spot | Stroma | Conidiophore | Conidium |  |  | Reference |
|---|---|---|---|---|---|---|---|
|  |  |  |  | Size | Septum | Shape |  |
| Korean isolates | irregular to angular<br>3–6 mm<br>amphigenous | 50 µm | 25–57 × 3–6 µm | 47–80 × 3–4 µm<br>hyaline | 1–6 | narrowly obclavate to subcylindrical |  |
| *Ps. rubigena* | irregular to angular<br>3–5 mm<br>amphigenous | 50 µm | 30–50 × 4–6 µm | (30–)45 –60(–70) × (3.5–) 4 µm<br>medium brown | 1–4 | subcylindrical | [ 2] |
| *Ps. rubi* | subcircular to irregular<br>2–10 mm<br>amphigenous | 20–60 µm | 13–50 × 2.5–4.0 µm | 30–132 × 2.5–4.0 µm<br>subhyaline to very pale olivaceous brown | 3–11 | obclavate to obclavate-cylindric | [ 5] |

The genus *Rubus* (Rosaceae; Rosoideae) encompasses approximately 700 species distributed across temperate and subtropical regions, including various shrubs, subshrubs, and herbs [19]. Many are economically important plants, commonly known as brambles, raspberries, or blackberries. *R. corchorifolius* is native to East Asia, including China, Korea, and Japan [20]. The phylogenetic and geographic range of *R. corchorifolius* closely aligns with the distribution of *Ps. rubigena*, first reported on *Rubus* sp. in China [2] and now confirmed in Korea. This overlap suggests a potential evolutionary relationship between host and pathogen, supporting the hypothesis that *Ps. rubigena* is a distinct species adapted to specific *Rubus* hosts.

This study reports a new finding of *Ps. rubigena* in Korea and identifies a new host record for this species globally. The findings provide strong evidence for the identification of *Ps. rubigena*, and the observed morphological variations may reflect environmental influences. Consequently, this work contributes to a deeper understanding of the taxonomy, phylogeny, and host range of *Ps. rubigena* for future research.

## CONFLICT OF INTERESTS

The authors declare no competing interests.

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