

Identification and Characterization of *Pseudocercospora pyricola* Causing Leaf Spots on *Aronia melanocarpa*

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Abstract Leaf spot disease on black chokeberry (*Aronia melanocarpa*) was observed at several locations in Korea during 2014–2015. Leaf spots were distinct, scattered over the leaf surface and along the leaf border, subcircular to irregular and brown surrounded by a distinct dark color, and were expanded and coalesced into irregularly shaped lesions. Severely infected leaves became dry and fell off eventually. The causative agent was identified as *Pseudocercospora pyricola*. Morphological observations and phylogenetic analyses of multiple genes, including internal transcribed spacer, translation elongation factor 1-alpha, actin, and the large subunit ribosomal DNA were conducted. The pathogenicity test was conducted twice yielding similar results, fulfilling Koch's postulates. To our knowledge, this is the first report on *P. pyricola* infection of *A. melanocarpa* globally.

Keywords *Aronia melanocarpa*, Multigene phylogenetic analysis, *Mycosphaerella*, *Pseudocercospora* leaf spot, *Pseudocercospora pyricola*

Aronia melanocarpa (Michx.) Elliott (black chokeberry), belonging to the family Rosaceae, is cultivated worldwide and is well known for its flavoursome juice with high levels of anthocyanins and flavonoids [1]. In recent years, black chokeberry has become a commercially popular crop in Korea used for producing juices, jams, and wines [2]. Recently, the cultivation area of black chokeberry as an alternative crop to blueberries (*Vaccinium* spp.) has increased in Korea (1,269 ha in 2015) (Rural Development Administration, unpublished). Several fungal pathogens have been known to be associated with leaf spot diseases of black chokeberry around the world, including *Cercospora*

pyri (USA), *Cercospora pirina* (Canada, USA), *Cercospora pyri* (Canada, USA), and *Mycosphaerella arbutifoliae* (USA) [3]. However, in Korea, black chokeberry has only been described in association with leaf spots caused by *Alternaria alternata*, *Alternaria mali*, and *Alternaria tenuissima* [4–6].

The genus *Pseudocercospora* is a well-known anamorph of *Mycosphaerella*, and its species are regarded as major pathogens of a wide variety of plants. *Pseudocercospora* can cause leaf spots, fruit spots, fruit rot, and blight disease, and are mostly found in tropical regions [7, 8]. The classification of *Pseudocercospora* is mainly based on a combination of characteristics such as morphological characteristics, host specificity, and molecular analyses. Morphological characteristics, which are presently acknowledged important in separating these genera include the structures of mycelia (presence or absence of superficial mycelium, and texture thereof), conidiophores (arrangement, branching, pigmentation), conidiogenous cells (placement, proliferation, scar type), and conidia (formation, shape, septation, and pigmentation) [9]. Molecular techniques are commonly used to overcome taxonomic problems posed by the limitation of morphological characteristics or in cases where morphological characteristics are in conflict, ambiguous, or missing [10]. Nuclear ribosomal DNA and internal transcribed spacer (ITS)-5.8S gene regions have been commonly used for inferring phylogenetic relationships for *Pseudocercospora* species [11]. Although the ITS region has been used as a

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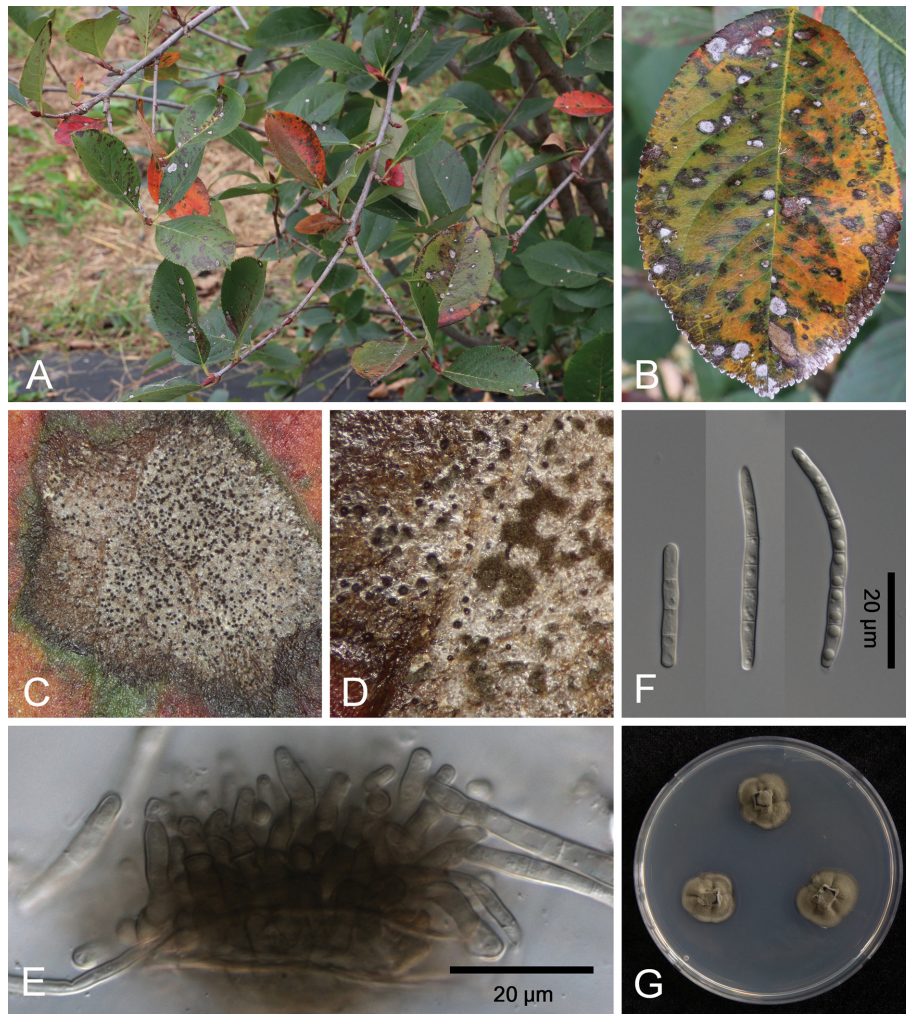


Fig. 1. *Pseudocercospora* leaf spots caused by *Pseudocercospora pyricola* on *Aronia melanocarpa* 'Nero'. A, Infected leaves decreasing vigor of the plants; B, Close-up view of leaf spots; C, D, Fructification of the fungus on the lesion, and grayish patches shown on the lesion due to heavy fructification; E, Conidiophores; F, Conidia; G, One-week-old colonies of *P. pyricola* growing on a potato dextrose agar plate (scale bars: E, F = 20 μ m).

universal DNA barcoding marker for fungi [12], its limited resolution has made it difficult to distinguish between different *Pseudocercospora* species [13]. Recently, the phylogeny of several *Pseudocercospora* species was evaluated using large subunit ribosomal DNA (LSU) and protein-coding genes, such as translation elongation factor 1-alpha (EF-1 α) and actin (ACT) [13]. This demonstrated that multi-gene analyses provide a more robust identification of *Pseudocercospora* species and, with a few exceptions, most of the *Pseudocercospora* species appear to be host-specific.

Leaf spot symptoms were observed on black chokeberry ('Nero') in the Jeollabuk-do areas, including Gochang, Sunchang, and Iksan, in Korea from July to October in 2014 and 2015. Black chokeberry shrubs exhibited leaf spots (approximately 70% disease incidence) with symptoms progressing from older to newer leaves. Initially, symptoms on leaves included distinct sub-circular to irregularly shaped brown spots surrounded by distinct dark, brownish-red

haloes, 2 to 5 mm in diameter, scattered over the surface and along the leaf border. Over time, these spots expanded and coalesced into irregularly shaped lesions. Severely infected leaves dried and eventually fell off (Fig. 1A and 1B). Evidence of *Pseudocercospora* fungus was consistently observed in tandem with disease symptoms. As *Pseudocercospora* leaf spot disease on black chokeberry has not been previously recorded, we have identified and characterized the causal agent based on morphological characteristics and molecular analyses.

Morphological characteristics of *Pseudocercospora pyricola*.

Fungal cells from fresh samples were mounted on a glass slide with a drop of water, and their structures were examined using bright-field and differential interference-contrast light microscopy. For measurements, an Olympus BX51 microscope (Olympus, Tokyo, Japan) and for imaging, a Zeiss AX10 microscope equipped with an AxioCam

MRC5 (Carl Zeiss, Göttingen, Germany) were used. Thirty measurements were taken under 100× and 1,000× magnification for each sample.

The representative specimen was submitted to the Korea University Herbarium (KUS) under accession number KUS-F27688. To obtain a pure isolate, spores were collected from infected leaf tissues by using a sterile needle under a dissecting microscope and were placed on a drop of sterile water on a glass slide. A conidial suspension was streaked onto 2% water agar plates supplemented with 100 mg/L of streptomycin sulfate, and the plates were incubated at 25°C for 4 days. Suitable germinated conidia were transferred to potato dextrose agar (PDA) plates using a sterile needle under a dissecting microscope. Two monoconidial isolates were deposited in the Korean Agricultural Culture Collection, Rural Development Administration, Wanju, Korea (accession Nos. KACC47656 and KACC48077).

After 7 days of incubation at 25°C on PDA plates in darkness, erumpent fungal colonies were formed with moderate aerial mycelia and smooth, lobate margins. The surface appearance was pale olivaceous-grey; reverse iron-grey (Fig. 1G). Stromata were not present or were weakly developed, subimmersed, globular, grayish dark brown, 20–100 µm, and visible as circular patches (Fig. 1C and 1D). Conidiophores were fasciculate, brown to pale brown in color, straight to curved, smooth, unbranched, apex rounded to truncate, 15–35 × 3–4 µm, and aseptate to septate (Fig. 1E). Conidiogenous cells were integrated, terminal, unbranched, brown to pale brown in color, smooth, and proliferating sympodially. Conidia were solitary, gray to subhyaline, guttulate, obconically truncate at the base, obtuse at the apex, 3- to 6-septate, non-constricted at the septa, 25–60 × 2.5–3.5 µm, and had un-thickened and not darkened hila (Fig. 1F). These morphological characteristics were close to those of *Pseudocercospora pyricola* (Sawada) J. M. Yen [14] and *Pseudocercospora photiniae* (Fukui) C. Nakash. & Tak. Kobay. [15], which have been recorded on several rosaceous trees and share common features with respect to conidiophores and conidia, suggesting that they might be synonymous. Since the former species is an older name, we concluded that the Korean isolate should be identified as *P. pyricola* (Sawada) J. M. Yen [14].

Phylogenetic analysis of *P. pyricola*. Genomic DNA was extracted from harvested mycelia from the cultures grown on PDA (KACC47656) using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). Four nuclear gene regions were targeted for PCR amplification and subsequent sequencing. Primers employed for the amplification of EF-1α included EF1-728F [16] and EF-2 [17], while ACT-512F [16] and ACT-2Rd [13] were used to amplify a portion of the ACT gene. The complete ITS region of ribosomal DNA was amplified using primers ITS-4 and ITS-5 [18], while primers used for amplification of the 28S LSU region were LSU1Fd [19] and LR5 [20]. All PCR reaction mixtures and conditions followed those outlined by Hunter *et al.* [21].

The amplified PCR products were separated on a 1.5% agarose gel, followed by purification with a PCR purification kit (Core-one; Core-Bio, Seoul, Korea). Both amplicon strands were sequenced using the same primers that were used for the initial amplification. The reactions were monitored using BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) as indicated by the manufacturer and were analyzed on an ABI 3130 automated DNA sequencer (Applied Biosystems). The possible identity of the isolates was established by comparing their ITS, LSU, EF-1α, and ACT sequences with those in the GenBank database (National Center for Biotechnology Information [NCBI], US National Institute of Health, Bethesda, MD, USA; <http://www.ncbi.nlm.nih.gov/BLAST>). Selected *Pseudocercospora* sequences, including EF-1α, ACT, ITS, and LSU, were retrieved from GenBank for the phylogenetic analysis. These retrieved sequences were included in the *Pseudocercospora* phylogenetic tree constructed by Crous *et al.* [13]. The obtained sequences were edited and assembled using the SeqMan software (Lasergene; DNASTAR, Madison, WI, USA). A neighbor-joining phylogenetic tree was constructed using the maximum composite likelihood method by MEGA6 [22].

The representative and resulting 490-bp EF-1α, 578-bp ACT, 525-bp ITS, and 861-bp LSU sequences obtained from KACC47656 were deposited in GenBank (accession Nos. KY048164, KY048162, KY048161, and KY048163). A BLAST search in GenBank using the ITS sequence revealed that the sequence showed over 99% identity with several sequences of *Pseudocercospora* species, including *P. paraquayensis*, *P. neriicola*, *P. lilacis*, and *P. rhododendrigena*. However, the EF1-α sequences varied between these species, even though they had the same ITS, ACT, and LSU sequences. The phylogenetic tree, created using a dataset of four combined sequences, translation elongation factor 1α (TEF-1α), ACT, ITS, and LSU, showed that *P. pyricola* from black chokeberry was included in a well-supported clade consisting of *Pseudocercospora* spp. (bootstrap value of 100%) that was distinct from other genera, such as *Passalora*, *Pallidocercospora*, *Pseudocercosporella*, *Septoria*, and *Cercospora* (Fig. 2).

Pathogenicity test. Tests in accordance with Koch's postulates were carried out to establish the pathogenicity of the isolated fungus. A conidial suspension with mycelial fragments (approximately 1 × 10⁴ propagules/mL) of isolate KACC47656 was prepared using a 7-day-old culture grown on V8 juice agar by flooding the plates with sterilized water. The mixed suspension was sprayed onto the young leaves of five healthy 2-year-old plants ('Nero') until it began to run off. Five control plants were sprayed with sterilized water. Treated and control plants were individually covered with polythene bags to maintain 100% relative humidity for 24 hr and then were transferred to separate compartments and maintained at 28 ± 2°C with high (> 80%) relative humidity in a greenhouse. Typical leaf spots were observed

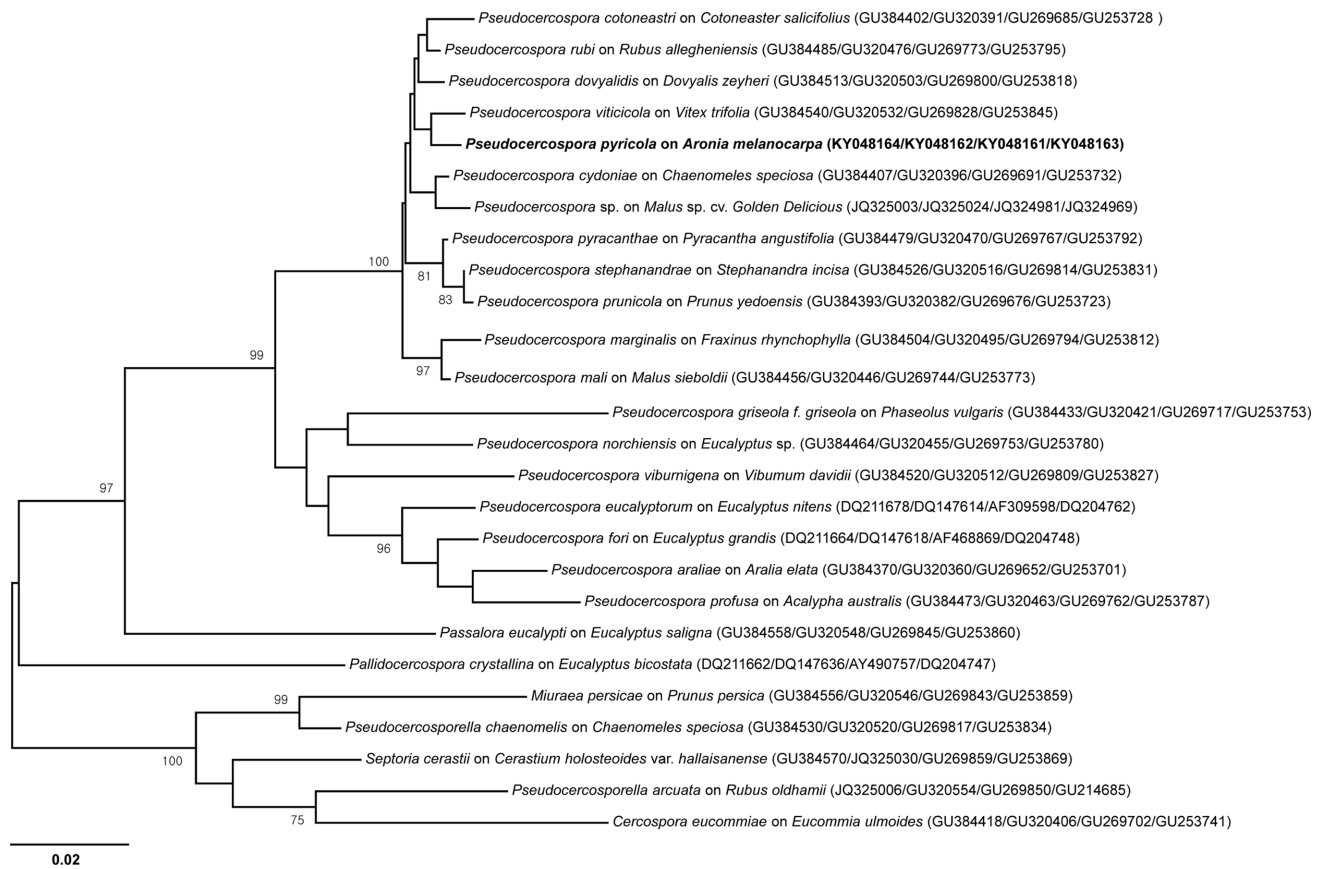


Fig. 2. Neighbor-joining tree of *Pseudocercospora pyricola* based on the multigenic dataset of translation elongation factor 1-alpha (EF-1 α), actin (ACT), internal transcribed spacer (ITS), and large subunit ribosomal DNA (LSU). In the phylogenetic tree, the numbers (bootstrap values) are above or under the nodes obtained from 1,000 replicates. The GenBank numbers are represented in the order of EF-1 α , ACT, ITS, and LSU, respectively. The isolate obtained in this study is highlighted in bold.

on the inoculated plants after 14 days of incubation, and were identical to those observed in the field. No symptoms were observed on the control plants. The fungus that was re-isolated from the lesions of the inoculated plants was identical to the original isolate and confirmed as *P. pyricola*. The pathogenicity tests were repeated twice with similar results, fulfilling Koch's postulates.

Identification and discussion. Leaf spots caused by *P. pyricola* have previously been reported on several species of *Pyrus* (Rosaceae), such as *P. communis* (China), *P. lindleyi* (China), *P. serotina* (Taiwan), *P. serrulata* (China), and *Pyrus* sp. (China) [3]. However, there have been no previous reports of infection on *Aronia melanocarpa*. Furthermore, these reports relied mainly on a combination of morphological characteristics and host specificity without any studies of molecular characteristics.

To date, *Cercospora* leaf spots which have been reported on black chokeberry worldwide have been known to be caused by *Cercospora pyri* (USA), *Cercosporiella pirina* (Canada, USA), and *Cercosporiella pyri* (Canada, USA) [3]. However, morphological and molecular characteristics of the fungus isolated from black chokeberry were not consistent

with the species of *Cercospora* and *Cercosporiella*. As shown above, this fungus is morphologically close to *P. pyricola* and *P. photiniae*. Although we concluded that this fungus should be identified as *P. pyricola* over *P. photiniae* based on morphological characteristics and taxonomic priority, further studies on the complexity of *Pseudocercospora* spp. on rosaceous host plants should be conducted.

To our knowledge, this is the first report on *P. pyricola* infection of black chokeberry. Since the sequences of *P. pyricola* and *P. photiniae* have never appeared in phylogenetic studies, this study provides a good platform for future studies on the taxonomy of *Pseudocercospora* and further investigations into *Pseudocercospora*-black chokeberry associations. Our observations in several orchards showed that *Pseudocercospora* leaf spots expanded rapidly on young leaves and detracted from the yield value of fruit.

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