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SHORT COMMUNICATION

Pseudocercospora leaf spot caused by *Pseudocercospora nymphaeacea* on *Nymphaea tetragona*

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Abstract Pygmy waterlily (*Nymphaea tetragona*, Nymphaeaceae) is a perennial aquatic herb with floating leaves and beautiful flowers. Leaf spot on pygmy waterlilies appeared in 2009 at several locations in Korea, e.g., Suwon in 2009, Yangpyeong in 2011, and Seoul in 2014. The leaf spots were circular to irregular, small, and brown in the center with a dark brown margin. The spots later became milky white. The causative agent of the leaf spot was identified as *Pseudocercospora nymphaeacea*. Morphological observations and multigene phylogenetic analyses of the internal transcribed spacer (ITS), the partial translation elongation factor 1-alpha (TEF-1α), and actin (ACT) regions are provided. The pathogenicity test was conducted twice with similar results, which fulfilled Koch's postulates. To our knowledge, this is the first report on *P. nymphaeacea* infection of *N. tetragona.*

Keywords Multigene phylogenetic analysis · *Mycosphaerella* · Pathogenicity · Pygmy waterlily

Pygmy waterlily (*Nymphaea tetragona* Georgi, Nymphaeaceae) is a perennial aquatic herb with floating leaves and beautiful flowers. This species has a broad distribution, and it is found in temperate and tropical Asia, Europe, northern America, and boreal regions of the Northern Hemisphere (Hu et al. 2009; Tandon et al. 2010). It is commonly planted in ponds and water gardens for ornamental purposes, and it has become commercially important in the aquaria industry. It is also used as a water-purifying plant because it can clean polluted water (Lu and Chen 2012; Peng et al. 2011). Although it has ornamental value and good purification abilities, this species is considered as rare and endangered in Meghalaya, India, because human activities have destroyed its natural habitats (Tandon et al. 2010).

The genus *Pseudocercospora* includes several major pathogens of a wide variety of plants. They cause leaf spot, fruit spot, fruit rot, and blight disease, and are mostly found in tropical regions (Chupp 1954; Crous et al. 2000). The taxonomy of *Pseudocercospora* species has generally been based on morphological characteristics, host specificity and molecular analyses. Several studies have used the internal transcribed spacer regions (ITS) of rDNA sequences for *Pseudocercospora* species (Crous et al. 2000; 2001). Although the ITS region has been used as a universal DNA barcoding marker for fungi (Schoch et al. 2012), its limited resolution has made it difficult to distinguish between different *Pseudocercospora* species (Crous et al. 2013). In a recent study, Crous et al. (2013) provided a broad framework and phylogeny for *Pseudocercospora*, by analyzing two additional genomic protein-coding genes, namely the partial gene regions of the translation elongation factor 1-alpha (TEF-1α) and actin (ACT). Their study showed that multigene analyses provided a more robust identification of them and most of the *Pseudocercospora* species appeared to be host-specific with a few, rare exceptions.

Extensive survey of cercosporoid fungi in South Korea revealed that leaf spot on pygmy waterlilies in several localities, e.g.,: Suwon in 2009, Yangpyeong in 2011, and Seoul in

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2014. These were circular to irregular, small, brown in the center with a dark brown margin and turned milky white with age. The leaf spots sometimes coalesced to blight the entire leaf in late September (Fig. 1a, b). Morphological observations and phylogenetic analyses were performed to clarify the causal agent of leaf spot on pygmy waterlilies.

Slides containing fresh fungal structures were mounted in water and observed with an Olympus BX51 microscope (Olympus). A Zeiss AX10 microscope equipped with an AxioCam MRc5 (Carl Zeiss) was used for imaging. Thirty measurements for each structure were taken at 100× to 1000× magnification. Three representative specimens were deposited in the Korea University Herbarium (KUS-F24349, F25686, and F28087).

To obtain a pure isolate, infected leaf tissue that showed abundant sporulation was excised and then placed in a drop of sterilized water on a glass slide using forceps. A loop of conidial suspension was streaked on the surface of 2 % water agar plates supplemented with 100 mg/L streptomycin sulfate using a disposable bacterial loop. After 2 days of incubation at 25 °C, a single conidial colony was transferred to potato dextrose agar (PDA) with a sterile needle under a dissecting microscope. Three monoconidial isolates were deposited in the Korean Agricultural Culture Collection, Rural Development Administration, Wanju, South Korea (KACC44783, KACC46113, and KACC47784).

Fructification of the fungus was mostly epiphyllous, grayish brown, effuse, and visible as circular patches (Fig. 1c). Stromata were small, rudimentary to slightly developed in the substomatal cavities, and occasionally erumpent through the cuticle. Conidiophores were fasciculate, olivaceous to pale brown, darker than conidia, geniculate to sinuous, 20–54×3–4 μm, and aseptate to 2-septate (Fig. 1d, e). Conidiogenous loci were inconspicuous, neither thickened nor darkened. Conidia were olivaceous to pale brown, cylindrical to obclavate, almost straight to mildly curved, obconically truncate at the base, obtuse at the apex, 3- to 8-septate, non-constricted at the septa, 40–230×2.5–4 μm, and had unthickened and not

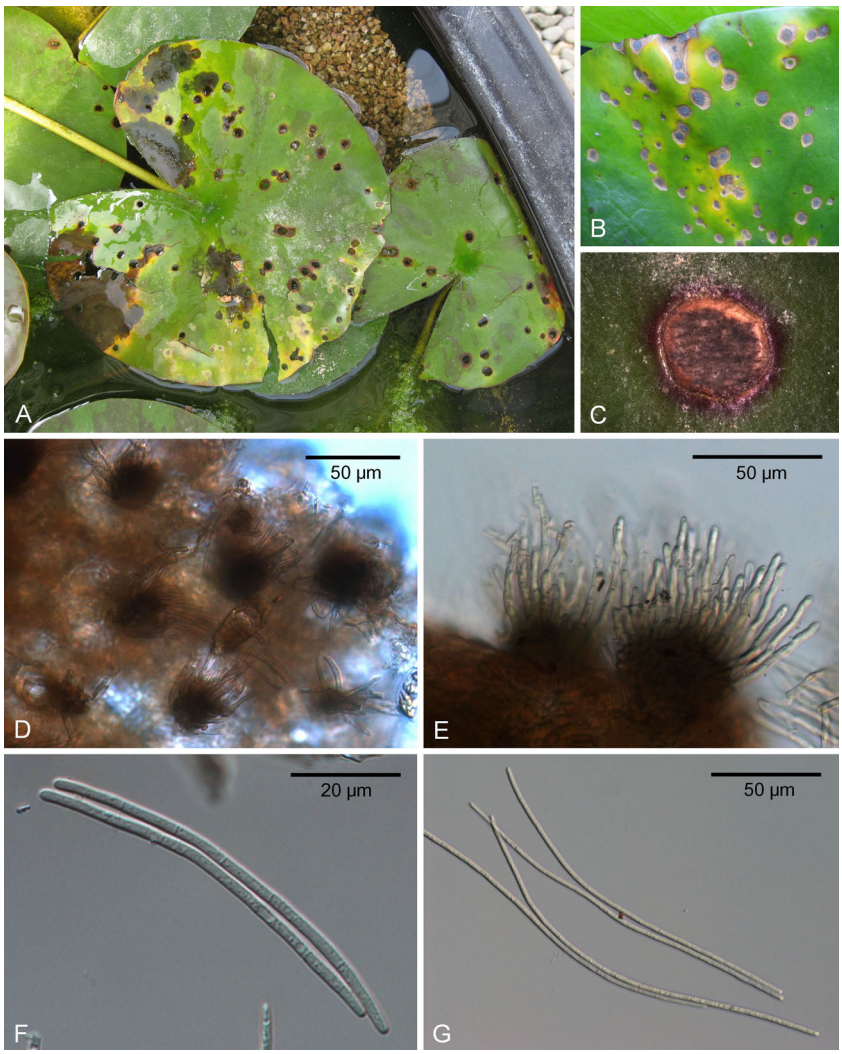

Fig. 1 *Pseudocercospora* leaf spot caused by *P. nymphaeacea* on *N. tetragona*. **a**. Infected leaves decrease the aesthetic value of the plant; **b**. Close-up view of leaf spots; **c**. Fructification of the fungus on the lesion; **d**–**e**. Conidiophores; **f**–**g**. Conidia

darkened hila (Fig. 1f, g). The morphological characteristics of the fungus were consistent with previous descriptions of *Pseudocercospora nymphaeacea* (Cooke & Ellis) Deighton (Chupp 1954; Guo and Hsieh 1995).

Genomic DNA was extracted from mycelia harvested from PDA grown cultures (KACC44783, KACC46113, and KACC47784) using a DNeasy Plant Mini Kit (Qiagen Inc.). Three targeted regions were chosen for multigene analysis. The ITS region was amplified using the primers pairs ITS1/ITS4 (White et al. 1990), the primers pair EF1-728F/EF1-968R were used to amplify the TEF-1α region, while ACT-512F/ACT-738R were used to amplify the ACT region (Carbone and Kohn 1999). The PCR products were visualized on 1.5 % agarose gels and then purified using a QIAquick PCR purification kit (Qiagen Inc.) following the manufacturer's protocols. Sequencing was carried out by the Macrogen Sequencing Service (Macrogen) with the same primers used for the PCR amplifications.

Both the ITS and ACT sequences obtained from the three isolates were identical. In the case of TEF-1α, the sequences were identical except for one isolate (KACC46113, 2-bp difference). Representative resulting 504-bp ITS, 314-bp TEF-1α, and 220-bp ACT sequences obtained from KACC47784 were deposited in GenBank (KT074354, KT074355, and KT074353, respectively). A BLAST search in GenBank using the ITS sequence revealed that the sequence showed 100 % identity with several sequences of *Pseudocercospora* species, including *P. marginalis*, *P. cercidis-chinensis*, and *P. chionanthi-retusi*. However, the ACT and TEF-1α sequences differed from these species, although having equivalent ITS sequences.

Selected *Pseudocercospora* sequences, including ITS, TEF-1α, and ACT, were retrieved from GenBank for the phylogenetic analysis. These sequences were included in the *Pseudocercospora* phylogenetic tree constructed by Crous et al. (2013) and were edited and assembled using the SeqMan software (Lasergene). A neighbor-joining phylogenetic tree was constructed using the maximum composite likelihood method in MEGA (Molecular Evolutionary Genetics Analysis) v.6.0 (Tamura et al. 2013). The phylogenetic tree, created using a combined sequence ITS, TEF-1α, and ACT dataset, showed that *P. nymphaeacea* was separate from other *Pseudocercospora* spp., including *P. marginalis*, *P. cercidis-chinensis*, and *P. chionanthi-retusi* (Fig. 2).

For the pathogenicity test, a mixed suspension of conidia and mycelial fragments (approximately 1×10^4 propagules/mL) was prepared in sterilized water from 2 weeks-old cultures that had been grown on V8 juice-agar. The suspension was sprayed onto the young leaves of five healthy plants until it began to runoff. The control plants were sprayed with sterilized water. Leaf spots appeared on the inoculated leaves 12 days after inoculation, and were identical to those observed in the field. *Pseudocercospora nymphaeacea* was re-isolated from lesions, fulfilling Koch's postulates. Control plants remained symptomless. The pathogenicity test was conducted twice with similar results.

The fungus had previously been recorded on several genera belonging to the Nymphaeaceae, e.g.,: *Nelumbo*., *Nymphaea*, *Nymphoides*, and *Victoria* (Chupp 1954; Farr and Rossman 2015). The occurrence of *Pseudocercospora* on species of *Nymphaea* has been reported in Brazil, Colombia, Dominican Republic, India, Jamaica, Kenya, Mauritius, Panama, Papua New Guinea, Thailand and the United States

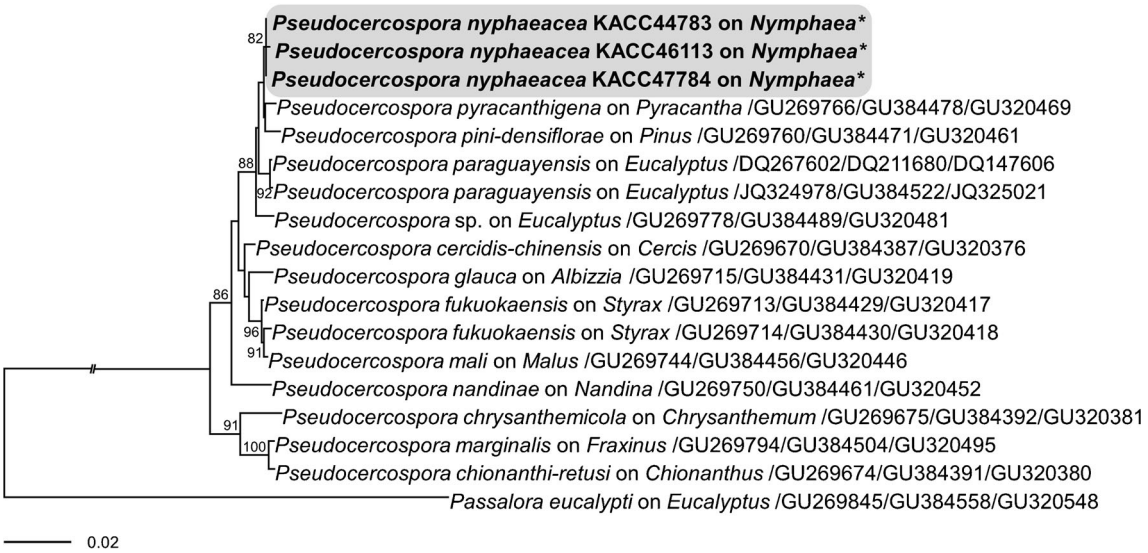

Fig. 2 Neighbor-joining tree of *Pseudocercospora nymphaeacea* based on the multigene dataset (ITS, TEF-1α, and ACT). The numbers above the nodes are the bootstrap values obtained from 1000 replicates. GenBank accession numbers are represented in the order of ITS, TEF-1α, and ACT. Isolates obtained from this study are indicated by an asterisk and highlighted in gray

(Farr and Rossman 2015), but this is the first report of *P. nymphaeacea* on *N. tetragona*.

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