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Identification of powdery mildew on *Prunus rufoides* in China, caused by *Podosphaera prunigena*

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Summary. *Prunus rufoides* is a deciduous wild tree, native to China, which is also used as an ornamental. From late March to December in the years 2018 to 2023, *P. rufoides* plants growing in Siming Mountain (29°71'08"N, 121°15'12"E), Ningbo, Zhejiang Province, were severely affected by powdery mildew. The disease initially emerged in late March each year, and was characterized as white, irregular mycelial patches on the adaxial surfaces of young leaves. Between July and August, the powdery mildew colonies on affected parts of leaves disappeared, leaving only irregular yellow-brown spots. The disease recurred in September and persisted until late December. Chasmothecia containing asci and ascospores were observed on the leaves in December. Morphological analyses of the chasmothecia indicated a *Podosphaera* sp. as the causative agent. Molecular identification, based on the internal transcribed spacer (ITS) region (primers ITS4/ITS5) confirmed the identity of the pathogen as *Podosphaera prunigena*. Koch's postulates were fulfilled by inoculation tests in which the same pathogen was identified from the inoculated leaf tissue. This study represents the first report confirming the powdery mildew disease on *P. rufoides* in China to be caused by *P. prunigena*.

Keywords. *Erysiphaceae*, morphology, molecular analysis, taxonomy.

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INTRODUCTION

Cerasus is classified as a subgenus of *Prunus* L. (*Rosaceae*), and is primarily distributed in tropical and subtropical regions (Willis, 1985; Mabberley, 1997). Most species within this subgenus are important for their ornamental and economic values, and these plants include *P. avium* (L.)L. (sweet cherry), *P. pseudocerasus* Lindl. (Chinese cherry), *P. serrulata* Lindl. (Japanese cherry), and *P. cerasoides* Buch.-Ham. ex D.Don (Lee and Wen, 2001). *Prunus rufoides* C.K.Schneid., originally described from China, is widely distributed and is an important flowering tree species in early spring (Wang, 2014; Zhu *et al.*, 2018).

Powdery mildews are widespread, common, and detrimental diseases on plant species, including *Prunus* spp., which can lead to substantial economic losses (Frye and Innes, 1998). Some species of *Podosphaera* are known to cause diseases on *Rosaceae* hosts. Notably, *Po. clandestina*, *Po. leucotricha*, and *Po. pannosa*, are important and frequently occurring species that cause powdery mildews on cultivated plants (Braun and Cook, 2012; Hubert *et al.,* 2012; Farr and Rossman, 2019). Among *Prunus* spp. hosts, *Po. tridactyla* (*s. lat*.) has been identified as the most common species causing powdery mildew (Braun and Cook, 2012; Meeboon *et al.,* 2015). In a recent morpho-phylogenetic analysis of *Po. tridactyla s. lat*., Meeboon *et al.* (2020) proposed dividing this organism into ten species, comprising seven new species and three previously recognized species. These authors identified *Po. prunigena* as one of the seven new species. *Podosphaera prunigena*, an Asian species on hosts of *Prunus* sub gen. *Cerasus*, is distinct from *Po. tridactyla s. str.* in morphology and phylogenetic characteristics. This pathogen has been reported on three *Prunus* host species including *P. apetala*, *P. leveilleana*, and *P. serulata* (Meeboon *et al.,* 2020). Shirouzu *et al.* (2020) reported *Po. prunigena* on *Cerasus kumanoensis* in Japan, and amended the circumscription of this powdery mildew species, including detailed description of the anamorph.

From 2018 to 2023, symptoms of powdery mildews were observed on *P. rufoides* trees in Siming Mountain, Ningbo, China. The disease predominantly affected 1-year-old plants in a nursery, with more than 80% of cherry plants infected by this fungus. To identify the pathogen, cherry leaf samples were collected and examined in a laboratory. Morphological, molecular, and pathogenicity tests were carried out, to provide information for development of effective disease management strategies.

MATERIALS AND METHODS

Plant material and pathogen Isolation

The infected leaf samples were deposited in the Herbarium of Ningbo City College of Vocational Technology, under the accession number NBCC2018016. Morphological analysis of the pathogen was carried out using the procedure outlined by Meeboon and Takamatsu, 2020). Mycelium and chasmothecia were taken from infected leaf surfaces using a clean needle and mounted on microscope slides. All samples were examined in water. Fifty conidia, chasmothecia, ascospores, and asci were measured, and photographs were taken using a light microscope (Leica DM3000), and dimensions of the fungal structures were measured.

Pathogenicity tests

Twenty 1-year-old healthy *P. rufoides* plants were each inoculated with a 1×10^8 mL^{-1} suspension of conidia. Ten healthy plants were treated with sterile distilled water served as negative experimental controls. The *P. rufoides* plants were grown in a greenhouse chamber for 1 year using the shoot cutting method. Prior to fungal inoculations, plant leaves were washed in sterilized water to remove adhering soil and were then air-dried. Inoculated plants were maintained in a greenhouse chamber at 25°C with a 12 h light 12 h dark daily cycle. Different treatment groups were separated to prevent cross-contamination. Symptoms were observed 9 to 10 d post-inoculation. Disease symptom observations, and morphological and molecular analyses of the fungus, were carried out as described above. All plant disease characteristics on inoculated leaves were compared with those of the initially affected greenhouse plants.

Pathogen DNA extraction, amplification, and sequencing

The genomic DNA was extracted separately from mycelium and chasmothecia, using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle, 1991). DNA concentrations were measured using a NanoDrop 2000 OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific). Polymerase chain reaction (PCR) and DNA sequencing were carried out as follows: DNA was diluted to a concentration of 100 ng μL^{-1} in water. Each 50 µL PCR reaction contained 2.5 µL of template DNA, 2.5 µL of each forward and reverse primer, 25 µL of Taq buffer, 5 µL of DNA polymerase, and 12.5 µL of dd H_2O (Li *et al.,* 2021). The primer pairs used were ITS4 and ITS5 (White *et al.,* 1990; Kusaba and Tsuge, 1995). The PCR amplification process included an initial denaturation at 95°C for 5 min, followed by 34 cycles each of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, a final extension at 72°C for 10 min, and a 4°C hold. The PCR products were visualized through gel electrophoresis on a 1.5% agarose gel with a TAE running buffer containing 4S Green Plus Nucleic Acid Stain (Sangon Biotech Co., Ltd.). The results were captured using a gel documentation system equipped with a digital camera (UVP GelDoc-It2 Image System). The expected bands were excised and purified using the SanPrep Column DNA Gel Extraction Kit (B518131-0050, Sangon Biotech Co., Ltd.,), following the manufacturer's instructions. The samples were subsequently sent for sequencing to Tsingke Biotechnology Co., Ltd. The obtained sequences were initially analyzed using NCBI-BLAST-Nucleotide BLAST, available at https://blast.ncbi.nlm.

Table 1. Specimen and accession numbers of sequences used for phylogenetic analyses.

Podosphaera spp. and vouchers	GenBank accession number of ITS
Podosphaera longiseta	AB000945.1
Podosphaera pruni-avium voucher 47899	MK530456.1
Podosphaera pruni-cerasoidis voucher 6861	MK530447.1
Podosphaera prunigena isolate WZL09	MT820800
Podosphaera prunigena TNS: F-89227	LC529409.1
Podosphaera prunigena	AB026147.1
Podosphaera prunigena voucher 5266	MK530446.1
Podosphaera pruni-japonicae	AB000943.1
Podosphaera pruni-lusitanicae voucher VPRI 22158	AY833655.1
Podosphaera prunina KUSF26292	JQ517296.1
Podosphaera prunina voucher 5209	MK530442.1
Podosphaera salatai	AB525929.1
Podosphaera sibirica MUMH303	AB026144.1
Podosphaera tridactyla	AF011318.1
Podosphaera tridactyla voucher 65909	MK530463.1
Podosphaera xanthii voucher MUMH68	D84387.1

nih.gov/Blast.cgi. The sequences generated from the ITS region were aligned with other *Podosphaera* sequences using ClustalW (Larkin *et al.,* 2007). The sequence data were deposited in GenBank, and the accession numbers were obtained from https://www.ncbi.nlm.nih.gov/genbank/. Phylogenetic analyses were conducted on the data using the Maximum likelihood method. The sequences were first aligned using the Clustal W method, and then the phylogenetic analysis was carried out in MEGA7, with a bootstrap value of 1000 (Kumar *et al.,* 2016).

RESULTS

Disease symptoms and morphological characterization

From 2018 to 2023, the *P. rufoides* plants growing in Siming Mountain exhibited severe powdery mildew symptoms. The disease began in late March to early April each year, mainly affecting the young cherry blossom plants, including leaves, petioles, and young shoots, especially the tender leaves which were most susceptible to the disease (Figure 1, A, B and C).The affected areas initially had chlorotic spots with indistinct margins, which gradually expanded, and white powdery mildew colonies developed on these spots (Figure 1 D). In severe cases, these patches merged, and caused defoliation. Between July and August, the white powdery mildew colonies on the affected parts disappeared, leaving irregular yellow-brown leaf spots (Figure 1 E). In September, white powdery mildew colonies re-appeared on the diseased leaves, and persisted until December. By the end of December, small brown dots were visible on the leaf spots, and were identified as the teleomorph of the pathogen (chasmothecia). These were accompanied

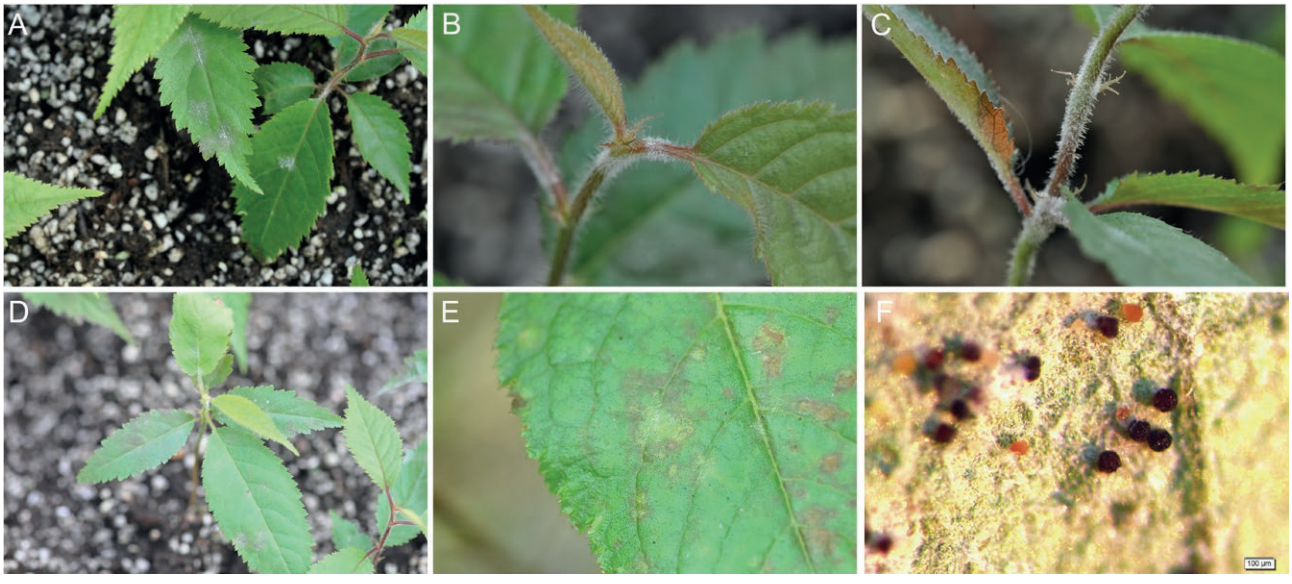

Figure 1. A, B and C. Disease symptoms on *Prunus rufoides* leaves, petioles and young shoots infected by *Podosphaera prunigena*. D. The affected areas show chlorotic spots with indistinct margins at edges the initial infection stage. E. Irregular yellow-brown spots on a leaf between July and August. F. Chasmothecia on the fallen cherry blossom leaf.

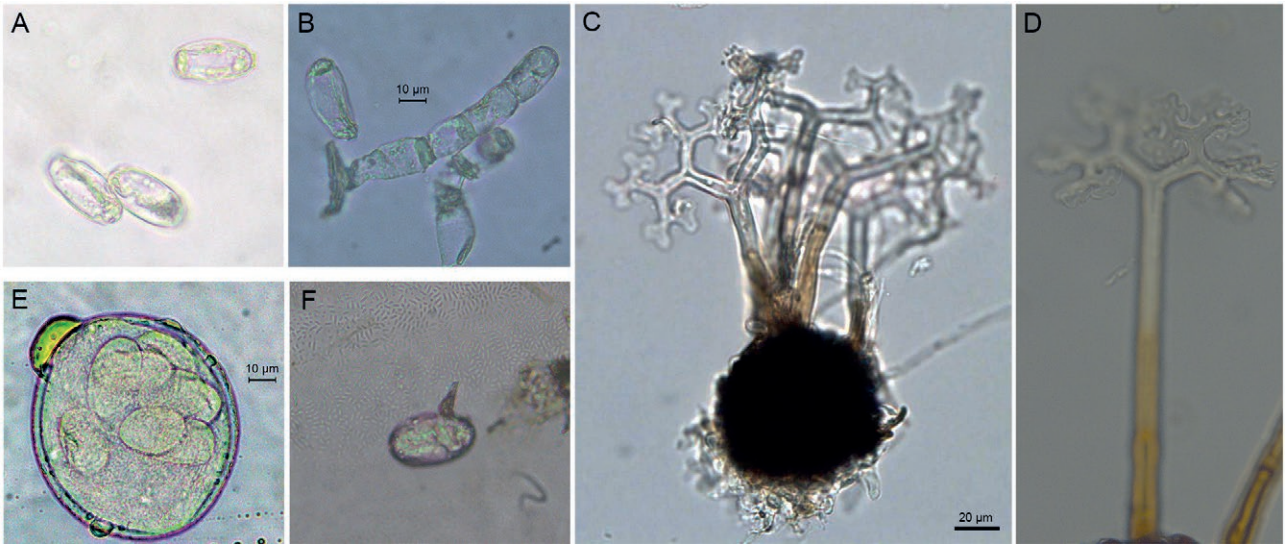

Figure 2. Morphology of *Podosphaera prunigena*, isolate WZL09. A, conidia. B, conidiophore with foot-cell. C, chasmothecium. D, chasmothecium appendage. E, ascus. F, ascospore with germ tube. Scale bars: A, B, E, and F = 10 µm; C and D = 20 µm.

by re-emergence of white mycelium until December, when the chasmothecia on fallen host leaves became mature, forming asci and ascospores (Figure 1 F). Leaves from diseased plants tended to detach earlier than those from healthy plants. The disease predominantly affected 1-year-old plants in a nursery, with more than 80% of the cherry plants infected by the fungus.

A voucher specimen affected by the pathogen was deposited at the in School Landscape Ecology of City College of Vocational Technology·Utilization of Ningbo (Accession WZL09).

To identify the causal agent of this disease, morphological examination was conducted on 20 infected leaves. The hyphae and conidia were hyaline. The foot cells of the conidiophores were straight, cylindrical, and measured 28–62 × 7–10 µm (n = 50) (Figure 2 B). The conidia were ellipsoid to ovate, measuring 20–32 × 14–21 µm (n = 50) (Figure 2 A). The conidia were produced in chains and contained well-developed fibrosin bodies. Conidium germ tubes were simple and lateral (Figure 2 F). Chasmothecia were each globose or depressed globose, with a diameters of 60–110 µm (average 81.9 µm; n = 100) (Figure 2 C), and had the chasmothecia had 2 to 5 appendages in the upper halves, and these were gregarious, straight or somewhat curved, were of dimensions 90–180 × 8–10 µm (n = 50), and each appendage had a 1–4 septate thick-walled stalk, with septae throughout or only in the lower half. The stalks were brown in the lower parts, and apices that were 3 to 5 times regularly and dichotomously branched. Tips of the ultimate branchlets were knob-like, and not recurved (Figure 2 D). Each ascus was subglobose to broad ellipsoid-ovoid, measuring 58–94 × 50–83 µm (n = 40), with a thick wall (4–5 µm), and contained eight ascospores (Figure 2 E). The ascospores were ellipsoid-ovoid to subglobose, 16–25 × 12–18 µm (n = 50), and colourless.

The morphological characteristics of the pathogen suggested it was a species of *Podosphaera* sect. *Podosphaera* subsect. *Tridactyla* [*Podosphaera tridactyla* complex] (Braun and Cook, 2012), and molecular identification was performed to determine the species of the pathogen.

Pathogenicity testing

All inoculated leaves developed powdery mildew within 9 to 10 d post-inoculation, while the control plants remained asymptomatic (Figures. 3, A and B). Specimens from the inoculated plants showed that the fungus on the infected leaves was identical to that originally observed on the naturally infected plants. Molecular analyses confirmed the identity of the fungus with that of the inoculated fungus. These results fulfilled Koch's postulates for the inoculated pathogen.

Phylogenetic analysis

To confirm the morphological identification of the pathogen, genomic DNA from mycelium and chasmothecia was extracted independently, and the internal transcribed spacer (ITS) region along with a portion

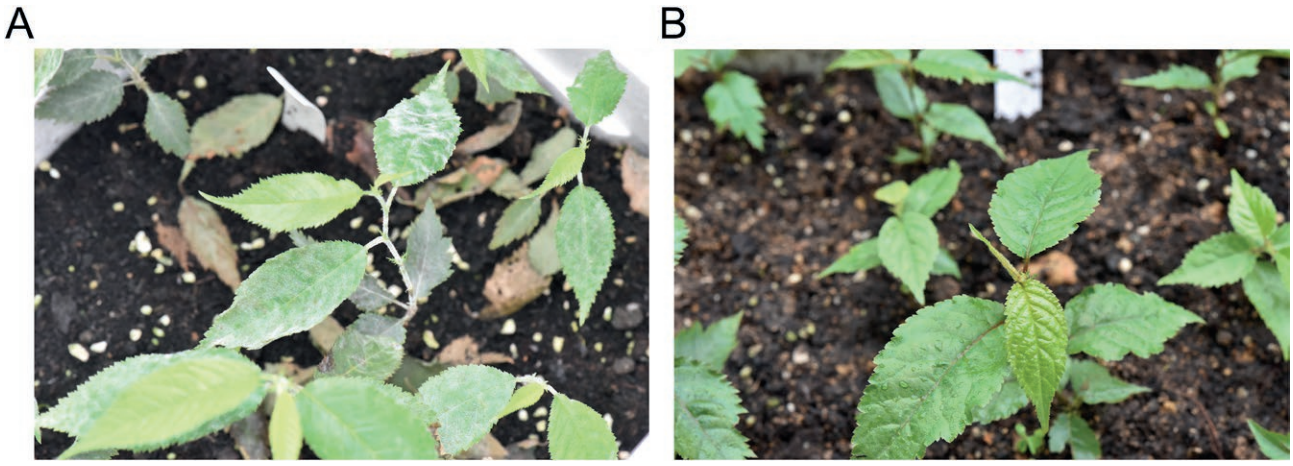

Figure 3. Pathogenicity test. Koch's postulates were fulfilled after inoculating 20 *Prunus rufoides* plants with powdery mildew (A) or with sterilized water (B). These photographs were taken at 10 d post inoculation.

of the 28S gene were amplified using polymerase chain reaction (PCR) and sequenced. The results indicated that the ITS sequences from mycelium and chasmothecia were identical, confirming that the anamorph and teleomorph were one species. The ITS sequence was deposited in GenBank (accession number MT820800). BLAST analysis showed that the ITS sequence had 99% similarity with *Po. prunigena* voucher 5266 (1166/1168, 99%, MK530446). To further confirm this phylogeny, ITS sequences from 15 other *Podosphaera* strains were retrieved from the NCBI database. A phylogenetic tree was constructed using bootstrap analysis with 1,000 replicates in MEGA 7. The other *Podosphaera* strains used in the phylogenetic analysis are listed in Table 1.

The fungus formed a strongly supported clade with *Po. prunigena* (GenBank: AB026147, LC529409 and MK530446) (Figure 4). Two additional strains, *Po. xanthii* and *Po. sibirica* MUMH303, were used as outgroups. In conjunction with the morphological analyses described above, it was concluded that the powdery mildew found in China on *Prunus rufoides* was caused by *Po. prunigena*.

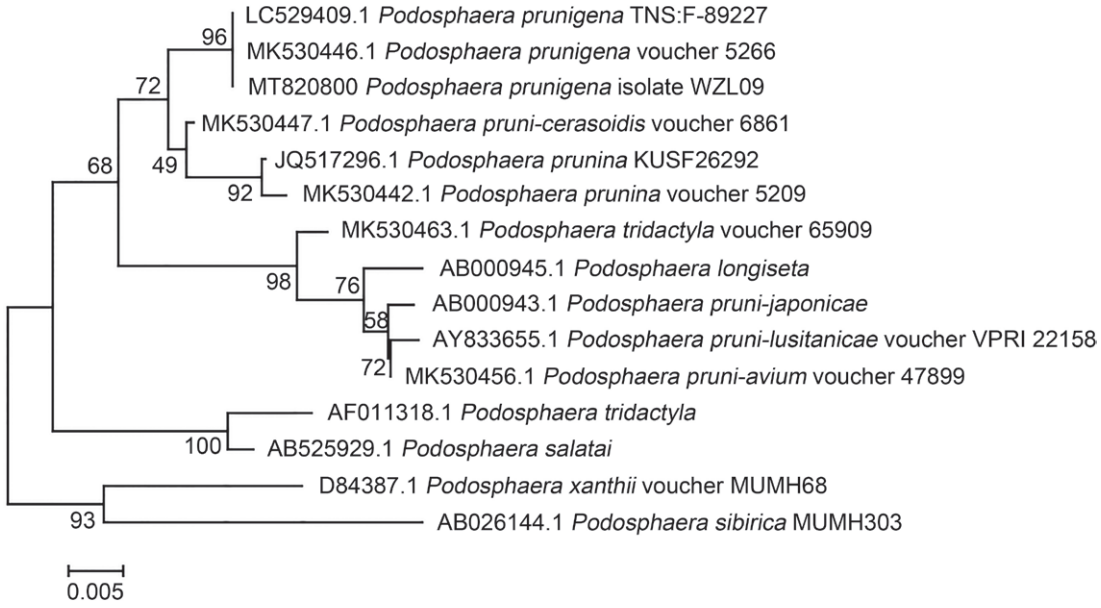

Figure 4. This phylogenetic tree was constructed in MEGA 7. These results inferred from the ITS sequences positioned the sequence obtained from powdery mildew on *Prunus rufoides* in China within the *Podosphaera prunigena* clade.

Table 2. Characteristics of five *Podosphaera* strains.

Name	Conidia	Foot-cells	Chasmothecia	Appendages	Ascus	Ascospores	Host	References
Podosphaera prunigena WZL09	20 to 32 × 14 to 21 µm	28 to 62 × 7 to 10 µm	60 to 110 µm	2 to 5, 90–180 × 8–10 µm	58–94 × 50–83 µm	16–25 × 12–18 µm	*Prunus rufoides*	The present study
Podosphaera prunigena Meeboon, S. Takam. & U. Braun, sp. nov.	-	-	100 to 130 µm	3 to 5, 85–205× 8–11.5 µm	87.5–100 × 73–78 µm	30–35 × 17–25 µm	*Prunus apetala*	Meeboon *et al.* (2020)
Podosphaera prunigena	-	-	75 to 95 µm	–	65–81 × 50–69 µm	18.5–27 × 10.5–12 µm	*Cerasus kumanoensis*	Shirouzu *et al.* (2020)
Podosphaera tridactyla voucher 65909 (= *Po. prunina*)	20 to 32 × 14 to 21 µm	28 to 62 × 7 to 10 µm	65 to 112 µm	2 to 4, –	66–86 × 47–76 µm	15–27 × 12–18 µm	*Prunus hypoleuca*	Bai *et al.* (2015); Meeboon *et al.* (2020)
Podosphaera wuyishanensis Z.X. Chen et Yao	-	-	80 to 110 µm	1 to 2, × 7.5–9 µm	75–97.5 × 67.5–90 µm	20–24 × 32.5–12.5 µm	*Prunus fukienensis* Yu et Ku	Chen and Yao, (1989)

DISCUSSION

Powdery mildews can cause widespread and severe diseases of wild and cultivated plants, and can reduce host growth and cause economic losses. Several studies have shown the pathogenicity of powdery mildews on *Prunus* spp. (Chen and Yao, 1989; Schulze-Lefert and Vogel, 2000; Bai *et al.,* 2015). In the present study, the powdery mildew WZL09 was examined, which caused severe disease on *P. rufoides* plants, primarily affecting 1-year-old cherry plants in a nursery in China. Based on morphology of the pathogen and results of sequence analyses, the causative agent of this powdery was identified as *Podosphaera prunigena*. This is the first unequivocal record of this species from China, with *P. rufoides* as a new host. The retrieved sequence clustered within the *Po. prunigena* clade. Morphology of chasmothecia found on *P. rufoides* agree with the original description of this species by Meeboon *et al.* (2020), except that the pathogen examined here had much smaller chasmothecia, which were described by Meeboon *et al.* (2020) to be 100 to 130 µm in diameter. However, Shirouzu *et al.* (2020) published an emended description for *Po. prunigena*, with chasmothecia of diameter 75 to 95 µm, which is similar to dimensions for the Chinese specimens. The amended description of *Po. prunigena* by Shirouzu *et al.* (2020) was based on a re-examination of type material of this species, and measurements of Japanese material found on *Cerasus kumanoensis*. This showed the size of the chasmothecia was incorrect in the Meeboon *et al.* (2020) description.

Podosphaera tridactyla and *Po. wuyishanensis* are in the *Po. tridactyla* complex that has also been reported on *Prunus* spp. in China. As shown in Table 1, a sequence obtained from a Chinese specimen of *Po. tridactyla s. lat.* on *Prunus hypoleuca* reported by Bai *et al.* (2015) was included the phylogenetic taxonomic revision of the *Po. tridactyla* complex, and was shown to belong to *Po. prunina*. Other older records of *Po. tridactyla s. lat.* cannot be compared with *Po. prunigena* since they refer to the heterogenous *Po. tridactyla* complex which has been divided into numerous species. Sequences for *Po. wuyishanensis* are not yet available in GenBank, but this species is unique and morphologically clearly distinguished from all other species of the *Po. tridactyla* complex by having only 1-2(-3) terminal cleithothecial appendages.

Flowering cherries, including *P. rufoides*, are popular ornamental plants, that have aesthetically beautiful flowers in early spring. The powdery mildew caused by *Po. prunigena* can infect cherry leaves from early spring to late autumn. This disease leads to early host defoliation and affects the growth and ornamental value of cherry plants, resulting in substantial losses for farmers and the industries involved. The present study has identified the pathogen causing powdery mildew on *P. rufoides*, which is information to support evaluation of appropriate methods to control this disease.

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