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NOTE

The first report of brown root rot disease caused by *Phellinus noxius* on *Reevesia formosana* which raises concerns on the conservation of *R. formosana* and *Formotosena seebohmi* (Hemiptera: Cicadoidea) in Taiwan

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ABSTRACT: Brown root rot disease on *Reevesia formosana* was firstly found in Chiayi County, Taiwan. The infected trees with foliage wilting, leaf discoloration, brown mycelial mat on root and basal stem, and brown mycelial networks in rotten diseased wood were observed. The infected trees wilted eventually. *Phellinus noxius* was identified as the causal pathogen by the symptoms and signs shown on the trees. Also, morphological and molecular identification of the fungal colony isolated from the diseased tissue was carried out. *P. noxius* was inoculated to 6-year-old *R. formosana* seedlings with *P. noxius* grown on the oat grain medium in the greenhouse. Inoculated seedlings were infected. The symptoms and signs shown on the seedlings were the same as in the field. The fungus was then re-isolated from the diseased tissue. This is the first report of *P. noxius* on the host *R. formosana* in Taiwan. Since *R. formosana* is the main food source of *Formotosena seebohmi*, the outbreak of brown root rot disease on *R. formosana* deteriorates the habitat of *F. seebohmi* and may threaten the population of *F. seebohmi* when no proper mitigation is applied.

KEY WORDS: Brown root rot disease, conservation, *Formotosena seebohmi*, *Phellinus noxius*, *Reevesia formosana*.

INTRODUCTION

Brown root rot disease caused by *Phellinus noxius* (Coner) Cunningham is one of the most common and serious tree diseases in Taiwan. It causes the death of trees and is commonly distributed in Taiwan with a wide host range (Chang *et al.*, 1999). It is also the most frequently reported tree disease via Forest Disease Information Center. There were 1282 out of 3087 (42%) and 1351 out of 3138 (43%) reported cases diagnosed as brown root rot disease in 2020 and 2021 (Forest Disease Information Center, 2022).

Reevesia formosana Sprague, commonly named Taiwan Reevesia, is in the family of Malvaceae which is a deciduous tree. It is one of the endemic, native, and rare tree species in Taiwan and is mainly distributed in the lower altitude of southern Taiwan (Li and Lo, 1993). There are 15 *Reevesia* spp. distributed in China and only one in Taiwan (Wu *et al.*, 2007). In 2017, it was officially listed in 'the Red List of Vascular Plant of Taiwan' and labeled as near threatened (NT) (Editorial Committee of the Red List of Taiwan Plants, 2017).

Moreover, there is a close relationship between *R. formosana* and *Formotosena seebohmi* (Distant, 1904). *F. seebohmi*, commonly named formosan giant cicada or seebom's giant cicada, is one of the endemic insects in Taiwan. It is also the largest cicada in Taiwan. According to the 'Wildlife Conservation Act Republic of China', *F. seebohmi* is listed in the category of 'Rare and Valuable Species'. Both commercial hunting and the development of lower-altitude forests have threatened its population (Shao, 2006). There were repeated records showed that the distribution of *F. seebohmi* was scattered and overlapped with *R. formosana*, for example, 3 hot spots in Mt. Kantou, Mt. Shihe (Tainan City), Caoshan (Chiayi County) (Fung, 2019). *F. seebohmi*, as an insect in Cicadidae, follows the life cycle of cicadas which hatches and burrows into the soil to suck the tree sap from the tree's root for growth, escapes from the soil at the final underground nymphs stage, metamorphoses into winged adult and forages on the tree trunk (Williams and Simon, 1995). Wang *et al.* (2020) reported that *F. seebohmi* in Chiayi mountain area did not randomly rest on different kinds of tree trunks. There was the highest count of *F. seebohmi* resting and foraging on *R. formosana*. They preferred resting on the tree trunks of *R. formosana.* Song *et al.* (2020) reported that *F. seebohmi* in the upstream watershed of Tsengwen Reservoir closely interacted with *R. formosana* throughout the cicada life cycle. Behaviors, such as resting, foraging, and egg laying on *R. formosana* were recorded as the highest count, which showed a strong preference towards *R. formosana*.

MATERIALS AND METHODS

Wood samples were collected from the root and butt of the diseased *Reevesia formosana* in Caoshan, Fanlu Township, Chiayi County in October 2021. The samples were then rinsed with tap water to remove soil on the surface. The samples were cut into small pieces (3 × 3 ×

3 mm^3) for surface sterilization. They were first soaked in 75% ethanol for 30 s, followed by 10% household bleach (5% NaOCl) for 1 min and blotted dry on a sterilized paper towel. After that, the samples were soaked in sterilized water and blotted dry again. The samples after surface sterilization were cultivated in the selective medium for *Phellinus noxius* (20 g/L malt extract, 20 g/L agar, 10 mg/L benomyl, 10 mg/L dicloran, 100 mg/L ampicillin, and 500 mg/L gallic acid) (Chang, 1995). The samples were cultured in the dark at 25℃ for 3 days. The colony grown was sub-cultured on MEA (malt extract agar) for further observation under a light microscope.

For molecular identification, 5 mg of isolated culture was used for DNA extraction. DNA extraction machine, LabAssist-32(64), with the program, L-BNA-PK-AUTO, and extraction kit, TANBead® Nucleic Acid Extraction Kit Fungal DNA Auto Plate (Taiwan Advanced Nanotech Inc.) were used according to the manufacturer's instructions. 24 µl of PCR reaction mixture containing 2.5 µl extracted DNA, 12.5 µl 2X Taq Master Mix (20 mM KCl, 4 mM $MgSO_4 \cdot 7H_2O$, pH8.8 40 mM tris-HCl, 0.2 % Triton X-100, 20 mM $(NH_4)_2SO_4$, 0.2 mg/ml bovine serum albumin (BSA), 0.4 mM dNTP mix, 100 U/ml Taq polymerase and stabilizers) (Genomics Bioscience and Technology Co., Ltd.), 0.5 µl of 10 µM specific primer G1-F (5' GCCCTTTCCTCCGCTTATTG 3') and 0.5 µl of 10 µM specific primer G1-R (5' CTTGATGCTG GTGGGTCTCT 3') (Wu *et al*., 2009) and 8 µl dd H_2O was used for PCR. PCR was run as an initial denaturation at 94 °C for 2 min, then at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 40 s by 30 cycles and a final extension at 72 °C for 5 min on a thermal cycler (Cole-Parmer Inc.). PCR products were checked on agarose gel containing 1.4% agarose and 0.5X Tris–acetate-EDTA (TAE) and stained with 5 µl/100 ml Healthview™ nucleic acid stain (Genomics Bioscience and Technology Co., Ltd.) under BluView Transilluminator (Major Science Co., Ltd). The extracted DNA was also undergone PCR by the above protocol but using 0.5 µl of 10 µM primer ITS 4 (5' TCCTCCGCT TATTGATAT 3') and 0.5 µl of 10 µM primer ITS 5 (5' CCTTCATTTTCAGCATTGTTCC 3') (White *et al*., 1990) instead. The PCR products were sent to Genomics Bioscience and Technology Co., Ltd. for purification and sequencing by Sanger Sequencing Method (ABI3730). The sequences were submitted to GenBank (NCBI).

The pathogenicity of *P. noxius* towards *R. formosana* was confirmed by the following inoculation. Pure culture of *P. noxius* was inoculated to autoclaved oat grains for 2 weeks and the oat grains were fully embedded with the mycelia of *P. noxius*. Six-year-old *R. formosana* seedlings from Zhuqi Nursery (Chiayi County) were used for inoculation. One seedling was inoculated and the inoculation was repeated 3 times by the following method for the experimental group and control group. The wound was fleshly cut by sanitized chisel to expose the woody tissue of the seedlings. The surface of the wound was wiped with 75% ethanol for surface sterilization. Inoculated oat grains were placed onto the wound and plastic wrap was applied to ensure the oats were clung to the wound. Same inoculation method but using autoclaved oat grains instead was carried out as the control group. All seedlings were placed in an incubator with 25℃ with 12 hr of light and watered every week. When the seedlings exhibited symptoms of foliage wilting, leaf discoloration and wilted, the above methods of pathogen isolation and morphological and molecular identification were carried out to confirm the pathogenicity of the inoculated pathogen.

RESULTS

Diseased or wilted *Reevesia formosana* were found in Caoshan, Fanlu Township, Chiayi County in 2021. Two locations with *R. formosana* in Caoshan were investigated. There were fallen trees, snags, and wilted bushes with mycelial mats at the basal part of plants. The roots and butts of three diseased or wilted *R. formosana* were dug out for detailed examination. According to the identification guideline listed in 'Diagnosis and Standard Operating Procedures for Controlling Brown Root Rot Disease' (Chang *et al*., 2009), the trees examined showed typical signs of brown root rot disease, including mycelial mats on the bark and brown network of mycelial cords in the woody tissue (Fig. 1).

Pure culture was isolated from the samples. The colonies were white and fast growing at the early stage and browning while aging on the selective medium. The colonies were also observed under a light microscope. Arthroconidia and trichocysts (Fig. 2) which are the microscopic characteristics of *Phellinus noxius* were observed (Chang *et al*., 1999, 2009).

At 1 month after inoculation, symptoms and signs including foliage wilting, leaf discoloration, defoliation, and mycelial mats around the area of inoculation were observed from all of the inoculated seedlings. At 6 months after inoculation, all the seedlings wilted. Rotten woody tissues were found in the basal part and the lignified roots of all the inoculated seedlings. Brown mycelial networks in the rotten wood and beneath the bark were observed (Fig. 3). Diseased wood tissues from the seedlings were sampled and the colonies of *P. noxius* were re-isolated. Control group seedlings showed no signs and symptoms of infection and a callus was found from each wound made on them after 6 months of inoculation.

The expected approximately 650-bp bands from the PCR products obtained with specific primers (G1-F/G1-R) of the pure culture from the diseased trees and the inoculated seedlings were shown after electrophoresis.

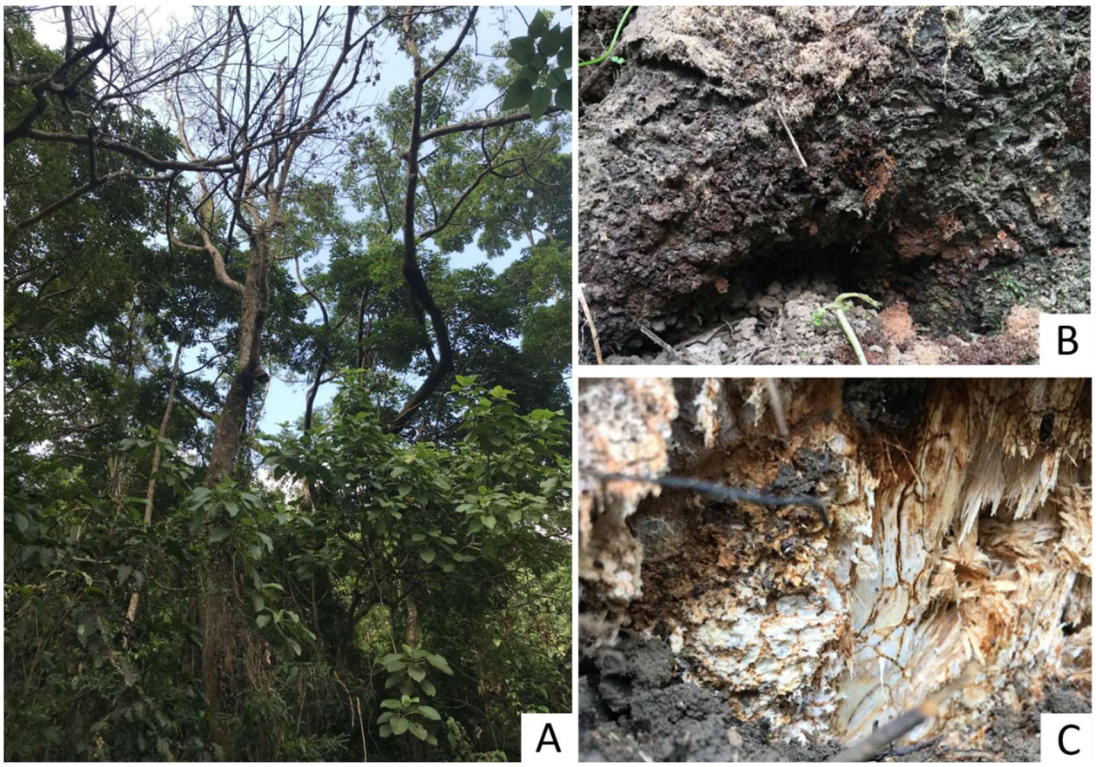

Fig. 1. Symptoms and signs of brown root rot disease shown from the diseased ***Reevesia formosana***. **A.** Defoliated ***R. formosana***. **B.** Mycelial mat on the butt. **C.** Brown mycelial network in the rotten woody tissue.

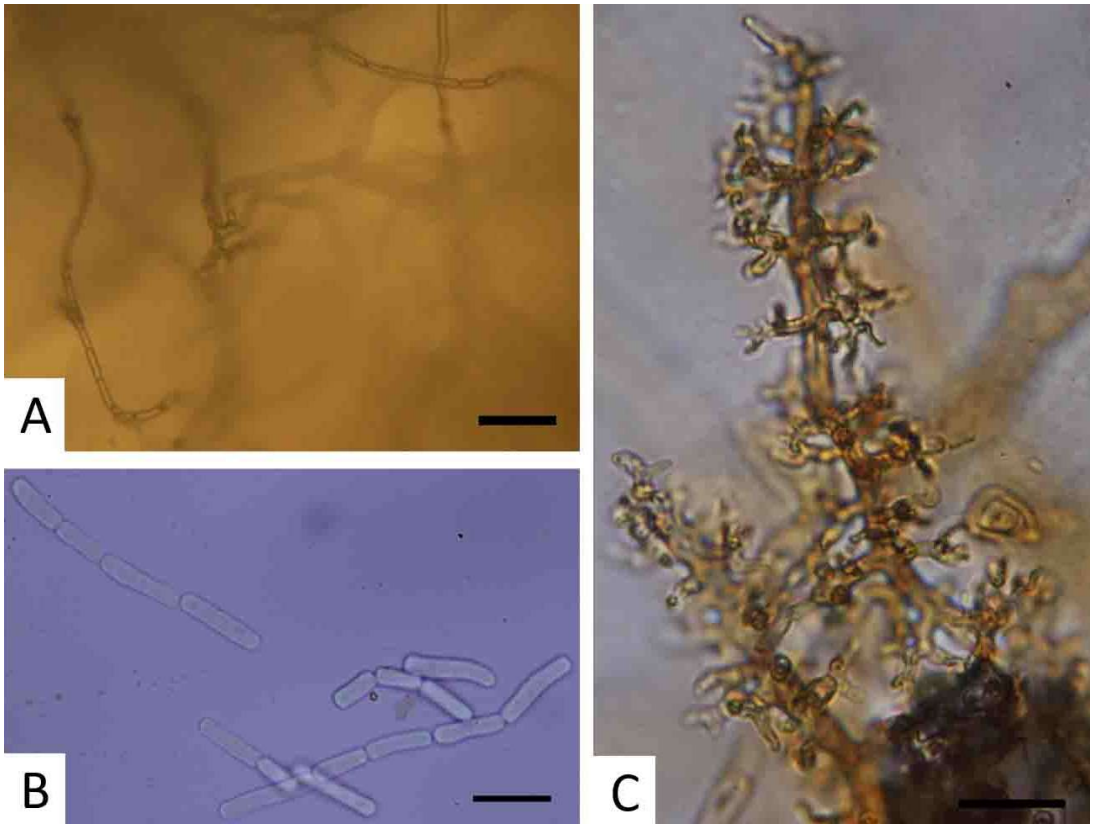

Fig. 2. Microscopic characteristics of ***Phellinus noxius*** culture on MEA. **A** and **B**. Arthroconidia. **C.** Trichocysts. (Scale bars: A: 25 µm; B and C: 10 µm)

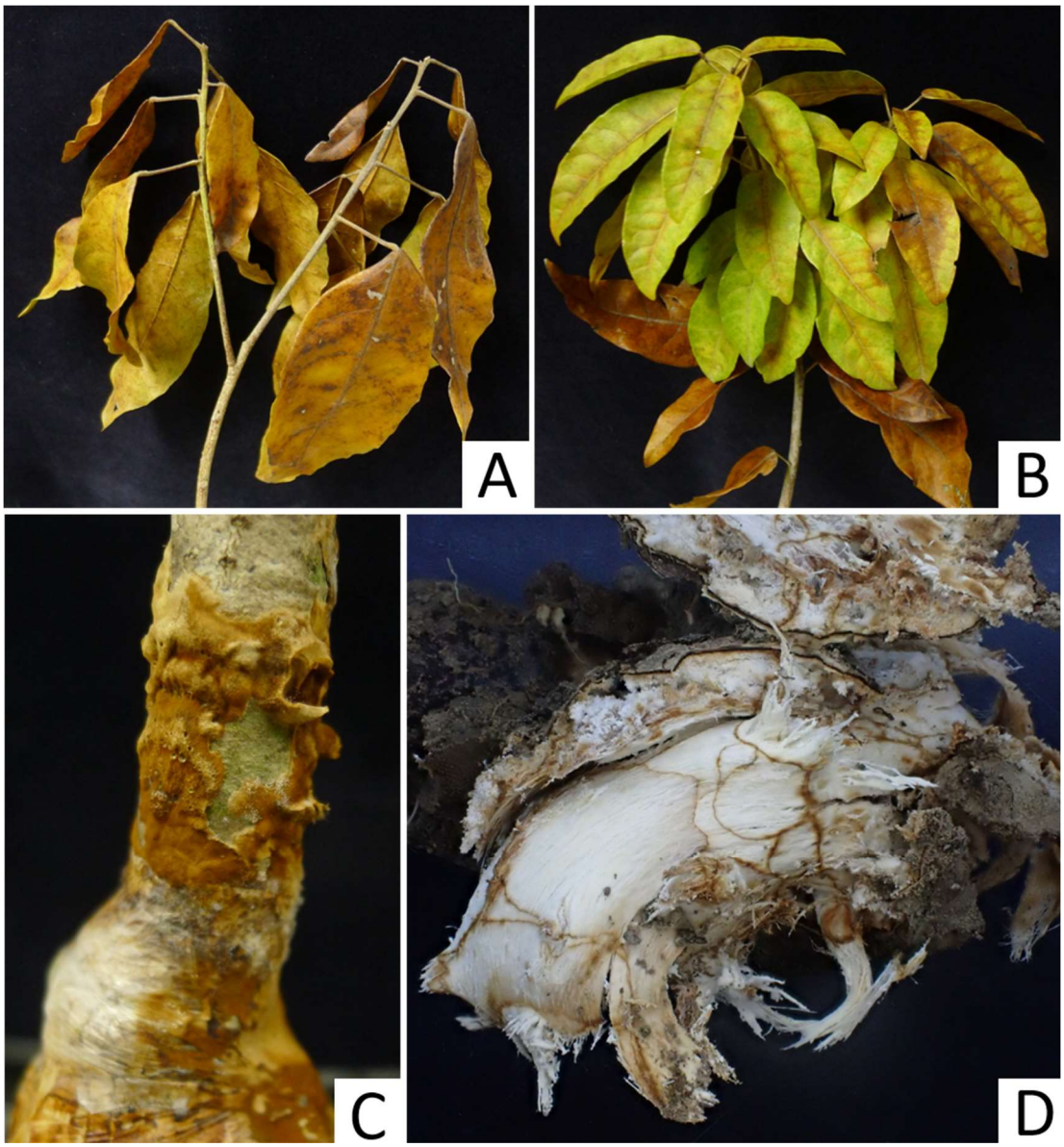

Fig. 3. Symptoms and signs of brown root rot disease shown from the inoculated ***Reevesia formosana*** seedlings. **A.** Foliage wilting. **B.** Leaf discoloration. **C.** Mycelial mat around the area of inoculation. **D.** Brown mycelial network in the rotten root. (A-C: 1 month after inoculation; D: 6 months after inoculation)

The pathogen obtained was confirmed as *P. noxius* by the use of specific primers (G1-F/G1-R) (Wu *et al*., 2009). The sequences of the pure culture from the diseased trees and the inoculated seedlings obtained with primers ITS4 and ITS5 were submitted to GenBank with accession numbers OQ946958-OQ946959 and OQ946960-OQ946961 (Fig. 4).

DISCUSSION

Reevesia formosana as an endemic, native, and rare tree species in Taiwan deserves input of resources for conservation and protection. In 2020, National Chiayi University was commissioned by Forestry and Nature Conservation Agency to conduct a tree and brown root rot disease survey in Caoshan area for the protection of the area. Trees were checked by visual inspection. Suspected trees were sampled and the samples were sent to Forest Disease Information Center for further identification and diagnosis. In the report of Huang and Song (2020), the samples shown in the photos were mostly collected from the rootlets of the suspected trees. The samples were diagnosed that they showed no sign of brown root rot disease by Forest Disease Information Center. However, photos in the report of Huang and Song (2020) which were captioned as browning and darkening of tree butts showed mycelial mats on the surface of tree butts. Those trees were likely to be infected. Because brown root rot

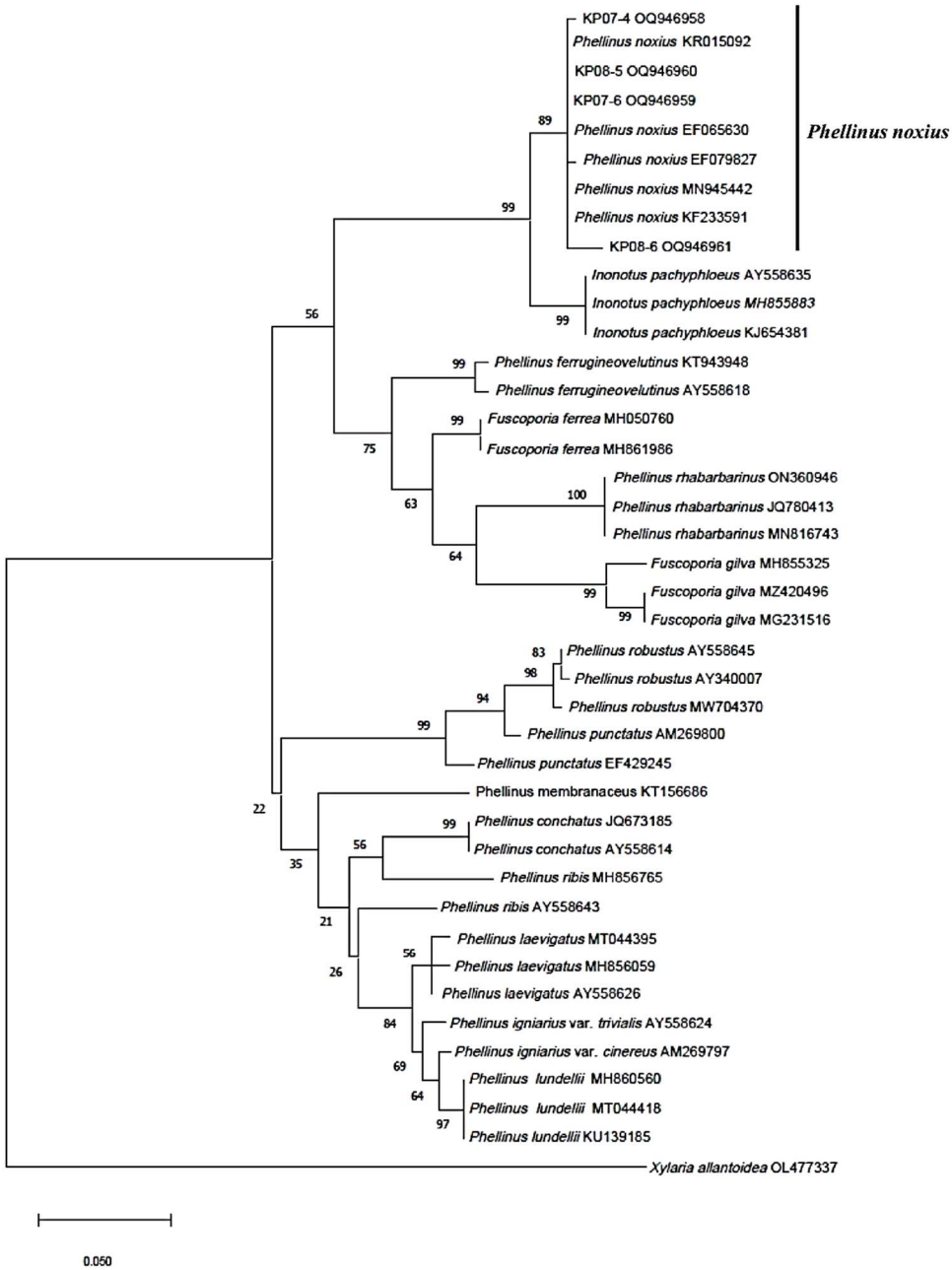

Fig. 4. Phylogenetic tree of ***Phellinus noxius*** and related ***Phellinus*** based on the ITS-rDNA sequences. ***Xylaria allantoidea*** was used as the outgroup taxa. The topology was constructed from maximum likelihood method. Numbers identify the bootstrap values and indicated near branches.

disease is not a systemic plant disease, the pathogen locates at the part where infection and rotting occured. The signs are easily covered by the soil or rarely observed on the rootlets of trees in the soil. To obtain valid samples for brown root rot disease identification, trained inspectors who are capable to identify the brown root rot disease in the field with proper sampling techniques are suggested to carry out the investigation or survey of brown root rot disease.

Furthermore, the behaviors of Taiwan-protected insect, *Formotosena seebohmi*, are closely related to *R. formosana*. Main threat toward the insect's population is habitat loss due to logging, agriculture, urbanization and habitat fragmentation due to transportation (Dar *et al.*, 2021). In other words, preserving the habitats of *F. seebohmi* would be an effective measure to maintain the population of *F. seebohmi*. For the conservation of a small-sized endemic cicada, *Tettigettalna mariae* (Quartau & Boulard, 1995), in Portugal, the preservation of pine forests with occasional planting and maintaining the herbaceous layer was advocated (Nunes *et al.*, 2014). In Caoshan, the habitat of *F. seebohmi* has deteriorated as

R. formosana was infected by *Phellinus noxius* and wilted. Fallen trees, snags, and wilted bushes around dead or diseased trees were found at two locations. The epidemic has been spreading out. Since the host range of *P. noxius* is broad and *P. noxius* spreads not only by root-to-root contact but also via spores after basidiocarps formed, increasing amount of *R. formosana* and other woody plants would eventually be infected and wilted over a greater area. Trees including *R. formosana* become snags and grassland will remain. *F. seebohmi* might lose *R. formosana* to emerge, feed, rest, and it gradually loses a woodland to inhabit. Therefore, effective mitigations to halt and control the spreading of *P. noxius* among *R. formosana* and other woody plants in the infected area are necessary as two valuable species can be conserved.

A complete brown root rot disease survey in Caoshan is required to estimate the diseased area for a more precise mitigation plan. Following the standard operating procedures for controlling brown root rot disease (Chang *et al.*, 2009), a huge impact on the population of *F. seebohmi* might be imposed due to the excavation of soil and soil fumigation at the diseased area. The population may gradually recover over time. Control measures may need to be adjusted, well-planned, and carried out carefully to impose a milder impact on the population. The diseased trees could be brought down in advance to ensure the safety of the forest road and the operation. The margin of the diseased area may need to be excavated first to confine the area and stop the spreading of brown root rot disease by the root-to-root contact, like the firebreak. Soil from the diseased area may be required to excavate gently so that most of the *F. seebohmi* nymphs could stay unharmed. The *F. seebohmi* nymphs could be picked up, rinsed with water, transferred to the root zone of other healthy *R. formosana*, and continue their life cycle. Replanting healthy *R. formosana* after the operation may be required to restore the population of *R. formosana* and the habitat of *F. seebohmi*.

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