A Novel *Phellinidium* sp. Causes Laminated Root Rot on Qilian Juniper (*Sabina przewalskii*) in Northwest China

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Abstract

Cui, B. K., Dai, Y. C., He, S. H., Zhou, L. W., and Yuan, H. S. 2015. A novel *Phellinidium* sp. causes laminated root rot on Qilian juniper (*Sabina przewalskii*) in northwest China. Plant Dis. 99:39-43.

A laminated root rot on Qilian juniper (*Sabina przewalskii*) caused by a species of *Phellinidium* (Basidiomycota) was observed in northwest China. Seventeen fungal samples collected from Qinghai Province during 2012 and 2013 were used for taxonomic and pathogenicity tests. The fungal pathogen was identified by morphological characters and phylogenetic analyses based on nLSU sequences. A new fungus is

described herein as *Phellinidium qilianense* sp. nov. One-year-old Qilian juniper seedlings were wound-inoculated under controlled conditions to test pathogenicity of the fungal species. The fungus was successfully reisolated from decayed tissue of tested seedlings. *P. qilianense* is a new forest pathogen on coniferous trees in China.

Phellinidium (Kotl.) Fiascon & Niemelä is a small genus with several species that are tree-pathogenic fungi. Two members of the *Phellinidium weirii* (Murrill) Y.C. Dai complex grow on living conifers: one is *P. weirii sensu stricto* (the cedar type), which is usually found on species of the Cupressaceae in North America; the other, *P. sulphurascens* (Pilát) Y.C. Dai (the Douglas-fir type) occurs on species of Pinaceae (6,9). Outside North America, *P. weirii* has been reported on *Juniperus* spp. in the Ural Mountains (2) and *P. sulphurascens* has been reported on *Picea*, *Abies*, *Larix*, and *Pinus* spp. in East Asia, including China (3). Besides the above-mentioned species, *Phellinidium noxium* (Corner) Bondartseva & S. Herrera is a serious pathogen on many angiosperm trees in tropical Asia (5,10). In North America, *P. weirii* causes significant root and butt rot of western red cedar (*Thuja plicata*), while *P. sulphurascens* is especially damaging to Douglas-fir (*Pseudotsuga menziesii*) (1,9,13,15,19). The pathogenicity of *Phellinidium* in China is poorly known.

The Qilian juniper, *Sabina przewalskii* Kom. (syn. *Juniperus przewalskii*), is an endemic species in China, and its natural distribution is around 32°35' to 39°05' N and 96°26' to 104°20' E. The species occurs mainly as pure stands in the Qilian Mountains of Qinghai Province in northwest China, at elevations ranging from 2,600 to 2,800 m. It is a very important tree species in western China because it is both highly drought tolerant and cold hardy and it plays an important role in preventing local soil erosion. In 2004, a fungus causing laminated root rot on *S. przewalskii* was found in the Qilian Mountains (2). Based on preliminary investigations, the pathogen was identified as *P. weirii* (2,4,5). Because the disease has increased in severity since 2004, further research on the pathogen and disease was carried out during 2012 to 2013 and these new results are reported here.

The objectives of our study were to 1) identify the pathogenic causal fungus based on molecular and morphological analyses, 2) determine the phylogenetic relationship of the fungus with other similar species, 3) provide a detailed description of the decay on

Qilian juniper caused by the fungus, and 4) evaluate the pathogenicity of the fungus on Qilian juniper.

Materials and Methods

Disease surveys. Healthy stands and diseased stands of *S. przewalskii* were investigated. Symptoms of the disease included slow growth, chlorotic foliage, wilt, and mortality. After cutting some roots of the diseased trees, cambial necrosis and decay were found. Five plots in the diseased stands of *S. przewalskii* on different slopes were investigated. Each plot was 100 × 100 m and all nonsymptomatic, symptomatic, and dead trees were calculated. Trees were considered infected if they had chlorotic foliage or the fruiting body of *Phellinidium qilianense* was found on its roots. The characteristics for laminated rot include a delamination where the wood separates at the annual rings. The decayed wood is usually soft, paler and lighter than sound wood.

Fungal collections and isolations. Seventeen fungal and/or decayed wood samples (IFP 018444, 018454, 018468, and 018481; and Dai YC 13303, 13304, 13305, 13306, 13307, 13308, 13310, 13311, 13312, 13315, 13316, 13317, and 13320) were collected from the roots of living, dying, and dead *S. przewalskii* trees occurring throughout the Xianmi Forest Farm, Qinghai Province, northwest China during 2012 and 2013 (Table 1). Five fungal strains (Dai YC 13304, 13308, 13311, 13315, and 13317) isolated from living, dying, or dead trees and/or fruiting bodies of the fungus (Table 1) were selected for deposition in the herbaria of the Institute of Applied Ecology, Chinese Academy of Sciences (IFP), and Beijing Forestry University (BJFC). These same fungal strains were preserved on PDA in the herbarium of the BJFC.

Morphological studies. The studied specimens were deposited at the herbaria of the BJFC and the IFP. Sections were studied at magnification up to 1,000× using a Nikon Eclipse 80i microscope and phase contrast illumination (Nikon, Tokyo). Drawings were made with the aid of a drawing tube. Microscopic features, measurements, and drawings were made from slide preparations stained with Cotton Blue and Melzer's reagent. Spores were measured from sections cut from the tubes. In presenting the variation in the size of the spores, 5% of measurements were excluded from each end of the range, and were given in parentheses. In the text, the following abbreviations were used: IKI = Melzer's reagent, IKI- = both inamyloid and indextrinoid, KOH = 5% potassium hydroxide, CB = Cotton Blue, CB- = acyanophilous, L = mean spore length (arithmetic average of all spores), W = mean spore width (arithmetic average of all spores),

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Accepted for publication 7 July 2014.

Q = variation in the L/W ratios between the specimens studied, and n (a/b) = number of spores (a) measured from given number (b) of specimens.

DNA extraction, PCR amplification, and phylogenetic analyses. A Phire Plant Direct PCR Kit (Finnzymes Oy, Finland) was used to perform PCR amplifications from fruiting bodies and mycelia on plates according to the manufacturer's instructions. These tissues originated from wood samples or fruiting bodies of nine isolates randomly selected from the 17 isolates we collected. About 50 mg samples were lysed in 30 µl dilution buffer for DNA extraction. After incubating 3 min at room temperature, 0.75 µl of the supernatant were used as template for a 30 µl PCR reaction. The nuclear large subunit (nLSU) region of the extracted DNA was amplified using the primers LR0R and LR7 (21). The PCR procedure was as follows: initial denaturation at 98°C for 5 min, followed by 39 cycles at 98°C for 5 s, 48°C for 5 s, and 72°C for 5 s, and a final extension of 72°C for 10 min. The PCR products were sequenced at the Beijing Genomics Institute, China, using the same primers as those used in our PCR procedure. All newly generated sequences are deposited in GenBank (<http://www.ncbi.nlm.nih.gov>; Table 1). The nLSU sequences of *P. sulphurascens* and *P. weirii* generated by Wagner and Fischer (22) and Lim et al. (14) were retrieved from GenBank to explore the phylogenetic relationships among these two species and the newly sequenced samples. *Phellinidium ferrugineofusum* (P. Karst.) Fiasson & Niemelä, the generic type, was selected as the outgroup (22) to use in our phylogenetic analysis. The nLSU dataset was aligned using MAFFT 7.110 (11,12) with Q-INS-i option. Sequence alignment was deposited at TreeBASE (<http://purl.org/phylo/treebase>; submission ID 15617). The best-fit evolutionary model for the resulting alignment was estimated as GTR model using jModelTest (8,17). Following this model, the maximum likelihood (ML) analysis was performed using raxmlGUI 1.2 (18,20) with auto FC option (16) in bootstrap (BS) replicates.

Pathogenicity tests. One-year-old seedlings of *S. przewalskii* obtained from a nursery in the Xianmi Forest Farm, Qinghai Province, northwest China, were planted into clay pots containing a sterile standard soil mixture of peat, perlite, and vermiculite (6:2:2 ratio by volume) in a greenhouse.

Four isolates of *P. qilianense* (Dai YC 13304, 13308, 13315, and 13317) that we isolated from roots of living, dying, and dead trees of *S. przewalskii* and/or fruiting bodies of the fungus were used to wound-inoculate 36 *S. przewalskii* seedlings (three seedlings in each pot, one isolate repeat in three pots). All four isolates were grown in petri dishes containing PDA for 10 days prior to inoculation. The seedlings were transplanted to pots after 1 week for inoculations, a random root was selected, and its connection part to the main root was excised using a sterile scalpel. An agar

plug with fresh mycelia was cut from each culture and placed onto the wounded tissue, and the wound was sealed with Parafilm. A total of nine control seedlings were wounded using a sterile scalpel prior to inoculation with sterile PDA plugs. Inoculations were conducted in August 2013. Seedlings were monitored weekly for symptom development such as chlorotic foliage, wilt, and mortality. Root decay was also examined when chlorotic foliage appeared after 2 months. A segment of about 2 mm decayed root was placed in petri dishes containing PDA and incubated. Reisolations were made in petri dishes containing PDA and after growth, their colony morphology (brownish generative hyphae with simple septa and hyphoid setae with sharp tips) was observed. nLSU sequences were then analyzed and compared with those of the inoculated cultures.

Results

Disease surveys, fungal collection, and morphological characterization. Dead and dying trees of *S. przewalskii* were found in many natural forests of the Qilian Mountains, Menyuan County, Qinghai Province (Fig. 1A). The trees were about 80 to 220 years old according to the stand data from the local forest department and occurred in almost pure stands. A disease patch or root rot center up to 5 ha was found consisting of standing dead and dying symptomatic trees (Fig. 1B); some fallen trees were also

Fig. 1. A, Dead trees in pure forest of *Sabina przewalskii*. B, Dead and dying trees of *S. przewalskii*. C, The fruiting body of *Phellinidium qilianense* (sample Dai 13309) on root of dying *S. przewalskii*. D, The fruiting body of *P. qilianense* (Sample Dai 13309). E, The laminated decay on wood of *S. przewalskii* caused by *P. qilianense*.

Table 1. Samples of *Phellinidium qilianense* on *Sabina przewalskii* in Qinghai Province used in this study

Samples	Host	Location	Year	Collector	GenBank no. (nLSU)
IFP 18444 ^a	Living tree	Sigou, Menyuan	2012	Yuan HS	KJ635808
IFP 18454 ^a	Dying tree	Sigou, Menyuan	2012	Yuan HS	KJ635809
IFP 18468 ^a	Fallen trunk	Donghai, Menyuan	2012	Yuan HS	KJ635810
IFP 18481 ^a	Stump	Donghai, Menyuan	2012	Yuan HS	—
Dai 13303 ^a	Living tree	Xianfeng, Menyuan	2013	Dai YC	—
Dai 13304 ^b	Living tree	Xianfeng, Menyuan	2013	Dai YC	—
Dai 13305 ^a	Living tree	Xianfeng, Menyuan	2013	Dai YC	—
Dai 13306 ^a	Living tree	Xianfeng, Menyuan	2013	Dai YC	—
Dai 13307 ^a	Dying tree	Xianfeng, Menyuan	2013	Dai YC	—
Dai 13308 ^b	Dying tree	Xianfeng, Menyuan	2013	Dai YC	—
Dai 13310 ^a	Dead tree	Xianfeng, Menyuan	2013	Dai YC	KJ635811
Dai 13311 ^a	Dying tree	Xianfeng, Menyuan	2013	Dai YC	KJ635812
Dai 13312 ^a	Dead tree	Xianfeng, Menyuan	2013	Dai YC	KJ635813
Dai 13315 ^b	Dead tree	Xianfeng, Menyuan	2013	Dai YC	KJ635814
Dai 13316 ^a	Dead tree	Xianfeng, Menyuan	2013	Dai YC	—
Dai 13317 ^b	Fallen trunk	Xianfeng, Menyuan	2013	Dai YC	KJ635815
Dai 13320 ^b	Fallen trunk	Xianfeng, Menyuan	2013	Dai YC	KJ635816

^a Indicates fruiting body.

^b Indicates isolate from wood or fruiting body.

occasionally present in the patch. Symptomatic trees showed reduced branch and leader growth, thin crowns, and chlorotic foliage. Based on our investigation on the five plots in the different diseased stands located on different slopes, the infected rate ranged from 40 to 90% while mortality ranged from 20 to 70%. But in another plot not included in the five plots, almost 100% of the trees were infected and mortality was approximately 80%. Fungal fruiting bodies were found at the base of many dead and dying trees (Fig. 1C), mostly on the underside of roots and stems of fallen infected trees. Fruiting bodies were perennial, resupinate, and dark brown, with poroid hymenophores (Fig. 1D). In the early stage of decay, *P. qilianense*-infected wood of dead trees and fallen trunks was woody hard, then became characteristically laminated in advanced stages of decay (Fig. 1E). Microscopically, the fungus had a monomitic hyphal system, hyphae without clamp connections, tramal tissue with hyphoid setae, and thin-walled, hyaline, smooth, ellipsoid basidiospores. The fungus belongs to *Phellinidium* (Hymenochaetaeaceae, Basidiomycota), and is closely related to *P. weirii* (3,7).

Phylogenetic analyses. A total of nine collections of the pathogens on *S. przewalskii* were nLSU-sequenced (Table 1) and sampled in phylogenetic analysis. The nLSU dataset, also including three nLSU sequences of *P. sulphurascens*, three of *P. weirii*, and one of *P. ferrugineofuscum*, resulted in an alignment with 912 characters. The ML tree tested by 650 BS replicates (Fig. 2) showed that the pathogenic fungus on *S. przewalskii* formed a distinct lineage that was separated from *P. sulphurascens* and *P. weirii*, and had a closer relationship with *P. weirii* than with *P. sulphurascens*.

Pathogenicity tests. The four fungal isolates of *P. qilianense* that we used to inoculate roots of 1-year-old *S. przewalskii* seed-

Fig. 2. Phylogenetic relationships among *Phellinidium qilianense* (in bold), *P. sulphurascens* and *P. weirii*.

Fig. 3. A, Symptoms of one year old seedlings of *Sabina przewalskii* infected by *Phellinidium qilianense* Dai 13309. B, Control.

lings colonized roots within 2 months, as evidenced by seedlings showing distinct chlorotic foliage (Fig. 3). Isolates of *P. qilianense* that we obtained from the decayed roots of inoculated seedlings (Fig. 4) were the same as those we used to inoculate seedlings, based on colony morphology (brownish generative hyphae with simple septa and plenty of hyphoid setae with sharp tips) and nLSU sequences. Recovery frequency from the decayed seedling roots was 88.8% for *P. qilianense*, 11.2% for *Fusarium* sp. and other fungi, and 0% for control treatment.

Taxonomic description. *Phellinidium qilianense* B.K. Cui, L.W. Zhou & Y.C. Dai, sp. nov. Figures 1D & 5.

Mycobank: MB 808449

Basidiocarps perennial, resupinate, inseparable, soft corky, without odor or taste when fresh, becoming corky to fibrous upon drying, up to 30 cm long, 14 cm wide, and 3 cm thick at center. Pore surface grayish brown to honey-yellow; margin cinnamon-buff, up to 5 mm wide; pores round or angular, 5 to 8 per mm; dissepiments thick, entire, or slightly lacerate. Subiculum cinnamon-buff, corky, very thin, about 1 mm thick. Tubes yellowish brown to cinnamon-buff, fibrous, new tubes distinctly paler contracting with old one, old tubes yellowish brown; tube layers up to 29 mm long, indistinctly stratified.

Hyphal system monomitic; generative hyphae simple septate; tissues darkening but otherwise unchanged in KOH. *Subiculum.* Generative hyphae hyaline to yellowish, thin- to slightly thick-walled, frequently branched and septate, interwoven, 2 to 5.5 μ m in diameter. *Hymenophoral trama.* Generative hyphae hyaline to yellowish, thin- to slightly thick-walled, occasionally branched, frequently septate, parallel along the tubes, 2.5 to 4.5 μ m in diameter. *Hyphoid setae* occasionally in subiculum, dominant in trama, dark brown, subulate, sharp-pointed, thick-walled with a narrow lumen, originating from the simple septum of yellowish, slightly thick-walled hyphae, parallel along the tubes, straight, occasionally penetrating into hymenium, sometimes apically to centrally encrusted by small rosettes of crystals, several hundreds of μ m long, 5 to 10 μ m in diameter. Hymenial setae absent. Cystidia hyaline, subulate,

Fig. 4. A, Symptoms on roots of one year old seedlings of *Sabina przewalskii* infected by *Phellinidium qilianense* Dai 13309. B, Control.

thin-walled, sharp-pointed, 17 to 30 × 3 to 5 µm; cystidioles absent. Basidia barrel-shaped, thin-walled, with four sterigmata and a simple septum at the base, 8 to 12 × 4 to 6 µm; basidioles in shape similar to basidia, but slightly smaller. Basidiospores hyaline, broadly ellipsoid, thin-walled, smooth, IKI–, CB–, (3.5–) 3.7 to 4.3 (–4.4) × 2.8 to 3.3 (–3.5) µm, L = 3.93, W = 3.06, Q = 1.27 to 1.3 (n = 60/2).

Etymology: *Qilianense* (Lat.): referring to the type locality, Qilian Mountains.

Materials studied. China. Qinghai: Menyuan County, Xianmi Forest Farm, Sigou, on base of living *S. przewalskii*, 7 September 2012, Yuan 6424 (holotype in IFP 18444); on base of dying *S. przewalskii*, Yuan 6441 (paratype in IFP 18454); Donghai, on fallen trunk of *S. przewalskii*, 7 September 2012, Yuan 6458 (paratype in IFP 18468). Xianfeng, on base of living *S. przewalskii*, 28 July 2013, Dai 13304 (paratype in BJFC 14792); on base of dead *S. przewalskii*, 28 July 2013, Dai 13310 (paratype in BJFC 14793); on fallen trunk of *S. przewalskii*, 28 July 2013, Dai 13320 (paratype in BJFC 14795).

Discussion

Laminated root rot of *S. przewalskii* was first found in the Xianmi Forest Farm, Qinghai Province, northwest China, about 20 years ago according to the local forestry department. The pathogen responsible for the disease was initially identified without molecular study as *P. weirii* (2,5). The present molecular analysis showed *P. weirii* and *P. qilianense* were in two distinct clades. Some samples of *P. weirii* from North America were also studied and morphological differences between the two species were found. Basidiospores of *P. weirii* are distinctly larger than those in *P. qilianense* (4.9 to 5.3 × 3.2 to 3.8 µm vs 3.7 to 4.3 × 2.8 to 3.3 µm). In addition, *P. weirii* occurs mainly on *Thuja plicata* in North America, while *P. qilianense* grows exclusively on *S. przewalskii* in northwest China.

P. sulphurascens is another pathogen on conifers in northeast, northwest, and southwest China (3,5), and it also causes a laminated root rot. Although *P. sulphurascens* has a wider distribution than *P. qilianense*, it mostly occurs in mature trees within old natural forests. According to our field investigation, *P. sulphurascens* did not cause serious disease on gymnosperm trees in China, and no distinct disease patches or root rot centers were found caused by this fungus. *P. sulphurascens* has an annual habit and larger pores (4 to 5 per mm), and attacks species of the Pinaceae, while *P. qilianense* has a perennial habit, smaller pores (5 to 8 per mm), and

infects species of Cupressaceae. Besides the morphology, the two species have different ecology; *P. sulphurascens* prefer moist environments, while *P. qilianense* favors dryer sites.

Based on phylogenetic analysis inferred from nLSU sequences, all nine pathogenic samples formed a distinct lineage with a high degree of support. They definitely belong to *Phellinidium*, and a new name, *P. qilianense*, is proposed in this paper. *P. qilianense* was found to be more closely related to *P. weirii* than any other species in the genus.

Laminated rot of roots caused by *P. qilianense* in natural forests of *S. przewalskii* was found in the Qilian Mountains of Qinghai Province. Many distinct disease patches were observed varying in size but usually about a few hectares. The characteristics of diseased trees are slow growth, thin crowns, chlorotic foliage, and tree decline. Although the number of infected trees in the plots was 40 to 90%, it may actually be found in greater amounts since it is difficult to diagnose during early stages of infection where neither symptoms nor fruiting body of the fungus are evident. During the early stages of the decay caused by *P. qilianense*, the wood color is almost the same as the wood in healthy tree, while in the advanced stages of decay, the wood separated along the annual ring because of localized attack within the annual rings. Usually the wood became softer, paler, and lighter than healthy wood, showing characteristics for laminated rot.

P. qilianense is an aggressive pathogen on *S. przewalskii* in the Qilian Mountains, northwest China. Although some trees may be infected for more than 20 years and show decline, the fungus can also kill trees. Pathogenicity testing was not done on mature trees in the field because it may take many years to get the results. Instead, small seedlings were inoculated in the greenhouse to demonstrate pathogenicity. The laminated root rot on Qilian juniper has been increasing in severity in recent years, and it appears that the disease was spread vegetatively from root to root. Further studies on disease progression, pathogenicity tests on trees in the field, and disease management shall be done in the future.

Acknowledgments

We express our gratitude to Mr. Jian-Hai Ma (the forest Pest Control Department of Qinghai Province, China) for helping in field investigation and to Prof. Robert A. Blanchette (University of Minnesota, United States) for improving the manuscript. The research was financed by the Fundamental Research Funds for the Central Universities (Project No. JC2013-1) and the National Natural Science Foundation of China (No. 31200015).

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Fig. 5. Microscopic structures of *Phellinidium qilianense* (drawn from specimen IFP 18444, holotype). **A**, Basidiospores. **B**, Basidia and basidioles. **C**, Cystidia. **D**, Hyphoid setae. **E**, Hyphae from trama. **F**, Hyphae from subiculum.

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