RESEARCH ARTICLE

Molecular diagnosis of the brown root rot disease agent *Phellinus noxius* on trees and in soil by rDNA ITS analysis

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Abstract: *Phellinus noxius* (*P. noxius*) is an important pathogen that causes brown root rot of trees in tropical and subtropical areas and has led to severe damage to trees. A quick and accurate diagnostic technique is essential to the timely confirmation of the pathogen and possible treatment. In this study, a fast, sensitive and accurate approach of molecular technique was used to diagnose the brown root rot pathogen on trees and in soils of subtropical Hong Kong. Two pairs of specific PCR primers were used to amplify the target rDNA internal transcribed spacer (ITS) region through polymerase chain reaction (PCR) for both tree tissues and soil samples. The amplified ITS fragments were then sequenced and analyzed phylogenetically for the diagnostic identification of the pathogen *P. noxius*. The results showed that 13 of 38 suspected trees in Hong Kong were infected with *P. noxius* through molecular detection. The pathogen showed no specific preference to any particular tree species. Quantitative PCR was applied to soils grown with trees identified both positive and negative for *P. noxius*, but the soils with healthy trees were also found positive for *P. noxius*. For the first time, *P. noxius* was reported to infect a wide range of tree species in Hong Kong and widely presented in soils, probably serving as a reservoir for the pathogen. Through this study, it is proposed that *P. noxius* is a soil-borne pathogen, which increases its infectivity when trees start to grow in the soil as a means in addition to the previously proposed root-to-root contact.

Keywords: *Phellinus noxius*, molecular diagnosis, brown root rot

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Received: June 22, 2015; **Accepted:** February 1, 2016; **Published Online:** April 8, 2016

Citation: Wang Y-F, Meng H, Gu V W, *et al*. 2016, Molecular diagnosis of the brown root rot disease agent *Phellinus noxius* on trees and in soil by rDNA ITS analysis. *Applied Environmental Biotechnology*, vol.1(1): 81–91. http://dx.doi.org/10.26789/AEB.2016.01.002.

1. Introduction

The fungus *Phellinus noxius*[1] is a pathogen that is responsible for the notorious brown root rot of landscape, orchard, and forest trees. This fungus has a wide range of hosts including more than 200 species in 59 families[2], and since then more new tree species have also been added to this list[3,4]. Despite favoring woody trees, *P. noxius* can also attack some herbaceous plants[2,5,6]. *P. noxius* has been found in many tropical and subtropical countries and districts, including Singapore, Japan, Indonesia, Malaysia, Hai-

nan Island in China, Macao, Taiwan, Africa, Australia, Central America, and the Caribbean[3–19]. The wide geographical distribution of this pathogen and the severe damage it has caused have drawn significant concerns.

Conventional diagnosis of brown root rot caused by *P. noxius* involves in observing symptoms and isolating the pathogen from diseased tissues[2,4]. Trees infected with *P. noxius* normally present wilting and discoloring of foliage[13]. The exposing roots and basal stem are often encrusted with a layer of yellowish-brown, brown, or brownish-black mycelial mat[13]. The inner surface of the infected bark is usually white with brown networks[2]. *P. noxius* can be isolated from infected tissues on malt-extract agar (MEA) medium amended by Chang[20]. Brown colonies can be formed on potato dextrose agar (PDA) medium accompanied by irregular dark brown lines or patches[2]. Although the traditional method is frequently applied to identify brown root rot caused by *P. noxius*, it is not quite reliable and practical. Firstly, other pathogens may produce similar symptoms which might lead to wrong conclusion[21]; secondly, *P. noxius* cannot always be obtained from the infected tissues through culturing; thirdly, the isolation process normally takes a long time, which may delay timely treatment.

Although several measures have been developed to control this pathogen[22–24], a quick and accurate diagnosis is the requisite condition for timely treatment. Therefore, a quick and reliable diagnosis is urgently needed. Molecular assay is a fast, sensitive and reliable method to identify *P. noxius*[25,26]. In this method, a pair of specific PCR primers were used to amplify rDNA internal transcribed spacer (ITS) fragments, the sequences of which can be subsequently applied to compare with the known sequences of *P. noxius* on GenBank database. Through this way, the pathogen can be identified as *P. noxius* or otherwise.

A quick and accurate diagnosis is critical to treat brown root rot of individual cases. It is also important to make clear the mechanism of dissemination approach of this pathogen to prevent the spread of brown root rot induced by *P. noxius*. However, the dissemination approach of *P. noxius* pathogen is still undetermined[2,3]. Currently, the dominant view is that this pathogen spread through root-to-root contact[2,3], but no solid evidence has been given.

P. noxius has not been reported in subtropical Hong Kong. However, our preliminary experimental data showed that some landscape trees in Hong Kong had been infected with *P. noxius*. In this study, we applied molecular technique to identify brown root rot infected with *P. noxius*. Firstly, the landscape trees in parks and roadsides around Hong Kong were scanned and then suspicious trees were sampled for molecular analysis in lab. For the first time, we determined the occurrence of *P. noxius* and their geographical distribution in Hong Kong. Besides the diagnosis of *P. noxius* in Hong Kong, we explored their dissemination mechanism by investigating the occurrence of *P. noxius* in soils. Through this investigation, we proposed that *P. noxius* probably spread through both pathogen-containing soil and root-to-root contact.

2. Materials and Methods

2.1 Wood and Soil Samples Collecting

From October 2013 to February 2014, all the landscape trees over Hong Kong were inspected by gardeners trained with the knowledge of brown root rot. Suspicious trees reported by gardeners were then sampled by members in our laboratory. A total of 38 suspicious trees were sampled (one tree was collected twice and another one were collected thrice). For each suspicious tree, at least 3 wood samples at different directions of the tree, weighting 5–10 g each, were collected aseptically from the roots and/or basal stem. The wood samples were then transported the same day to lab for genomic DNA extracting.

Soils between a dead tree and a diseased tree caused by *Phellinus noxius* in Kowloon Park were collected. The dead tree had already been removed on the day of soil collecting. The two trees were 15 m apart and soils between the two trees were collected every 2 m. A total of 9 soils were collected (including 2 soils at the basal stems of the two trees). Another 5 soils under trees that were not infected with *P. noxius* were also collected. Among these 5 soils, 2 soils were under Tree008 and Tree036; another 3 soils, which were named HKU soil1, HKU soil2, and HKU soil3, were under three healthy trees on campus of The University of Hong Kong. HKU soil1 and HKU soil2 were both under tree species *Ficus microcarpa*, but in different places; HKU soil3 was under tree species *Bauhinia* sp.

2.2 Total Genomic DNA Extraction

For wood samples, the extraction protocol was revised from Ding *et al.*[27]. Briefly, 0.5 g of the wood tissue was cut from the sample. The wood tissue was then

washed with Milli-Q water three times and blot dried. The dry tissue was then put into a sterile mortar and frozen by liquid nitrogen (–196°C) and ground into powder. About 0.2 g of the powder was then transferred into a sterile 1.5 mL Eppendorf tube for subsequent DNA extraction. The total DNA was extracted with E.Z.N.A® Forensic DNA kit (Omega Bio-Tek, USA) according to the manual of the manufacturer.

For soil samples, 0.25 g of soil was weighted for DNA extraction. The soil was extracted in duplicate using the PowerSoil® DNA isolation kit (MO BIO Laboratories, Inc. USA) according to the manual of the manufacturer, which was demonstrated to produce ideal result elsewhere[28,29]. The duplicate DNA extractions from the same soil sample were then pooled together and stored at −20°C for subsequent molecular analysis.

2.3 PCR Amplification

The genomic DNA extracted from the wood samples were amplified using the primer pair PN-1F (5' – AGTTTGCGCTCATCCATCTC – 3') and PN-2R (5' – AGCCGACTTACGCCAGCAG – 3')[25]. The optimized polymerase chain reaction (PCR) mixture contained in a final volume of 50 μL was as the following: 1 μL of DNA (20 ng μL^{-1}), 10 μL of 5 × GoTaq Flexi buffer (Promega, Hong Kong) and 4 μL of $MgCl_2$ (25 mM, Promega), 1 μL of dNTPs (10 mM of each, Promega), 1 μL of each forward and reverse primers (20 μM) and 0.25 μL of GoTaq Flexi polymerase (5 U μL^{-1}, Promega), 5 μL of BSA (0.1%). PCR conditions were set as follows: 95°C for 3 min; 30 cycles of 95°C for 45 s, 60°C for 45 s, followed by 72°C for 1 min; and finally 72°C for 10 min.

When using PN-1F/PN-2R to amplify target fragments from genomic DNA of soils, only smear bands were formed. But when utilizing the primer pair G1-F (5' – GCCCTTTCCTCCGCTTATTG – 3') and G1-R (5' – CTTGATGCTGGTGGGTCTCT – 3')[26], the PCR result was much better. Therefore G1-F/G1-R was used to amplify soil genomic DNA in this study. The optimized PCR reaction mixture contained in a final volume of 50 μL was as the following: 1 μL of DNA (20 ng μL^{-1}), 10 μL of 5× GoTaq Flexi buffer (Promega, Hong Kong) and 4 μL of $MgCl_2$ (25 mM, Promega), 1 μL of dNTPs (10 mM of each, Promega), 1 μL of each forward and reverse primers (20 μM) and 0.25 μL of GoTaq Flexi polymerase (5 U μL^{-1}, Promega), 5 μL of BSA (0.1%). PCR conditions were set as follows: 94°C for 4 min; 38 cycles of 94°C for 45 s, 56°C for 45 s, followed by 72°C for 45 s; and finally 72°C for 10 min.

PCR products were checked by electrophoresis in a 1% agarose gel stained with GelRed™ (Biotium, Hayward) at 1:10,000. The target gene fragments by PN-1F/PN-2R and G1-F/G1-R were 414/422 and 645/651/653 bp long respectively. If the result of gel check is positive, the PCR products were then purified using Gel Advance™ Gel Extraction System (Viogene-Bio Tek Co., Taiwan, ROC) and subsequently sent for sequencing. If the sequencing quality was not desirable (e.g. the PCR product was not a single clone), a clone library was then constructed.

2.4 Cloning and Sequencing

When a clone library was needed, the purified products were ligated into the pMD18 T-vector (Takara, Japan) and then transformed into the host *Escherichia coli* competent cell DH5α (Takara, Japan) in accordance with the manufacturer's instructions. A total of 5–10 clones were randomly selected for culturing in solution. Plasmid DNA was subsequently extracted from the culturing solution. The target gene in the extracted plasmid DNA was then sequenced with ABI 3730xl DNA Analyzer (Applied Biosystems).

2.5 Phylogenetic Analysis

The retrieved sequences were analyzed against those in GenBank with BLAST[30]. After chimeric check and aligned, all the sequences were cropped to 360/368 bases long to construct the phylogenetic tree using MEGA, version 5.1[31]. Some sequences from other studies were downloaded from GenBank as reference. The sequence of *Phellinus lamaoensis* was used as the outgroup. The phylogenetic tree was constructed with the neighbor-joining method with 1000 bootstraps to estimate the confidence of the tree topologies.

2.6 Quantitative Real-time PCR Analysis

The abundances of *Phellinus noxius* ITS genes in the soils between a dead tree and an infected tree in Kowloon Park were determined in quadruplicate with quantitative real-time PCR amplification using SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Takara, Japan). Real-time qPCR was performed in 96-well optical plates placed in the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). The primer set

composed of G1-F and G1-R was used for the amplification of target genes. The final reaction volume was 20 μL and the reaction composition and cycling conditions were in accordance with the manual.

The specificity of the PCR amplification was determined by the melting curve and the gel electrophoresis. Cycle thresholds were determined by comparing with the standard curves constructed using a 10 fold serial dilution (10^2–10^7 gene copies μL^{-1}) of newly extracted plasmids containing ITS gene fragments. Relative copy numbers among target groups were evaluated and some replicates of apparent discrepancy were excluded in order to decrease standard error. The correlation coefficient R^2 value was greater than 0.99 for the standard curve.

2.7 Scanning Electron Microscope Sample Preparation

The diseased root samples were fixed in 0.2 M sodium cacodylate for about 24 hours. The fixative was prefiltered through a 0.2 μm pore size cellulose acetate membrane filter (Toyo Roshi Kaisha Ltd., Japan). Samples were then rinsed with Milli-Q water three times. The samples were dehydrated in an ethanol-Milli-Q water series of 40, 60, 70, 80, 85, 90, 95 and 100% ethanol[32]. Samples were stored in 100% ethanol at 4°C before further treatment. The samples were cold air-dried and then coated with gold-palladium and viewed under the Hitachi S-4800 field emission scanning electron microscope.

2.8 Nucleotide Sequence Accession Numbers

The rDNA ITS sequences are available in GenBank under accession numbers KM251804 to KM251851.

3. Results

3.1 The Distribution of *Phellinus noxius* in Hong Kong

A total of 38 suspected trees were sampled and diagnosed with molecular technique from October 2013 to February 2014 in Hong Kong (Table 1). Thirteen of the trees were confirmed for infection by *P. noxius* after PCR amplification of the ITS regions of the pathogen in tree tissue samples collected from various trees. Most infected tree species were *Ficus microcarpa*; another two were *Celtis sinensis* and *Khaya senegalensis*. No specificity to a selected group of tree species can be ascertained in this study. The infected trees were distributed in different districts in Hong Kong without any apparent pattern or concentration (Figure 1).

3.2 Symptoms and Ultrastructure of Diseased Tissue Infected with *Phellinus Noxius*

The brown root rot caused by *P. noxius* was normally encrusted with a layer of mycelial mat that presented brown or dark brown on surface of roots in this study (Figure 2A). Scanning electron microscopic (SEM) micrographs showed that the surface of the mycelial mat was covered with a layer of substance so that no hyphae could be seen clearly (Figure 2B), but unidentified bacteria could be found on the surface of the mycelial mat of the tree tissue. The pathogen may invade the host tissues and reside inside without showing apparent morphological features of the fungus. Hyphae are capable of penetrating into the wood cells, and the hyphae can also form branches during growth (Figure 2C and 2D). Unknown bacteria can also be found coexisting with *P. noxius* in the diseased tissues (Figure 2C and 2D).

3.3 Phylogeny of *Phellinus noxius*

PCR amplified ITS DNA sequences of *P. noxius* obtained from the tree tissues sampled in this study were analyzed. The phylogenetic tree of *P. noxius* showed that the majority of the sequences retrieved in this study had known related sequences already obtained in other countries or districts, except those in Clade 5 (Figure 3), so the pathogen is similar to those reported elsewhere and local previously. The distribution pattern of *P. noxius* showed that the same clade included sequences from different countries or districts indicating their wide spread. Clade 1 includes sequences obtained from Taiwan, Hainan of China, Malaysia, and Hong Kong of China in this study (Figure 3). Another phenomenon was that the sequences from the same sample can fall into different clades. For example, sequences obtained from Tree003 fell into both Clade 2 and Clade 3; sequences obtained from Tree-009 fell into both Clade 1 and Clade 3; and sequences obtained from Tree027 fell into Clade 1, Clade 3, and Clade 5 (Figure 3). It seems that *P. noxius* is a pathogen with little divergence across a large geographic region but at the same time the variation among the region can be localized on a particular tree tissue. Such observation may be related to the survival strategies and also dissemination means used by the pathogen.

Table 1. Information of suspicious trees and results of molecular diagnosis

Tree code	Collecting Date	Tree species	Location	OVT[Δ] No.	*P. noxius*
Tree001	21/10/2013	*Celtis sinensis*	Kowloon Park	Non OVT	Yes
Tree002	24/10/2013	*Ficus microcarpa*	Lei Yue Mun Park	LCSD E/4	Yes
Tree003	24/10/2013	*Khaya senegalensis*	Lai Chi Kok Park	Non OVT	Yes
Tree004	24/10/2013	*Khaya senegalensis*	Lai Chi Kok Park	Non OVT	Yes
Tree005*	29/10/2013	*Ficus microcarpa*	Kowloon Park	LCSD YTM/65	Yes
Tree039*	10/02/2014				
Tree006[#]	29/10/2013				
Tree028[#]	02/01/2014	*Ficus microcarpa*	Kowloon Park	LCSD YTM/97	Yes
Tree038[#]	10/02/2014				
Tree007	30/10/2013	*Melia azedarach*	Kwai Tsing	Non OVT	No
Tree008	21/11/2013	*Ficus microcarpa*	Pokfield Road	LCSD CW/4	No
Tree009	26/11/2013	*Ficus microcarpa*	Hong Kong Observatory	Non OVT	Yes
Tree010	11/12/2013	*Ficus virens* var. *sublanceolata*	Former Central Government	LCSD CW/95	No
Tree011	11/12/2013	*Heteropanax fragrans*	Former Central Government	LCSD CW/96	No
Tree012	11/12/2013	*Ficus microcarpa*	Hong Kong Park	LCSD CW/107	No
Tree013	11/12/2013	*Ficus virens* var. *sublanceolata*	Hong Kong Park	LCSD CW/108	No
Tree014	11/12/2013	*Ficus elastica*	Hong Kong Park	LCSD CW/109	No
Tree015	19/12/2013	*Ficus microcarpa*	Nathan Road	LCSD YTM/21	No
Tree016	19/12/2013	*Ficus microcarpa*	Nathan Road	LCSD YTM/22	No
Tree017	19/12/2013	*Ficus microcarpa*	Nathan Road	LCSD YTM/27	No
Tree018	19/12/2013	*Ficus microcarpa*	Nathan Road	LCSD YTM/28	No
Tree019	19/12/2013	*Ficus microcarpa*	Nathan Road	LCSD YTM/29	No
Tree020	23/12/2013	*Ficus microcarpa*	Nathan Road	LCSD YTM/1	No
Tree021	23/12/2013	*Ficus microcarpa*	Nathan Road	LCSD YTM/17	No
Tree022	23/12/2013	*Ficus microcarpa*	Nathan Road	LCSD YTM/19	No
Tree023	23/12/2013	*Ficus microcarpa*	Lo Wai Road	LCSD TW/3	No
Tree024	23/12/2013	*Ficus microcarpa*	Tsing Yi Park	LCSD KTW/9	No
Tree025	23/12/2013	*Ficus microcarpa*	Tsing Yi Park	LCSD KTW/10	No
Tree026	02/01/2014	*Mangifera indica*	Hong Kong Park	Non OVT	No
Tree027	02/01/2014	*Ficus microcarpa*	Lam Tsuen	Non OVT	Yes
Tree029	06/01/2014	*Ficus microcarpa*	Tin Sam Garden	LCSD ST/13	Yes
Tree030	06/01/2014	*Ficus microcarpa*	Pak Fuk Children's Playground	LCSD N/38	Yes
Tree031	06/01/2014	*Ficus microcarpa*	Shui Mei Village Playground	LCSD YL/6	Yes
Tree032	06/01/2014	*Ficus microcarpa*	Tuen Mun Park	LCSD TM/5	Yes
Tree033	13/12/2013	*Phoenix canariensis*	Magic road at Penny's Bay	Non OVT	No
Tree034	15/01/2014	*Ficus microcarpa*	Essex Crescent Rest Garden	LCSD KC/3	No
Tree035	15/01/2014	*Ficus microcarpa*	Essex Crescent Rest Garden	LCSD KC/4	No
Tree036	23/01/2014	*Ficus microcarpa*	Caine Road near Ladder Street	LCSD CW/32	No
Tree037	23/01/2014	*Ficus microcarpa*	Caine Road near Ladder Street	LCSD CW/33	No
Tree040	10/02/2014	*Ficus microcarpa*	Cemetery beside Stubbs Road	Non OVT	Yes
Tree041	14/02/2014	*Gleditsia fera*	Victoria Park	LCSD E/36	No

*Tree005 and Tree039 were the same tree that was sampled twice in different days

[#]Tree006, Tree028 and Tree038 were the same tree that was sampled trice in different days

[Δ]OVT: old and valuable tree

Figure 1. Sketch map indicating locations of trees infected with *Phellinus noxius* in Hong Kong. The Arabic numbers in red are the codes of the diseased trees infected with *P. noxius*, indicating the approximate locations of the trees on the map. The detailed information about the trees are listed in Table 1.

3.4 The Occurrence of *Phellinus Noxius* in Soils

In Kowloon Park, two trees with 15 m apart were confirmed positive for infection by *P. noxius* before. One of them was dead and had been removed. In this study, soils between the dead tree and the standing diseased tree (i.e. Tree006) were assayed. Eight of the nine samples collected were positive when the genomic DNA was tested with the specific primer pair PN-1F/PN-2R (data not shown), suggesting the eight samples contained *P. noxius*. Three of the positive samples were selected for constructing clone library and all of them were later confirmed to contain *P. noxius* (Figure 3). Most of the sequences acquired from the soils were related to the sequences from the diseased tree (they all fell into Clade 1) to support the association of pathogen identity between soil and trees growing in the soil. But one sequence of the soils fell into Clade 3 that did not contain sequences from Tree006.

Through quantitative real-time PCR, the abundances of *P. noxius* in soils between the dead tree and the diseased tree were quantified. The abundances of *P. noxius* ITS gene copies were between 1.5×10^4 and 2.7×10^5 gene copies g^{-1} soil for the eight of the nine samples (Figure 4). However, no clear trend in the abundance of *P. noxius* was found for the soils between the two trees.

Another 5 soils under trees that were not infected with *P. noxius* were collected for testing the occurrence of *P. noxius*. Two soils were under Tree008 and Tree036, which were not infected with *P. noxius* through molecular analysis. The other three soils (HKU soil1, HKU soil2 and HKU soil3) were collected from three healthy trees on campus of The University of Hong Kong. The soil under Tree036 and two soils on campus of The University of Hong Kong (HKU soil2 and HKU soil3) were confirmed to contain *P. noxius* with molecular analysis (Figure 2). The other two soils (soil under Tree008 and HKU soil1) were tested negative (data not shown), indicating no *P.*

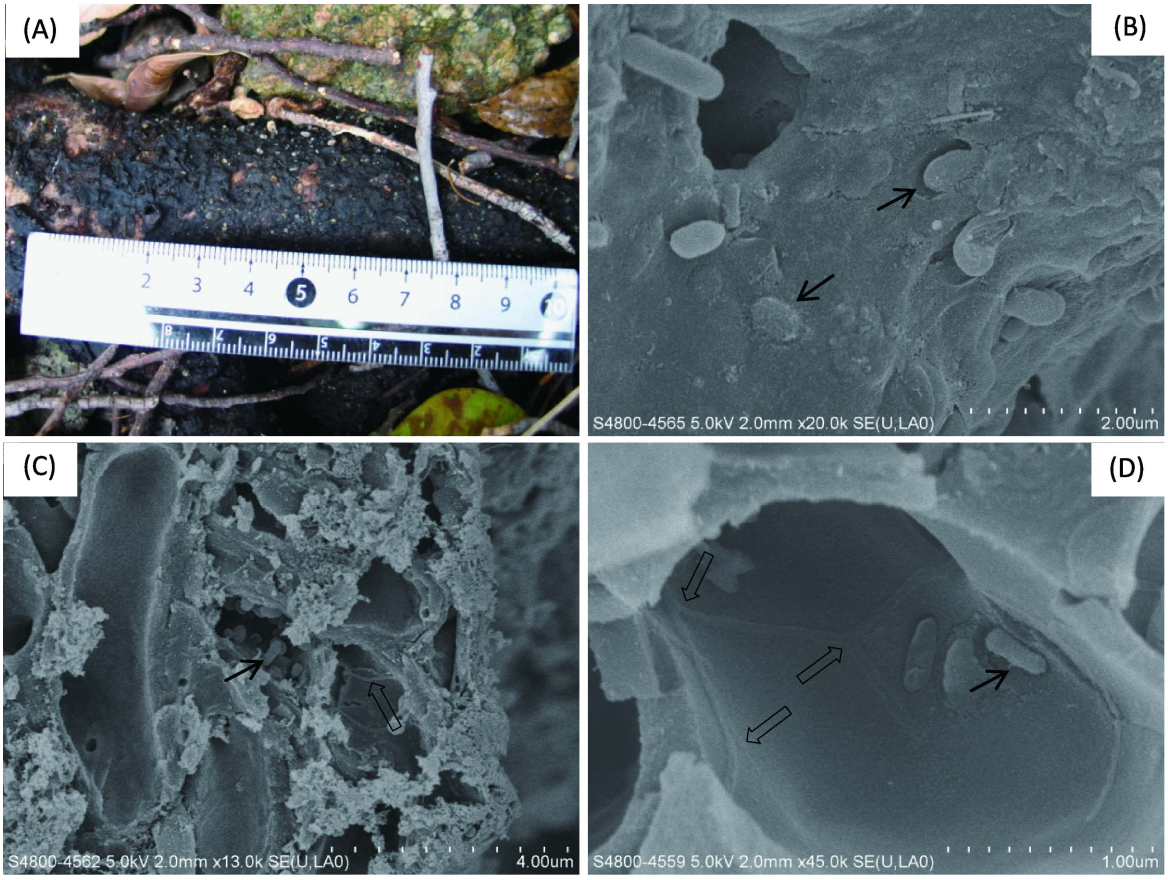

Figure 2. Symptoms of brown root rot caused by *Phellinus noxius* (**A**) and ultrastructure of diseased tissue (**B**, **C**, **D**). Picture (**A**) shows the symptoms of the root infected with *P. noxius* collected from Tree040; picture (**B**) shows the surface of mycelial mat; pictures (**C**) and (**D**) show the inner part of diseased root (⇨ indicates hyphae, → indicates bacteria that co-occurred in the diseased tissue).

noxius occurred in these two soils. These results show that the pathogen *P. noxius* is widely occurring in soils and the soil may be an important reservoir for its survival and infection.

4. Discussion

4.1 Molecular Technique for *Phellinus Noxius* Diagnosis

Traditional approach to identify *P. noxius* involves observing symptoms in combination with culturing and isolating the pathogens on agar plates[3,14]. More convincingly, researchers utilized the isolated pathogen to test pathogenicity according to the Koch's Postulates[2,18]. However, the culturing procedure is usually time-consuming and tedious, which is not practical for quick diagnosis when confirmation is the first key information in need before a decision can be made accordingly on subsequent handling. The conventional diagnosis can be simplified by omitting pathogenicity test to save time, but it still needs about two weeks to culture *P. noxius* on the defined medium agar plates. Moreover, the simplified conventional diagnosis is neither sensitive nor specific enough because of the sample size on agar plates and also the selectivity of the medium composition.

Molecular approach is a quick and accurate way to identify *P. noxius*[25,26]. When the tissue is infected with the same type of *P. noxius*, all the sequences of the PCR amplified products are identical and thus can be sequenced directly. The whole process of diagnosis normally takes only two days to reach a final conclusion. When the tissue is infected with more than two types of *P. noxius*, a clone library can be built for the PCR amplified ITS fragments, which will take about a total of one week to achieve a final conclusion. Thus, the molecular approach can shorten the time of diagnosis, which makes it a practical way for diagnosis of this pathogen. Moreover, molecular approach is sensitive, which can detect the pathogen at very low abundance

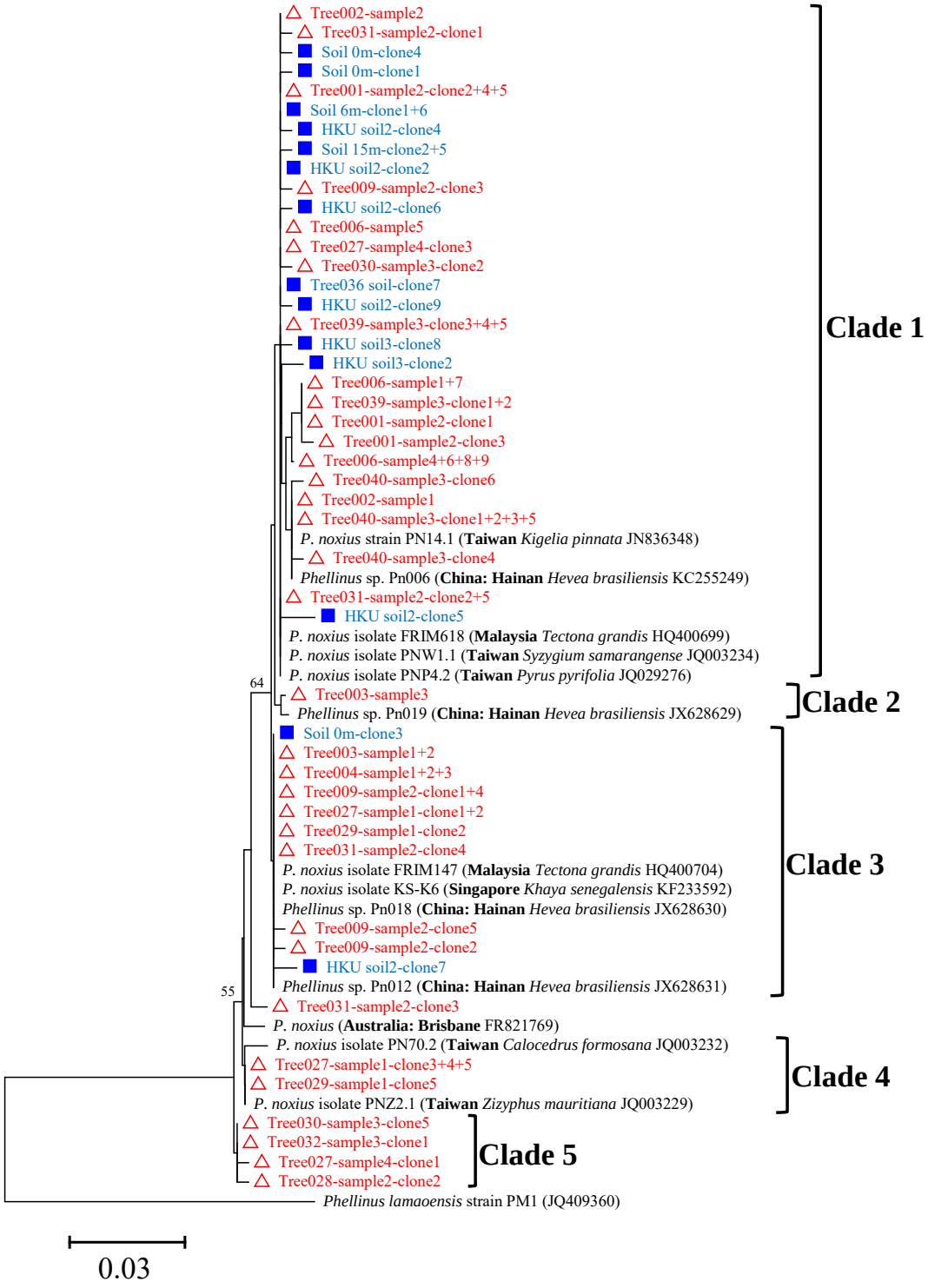

Figure 3. Phylogenetic tree of *Phellinus noxius* based on rDNA internal transcribed spacer (ITS) sequences retrieved from trees and soils in subtropical Hong Kong. The phylogenetic tree was reconstructed based on ITS sequences of 360/368 nucleotides. The corresponding sequence of *Phellinus lamaoensis* was used as the out group. If more than two identical sequences existed in one individual sample, only one sequence was used for phylogenetic analysis. For the reference sequences acquired from the GenBank data, the collection location, host, and accession number in the GenBank were indicated in the parentheses. The phylogenetic tree was constructed with the neighbor-joining method with 1000 bootstrapping to estimate the confidence of the tree topologies. Bootstrap values (> 50%) are indicated at the branch points. The scale bar represents 0.03 sequence divergence. The open triangles (Δ) in red represent tree samples, while the closed squares (■) in blue represent the soil samples.

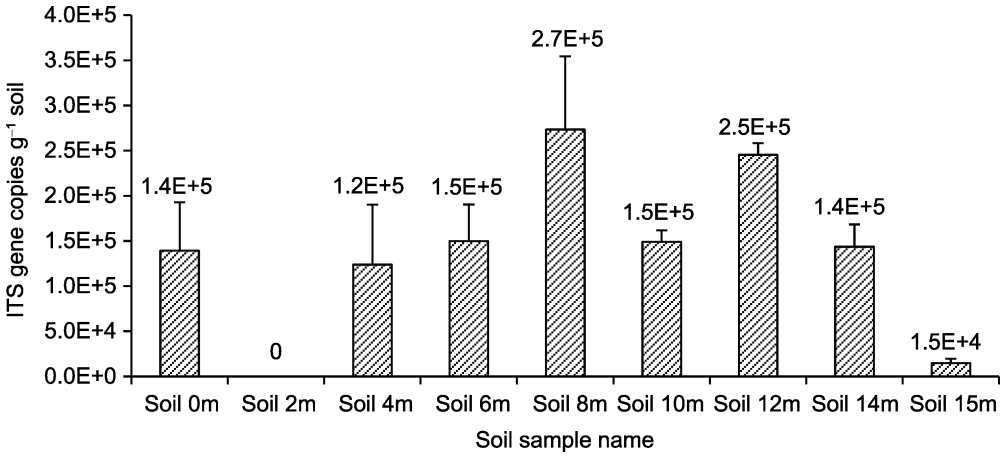

Figure 4. Internal transcribed spacer (ITS) gene copy numbers of soils between a dead tree and a diseased tree caused by *P. noxius*. The dead tree had been removed before the soils were collected. The two trees were 15 m apart and soils between the two trees were collected every 2 m. The soil names indicate the distances from the dead tree.

from complex matrices of samples. This makes it possible to identify brown root rot at its early phase when no obvious symptoms appear and the pathogen loading is low. The early diagnosis is very important for timely treatment and management. Traditional diagnosis, however, does not possess this priority features because when symptoms are obvious, the tree is usually seriously infected by *P. noxius* and effective treatments are virtually not available[2,33]. In the present study, we analyzed 38 suspected trees and found 13 trees of them were positive for infection with *P. noxius*, suggesting that symptom observation can only serve as an auxiliary means for accurate diagnosis and for this particular detection, a 66% incorrect diagnosis was resulted when only symptom observation was applied.

4.2 Dissemination Mechanism of *Phellinus noxius*

Despite *P. noxius* has been discovered for more than 8 decades[1], no agreement has been reached on the mechanism of *P. noxius* dissemination in ecosystems. The dominant hypothesis is that *P. noxius* spread through root-to-root contact[2]. One reason is that infected soils and tissues could lead to the further infection in healthy plants, which has been confirmed through experiments[13]. Another reason is that when one tree was affected with this pathogen, some surrounding tree/trees might also be infected later[3]. This hypothesis ruled out that airborne spores spreading is the main approach of dissemination because basidiocarps are rarely found in natural ecosystems[2].

In the present study, however, all evidence suggested that *P. noxius* reside in soils as a reservoir for infection and possible spread. Firstly, the diseased trees caused by *P. noxius* were distributed separately all over Hong Kong (Figure 1), suggesting that they were not infected through a single source, because if so, the diseased trees should be infected by one type of *P. noxius*. Moreover, *P. noxius* has been reported in many countries and different continents in tropical and subtropical regions, which indicated it is impossible that *P. noxius* spread only through root-to-root contact in nature and human may facilitate the spread of this pathogen through transport of seedlings legally and illegally[2]. This hypothesis needs further test to prove the mechanisms involved.

Secondly, a single diseased tree contains clones of *P. noxius* belonging to different clades of the phylogenetic tree (Figure 3), suggesting that the diseased tree was infected by different clones of *P. noxius* simultaneously or at different times. The possible reason is that the tree was infected by the pathogens in soils as the pathogens were detected in all soil samples regardless of the tree infection in this study and the microbial community is complex. In addition, our study also showed that diseased trees in separate sites can have the similar type of *P. noxius*, suggesting that these *P. noxius* might come from the same origin or source. As the diseased trees are separated distantly from each other, the spread of this pathogen might be through human or air transport of soil and spores. This viewpoint is also in line with the fact that diseased trees in different countries have the same type of *P. noxius* (Figure 3).

Thirdly, *P. noxius* was detected in the soils grown with the healthy trees in this study (Figure 3), sug-

gesting this pathogen in the soils did not come from the trees. When examining the occurrence of *P. noxius* in the soils between a dead tree and an infected one, eight of the nine soil samples between these two trees contained *P. noxius* (Figure 4). Then a question rose: was *P. noxius* in the soils really from the infected trees or the other way around? We could not answer this question directly. Instead, we further examined 5 soil samples grown with healthy trees distributed in different location in Hong Kong and three of the five soils tested positive for *P. noxius*, suggesting the pathogen is commonly residing in the soils. We hypothesize that the more plausible dissemination way of the pathogen over a long distance is through the spread of soil particles containing the spores or hyphae as the infection of *P. noxius* is always on roots or in the lower part of the trunk of trees. Thus, soils are more likely the major source of *P. noxius* to infect trees when root tips are in contact with the pathogens in spores or vegetative forms.

Although *P. noxius* may spread by airborne routes in natural systems, direct contact between the pathogen and roots and root-to-root is the key initial step before effective infection can be realized. Airborne spores might widely exist in soils, but only under selective conditions these spores can infect the trees and germinate. For example, the young root tips of the trees are most likely the entry point of infection because matured roots do not allow penetration by germinated spores unless physical damages are available. Once a tree is infected, *P. noxius* might infect other trees through root-to-root contact when extension of roots makes this possible. It is a normal phenomenon that trees are infected frequently in patches in ecosystems most likely due to the availability of pathogens and also the root-to-root contacts.

In summary, a molecular based testing was tested and observation can overestimate the brown root rot by as high as 66%. *P. noxius* found on various trees in Hong Kong may be originated from widely different sources.

Conflict of Interest and Funding

No conflict of interest is reported by the authors. This project was supported by Development Bureau of Hong Kong SAR Government (Contract No. WQ/027/13; Q072/2010). The views expressed are those of the authors and no implication for the role of government on the information reported should be made.

Acknowledgements

We would like to thank the arborist Mr. Ching Wai for assisting sample collection in the field. We are grateful to Ms. Kelly Lau for support in laboratory work.

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