Molecular Characterization and Development of Real-Time PCR Assay for Pine-Wood Nematode

Bursaphelenchus xylophilus (Nematoda: Parasitaphelenchidae)

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Abstract

Bursaphelenchus xylophilus, the pine-wood nematode (PWN), is the causal agent of pine wilt disease, one of the most damaging emerging pest problems to forests around the world. It is native to North America where it causes relatively minor damage to native conifers but is labeled an EPPO-A-2 pest and a quarantine nematode for many countries outside of the United States because of its potential for destruction to their native conifers. Exports of wood logs and commodities involving softwood packaging materials now require a lab test for the presence/absence of this regulated nematode species. We characterized the DNA sequences on the ribosomal DNA small subunit, large subunit D2/D3, internal transcribed spacer (ITS) and mitochondrial DNA cytochrome oxidase subunit one on the apherlenchid species and described the development of a real-time-PCR method for rapid and accurate identification of PWN targeting the ITS-1. A total of 97 nematode populations were used to evaluate the specificity and sensitivity of this assay, including 45 populations of B. xylophilus and 36 populations of 21 other species of Bursaphelenchus which belong to the abietinus, cocophilus, eggersi, fungivorus, hofmanni, kevini, leoni, sexdentati, and xylophilus groups and one unassigned group from a total of 13 groups in the genus Bursaphelenchus; 15 populations of Aphelenchoides besseyi, A. fragariae, Aphelenchoides species and Aphetlenchus avenueae; and one population of mixed nematode species from a soil sample. This assay proved to be specific to B. xylophilus only and was sensitive to a single nematode specimen regardless of the life stages present. This approach provides rapid species identification necessary to comply with the zero-tolerance export regulations.

Introduction

The pine-wood nematode (PWN), Bursaphelenchus xylophilus (Steiner & Buhrer, 1934) Nickle, 1970 (= B. lignicola Mamiya & Kiyohara, 1972), first recorded and described in Louisiana as Aphelenchoides xylophilus [1], is native to North America (USA, Canada and Mexico) [2,3] and is a serious invasive and destructive species to coniferous forests in countries where it has been introduced. This nematode has been considered the causal agent for pine wilt disease since 1971 [4] being transmitted from tree to tree by wood-inhabiting longhorn beetles that belong mainly to the genus Monochamus (Coleoptera: Cerambycidae) [5]. PWN was introduced in Japan at the beginning of the 20th century [6] and later in mainland China [7], Taiwan [8,9] and Korea [10] which caused massive mortality of native pine trees. PWN was first recorded in Europe (Portugal) in 1999 [11]; later on the Portuguese island of Madeira, 900 km SW from the European continent in 2010 [12]; and more recently in three locations in Spain close to the Portuguese border [13]. The international spread of PWN occurs mainly through the movement of infested logs, untreated wood products and wood-packaging material. To prevent further spread and new introductions, China considers the nematode as a quarantine organism and the European and Mediterranean Plant Protection Organization has placed it on the A2 list (EPPO, http://www.eppo.int). A2 pests are locally present in the EPPO region, and EPPO recommends that its member countries regulate them as quarantine pests. During the 1990 s, a quarantine on green lumber exports to Europe caused an estimated annual loss to the American forest industry of US $100 million [14].

The genus Bursaphelenchus currently contains nearly ninety species [15–17], which are split into 13 typological groups: namely abietinus, africanus, cocophilus, eggersi, erumeus, fungivorus, hofmanni, kevini, leoni, okinawensis, sexdentati, sinensis, xylophilus and one unassigned group [17]. The xylophilus group contains B. bangardi Walia, Negi, Bajaj & Kalia, 2003; B. comandatus Kanazaki, Tsuda & Futai, 2000;
B. trypophloei

The DNA sequences of with the issuing of phytosanitary certificates for exported pine-

specified for a wide range of species.

available, none of the tests can be implemented directly in a lab

post-PCR-agarose-gel electrophoresis. Although these methods are

Real-time PCR offers an advantage over conventional PCR in

isothermal amplification [37]; and real-time PCR [35,38–44].

spacer [32]; satellite DNA [33]; heat-shock-protein 70 [34];

identification of PWN is required in order to comply with

requires a high level of expertise [15–17] and can be very time-

Two holes, up to six inches (15 cm) deep, were drilled per log at six

inches (15 cm) from both ends using a 2.125-inch (5.4-cm), self-

feeding-wood bit. The wood shavings from two logs were mixed
together, and a minimum 200 g of wood shavings were collected as
one lab sample. Samples were shipped overnight to NCD&A&CS for

nematode analysis. Some nematode samples were collected from Europe, North America, Central America and Asia. They were reared on cultures of the fungus Monilinia fructicola on potato dextrose agar plates, except for Bursaphelenchus cocophilus (Cobb, 1919) Baujard, 1989, which was extracted from infested hosts and killed and shipped in 95% ethanol before subsequent DNA extraction.

Extraction of Nematodes

Each wood sample was weighed and assigned a unique lab ID

number. The wood shavings for each sample were placed in a

single layer inside a wire basket lined with a large, single-folded

Kimwipe (37 cm x 42 cm, Kimberly-Clark Professional, Neenah,

WI, USA) and completely wrapped. The baskets were then placed

into plastic containers (36 cm L x 24 cm W x 14 cm H). Tap water

was added until the wood shavings were completely submerged.

After incubation for 24 hours at room temperature to allow

nematodes to move out [45], the wood-containing baskets were

removed gently and the supernatant water was vacuumed out

slowly using a H2O Pro electrical pump (#50AC110B, FM

Industries, Milwaukee, WI) [45]. Then the remaining nematode

suspension was left to settle for 30 minutes at a slant, approximately 45 degrees, after which additional supernatant water was vacuumed. Approximately 100 ml of the remaining nematode solution was decanted into beakers and allowed to settle for 30 minutes. The supernatant water was then vacuumed with a water-faucet-vacuum-aspirator apparatus to approximately 20 ml. No sieve was used in the nematode extraction to avoid cross contamination between samples. The sample was poured into a counting dish (7.5 cm L x 3 cm W x 1.5 cm H), and the nematodes present were identified and counted under a Nikon Diaphot 200 inverted microscope (Tokyo, Japan). Further species confirmation was performed with a Leica DM2500 compound microscope (Leica Microsystems Inc., Buffalo Grove, IL) with interference contrast up to 1,000x magnification.

DNA Preparation

One to ten nematodes were transferred to a glass microscope slide (7.5 cm x 2.5 cm), squashed using a pipette tip in about 5 μl of AE buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0), and then placed in a 1.5-ml microtube. AE buffer was added up to 30 μl. DNA extracts were stored at -20°C until used as PCR template.
| Species (group) | Sample No. | Locality | Host | GenBank Accession Numbers | Threshold Cycle (Ct) by PWN-Specific Primer/Probe | C<sub>0</sub> by Nematode-Universal Primer/Probe |
|----------------|------------|----------|------|---------------------------|-----------------------------------------------|-----------------------------------------------|
| Bursaphelenchus abietinus (abietinus group) | 137 | Austria | Abies alba | AY508011, AY508074, AY508037 | 0 | 23.00 |
| B. abruptus (unassigned group) | 136 | MD, USA | Anthophora abrupta | AY508010, AY508073, AY508036 | 0 | 31.22, 29.18<sup>a</sup> |
| B. anatolius (kevini group) | 170 | Turkey | Halictus sp. | AY508025, AY508093, AY508056 | 0 | 24.89 |
| B. barealis (leonii group) | 138 | Germany | Picea abies | AY508012, AY508075, AY508038 | 0 | 22.41 |
| B. coccophilus (coccophilus group) | 140 | Costa Rica | Elaeis guineensis | AY508076 | 0 | 27.07 |
| B. eggersi (eggersi group) | 146 | Germany | Pinus sylvestris | AY508013, AY508078, AY508040 | 0 | 12.48 |
| B. fraudulentus (xylophilus group) | 148 | Hungary | Quercus sp. | AY508079, AY508042 | 0 | 17.98 |
| B. fungivorus (fungivorus group) | 153 | Germany | Greenhouse soil | AY508016, AY508082, AY508045 | 0 | 27.07 |
| B. gerberae (hofmanni group) | 169 | Trinidad | Cocos nucifera | AY508024, AY508092, AY508055, KF025320 | 0 | 27.96 |
| B. hoffmanni (abietinus group) | 154 | Germany | Pinus brutia | AY508017, AY508083, AY508046 | 0 | 31.94, 29.97 |
| B. hylobianum (abietinus group) | 160 | Russia | Larix sibirica | AY508019, AY508085, AY508048 | 0 | 24.38 |
| B. kevini (kevini group) | 355 | Santa Cruz Island, CA, USA | Halictus farinosus | AY508017, AY508083, AY508046 | 0 | 24.44 |
| B. mucronatus (xylophilus group) | 163 | Norway | Pinus sylvestris | AY508018, AY508084, AY508047 | 0 | 23.00 |
| B. paracorneolus (hofmanni group) | 172 | Germany | Picea abies | AY508022, AY508090, AY508053 | 0 | 29.08, 29.97, 32.71 |
| B. platzeri (coccophilus group) | 171 | CA, USA | Carphophilus humeralis | AY508023, AY508091, AY508054, KF025318 | 0 | 24.72, 32.54, 23.04 |
| B. poligraphi (sexdentati group) | 173 | Germany | Picea abies | AY508028, AY508096, AY508059 | 0 | 30.30, 27.19 |
### Table 1. Cont.

| Species (group) | Sample No. | Locality | Host | GenBank Accession Number | Threshold Cycle (Ct) by PWN-Specific Primer/Probe | C_t by Nematode-Universal Primer/Probe |
|----------------|------------|----------|------|--------------------------|-----------------------------------------------|--------------------------------------|
|                |            |          |      | SSU LSU mtCOI ITS        |                                               |                                      |
| *B. rufipennis* (hofmanni group) | 727        | WI, USA  | Spruce bark beetle (Dendroctonus rufipennis) from Picea sp. | AY508097 AY508060 0 22.17 |                                               |                                      |
| *B. seani* (fungivorus group)   | 174        | CA, USA  | Anthophora bomboides | AY508097 AY508060 0 25.25 |                                               |                                      |
|                                | 175        | CA, USA  | Anthophora bomboides | AY508097 AY508060 0 31.47 |                                               |                                      |
| *B. sexdentati* (sexdentati group) | 176        | CA, USA  | Anthophora bomboides | AY508097 AY508060 0 24.7 |                                               |                                      |
|                                | 177        | Greece   | Pinus nigra   | AY508100 AY508063 0 25.35 31.46 |                                               |                                      |
|                                | 178        | Greece   | Pinus nigra   | AY508097 AY508060 0 24.87 |                                               |                                      |
|                                | 179        | Greece   | Pinus radiata | AY508031 AY508065 0 23.93 |                                               |                                      |
|                                | 180        | Italy    | Pinus pinea   | AY508032 AY508066 0 29.08 |                                               |                                      |
| *B. tusciae* (eggersi group)    | 183        | Italy    | Pinus pinea   | AY508033 AY508067 0 27.68 |                                               |                                      |
| *B. xylaphillus* (xylaphillus group) | 185        | Canada   | Pinus banksiana | AY508015 AY508068 KF025325 15.98 16.36 |                                               |                                      |
|                                | 186        | Japan    | Pinus densiflora | AY508034 AY508069 KF025326 10.14 13.37 15.18 |                                               |                                      |
|                                | 187        | NB, Canada | Pine tree     | AY508017 AY508070 KF025327 10.53, 8.69, 12.02 12.80 |                                               |                                      |
|                                | 188        | QC, Canada | Pinus/Picea   | AY508018 AY508071 KF025328 10.27 14.97 |                                               |                                      |
|                                | 345        | MO, USA  | Pinus sylvestris | KF025324 24.62 29.18 |                                               |                                      |
| N18                          | China      |          | Pinus kesiya  | KF025321 27.86, 27.93 28.73, 25.16 |                                               |                                      |
| 2008-00610                    | USA        |          | Pine tree     | KF025321 27.86, 27.93 28.73, 25.16 |                                               |                                      |
| 2008-01182                    | USA        |          | Pine tree     | KF025321 27.86, 27.93 28.73, 25.16 |                                               |                                      |
| 2008-12108                    | USA        |          | Wood chips    | KF025321 27.86, 27.93 28.73, 25.16 |                                               |                                      |
| 2008-12140                    | USA        |          | Wood chips    | KF025321 27.86, 27.93 28.73, 25.16 |                                               |                                      |
| 2008-26471                    | USA        |          | Wood chips    | KF025321 27.86, 27.93 28.73, 25.16 |                                               |                                      |
| 2008-26479                    | USA        |          | Wood chips    | KF025321 27.86, 27.93 28.73, 25.16 |                                               |                                      |
| 2008-26522                    | USA        |          | Wood chips    | KF025321 27.86, 27.93 28.73, 25.16 |                                               |                                      |
| 2009-00185                    | USA        |          | Wood chips    | KF025321 27.86, 27.93 28.73, 25.16 |                                               |                                      |
| 2009-00211                    | USA        |          | Wood chips    | KF025321 27.86, 27.93 28.73, 25.16 |                                               |                                      |
| Species (group) | Sample No. | Locality | Host | GenBank Accession Number | Threshold Cycle (Ct) by PWN-Specific Primer/Probe | C<sub>q</sub> by Nematode-Universal Primer/Probe |
|----------------|------------|----------|------|--------------------------|-----------------------------------------------|------------------------------------------|
|                | 2009-00251 | USA      | Wood chips | KF025321 | 20.40, 20.96 | 21.00 |
|                | 2009-00740 | USA      | Pine tree | KF025321 | 21.91, 22.54, 23.55 | 22.28 |
|                | 2009-01010 | USA      | Wood chips | KF025321 | 28.81 | 23.58 |
|                | 2009-01042 | Beaufort, NC, USA | Pine tree | KF025321 | 21.24, 28.56 | 22.77 |
|                | 2009-12070 | USA      | Wood chips | KF025321 | 24.56 | 27.43 |
|                | 2009-23917 | USA      | Pine tree | KF025321 | 22.33 | 25.05 |
|                | 2010-00489 | Beaufort, NC, USA | Japanese black pine | KF025321 | 24.83, 25.61 | 31.98 |
|                | 2010-00695 | Wilmington, NC, USA | Japanese black pine | KF025317 | 24.14 | 24.56 |
|                | 2012-05740 | Emerald Isle, NC, USA | Japanese black pine | KF025321 | 20.86, 22.88 | 22.40 |
|                | 2012-08812 | Seven Spring, NC, USA | Pine-wood log | KF025321 | 28.81 | 22.87 |
|                | 2012-19124 | Atlantic Beach, NC, USA | Japanese black pine | KF025330 | 20.86, 21.91, 22.04, 23.58, 23.95, 25.97 | 23.26, 23.96, 24.95, 25.80 |
|                | 2012-33423 | USA      | Pine-wood log | KF025321 | 24.22, 26.61 | 26.63, 31.32 |
|                | 2012-33666 | USA      | Pine-wood log | KF025323 | 24.21 | 24.58 |
|                | 2012-34017 | USA      | Pine-wood log | KF025323 | 28.00 | 22.67 |
|                | 2013-00850 | USA      | Pine-wood log | KF025323 | 24.49 | 22.82 |
|                | 2013-00930 | USA      | Pine-wood log | KF025319 | 21.96 | 21.49 |
|                | 2013-02091 | USA      | Pine-wood log | KF025321 | 22.84 | 22.58 |
|                | 2013-02478 | USA      | Pine-wood log | KF025321 | 29.96 | 26.18 |
|                | 2013-03266 | USA      | Pine-wood log | KF025321 | 30.40 | 30.19 |
| Species (group) | Sample No. | Locality | Host | SSU | LSU | mtCOI | ITS | Threshold Cycle (Ct) by PWN-Specific Primer/Probe | C<sub>t</sub> by Nematode-Universal Primer/Probe |
|----------------|------------|----------|------|-----|-----|-------|-----|-----------------------------------------------|-----------------------------------------------|
| Aphelenchoides besseyi | 98 | FL, USA | Strawberry (Fragaria ananassa) | AY508035 | AY508109 | AY508072 | 0 | 23.77, 30.44 |
| Aphelenchoides fragariae | 2010-02011 | Raleigh, NC, USA | Ornamental plant | | | | 0 | 22.39 |
| | 2010-02016 | Raleigh, NC, USA | Ornamental plant | | | | 0 | 21.65 |
| | 2012-08056 | Raleigh, NC, USA | Fern (Woodia obtusa) | | | | 0 | 27.91, 29.14 |
| | M112 | Raleigh, NC, USA | Lantana | | | | 0 | 31.67 |
| Aphelenchoides sp. | 757 | NC, USA | Soil around pine tree | | | | 0 | 22.04, 30.18 |
| | 2008-01707 | USA | Pine tree | | | | 0 | 27.83 |
| | 2010-00129 | Crane, IN, USA | Pine-wood-packaging material | | | | 0 | 22.37 |
| | 12-6370 | Crane, IN, USA | Pine-wood-packaging material | KF032031 | KF032032 | KF032031 | 0 | 29.19 |
Polymerase Chain Reaction (PCR) and DNA Sequencing

PCR for ribosomal DNA near-full-length-small subunit (SSU), ITS and cytochrome-oxidase-gene subunit I (mtCOI) amplification was conducted using various combinations of universal forward and reverse primers (Table 2). These primers were based on the conserved sites from a multiple alignment of many *Bursaphelenchus* species and some aphelenchids from GenBank and their approximate positions were shown in Fig. 1. The primer selection criteria were as follows: Tm (melting temperature) 55 to 60°C, primer length 18 to 22 bp, and absence of secondary structure when possible. Primers for partial ribosomal-DNA-large-subunit D2/D3 (LSU D2/D3) were forward-primer D2a (5' ACAAGTACCGTGAGGGAAAGT 3') and reverse-primer D3b (5' TGCCGAAGGAACCGAGCTACTA 3') [46]. The 25-μl PCR was performed using Apex-Taq-red-master-mix DNA polymerase (Genesee Scientific Corporation, San Diego, CA, USA) according to the manufacturer’s protocol in a Veriti™ thermocycler (Life Technologies, Carlsbad, CA). The thermal cycler program for PCR was as follows: denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min. PCR products were cleaned using ExoSap-IT (Affymetrix, Inc., Santa Clara, CA, USA) according to the manufacturer’s protocol and were sequenced by Genomic Sciences Laboratory in North Carolina State University using a 3730 XL DNA Analyzer (Life Technologies, Carlsbad, CA). The molecular sequences were compared with other nematode species available at the GenBank sequence database using the BLASTn homology search program. The sequences were deposited into GenBank database.

Real-time PCR

A fluorescent probe (BxITSP) specific for PWN targeting ITS1 was labeled with reporter dye 6-carboxy-fluorescein (FAM) (518 nm maximum emission) at the 5’ end, and the 3’ end was modified with nonfluorescent quencher (NFQ) (Table 2). The forward and reverse primer sequences (BxITSF, BxITSR) yield an amplicon of 140 bp. The design of this probe and these primers (BxITSF, BxITSP, BxITSR) is based on a multiple alignment of ITS1 sequences of some representative species of *Bursaphelenchus* species from GenBank and our sequences. These sequences included all 13 species in the *xylophilus* group, except *B. baujardi*. The 21-bp probe sequence is PWN-specific, providing 100% identity with 100% coverage for 52 PWN sequences from GenBank using BLASTn search but low matches for all other nematode species included all available species in the *xylophilus* group. Except *B. bayardi*. The 21-bp probe sequence is PWN-specific, providing 100% identity with 100% coverage for 52 PWN sequences from GenBank using BLASTn search but low matches for all other nematode species included all available species in the *xylophilus* group. Fig. 2 shows the alignment and priming sites of two representative sequences of *Bursaphelenchus* species from GenBank and our sequences. These sequences included all 13 species in the *xylophilus* group, except *B. baujardi*. The 21-bp probe sequence is PWN-specific, providing 100% identity with 100% coverage for 52 PWN sequences from GenBank using BLASTn search but low matches for all other nematode species included all available species in the *xylophilus* group. 21-bp probe sequence is PWN-specific, providing 100% identity with 100% coverage for 52 PWN sequences from GenBank using BLASTn search but low matches for all other nematode species included all available species in the *xylophilus* group. Fig. 2 shows the alignment and priming sites of two representative sequences of *Bursaphelenchus* species from GenBank and our sequences. These sequences included all 13 species in the *xylophilus* group, except *B. baujardi*. The 21-bp probe sequence is PWN-specific, providing 100% identity with 100% coverage for 52 PWN sequences from GenBank using BLASTn search but low matches for all other nematode species included all available species in the *xylophilus* group.
forward primer, 18 µl of 100-µM reverse primer, 5 µl of 100-µM probe and 159 µl of 1× TE buffer.

The second nematode-universal primer/probe set (Ne18SF, Ne18SP and Ne18SR) was designed based on the conserved sites of SSU from a multiple alignment of 54 species of nematodes in the genera *Anguina*, *Aphelenchoidea*, *Aphelenchus*, *Aseris*, *Bursaphelenchus*, *Caenorhabditis*, *Cephalobus*, *Cryptaphelenchus*, *Ditylenchus*, *Ekathaphelenchus*, *Globodera*, *Heterodera*, *Laimaphelenchus*, *Longidorus*, *Meloidogyne*, *Myolaimus*, *Pristionchus*, *Rhabdaphelenchus*, *Schistaphelenchus* and *Seinura* as an internal positive control. The double-quenched probe Ne18SP is labeled with a different dye HEX (536-nm maximum emission) to allow for duplex-real-time PCR. Real-time PCR SciTool (PrimeQuest and OligoAnalyzer) by Integrated DNA Technologies, Inc. (Coralville, IA, USA) (http://www.idtdna.com/Scitools/Applications/RealTimePCR/) was used for primer and probe design. The primers amplify a 142-bp region of ribosomal DNA SSU for a variety of nematode species. These primers and probe were synthesized by Integrated DNA Technologies, Inc. and prepared in 10^(-6) working solution.

### Table 2. PCR Primers and Real-Time PCR Primers and Probes.

| No. | Primer   | Gene | Direction[a] | Sequence                                                                 |
|-----|----------|------|--------------|---------------------------------------------------------------------------|
| 1   | B18S1F[b] | SSU  | F            | ATACGCATGTCTAAGTGGAG                                                      |
| 2   | B18S570F  | SSU  | F            | GCAGGATTACTTTGAAGGCTC                                                    |
| 3   | B18S750F  | SSU  | F            | AAATCGTGAGCGGTAGCC                                                       |
| 4   | B18S750R  | SSU  | R            | GTGAGCCGGTCGAAAGCC                                                       |
| 5   | B18S930F  | SSU  | F            | AATTCTGGAGCCGTGGAG                                                       |
| 6   | B18S930R  | SSU  | R            | CTGCTACGGTGACGGA                                                         |
| 7   | B18S1000F | SSU  | F            | GTCAGAGGGTCGAGGG                                                        |
| 8   | B18S1000R | SSU  | R            | CCGCTCGAAGCTCT                                                       |
| 9   | B18S1300F | SSU  | F            | GCATGCCGTTCTCTAGTT                                                      |
| 10  | B18S1480F | SSU  | F            | GGGTGACGCGTGGG                                                        |
| 11  | B18S1480R | SSU  | R            | ATGTGACGCGTGGG                                                        |
| 12  | B18S1820R | SSU  | R            | CTGCTACGGTGACGGA                                                         |
| 13  | BITSF     | ITS  | F            | ATCCGCTGGCTGAAACG                                                       |
| 14  | B58SF     | ITS  | F            | AATCCGACTGAGG                                                          |
| 15  | B58SR1    | ITS  | R            | CTCATAATCTGTAATC                                                       |
| 16  | B58SR2    | ITS  | R            | AACTACCCCTCTGGA                                                       |
| 17  | BITS2R    | ITS  | R            | TCTCTGCTCTAGTT                                                                         |
| 18  | BCOIF     | mtCOI| F            | GGTGTTITGTTGAAT                                                         |
| 19  | BCOIR     | mtCOI| R            | AACTAACTAATAC                                                                  |
| 20  | Ne18SF    | SSU  | F            | ATGTGACGCGGAGG                                                         |
| 21  | Ne18SP    | SSU  | F            | 5'--/SHEX/TGCCCTTA/ZEN/ATGTGACGACCCGG/3IABkFQ/3--3'                     |
| 22  | Ne18SR    | SSU  | R            | GAAGGCAGTCGAC                                                        |
| 23  | BxITSF    | ITSI | F            | GATGCCCCCTGATTG                                                        |
| 24  | BxITSF    | ITSI | R            | FAM AACTCAAAACAGCAGTAGA MGBNFQ                                         |
| 25  | BxITSR    | ITSI | R            | TGGCTGGCTCTATCTG                                                        |

[a]: F: forward, R: reverse. [b]: number after 18S represents the relative primer position in rDNA SSU gene.

The second nematode-universal primer/probe set (Ne18SF, Ne18SP and Ne18SR) was designed based on the conserved sites of SSU from a multiple alignment of 54 species of nematodes in the genera *Anguina*, *Aphelenchoidea*, *Aphelenchus*, *Ascaris*, *Bursaphelenchus*, *Caenorhabditis*, *Cephalobus*, *Cryptaphelenchus*, *Ditylenchus*, *Ekathaphelenchus*, *Globodera*, *Heterodera*, *Laimaphelenchus*, *Longidorus*, *Meloidogyne*, *Myolaimus*, *Pristionchus*, *Rhabdaphelenchus*, *Schistaphelenchus* and *Seinura* as an internal positive control. The double-quenched probe Ne18SP is labeled with a different dye HEX (536-nm maximum emission) to allow for duplex-real-time PCR. Real-time PCR SciTool (PrimeQuest and OligoAnalyzer) by Integrated DNA Technologies, Inc. (Coralville, IA, USA) (http://www.idtdna.com/Scitools/Applications/RealTimePCR/) was used for primer and probe design. The primers amplify a 142-bp region of ribosomal DNA SSU for a variety of nematode species. These primers and probe were synthesized by Integrated DNA Technologies, Inc. and prepared in 10^(-6) working solution.

The 10-µl real-time PCR contained 5 µl of 2× TaqMan® real-time PCR master mixes (Life Technologies), 1 µl of 10× primer and probe mix, 3-µl water and 1-µl DNA template. This provides for 900 nM of each primer and 250 nM of the probe at the final 1× concentration. A two-step, thermal-cycling program was used: denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min in Applied Biosystems® 7500 Real-Time PCR Systems (Life Technologies).

![Figure 2](https://example.com/f2.png)

**Figure 2.** Primer (BxITSF, BxITSR) and probe (BxITSP) design based on the multiple alignment of ITS1 DNA sequences of *Bursaphelenchus species*. Data only show two representative species, *B. xylophilus* (EU259322) and *B. mucronatus* (DQ841162).

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One sample (2013–33795) with three life stages (single female, single male and single juvenile) in separate tubes was prepared in 50-μl AE buffer, and a 5-, 10- and 20-fold dilution was prepared and tested for the sensitivity of the real-time PCR assay by PWN-specific primer and probe mix.

Duplex-Real-time PCR

The real-time PCR contained 5 μl of 2× TaqMan® real-time PCR master mixes, 1 μl of 10× PWN-specific primer and probe mix, 1 μl of 10× nematode-universal primer and probe mix, 2-μl water and 1-μl DNA template. The same two-step, thermal-cycling program was used.

Results and Discussion

Morphological Identification of PWN

PWN is a gonochoristic species that can be typologically characterized by the presence of a vulva flap and broad tail with rounded tip in the female, and large, arcuate spicules in the male that are trapezoidal in lateral view, with a sharply pointed prominent rostrum and cucullus (disc-like projection) at the distal ends. Other characters and measurements are available from the original description by Mamiya & Kiyohara [5] and EPPO diagnostic standard [45]. A quick guide for distinguishing PWN from other wood-inhabiting aphelenchids is presented in Fig. 3. Typological species identification is not so challenging for wood logs and products for export from the USA because none of the

Figure 3. Morphological comparisons between *Bursaphelenchus xylophilus* (A-C) and other wood-inhabiting aphelenchids (D-M). A. Posterior female end showing vulva flap, anus and blunt tail. B. Male spicule and tail end. C. Female tail showing mucro. D-E. Vulva without flap. F-H. Female pointed tail. I-M. Spicule and tail end of male. doi:10.1371/journal.pone.0078804.g003
other closely related species in the xylophilus group are known to be present in the USA.

**DNA Sequencing**

The ribosomal DNA SSU, LSU D2/D3, ITS and mitochondrial-DNA COI were sequenced, and their accession numbers from GenBank are presented in Table 1. Some of data were collected from our previous study [47]. Sequencing analysis revealed PWN has unique sequences in all these markers and is closest to its sister species B. mucronatus. Molecular phylogenetic relationships of Bursaphelenchus species are available in Ye et al. [47] and Kanzaki et al. [20]. The multiple sequence alignment revealed the protein-coding-gene mtCOI has no insertion/deletions, but only site variations, except for B. cocophilus with a 3-bp deletion. SSU and LSU D2/D3 have few insertions/deletions and some site variations, and ITS is the most variable with considerable insertions/deletions and site variations. Therefore, ITS1 was chosen as the real-time-PCR marker to ensure the specificity. The primer/probe design based on the variable sites is shown in Fig. 2.

**Real-time PCR**

Using the PWN-specific-primer/probe set, all assays were 100% specific and accurate for the detection of PWN with a FAM threshold cycle (Ct value) from 8.69–30.94. No fluorescent signals were obtained for samples other than PWN. The

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**Amplification Plot**

Figure 4. Example of a real-time-PCR result for testing sample 2013-33423 by PWN-specific- and nematode-universal- primer/probes.
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**Multicomponent Plot**

Figure 5. Multi-component plot of a real-time-PCR result. This plot shows increased reference dye ROX (Curve 2) and non-increased ROX (Curve 3), increased reporting dye HEX (Curve 1) and non-increased HEX (Curve 4) for Bursaphelenchus mucronatus (sample 167, Curves 1 and 2) and negative control (Curves 3 and 4).
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nematode-universal primer/probe set showed all PWN samples and all other non-PWN samples to be positive for the presence of nematode SSU with HEX Ct from 12.48–32.99 (Table 1). This nematode-universal marker also works for a number of mixed species from the soil nematode sample (2013–33843) from a centipede lawn. Fig. 4 is the amplification plot of an example assay to test sample 2012–33423. In this assay, samples 2008–12108, 2009–00211 and 2012–19124, which were previously identified as PWN, were used as positive controls. Water was used as the negative control. This assay revealed the single female and single male of 2012–33423 were positive using PWN-specific primer/probe, and the single female was positive using the nematode-universal primer/probe. The fact that all three positive controls were positive and the negative control was negative demonstrates that the reactions were successful and valid. If a sample tested PWN-negative, it then should be nematode-positive to be considered a valid assay. If it tested nematode-negative, the nematode DNA preparation and/or real-time PCR should be repeated. In any assay, the multi-component plot was examined to ensure the reaction was set up correctly and the reaction mix had not evaporated during the approximately 90-minute-long-PCR amplification.

Fig. 5 is an example of a test showing one sample 167 (curves 1 and 2) and negative control (curves 3 and 4) using the nematode-universal primer/probe. The starting fluorescence of ROX in red (curves 2 and 3) were close to each other at ca 220,000, and the starting fluorescence of HEX in green (curves 1 and 4) was ca 74,000. This revealed the pipetting for adding 2× TaqMan® real-time PCR master mixes containing background-dye ROX and adding primer/probe containing reporting-dye HEX was approximately even. At the end of the amplification at cycle 40, the ROX (curve 2) on sample 167 had increased to 250,000, indicating evaporation in the reaction tube due to problems with the cap seal. The ROX (curve 3) on the negative control remained the same, demonstrating that no evaporation occurred. The reporting-dye HEX (curve 1) on sample 167 increased considerably to 475,000, indicating a positive result. Lack of amplification in the negative control as indicated by the HEX (curve 4) corroborates the validity of the test. In this assay, although the reaction tube of sample 167 was not completely enclosed, the amplification of a positive result was still considered successful and valid, and the fluorescent signal of HEX could be normalized automatically by the reference to the ROX dye in the amplification plot. In any test, the multi-component plot should be reviewed and all ROX curves in a test should be without an increase ideally, and any test with odd results should be repeated.

The use of the PWN-specific primer/probe with real-time PCR yielded similar results regardless of the life stages used (Table 3). The test could detect a single nematode with a tiny amount of nematode DNA, demonstrated by testing 1/5, 1/10 and 1/20 dilutions, but the dilution extended the Ct value up to three cycles when diluted to 1/20× (Fig. 6, Table 3). This result revealed the real-time PCR is highly sensitive, i.e., it can detect a 1-ml nematode template even if a single nematode was squashed and dissolved in 1,000 ml of buffer. This highly diluted DNA template is sufficient to run many molecular tests and replicates. In a real-world application, a single nematode would always be available for preparation in 50 ml buffer or even up to 1,000 ml buffer for real-time-PCR assay.

### Duplex-Real-time PCR

Duplex-real-time PCR was performed on a subset of samples including 12 nematode species and 15 populations of PWN from a single nematode (female, male or juvenile) up to 20 nematodes (Table 4). All samples were positive regardless of the nematode species with the internal-positive-control marker using the nematode-universal primer/probe, but only positive for PWN.

**Table 3.** Real-time PCR results for nematode sample no. 2013–33795 with different life stages and dilutions.

| Life stage | Dilution | 1× | 1/5× | 1/10× | 1/20× |
|------------|----------|----|------|-------|-------|
| 1 female   | 28.93    | 29.34| 32.83| 32.40 |
| 1 male     | 29.89    | 33.28| 33.24| 33.99 |
| 1 juvenile | 28.74    | 30.40| 29.63| 31.06 |

Figure 6. Amplification plot of a real-time-PCR result with different dilutions of a male of *Bursaphelenchus xylophilus* (2013–33795).

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samples using the PWN-specific primer/probe (Table 4). A positive amplification in duplex-real-time PCR is represented by two sigmoid curves (PWN dye and nematode dye) in the amplification plot (Fig. 7A) and two sigmoid curves (PWN dye and nematode dye) and no increase in the ROX curve (Fig. 7B) in a multi-component plot. A negative amplification in duplex-real-time PCR is represented by one sigmoid curve (nematode dye) as an internal-positive control, but no amplification in PWN dye in the amplification plot (Fig. 7C) and one sigmoid curve (nematode dye), no increase in the ROX curve and no increase in the PWN curve (Fig. 7D) in the multi-component plot. This duplex PCR further confirmed that the assay is sensitive to any stage of nematode and is sensitive to a single nematode. In a few cases, even in a negative control, a later amplification was observed with C\textsubscript{T} greater than 35 (Fig. 7E), but the amplification of the reporting dye was not strong (Fig. 7F). This false-positive result is probably due to primer-dimmer formation and/or degradations of the probe.

| Species                  | Sample No. | Number of Nematode and Life Stage | Threshold Cycle (C\textsubscript{T}) by PWN-Specific Primer/Probe | C\textsubscript{T} by Nematode-Universal Primer/Probe |
|--------------------------|------------|-----------------------------------|---------------------------------------------------------------|-----------------------------------------------------|
| Bursaphelenchus abruptus | 136        | 10 nematodes                      | 0                                                             | 31.23                                               |
| B. anatolius             | 170        | 10 nematodes                      | 0                                                             | 27.83                                               |
| B. fraudulentus          | 150        | 10 nematodes                      | 0                                                             | 26.47                                               |
| B. gerberae              | 169        | 10 nematodes                      | 0                                                             | 30.44                                               |
| B. hylobianum            | 160        | 10 nematodes                      | 0                                                             | 27.33                                               |
| B. mucronatus            | 163        | 1 female                          | 0                                                             | 31.60                                               |
|                          | 167        | 1 female                          | 0                                                             | 31.64                                               |
|                          | 168        | 1 female                          | 0                                                             | 27.16                                               |
| B. paracornelos          | 172        | 10 nematodes                      | 0                                                             | 30.12                                               |
| B. tusciae               | 183        | 10 nematodes                      | 0                                                             | 30.00                                               |
| B. xylophilus            | 345        | 1 male                            | 31.99                                                         | 29.44                                               |
|                          | 2009-00251 | 10 nematodes                      | 23.90                                                         | 23.09                                               |
|                          | 2009-00740 | 10 nematodes                      | 23.06                                                         | 22.98                                               |
|                          | 2010-00489 | 5 nematodes                       | 26.06                                                         | 26.23                                               |
|                          | 2009-01010 | 6 nematodes                       | 32.33                                                         | 32.16                                               |
|                          | 2012–19124 | 1 juvenile                        | 26.25                                                         | 26.35                                               |
|                          | 2012–33423 | 1 female                          | 29.95                                                         | 26.79                                               |
|                          | 2013–09254 | 20 nematodes                      | 27.64                                                         | 26.66                                               |
|                          | 2013–33795 | 1 male                            | 30.45                                                         | 31.04                                               |
|                          | 2013–33795 | 7 nematodes                       | 25.02                                                         | 25.55                                               |
|                          | 2013–34160 | 3 nematodes                       | 23.91                                                         | 23.34                                               |
|                          | 2013–34252 | 2 nematodes                       | 27.84                                                         | 25.57                                               |
|                          | 2013–34362 | 7 nematodes                       | 26.92                                                         | 24.95                                               |
|                          | 2013–34693 | 10 nematodes                      | 25.94                                                         | 25.23                                               |
|                          | 2013–34814 | 1 female                          | 22.99                                                         | 22.18                                               |
|                          | 2013–34973 | 6 nematodes                       | 26.43                                                         | 25.08                                               |
| Aphelenchoides besseyi   | 98         | 10 nematodes                      | 0                                                             | 30.37                                               |
| Aphelenchoides sp.       | 2013–34187 | 1 female                          | 0                                                             | 28.96                                               |
|                          | 2013–34814 | 1 female                          | 0                                                             | 28.68                                               |
| Mixed species            | 2014–33843 | Many nematodes                    | 0                                                             | 26.13                                               |

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In conclusion, this study characterized DNA sequences on ribosomal DNA LSU, SSU D2/D3, ITS and mtCOI on a wide range of species in Bursaphelenchus and other aphelenchids. Universal primers were developed to perform DNA sequencing on this group of nematodes. Through extensive DNA analysis of these genes, ITS1 was chosen as the marker to develop PWN-real-time PCR. All assays were highly robust and specific for detection of PWN and sensitive for a single nematode regardless of the life stage. This real-time-PCR assay has been successfully applied in export and diagnostic assay services in a high-throughput-nematode-assay lab. One nematode prepared in 50 μl of DNA template provided sufficient material for molecular diagnosis. It is extremely sensitive even when a single nematode was prepared in 1,000 μl of buffer, which allowed numerous replications and long-term storage in the freezer for future confirmation and reference. Compared with other real-time-PCR applications [35,38–44], this assay tested more species in Bursaphelenchus which include many representative species in nine groups from a total of 13 groups in
the genus *Bursaphelenchus*, and more populations of PWN, especially American populations where the nematode originated, and other aphelenchid species. Both PWN-specific and nematode-universal-primer/probe sets using different fluorescent dyes were developed, and the test could be implemented through either simplex- or duplex-real-time PCR. The nematode-universal-primer/probe set for real-time-PCR amplification was included as a nematode endogenous control to detect the presence of nematode-ribosomal-SSU gene, so that a PWN-negative sample can still be evaluated to exclude false negatives due to instrument, pipetting, reagent, and/or reaction failure. In addition, many of the real-time-PCR results were further confirmed by DNA sequencing with GenBank accession numbers (Table 1). This real-time-PCR assay is rapid (<3 h) and therefore ensures a short turnaround time for phytosanitary certification. If a sample of any of these tests is negative for PWN, this study provided a PCR and DNA sequencing approach on ribosomal DNA SSU, LSU D2/D3, ITS and mtCOI genes to help determine the species. 

**Author Contributions**

Conceived and designed the experiments: WY RMGD. Performed the experiments: WY. Analyzed the data: WY. Contributed reagents/materials/analysis tools: WY RMGD. Wrote the paper: WY RMGD.

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**Figure 7. Duplex real-time-PCR result.** A. A positive result with two sigmoid FAM and HEX curves in amplification plot. B. A positive result with two sigmoid FAM and HEX curves and non-increased ROX in multi-component plot. C. A negative result with one sigmoid HEX curve, and non-increased FAM curve and non-increased ROX in amplification plot. D. A negative result with one sigmoid HEX curve, and non-increased FAM curve and non-increased ROX in multi-component plot. E. A false-positive result with a slightly later-increased HEX curve (Ct>35) in amplification plot. F. A false-positive result with a slightly later-increased HEX curve (Ct>35) and non-increased FAM and ROX curves in multi-component plot. doi:10.1371/journal.pone.0078804.g007
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