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Pinewood Nematode Assay and Development of Real-Time PCR for Species Identification in North Carolina Department of Agriculture & Consumer Services

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Bursaphelenchus xylophilus, the pinewood nematode (PWN), is the causal agent of pine wilt disease, one of the most damaging emerging pest problems to forests around the world. PWN was introduced in Japan at the beginning of the 20th century (Yano 1913) and later in mainland China (Cheng *et al*. 1983), Taiwan (Chang & Lu 1996) and Korea (Yi *et al*. 1989), causing massive mortality of native pine trees. PWN was first recorded in Europe (Portugal) in 1999 (Mota *et al*. 1999); later on the Portuguese island of Madeira, 900 km SW of the European continent in 2010 (Fonseca *et al*. 2012); and more recently in three locations in Spain close to the Portuguese border (Robertson *et al*. 2011). The international spread of PWN occurs mainly through the movement of infested logs, untreated wood products and wood-packaging material. 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.

The Agronomic Division of the N.C. Department of Agriculture & Consumer Services operates a high-throughput and publicly operated nematode assay lab. Recently, due to more strict regulations on PWN, a large number of pine-wood samples were submitted to our lab (Table 1). In fiscal year 2013, 3,934 pine-wood samples were analyzed and 233 reports were generated for USDA/APHIS/PPQ in connection with the issuing of phytosanitary certificates for exported pine-wood logs to China; this workload represented a more than six-fold increase over the previous year. Although in the first two

months of fiscal year 2014, NCDA&CS assayed 1,139 samples for pinewood nematode—accounting for 55% of the sample total to date, July and August are during our non-busy season in receiving samples. PWN prevalence in pine-wood samples was 0.82%, 0.89% and 4.48% for fiscal year 2012, fiscal year 2013 and the first two months of fiscal year 2014, respectively. These results indicate the low presence of PWN in exported pine-wood logs in the USA and the importance of regulatory measures and laboratory testing 0.82% 0.89% 4.76%

Identification of these species using traditional morphology requires a high level of expertise and can be very time-consuming and inconclusive. However, rapid and accurate identification of PWN is required in order to comply with quarantine regulations and to prevent its movement between countries. Molecular diagnosis is potentially simple, rapid, sensitive and reliable and can be used with high precision to determine the presence of PWN in wood. We characterized the DNA sequences of the ribosomal DNA small subunit, large subunit D2/D3, internal transcribed spacer and mitochondrial DNA cytochrome oxidase subunit one of a large collection of Aphelenchid species. This allowed the development of a real-time-PCR method by either simplex (Figure 1) or duplex (Figure 2) 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*, *A.* spp. and *Aphelenchus avenae*; 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 the rapid species identification necessary to comply with the zero-tolerance export regulations.

REFERENCES

Chang R J; Lu S S (1996). Investigation of the occurrence of pine wilt disease and its naturally infected hosts in Fushan Botanical Garden. *Taiwan Journal of Forest Science* 11, 201207.

Cheng H; Lin M; Li W; Fang Z (1983). The occurrence of a pine wilting disease caused by a nematode found in Nanjing. *Forest Pest and Disease* 4, 15.

Fonseca L; Cardoso J M S; Lopes A; Pestana M; Abreu F; Nunes N; Mota M; Abrantes I (2012). The pinewood nematode, *Bursaphelenchus xylophilus*, in Madeira Island. *Helminthologia* 49, 96-103.

Mota M M; Braasch H; Bravo M A; Penas A C; Burgermeister W; Metge K; Sousa E (1999). First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology* 1, 727-734.

Robertson L; Cobacho Arcos S; Escuer M; Santiago Merino R; Esparrago G; Abelleira A; Navas A (2011). Incidence of the pinewood nematode *Bursaphelenchus xylophlius* Steiner & Buhrer, 1934 (Nickle, 1970) in Spain. *Nematology* 13, 655-757.

Yano M (1913). Investigations on the cause of pine mortality in Nagasaki prefecture. Sanrin-Koho 4, 1-14.

Yi C; Byun B; Park J; Yang S; Chang K (1989). First finding of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle and its insect vector in Korea. *Research Reports of the Forestry Research Institute (Seoul)* 38, 141-149.

Table 1. Pinewood nematode assay summary in NCDA&CS

	FY2012 (7/1/11-6/30/12)	FY2013 (7/1/12-6/30/13)	FY2014 (7/1/13-8/31/13)	Total
Total pine-wood samples	613	3934	1139	5686
Total pine-wood reports	31	234	80	344
Positive PWN samples	5	35	51	91
Positive PWN reports	3	16	16	35
Yearly nematode samples	34129	35012	2090	71231
Yearly nematode reports	4606	4744	279	9629
Pine-wood-sample percentage	1.80%	11.29%	54.50%	7.98%
Pine-wood-report percentage	0.67%	4.97%	28.67%	3.57%
PWN prevalence in pine-wood samples	0.82%	0.89%	4.48%	1.60%

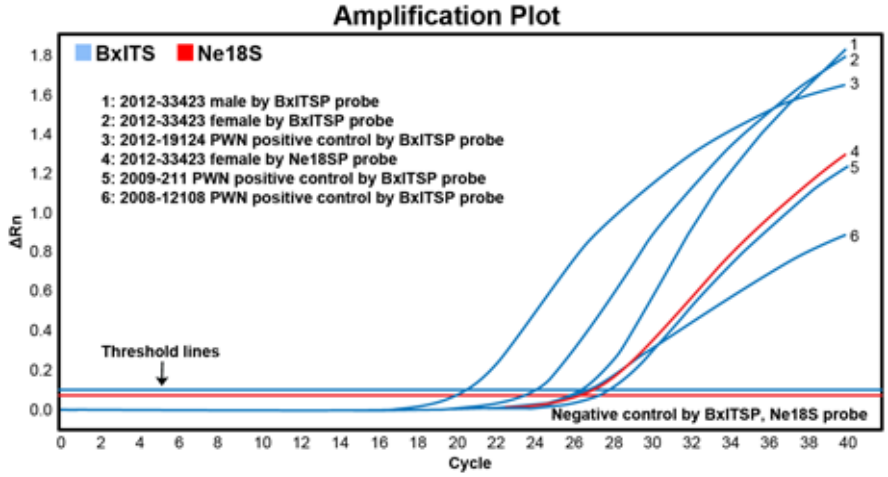

Figure 1. Example of a real-time-PCR result for testing sample 2013-33423 by Pinewood-nematode -specific and nematode-universal primer/probes.

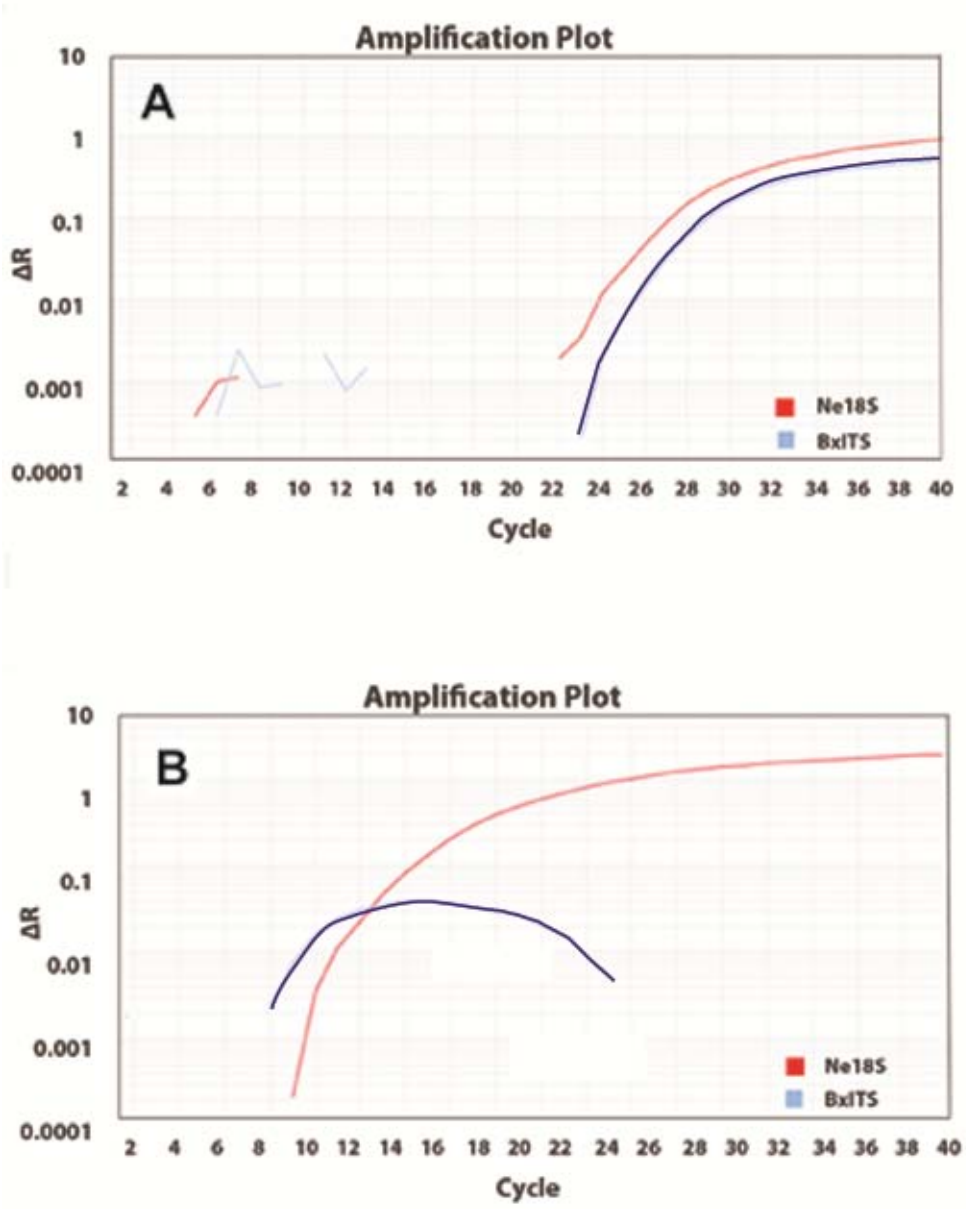

Figure 2. Duplex real-time-PCR result. A. A positive result with two sigmoid FAM (Pinewood-nematode-specific) and HEX (nematode-universal) curves in amplification plot. B. A negative result with one sigmoid HEX curve, and nonincreased FAM curve.