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First report of *Bursaphelenchus antoniae* from *Pinus strobus* in the U.S.

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Abstract

Juvenile, female and male nematodes were discovered in wood chips of white pine *Pinus strobus* from Ashley Falls, MA. Initial observations suggested these nematodes might be PWN, but closer morphological and molecular characterization proved otherwise. Comparison of measured features with those in the literature indicated this nematode population had some unique characteristics. The specimens were identified as *Bursaphelenchus antoniae* Penas et al., 2006 based on 18S rDNA molecular sequence vs only 95% similarity with PWN *B. xylophilus*. Compared to the previously described Portuguese population of *B. antoniae*, the sequences generated for the MA population were 98.3% similar in the ITS1, 2 rDNA and 99.9% similar for 28S rDNA. There was 99.2% similarity between the COI sequences of the US and Portuguese isolates of *B. antoniae*. This population has morphology consistent with that of Penas et al., 2006; however, the female tail on this MA pine population is mucronate and more attenuated than in *B. antoniae* from Portuguese *P. pinaster* found in association with *Hylobius* sp. Ecological associations of both populations of *B. antoniae* are discussed.

Key words

DNA extraction, Nematode taxonomy, Molecular identification.

Juvenile, female and male nematodes were discovered in wood chips of white pine *Pinus strobus* from Ashley Falls, MA. The white pine specimen was submitted to the University of Massachusetts Nematology Lab to examine for the pine wood nematode (PWN), *Bursaphelenchus xylophilus*, as required for shipment of pine logs to an Asian trading partner. Initial observations suggested these nematodes might be PWN, but closer morphological and molecular characterization proved otherwise. Comparison of measured features with those in the literature indicated this nematode population had some unique characteristics. Female nematodes having a vulval flap but an acute tail did not agree with PWN *B. xylophilus* that has a rounded tail. Specimens were characterized microscopically and with four molecular markers to identify this population.

Materials and methods

Individual specimens from white pine trees in Massachusetts, and specimens of *B. antoniae* from Portugal were mechanically disrupted in 20 µl of extraction buffer (Thomas, 2011) then stored in PCR tube at −80°C until needed. Each extract was prepared by incubating the tubes at 60°C for 60 min, followed by 95°C for 15 min to deactivate proteinase K.

PCR amplification: Each 25 µl PCR reaction was prepared with 2 µl of the extract and 23 µl of the PCR master mix containing 0.625U TaKaRa EX Taq (Takara Bio USA, Inc., Mountain View, CA) according to the manufacturer's protocol. The ribosomal 18S SSU DNA, ribosomal 28S LSU DNA, internal transcribed spacer (ITS) and cytochrome c oxidase I (COI) were amplified by PCR with the primer sets described in Table 1. The PCR condition for the 18S was 95°C for 3 min; 36 cycles of 95°C for 30 sec, 50°C for 40 sec, and 72°C for 70 sec; and final extension at 72°C for 5 min, for the 28S was 95°C for 3 min; 36 cycles of 95°C for 30 sec, 58°C for 45 sec, and 72°C for 70 sec; and final extension at 72°C for 5 min, for the ITS was 95°C for 3 min; 36 cycles of 95°C for 30 sec, 55°C for 60 sec, and 72°C for 105 sec; and final extension

at 72°C for 5 min, and for the COI was 1X (94°C for 1 min), 5 X (94°C for 40 sec, 45°C 45 sec, 72°C 1 min), 35 X (94°C for 40 sec, 51°C 45 sec, 72°C 1 min), and final extension 72°C for 5 min. PCR products were visualized with the Lonza FlashGel™ DNA system (VWR International, Radnor, PA) and then treated with ExoSAP-IT reagent (Affymetrix, Inc, Santa Clara, CA) according to the manufacturer's protocol. Direct DNA sequencing was performed bidirectionally with the primers (Table 1) and an ABI BigDye Terminator v3.1 kit and in an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) owned by the USDA Systematic Entomology Lab, Beltsville, MD.

Phylogenetic analysis was performed with Geneious ver. 7.1.7 (Biomatters, Auckland, NZ), using Clustal W alignment (Thompson et al., 1994) with default parameters and Bayesian likelihood tree constructed with the MRBAYES plugin (Huelsenbeck and Ronquist, 2001). Sequences from GenBank used in phylogenetic trees for 18S rDNA and 28S rDNA are given in Tables 2,3. Sequences generated were submitted to GenBank under accession numbers (18S: MK160127, MK160128, 28S: MK160125, MK160126, ITS: MK160122, COI MA: MK160123, MK160124, COI Portugal: MK174262, MK174263).

Results and discussion

Bursaphelenchus antoniae females (Fig. 1A) and males (Fig. 1B) were found for the first and only time in North America since its species description from Portugal (Penas et al., 2006a). All standard morphometric measurements were within the bounds of the original population from Europe.

Female *n* = 5: L = 597.5 ± 44.6 (527.5–650.5) µm, body width = 21.6 ± 1.3 (19.8–23.1) µm, pharynx length = 67.8 ± 3.7 (63.2–73.2) µm, tail length = 44.2 ± 2.1 (41.3–46.8) µm, ABD = 11.0 ± 1.6 (8.6–12.3) µm, stylet length= 14.3 ± 0.6 (13.3–14.8) µm, a = 27.7 ± 1.9 (26.4–30.7), b = 8.8 ± 0.6 (7.9–9.6), c = 13.5 ± 0.8 (12.3–14.4), c' = 4.1 ± 0.5 (3.7–5.0), V = 71 ± 1.1 (69–72)%.

Male *n* = 5: L = 568 ± 71 463-654) µm, body width = 20.3 0.4 (20.1–20.6) µm, e = 71.4 ± 1.8 (70.2–72.7) µm, tail length = 30.0 ± 3.5 (28.3–36.6) µm, ABD = 17.0 ± 0.0 (17.0–17.0) µm, stylet length = 17 ± 1 (13–15) µm, spicule length = 15± 1.0 (41–21) µm, a = 30.2 ± 2.22 (28.7–31.8), b = 8.6 ± 1.00 (76.5–9.3), c = 16.9 ± 1.75 (15.7–18.2), c' = 2.3 ± 0.2 (2.11–2.6).

This population is part of a species complex within a clade of other weevil-vectored *Bursaphelenchus* (Penas et al., 2006a, 2006b, 2007) within the *Hylobius* species group of *Bursaphelenchus* species associated with weevil vectors. This group is phylogenetically distinct from the *Xylophilus* group (Kanzaki et al., 2015).

The female tail tip in *B. antoniae* was clearly pointed (Penas et al., 2006a) while in this US population the tail tip was mucronate (Fig. 1C) and not acute. The closely related species *B. parantoniae* (Munawar et al., 2015) had a bluntly rounded tail tip. These female tail tip shapes may represent genetic, epigenetic or environmental polyphenisms (Duncan et al., 2014; Susoy et al., 2015). These possibilities would be clarified if cultures of both populations could be crossed to assess the stability of these phenotypes.

Table 1. Primers used for PCR and sequencing.

Primers	Direction	Sequence (5'-3')	Loci	PCR	Sequencing	Reference
18S-CL-F3	F	CTTGTCTCAAAGATTAAGCCATGCAT	18S	√	√	Carta and Li, 2019
1912R	R	TTTACGGTCAGAACTAGGG	18S	√	√	Holterman et al. (2006)
18S-530R	R	GCGGCTGCTGGCACCACACTT	18S		√	Thomas (2011)
530F	F	AAGTCTGGTGCCAGCAGCCGC	18S		√	Thomas (2011)
D2A	F	ACAAGTACCGTGAGGGAAAGTTG	28S	√	√	Nunn (1992)
D3B	R	TCGGAAGGAACCAGCTACTA	28S	√	√	Nunn (1992)
D3A	F	GACCCGTCTTGAAACACGGA	28S		√	Nunn (1992)
ITS-CL-F2	F	ATTACGTCCCTGCCCTTTGTA	ITS	√	√	This study
VRAIN 2R	R	TTTCACTCGCCGTTACTAAGGGAATC	ITS	√	√	Vrain et al. (1992)
rDNA1.58S	R	ACGAGCCGAGTGATCCACCG	ITS		√	Cherry et al. (1997)
COI-CL-F8	F	AGAGAGTTCTAATCATAAAGATATTGG	COI	√	√	This study
COI-R2	R	GTAGCAGCAGTAAAATAAGCACG	COI	√	√	Kanzaki and Futai (2002)
COI-F2	F	CCTGTCTTGGCTGGTGCTATTAC	COI		√	Kanzaki and Futai (2002)

Table 2. Summary of 18S rDNA sequences in Figure 2 tree.

Taxon	Isolate/Strain	Accession	Length (bp)	Locality
Bursaphelenchus antoniae	104F33		978	MA, USA
Bursaphelenchus abietinus	137	AY508011	1,706	Austria
Bursaphelenchus antoniae	–	AM279709	1,650	Portugal
Bursaphelenchus borealis	138	AY508012	1,698	Germany
Bursaphelenchus chengi	–	KT599480	1,748	Taiwan
Bursaphelenchus crenati	PL-21	KU683736	1,676	Poland
Bursaphelenchus gerberae	169	AY508024	1,653	Trinidad & Tobago
Bursaphelenchus hellenicus	154	AY508017	1,706	Greece
Bursaphelenchus hylobianum	160	AY508019	1,709	China
Bursaphelenchus niphades	NK203	AB849465	1,564	Japan
Bursaphelenchus parantoniae	JH-2015	KT223041	1,748	Belgium
Bursaphelenchus paraparvispicularis	38717	GQ421483	1,642	Hong Kong, China
Bursaphelenchus parapinasteri	Zhoushan	KT878515	1,648	China
Bursaphelenchus rainulfi	Ne27/04	AM397017	1,687	Brazil
Bursaphelenchus rufipennis	–	AM397017	1,699	Alaska, USA
Bursaphelenchus sakishimanus	–	LC027461	1,699	Ishigaki Is., JP
Bursaphelenchus sinensis	–	AB232162	2,525	Japan

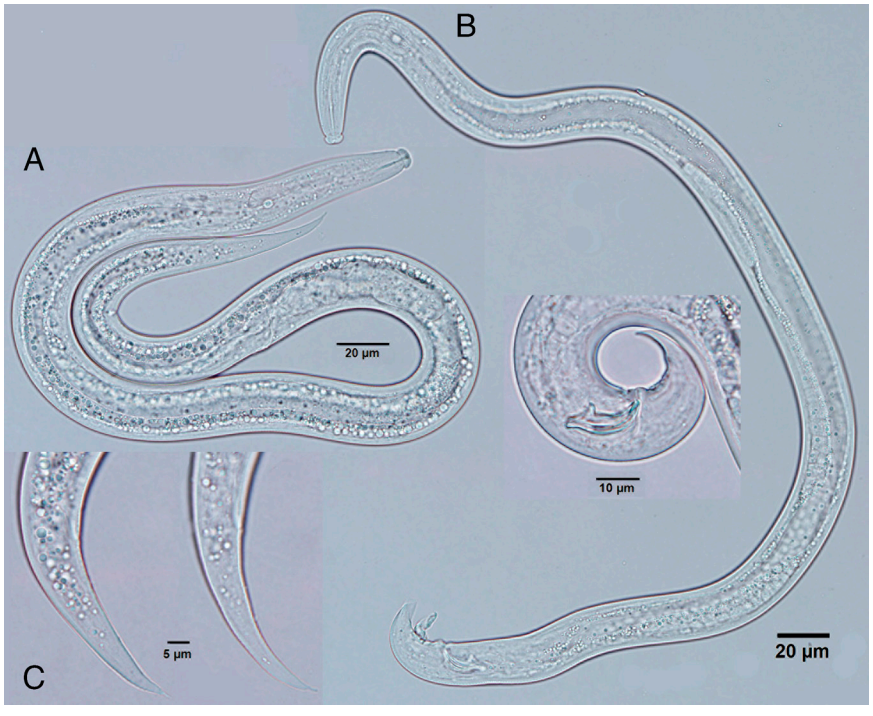

Figure 1: A. Female body, B. Male body, C. Female Tail, D. Male Tail.

Table 3. Summary of 28S rDNA sequences in Figure 3 tree.

Taxon	Isolate/Strain	Accession	Length (bp)	Locality
Bursaphelenchus abietinus	137	AY508074	724	Austria
Bursaphelenchus antoniae	104F25F3			MA, USA
Bursaphelenchus antoniae		AM279710		Portugal
Bursaphelenchus chengi	HLi104111UGMD	EU107359	741	Taiwan
Bursaphelenchus hellenicus	154	AY508083	724	Greece
Bursaphelenchus hofmanni	155	AY508084	725	Germany
Bursaphelenchus hylobianum	Ne-2-98	KT806477	782	China
Bursaphelenchus niphades	BnFFPRI	AB849479	708	Japan
Bursaphelenchus parantoniae	JH2015	KT223042	786	Belgium
Bursaphelenchus rainulfi	BrBRA	KF978102	785	Brazil
Bursaphelenchus rufipennis		AB368530	1,241	Alaska, USA

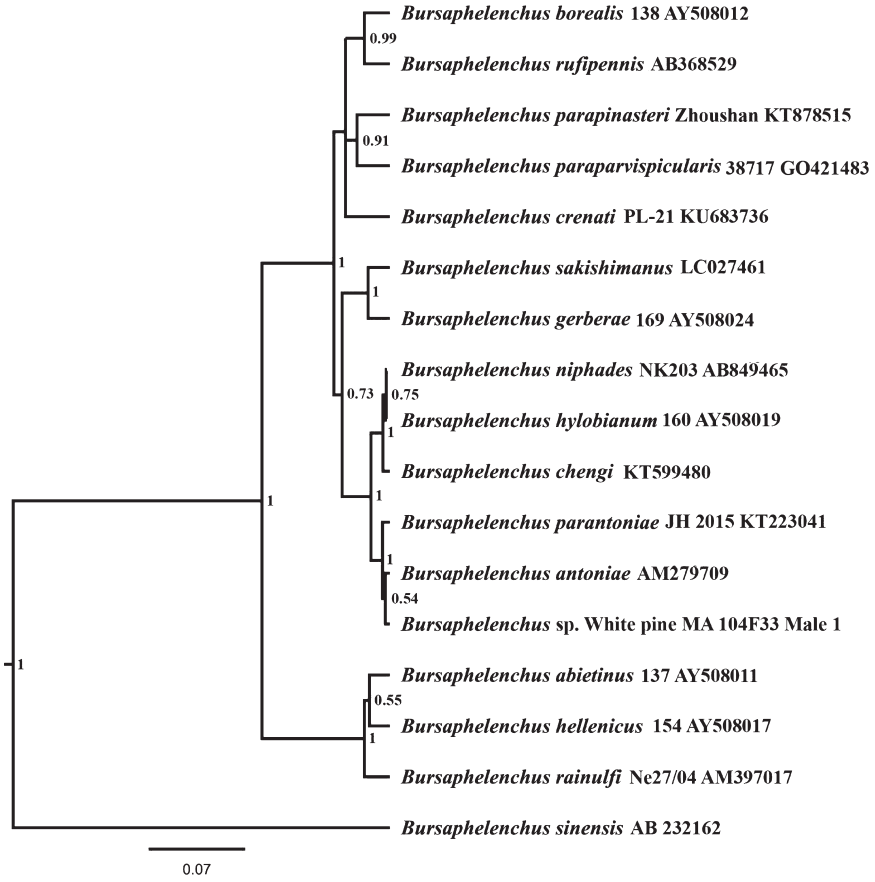

Figure 2: 18S, MrBayes tree with posterior probabilities on branches of *Bursaphelenchus antoniae* and close relatives within the '*B. hylobianum* species group' (in Clade I of Kanzaki et al., 2015) based on a Clustal W alignment implemented in Geneious ver. 7.1.7 (Biomatters, Auckland, NZ) using the MRBAYES plugin with Chain Length 1,100,000, Burnin 110,000, mean -LnL - 7438.56.

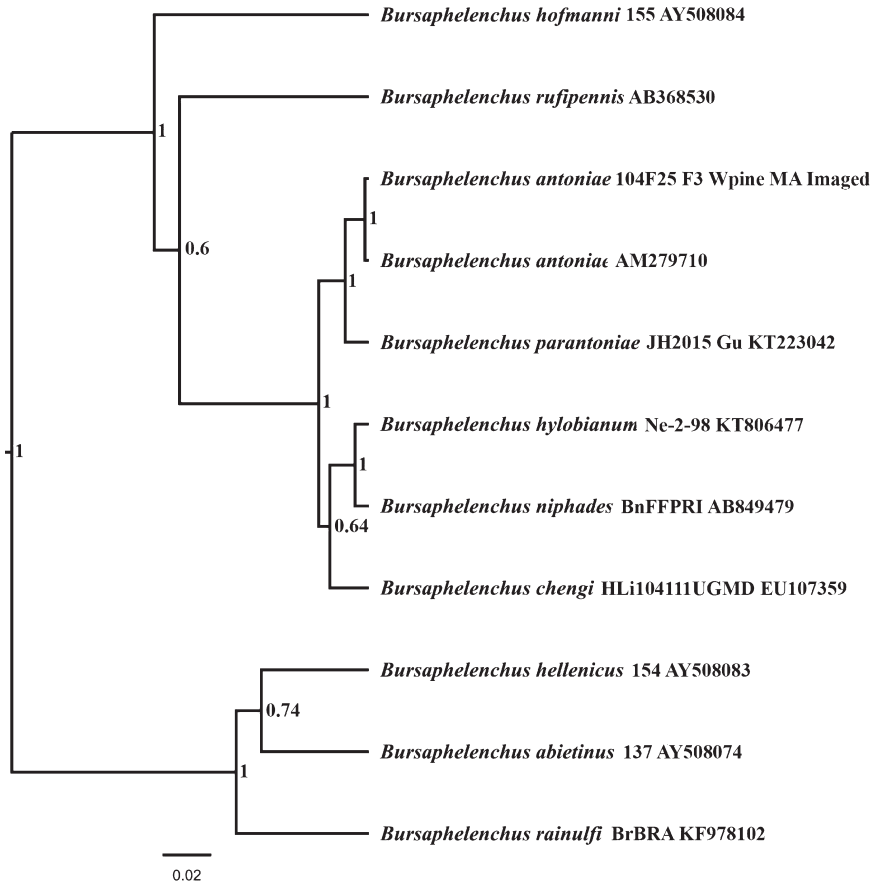

Figure 3: 28S MrBayes tree with posterior probabilities on branches of *B. antoniae* based on a Clustal W alignment implemented in Geneious ver. 7.1.7 (Biomatters, Auckland, NZ) with ChainLength 1,100,000, Burnin 110,000, mean -LnL 3407.0.

In North America, the pathogenic form of *Bursaphelenchus xylophilus* "r" has a round tail and usually occurs in pine species (Bolla et al., 1986). The generally non-pathogenic form "m" (or mucro) has a pointed tail. However, since this form can be environmentally induced (Tsai et al., 2016), and mucronate, pathogenic populations exist (Gu et al., 2011), tail form is not a very reliable indicator of potential pathogenicity of an isolate. Therefore the stability of these tail variations is important to understand in greater detail.

The 18S sequence was 99.9% similar to the Portuguese population of *B. antoniae* and 99.7% similar to and *B. parantoniae* (Fig. 2). The 28S sequence showed 97.8% similarity to *B. parantoniae* (Fig. 3). The ITS rDNA was 98.3% similar to *B. antoniae* Portugal. There were 7/834 bp differences and 99.2% similarity between the COI sequences of the US and Portuguese isolates of *B. antoniae*. The COI sequence was only 88% similar to *B. mucronatus* simply because there are very few COI sequence accessions for *Bursaphelenchus* species in GenBank.

Determining whether a given species is native or introduced is an important question when dealing with an apparently known species occurring on a new continent. *Bursaphelenchus luxuriosae* described in Japan was identified in Portugal. This was the third member of the *xylophilus* group in Portugal "It is difficult to ascertain whether *B. luxuriosae* was introduced, together with its insect vector, or already occurred as a native species (Inácio et al., 2017)." There may be an endemic association of US *B. antoniae* with another *Hylobius* in the USA, (Salom, 1997) such as the relatively common pales weevil, *H. pales,* in eastern North America (www.na.fs.fed.us/spfo/pubs/fidls/pales/fidl-pales.htmPales weevil). Alternatively, the nematode may have been introduced with the regulated ecological counterpart *H. abietis*, commonly found in Europe (Leather et al., 1999). Many *Hylobius* spp. have been intercepted at US borders over recent years (USDA-APHIS, AQAS database), and others may have managed to get through yet remain undetected. Beetle-targeted surveys in MA/CT are needed to determine whether the

pales weevil actually carries *B. antoniae* in the USA. If *B. antoniae* was an introduced species it might conceivably be pathogenic to some US pines.

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