RESEARCH/ INVESTIGACIÓN

DETECTION OF *BURSAPHELENCHUS MUCRONATUS* ASSOCIATED WITH *ACANTHOCINUS GRISEUS* ON *PINUS NIGRA* IN THE NORTHWESTERN TURKEY

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ABSTRACT

Tülek, A., I. Kepenekci, M. İmren, S. Akbulut, B. Tülek, A.S. Koca, and H. Özdikmen. 2019. Detection of *Bursaphelenchus mucronatus* associated with *Acanthocinus griseus* on *Pinus nigra* in the northwestern Turkey. Nematropica 49:220-228.

A survey was conducted in Edirne Province, Turkey for the pinewood nematode, *Bursaphelenchus xylophilus*. Wood samples were collected from declining black pine trees. Nematodes were extracted from wood samples and insect specimens were collected. Nematodes were observed and identified microscopically and molecularly with the internal transcribed spacer (ITS) of ribosomal DNA. *Bursaphelenchus xylophilus* was not detected, but *B. mucronatus* was found in very high numbers with an average up to 300 nematodes/g of wood shaving. *Acanthocinus griseus* (Coleoptera: Cerambycidae) was detected to be associated with *B. mucronatus* as a potential vector. This is the first report of *B. mucronatus* association with *A. griseus* in Turkey.

Key words: *Acanthocinus griseus*, black pine, *Bursaphelenchus mucronatus*, insect vector, pine wilt disease

RESUMEN

Tülek, A., I. Kepenekci, M. İmren, S. Akbulut, B. Tülek, A.S. Koca, and H. Özdikmen. 2019. Detección de *Bursaphelenchus mucronatus* asociada con *Acanthocinus griseus* en *Pinus nigra* en el noroeste de Turquía. Nematropica 49:220-228.

Se realizó una encuesta en la provincia de Edirne ubicada en la parte noroeste de Turquía para el nematodo de madera de pino. Se tomaron muestras de madera de pinos en declive. Se extrajeron nematodos de muestras de madera y se recolectaron muestras de insectos. Se observaron e identificaron nematodos. No se detectó *Bursaphelenchus xylophilus*, pero se encontraron especies de *B. mucronatus* en cantidades muy altas, con un promedio de hasta 300 nematodos por 1 g de viruta de madera, obtenida de pinos negros. *Acanthocinus griseus* (Coleoptera: Cerambycidae) se detectó como un vector de *B. mucronatus.* Este es el primer informe de la asociación entre *B. mucronatus* y *A. griseus* de Turquía.

Palabras clave: Acanthocinus griseus, *Bursaphelenchus mucronatus*, enfermedad del marchitamiento del pino, insecto vector, pino negro

INTRODUCTION

Pine wilt disease causes serious damage in susceptible conifer forests of eastern Asia and recently in Europe (Mota and Vieira, 2008). The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle, 1970 (Nematoda: Parasitaphelenchidae) is the causal agent of pine wilt disease, and *Monochamus* (Coleoptera: Cerambycidae) is the main vector of the nematode. Since the first detection of the disease in Japan (Yano, 1913), the nematode and disease have been spreading to new geographic regions. The recent detection of the *B. xylophilus* was in *Pinus nigra* black pine trees (Inacio *et al*., 2015) and in addition to *P. pinaster* in Portugal, indicating that the nematode continues to increase both it's distribution and host range.

Turkey is located at a very specific transitional area between Europe and Asia, which increases the possibility of invasion of both forest and agricultural pests. The invasion potential of *B. xylophilus* into Turkey is very high because of wood importation from several countries, suitable climatic conditions, richness of host trees, and the presence of vector insects. Total forested areas, about 21.2 million ha, cover 27% of the country. Forestland is occupied by approximately 60% coniferous and 40% broadleaf tree species. Oak species predominate in broadleaf forests while *P. brutia*. (5.4 million ha; 25%) and *P. nigra* (4.2 million ha; 19.8%) dominate in coniferous forests (Anonymous, 2018).

Because of the aforementioned reasons, a survey of *B. xylophilus* in Turkey were initiated in 2003. Several *Bursaphelenchus* species were reported for the first time from Turkey (Akbulut *et al*., 2006, 2007, 2008a, 2008b, 2013; Dayı *et al*., 2014). Turkey follows the phytosanitary regulations of EPPO for quarantine organisms which requires special attention to the possible introduction of quarantine organisms including *B. xylophilus*.

In 2017, typical symptoms of pine wilt disease (needle discoloration starting from the top of the tree, death of branches, and presence of blue-stain fungi in cross-section of tree trunks) were observed in black pine trees in the northwestern part of Turkey close to the border with Bulgaria. Therefore, the coniferous forests of Edirne Province were surveyed to determine the presence of *Bursaphelenchus* spp. and to collect possible vector insect species. *Bursaphelenchus* species was identified based on morphological and molecular identification.

MATERIALS AND METHODS

In 2018, *P. nigra* trees (approximately 20-year-old) in a forest in Edirne Province, Turkey (bordering Bulgaria) were investigated for the presence of *B. xylophilus*.

Nematode morphological observation

Wood shaving samples, 2.0-2.5 cm long and 1 mm thick, were obtained using a planer from the trunk of declining or wilted trees (Fig. 1). Samples were stored in polyethylene bags. Nematodes were extracted from samples using a modified Baermann funnel technique (Southey, 1986) with wood shavings immersed in water for 48 hr. Nematodes that migrated from the wood shavings into the water were collected from the bottom of the funnel. Samples were stored in water in conical flasks and nematodes were then transferred into 20-ml tubes. The nematodes from two 1-ml aliquots of water from each extraction were placed in counting dishes and enumerated using a binocular stereo microscope, Leica MZ16 (Leica Camera AG, Wetzlar, Germany). The average number of nematodes was calculated and used to calculate the number of nematodes/g of wood chips.

Specific insect traps were placed by the Regional Forestry Directorate and monitored for the presence of insects carrying nematodes. In order to capture beetles from Cerambycidae, multifunnel traps were placed in pine stands along pathways. A mixture of kairomone and pheromone lure (1,500 mg metil-butenal + 100 mg cis-verbenol + 30 mg Ipsdienol, Tripheron, Ortero®) was added to multifunnel traps. Captured insects

Figure 1. Pine wilt disease symptoms on a pine tree (left) and blue stain on cross section (right).

were brought to the Nematology lab of Trakya Agricultural Research Institute (TARI). Before nematode extract, morphological identification of insect species was performed and the specimens were sent for additional molecular analysis. Nematodes were extracted according to the EPPO PM 7/4(3) protocol (Anonymous, 2013). Each insect was cut into pieces and nematodes were extracted from the body of each insect for 24 hr by the modified Baermann funnel technique.

Bursaphelenchus species identification was based on morphological characters, including vulval flap, shape of spicules, female tail, and body measurements including length (L), L/maximum body width (a), L/oesophageal length (b), L/tail length (c), stylet length, (distance from head end to vulva/L) × 100 (V), spicules length (S). Each individual was measured using ImageJ software (Worthington, OH) calibrated with a stage micrometer.

Molecular identification

For each nematode population obtained from wood shavings, single third-stage juveniles (J3) were transferred into 45 µl of double-distilled water (ddH_2O) in an Eppendorf tube and crushed using a microhomogeniser (Vibro Mixer). After centrifugation of the crushed J3, 40 µl was transferred to a 0.2-ml PCR tube. An aliquot of 50 µl of worm lysis buffer and 10 µl of Proteinase K (20 mg/ml) was added, and the tube was frozen at -80°C for at least 10 min and then incubated at 65°C for 1 hr and 95°C for 10 min consecutively in a thermocycler. After incubation, the tubes were centrifuged for 1 min at 14,000 rpm and held at 20°C (Tanha Maafi *et al*., 2003; Waeyenberge *et al*., 2009).

Due to the small amount of the extracted DNA, whole genome amplification was conducted. One µl of the DNA extract was processed following a purification step using the alcohol precipitation method as described in the manufacturer's instructions (Illustra™ GenomiPhi V2 DNA Amplification Kit, GE Healthcare, UK) (Skantar and Carta, 2005). The DNA concentration was measured using a UV spectrophotometer (Nanodrop ND1000, Isogen Life Sciences, Sint-Pieters-Leeuw, Belgium) and 1 ng DNA was used for PCR amplification. The remainder of the crude and amplified DNA extract was stored at -20°C.

For molecular identification, the ITS region of rDNA was amplified. One ng of DNA was added to a PCR reaction mixture containing 23 µl ultra-pure sterile water, 25 µl 2X DreamTaq PCR Master Mix (Fermentas Life Sciences, Germany), and 0.5 µl of forward primer (5-CGTAACAAGGTAGCTGTAG-3) (Ferris *et al*., 1993) and reverse primer (5-TTTCACTCGCCGTTACTAAGG-3) (Vrain *et al*., 1992). The amplification program consisted of 3 min at 94°C; 35 cycles of 94°C for 30 sec, 55°C for 45 sec, and 72°C for 60 sec followed by a final elongation step of 10 min at 72°C. After PCR amplification, 5 µl of each PCR product was mixed with 1 µl of 6× loading buffer (Fermentas Life Sciences, Germany) and loaded on a 1.5% standard 1x TAE buffered agarose gel. After electrophoresis (100 V for 40 min), the gel was stained with ethidium bromide (0.1 µg/ml) for 15 min, visualized, and photographed under UV light. The remaining PCR product was stored at –20°C.

Sequencing and phylogenetic tree

The remaining PCR product (two times 45 µl) was loaded on a 1% agarose gel for electrophoresis (100 V, 40 min). The purification process was conducted as described in the manufacturer's instructions (Wizard® SV Gel and PCR Clean-Up System Kit, Promega). DNA from each sample was sequenced by a commercial company (Refgen, Ankara, Turkey) in both directions to obtain overlapping sequences of both DNA strands. The sequences were edited and analyzed using Chromas 2.00 (Technelysium, Helensvale, QLD, Australia) and BioEdit 7.0.4.1 (Hall, 1999).

The sequences were aligned with ClustalW (Thompson *et al.,* 1994). The sequences were analyzed, and BLAST searched against GenBank (http://blast.ncbi.nlm.nih.gov/) to identify the closest available reference sequences in the complete National Center for Biotechnology Information (NCBI) nucleotide collection (http://blast.ncbi.nlm.nih.gov/Blast). Phylogenetic analyses of the collected populations and reference isolates available in GenBank database including *B. koreanus* (Korea, JX154583.1), *B. paraluxuriosae* (JF966205.1), *B. paraburgeri* (HQ727724.1), *B. masseyi* (JQ287494.1), *B. mucronatus* (JF912332.1), *B. xylophilus* (MF397917.1), *B. braaschae* (GQ845407.1), *B. populi* (FJ888483.1), *B. singaporensis* (AY850162.1), *B. minutus* (KU645400.1), *B. borealis* (KP292902.1), *B. sinensis* (MG934676.1), *B. firmae* (AB663192.1), *B. doui* (AB299224.1), *B. abruptus* (AM400244.1), *B. borealis* (AM179511.1), and *B. xylophilus* (AB050051.1) was performed with MEGA X software (Kumar *et al.,* 2018) for the ITS region. A neighbor-joining tree was constructed using Tamura and Nei (1993) model with 1,000 bootstrap replicates.

RESULTS

Wood shaving samples collected from *P. nigra* trees were analyzed for the presence of *Bursaphelenchus* sp. While *B. xylophilus* was not detected, *B. mucronatus* was found in very high numbers, with an average of 300 nematodes/g of wood. Morphological identification showed the species to be *B. mucronatus* (Table 1). Male specimens had the typical, strongly curved, cucullus bearing spicules of the *xylophilus*-group. The females had a vulva postmedian with prominent vulvar flap. The tail was conoid with a mucro (Fig. 2).

Two cerambycid species were collected from pheromone traps and identified as *Acanthocinus griseus* (Fabricius, 1792) and *Monochamus galloprovincialis* (Olivier, 1795) (Coleoptera, Cerambycidae). A total of 115 male and 53 female individuals of *A. griseus* captured from 3 locations (Edirne; Suakacağı, Doğanköy, Vaysal) were used for nematode extraction. It was found that 4

Table 1. Measurements of female and males of *Bursaphelenchus mucronatus*.

Morphometric characters[y]	Female	Male
n	20	20
L	653.11±10.23 (638.45-666.00)[z]	614.57±11.64 (601.00-634.88)
a	48.33±1.10 (46.97-50.53)	43.6±1.82 (42.92-45.40)
b	13.66±0.55 (12.40-14.20)	11.81±0.37 (11.24-12.50)
c	22.97±2.34 (19.53-25.61)	19.24±1.80 (16.69-21.11)
c′	3.82±0.80 (2.64-5.14)	2.90±0.50 (2.03-3.20)
Stylet	11.59±0.47 (10.93-12.3)	12.11±0.82 (11.00-13.42)
Tail	30.36±3.32 (26.00-34.15)	32.45±3.32 (29.30-36.77)
V	72.26±0.89 (70.95-74.00)	-
S	-	19.55±3.71 (13.65-23.50)

[y]n = Number of examined specimens; L = body length; a = L/maximum body width; b = L/oesophageal length; c = L/tail length; c′ = tail width / tail length; Stylet = stylet length; Tail = tail length; V = (distance from head end to vulva/L) × 100; S = spicules length.

[z]All measurements are in micrometers (mean ± standard deviation, with range in parenthesis).

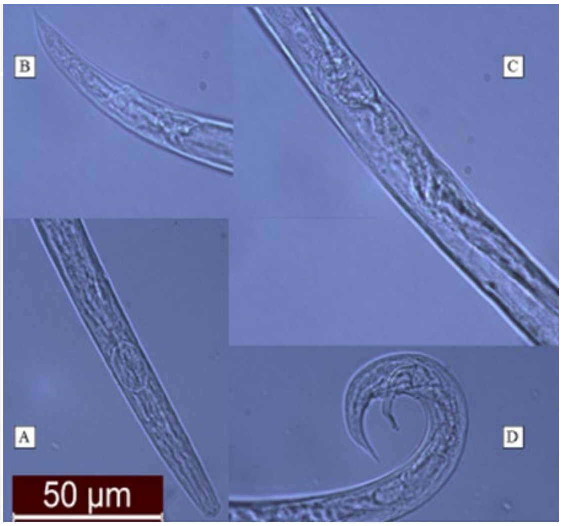

Figure 2. Light micrographs of *Bursaphelenchus mucronatus*. A: anterior region; B: female tail; C: vulval region; D: male tail.

individuals from 115 males (134-816 nematodes per beetle) and 1 individual from 53 females (111 nematodes per beetle) of *A. griseus* carried *B. mucronatus.* It appears that *A. griseus* has an association with *B. mucronatus*.

Phylogenetic analysis of Bursaphelenchus mucronatus *populations*

PCR amplification of the ITS region of rDNA of all *Bursaphelenchus* populations produced a single fragment of approximately 800 bp. All populations were identified as *B. mucronatus*, and 10 sequences from *B. mucronatus* were obtained. One consensus sequence was derived from these sequences and was used for further phylogenetic analysis. The sequences of *B. mucronatus* were deposited in GENBANK with accession numbers from MH923195 to MH923204 (Table 2).

The ITS sequences of *B. mucronatus* isolates were 98% similar to sequences of each other indicating little intraspecific polymorphism exists among the detected nematode populations. Based on the ITS-rDNA sequences, *B. mucronatus* populations showed some intraspecific polymorphism. *B. mucronatus* populations grouped and were supported by a high bootstrap value (Fig. 3). Sequences had a high similarity with the sequences of closely related species documented in the GenBank.

DISCUSSION

Previously, seven *Bursaphelenchus* species isolated from different pine species were reported from Turkey (Akbulut *et al*., 2006, 2007, 2008a, 2008b, 2013; Dayı *et al*., 2014). *Bursaphelenchus mucronatus* was also previously reported from different parts of Turkey (Akbulut *et al*., 2006, 2008b). According to the results of the current study, this is the first time *B. mucronatus* has been reported from Edirne Province of Turkey.

Several insect vectors of *B. mucronatus* have been reported from other countries. Similar to *B. xylophilus,* the main vectors of *B. mucronatus* are beetles from the genus *Monochamus*. *Monochamus alternatus, M. galloprovincialis, M. sutor, M. saltuarius* (Ryss *et al*., 2005, Abelleira *et al*., 2015), *M. urussovi* (Togashi *et al*., 2008), and *M. nitens* (Kanzaki and Akiba, 2014) have all been reported as vectors of the nematode. *Arhopalus rusticus* and *Spondylis buprestoides* (Ryss *et al*., 2005, Zhao *et al*., 2004) were also reported as vector insects of *B. mucronatus* in China. Previously, *M. galloprovincialis* (Akbulut *et al*., 2010) and *Ips sexdentatus* (Dayı and Akbulut, 2018) were reported as potential vectors of *B. mucronatus*. According to the literature, *A. griseus* has not been associated with *B. mucronatus,* but it was reported as a vector of *B. xylophilus* in Japan (Ryss *et al*., 2005; Linit, 1988). Linit (1988) stated that *A. griseus* only carried *B. xylophilus* without

Table 2. *Bursaphelenchus mucronatus* identified using ITS-rDNA with their GenBank accession numbers.

Code	Accession Number
BO1[y]	MH923195
BO2	MH923196
BO3	MH923197
BO4	MH923198
BO5	MH923199
TO1[z]	MH923200
TO2	MH923201
TO3	MH923202
TO4	MH923203
TO5	MH923204

[y]BO: Samples obtained from logs that originated from Bulgaria.

[z]TO: Samples obtained from black pine trees, Edirne, Turkey.

transmission to new host trees. To be a vector of *Bursaphelenchus*, the insect should both carry and transmit the nematodes to new host trees. Therefore, it is important to prove that *A. griseus* is a vector of *B. mucronatus* by undertaking transmission studies. This finding also indicates that there is an association between *B. mucronatus* and *A. giresus* that needs further studies to clarify the status of this association among the beetle, nematode, and host trees.

Pinus nigra is a native species in Europe, Asia and Turkey. The tree represents 19% of coniferous

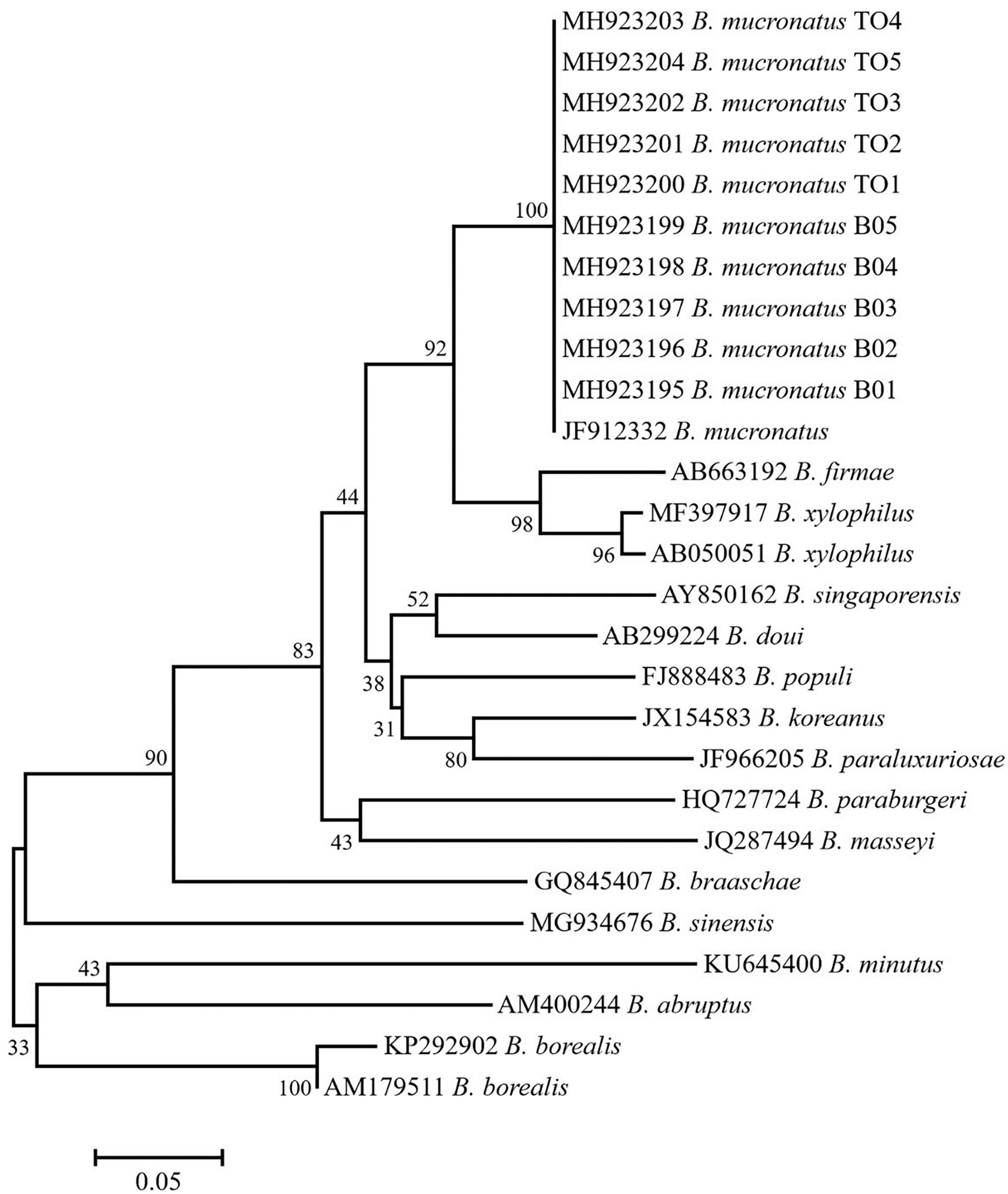

Figure 3. The neighbor-joining phylogenetic tree obtained ITS sequences from *Bursaphelenchus mucronatus* populations obtained from *Pinus nigra* and reference sequences from the GenBank. Populations are denoted using the codes described in Table 2. Bootstrap values >60 are given.

forests in Turkey (Anonymous, 2018). Several early studies indicated that *P. nigra* is susceptible to PWN under both natural (Wingfield *et al*., 1982; Wingfield *et al*., 1986; Inacio *et al*., 2015) and artificial conditions (Dropkin and Foudin, 1979). In Turkey, several pathogenicity studies were conducted using pine seedlings including *P. nigra*. Some *Bursaphelenchus* species have different levels of pathogenic effects on *P. nigra* seedlings (Akbulut *et al*., 2007; Dayı and Akbulut, 2012). Therefore, it is important to monitor *P. nigra* forests in case *B. Xylophilus* is introduced, as well as the presence and effects of *B. mucronatus* on forests.

In conclusion, *B. mucronatus* populations from Turkey, Bulgaria, and a reference isolate from GenBank, were distinctly clustered, based upon phylogenetic analyses of the ITS region of rDNA. All *B. mucronatus* populations in this study had a high homology with *B. mucronatus* JF912332 (Fig. 3).

Surveys for *Bursaphelenchus* species in pine forests of Turkey should be repeated periodically. To date, the association of the nematode and unknown *Pinus* species was found in some imported logs that originated from Bulgaria and was described as *B. mucronatus*. It is particularly important to conduct surveys near the European border of Turkey and to monitor custom gates and ports for possible introduction of *B. xylophilus* and other congeneric species in the light of new high-resolution COI region mtDNA capable of detecting interspecific hybrids (Matsunaga *et al.,* 2019).

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