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Survey for the detection of *Bursaphelenchus* insect-vector species in the western part of Turkey

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Abstract

Aim of the study: This study aimed to identify the insect vectors of these *Bursaphelenchus* species in conifer forests of the western Turkey.

Area of study: Sampling was performed in conifer forests of the western part of Turkey.

Material and Methods: Two different methods were used, trap trees and pheromone traps with specific lure combinations, to catch insects.

Main results: A total number of 11,076 insects in the first year (2012), and 226,205 insects in the second year (2013) surveys were captured. The most represented insect order was Coleoptera and the most common insect species found in all sampling areas was *Orthotomicus erosus*. After morphological and molecular studies, *B. mucronatus* was found to be associated with insect bulk samples of *Ips sexdentatus*, while *B. sexdentati* was found to be associated with individual and insect bulk samples of *O. erosus*, *Acanthocinus aedilis*, and *Arhopalus rusticus*.

Keywords: Pine Wilt Disease, *Bursaphelenchus* species, conifer forests, insect vectors, insect traps.

Türkiye`nin batı bölgelerinde *Bursaphelenchus* türlerinin taşıyıcı böceklerinin tespitine yönelik arazi taramaları

Özet

Çalışmanın amacı: Bu çalışmada, Türkiye'nin batı bölgelerindeki *Bursaphelenchus* türlerinin taşıyıcı böceklerinin tespit edilmesi amaçlanmıştır.

*Çalışma alanı:*Türkiye`nin batı bölgesinde iğne yapraklı ormanlarda çalışma yürütülmüştür.

Materyal ve Yöntem: Böcekleri yakalamak için tuzak ağaçları ve özel koku karışımlarından oluşan feromon tuzakları olmak üzere iki farklı yöntem kullanılmıştır.

Temel Sonuçlar: Çalışmanın ilk yılında (2012) toplamda 11,076 böcek yakalanırken, ikinci yılda (2013) ise 226,205 böcek yakalanmıştır. Tüm örnekleme sahalarında en fazla yakalanan böcek takımı Coleoptera olurken, en fazla yakalanan böcek türü ise *Orthotomicus erosus* olmuştur. Nematodların morfolojik ve moleküler açıdan teşhis çalışmalarından sonra, *B. mucronatus*'un *Ips sexdentatus* ile, *B. sexdentati*'nin ise *O. erosus*, *Acanthocinus aedilis* ve *Arhopalus rusticus* böcekleriyle ilişkili olduğu tespit edilmiştir

Anahtar kelimeler: Çam Kuruma Hastalığı, *Bursaphelenchus* türleri, iğne yapraklı ormanlar, vektör böcekler, böcek tuzakları.

Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* ((Steiner and Buhrer, 1934) Nickle, 1970) is the causal agent of pine wilt disease (PWD), which causes severe ecological and economical damages in conifer forests in East Asia and southern Europe (Mota and Vieira, 2008). The insect vectors belonging to the genus *Monochamus* Dejean 1821 carry this nematode from symptomatic to new and healthy pine trees (Akbulut and Stamps, 2012). The disease was first reported from Japan in the beginning of the 20th century (Yano, 1913) further spreading to China and Korea (Yang, 2004; Yi., Byun, Park, Yang and Chang, 1989) and to Europe (Abelleira, Picoaga, Mansilla, Aguin, 2011; Fonseca et al., 2012; Mota et al., 1999; Vicente, Espada, Vieira, Mota, 2012). These new reports increased the concerns of a new introduction/spread of the PWN into other uninfected pine forests in Europe. Due to the presence of susceptible pine species (e.g. *Pinus brutia* Ten, *Pinus nigra* Arnold and *P. sylvestris* L.), the insect vector (*Monochamus galloprovincialis*) and geographic position and Mediterranean climate, the possible

introduction of the PWN is a constant threat to conifer forests of Turkey (Akbulut et al., 2006). In Turkey, the survey for the presence of the PWN in conifer forests started in 2003 (Akbulut et al., 2006). As a result of these surveys, several *Bursaphelenchus* Fuchs, 1937 species have been found, namely *B. anamurius*, Akbulut, Braasch, Baysal, Brandstetter, Burgermeister, 2007, *B. andrasyyi* Dayi, Calin, Akbulut, Gu, Schroder, Vieira, Braasch, 2014, *B. hellenicus* Skarmoutsos, Braasch, Michalopoulou, 1998, *B. mucronatus* Mamiya, Enda, 1979, *B. pinophilus* Brzeski, Baujard, 1997, *B. sexdentati* Ruhm, 1960 and *B. vallesianus* Schonfeld, Polonski, Burgermeister, 2004 (Akbulut *et al*., 2006, Akbulut, Braasch, Baysal, Brandstetter, Burgermeister, 2007; Akbulut, Elekçioğlu, Keten, 2008a; Dayi et al., 2014). So far, *B. xylophilus* has not been detected in Turkey.

In nature, the spread of *Bursaphelenchus* species is totally dependent on its insect vectors. The association established between *Bursaphelenchus* species and its insect vector can be more or less specific (Ryss, Vieira, Mota, Kulinich, 2005). For example, species belonging to the *Xylophilus* group are mainly associated with *Monochamus* species (Cerambycidae), while species belonging to other different morphological groups, such as the *sexdentati*, *egersi* or *eremus* can be vectored mainly by Scolytid species (Curculionidae: Scolytinae) (Braasch, Burgermeister, Gu, 2009; Ryss et al. 2005). In Turkey, the vector species of *Bursaphelenchus* from different morphological groups of *Bursaphelenchus* species has not been studied. There are only few reports related to insect vectors of *Bursaphelenchus* (Akbulut et al., 2010; Giblin-Davis et al., 2005). Therefore, the main goal of this study was to determine the potential insect vectors of *Bursaphelenchus* species previously recorded in the western parts of Turkey.

Material and Methods

Insect surveys

Insect sampling studies were carried out in the pine forests of Muğla, İzmir, Burdur, Isparta, Denizli and Manisa. Studied forest areas consisted of two main pine species, *Pinus brutia* and *P. nigra*. The average altitudes were 500 m and 1200 m for *P. brutia* and *P. nigra* stands, respectively. All study locations were established by using the same GPS (Global Positioning System) coordinates of previously known areas for the presence of *Bursaphelenchus* species (Akbulut et al., 2006; Akbulut et al., 2007; 2008a; Dayi et al., 2014).

For the 2012 survey, trap trees were prepared before the flight period of possible insect vector species (as early as at the beginning of March). For each location, four trap trees (20-30 m length and 12-15 cm diam.) were selected from healthy trees: *P. brutia* in the regions of İzmir, Manisa and Muğla; *Abies cilicica* Ant. and Kotschy Carriére, and *P. brutia* in the region of Burdur, and *P. nigra* in the regions of Isparta and Denizli, representing the forest type of the study areas. A total of 24 trap trees were used in this study. For each location, trees were felled while retaining their branches to prevent water loss. Trap trees were kept in the field to attract insect species from March to September and checked periodically for insect activities, such as oviposition marks, larval feeding (presence of frass), or insect emergence. Several log samples (50-60 cm in length) (about 5 logs of each tree) were taken from trap trees showing any insect activities and sent to the lab via cargo from March to September. The logs were kept under constant conditions (25-28^{o}C, 65-75 % RH, and a 12L / 12D photoperiod) until emergence of adult insects in the lab. Before transferring each log into its own PVC container (50-65 cm in length, 15-30 cm in diameter and the top part with a wire screen), both ends of each log were waxed with hot paraffin to reduce desiccation. Emerged insects were collected daily and identified to species level. Then, nematodes were extracted using a Baermann funnel or by crushing (or dissecting) the insects in Petri dishes containing water for 24 h. Extracted nematodes were checked under a stereo-microscope (Olympus SZX-12), nematodes were transferred into a 2 ml tube containing DESS solution (Yoder et. al. 2006), and kept at room temperature for morphological and molecular studies.

For the 2013 survey, three different pheromone-baited traps were used at each location; radiator (Witasek-Germany), Scandinavian (VIT-Turkey) and multi-funnel (SMC-Turkey). For each location three pheromone traps (radiator, Scandinavian and multi-funnel) were placed in open areas close to location where *Bursaphelenchus* species were reported in previous studies mentioned in the introduction section. A total of 18 pheromone traps were used. Each trap was placed 100 m away from each other, and a combination of three pheromone lures was used: α-pinene (20 mg), ipsdienol (95 mg) and 2-methyl-3-buten-2-ol (MBO) (1500 mg) and kept from March to September in the field. The traps were checked weekly, all insects were collected and sent to the lab in small cups via cargo. Each pheromone lure was periodically changed within a 20 day-interval with a new one. Collected insects were identified to species level (when possible) then dissected or crushed for nematode extraction as described above. Due to the great number of collected insects in some samples (mainly for bark beetles), insects from the same trap were separated and processed for nematode extraction using bulk samples of approximately 100 individuals. Insects from other families (big-sized insects belonging to the families such as Cerambycidae or Buprestidae) were separated and processed individually.

Morphological identification and molecular analysis of nematodes

Morphological and molecular studies to identify nematodes species isolated from insects were done at University of Evora (Portugal) in support of a fellowship supported by COST (European Cooperation in Science and Technology).

Isolated nematodes were observed under a light microscope (Olympus BX50). For specimens belonging to the genus *Bursaphelenchus*, nematodes isolated from individual insects or bulks of insects were identified according to Braasch et al., (2009) and Ryss et al., (2005) and and measured using the software Cell^D (Imaging software for life sciences microscopy, Olympus). As *Bursaphelenchus* species were the main focus of this project, other nematode species found are classified here as non-*Bursaphelenchus* only.

The same nematodes were then used for single-nematode genomic DNA extraction using the Purelink Genomic DNA kit following the manufacturer's instructions (Life technologies–Invitrogen). For amplification of the ITS1/ITS2 region of the rDNA the procedure used by Cermak et al., (2013) was followed. For amplification of the ITS1/ITS2 region of the rDNA the forward primer 5'-CGTAACAAGGTAGCTGTAG-3' (Ferris, Ferris and Faghihi, 1993) and reverse primer 5'-TTTCACTCGCCGTTACTAAGG-3' (Vrain, 1993) were used. Each PCR reaction contained 1X Taq buffer, 2.5 mM $MgCl_2$, 0.6 mM of each primer, 0.2 mM dNTPs, 2 units Taq DNA Polymerase (Fermentas, ThermoScientific), and 5 µl of template DNA. The PCR reaction consisted of a denaturation step at 94°C for 4 minutes, followed by 35 cycles at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. The amplified PCR products were analyzed by electrophoresis in a 1.5 % agarose gel. Posteriorly, the remaining PCR products were purified using a combination of two enzymes: Exonuclease I (0.5 µl) and Fast AP (Alkaline phosphatase) (1 µl) (Fermentas, ThermoScientific), and sent for sequencing (Macrogen). The obtained sequences were proofread manually using CLC Workbench v. 7 software (CLC Bio, Qiagen). The ITS rDNA region corresponding sequences were compared with a set of reference sequences from different species of *Bursaphelenchus* selected from GenBank (NCBI). The tree topology was obtained with the neighbour-joining (NJ) analysis with 1000 bootstrap replications using CLUSTAL X.

The phylogenetic tree was visualized and annotated using the program FigTree (http://tree.bio.ed.ac.uk/software/fig-tree/). ITS-RFLP profiles presented for species of the *sexdentati* group were calculated from the ITS1/2 sequences available at GenBank (NCBI), using the software EnzymeX 3 (www.mekentosj.com/science/enzymex).

Results

Insect captures

In the 2012 survey, a total of 11,076 insects were captured, and the most represented order was Coleoptera with most of the specimens belonging to the family Curculionidae followed by the family Cerambycidae and only one specimen was from the order Isoptera.

The total number of insects was distributed among thirteen families of the order Coleoptera, with most of the specimens belonging to the family Curculionidae (twelve species), followed by the family Cerambycidae with five species. Insects belonging to the families of Cerambycidae and Curculionidae were captured in all sampling areas - 170 and 10,875 specimens, respectively. *Orthotomicus erosus* Wollaston (Curculionidae) was the most common species found, followed by *Ips sexdentatus* Boerner (Curculionidae).

In the 2013 survey, a total of 226,205 insects were captured. Almost all of the collected specimens were distributed within the family Curculionidae (225,950 individuals). The most common insect species found in all areas was *O. erosus* followed by *I. sexdentatus* with few specimens of *M. galloprovincialis* also captured.

Even though the number of families collected was higher in 2012, the number of insects captured increased dramatically in 2013, with a total of 226,205 in comparison to 11,076 individuals in 2012. In both survey years, the most common scolytid species was *O. erosus*, while the most common cerambycid species was *Acanthocinus aedilis* Linnaeus and *Arhopalus rusticus* Linnaeus in 2012 and 2013, respectively. Interestingly, *M. galloprovincialis* Olivier was only captured, during the survey of 2013.

Insect-nematode association and identification

From the total number of insects collected, nematodes were only found in 11 individual insects and 73 insect bulk samples (100 individuals per bulk) from all sampling locations. Nematodes were found to be associated with Curculionidae and Cerambycidae species only. The majority of nematode species extracted from insect samples were free-living nematodes, herein classified as non-*Bursaphelenchus* species. Nematode carrying insect species were *A. rusticus, O. erosus, Tomicus piniperda* Linnaeus, *I. sexdentatus, Spondylis buprestoides* Linnaeus and *A. aedilis*.

All *Bursaphelenchus* specimens were identified based on the type of spicule and assigned to one of the morphological group of species established for this genus, namely the *sexdentati*- and *xylophilus*-group (Table 1) and measured.

Table 1. *Bursaphelenchus* species associated with insects captured in six different locations in the western part of Turkey.

The group of nematodes	Nematode species	Location	Insect	Number of total individuals	Number of females	Number of males	Number of juveniles/dauer larvae
The *xylophilus* group	*Bursaphelenchus* sp.	Burdur	*Orthotomicus*[1] *erosus*	3 (2)	0	3	0
The *xylophilus* group	*Bursaphelenchus* sp.	Burdur	*O. erosus*[1]	4 (1)	0	4	0
The *sexdentati* group	*Bursaphelenchus sexdentati*	Denizli	*Arhopalus rusticus*[2]	4 (1)	1	3	0
The *sexdentati* group	*Bursaphelenchus sexdentati*	Izmir	*Aconthocinus aedilis*[2]	3 (2)	1	2	0
The *sexdentati* group	*Bursaphelenchus sexdentati*	Izmir	*O. erosus*[1]	16 (2)	15	1	0
The *xylophilus* group	*Bursaphelenchus mucronatus*	Isparta	*Ips sexdentatus*[1]	1 (1)	0	1	0

Note. The number of nematodes found in individual insect species is presented between parentheses. In the Insect column, the asterisk 1 represents the method "crashing", and the asterisk 2 represents the method "dissecting" for nematode extraction.

Specimens belonging to the *sexdentati*-group presented stout and curved spicules with prominent rostrum and condylus, the presence of seven caudal papillae, and females with a small vulval flap. The specimens belonging to the *xylophilus*-group presented the typical narrow and long spicules, with flattened capitulum, and presence of cucullus, and seven caudal papillae.

Only 3 individual and 6 bulk insect samples were found to carry *Bursaphelenchus* specimens (Table 1). The number of *Bursaphelenchus* specimens extracted was very limited, varying from 1 to 16 individuals (Table 1).

Bursaphelenchus individuals extracted from all insect samples were female or male stages, with no juveniles or dauer larvae found within these nematode-insect associations (Figure 1).

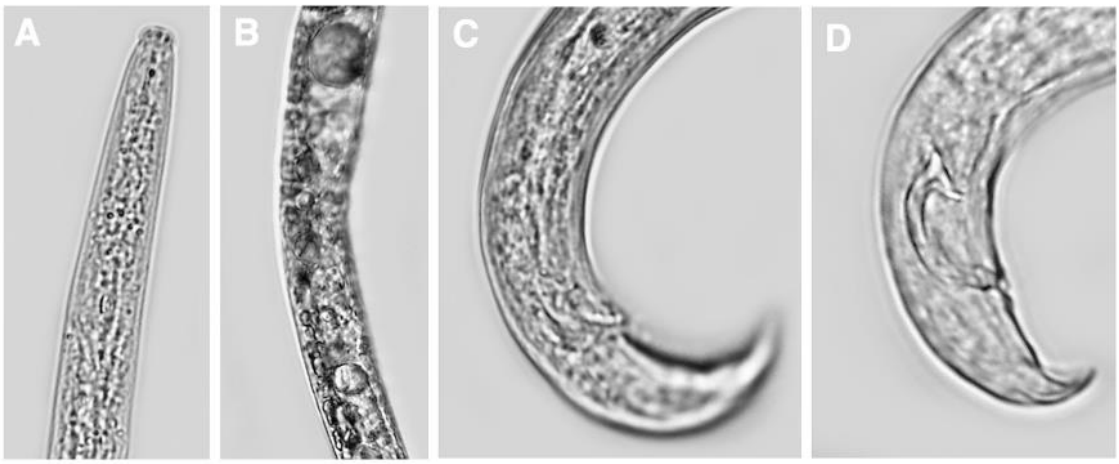

Figure 1. Light microscope observations of *Bursaphelenchus* specimens found in association with insects captured during the surveys of 2012 and 2013 in the western part of Turkey. A: anterior region of a specimen belonging to the genus *Bursaphelenchus*; B: female vulva region of *B. sexdentati*; C: male tail of *B. sexdentati*; D: male tail of *B. mucronatus*. Scale bars: 10 µm.

B. sexdentati was collected from individual insects of *A. rusticus* and *A. aedilis* species (Cerambycidae), while the remaining *Bursaphelenchus* specimens identified were collected from insect bulk samples of *O. erosus* and *I. sexdentatus* (Curculionidae) (Table 1).

For each sample containing *Bursaphelenchus* specimens, a molecular characterization was performed based on the ITS rDNA region using single nematode specimens. Amplification of the ITS1/2 region (including a partial region of both 18S and 28S rRNA genes) was obtained for five individuals out of four samples containing *Bursaphelenchus* specimens. Amplification of this region resulted in a PCR product of 981 bp for nematodes collected from the regions of Denizli and İzmir that were identified as *B. sexdentati*, and 920 bp for a single nematode used for Isparta region and identified as *B. mucronatus* (Table 2). The nematodes belonging to *B. sexdentati* clustered together with other isolates previously identified has belonging to the South European type. The ITS rDNA sequences obtained for *B. sexdentati* nematodes collected from the areas of Denizli and Izmir also displayed several point mutations in both ITS1 and ITS2 regions, including a point nucleotide substitution at the 759 bp in the ITS2 region for two of the isolates, which led to the loss of the *Msp*I recognition site (Table 2), generating a different ITS-RFLP pattern for this enzyme, in comparison to the established

ITS-RFLP pattern for this species (Burgermeister et al., 2009).

Table 2. ITS-RFLP pattern of *Bursaphelenchus* species found in different forest areas of western part of Turkey. The ITS-RFLP pattern was generated using the sequencing results obtained for the ITS/ITS2 rDNA region.

Bursaphelenchus sp.	PCR product (bp)	Restriction fragments (bp)				
		*Rsa*I	*Hae*III	*Msp*I	*Hinf*I	*Alu*I
B. sexdentati Izmir	981	545	584	981	466	968
		414	279		279	13
		22	118		211	
					25	
B. sexdentati Denizli and Izmir	981	545	863	981	466	968
		414	118		279	13
		22			211	
					25	
B. mucronatus Isparta	920	487	620	354	408	275
		411	300	303	323	245
		22		263	120	
					86	
					49	
					25	

The isolate belonging to the *xylophilus*-group was identified as *B. mucronatus* showing similar pattern of the ITS-RFLP established for this species.

We were unable to successfully amplify the respective rDNA region of the nematodes collected from the Burdur area, which belong to the *xylophilus* group of species based on the male spicules morphology (Ryss et al., 2005).

Discussion

In this study, insect vectors of previously reported *Bursaphelenchus* species were investigated in six different locations of western forest sites of Turkey. The majority of the insect species collected mainly belong to the families of Curculionidae and Cerambycidae. The most common species are *O. erosus* and *I. sexdentatus* in all study locations.

There is a huge difference in terms of the number of insects captured between both type of traps herein used. The number of insects captured with pheromone-based traps was 20 times higher than those of trap trees. The wide range dispersal of the pheromones in these types of traps compared to the trap trees may explain this high difference. In addition, this high number of captures may also be related to the specific combinations of pheromones, such as α-pinene, ipsdienol and 2-methyl-3-buten-2-ol. Pheromone lures were renewed periodically, which may have increased the number of insects captured by the pheromone traps compared to the trap trees. Dodds, Darrel, Daterman, (2000) discussed that trap trees have a finite capacity for trapping insects due to their limited area. On the other hand, in the pheromone traps, captured insects were removed periodically and the traps were ready to capture new insects. However, the richness of insect species/families was higher

for trap trees than pheromone traps. Regarding to trap trees, different wood inhabiting insects were collected, whereas in pheromone traps only insects attracted to the specific pheromone lures were collected which could explain the reduced richness of species/families. The differences in the number of insect species/families among the studied locations can be also related to the stand types and tree species distributed among the different locations. In Burdur area, three different conifer species (*Abies cilicica, Cedrus libani* and *P. brutia*) were identified in the stand, while in the other locations only one pine species was represented, either *P. brutia* or *P. nigra*. Sarikaya and Avci (2011) reported that *O. erosus, I. sexdentatus* and *T. minor* were found as the most common species associated with *P. brutia* and *P. nigra* forests in the western part of Turkey, such as Antalya, Burdur, Denizli, Isparta, Muğla and Afyonkarahisar. In the current study, similarly, *O. erosus* and *I. sexdentatus* were the most common species, followed by *T. piniperda*.

The species of the genus *Bursaphelenchus* are often found in association with different insect vector species (Penas et al., 2006; Ryss et al., 2005). In Turkey a total of seven *Bursaphelenchus* species have been identified associated with conifer trees, namely *B. anamurius*, *B. andrassyi*, *B. hellenicus*, *B. mucronatus*, *B. pinophilus*, *B. sexdentati* and *B. vallesianus*, (Akbulut et al., 2006, Akbulut et al., 2007; Akbulut et al., 2008a; Akbulut et al., 2008b; Akbulut et al., 2013, Dayi et al., 2014). However, no insect species have been reported as vectors of these *Bursaphelenchus* species in Turkey except the report of *M. galloprovincialis* as the vector of *B. mucronatus* (Akbulut et al., 2010). From the total of 237,281 insect specimens captured during surveys, only a very small percentage of insects (11 individual and 73 bulk insect samples) were found to be associated with nematodes.

In the particular case of *Bursaphelenchus*, only 3 individual and 6 bulk insect samples showed the presence of *Bursaphelenchus* with limited number of individuals. This may be caused by low population density or low distribution frequency of these nematodes in sampling areas. Thus, the insects may have carried few or no nematodes.

Similar results were also observed during the survey of *B. xylophilus*. Since 2002, over 3000 wood samples around Turkey were taken from dead or dying conifer species, and only 5 % of the total samples was contained *Bursaphelenchus* species with limited number of individuals (Akbulut et al., 2006; Akbulut et al., 2007; Akbulut et al., 2008a; Akbulut et al., 2013). All reported *Bursaphelenchus* species are believed to be native to Turkish conifer forests, and no extensive wilting symptoms caused by these *Bursaphelenchus* species have been observed so far.

Nematodes belonging to the *xylophilus* group were found in Burdur and Isparta regions, and associated with bulk samples of *O. erosus* and *I. sexdentatus*, respectively. Nematodes belonging to the *sexdentati* group were found in Denizli and İzmir regions and isolated from individual insects belonging to the species *A. rusticus*, *A. aedilis*, as well as bulk samples of *O. erosus*, respectively (Table 1). Interestingly, only adult stages of *Bursaphelenchus* were isolated from these insects. This pattern of nematode stage-insect association is not usual, as the most common *Bursaphelenchus* stage found with other insect species is called as “dauer larvae” (Ryss et al., 2005). The occurrence of adult stages (and in such reduced number of individuals) has been previously reported for other *Bursaphelenchus* species, for example *B. hellenicus* in association with *Hylurgus ligniperda*, and *B. leoni* in association with a *Pityogenes* sp. (Penas et al., 2006).

During this study, 39 individuals of *M. galloprovincialis*, the vector of *B. xylophilus* in Portugal (Sousa et al., 2001), were also collected, but none of them carried nematodes. *M. galloprovincialis* is known as a vector of *B. xylophilus* (Sousa et al., 2001) and *B. mucronatus* (Ryss et al., 2005). The number of captured individuals was very low compared to other insects. Since it has not been considered to be a pest for pine forests, there is no study related to population density of *M. galloprovincialis* in the field. In sampling areas, previous studies on cerambycid and curculionid species focused on distribution of these species in conifer

forests and little is known as their abundance (Özdikmen, 2007; 2008, Sarıkaya and Avcı 2011). Several other insect species such as *A. griseus*, *Ips* spp., *S. buprestoides* and *A. rusticus*, were previously reported as potential carriers of *B. xylophilus* in the USA, although as a non-specific/casual association (Linit, 1988; Ryss et al., 2005). Both *A. rusticus* and *S. buprestiodes* have a wide distribution in Turkish pine forests (Çanakçıoğlu and Mol 1998), including the areas of Burdur, Denizli, İzmir, Muğla and Isparta sampled during the current study.

In this study, *Bursaphelenchus*-vector associations show differences when compared to the literature. For instance, a *Bursaphelenchus* species of the *sexdentati* group, *B. sexdentati*, was found in association with a cerambycid insect, *A. aedilis*. Moreover, a *Bursaphelenchus* species of the *xylophilus* group, *B. mucronatus*, was found to be associated with a curculinid insect, *I. sexdentatus.* These results suggest that vector specificity in *Bursaphelenchus* species may vary depend on insect species in trees. Due to the critical geographical location, suitable climatic conditions, presence of vectors and highly succeptible host trees for *B. xylophilus*, these surveys not only emphasize our lack of knowledge related to the potential vectors of Bursaphelenchus species, but enable recognition of priority areas where PWN might succcesfully establish.

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