



# First finding of *Bursaphelenchus xylophilus* in pine plantations of the Republic of Armenia

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## Abstract

In 2022-2023, a survey of drying coniferous plantations in 5 regions of the Republic of Armenia was conducted. Investigations were carried out in plantations of Scots pine *Pinus sylvestris* L. and Crimean pine *Pinus nigra* subsp. *pallasiana* (Lamb.) Holmboe. A total of 125 wood samples were collected and analyzed. For the first time in the territory of the Republic of Armenia, single individuals of pinewood nematode *Bursaphelenchus xylophilus* (Steiner and Buhner 1934) Nickle 1981 were found near Hankavan village (Kotayk Province). The nematodes were found in samples from several trees with signs of browning needles. Identification by morphological features corresponded to the description of nematodes of the genus *Bursaphelenchus* Fuchs, 1937 of *xylophilus* group. The results of molecular genetic analysis of a fragment of the *mitochondrial* cytochrome oxidase I (mtCOI) gene showed 100% compliance with the species *B. xylophilus*. The phylogenetic analysis showed that the identified nematodes are grouped into one cluster with isolates from China, Korea, Portugal, and Japan. It is necessary to thoroughly examine pine plantations on the territory of Armenia to accurately determine the size of the outbreak of this quarantine species.

## Keywords

artificial pine plantations, *Bursaphelenchus*, first finding, pines, pine wilt disease (PWD), pinewood nematode (PWN), Republic of Armenia

## Introduction

Pinewood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner and Buhner 1934) Nickle 1981 is a causative agent of the withering of coniferous species. It is one of the most dangerous forest quarantine organisms in many world countries. From North America, which is considered the country of origin of *B. xylophilus*, the species first invaded the countries of East Asia, such as Japan (1905), China (1982), Taiwan (1985) and South Korea (1988), where it caused huge damage to forestry (Dropkin et al., 1981; Bergdahl, 1988; Mamiya, 1984; Shin, 2009; Zhao, 2008; Futai, 2021). In 1999, PWN was first registered in Europe and discovered in southern Lisbon, Portugal (Mota et al., 1999; Mota and Vieira,

2008). Since its discovery, large-scale nematode control measures have been carried out in infected areas, but despite this, in 2008, PWN was detected in Spain and 2009 on the island of Madeira (Abelleira et al., 2011; Fonseca et al., 2012; Zamora et al., 2015). These circumstances were the reason for the annual surveys of coniferous forests in the territory of the European Union (Anonymous, 2009; FAO, 2012). The distribution and introduction of PWN over long distances occur during international trade in coniferous wood, as well as with bark, sawdust, and wood packaging materials (Douma et al., 2017; EFSA et al., 2020; Arbuzova et al., 2023; Sousa et al., 2011; Hopf-Biziks et al., 2017).

The territory of Armenia is located in the north of West Asia and borders Georgia, Azerbaijan, Turkey,

and Iran. The total area of forests in Armenia is more than 330 thousand hectares, or 11% of the country's total area, and is of great natural and economic importance (Bondarev, 2020) (Fig. 1). The distribution of forests in Armenia is uneven. Currently, 62% of the forest cover is located in the north and northeast (Lori and Tavush Provinces), 36% in the southeast (Syunik Province), and only 2% in the central region of the country (Mkrtchyan and Grigoryan, 2014).

According to "Flora of Armenia" (Takhtajyan, 1954), the relict conifer (Coniferales) flora is presented in the country by only seven species, including pines (*Pinus kochiana*, currently considered synonym of *Pinus sylvestris* var. *hamata*), junipers (5 species of *Juniperus*) and yew tree (*Taxus baccata*) and has an important nature conservation role. Of the main forest-forming species, pine plantations make up the smallest percentage of the forested area of Armenia. Numerous varieties of pines grow in the country. According to Alaverdyan (2002) natural forests and artificial forest plantations of the Caucasian pine *P. sylvestris* var. *hamata* (the author provided it as *P. hamata*) on the territory of Armenia occupy an area of about 17.7 thousand hectares – 5.3% of the countries' whole forested area, of which artificial plantations represent 94.9%, and only 5.15% are natural. *Pinus sylvestris* var.

*hamata* makes up most of the artificial pine plantations in Armenia; somewhere, it is mixed with small plots of *Pinus nigra* subsp. *pallasiana*.

In recent years, pine trees have been dried in Armenia, mainly in artificial plantations. This has caused serious concern among environmentalists, scientists, and residents of the country and has led to large-scale comprehensive surveys of pine pests in the territories where the mass drying was observed and carried out by a research group of the Scientific Center of Zoology and Hydroecology NAS RA since 2020. One task for our research group was to reveal the current state of the fauna of pathogenic nematodes in the pine forests of Armenia, for which reconnaissance surveys were performed in 2022-2023.

## Materials and Methods

**Wood sampling.** In the period 2022-2023 in five Provinces of Armenia (Kotayk, Gagharunik, Tavush, Lori, Aragatsotn) (see Map on Fig. 1), where the signs of pine wilt disease were observed, pine plantations were examined, most of which consisted of varieties of *Pinus sylvestris* var. *hamata*, somewhere mixed with small plots



Figure 1: Map with sampling sites and locality of the findings of *Bursaphelenchus xylophilus* in Armenia.

of *Pinus nigra* subsp. *pallasiana*. To determine the infestation of pine plantations with nematodes, including *B. xylophilus*, both completely dried trees, and trees at different stages of drying out, were studied. The wood samples were collected from April to November from the trunks of the trees, or drying branches of the first and second orders, as well as in places containing larval passages of stem pests and in areas with signs of damage by wood-coloring fungi (bluing of wood, etc.). In total, wood samples from 125 trees were collected and processed.

Wood chips with 100-150 cm<sup>3</sup> volume were drilled from each selected tree using a cordless drill. After taking each sample, the parts of the instruments in contact with wood were sterilized with ethanol and burned with a flame.

The Baermann funnel method was used to isolate nematodes (Baermann, 1917; EPPO, 2013). The exposure time of wood tissues in water was 24 hours at room temperature, after which the resulting suspension was viewed using a Zeiss Stemi 508 stereomicroscope. After the preparation of temporary preparations (FAO, 2016), the detected nematodes were photographed with a microscope Zeiss Axio Imager A2 equipped with AxioCam 208 color camera.

A morphological method was used to identify nematodes. Identification to the species level was carried out in accordance with Diagnostic protocols for related pests (FAO, 2016) and PM 7/4(4) (EPO, 2023) using keys from Nickle et al. (1981), Kulinich and Ryss (2006), Hunt (2008), Fonseca et al. (2008), Braasch and Schönfeld (2015).

Morphological diagnostics were combined with molecular diagnostics to obtain a reliable result since it is extremely difficult to differentiate closely related species by morphological characteristics.

**DNA extraction, PCR amplification, and sequencing.** The nematodes were collected in Eppendorf tubes for further DNA extraction, which was carried out using a DNA-Extra-2 kit (Syntol, Russia) according to the manufacturer's recommendation. The method is based on treating the sample with proteinase K, followed by removing proteins without extraction with organic solvents. DNA precipitation from the purified solution was carried out with isopropyl alcohol using glycogen as a precipitator. The concentration and purity of the isolated DNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

The amplification of the mitochondrial gene fragment (mtCOI) was performed for all samples using the forward primer COI-F1 (5-CCTACTATGAT

TGGTGGTTTTGGTAATTG-3) and the reverse primer COI-R2 (5'-GTAGCAGCAGTAAAAAAGCACG-3) (Kanzaki and Futai, 2002; Ye et al., 2007). The PCR mixture (50 µl) contained 5 µl DNA, 1.5 µl of each primer, 10 µl 5X ScreenMix (Evrogen, Russia), and 32 µl of sterilized distilled water. Amplification was conducted using a T100 Thermal Cycler (Bio-Rad, USA). The thermal cycling program performed denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 45 s, and extension at 72°C for 2 min. A final extension was performed at 72°C for 10 min. The PCR products were purified (QIAquick PCR Purification Kit, Qiagen, Germany) and sequenced. The sequencing reaction was performed using the Sanger method using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) reagents according to the manufacturer's instructions, followed by fragment separation on an automatic genetic analyzer ABI PRISM 3500 (Applied Biosystems).

**Samples and sequence alignment.** Nucleotide sequences obtained in this study were edited and aligned using BioEdit software (Hall, 1999). After that, using the BLAST program, a search for similar nucleotide sequences was carried out in the GenBank database (NCBI) (<http://blast.ncbi.nlm.nih.gov/>).

22 additional DNA barcodes of *Bursaphelenchus* including 10 specimens of *B. xylophilus* (3 from China, 3 from Japan, 2 from Portugal, 1 from South Korea, 1 from USA), 10 specimens of *B. mucronatus* (2 from South Korea, 5 from Japan, one specimen each from Ukraine, Brazil and Finland), *B. sexdentati* (1 from Germany and 1 from Greece) were obtained from GenBank and added to the alignment. *Aphelenchoides besseyi* was added to the alignment as the out-group to root the tree. The data of specimens used in our phylogenetic analyses are presented in Table 1.

The maximum Likelihood phylogenetic tree was constructed using the Kimura 2-parameter model with 1000 bootstrap replicates in MEGA 7 (Kimura, 1980). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 24 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 510 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2016).

**Table 1: Specimens of the *Bursaphelenchus* spp. and outgroup sample used in phylogenetic analysis.**

| Species                       | Country     | Plant host                | GenBank Accession No. mtCOI | Reference                   |
|-------------------------------|-------------|---------------------------|-----------------------------|-----------------------------|
| <i>B. xylophilus</i>          | China       | <i>Pinus koraiensis</i>   | MT808403                    | GenBank                     |
|                               | China       | <i>Pinus koraiensis</i>   | MT808404                    |                             |
|                               | China       | <i>Pinus tabuliformis</i> | MT808405                    |                             |
|                               | USA*        | Pinus packaging wood      | JF317257                    |                             |
|                               | Japan       | Unknown                   | LC316944                    | GenBank                     |
|                               | Japan       | Unknown                   | LC311456                    | GenBank                     |
|                               | Japan       | <i>Pinus</i> spp.         | AB067766                    | Kanzaki and Futai, 2002     |
|                               | Portugal    | <i>Pinus pinaster</i>     | JQ514068                    | Moreira et al., 2014        |
|                               |             |                           | JQ429761                    |                             |
|                               | South Korea | Unknown                   | GQ332424                    | Sultana et al., 2013b       |
| <i>B. mucronatus</i>          | Armenia     | <i>Pinus sylvestris</i>   | PP989924                    | This study                  |
|                               | South Korea | Unknown                   | NC021120                    | Sultana et al., 2013a       |
|                               | South Korea | Unknown                   | GU177865                    | Sultana et al., 2013b       |
|                               | Japan       | <i>Pinus</i> spp.         | AB067765                    | Kanzaki and Futai, 2002     |
|                               | Japan       | Unknown                   | AB595193                    | GenBank                     |
|                               | Japan       | <i>Pinus thunbergii</i>   | AB634848                    | Matsunaga and Togashi, 2004 |
|                               | Japan       | <i>Pinus thunbergii</i>   | AB634847                    | Matsunaga and Togashi, 2004 |
|                               | Japan       | Unknown                   | AY508049                    | Ye et al., 2007             |
|                               | Ukraine*    | Pinus packaging wood      | JF317258                    | Gu et al., 2011             |
|                               | Brazil      | Pinus packaging wood      | JF317259                    | Gu et al., 2011             |
| <i>B. sexdentati</i>          | Finland     | <i>Pinus sylvestris</i>   | AY508051                    | Ye et al., 2007             |
|                               | Germany     | <i>Pinus sylvestris</i>   | MN101135                    | Gu et al., 2020             |
|                               | Greece      | <i>Pinus nigra</i>        | AY508063                    | Ye et al., 2007             |
| <i>Aphelenchoides besseyi</i> | USA         | <i>Fragaria ananassa</i>  | AY508072                    | Ye et al., 2007             |

\*The country of origin of these specimens remains unclear since the nematodes were found in packaging wood, a circulating product that is repeatedly used.

## Results

As a result of the conducted surveys, *Bursaphelenchus xylophilus* was reliably detected in samples collected from only one locality, namely, single individuals of nematodes were isolated from the wood chippings of several pines with signs of drying out near the village of Hankavan (in 2022, N40.63029 E44.47296, 1999 m; in 2023, N40.63030 E44.47299,

1994 m). Over the two years of our investigation, only six individuals were identified: in 2022, one sexually mature female and two males, while in 2023, two females and one male were discovered. Using light microscopy, the main morphological features of the identified nematodes were photographed (Fig. 2). Characteristic features of *Bursaphelenchus* include the typical head shape with a stylet. The vulval flap was clearly visible on the vulva of the female, which



is located at 75% of the body length from the anterior end (Fig. 2b). The female has a round tail end (Fig. 2c). The male's spicule is curved and has a characteristic cucullus in the form of a flattened disk (Fig. 2d). The morphometric characteristics of nematodes identified in Hankavan in 2023 are shown in Table 2.

According to the results of the sequence of the mtCOI gene and using the BLAST sequence analysis tool, nematodes from Armenia were clearly identified as representatives of the species *B. xylophilus*, showing exact similarity to the Chinese isolate MT808405. The nucleotide sequence we obtained from the mtCOI

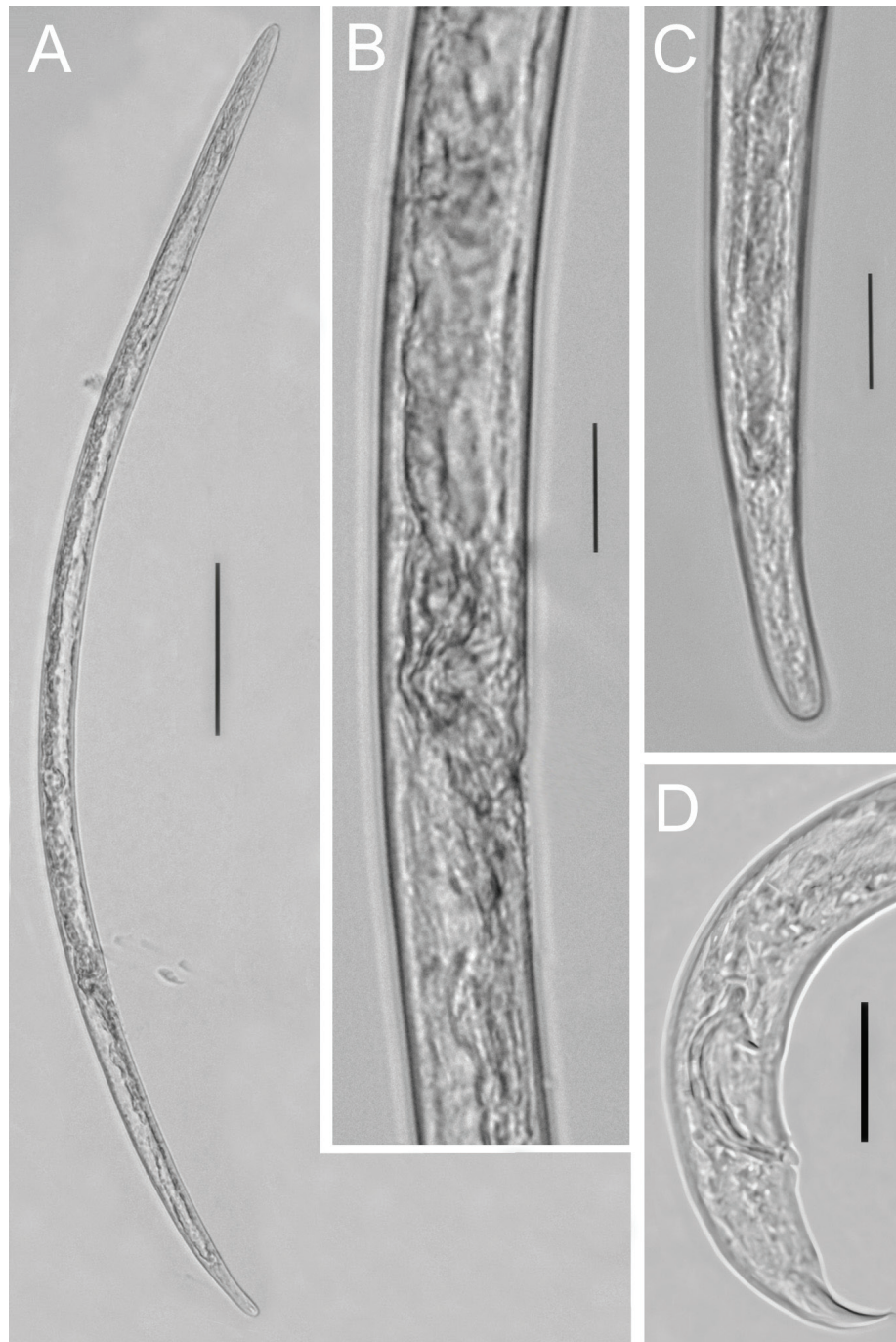


Figure 2: Light micrographs of *Bursaphelenchus xylophilus* from Armenia, env. Hankavan. A – female (100 µm); B – female vulval region (20 µm); C – female rounded tail terminus (20 µm); D – male tail with spicule (20 µm).

**Table 2: Morphometric characteristics of *Bursaphelenchus xylophilus* (Armenia, env. Hankavan vill., collected in 2023) in comparison with the typical populations from USA, Japan, and Portugal.**

| Character                         | Males                        |                                          |                                                   |                                              | Females                      |                                          |                                                   |                                           |
|-----------------------------------|------------------------------|------------------------------------------|---------------------------------------------------|----------------------------------------------|------------------------------|------------------------------------------|---------------------------------------------------|-------------------------------------------|
|                                   | Armenia<br>this study<br>n=1 | USA<br>(Nickle et al.,<br>1981)<br>n = 5 | Japan<br>(Mamiya and<br>Kiyohara, 1972)<br>n = 30 | Portugal<br>(Mota et al.,<br>1999)<br>n = 12 | Armenia<br>this study<br>n=2 | USA<br>(Nickle et al.,<br>1981)<br>n = 5 | Japan<br>(Mamiya and<br>Kiyohara, 1972)<br>n = 30 | Portugal (Mota et<br>al., 1999)<br>n = 12 |
| <b>L, <math>\mu</math>m</b>       | 0.85                         | 0.56<br>(0.52-0.60)                      | 0.73<br>(0.59-0.82)                               | 1.03<br>(0.80-1.30)                          | 0.98<br>(0.97-1.00)          | 0.52<br>(0.45-0.61)                      | 0.81<br>(0.71-1.01)                               | 1.05<br>(0.89-1.29)                       |
| <b>Stylet, <math>\mu</math>m</b>  | 11.9                         | 13.3<br>(12.6-13.8)                      | 14.9<br>(14.0-17.0)                               | 12.6<br>(11.0-16.0)                          | 12.4<br>(12.7-12.1)          | 12.8<br>(12.6-13.0)                      | 15.9<br>(14.0-18.0)                               | 12.3<br>(11.0-15.0)                       |
| <b>Spicule, <math>\mu</math>m</b> | 24.8                         | 21.2<br>(18.8-23.0)                      | 27.0<br>(25.0-30.0)                               | 24.0<br>(22.0-25.0)                          | -                            | -                                        | -                                                 | -                                         |
| <b>Vulva position,<br/>% of L</b> | -                            | -                                        | -                                                 | -                                            | 75.4<br>(75.0-75.7)          | 74.7<br>(73.0-78.0)                      | 72.7<br>(67.0-78.0)                               | 73.3<br>(70.0-76.0)                       |

L – body length, n – number of specimens.

fragment with a length of 702 bp was deposited in GenBank (Accession No. PP989924). Maximum Likelihood (ML) phylogenetic analysis showed that the sequence of the Armenian nematodes was grouped to the clade with sequences of Portuguese (JQ429761, JQ514068), Chinese (MT808403 – MT808405), Korean and Japanese isolates of *B. xylophilus* (Fig. 3).

The tree with the highest log likelihood (-1916.33) is shown. The percentage of trees in which the associated taxa clustered is shown next to the branches.

## Discussion

It is believed that the manifestation of pine wilt disease (PWD) caused by the pinewood nematode *B. xylophilus* occurs only in areas with an average summer temperature above 20-25°C, and in colder climates, it is rare or completely absent (Evans et al., 2008; Jones et al., 2008). On the other hand, nematode itself can survive with asymptomatic carriers in lower temperature conditions without manifestation of PWD; for example, in Japan, in the territories with the hottest summer month below 20°C average where *B. xylophilus* is present, the manifestation of PWD was not observed (Rutherford and Webster, 1987). The same can be assumed for Armenia. The climate in the country can be described as dry continental, where the average temperature ranges from 2.7 to 14°C (Fayvush et al., 2011). The average annual precipitation is about 600 mm (250 to 1000 mm depending on the altitude above sea level) (Second National Communication of the Republic of Armenia under the United Nations framework convention on climate change, 2010). According to the climatic data for Hrazdan town, the locality closest to Hankavan from which such data are available, the mean daily temperature for January ranges from -1 to -8°C and for July and August – from 13 to 21°C, reaching the hottest days 25–26°C (Simulated data for Hrazdan, 2024). Such conditions are suitable for the survival of PWN but not for its mass reproduction (Rutherford and Webster, 1987; Futai, 2013; Tuomola et al., 2021); thus, the presence of PWN is difficult to detect in these conditions, which can explain the identification of only single PWN individuals. The nematodes identified during reconnaissance surveys in the sawdust of *P. sylvestris* pine and described in this study had diagnostic signs (vulva and tail section of the female, the shape of the male's spicules) and morphometric parameters characteristic of *B. xylophilus* (Zamora et al., 2015; Fonseca et al., 2008; EPPO, 2023). The morphological identification of PWN was unambiguously confirmed by the

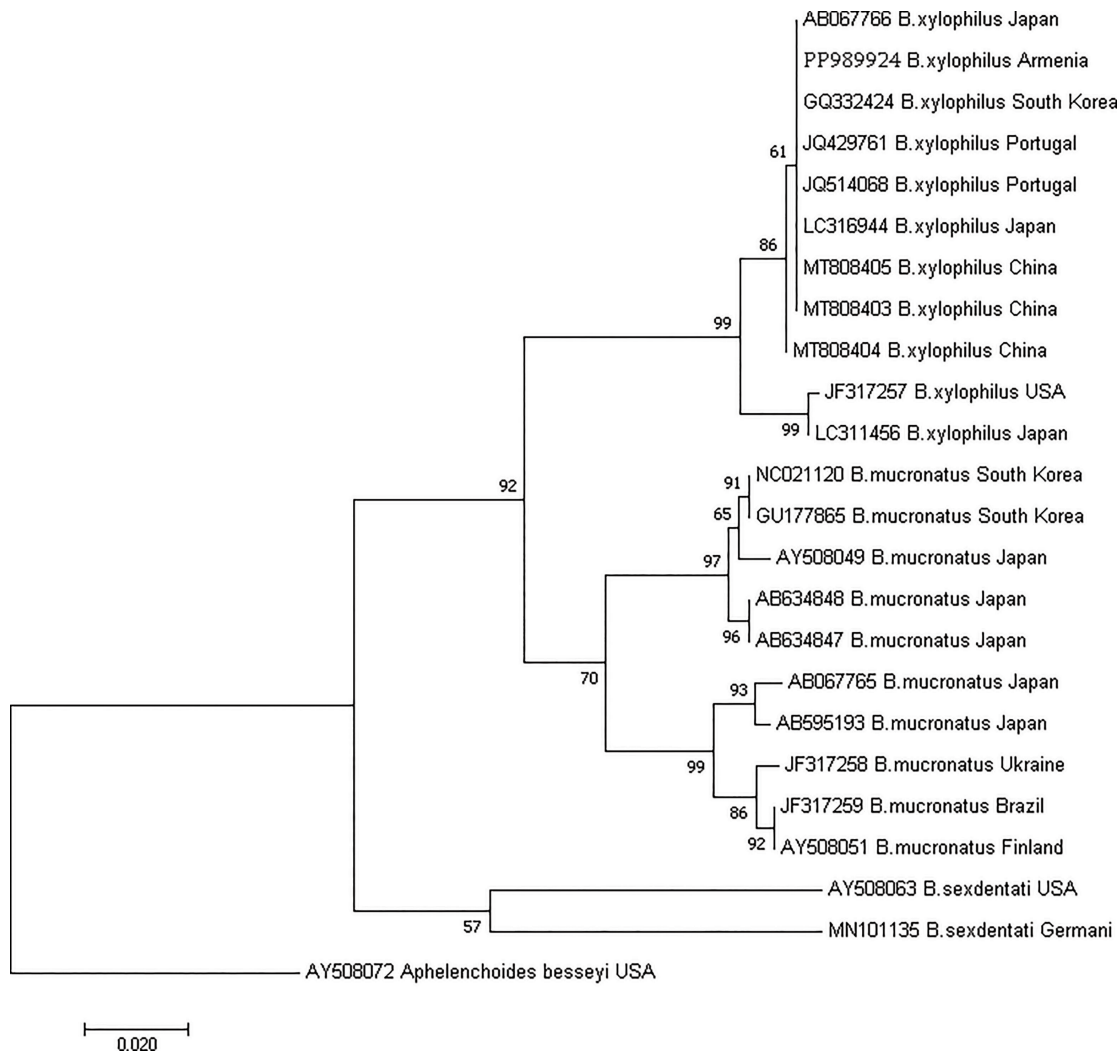


Figure 3: Molecular phylogenetic analysis by Maximum Likelihood method for 23 *Bursaphelenchus* isolates inferred from the alignment of mtCOI sequences.

sequencing results by the mitochondrial cytochrome oxidase subunit I (mtCOI).

It is impossible to say whether this species has been in the country for some time or it has appeared recently since there has been no real control over imported wood. Like in other countries, the species could be imported with wood or wood packaging material. However, the quarantine and forest services of Armenia have no capacity to detect PWN. Unclear also is the question of vector species of PWN in the locality where it was revealed. As far as it is known, the main vector of PWN is considered the species of the genus *Monochamus* (Naves et al., 2016). In Armenia, only one species of the genus, *M. galloprovincialis*, was reported as being most probably invasive for the country as it was found only in the cities, such as Yerevan and Gyumri (Plavilshnikov, 1948) and in Khosrov reserve (Danilevsky, 2023) where

pine plantations are completely absent. This allows us to suppose that the beetles collected in the localities above mentioned emerged from imported wood. During our survey, we found the species in natural conditions only in artificial pine plantations in most Northeastern parts of Armenia, but not in the locality where PWN was detected. On the other hand, several beetle species other than *Monochamus* were found transporting PWN (Naves et al., 2016). In pine plantations near Hankavan some beetles listed by these authors were revealed, namely *Acanthocinus*, *Spondylis*, *Arhopalus*, *Asemum*. Some of these species could serve as a vector in the area.

Considering the fact of the discovery of quarantine object *B. xylophilus* in the vicinity of Hankavan village, it is necessary to change the focus of phytosanitary measures. It is important to take preventive measures

by removing dead or dying trees. Isolation of the infected area, burning or recycling of infected trees is necessary. Any weakened and shrinking tree can become a reservoir of nematodes, as well as insect vectors that will carry nematodes over long distances (Schrader and Unger, 2003; Jones et al., 2008). In addition, an urgent national, widespread study of pine plantations is needed to assess the prevalence of *B. xylophilus* and its vector beetles in the country. It is also advisable to conduct an analysis of the phytosanitary risk of PWN for the territory of Armenia in order to determine damage in these climatic conditions new to *B. xylophilus* and possible ways of introducing this dangerous pathogen.

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