Chapter 3

Systematic Identification of the *Xylophilus* Group in the Genus *Bursaphelenchus*

Jianfeng Gu

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.77096

Abstract

The pine wood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970 is the agent responsible for pine wilt disease (PWD). This nematode has been killing native pine trees (*Pinus densiflora*, *P. thunbergii*, *P. luchuensis*) in Japan since the early twentieth century. It is the number one forest pest in Japan and has been spread to China, Korea, Portugal, and Spain. The nematode is native to North America (Canada, USA, Mexico) and is thought to have been carried to Japan at the beginning of the twentieth century on timber exports. Up to now, the genus *Bursaphelenchus* Fuchs, 1937 comprises nearly 120 species (14 groups). Around 14 species very similar to *B. xylophilus* are put together and named the *xylophilus* group. This chapter presents the grouping history, subspecies or genetic types in species of the *xylophilus* group, and an identification key for 14 species of the *xylophilus* group, ITS-RFLP identification, and other molecular identification methods are also discussed.

Keywords: morphology, molecular, ITS-RFLP, DNA barcoding

1. Introduction

Pine wilt disease (PWD), which is caused by pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer [6]) Nickle [1], has been devastating Japanese pine forests since the beginning of the twentieth century. For many years, the mass mortality of pine trees was supposed by attacks of beetles. Until 1971, *Bursaphelenchus* sp. was demonstrated as the causal agent of PWD by inoculation tests on *Pinus* spp. [2], and subsequently the nematode was described as *Bursaphelenchus lignicolus* [3]. After that, the PWN was first reported in the United States in 1979 [4]. Extensive surveys revealed the widespread distribution of the

nematode throughout the country [5], but no epidemic was found, and the disease occurred only on a few exotic pine species. The PWN was later proven to be the same species as the one described in Florida in 1934 [6], the name was then changed from *B. lignicolus* to *B. xylophilus* [7], and it has been indigenous to North America [8].

Later, the disease has spread into China in 1982, Korea in 1988, Mexico in 1993, Portugal in 1999, and Spain in 2011 [9], and it is now still a potential threat to pine forests worldwide.

In nature, *B. xylophilus* is spread from tree to tree through the activity of adult stages of wood-inhabiting longhorn beetles of the genus *Monochamus* (Coleoptera: Cerambycidae) for short distance. This transmits the nematode either to the shoots of living trees during maturation feeding either by sex or by oviposition of females. But human activity is responsible for the long-distance spread. It is widely accepted that national and international trade of pine logs and related packaging wood is the causal of PWN spreading, so national and international regulations (e.g., ISPM 15: FAO, 2003, revised 2009) were accompanied by intensive sampling and laboratory investigations for the presence of PWD in imported wood worldwide in order to significantly reduce the risk of the pest's spread. So, it is important to identify *B. xylophilus* to manage its further spreading and conduct early eradication plan.

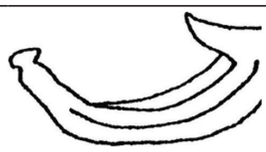
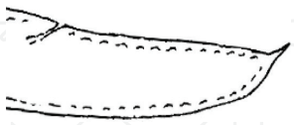
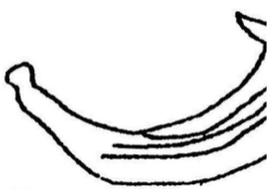
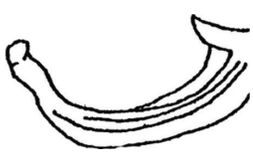
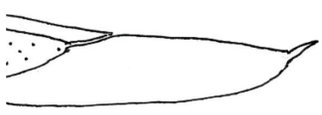
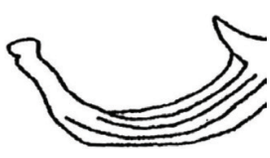
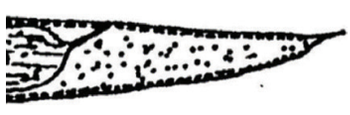
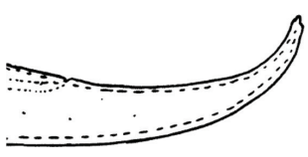
Before 2000, there were only other two closely related species: *B. fraudulentus* Rühm [10] and *B. mucronatus* Mamiya and Enda [11] (*B. kolymensis* Korenchenko [12] was later considered as being synonymous with *B. mucronatus*). For a long time, in diagnostic protocol of *B. xylophilus*, it was morphologically compared with only *B. mucronatus* and *B. fraudulentus*, many PCR-based methods also used only these three species samples.

Since 2000, with further study of packaging wood and phoretic insects, more *Bursaphelenchus* species were discovered. Now, there are 110–120 known species in this genus [9] and 14 species in the *xylophilus* group. *B. xylophilus* (Steiner & Buhrer [6]) Nickle [1]; *B. fraudulentus* Rühm [10] (J. B. Goodey, 1960); *B. mucronatus* Mamiya and Enda [11]; *B. conicaudatus* Kanzaki et al. [13]; *B. baujardi* Walia, Negi et al. [14]; *B. luxuriosae* Kanzaki and Futai [15]; *B. doui* Braasch et al. [16]; *B. singaporensis* Zhang et al. [17]; *B. macromucronatus* Gu et al. [18]; *B. populi* Tomalak and Filipiak [19]; *B. paraluxuriosae* Gu et al. [20]; *B. firmae* Kanzaki et al. [21]; *B. koreanus* Gu et al. [22]; and *B. gillanii* Schönfeld et al. [23].

2. Grouping history

Giblin and Kaya [24] first separated five groups within *Bursaphelenchus* mainly according to spicule morphology; the *xylophilus* group contains three species, namely, *B. xylophilus*, *B. mucronatus*, and *B. fraudulentus*, all have large, paired, arcuate spicules with a sharply pointed rostrum, and a disk-like expansion, cucullus, and females of this group have a vulval flap (**Table 1**). Braasch [25] studied the morphological relationship between European *Bursaphelenchus* species in order to provide key characters for their taxonomic identification. She considered the number of incisures in the lateral field as a basic grouping feature, together with other features like spicule shape, number and position of caudal papillae, presence and size of a vulval flap, and the shape of female tail. Among the 28

http://dx.doi.org/10.5772/intechopen.77096

Species	Main characters	Typical spicule shape	Typical female tail
B. xylophilus (R form)	Female tail cylindrical, terminus broadly rounded, without mucro (if there's a mucro, usually less than 2 μm)		
B. xylophilus (M form)	Like R form, but all females have a mucro, less than 3 μm on average (1.5–4.2 μm)		
B. fraudulentus	Spicule cucullus not clearly expanded, female tail cylindrical, c′ = 2.7–3.4, mucro usually present, about 1.5~2.6 μm		
B. mucronatus kolymensis	Female tail cylindrical or subcylindrical, mucro usually offset from the tail, mean length more than 4 μm		
B. mucronatus mucronatus	Female tail subcylindrical or conical, mucro not offset from the tail, about 4~7 μm		
B. conicaudatus	Female tail conical, ventrally bent, mucro about 2~3 μm, at the ventral position		
B. baujardi	Similar to *B. conicaudatus*, female tail conical, a small mucro present, length not clear		
B. luxuriosae	Female tail conical and clearly ventrally bent, terminus without mucro, roughed or irregular		
B. paraluxuriosae	Similar to *B. luxuriosae*, but female tail only slightly bent, without mucro, spicule without cucullus		

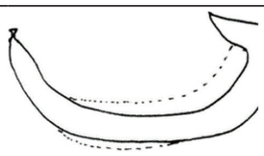
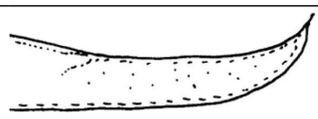
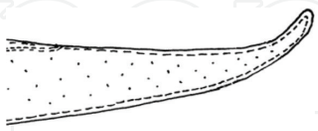
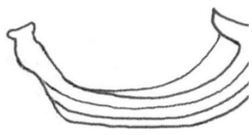
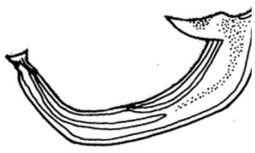
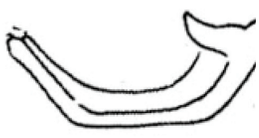
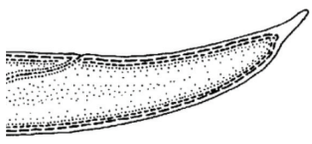
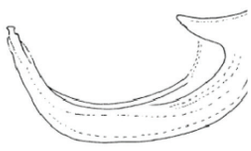
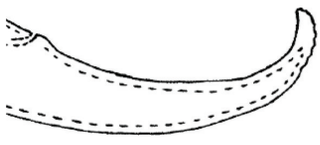
Species	Main characters	Typical spicule shape	Typical female tail
B. doui	Spicule length in chord 34~44 μm, the middle part nearly straight, female tail variable, usually show a mucro at the ventral position, about 2~4 μm		
B. singaporensis	Female tail without mucro, spicule length along the curved median line 41–48 μm, condylus continuous with the dorsal spicule line		
B. macromucronatus	Female tail conical, straight mucro usually continuous with tail, about 4.5 μm(2.5~6.5 μm)		
B. populi	Vulval flap ventrally bent with its distal half sunken in a conspicuous, sharp depression immediately posterior to the vulva		
B. firmae	Female mucro thick, terminus bluntly pointed		
B. koreanus	Spicule length along the curved median line 35–44 μm, condylus set off from dorsal spicule line, female tail conical and ventrally bent with slightly pointed, irregular, or roughened terminus		
B. gillanii	Female tail conical, mucro 5–7 μm, wide at the base		

Table 1. Main morphological characters of the ***xylophilus*** group.

conifer-inhabiting European species, she proposed eight groups. The *xylophilus* group (*B. xylophilus*, *B. mucronatus*, and *B. fraudulentus*) can easily be separated from all other species by the presence of four incisures, the typical shape of spicules, the special position of the caudal papillae, and the large vulval flap of females.

Ryss [26] considered that those characters like lateral lines, number and position of caudal papillae, and vulval flap are available for only some of the nominal species; thereby, their utility is limited. So, he studied 75 valid species of the genus *Bursaphelenchus* known that time. Only based on spicule structure, he sorted this genus into six groups: *hunti*, *aberrans*, *eidmanni*, *borealis*, *xylophilus*, and *piniperdae* groups. For the *xylophilus* group, its spicule is characterized by capitulum flattened anteriorly, small condylus, dorsal contour of the lamina distinctly angular in last third, and cucullus usually present (except in *B. crenati*). He listed ten species: *B. xylophilus*, *B. abruptus*, *B. baujardi*, *B. conicaudatus*, *B. crenati*, *B. eroshenkii*, *B. fraudulentus*, *B. kolymensis*, *B. luxuriosae*, and *B. mucronatus*. Later study showed that *B. abruptus*, *B. crenati*, and *B. eroshenkii* were definitely different from the *xylophilus* group [27]. *B. crenati* has a different position of the caudal papillae (the double pair in front of the bursa is missing), the presence of a vulval flap is questionable, and the spicules do not show a cucullus. Additionally, it is transmitted by a bark beetle, a scenario not typical for the *xylophilus* group. *B. eroshenkii* has five incisures in the lateral field, only five caudal papillae (seven in the *xylophilus* group) and no vulval flap [28]. The spicule shape of *B. abruptus* is not typical.

Braasch [29] stated that the *xylophilus* group of the genus *Bursaphelenchus* can be clearly distinguished from other species of the genus by the presence of four lateral lines, the presence of a vulval flap in females, a characteristic shape of the male spicules, and the arrangement of the seven caudal papillae. An identification key of the nine species of the *xylophilus* group was presented, and *B. kolymensis* was considered to be the European type of *B. mucronatus*.

Later, with the development of the molecular methods, especially sequencing technique, more *Bursaphelenchus* sequences are available in the GenBank. Based on morphological characters and phylogenetic analysis [27], the genus is divided into eight groups with four incisures in the lateral field (*xylophilus*, *okinawaensis*, *africanus*, *fungivorus*, *cocophilus*, *kevini*, *tokyoensis* and *sexdentati* groups), four groups with three incisures (*eggersi*, *eremus*, *hofmanni*, and *leoni* groups), and two groups with two incisures (*abietinus* and *sinensis* groups). Most of the groups are well separated by both morphological and molecular studies.

3. Subspecies or genetic types in species of the *xylophilus* group

Bursaphelenchus mucronatus Mamiya and Enda [11] was first found from pine trees in Japan. Braasch [30] reported for the first time *B. mucronatus* in timber imports from Siberia and in forest trees in Germany. These populations (later on named "European genotype" or "European type") had shown morphological and morphometric deviations from Japanese *B. mucronatus* isolate. Separate species status for Japanese and European *B. mucronatus* was postulated on the basis of sequence differences of an amplified fragment of the heat shock 70A gene [31]. However, successful mating experiments of a European *B. mucronatus* with a Japanese isolate argued against this idea [32].

Braasch et al. [33] proposed the two *Bursaphelenchus mucronatus* types to be subspecies. The European type is named *B. mucronatus kolymensis*, and the East Asian type is named *B. mucronatus mucronatus*. The earlier described *Bursaphelenchus kolymensis* corresponds to *B. mucronatus kolymensis* in morphological characters. The two subspecies show morphological

differences in the shape of female tail, length of mucro, position of excretory pore, and also small differences in spicule shape. They can be distinguished by their ITS-RFLP patterns based on restriction fragments obtained with enzymes *Rsa* I and *Hae* III. Based on sequence analysis of ribosomal ITS1/ITS2, LSU D2/D3, and mitochondrial COI regions, a clear subdivision of the two isolate groups (subspecies) has been confirmed.

Since the report of a mucronate ("M") form of *B. xylophilus* detected from balsam fir (*Abies balsamea*) in Minnesota and Wisconsin, USA [34], uncertainty in morphological distinction of *B. xylophilus* from related species became evident. For a long time, it is morphological and molecular characters are not clear. Gu et al. [35] made a morphological and molecular study based on five isolates of "M" form of *Bursaphelenchus xylophilus*, together with the round-tailed ("R") form of *B. xylophilus* and *B. mucronatus* (both subspecies), and founded that the spicules of these species (types or forms) are similar. The "M" form of *B. xylophilus* is distinguished from the "R" form of *B. xylophilus* by a distinct mucro at the female tail end. It differs from the *B. mucronatus kolymensis* by slightly shorter female tail mucro and position of excretory pore. It is distinguished from *B. mucronatus mucronatus* by female tail shape and shorter female tail mucro. The conventional five restriction endonucleases (*Rsa* I, *Hae* III, *Msp* I, *Hinf* I, and *Alu* I) used for obtaining ITS-RFLP patterns of *Bursaphelenchus* species cannot distinguish the "M" and "R" form of *B. xylophilus*, but the two forms can be differentiated by the use of two additional restriction endonucleases (*Hpy*188 I and *Hha* I). The molecular phylogenetic analysis based on the sequences of D2D3 LSU rDNA, ITS1/2 region, and mtCOI revealed that the "M" form of *B. xylophilus* is genetically closest to the "R" form of *B. xylophilus*, and that their sequence divergence is small.

4. Morphological characters of the *xylophilus* group

According to Braasch et al. [27], the *xylophilus* group is characterized by four lateral lines; seven caudal papillae; conspicuous P4, P3, and P4 papillae adjacent to each other (double pair) just anterior to bursa; spicules long, slender, and semicircular with angular lamina in posterior third; capitulum fattened with small condylus and distinct rostrum; cucullus present (for *B. fraudulentus* and *B. paraluxuriosae*, spicule cucullus is not clearly visible); and large vulval flap.

But lateral lines and caudal papillae are not easy to be seen sometimes, so typical male spicule shape and female vulval flap should be the main grouping characters [35]. In all known *Bursaphelenchus* species, only *B. masseyi*, *B. trypophloei*, and *B. abruptus* may be confused with *B. xylophilus* group. All their females have a vulval flap, but their spicules are not typical. *B. trypophloei* and *B. masseyi* differ in having relatively short rostrum, and the angular contour of the dorsal lamina is usually indistinct. *B. abruptus* differs in different ventral curvatures of the spicules.

5. Morphological identification of *B. xylophilus* with a key

Usually, R form of *B. xylophilus* is distinguished from other species by cylindrical female tail with bluntly rounded terminus, without mucro, or in some cases, some females will show a mucro, which is less than 2 μm.

http://dx.doi.org/10.5772/intechopen.77096

But the mucro character of the R form of *B. xylophilus* is not always stable; it depends on different hosts and environmental situations. Braasch [36] reported that when an R form *B. xylophilus* isolate (US15) was re-extracted from trees 3 months after inoculation experiment, 35% of females were round-tailed, 8% had conical tails, and 17% had a distinct mucro (up to 4–5 μm), whereas 40% had a very small mucro of 1 μm length. Zheng et al. [37] reported that an R form *B. xylophilus* was detected from a pine tree in Ningbo, China; all females had a distinct mucro, ranging from 0.5 to 2.9 μm (mean 1.7 μm), but the mucro disappeared after culturing on *B. fuckeliana*. Gu et al. [35] also reported an R form *B. xylophilus* isolate (4049); about half of the females detected from packaging wood had a round tail, and the other half showed a

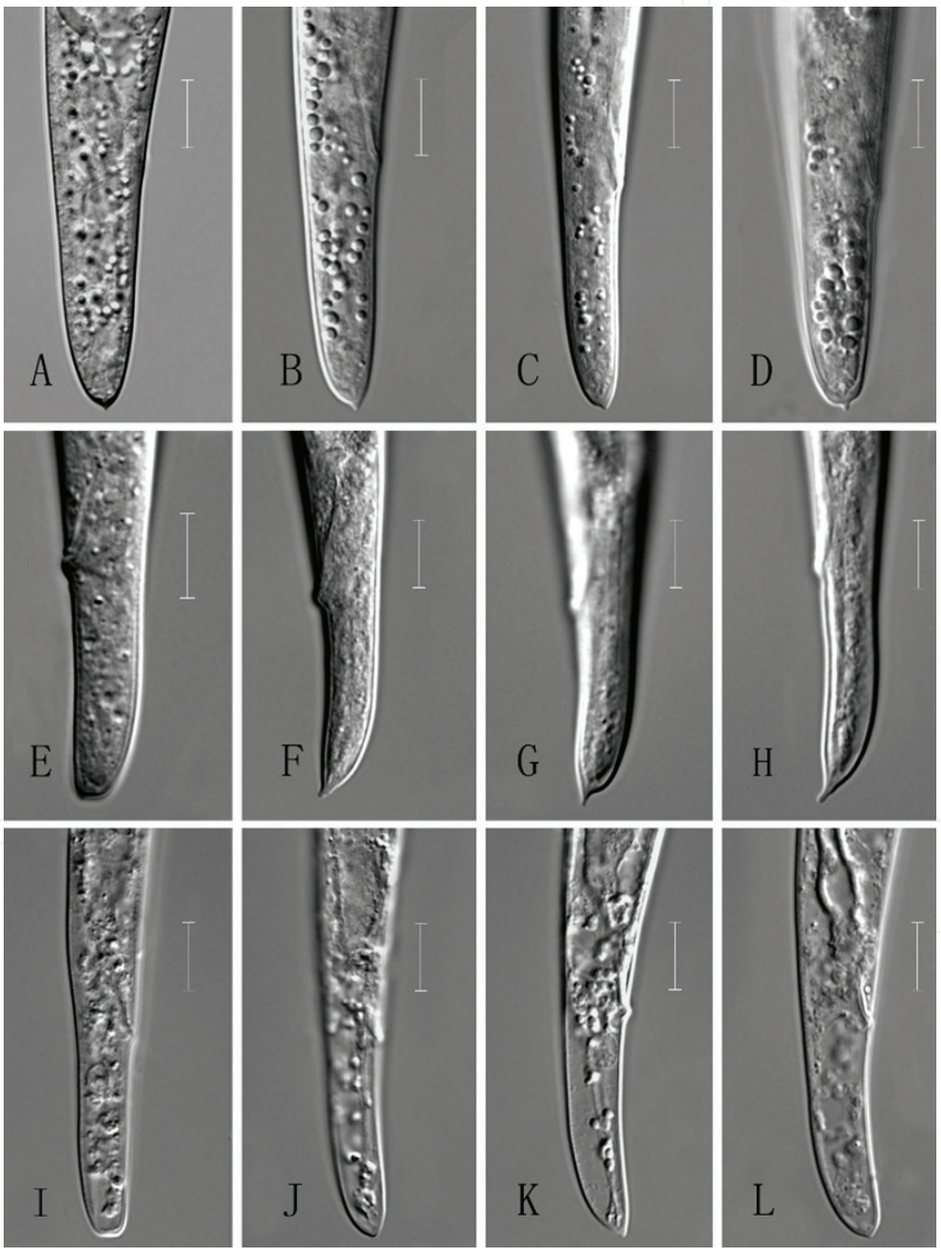


Figure 1. Light photomicrographs of female tails of "R" form of *Bursaphelenchus xylophilus* (isolate 4049) in different situations: A–D, detected from the packaging wood; E–H, after culturing on *B. fuckeliana*; and I–L, after culturing on *Pestalotiopsis* sp. (scale bars = 10 μm).

very small mucro about 0.5–1 μm long. But after culturing on *B. fuckeliana* for 1 month, more than half of females showed a mucro of about specimens, a mucro of less than 0.5 μm long, or no mucro. However, after being cultured on *Pestalotiopsis* sp., apart from some round-tailed females, most females had a bluntly pointed tail terminus (**Figure 1**).

Typical R form of *B. xylophilus* can be distinguished from other species of the *xylophilus* group by the female tail shape. *B. populi* sometimes also shows a cylindrical female tail without mucro, but they can be separated by the vulval flap ventrally bent with its distal half sunken in a conspicuous, sharp depression. Identification of the M form of *B. xylophilus* is more difficult. Females in mucronate populations generally show a mucro on the female tail end, on average 2.2–3.0 μm long (1.5–4.2 μm). Its mucro shape does not change even after culturing for many years. The M form of *B. xylophilus* is morphologically most similar to the *B. mucronatus kolymensis*. It is distinguished from it by slightly shorter mucro on female tail (mean 2.2–3.0 μm *vs.* 3.0–5.0 μm) and the position of excretory pore. Up to now, M form of *B. xylophilus* has only been reported in North America, and its report in China and Taiwan is still questionable. Due to a certain variation in characters between populations and different hosts and environmental situations, it is essential to perform molecular test in case of doubt.

The following dichotomous key of species of the *xylophilus* group is based on the female tail shape (conical or cylindrical, with or without mucro, and mucro length), vulval flap shape (straight or bent), and spicule size and shape (with or without cucullus).

1.	(a) Posterior to the vulva	*B. populi*
	(b) Vulval flap bent and to the vulva not clear	2
2.	(a) Spicule cucullus not clearly expanded	3
	(b) Spicule cucullus expanded	4
3.	(a) Female tail cylindrical, c′ = 2.7–3.4, mucro present	*B. fraudulentus*
	(b) Female tail conical, c′ = 4–5, without mucro	*B. paraluxuriosae*
4.	(a) Average c′ > 4, female tail conical	5
	(b) Average c′ < 4, female tail cylindrical, subcylindrical, or conical	9
5.	(a) Female tail without mucro	6
	(b) Female tail with mucro	8
6.	(a) Spicule length along the curved median line 27–30 μm	*B. luxuriosae*
	(b) Spicule length along the curved median line more than 35 μm	7
7.	(a) Spicule length along the curved median line 35–44 μm, condylus set off from dorsal spicule line	*B. koreanus*
	(b) Spicule length along the curved median line 41–48 μm, condylus continuous with the dorsal spicule line	*B. singaporeinsis*
8.	(a) Stylet with small knob, excretory pore ranging from median bulb to hemizonid, c′ = 3.6–5	*B. conicaudatus*
	(b) Stylet without small knob, excretory pore at the position of median bulb, c′ = 3–4	*B. baujardi*

9.	(a) Spicule length in chord 34~44 μm, the middle part nearly straight	*B. doui*
	(b) Spicule length in chord <34 μm, the middle part slightly ventrally curved	10
10.	(a) Female tail cylindrical, terminus broadly rounded, without mucro (some females may possess a short process at the tail terminus, usually less than 2 μm	*B. xylophilus* (R form)
	(b) Female tail cylindrical, subcylindrical, or conical, terminus with mucro, more than 2 μm	11
11.	(a) Mucro usually continuous with tail	12
	(b) Mucro usually offset from tail	15
12.	(a) Spicule condylus dorsally not offset, body slim (a > 40)	*B. mucronatus mucronatus*
	(b) Spicule condylus dorsally offset, body stout (a < 40)	13
13.	(a) Female mucro terminus pointed	14
	(b) Female mucro terminus bluntly pointed	*B. firmae*
14.	(a) Female tail straight	*B. macromucronatus*
	(b) Female tail slightly bent, dorsally stronger bent than ventrally	*B. gillanii*
15.	(a) Mucro mean length more than 4 μm	*B. mucronatus kolymensis*
	(b) Mucro mean length less than 3 μm	*B. xylophilus* (M form)

6. Identification of the *xylophilus* group species with ITS-RFLP method

Application of ITS-RFLP analysis to *Bursaphelenchus* species identification was first described in 1998 [38, 39]. In this technique, a region of ribosomal DNA (rDNA), containing the internal transcribed spacer regions ITS1 and ITS2, is amplified by PCR method with forward primer F194 5′-CGTAACAAGGTAGCTGTAG-3′ (Ferris et al.) and reverse primer 5368 5′-TTTCACTCGCCGTTACTAAGG-3′ (Vrain) [40, 41], and, subsequently, the PCR products were digested with five restriction endonucleases *Alu* I, *Hae* III, *Hinf* I, *Msp* I, and *Rsa* I to get the restriction fragment length polymorphisms. Using the same set of five restriction enzymes, species-specific ITS-RFLP reference patterns were compiled for 11 *Bursaphelenchus* species in 1999 [42] and extended to 26 species in 2005 [43]. The technique has proven to be a valuable tool in identification of nematodes isolated from imported wood in quarantine control or forest surveys [44–47]. Wolfgang et al. (2009) produced ITS-RFLP reference profiles of 44 *Bursaphelenchus* species [48], including two intraspecific types in each of *B. mucronatus* and *B. leoni*. Though in the case of *B. corneolus*, *B. lini* (later identified as *Deviburs aphelenchus lini*), *B. singaporensis*, *B. sexdentati*, and *B. doui* [49], additional bands in the patterns of certain isolates or individual nematodes were observed which may be explained by ITS sequence microheterogeneity, i.e., the presence of ITS sequence variants within the number of rDNA tandem repeats, but they did not seriously impair identification of species based on the overall reference patterns. ITS-RFLP analysis has proven valuable not only for differentiation of the pathogenic pine wood nematode, *B. xylophilus*, from related species but also useful in

other *Bursaphelenchus* identifications. In many recent descriptions of new *Bursaphelenchus* species, ITS-RFLP profiles have been used as additional species identification criteria.

The abovementioned traditional ITS-RFLP method cannot separate M and R form of *B. xylophilus*, but according to Gu et al. [33], the two forms can be differentiated by the use of two additional restriction endonucleases (*Hpy188* I and *Hha* I).

7. Other molecular identification methods

Besides RFLP method, many species-specific PCR and real-time PCR methods were developed for *B. xylophilus* identification [50–58]. By real-time PCR [59] or loop-mediated isothermal amplification (LAMP) methods [60], *B. xylophilus* can be detected directly from wood. But we should notice that those methods were developed years ago, now more species in the *xylophilus* group are known, and the results may be questionable. And when molecular tests are used for quarantine purposes to detect *B. xylophilus* in wood products, it is essential to recognize that both live and dead nematodes can be detected by these tests.

More recently, Ye et al. [60] developed a real-time PCR assay for PWN identification [61]. Based on DNA sequence analysis on the ribosomal DNA small subunit, large subunit D2/D3, internal transcribed spacer (ITS), and mitochondrial DNA cytochrome oxidase subunit one on the aphelenchid species, they developed a rapid and accurate PWN identification method 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*; 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*, *Aphelenchoides* species, 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 rapid species identification necessary to comply with the zero-tolerance export regulations.

Nucleic acid sequencing methods have undergone tremendous advances over the past decade. Now, many 18S, ITS, and 28S gene sequences have been determined for *Bursaphelenchus* species, and they are deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/). In general, the comparison of those genes with reference data using sequence and phylogenetic analysis allows classification of nematode samples and establishing identification. Determinations of clades to which samples belong and the level of the interspecific variation are two approaches used together for molecular identification.

DNA sequencing method has been used widely in the last decade. But this method is not standard: different target genes and different primers are used, and sequences are analyzed with different methods in different labs.

DNA barcoding is a generic diagnostic method that uses a short standardized genetic marker in an organism's DNA to aid species identification. An organism is identified by finding the closest matching reference record in a database containing large amounts of barcode sequence

data. The first genetic marker to be described as a "barcode" was the mitochondrial cytochrome c oxidase I (COI) gene which is used for species identification in the animal kingdom [62]. According to Quarantine Barcoding Of Life (QBOL) project financed by the Seventh Framework Program of the European Union (www.q-bank.eu), first, a 1600 bp fragment of the small subunit (SSU) 18S rDNA gene can be PCR amplified and sequenced using primers 988F, 1912R, 1813F, and 2646R [63]. The obtained sequence data is used for identification to the genus and sometimes to species level. However, in some cases the SSU does not contain sufficient variation for identification to the species level, and additional sequences of the LSU (28S) rDNA or COI gene may be required to confirm the identification.

He and Gu [64] evaluated the applicability of 28S, 18S, and ITS loci as candidate DNA barcode markers for the *xylophilus* group of the genus *Bursaphelenchus*; they demonstrated that the average intraspecific divergences of 28S (not distinguishing two subspecies of *B. mucronatus*), 28S (distinguishing two subspecies of *B. mucronatus*), 18S, and ITS were 0.0071, 0.0030, 0.0007, and 0.0043, respectively, and, for interspecific divergences, were 0.0476, 0.0454, 0.0052, and 0.1556, respectively. The genetic distances between intraspecific and interspecific divergences of 28S and 18S loci showed some overlapping, but ITS loci had some degree of barcoding gap. The NJ trees from 28S and ITS loci with reliable bootstrap value could effectively separate 14 species of the *B. xylophilus* group into an independent branch. Furthermore, 28S locus could identify two subspecies of *B. mucronatus* well. The NJ tree of 18S locus demonstrated that *B. gillanii*, *B. firmae*, and *B. mucronatus* were mixed and difficult to be separated each other. In conclusion, 28S and ITS loci were suggested as candidate barcode genes for the *B. xylophilus* group due to their larger barcoding gap and higher species resolution.

When sequencing is more easy, quick, and cheap, and more sequences are available in the database, DNA barcoding will be the best way for species identification for genus *Bursaphelenchus*, even for other genera in the future.

8. Conclusion

After devastating a vast area of pine forests in Asian countries, the pine wilt disease was spread into European forests in 1999 and was causing a worldwide concern. To date, about 120 species of the genus *Bursaphelenchus* have been described, and 14 groups is suggested. About 14 species very similar to *B. xylophilus* are put together and named the *xylophilus* group. The *xylophilus* group is characterized by four lateral lines; seven caudal papillae; conspicuous P4, P3, and P4 papillae adjacent to each other (double pair) just anterior to bursa; spicules long, slender, and semicircular with angular lamina in posterior third; capitulum fattened with small condylus and distinct rostrum; cucullus present or not clearly visible; and large vulval flap. Subspecies (*B. mucronatus kolymensis* and *B. mucronatus mucronatus*) and two genetic types ("M" form and "R" form of *B. xylophilus*) exist in the group, and the mucro character of *B. xylophilus* is not always stable, which depends on different hosts and environmental situations, making identification complicated. Usually, R form of *B. xylophilus* is distinguished from other species by cylindrical female tail with bluntly rounded terminus, without mucro, or in some cases, some females will show a mucro, which is less than 2 μm. Due to a certain variation in characters between populations and different hosts and environmental situations,

it is essential to perform molecular test in case of doubt. ITS-RFLP identification and other molecular identification methods are also discussed; DNA barcoding by using the 28S and ITS loci will be a reliable and convenient method in the future.

Acknowledgements

The research was supported by the State Key Research and Development Plan (2016YFC1202104), Ningbo Science and Technology Innovation Team (2015C110018), and General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (AQSIQ) Science Program (2016IK168).

Author details

Jianfeng Gu

Address all correspondence to: jeffgu00@qq.com

Technical Centre, Ningbo Entry-Exit Inspection and Quarantine Bureau, Ningbo, Zhejiang, P. R. China

References

[1] Nickle WR. A taxonomic review of the genera of the *Aphelenchoidea* (Fuchs, 1937) Thorne, 1949 (Nematoda: Tylenchida). Journal of Nematology. 1970;**2**:375-392

[2] Kiyohara T, Tokushige Y. Inoculation experiments of a nematode, *Bursaphelenchus* sp., onto pine trees. Japanes Forestry Society. 1971;**53**:210-218

[3] Mamiya Y, Kiyohara T. Description of *Bursaphelenchus lignicolus* n. Sp. (Nematoda: Aphelenchoididae) from pine wood and histopathology of nematode-infected trees. Nematologica. 1972;**18**:120-124. DOI: 10.1163/187529272X00296

[4] Dropkin VH, Foudin AS. Report of the occurrence of *Bursaphelenchus lignicolus*–induced pine wilt disease in Missouri. Plant Disease Report. 1979;**63**:904-905

[5] Dropkin VH, Linit M, Kondo E, Smith M. Pine wilt associated with *Bursaphelenchus xylophilus* (Steiner&Buhrer, 1934) Nickle, 1970, in the United States of America. In: Proceedings of the IUFRO World Congress, 17th; 1981; Kyoto. Kukizaki, Japan: IUFRO Congress Committee. pp. 265-268

[6] Steiner G, Buhrer EM. *Aphelenchoides xylophilus* n. sp. a nematode associated with blue-stain and other fungi in timber. Journal of Agricultural Research. 1934;**48**:949-951

[7] Nickle WR, Golden AM, Mamiya Y, Wergin WP. On the taxonomy and morphology of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer 1934) Nickle 1970. Journal of Nematology. 1981;**13**:385-392

[8] Robbins K. Distribution of the pinewood nematode in the United States. In: Proceedings of the National Pine Wilt Disease Workshop. Illinois. Natural History Survey, Springfield: Illinois Department of Natural Resources; 1982. pp. 3-6

[9] Futai K. Pine wood nematode, *Bursaphelenchus xylophilus*. Annual Review of Phytopathology. 2013;**51**:61-83. DOI: 10.1146/annurev-phyto-081211-172910

[10] Rühm W. Die Nematoden der Ipiden. Parasitologische Schriftenreihe. 1956;**6**:1-435

[11] Mamiya Y, Enda N. *Bursaphelenchus mucronatus* sp. n. (Nematoda: Aphelenchoididae) from pinewood and its biology and pathogenicity to pine trees. Nematologica. 1979; **25**:353-361. DOI: 10.1163/187529279X00091

[12] Korenchenko EA. New species from the family Aphelenchoididae, parasites of stem pests of the Dahurian larch. Zoologichesky Zhurnal. 1980;**59**:1768-1780

[13] Kanzaki N, Tsuda K, Futai K. Description of *Bursaphelenchus conicaudatus* n. Sp. (Nematoda: Aphelenchoididae), isolated from the yellow-spotted longicorn beetle, *Psacothea hilaris* (Coleoptera: Cerambycidae) and fig trees, *Ficus carica*. Nematology. 2000;**2**:165-168. DOI: 10.1163/156854100509051

[14] Walia KK, Sapna N, Bajaj HK, Kalia DC. Two new species of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoididae) from pine wood and insect frass from India. Indian Journal of Nematology. 2003;**33**:1-5

[15] Kanzaki N, Futai K. Description and phylogeny of *Bursaphelenchus luxuriosae* n. sp. (Nematoda: Aphelenchoididae) isolated from *Acalolepta luxuriosa* (Coleoptera: Cerambycidae). Nematology. 2003;**5**:565-572. DOI: 10.1163/156854103322683283

[16] Braasch H, Gu J, Burgermeister W, Zhang J. *Bursaphelenchus doui* sp. n. (Nematoda: Parasitaphelenchidae) in packaging wood from Taiwan and South Korea—A new species of the *xylophilus* group. Russian Journal of Nematology. 2004;**12**:19-27

[17] Gu J, Zhang J, Braasch H, Burgermeister W. *Bursaphelenchus singaporensis* sp. n. (Nematoda: Parasitaphelenchidae) in packaging wood from Singapore—A new species of the *B. xylophilus* group. Zootaxa. 2005;**988**:1-12. DOI: 10.11646/zootaxa.988.1.1

[18] Gu J, Zheng W, Braasch H, Burgermeister W. Description of *Bursaphelenchus macromucronatus* sp. n. (Nematoda: Parasitaphelenchidae) in packaging wood from Taiwan and India—A new species of the '*xylophilus*' group. Journal of Nematode Morphology and Systematics. 2008;**11**:31-40

[19] Tomalak M, Filipiak A. Description of *Bursaphelenchus populi* sp. n. (Nematoda: Parasitaphelenchidae), a new member of the *xylophilus* group from aspen, *Populus tremula* L., in Europe. Nematology. 2010;**12**:399-416. DOI: 10.1163/138855409X12559479584963

[20] Gu J, Wang J, Braasch H, Burgermeister W, Schröder T. *Bursaphelenchus paraluxuriosae* sp. n. (Nematoda: Parasitaphelenchidae) in packaging wood from Indonesia. Nematology. 2012;**14**:787-798. DOI: 10.1163/156854112X627309

[21] Kanzaki N, Maehara N, Aikawa T, Matsumoto K. *Bursaphelenchus firmae* n. sp. (Nematoda: Aphelenchoididae), isolated from *Monochamus grandis* waterhouse that emerged

from dead firs, *Abies firma* Sieb. Et Zucc. Nematology. 2012;**14**:395-404. DOI: 10.1163/156854111X602974

[22] Gu J, Wang J, Chen X. *Bursaphelenchus koreanus* sp.n. (Nematoda: Parasitaphelenchidae) in packaging wood from South Korea. Nematology. 2013;**15**:601-609

[23] Schönfeld U, Braasch H, Riedel M, Gu J. *Bursaphelenchus gillanii* sp. n. (Nematoda: Aphelenchoididae)–a new species of the xylophilus group in packaging wood imported from China. Nematology. 2014;**16**:53-62. DOI: 10.1163/15685411-00002744

[24] Giblin RM, Kaya HK. *Bursaphelenchus seani* n. Sp. (Nematoda, Aphelenchoididae), a phoretic associate of *Anthophora bomboides stanfordiana* Cockerell, 1904 (Hymenoptera, Anthophoridae). Revue de Nématologie. 1983;**6**:39-50

[25] Braasch H. *Bursaphelenchus* species in conifers in Europe: Distribution and morphological relationships. EPPO Bulletin. 2001;**31**:127-142. DOI: 10.1111/j.1365-2338.2001.tb00982.x

[26] Ryss A, Vieira P, Mota M, et al. A synopsis of the genus *Bursaphelenchus* Fuchs, 1937 (Aphelenchida: Parasitaphelenchidae) with keys to species. Nematology. 2005;**7**:393-458. DOI: 10.1163/156854105774355581

[27] Braasch H, Burgermeister W, Gu J. Revised intra-generic grouping of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoididae). Journal of Nematode Morphology and Systematics. 2009;**12**:65-88

[28] Kollosova NV. *Bursaphelenchus eroshenkii* sp. n. (Nematoda: Aphelenchoididae) from the Russian Far East, with a key to some species of *Bursaphelenchus* Fuchs, 1937. Russian Journal of Nematology. 1998;**6**:161-164

[29] Braasch H. The enlargement of the *xylophilus* group in the genus *Bursaphelenchus*. In: Mota M, Vieira P, editors. Pine Wilt Disease: A Worldwide Threat to Forest Ecosystems. Germany: Springer; 2008. pp. 139-149. DOI: 10.1007/978-1-4020-8455-3_12

[30] Braasch H. Erster Nachweis von *Bursaphelenchus mucronatus* Mamiya und Enda, 1979 in der DDR und sein Vorkommen in Holzimporten aus der UdSSR nebst Ergänzungen zur Beschreibung dieser Art. Archiv für Phytopathologie und Pfanzenschutz. 1991;**27**:209-218

[31] Bechenbach K, Smith JM, Webster JM. Taxonomic affinities and intra-and interspecific variation in *Bursaphelenchus* spp. as determined by polymerase chain reaction. Journal of Nematology. 1992;**24**:140-147

[32] Braasch H. Intra- und interspezifsche Kreuzungsexperimente mit einem deutschen und einem sibirischen Isolat von *Bursaphelenchus mucronatus* (Nematoda, Aphelenchoididae) und verwandten Bursaphelenchus-Arten. Nachrichtenblatt des Deutschen Pfanzenschutzdienstes. 1994;**46**:276-281

[33] Braasch H, Gu J, Burgermeister W. *Bursaphelenchus mucronatus kolymensis* comb. n.—New definition of the "European type" of *B. mucronatus*. Journal of Nematode Morphology and Systematics. 2011;**14**(2):77-90

[34] Wingfield MJ, Blanchette A. The pine-wood nematode, *Bursaphelenchus xylophilus*, in Minnesota and Wisconsin: Insect associates and transmission studies. Canadian Journal of Forest Research. 1983;**13**:1068-1076

[35] Gu J, Wang J, Braasch H, Burgermeister W, Schröder T. Morphological and molecular characterization of mucronate isolates ("M" form) of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). Russian Journal of Nematology. 2011;**19**(2):103-119

[36] Braasch H. Variability and morphological differentiation between *Bursaphelenchus xylophilus* (r and m form) and *B. mucronatus.* Abstr. Third International Nematology Congress; 7-12 July 1996; Gosier, Guadeloupe. Nematropica. 1996;**26**:245-246

[37] Zheng W, Gu J, Wu H, Be S. Morphological change of *Bursaphelenchus xylophilus* in *Pinus massoniana* in Ningbo. Journal of Zhejiang Forestry Science and Technology. 2007; **27**:38-44

[38] Hoyer U, Burgermeister W, Braasch H. Identification of *Bursaphelenchus* species (Nematoda Aphelenchoididae) on the basis of amplified ribosomal DNA(ITS-RFLP). Nachrichtenblatt des Deutschen Pflanzenschutzdienstes. 1998;**50**:273-277

[39] Iwahori H, Tsuda K, Kanzaki N, et al. PCR-RFLP and sequencing analysis of ribosomal DNA of *Bursaphelenchus* nematodes related to pine wilt disease. Fundamental and Applied Nematology. 1998;**21**:655-666

[40] Ferris VR, Ferris JM, Faghihi J. Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes. Fundamental and Applied Nematology. 1993;**16**:177-184

[41] Vrain TC. Restriction fragment length polymorphism separates species of the *Xiphinema americanum* group. Journal of Nematology. 1993;**25**:361-364

[42] Braasch H, Metge K, Burgermeister W. *Bursaphelenchus* species (Nematoda, Parasitaphelenchidae) found in coniferous trees in Germany and their ITS-RFLP patterns [in German]. Nachrichtenblatt des Deutschen Pflanzenschutzdienstes. 1999;**51**:312-320

[43] Burgermeister W, Metge K, Braasch H, Buchbach E. ITS-RFLP patterns for differentiation of 26 *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) and observations on their distribution. Russian Journal of Nematology. 2005;**13**:29-42

[44] Gu J, Braasch H, Burgermeister W, Brandstetter M, Zhang J. Description of *Bursaphelenchus yongensis* sp. n. (Nematoda: Parasitaphelenchidae) isolated from *Pinus massoniana* in China. Russian Journal of Nematology. 2006;**14**:91-99

[45] Mota MM, Braasch H, Bravo MA, Penas AC, Burgermeister W, Metge K, Sousa E. First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. Nematology. 1999;**1**:727-734. DOI: 10.1163/156854199508757

[46] Polomski J, Schönfeld U, Braasch H, Dobbertin M, Burgermeister W, Rigling D. Occurrence of *Bursaphelenchus* species in declining *Pinus sylvestris* in a dry Alpine valley in Switzerland. Forest Pathology. 2005;**35**:1-9. DOI: 10.1111/j.1439-0329.2006.00437.x

[47] Schönfeld U, Braasch H, Burgermeister W. *Bursaphelenchus* spp. (Nematoda: Parasitaphelenchidae) in wood chips from sawmills in Brandenburg and description of *Bursaphelenchus willibaldi* sp. n. Russian Journal of Nematology. 2006;**14**:119-126

[48] Burgermeister W, Braasch H, Metge K, et al. ITS-RFLP analysis - an efficient tool for differentiation of *Bursaphelenchus* species. Nematology. 2009;**11**:649-668. DOI: 10.1163/156854108/399182

[49] Kanzaki N, Aikawa T, Maehara N, et al. *Bursaphelenchus doui* Braasch, Gu, Burgermeister & Zhang, 2005 (Aphelenchida: Parasitaphelenchidae), an associate of *Monochamus subfasciatus* Bates (Coleoptera: Cerambycidae) and *Pinus densiflora* Sieb. & Zucc. Nematology. 2008;**10**:69-78. DOI: 10.1163/156854108783360195

[50] Harmey JH, Harmey MA. Detection and identification of *Bursaphelenchus* species with DNA fingerprinting and polymerase chain reaction. Journal of Nematology. 1993;**25**:406-415

[51] Zheng J, Subbotin SA, He S, Gu J, Moens M. Molecular characterisation of some Asian isolates of *Bursaphelenchus xylophilus* and *B. mucronatus* using PCR-RFLPs and sequences of ribosomal DNA. Russian Journal of Nematology. 2003;**11**:17-22

[52] Kang JS, Choi KS, Shin SC, Moon IS, Lee SG, Lee SH. Development of an efficient PCR-based diagnosis protocol for the identification of the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelechoididae). Nematology. 2004;**6**:279-285

[53] Matsunaga K, Togashi K. Among-tree difference in the inhibition of systemic dispersal of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) by Pinus densiflora. Applied Entomology and Zoology. 2004;**39**:271-277. DOI: 10.1303/aez.2004.271

[54] Cao AX, Liu XZ, Zhu SF, Lu BS. Detection of the pinewood nematode, *Bursaphelenchus xylophilus*, using a real time polymerase chain reaction assay. Phytopathology. 2005; **95**:566-571. DOI: 10.1094/PHYTO-95-0566

[55] Castagnone C, Abad P, Castagnone-Sereno P. Satellite DNA-based species specific identification of single individuals of the pinewood nematode *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). European Journal of Plant Pathology. 2005;**112**:191-193

[56] Jiang LQ, Zheng JW, Waeyenberge L, Subbotin SA, Moens M. Duplex PCR based identification of *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970. Russian Journal of Nematology. 2005;**13**:115-121

[57] Leal I, Green M, Allen E, Humble L, Rott M. An effective PCR-based diagnostic method for the detection of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) in wood samples from lodgepole pine. Nematology. 2005;**7**:833-842. DOI: 10.1163/156854105776186398

[58] Takeuchi Y, Kanzaki N, Futai K. A nested PCR-based method for detecting the pine wood nematode, *Bursaphelenchus xylophilus*, from pine wood. Nematology. 2005;**7**:775-782. DOI: 10.1163/156854105775142928

[59] Francois C, Castagnone C, Boonham N, Tomlinson J, Lawson R, Hockland S, Quill J, Vieira P, Mota M, Castagnone-Sereno P. Satellite DNA as a target for TaqMan real-time PCR detection of the pinewood nematode, *Bursaphelenchus xylophilus*. Molecular Plant Pathology. 2007;**8**:803-809. DOI: 10.1111/j.1364-3703.2007.00434.x

[60] Ye W, Giblin-Davis RM, Lin B. Molecular characterization and development of real-time PCR assay for Pine-Wood Nematode, Bursaphelenchus xylophilus (Nematoda: Parasitaphelenchidae). PLoS One. 2013;**8**(11):e78804. DOI: 10.1371/journal.pone.0078804

[61] Kikuchi T, Aikawa T, Oeda Y, Karim N, Kanzaki N. A rapid and precise diagnostic method for detecting the pinewood nematode *Bursaphelenchus xylophilus* by loop-mediated isothermal amplification. Nematology. 2009;**99**(12):1365-1369

[62] Hebert PDN, Cywinska A, Ball SL, deWaard JR. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London, Series B: Biological Sciences. 2003;**270**:313-321. DOI: 10.1098/rspb.2002.2218

[63] Holterman M, Van der Wurff A, Van den Elsen S, Van megen H, Bongers T, Holovachov O, Bakker J, Helder J. Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades. Molecular Biology and Evolution. 2006;**23**:1792-1800. DOI: 10.1093/molbev/msl044

[64] He J, Gu J. Screening DNA barcode markers for the xylophilus group of the genus Bursaphelenchus. Plant Quarantine. 2016;**30**(1):14-20

IntechOpen