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Original article

Occurrence of *Bursaphelenchus mucronatus* (Nematoda; Aphelenchoididae) in France and association with *Monochamus galloprovincialis* (Coleoptera: Cerambycidae)

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Abstract – As a consequence of the recent introduction of the pine wood nematode *Bursaphelenchus xylophilus* in Portugal, nematodes of the genus *Bursaphelenchus* were looked for in various French pine forests, in trees attacked by *Monochamus galloprovincialis*, the vector insect of *B. xylophilus*, and in the insects themselves. Trap trees were felled in 12 localities distributed all over the country. Nematodes were extracted from transversal stem discs; insects emerging from the trap trees were studied. *B. hellenicus*, *B. leoni*, *B. mucronatus* and *B. sexdentati* were isolated, but not *B. xylophilus*. The presence of *B. mucronatus* and the absence of *B. xylophilus* were confirmed by molecular markers. *B. mucronatus* was isolated from several regions with an average prevalence of 19%. The infestation of *M. galloprovincialis* by *B. mucronatus* reached 26.7%. The wide distribution of *B. mucronatus* in France could have an effect on the extension of *B. xylophilus* in a case of an introduction.

pinewood nematodes / *Bursaphelenchus* spp. / vector insect / morphology / ITS-RFLP

Résumé – Présence de *Bursaphelenchus mucronatus* (Nematoda ; Aphelenchoididae) en France et association avec *Monochamus galloprovincialis* (Coleptera : Cerambycidae). Suite à la récente introduction du nématode du pin *Bursaphelenchus xylophilus*, au Portugal, les nématodes du genre *Bursaphelenchus* ont été recherchés dans diverses forêts de pins françaises, sur les arbres attaqués par *Monochamus galloprovincialis*, le vecteur de *B. xylophilus*, et sur les insectes eux-mêmes. Des arbres pièges ont été abattus dans 12 localités réparties sur le territoire national. Les nématodes ont été extraits de sections transversales de tronc, et les insectes émergeant des arbres pièges ont été étudié. *B. hellenicus*, *B. leoni*, *B. mucronatus* et *B. sexdentati* ont été isolés, mais pas *B. xylophilus*. La présence de *B. mucronatus* et l'absence de *B. xylophilus* ont été confirmées par des marqueurs moléculaires. *B. mucronatus* a été isolé de plusieurs régions avec une fréquence moyenne de 19 %. Le taux de contamination de *M. galloprovincialis* par *B. mucronatus* atteignait 26,7 %. La vaste distribution de *B. mucronatus* en France pourrait avoir un effet sur la propagation de *B. xylophilus* dans le cas d'une introduction accidentelle.

nématodes du pin / *Bursaphelenchus* spp. / insecte vecteur / morphologie / ITS-RFLP

1. INTRODUCTION

Spread of non-indigenous species across their natural dispersal barriers by international travel and trade can lead to replacement of native species, causing disturbance of ecosystems [16, 17]. Non-indigenous species are recognised as one of the leading global threats to native biodiversity and ecosystem function [42] and the pinewood nematode, *Bursaphelenchus xylophilus* [26, 39] is one of these species. It is a destructive pest of pines and is inferred to have been introduced early in the 1900s from North America into Japan [21] where it has been responsible for severe epidemic damage on Japanese black pine (*Pinus thunbergii* Palatore) and red pine (*Pinus densiflora* Siebold and Zuccarini) in central and south-western Japan for many years [24, 41]. Pine trees in the north-western Pacific area (China, Korea, and Taiwan) have also been infested [22]. Its recent introduction in Portugal and its establishment in *Pinus pinaster* [25] represent a real threat for the European forests and raise the question of its possible introduction or extension in other European countries. The susceptibility of European pine species (*Pinus sylvestris* L., *Pinus pinaster* Ait and *Pinus nigra* Arnold) [12] has led *B. xylophilus* to be listed as a quarantine pest in Europe. The infection process of the nematode to trees requires an insect vector with larval stages occurring in dying trees. Beetles of the genus *Monochamus* (Coleoptera: Cerambycidae) are the most important vectors of the nematode worldwide [18] and *Monochamus galloprovincialis* Olivier is its vector in Portugal [38]. In France, *B. xylophilus* has never been observed but three species of *Monochamus* (*M. galloprovincialis*, *M. sartor* and *M. sutor*) are present, the most frequent being *M. galloprovincialis* on different pine species [4].

Monitoring of *B. xylophilus* is difficult because of the taxonomic confusion within the genus *Bursaphelenchus* [2, 24] due to very similar morphological characters in several

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Table I. Description of the localities investigated for studying the occurrence of *Bursaphelenchus mucronatus*. Last column indicates *M. galloprovincialis* occurrence from the 2003-2004 trap-campaign: (+) capture of *M. galloprovincialis*, (0) no capture of *M. galloprovincialis*.

Locality		Coordinates	Altitude (m)	Host	Date of felling	Date of collect	Number of logs	*M. galloprovincialis*
1	Val de la Haye	49° 23' N / 0° 59' E	145	*P. sylvestris*	05/07/2004	07/10/2005	20	0
2	Saint-Jean-aux-bois	49° 24' N / 2° 52' E	50	*P. strobus*	28/06/2004	11/10/2005	20	+
3	Sturzelbronn	49° 04' N / 7° 34' E	304	*P. sylvestris*	30/06/2004	06/07/2005	16	+
4	Cleebourg	49° 01' N / 7° 53' E	289	*P. sylvestris*	28/06/2004	05/07/2005	15	0
5	Schirrhein	48° 50' N / 7° 49' E	158	*P. sylvestris*	21/06/2004	05/07/2005	16	0
6	Lorris	47° 53' N / 2° 24' E	129	*P. sylvestris*	25/06/2004	23/02/2005	19	+
7	Savigny-les-Beaunes	47° 04' N / 4° 48' E	399	*P. nigra austriaca*	08/07/2004	07/07/2005	17	+
8	St-Alban-des-Hurtières	45° 28' N / 6° 17' E	352	*P. sylvestris*	30/06/2004	28/04/2005	16	0
9	Vendays-Montalivet	45° 19' N / 1° 09' W	14	*P. pinaster*	26/08/2004	05/06/2005	13	+
10	Bréau-et-Salagosse	43° 59' N / 3° 32' E	410	*P. nigra*	29/06/2004	26/04/2005	16	+
11	St-André-les-Alpes	44° 01' N / 6° 29' E	1100	*P. sylvestris*	28/06/2004	27/04/2005	15	+
12	Meyrargues	43° 37' N / 5° 30' E	321	*P. halepensis*	29/06/2004	27/04/2005	18	+

species inhabiting pine wood. Techniques employing sex pheromones [31] as well as DNA analysis [6, 8, 13, 14, 30] have helped clarify the taxonomy of this group. PCR-RFLP of nuclear genes, especially ITS (Internal Transcribed Spacer) proved to produce appropriate markers for species identification, in particular to distinguish between *B. mucronatus* and *B. xylophilus* [7,13,14]. About 25 *Bursaphelenchus* species associated with conifers have been reported in Europe [5], among which *Bursaphelenchus mucronatus* [23] was the most abundant and present in many European countries [33]. In France, since its discovery in the Southwest on *P. pinaster* by Baujard in 1979 [3], *B. mucronatus* was never observed in any other pine species or locality. The European *B. mucronatus* has a low virulence in comparison to *B. xylophilus* [23] but displayed a moderate pathogenicity on pine seedlings during laboratory tests, although it never induced any visible damage on adult trees under natural conditions [34]. *B. mucronatus* displays similar morphological and biological characters than *B. xylophilus* [23] and occupies the same ecological niche in pine forest ecosystems. Moreover, the infection biology of the two species is also similar. Dispersal fourth-stage juveniles of both species are transported from one host tree to another by beetles of the genus *Monochamus* [18, 23, 24].

In general, host species can harbour several species of parasites [28]. Nevertheless, because a host is a finite resource, parasites that share the same host species can adversely affect each other's densities through interspecific competition [10]. Thus, when *B. xylophilus* is introduced in a new area where *B. mucronatus* is already present, it must share resources, such as food and vector species, with *B. mucronatus*. Consequently, studying the distribution of *B. mucronatus* in the absence of *B. xylophilus* could give useful information on the modalities of a possible dissemination of this latter in case it would be introduced.

The aim of this study was to determine the infestation level by *B. mucronatus* of different pine species in France, and its phoretic relationships with *M. galloprovincialis*. The study focused mainly on *Pinus sylvestris*, *Pinus halepensis*, *Pinus pinaster* and *Pinus nigra*, because these species are representative of the French pine forests.

2. MATERIALS AND METHODS

2.1. Nematode sampling and extraction

In summer 2004, four healthy pine trees were felt in each of twelve localities in France during the flight period of *M. galloprovincialis* adults, to be used as trap trees for the beetle. As much as possible but also depending on practical possibilities in the field, these localities were chosen so that they are representative of the different types of coniferous forests in France. The choice also resulted from a preliminary sampling of *Monochamus galloprovincialis* in 40 sites, and nematode sampling was done mainly in localities where *M. galloprovincialis* had been previously found. The characteristics and locations of the localities are presented in Table I and Figure 1. Entire trees were left in the field until April 2005. In May, four to five logs (about 45 cm long and 9 cm mid-diameter) were cut off mainly from the top of each tree (201 logs in total). Each log was checked for the presence of *M. galloprovincialis* (larval and adult feeding). A 1 cm thick stem disc was taken from each log to further extract nematodes, and the logs were then placed individually in 70 cm diameter × 70 cm deep plastic containers covered with tulle. The containers were placed at 20 °C and checked two times a week from May to September until emergence of *M. galloprovincialis* (Tab. II). The number of emergence holes was counted. Beetles were dissected and crushed in sterilised water and then kept in water at 20–24 °C during 24 h for the nematode juveniles to gather in water. The discs taken from the logs were kept in a plastic bag at room temperature during one to three months, until nematode extraction. Nematodes were extracted from the discs during 48 h with a modified Baermann funnel technique [27], involving immersion of pieces of wood in water. Nematodes were collected from the closed bottom of a funnel, after their migration out of the wood.

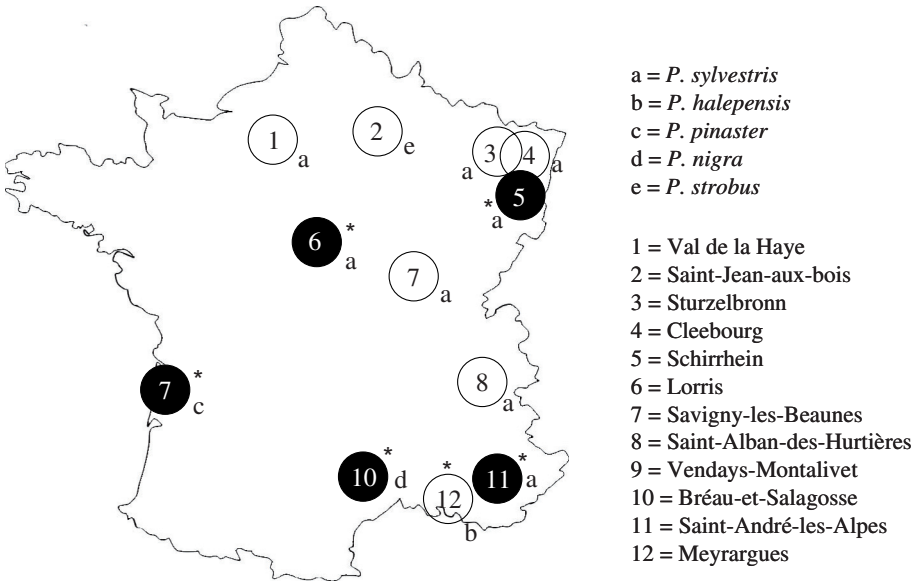

Figure 1. Map of the sampling sites (see Tab. I). Black disks = areas where *Bursaphelenchus mucronatus* was present; white disks = areas where *Bursaphelenchus mucronatus* was not found. * Localities where *Monochamus galloprovincialis* emergence holes were found.

Table II. Contingency table: occurrence of *Bursaphelenchus mucronatus* in relation to the presence of *Monochamus galloprovincialis* grub holes in all the localities.

	Number of trees with *M. galloprovincialis* emergence holes	Number of trees without *M. galloprovincialis* emergence holes	Total
B. mucronatus present	8	1	9
B. mucronatus absent	4	35	39
Total	12	36	48

c2 = 24.114; $p = 1.08.10^{-5} << 0.05$ (significant).

2.2. Nematode preparation and morphological observations

Nematodes present in water were transferred with a pipette and a lash, to a glass slide, under a stereomicroscope. Some *Bursaphelenchus* specimens were fixed in FA 4:1 hot solution (40% formaldehyde, glacial acid acetic), using the glycerol-ethanol method [35] then fixed in anhydrous glycerin for identification. Observations were made from alive and fixed adult specimens using a digital biological microscope (Model DMWB1-223) and Motic Images Plus 2.0 software. *Bursaphelenchus* identification was based on morphological characters, particularly vulval flap, shape of spicules and female tail [5, 33, 44], and measurements. Measurements concerned body length (= L), L/maximum body width (= a), L/oesophageal length (= b), L/tail length (= c), stylet length, (distance from head end to vulva/L) × 100 (= V), spicules length (= S). Each individual was measured using Image J software calibrated with a stage micrometer.

Nematode species extracted from pines, with morphometric characteristics similar to *B. xylophilus* (belonging to the *B. xylophilus*-group [33]), and juveniles J_{IV} extracted from insects, were identified by molecular methods: 15 individuals/locality extracted from pines were analysed for Schirrhein, Lorris, Bréau-et-Salagosse and 5 for Vendays-Montalivet and Saint-André-les-Alpes; 10 individuals/insect were analysed.

2.3. Molecular methods

Nematodes were prepared according to the single worm PCR procedure modified by Castagnone et al. [8]. Single nematodes were transferred to a dry thin walled PCR tube, covered with 15 µL lysis buffer (1X Buffer PCR, 6 $\mu g.\mu L^{-1}$ proteinase K). Tubes were put at –80 °C for 60 min and immediately transferred to 60 °C for 60 min and then 95 °C for 15 min in the thermocycler.

Extracted nematode DNA was compared with ITS-RFLP markers (Internal Transcribed Spacer-Restriction Fragment Length Polymorphism). The ITS regions of rDNA were amplified using primers F194 (5'- CGTAACAAGGTAGCTGTAG-3') and P5368 (5'-TTTCACTCGCCGGTTACTAAGG-3') as described by Ferris et al. [11] and Vrain [43], respectively. All polymerase chain reactions were performed in a final volume of 25 µL using 10 ng/µL of template DNA, 1 µM of each primer, 0.2 µM of dNTPs, 2 U of Taq DNA polymerase, 1× Reaction Buffer and 1.25 mM of MgCl2. The reaction consisted of one denaturation step at 94 °C for 1 min, 35 cycles at 94 °C for 1 min, 51 °C for 1 min, and 72 °C for 2 min, followed by a final extension step at 72 °C for 5 min. Digestion of the amplified ITS region was performed with HaeIII, MspI and HinfI endonucleases, using an aliquot of 4 µL of the PCR product and 10 U of each enzyme. Patterns observed on 2% agarose gel after the digestion by endonucleases were compared to *Bursaphelenchus* species

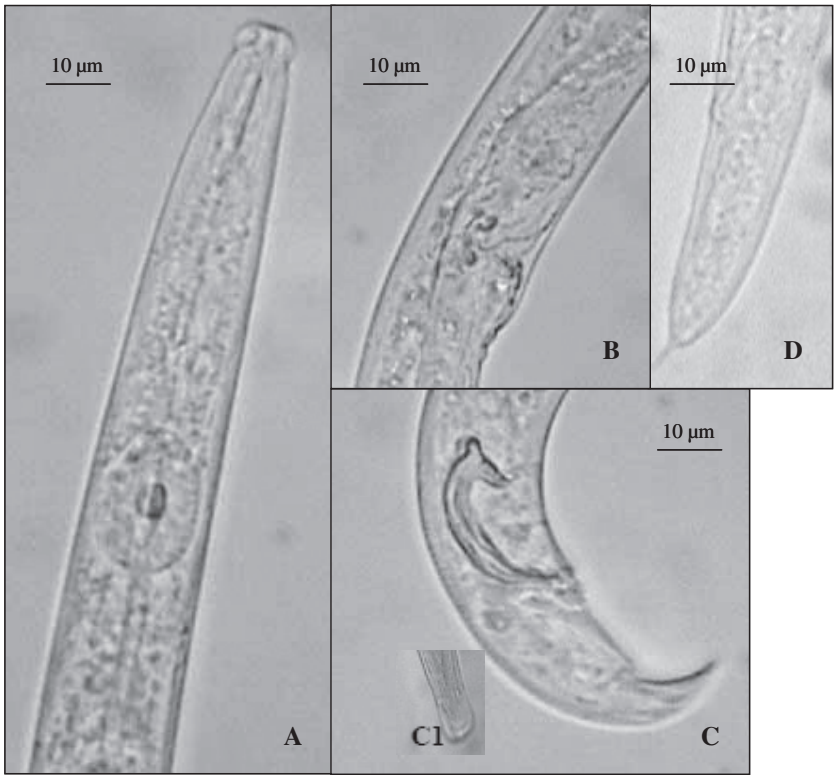

Figure 2. Light micrographs of *Bursaphelenchus mucronatus*. A: anterior region; B: vulval region; C: male tail, with bursa in inset (C1); D: female tail.

reference patterns [7]. Fragment sizes were estimated by comparison with a DNA size marker (Smart Ladder, Eurogentec).

2.4. Statistics

The percentage of trees contaminated by *B. mucronatus*, in the localities where this nematode was present, as well as the percentage of infested insects, were tested with a χ^2 test. The association between the presence of *M. galloprovincialis* emergence holes and the presence of *B. mucronatus* was assessed in a contingency table using a Fisher exact test throughout the trees analysed. All results were evaluated using the SPSS statistical software package (SPSS, Chicago, IL, USA). For each morphological measurement, the mean was expressed with its standard deviation. All statistical tests were considered significant when $P \leq 0.05$.

3. RESULTS

3.1. Identification of *B. mucronatus* populations

B. xylophilus-group individuals observed in our samples (Fig. 2, Tabs. III and IV) corresponded to the description of *B. mucronatus*, done by Mamiya and Enda [23]. Our male specimens showed the typical, strongly curved, cucullus bearing spicules of the *xylophilus*-group [5, 33]. The females had a vulva postmedian with a proeminent vulvar flap. The tail was conoid with a mucro.

Amplification of ITS regions of studied samples produced a single DNA fragment of 950 bp. The same 950 bp amplicon was also obtained with *B. mucronatus, B. xylophilus and B. eggersi* [7]. Subsequent analysis of ITS regions with HaeIII, MspI and HinfI endonucleases produced characteristic patterns for all individuals studied (Fig. 3) similar to those obtained with the European type of *B. mucronatus* [7].

3.2. Occurrence of *B. mucronatus*

M. galloprovincialis emergence holes were observed from the collected logs in six localities (Fig. 1): Schirrhein (*P. sylvestris*), Lorris (*P. sylvestris*), Vendays-Montalivet (*P. pinaster*), Bréau-et-Salagosse (*P. nigra*), Saint-André-les-Alpes (*P. sylvestris*) and Meyrargues (*P. halepensis*). *B. mucronatus* was detected from logs in five localities, all where *M. galloprovincialis* emergence holes had been observed (Fig. 1). Half of trees was contaminated by *B. mucronatus* in Schirrhein, Lorris and Bréau-et-Salagosse, and a quarter in Vendays-Montalivet and Saint-André-les-Alpes, without significant difference among localities ($\chi^2 = 0.8$; $p = 0.371$) and corresponding to an averaged prevalence of 40%. The number of individuals extracted from logs was always low: 80 g^{-1} of wood at Lorris, 30 g^{-1} at Schirrhein, 45 g^{-1} at Bréau-et-Salagosse, less than 10 g^{-1} at Vendays-Montalivet and Saint-André-les-Alpes.

Table III. Measurements on males of *Bursaphelenchus* spp. isolated in France, compared to Allotypes. All measurements are in µm (mean ± standard deviation, with range in parenthesis).

Species		Morphometric characters					
Locality or Reference	*n*	*L*	*a*	*b*	*c*	*Stylet*	*S*
B. mucronatus							
Schirrhein	30	802 ± 14.5 (644–994)	48.3 ± 0.9 (39.8–57.0)	10.2 ± 0.2 (8.8–12.6)	22.4 ± 0.4 (17.8–27.6)	15.5 ± 0.3 (12.5–18.0)	25.3 ± 0.4 (21.4–31.8)
Lorris	53	810 ± 15.7 (671–1086)	43.1 ± 0.8 (32.0–55.9)	10.9 ± 0.2 (9.0–13.8)	22.0 ± 0.4 (13.4–25.6)	15.2 ± 0.3 (13.0–17.9)	26.8 ± 0.4 (21.6–31.3)
St-André-les-Alpes	5	763 ± 30.2 (676–845)	46.5 ± 2.3 (41.1–54.8)	10.8 ± 0.4 (9.6–12.0)	23.0 ± 0.6 (21.5–24.6)	13.3 ± 0.4 (12.5–14.2)	24.7 ± 0.6 (22.5–26.0)
Vendays-Montalivet	2	798 ± 82.7 (715–881)	43.5 ± 1.3 (42.1–44.8)	9.1 ± 0.9 (8.2–9.9)	21.2 ± 1.4 (19.8–22.7)	14.8 ± 1.4 (13.5–16.2)	29.5 ± 0.2 (29.2–29.8)
Bréau-et-Salagosse	28	840 ± 15.8 (698–1000)	46.5 ± 0.8 (38.4–58.0)	12.2 ± 0.3 (9.0–15.4)	23.9 ± 0.5 (18.3–28.0)	15.3 ± 0.3 (12.2–17.5)	26.8 ± 0.4 (23.3–31.7)
B. mucronatus – Allotype (Mamiya & Enda, 1979)	–	830	46.3	12.0	30.3	15.0	26.5
B. lignicolus – France (= *B. mucronatus*) (Baujard et al., 1979)	–	692	42.2	10.4	25.8	13.0	25.3
B. mucronatus (min-max) (Braasch, 2001)	–	(480–1103)	(25–59)	–	(15–36)	(12–18)	(16–33)
B. sexdentati Syn. : *B. naujaci* Baujard, 1980							
Lorris	8	866 ± 18.7 (784–915)	41.7 ± 0.8 (39.6–45.1)	14.4 ± 0.4 (12.2–16.1)	27.4 ± 3.2 (22.7–49.3)	17.0 ± 0.4 (14.8–18.0)	14.9 ± 0.3 (13.6–15.7)
St-André-les-Alpes	7	776 ± 18.3 (720–862)	45.0 ± 1.6 (39.2–51.6)	12.5 ± 0.3 (11.3–13.9)	22.1 ± 0.8 (18.9–24.1)	13.4 ± 0.3 (12.6–14.9)	14.1 ± 0.3 (13.1–14.9)
Vendays-Montalivet	19	1059 ± 26.9 (856–1234)	48.1 ± 1.3 (35.9–59.9)	14.3 ± 0.4 (11.9–18.3)	26.4 ± 1.0 (20.6–35.7)	16.1 ± 0.3 (13.4–18.0)	15.5 ± 0.5 (12.2–20.1)
B. sexdentati – Allotype (Rühm, 1960)	–	924	41.7	13.6	25.4	18.0	20.5
B. naujaci – France (Baujard, 1980)	–	890	42.0	13.0	24.0	15.0	17.0
B. sexdentati (min-max) (Braasch, 2001)	–	(541–1154)	(36–59)	–	(25–36)	(12–18)	(13–22)
B. naujaci (min-max) (Braasch, 2001)	–	(560–1200)	(33–63)	–	(17–30)	(13–18)	(13–22)
B. leoni							
Lorris	1	712	36.7	10.2	23.3	13.7	15.9
B. leoni – Allotype – France (Baujard, 1980)	_	760	47	10	25	13	16
B. leoni (min-max) (Braasch, 2001)	_	(510–1060)	(26–56)	–	(16–30)	(12–17)	(10–21)
B. hellenicus							
Bréau-et-Salagosse	16	695 ± 17.4 (565–798)	30.5 ± 0.7 (26.0–34.2)	8.7 ± 0.2 (7.4–9.9)	21.4 ± 0.6 (18.6–27.1)	14.7 ± 0.2 (13.5–17.3)	14.9 ± 0.3 (12.8–6.7)
B. hellenicus – Allotype (Skarmoutsos et al., 1998)	–	770	33.0	8.3	21.0	16.0	15.0
B. hellenicus (min-max) (Braasch, 2001)	–	(640–820)	(22–38)	–	(19–30)	(13–17)	(12–18)

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

Table IV. Measurements on females of various *Bursaphelenchus* spp. isolated in France, compared to Allotypes. All measurements are in µm (mean ± standard deviation, with range in parenthesis).

Species		Morphometric characters					
Locality or Reference	*n*	*L*	*a*	*b*	*c*	*V*	*Stylet*
B. mucronatus							
Schirrhein	17	825 ± 24.16 (701–1071)	42.7 ± 0.72 (36.9–57.0)	10.4 ± 0.20 (9.2–12.1)	24.4 ± 0.58 (17.8–27.0)	72.9 ± 0.28 (71.3–74.7)	15.9 ± 0.3 (12.5–17.8)
Lorris	25	993 ± 42.37 (699–1187)	45.2 ± 1.34 (38.7–49.9)	13.4 ± 0.63 (10.4–15.3)	22.9 ± 0.65 (20.3–25.7)	75.6 ± 0.42 (73.5–77.2)	14.9 ± 0.33 (13.2–16.8)
St-André-les-Alpes	4	838 ± 8.41 (821–856)	44.4 ± 1.87 (40.9–49.1)	12.7 ± 0.36 (11.8–13.3)	25.7 ± 0.90 (24.0–28.2)	73.3 ± 0.59 (72.0–74.9)	14.5 ± 0.79 (13.2–16.5)
Vendays-Montalivet	2	833 ± 45.12 (788–878)	39.7 ± 0.36 (39.3–40.0)	9.7 ± 0.31 (9.4–10.0)	25.3 ± 3.82 (21.5–29.1)	72.2 ± 0.79 (71.4–73.0)	15.7 ± 0.98 (14.8–16.7)
Bréau-et-Salagosse	7	887 ± 53.51 (652–1057)	42.7 ± 1.99 (36.0–50.6)	11.7 ± 0.81 (9.0–14.7)	29.1 ± 1.50 (25.6–37.7)	74.2 ± 0.42 (72.8–75.5)	15.3 ± 0.64 (13.6–17.8)
B. mucronatus – Allotype (Mamiya & Enda, 1979)	–	870	42.9	12.3	25.7	74.0	15.0
B. lignicolus = *B.mucronatus* – France (Baujard et al., 1979)	–	753	43.1	11.0	26.9	73.4	13.5
B. mucronatus (min-max) (Braasch, 2001)	–	(560–1124)	(25–51)	–	(17–35)	–	(12–17)
B. sexdentati Syn. : *B. naujaci* Baujard, 1980							
Lorris	4	922 ± 85.2 (725–1112)	43.1 ± 2.4 (36.2–46.9)	14.7 ± 1.4 (12.8–17.7)	24.8 ± 1.6 (21.3–28.1)	74.5 ± 0.5 (73.1–75.6)	15.5 ± 0. (13.6–15.7)
Vendays-Montalivet	7	1186 ± 35.2 (1038–1290)	51.5 ± 1.5 (46.5–56.2)	16.3 ± 0.2 (15.4–16.8)	26.4 ± 1.0 (27.5–29.5)	75.5 ± 0.3 (74.0–76.2)	15.5 ± 0.5 (12.2–20.1)
B. sexdentati – Allotype (Rühm, 1960)	–	1078	39.8	16.6	26.0	73.8	18.5
B. naujaci – France (Baujard, 1980)	–	900	43.0	13.0	24.0	74.0	15.0
B. sexdentati (min-max) (Braasch, 2001)	–	(599–1314)	(32–57)	–	(17–36)	–	(13–19)
B. naujaci (min-max) (Braasch, 2001)	–	(620–1300)	(31–53)	–	(19–32)	–	(13–18)
B. hellenicus							
Bréau-et-Salagosse	1	688	33.9	–	–	73.6	13.5
B. hellenicus – Allotype (Skarmoutsos et al., 1998)	–	–	–	–	–	–	–
B. hellenicus (min-max) (Braasch, 2001)	–	(680–920)	(22–36)	–	(17–31)	–	(13–17)

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

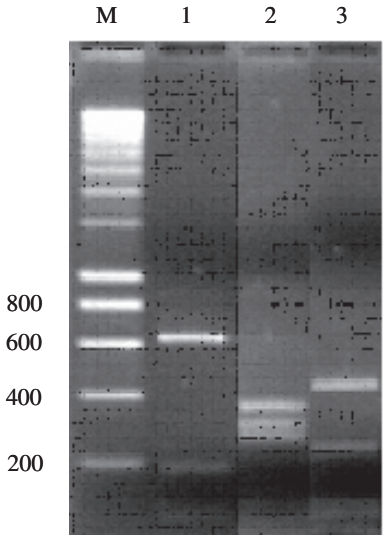

Figure 3. ITS-RFLP patterns of a *Bursaphelenchus mucronatus* individual from Bréau-et-Salagosse (France). Restriction fragments were obtained by digestion of the amplified rDNA fragment with Hae III (1), Msp I (2), Hinf I (3). M: DNA marker.

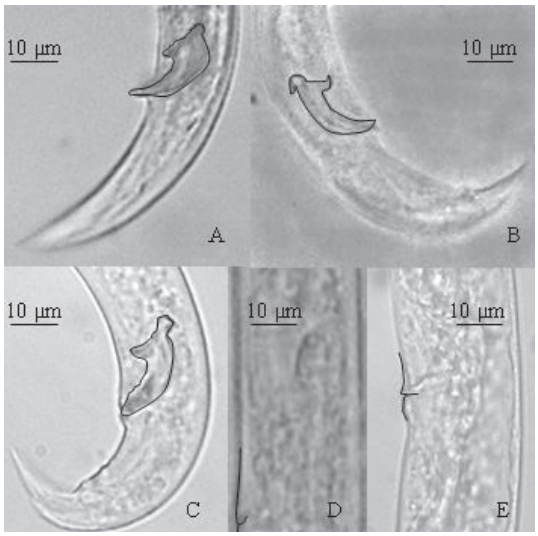

Figure 4. Light micrographs of the 3 other *Bursaphelenchus* species. A: *B. hellenicus* male tail; B: *B. leoni* male tail; C: *B. sexdentati* male tail; D: *B. sexdentati* vulval region; E: *B. hellenicus* vulval region. Morphological characters as spicules and vulvar lips were accentuated by black lines.

In Bréau-et-Salagosse and Lorris, the emerging insects were infested with *B. mucronatus* at a rate of 12.5% and 26.7%, respectively (difference not significant: $\chi^2 = 0.672$; $p = 0.41$). No nematode was extracted from the 89 insects emerged from *P. halepensis* at Meyrargues and from the 4 insects at Vendays-Montalivet. Unfortunately at Schirrhein and Saint-André-les-Alpes, the insects had already emerged when the logs were brought to the lab and thus no *M. galloprovincialis* could be collected. Considering all localities, 66% of trees with *M. galloprovincialis* emergence holes and 3% of trees without *M. galloprovincialis* emergence holes were infested by *B. mucronatus*. The occurrence of *B. mucronatus* was significantly associated with the presence of *M. galloprovincialis* emergence holes on trees ($\chi^2 = 24.114$; $p << 0.05$) (Tab. II).

3.3. Occurrence of other *Bursaphelenchus* species

Besides *B. mucronatus*, other *Bursaphelenchus* species were collected sporadically and isolated from logs. Because of the low number of individuals, they were described by using morphological characters (Fig. 4) which make them resembling to *B. leoni, B. sexdentati* and *B. hellenicus* and were concordant to the previous species description [2,32,37]. Morphometrical characters were also used and were concordant with the allotypes and fitted within the limits found by Braasch [5] (Tabs. III and IV).

4. DISCUSSION

Most individuals found in our localities resembled morphologically to nematodes of the *B. xylophilus*-group [33]. However, the use of the ITS-molecular marker demonstrated unequivocally that all these specimens belonged to the species *B. mucronatus*.

Therefore our results support the hypothesis that the presence of *B. xylophilus* in France is very unlikely.

The direct isolation of *B. mucronatus* from individuals of *M. galloprovincialis* and the existence of a significant association in logs between *M. galloprovincialis* emergence holes and *B. mucronatus* positive wood samples demonstrate that these species are associated such an association has already been reported several times [33].

Our investigations on fallen trees showed the occurrence of *B. mucronatus* in five among twelve localities where its insect vector *M. galloprovincialis* had been recorded. *B. mucronatus* has already been reported from south-western France in *P. pinaster* [3], where it was first misidentified as *B. xylophilus* [9]. *B. mucronatus* found in our samples displayed similar size (Tab. III) and morphology (mucronate tail) as *B. mucronatus* specimens found in 1979 in south-western France in *P. pinaster*. *B. mucronatus* thus seems to be widely distributed in France. Nineteen percent of the analysed trees contained *B. mucronatus*. The nematode was found on three pine species: *P. nigra*, *P. pinaster* and *P. sylvestris*. It has already been identified in these species in other European countries [29,33]: in *P. nigra* and *P. sylvestris* in Austria and Spain, in *P. pinaster* in Portugal and Italy and in *P. sylvestris* in Norway, Sweden, Finland, Poland, Germany and Switzerland.

Although there was no significant difference between localities in the prevalence of *B. mucronatus*, there were contrasted results regarding the abundance of *B. mucronatus* individuals per gram of wood. Factors affecting the number of nematodes transmitted to trees by beetles can certainly interfere, for example through initial nematode load and longevity of insect vector, which can themselves depend on humidity

and fungal flora in the beetle galleries, as shown for *B. xylophilus* [19, 20, 36, 40]. The local abundance of the main vector also certainly plays a role in the intensity of infestation by *B. mucronatus* since, when considering the localities where *B. mucronatus* was found in our results, the number of wood samples containing *B. mucronatus* was directly related to the number of *M. galloprovincialis* emergence holes. However, other factors are also certainly involved, such as differences in site locations and host trees, and possibly associated fungi. For *B. xylophilus*, intense blue-stain on the pupal chamber walls of *M. alternatus* increased the number of pinewood nematodes [20].

Morphometric characters allowed suggesting the existence of populations of *B. mucronatus*, *B. sexdentati*, *B. leoni* and *B. hellenicus*. However within each species, it was not possible, with these parameters, to separate the populations geographically or according to their pine host. Only *B. mucronatus* was found in association with *M. galloprovincialis*. The others species were found directly on pines; these three species were known to be associated with Scolytinae such as *Ips sexdentatus*, *Tomicus piniperdae*, *Dryocoetes autographus* and *Hylurgops palliatus* [5, 7] which are common in French pine forests [1]. Some sampling trees were probably attacked by Scolitydae contaminated with nematodes which then migrated inside the tree. *B. sexdentati* (syn. *B. naujaci*) and *B. leoni* were already found in France (Les Landes) on *P. pinaster* [2] and correspond to the measurements of our specimens.

In conclusion, the wide distribution of *B. mucronatus* in France is interesting under the hypothesis that its presence in *M. galloprovincialis* and in pines can, as a possible result of competition, lower the initial load of *B. xylophilus* in the insects, and thus impede the geographical extension of this latter species. An inhibitory effect of *B. mucronatus* on the number of *B. xylophilus* in its vector has already been shown for *Monochamus alternatus* [15]. It would be interesting to test the interaction between *B. mucronatus* (French samples) and *B. xylophilus* (Portuguese samples) in the transmission process by *M. galloprovincialis* to trees. Interestingly in Portugal, *B. mucronatus* does not seem to occur in the quarantine area where damage is caused by *B. xylophilus* [38]. No *B. mucronatus* was recorded on *P. halepensis* at Meyrargues despite the high number of *M. galloprovincialis* in this locality. Furthermore, no *B. mucronatus* was found either in the beetles collected in this area. This observation is in agreement with the fact that *B. mucronatus* seems to be rare in the Mediterranean area [5], although its main vector, *M. galloprovincialis*, is abundant. This empty niche and locally high number of potential vectors could make Meyrargues a locality where the establishment of *B. xylophilus* would be facilitated.

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