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Morpho-biometrical characterisation of Portuguese *Bursaphelenchus xylophilus* isolates with mucronate, digitate or round tailed females

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Summary. Morpho-biometrical studies were conducted on 12 *Bursaphelenchus xylophilus* isolates collected from maritime pine, *Pinus pinaster,* in Portugal. The studies were carried out on 20 females and 20 males from each isolate. A wide variation in the female tails, from round, digitate to mucronate was detected in all isolates, confirming the occurrence of mucronate tails in some females of *B. xylophilus*. The presence of mucronate tailed females in the Portuguese isolates of *B. xylophilus* clearly makes the identification of *B. xylophilus* by this morphological character difficult since other non-pathogenic *Bursaphelenchus* species also have mucronate tailed females. Amplification of satellite DNA of single specimens using species-specific primers confirmed the identity of the mucronate tailed females in the Portuguese isolates as *B. xylophilus*. The satellite DNA technique was also useful in the identification of juveniles of *B. xylophilus* from *P. pinaster* wood samples.

Key words: morphometry, morphology, pinewood nematode, satellite DNA.

Introduction

The pinewood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle 1970, is the causal agent of pine wilt disease. This nematode is listed as an A1 quarantine pest by the European and Mediterranean Plant Protection Organisation (EPPO) (EPPO/OEPP, 2001).

After the detection of PWN in Portugal in 1999, in the Setubal Peninsula (south-east of Lisbon), associated with maritime pine, *Pinus pinaster* Aiton., official measures were taken to eradicate and control the nematode as part of a National Eradication Programme for the Pinewood Nematode (PROLUNP) (Mota *et al*., 1999; Rodrigues, 2006). Nevertheless, all pine forests in continental Portugal were declared affected by the disease in June 2008. Strict requirements have been imposed on wood movement from Portugal to other countries, and this has had a significant impact on the Portuguese economy (Portaria no. 553/B/2008; Commission Decision 2008/684/EC).

The economic importance of the introduction of the PWN into new areas has reinforced the need for an accurate diagnosis of the species. Reliable identification and detection is fundamental to define aspects of PWN control and management, to improve quarantine regulations and to prevent the further spread of the disease.

Morphologically, *B. xylophilus* has been identified by three characters: in the males, (a) the flattened spicules with a disc-like projection (cucullus) at the distal extremity; and in the females, (b) the anterior vulval lip with a distinct overlapping flap and (c) the tail terminus, which is usually round in the females of

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this species (Braasch, 2001; EPPO/OEPP, 2001; Penas *et al*., 2004; Ryss *et al*., 2005). The character of a round tail has been used to distinguish *B. xylophilus* from *B. mucronatus* Mamiya and Enda, 1979, a closely related but non-pathogenic species, in which the female has a mucronate tail. However, females of *B. xylophilus* isolates with a mucronate tail have been reported from Japan, Portugal and the USA (Wingfield *et al*., 1983; Guiran and Bruguier, 1989; Bolla and Boschert, 1993; Fonseca *et al*., 2004; Penas *et al*., 2004) and this characteristic is not reliable to distinguish *B. xylophilus* from the non-pathogenic species.

Morphometrical studies have been described for several characters of male and female of PWN isolates from Japan, North America and Portugal (Mamiya and Kiyohara, 1972; Nickle *et al*., 1981; Mota *et al*., 1999). In some cases, however, morphological and morphometrical characters are so variable that an accurate identification would be difficult or impossible. With the expansion of DNA-based methods, new tools have been used for the taxonomy and diagnosis of plant parasitic nematodes (Abrantes *et al*., 2004; Powers, 2004; Hockland *et al*., 2006).

Satellite DNA (satDNA) has been isolated from plants and animal species, including plant parasitic nematodes. The abundance of satDNA and the sequence variations it exhibits makes it a valuable tool for species identification (Tarés *et al*., 1993, 1994; Grenier *et al*., 1997). A satDNA sequence from *B. xylophilus* was previously isolated and characterised and had repeats organised in tandem arrays containing a sequence with a monomeric unit of 160 bp. Positive identification was achieved from single specimens using J10-1 and J10-2Rc primers designed close to both ends of the sequence of the 160 bp monomers (Tarés *et al*., 1994; Castagnone *et al*., 2005). The main objectives of the present work were to characterise Portuguese PWN isolates using morpho-biometric analysis and to evaluate intraspecific variability in the female tail termini of the isolates.

Materials and methods

Portuguese *B. xylophilus* isolates

Bursaphelenchus xylophilus isolates were obtained from *P. pinaster* wood samples collected in collaboration with the Autoridade Florestal Nacional (ex-Direcção Geral dos Recursos Florestais), the Portuguese forestry authority, from three sites in the Setúbal Peninsula: Herdade da Comporta (Álcacer do Sal); Vale Amada (Grândola) and Pinheiro do Cravo (Melides). Three other PWN isolates, obtained from *P. pinaster* from unknown locations in the Setúbal Peninsula were also examined (Table 1). Wood samples of 20–40 g each were collected from each pine tree 1.5 m from the base of the trunk using a low-speed drill; the samples were stored in plastic bags. Nematodes were extracted from the samples using the Baermann funnel method with modifications (Abrantes *et al*., 1976). After 48 h, 20 ml of water was collected and observed using an inverted stereomicroscope. One to three dispersal third-stage juveniles from each wood sample were subjected to satDNA species-specific identification to confirm the identity of the nematode as *B. xylophilus* using the primers and methods that have been developed (Castagnone *et al*., 2005). Ten dispersal third-stage juveniles were hand-picked, washed several times with sterilised distilled water, transferred to cultures of *Botrytis cinerea* Pars. grown on malt extract agar (MEA), and incubated at 25ºC. After three weeks, the nematodes were removed with sterilised distilled water from the fungal cultures. Males and females from each of the 12 Portuguese PWN isolates were hand-picked for molecular and morpho-biometrical studies.

Subcultures of each nematode isolate were made at three-week intervals by transferring small plugs with nematodes to new MEA colonised with *B. cinerea*.

Table 1. Portuguese *Bursaphelenchus xylophilus* isolates maintained axenically on *Botrytis cinerea* grown on malt extract agar medium at 25ºC.

Origin of the *B. xylophilus* isolate	Isolate code
Unknown	BxPt1, BxPt2, BxPt3
Herdade da Comporta	BxPt4, BxPt5, BxPt6, BxPt9, BxPt10, BxPt11
Vale Amada	BxPt12, BxPt13
Pinheiro do Cravo	BxPt14

Species-specific identification using satellite DNA

DNA extraction

Single and three specimens of dispersal third-stage juveniles collected directly from *P. pinaster* wood samples, and single and three specimens of PWN females with round, digitate or mucronate tails, collected from each isolate culture were transferred to 0.2 ml microtubes containing 2.5 μl of lysis buffer

(50 mM KCl, 10 mM Tris pH 9.0, 1.5 mM $MgCl_2$, 1% TritonX-100, 0.2 mg ml^{-1} BSA and 6 μg ml^{-1} proteinase K). The tubes were kept at -80ºC for 15 min, and then immediately heated to 60ºC for 60 min and to 95ºC for 15 min in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA), and maintained at 4ºC until use (Castagnone *et al*., 2005).

Amplification of satDNA

SatDNA was amplified following a satDNA species-specific procedure using the primers J10-1 (5'-GGTGTCTAGTATAATATCAGAG-3') and J10-2Rc (5'-GTGAATTAGTGACGACGGAGTG-3'), designed according to a sequence derived from a satDNA family previously characterised for PWN (Tarès *et al*., 1993, 1994; Castagnone *et al*., 2005). PCR was carried out in a 25 μl reaction mixture containing 2.5 μl lysis buffer (and nematode DNA); 2.5 μl *Taq* buffer (Q-Biogene, Illkirch, France); 2.5 μl of dNTPs (2 mM); 1 μl of each primer (100 ng ml^{-1}); and 2.5 units of *Taq* DNA polymerase (Q-Biogene). The reaction was heated to 94ºC for 5 min and cycled 15 times: 94ºC for 30 s, 63ºC for 1 min, 72ºC for 2 min with a final extension step at 72ºC for 5 min. After the PCR was completed, 15 μl of amplified product from each sample was analysed by electrophoresis in a 1% (w:v) agarose gel stained with ethidium bromide. The gel was analysed under UV light. Single and three specimens of a Japanese *B. xylophilus* (BxJ10) and German *B. mucronatus* (BmG) isolates were also included in each PCR reaction as controls.

Morpho-biometrical characterisation of Portuguese *B. xylophilus* isolates

Twenty males and 20 females of *B. xylophilus* from each isolate in culture were killed by heat in a drop of water on a cavity glass slide, mounted in water, viewed, photographed and immediately measured. Photographs were taken with a Leitz Dialux 20 bright field light microscope (Ernst Leitz Ltd., Midland, Ontario, Canada) using a Wild Photoautomat MPS45 camera (Wild Heerburg Ltd., Heerburg, Switzerland), and measurements were performed on a Leitz Dialux 20 bright field light microscope with the help of a drawing tube.

Results

Species-specific identification using satellite DNA

A ladder pattern of ca. 160 bp monomers was amplified from the DNA of dispersal third-stage juveniles extracted directly from *P. pinaster* wood samples. No amplification was obtained with *B. mucronatus* DNA (Fig. 1). DNA from females with mucronate, digitate or round tails collected from *B. cinerea* cultures was also amplified, and a ladder of ca. 160 bp monomers was obtained (Fig. 2).

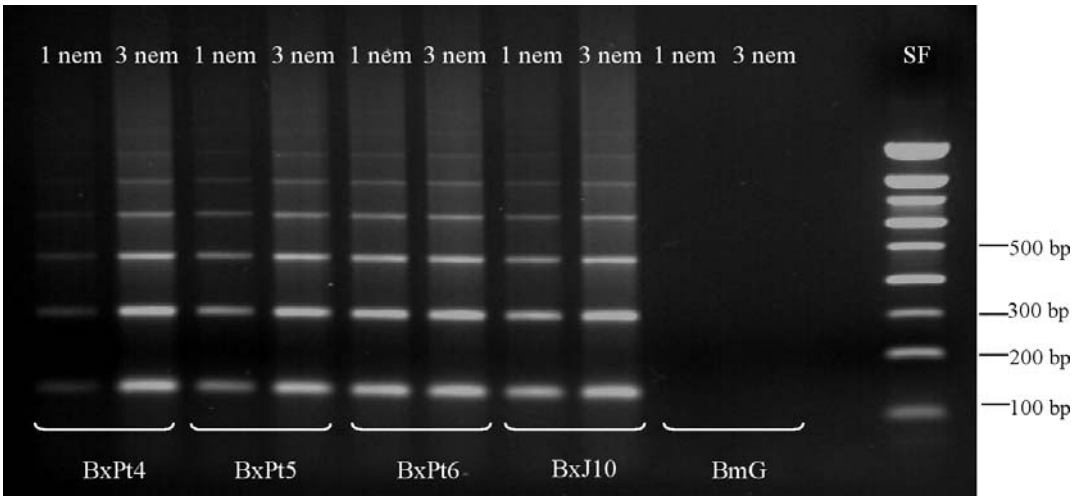

Fig. 1. Satellite DNA amplification using a set of primers specific for *Bursaphelenchus xylophilus*. BxPt4, BxPt5 and BxPt6: dispersal third-stage juveniles extracted from *Pinus pinaster* wood samples; BxJ10: Japanese *B. xylophilus* isolate; BmG: German *B. mucronatus* isolate. SF, DNA marker (Smart Ladder SF, 100 bp ladder, Eurogentec SA, Seraing, Belgium).

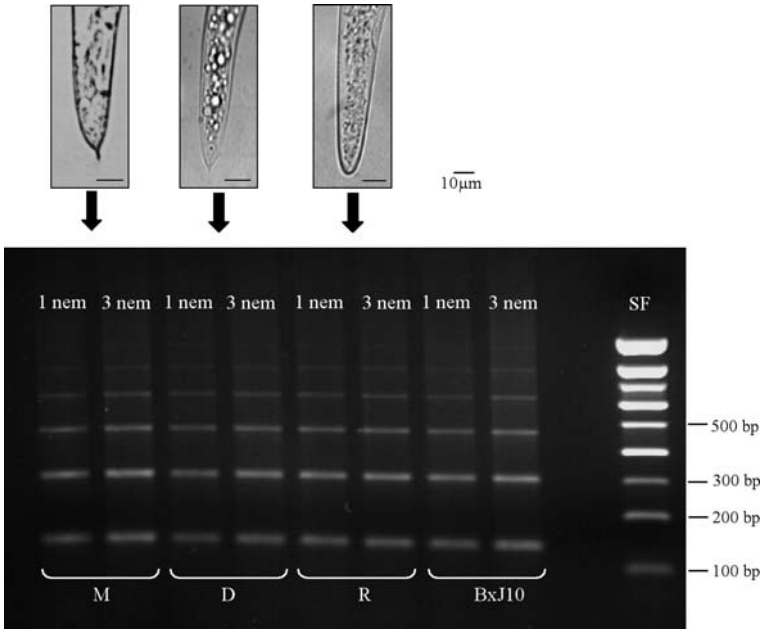

Fig. 2. Satellite DNA amplification using a set of primers specific for *Bursaphelenchus xylophilus*. M, Portuguese *B. xylophilus* females with mucronate tail terminus; D, Portuguese *B. xylophilus* females with digitate tail terminus; R, Portuguese *B. xylophilus* females with round tail terminus; BxJ10, Japanese *B. xylophilus* isolate; SF, DNA marker (Smart Ladder SF, 100 bp ladder, Eurogentec SA).

Morpho-biometrical characterisation of Portuguese *B. xylophilus* isolates

Morphometric analysis of males and females of the 12 PWN isolates are shown in Tables 2, 3, 4 and 5. Both males and females of the 12 isolates had a short stylet; a high cephalic region with offset lips; and a well-developed medium bulb (Fig. 3a). The male tail was curved ventrally, with a small terminal bursa, which could be seen in the dorso-ventral position, and the spicules were narrow, evenly arcuate, with a sharply pointed rostrum, capitullum flattened, condylus small, lamina angular in the last third and a cucullus at the distal end (Fig. 3b). The female vulva clearly showed a distinct overlapping anterior lip (vulval flap) (Fig. 3c), usually located at 70–80% of the body length. Wide variation in the forms of the female tail, which included both round, digitate and mucronate termini, was detected in all isolates (Fig. 4 and 5).

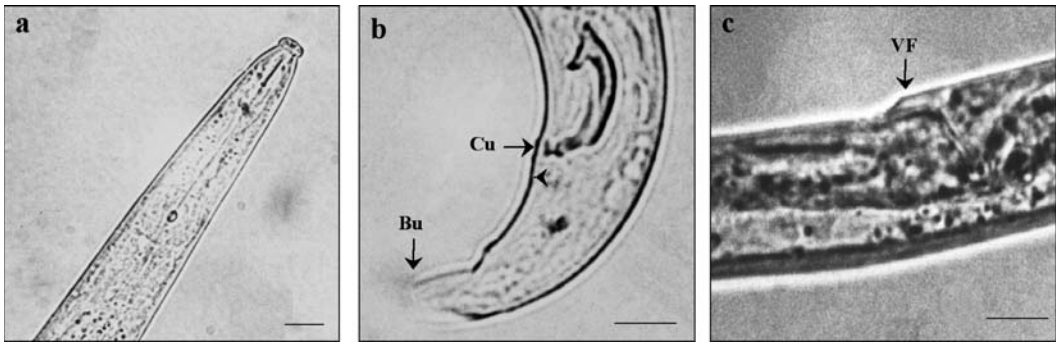

Fig. 3. Light microscope photographs of Portuguese *Bursaphelenchus xylophilus*: a, anterior region; b, male tail; c, vulval region; Bu, bursa; Cu, cucullus; VF, vulval flap. Scale bars = 10 μm.

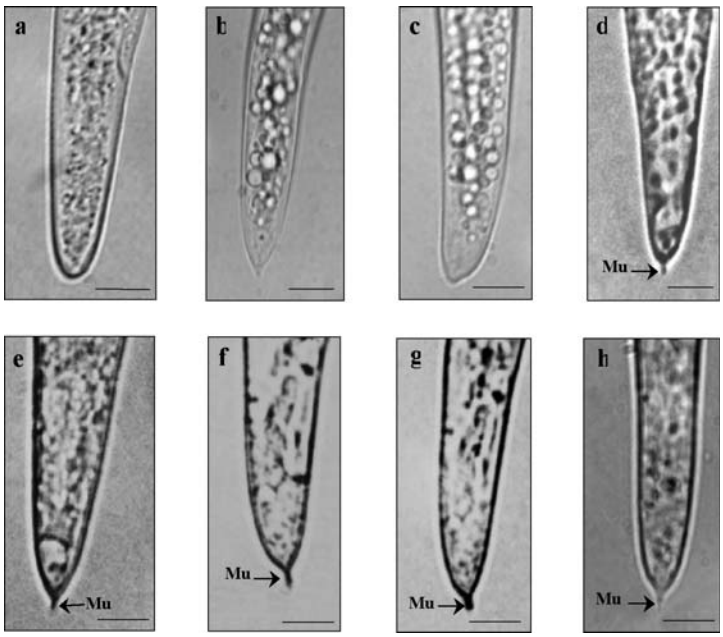

Fig. 4. Light microscope photographs of female tails of Portuguese *Bursaphelenchus xylophilus*. a, round tail terminus; b-c, digitate tail terminus; d-h, mucronate tail terminus; Mu, mucro. Scale bars = 10μm.

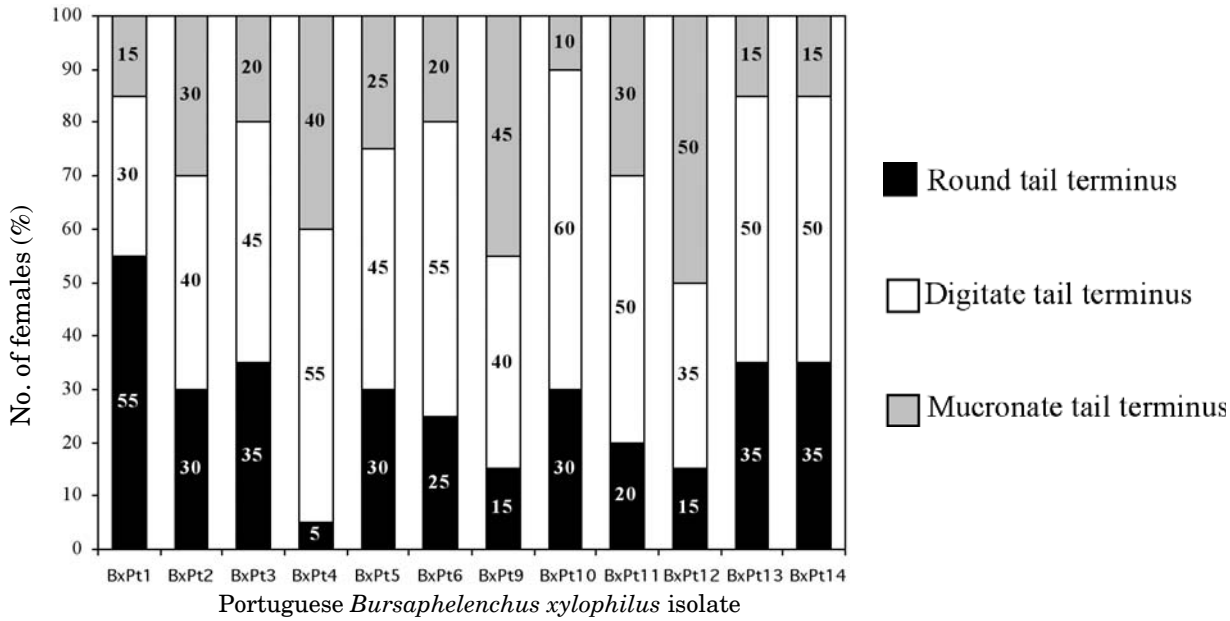

Fig. 5. Percentage of females with round, digitate and mucronate tail termini detected in the Portuguese *Bursaphelenchus xylophilus* isolates.

Table 2. Morphometrics of males of Portuguese isolates of *Bursaphelenchus xylophilus* (BxPt1, BxPt2, BxPt3, BxPt4, BxPt5 and BxPt6). Values are mean±SD. Values in parentheses indicate the minimum and maximum.

Character	*B. xylophilus* isolate					
	BxPt1 (n=20)	BxPt2 (n=20)	BxPt3 (n=20)	BxPt4 (n=20)	BxPt5 (n=20)	BxPt6 (n=20)
Linear (μm)						
Body length (L)	761.2±60.7 (613.3–838.3)	665.5±58.6 (571.7–791.7)	793.3±74.8 (700.0–940.0)	666.4±42.4 (585.0–740.0)	694.3±72.7 (558.3–866.7)	748.5±85.8 (640.0–926.7)
Stylet length	13.0±0.3 (12.6–13.7)	12.4±0.5 (11.6–13.4)	13.4±0.8 (12.4–14.7)	12.2±0.3 (11.6–12.6)	12.4±0.4 (11.6–13.2)	12.7±0.6 (12.1–14.2)
Greatest body width	21.2±1.6 (16.7–23.3)	18.1±1.6 (16.7–21.7)	22.3±1.8 (20.0–26.7)	18.0±1 (16.0–20.0)	18.9±1.9 (16.7–23.3)	20.5±2.1 (16.7–23.3)
Distance from anterior end to end of median bulb	71.6±3.1 (66.3–79.5)	67.4±5.7 (58.4–79.0)	72.0±3.6 (66.3–79.0)	64.7±4.3 (57.9–74.2)	67.1±6.3 (56.3–75.8)	69.9±4.6 (62.1–76.8)
Tail length	33.9±2.5 (29.7–37.9)	31.0±2.5 (26.4–37.2)	35.0±2.4 (31.8–40.5)	31.3±2.5 (27.0–35.3)	31.4±2.7 (24.3–35.1)	34.6±3.7 (30.0–44.6)
Body width at anus	15.8±1.4 (12.8–17.6)	13.7±1.1 (12.2–15.5)	14.7±1.2 (13.5–16.9)	13.5±1.1 (10.8–15.5)	14.0±2.0 (10.8–18.9)	15.1± 1.8 (12.2–18.9)
Spicule length	24.8±1.1 (21.6–26.8)	23.5±0.9 (22.1–25.3)	27.1±2.0 (24.2–31.1)	24.3±1.2 (21.6–26.6)	24.4±1.5 (21.6–29.2)	25.0±2.0 (21.3–29.5)
Ratio						
a	36.0±2.0 (31.7–39.7)	36.9±2.5 (32.8–40.4)	35.7±2.6 (31.0–40.8)	37.2±1.8 (33.8–39.5)	36.7±2.1 (33.5–41.6)	36.5±2.1 (32.7–39.8)
b_1	10.6±0.9 (9.0–12.0)	9.9±0.7 (8.8–11.0)	11.0±1.0 (9.7–13.4)	10.3±0.5 (9.4–11.4)	10.4±0.9 (9.3–12.5)	10.7±0.7 (10.0–12.7)
c	22.4±1.2 (20.2–24.8)	21.5±0.7 (20.6–23.1)	22.7±1.3 (20.3–25.1)	21.3±0.8 (20.0–23.5)	22.1±1.2 (20.6–25.7)	21.7±0.9 (20.4–23.5)
c’	2.2±0.2 (1.8–2.6)	2.3±0.2 (1.9–2.5)	2.4±0.1 (2.1–2.6)	2.3±0.2 (2.0–2.8)	2.3±0.2 (1.8–2.6)	2.3±0.2 (2.1–2.8)

Table 3. Morphometrics of males of Portuguese isolates of *Bursaphelenchus xylophilus* (BxPt9, BxPt10, BxPt11, BxPt12, BxPt13 and BxPt14). Values are mean±SD. Values in parentheses indicate the minimum and maximum.

Character	*B. xylophilus* isolate					
	BxPt9 (n=20)	BxPt10 (n=20)	BxPt11 (n=20)	BxPt12 (n=20)	BxPt13 (n=20)	BxPt14 (n=20)
Linear (μm)						
Body length (L)	718.9±68.6 (610.0–835.0)	712.9±32.2 (651.7–771.7)	754.8±55.1 (690.0–883.3)	658.1±65.6 (566.7–823.3)	752.9±55.6 (640.0–926.7)	758.7±76.8 (643.3–940.0)
Stylet length	11.7±0.6 (11.1–12.9)	12.5±0.3 (12.1–13.2)	12.7±0.4 (12.1–13.4)	12.3±0.4 (11.6–13.2)	12.7±0.4 (12.1–13.6)	12.8±0.5 (12.1–14.2)
Greatest body width	20.2±2.1 (16.7–23.3)	19.0±1.1 (16.7–21.7)	20.2±1.5 (16.7–23.3)	17.7±1.6 (15.0–20.0)	20.6±1.7 (18.3–23.3)	20.5±2.4 (16.7–25.0)
Distance from anterior end to end of median bulb	69.7±3.7 (63.2–75.8)	70.2±1.5 (67.4–72.6)	68.8±4.7 (61.6–79.7)	65.7±6.9 (52.6–81.0)	70.2±4.5 (63.2–77.9)	72.2±3.6 (65.8–77.9)
Tail length	32.6±2.3 (29.1–36.5)	32.5±1.9 (28.4–35.5)	34.0±2.3 (30.0–38.9)	30.8±2.6 (26.6–35.3)	32.3±2.1 (29.1–36.5)	34.5±2.6 (30.4–40.5)
Body width at anus	14.5±1.0 (12.2–16.2)	15.0±1.1 (12.8–16.9)	16.3±1.7 (13.5–19.5)	12.5±1.4 (9.5–15.3)	13.9±2.4 (9.9–16.9)	14.3±1.6 (12.2–17.6)
Spicule length	24.7±1.3 (22.6–27.1)	23.9±1.0 (22.4–25.8)	25.1±1.0 (23.4–26.8)	23.7±1.3 (22.1–27.6)	25.8±1.1 (23.9–27.9)	25.3±1.5 (22.6–28.4)
Ratio						
a	35.6±2.3 (31.9–40.6)	37.6±2.4 (33.5–42.1)	37.5±2.0 (34.5–43.4)	37.3±2.6 (31.8–41.2)	36.7±1.5 (34.6–39.7)	37.2±3.0 (33.2–42.5)
b_1	10.3±1.1 (8.5–11.8)	10.2±0.5 (9.4–11.0)	11.0±1.1 (9.2–13.7)	10.1±0.6 (8.7–11.1)	10.8±1.0 (9.1–13.1)	10.5±0.8 (9.2–12.6)
c	22.1±1.6 (20.1–26.5)	22.0±1.2 (20.0–23.9)	22.2±1.5 (20.2–24.7)	21.3±0.9 (20.1–23.4)	23.4±1.2 (21.9–27.1)	22.0±1.1 (20.0–24.0)
c'	2.2±0.1 (2.0–2.5)	2.2±0.2 (1.9–2.5)	2.1± 0.3 (1.7–2.6)	2.5±0.2 (2.2–2.9)	2.5±1.0 (2.0–6.7)	2.4±0.2 (2.3–2.9)

Table 4. Morphometrics of females of Portuguese isolates of *Bursaphelenchus xylophilus* (BxPt1, BxPt2, BxPt3, BxPt4, BxPt5 and BxPt6). Values are mean±SD. Values in parentheses indicate the minimum and maximum.

Character	*B. xylophilus* isolate					
	BxPt1 (n=20)	BxPt2 (n=20)	BxPt3 (n=20)	BxPt4 (n=20)	BxPt5 (n=20)	BxPt6 (n=20)
Linear (μm)						
Body length (L)	809.1±77.3 (723.3–966.7)	714.7±58.8 (643.3–856.7)	787.5±85.2 (655.0–900.0)	719.0±84.3 (529.8–840.0)	737.6±58.1 (613.3–816.7)	796.4±70.8 (703.3–983.3)
Stylet length	13.2±0.8 (12.1–14.9)	12.5±0.4 (11.8–13.7)	12.8±0.7 (11.6–14.0)	12.5±0.5 (11.6–13.2)	12.6±0.4 (11.6–13.2)	12.9±0.5 (12.4–14.2)
Greatest body width	23.4±2.5 (20.0–26.7)	20.3±2.1 (16.7–25.0)	22.8±3.3 (16.7–28.3)	19.0±2.4 (14.9–23.3)	19.8±1.8 (16.7–23.3)	22.6±2.4 (20.0–26.7)
Distance from anterior end to end of median bulb	68.5±3.6 (62.4–75.8)	66.3±2.8 (62.4–73.2)	66.6±4.2 (57.9–72.9)	64.0±7.4 (47.4–75.8)	67.9±3.7 (63.2–75.3)	69.8±2.8 (64.7–76.3)
Distance from anterior end to vulva	587.5±59.0 (513.3–698.0)	521.9±38.8 (476.7–618.3)	562.5±59.2 (466.7–653.3)	515.7±60.1 (383.0–615.0)	528.7±45.2 (431.7–590.0)	577.8±49.0 (505.0–701.7)
Distance from vulva to anus	187.8±19.6 (160.8–221.0)	162.4±18.1 (135.1–200.0)	191.3±25.9 (147.3–229.7)	170.9±25.4 (119.6–215.5)	174.8±13.5 (150.0–194.6)	186.2±21.1 (158.1–237.8)
Tail length	34.9±3.1 (30.4–43.2)	31.2±2.9 (27.0–37.8)	34.6±3.7 (28.4–39.5)	32.1±4.3 (23.7–40.0)	32.3±2.5 (28.4–36.5)	34.7±2.6 (31.1–41.2)
Body width at anus	11.6±1.7 (9.5–14.9)	9.7±0.9 (8.1–12.2)	11.3±1.1 (9.5–13.5)	9.8±1.1 (8.1–11.5)	10.0±0.6 (9.5–11.5)	11.1±1.1 (9.5–13.5)
Ratio						
a	34.7±3.0 (29.7–40.4)	35.3±2.1 (31.4–39.9)	34.9±3.3 (30.0–41.2)	38.0±3.5 (33.8–45.8)	37.5±2.6 (33.5–42.4)	35.4±2.3 (31.6–39.3)
b_1	11.8±0.9 (10.4–13.5)	10.8±0.8 (9.3–12.7)	11.8±1.0 (9.7–13.4)	11.3±0.9 (9.6–13.7)	10.9±0.8 (9.6–12.2)	11.4±0.8 (10.2–13.0)
c	23.2±1.7 (20.7–26.8)	22.9±1.2 (21.5–26.2)	22.8±1.3 (20.7–25.2)	22.5±1.2 (20.7–24.8)	22.9±1.1 (21.4–25.9)	23.0±1.7 (20.8–26.9)
c'	3.1±0.3 (2.6–3.7)	3.2±0.3 (2.8–3.7)	3.1±0.3 (2.5–3.6)	3.3±0.4 (2.8–3.9)	3.2±0.3 (2.9–3.9)	3.1±0.3 (2.7–3.7)
Percentage						
V	72.6±1.1 (70.7–75.1)	73.1±1.1 (70.7–74.5)	71.5±1.3 (69.5–73.7)	71.8±1.6 (67.9–74.0)	71.6±1.4 (68.6–73.6)	72.6±1.3 (70.6–75.6)

Table 5. Morphometrics of females of Portuguese isolates of *Bursaphelenchus xylophilus* (BxPt9, BxPt10, BxPt11, BxPt12, BxPt13 and BxPt14). Values are mean±SD. Values in parentheses indicate the minimum and maximum.

Character	*B. xylophilus* isolate					
	BxPt9 (n=20)	BxPt10 (n=20)	BxPt11 (n=20)	BxPt12 (n=20)	BxPt13 (n=20)	BxPt14 (n=20)
Linear (μm)						
Body length (L)	820.6±92.1 (688.3–1058.3)	751.1±40.5 (680.0–830.0)	802.3±87.1 (676.7–1013.3)	732.8±75.4 (570.0–870.8)	857.2±55.7 (736.7–990.0)	852.8±56.2 (766.7–950.0)
Stylet length	13.0±0.6 (12.1–14.2)	12.6±0.4 (11.6–13.2)	13.0±0.7 (12.1–14.7)	12.6±0.6 (11.6–13.7)	13.3±0.7 (12.4–14.5)	13.3± 0.5 (12.6–14.3)
Greatest body width	22.8±2.2 (20.0–26.7)	21.3±1.8 (18.3–23.3)	21.0±2.4 (16.7–26.7)	20.3± 1.9 (16.7–25.0)	23.6±2.5 (20.01–28.30)	23.1±1.8 (20.0–26.7)
Distance from anterior end to end of median bulb	70.1±3.2 (63.9–76.8)	67.3±4.3 (54.0–73.2)	71.9±4.7 (60.8–80.0)	66.1±3.6 (56.9–72.1)	72.6±2.3 (66.8–76.8)	71.7±3.1 (65.0–78.9)
Distance from anterior end to vulva	596.4±66.4 (503.3–750.0)	548.0±29.0 (503.3–605.0)	586.0±64.6 (480.0–740.0)	536.5±57.3 (411.7–650.0)	612.8±39.7 (536.7–720.0)	609.8±38.8 (546.7–690.0)
Distance from vulva to anus	194.0±27.1 (162.2–277.0)	171.2±13.8 (143.9–192.6)	182.0±19.5 (159.5–229.7)	170.9±21.6 (136.5–207.4)	213.4±20.8 (173.0–267.6)	210.1±19.8 (177.0–240.5)
Tail length	36.2±2.6 (32.4–40.5)	32.2±1.8 (28.2–34.5)	34.1±3.1 (29.1–42.1)	32.9±3.7 (23.2–39.9)	35.4±2.9 (31.1–41.8)	36.5±1.7 (33.1–39.2)
Body width at anus	11.1±0.9 (9.5–13.5)	10.7±0.8 (9.5–12.2)	9.8±1.0 (8.1–12.2)	10.2±1.1 (7.4–11.5)	10.5±1.1 (9.3–12.9)	10.8±1.0 (9.5–12.2)
Ratio						
a	36.0±2.5 (32.7–41.4)	35.3±2.0 (32.9–40.6)	38.3±2.0 (34.6–41.5)	36.2±2.0 (33.3–40.0)	36.5±2.6 (32.1–41.5)	37.1±2.9 (33.6–46.0)
b_1	11.7±1.0 (10.1–14.0)	11.2±0.7 (10.0–12.9)	11.2±0.8 (9.9–12.7)	11.1±0.7 (10.0–12.3)	11.8±0.7 (10.9–13.5)	11.9±0.8 (10.2–13.1)
c	22.6±1.3 (20.2–26.1)	23.4±1.0 (21.8–25.1)	23.6±1.5 (20.0–25.4)	22.3±1.4 (20.1–25.3)	24.3±1.8 (21.0–28.1)	23.4±1.5 (21.2–27.8)
c'	3.3±0.3 (2.9–3.9)	3.0±0.3 (2.6–3.5)	3.5±0.2 (3.2–4.0)	3.3±0.3 (2.6–4.0)	3.4±0.3 (2.7–3.9)	3.4±0.3 (3.1–3.9)
Percentage						
V	72.7±1.2 (70.6–74.8)	73.0±1.2 (70.8–74.8)	73.0±0.9 (70.9–74.1)	73.2±1.5 (71.0–75.9)	71.5±1.3 (68.0–73.0)	71.5±1.3 (68.8–73.0)

Discussion

The morpho-biometrical studies of the Portuguese *B. xylophilus* isolates showed that the spicule of the males and the morphology of the vulval region of the females were characteristic of the species. Most of the morphometric data for the males and females of the PWN isolates (Tables 2–5) were within the range of the other *B. xylophilus* isolates, with the exception of the body length and the *a* ratio, which were smaller than those previously described for a Portuguese isolate from *P. pinaster* (Mota *et al*., 1999). The form of the female tail (round, digitate or mucronate) was however not characteristic of this species (Fig. 4). In the Portuguese PWN isolate described by Mota *et al*. (1999) a minute inconspicuous terminus resembling a mucro was detected in some females. However, the frequency of this variation in the female tail shape within the same isolate has not been reported. In our study, this variation was detected in all 12 Portuguese PWN isolates. Some isolates (BxPt4, BxPt9 and BxPt12) had a high percentage of mucronate tailed females (>40%). Only one of the isolates (BxPt1) had >50% of the females with a round tail (Fig. 5).

The female tail terminus is the only morphological character that has been considered to distinguish *B. xylophilus* from *B. mucronatus* isolates. The occurrence of mucronate tailed females in Portuguese PWN isolates therefore clearly made it difficult to identify this species using this morphological character. Identification by morphological characters can thus lead to a wrong diagnosis and hence inadequate phytosanitary measures, so that molecular based techniques are advisable.

A simple DNA-based species-specific identification of single individuals of *B. xylophilus*, based on a set of primers from the sequence of a satDNA family, was developed by Castagnone *et al*. (2005). This species-specific marker was very useful in the detection and identification of PWN from *P. pinaster* wood samples where only juvenile stages were found. The juvenile stages of *Bursaphelenchus* spp. are morphologically indistinguishable; the DNA based method therefore increases the speed of diagnosis by eliminating the need for culturing the juveniles until they reach the adult stage. Furthermore, it allowed specific DNA amplification from a single specimen and it confirmed the intraspecific variability of the tail shape among the females in the Portuguese isolates of PWN (Fig. 2).

The economic impact of *B. xylophilus* on the Portuguese economy clearly reinforces the need for the accurate identification of this nematode. Morphological characters to identify PWN in Portugal have some limitations due to the variability in the shape of the female tails . Therefore, molecular-based methods using species-specific probes should be used in conjunction with taxonomic studies for diagnostic purposes.

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