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Morphological characterisation and diagnostics of *Xiphinema* non-*americanum* group species (Nematoda: Longidoridae) from Romania using mutiplex PCR

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Summary

Additional data on the occurrence and distribution of *Xiphinema* non-*americanum* group species in Romania are provided. *Xiphinema diversicaudatum, X. index*, *X. vuittenezi* and *X. italiae* were recovered from vineyards and cherry fruit trees; adults and juvenile stages were described and analysed and the morphology/variability discussed. Multiplex PCR diagnostic test using species-specific primers designed by Wang *et al*. (2003) yielded amplification products with expected lengths for all screened populations of these four species. Two ribosomal markers (D2-D3 28 LSU rDNA and ITS) were sequenced and ITS RFLP patterns were obtained from two *X. vuittenezi* populations, which have shown some morphological differences. Comparatively low level of interpopulation genetic dissimilarity (<1 %) was revealed for both markers (for D2D3 – 0.5 %; for ITS – 0.7 %). Both populations of *X. vuittenezi* studied produced identical ITS-RFLP specific pattern that clearly identify this species.

Keywords: distribution; juvenile stages; D2-D3; ITS; RFLP

Introduction

Xiphinema index Thorne & Allen 1950, *X. diversicaudatum* (Micoletzky, 1927), *X. vuittenezi* Luc, Lima, Weischer & Flegg, 1964 and *X. italiae* Meyl, 1953 are ectoparasitic species known or suspected to be vectors of nepo-viruses: *X. index* transmits Grapevine Fanleaf Virus (virus that cause serious disease in viticulture regions) and *X. diversicaudatum* – Arabis Mosaic Virus and Strawberry Latent Ringspot Virus that have wide range of hosts among annual and perennial crops (MacFaralne *et al*., 2002). *Xiphinema italiae, X. index* and *X. vuittenezi* have been recorded from grapevine in different regions of Romania (Romaşcu, 1971; Romaşcu & Zinca, 1974); *X. diversicaudatum* was recorded also from the rhizosphere of strawberry, cherry, peach and plum trees (Romaşcu, 1981), however the data on their morphology and variability are limited and developmental stages were not described.

The purposes of this work are 1) to provide data on the morphology of adults and juvenile stages of these species from Romania; 2) to test the multiplex polymerase chain reaction using species-specific primers (Wang *et al*., 2003) and 3) to study the genetic variability of two *X. vuittenezi* populations which have shown differences in their morphology.

Materials and methods

Soil samples were collected from the rhizosphere of grapevine and cherry trees at a depth of 20 – 40 cm, from different regions of the country. Nematodes were extracted from 200 g of soil by using the Baerman funnel method for 48 hours exposition, killed by gentle heat, and fixed in 4 % formalin. The extracted specimens were processed in anhydrous glycerine by the Seinhorst method (1959) and mounted on permanent slides. Photographs were taken using an AxioImager.M2 – Carl Zeiss compound microscope equipped with digital camera (ProgRes C7) and specialised software (CapturePro Software 2.8). Measurements were made using an Olympus BX 41 light microscope with a drawing tube and digitizing tablet (CalComp Drawing Board III, GTCO CalCom Peripherals, Scottsdale, AZ, USA) and Digitrak 1.0f computer program (Philip Smith, John Hutton Institute Dundee, UK).

Multiplex PCR

DNA isolation was carried out following the procedure described by Wang *et al*. (2003) by placing 4 nematodes in

10 µl of lysis buffer (1X Platinum Taq DNA polymerase/Invitrogen and 60 µg of proteinase K per ml) between two glass slides and crushed gently. The homogenate was taken up carefully with a pipette, transferred to 0.2 ml Eppendorf tubes and frozen at -80 °C for 15 min. Subsequently, the tubes were incubated at 60 °C for 1 h and 95 °C for 15 min.

Amplification was carried out in a 25-µl reaction mixture containing the 2.5 µl lysis buffer (nematode lysate as PCR template), 1x Platinum *Taq* DNA polymerase buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Sigma 10mM), 0.8 pmol each primer, and 0.5 units of Platinum *Taq* DNA polymerase (Invitrogen). The following primers A-ITS1, I27, D24, V18, ITA26 were used (Wang *et al*., 2003). Amplifications were performed in a thermal cycler (Master cycler Pro S – Eppendorf), with the following cycling conditions: 95 °C for 3 min followed by 39 cycles at 94 °C for 1 min, 55 °C

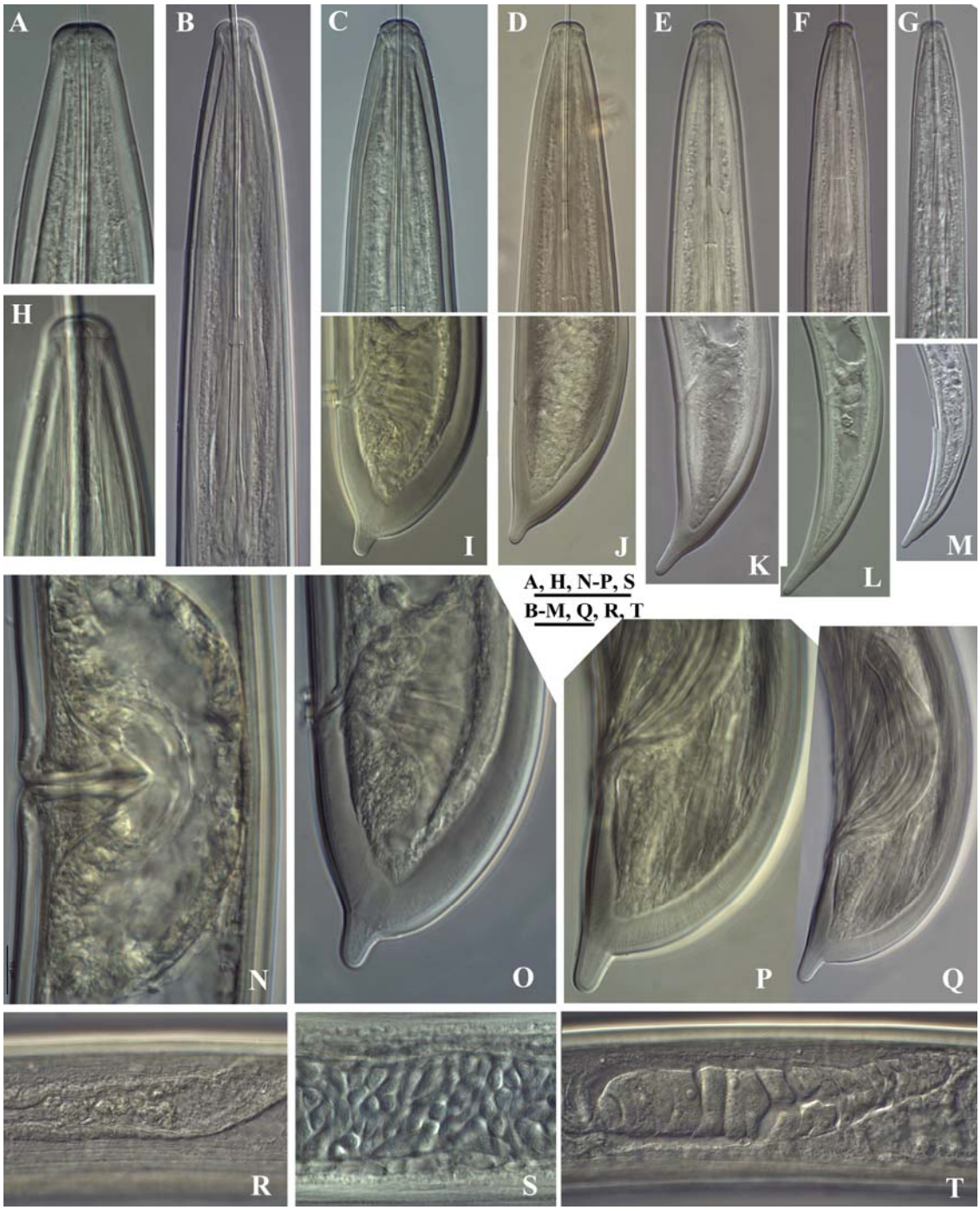


Fig. 1. *Xiphinema diversicaudatum. Females, males and juveniles.* A – G, Anterior ends of male (A, B), female (C) fourth to first juvenile stages (D – G); H, Amphid of male; I – M, Tails of female (I) and fourth- to first- juvenile stages (J – M); N, Vaginal region; O, Female tail; P, Male tail; Q, Male tail and spicules, R, Z-differentiation; S, Sperms in the testis; T, Ovary; Scale bars: 20 µm.

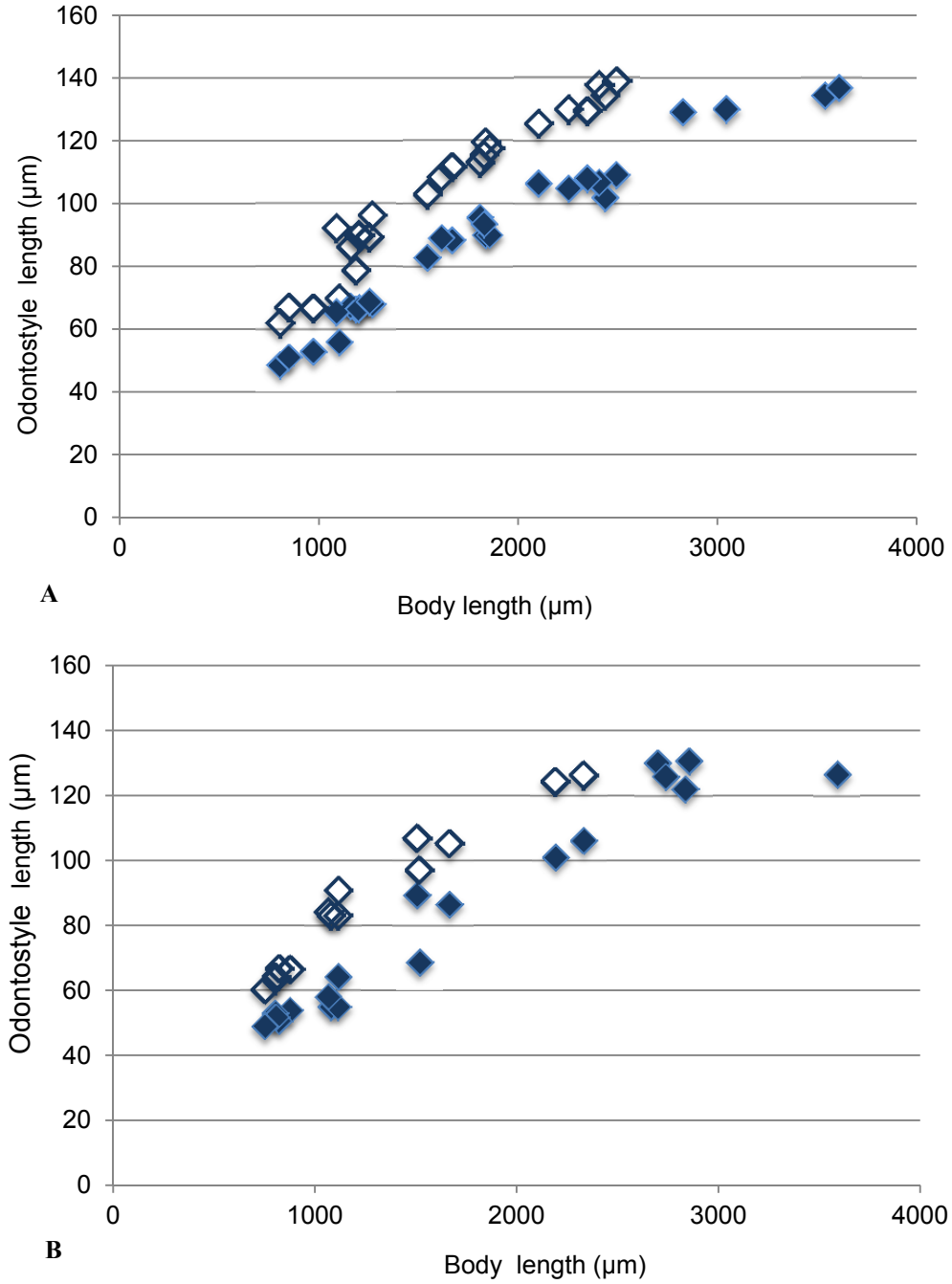


Fig. 2. Scatter plot of the functional (◆) and replacement (◇) odontostyle in relation to body length of the juvenile stages and adults. A, *Xiphinema diversicaudatum* B, *Xiphinema index*

for 1 min, and 72 °C for 1 min 30 s, and ending with 1 cycle at 72 °C for 5 min and storage at 4 °C.

Amplification products (10 µl PCR product) were separated on 1.5 % agarose gel (Sigma) in 0.5X TBE at 100V for 40 minutes and visualized with GENi photo documentation system (Syngene).

DNA extraction, PCR amplification, sequencing and RFLP analysis

The following analyses were performed for both *X. vuittenezi* populations, which have shown differences in morphometrics. Two ribosomal genes (D2-D3 28LSU rDNA and ITS) were sequenced and ITS RFLP pattern was obtained. Genomic DNA was extracted from 10 individual nematodes as described by De Luca *et al.* (2004). The crude DNA isolated from each individual nematode was directly amplified by using two sets of universal primers. The ITS containing region was amplified using the forward primer 18S (5-TGATTACGTCCCTGCCTTT-3) and the reverse primer 26S (5-TTTCACTCGCCGTTACTAAGG-3) (Vrain *et al*., 1992), while the D2-D3 region using the forward primer D2A (5-ACAAGTACCGTGAGGGAAAG

Table 1. Measurements of females, males and juvenile stages of *Xiphinema diversicaudatum* from Voineşti (mean ± standard deviation, with range). All measurements in micrometers except L in mm.

Character	Females	Males	J1	J2	J3	J4
n	n = 2	n = 2	n = 4	n = 7	n = 7	n = 6
L	3.54, 2.83	3.61, 3.04	0.93 ± 0.13 0.816 – 1.10	1.19 ± 0.595 1.08 – 1.26	1.74 ± 0.125 1.54 – 1.86	2.34 ± 0.14 2.10 – 2.49
a	59.7, 52.2	70.5, 64.1	41.9 ± 3.3 38.7 – 45.9	42.8 ± 1.9 40.4 – 45.1	47.7 ± 2.6 43.9 – 51.0	51.0 ± 7.9 43 – 61
b	7.7	7.6, 6.6	3.9 ± 0.5 3.4 – 4.5	4.1 ± 0.3 3.7 – 4.5	4.7 ± 0.2 4.4 – 4.9	5.5 ± 0.2 5.2 – 5.9
c	65.8, 65.2	58.4, 60.0	16.0 ± 1.8 14.1 – 17.8	18.4 ± 1.7 16.6 – 21.1	28.8 ± 3.5 23.5 – 33.7	49.9 ± 9.8 40.7 – 65.4
c'	1.1, 1.0	1.4, 1.1	4.0 ± 0.3 3.8 – 4.4	3.1 ± 0.3 2.6 – 3.4	2.1 ± 0.3 1.8 – 2.6	1.3 ± 0.3 1.0 – 1.7
V (%)/ Spicules length	41.7, 44.4	74, 75				
G1 (%)	18.9, 22.4					
G2 (%)	22.0, 22.4					
d	7.7, 7.9	9.0, 8.2	5.4 ± 0.5 4.8 – 6.1	6.2 ± 0.4 5.6 – 6.6	7.6 ± 0.5 7.0 – 8.6	8.3 ± 0.6 7.3 – 9.2
d'	3.0, 3.1	2.9, 2.7	2.2 ± 0.1 2.0 – 2.4	2.5 ± 0.2 2.2 – 2.8	2.7 ± 0.1 2.6 – 3.0	3.0 ± 0.2 2.8 – 3.3
Anterior end to guide ring	103, 97	113, 109	40.9 ± 3.4 37 – 45	53.3 ± 2.3 50 – 57	74.1 ± 5.1 69 – 84	91.2 ± 4.9 84 – 96
Odontostyle	135, 129	137, 130	52.3 ± 3.1 49 – 56	67.2 ± 1.1 66 – 69	90.1 ± 4.0 83 – 96	106.2 ± 2.5 102 – 109
Replacement odontostyle			66.6 ± 3.2 62 – 70	89.1 ± 5.4 79-96	112.8 ± 5.6 103 – 120	132.9 ± 5.2 126 – 139
Odontophore	89, 78	82.5, 81	35.9 ± 1.0 34 – 37	50.1 ± 3.6 43 – 53	60.2 ± 3.4 55 – 64	70.0 ± 3.8 66 – 76
Pharynx length	460, –	475, 464	241.5 ± 2.5 238 – 244	292.6 ± 16.8 263 – 307	369.1 ± 19.5 337 – 391	425.3 ± 21.0 400 – 447.5
Tail	54, 43	62, 51	58.2 ± 3.6 54 – 62	65.0 ± 4.7 60 – 71	60.6 ± 5.1 55 – 71	48.0 ± 7.1 37 – 55
Hyaline part of tail	19, 19	23, 17	10.5 ± 1.2 9 – 12	15.7 ± 2.1 12.5 – 19	19.3 ± 1.7 17 – 22.5	17.7 ± 1.8 16 – 20
Body diameter at: – lip region	13, 12	12.5, 13	7.6 ± 0.2 7 – 8	8.6 ± 0.4 8 – 9	9.7 ± 0.5 9 – 10	11.0 ± 0.6 10 – 12
– guide ring	41, 38	37, 36	16.3 ± 0.8 15 – 17	21.3 ± 1.0 19 – 22	26.4 ± 0.8 25 – 27.5	33.2 ± 2.1 30 – 36
– base of pharynx	50, 49	46, 45	21.6 ± 0.7 21 – 23	26.6 ± 1.2 25 – 28	35.0 ± 3.0 30 – 39	44.4 ± 5.3 38 – 50
– mid-body/at vulva	59, 54	51, 47.5	22.2 ± 1.4 21 – 24	27.8 ± 1.5 26 – 30	36.5 ± 3.7 30 – 42	46.6 ± 6.2 38 – 54
– anus	48, 44	43, 44.5	14.6 ± 1.2 14 – 16	21.0 ± 1.4 19 – 23	29.0 ± 2.2 25 – 32	38.5 ± 4.1 33 – 44
– hyaline part	27, 33	26, 19	5.8 ± 0.8 5 – 7	7.8 ± 0.8 6.5 – 9	11.1 ± 1.1 9 – 12	18.3 ± 2.3 16 – 21

TTG-3) and the reverse primer D3B (5-TCGGAAGGAAC CAGCTACTA-3) (Castillo *et al.*, 2003). PCR cycling conditions used for amplification of both segments were identical: an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 50 s, annealing at 55 °C for 50 s and extension at 72 °C for 1 min and a final step at 72 °C for 7 min. The size of amplification products was determined by comparison with the molecular weight marker ladder 100 (Fermentas, St Leon-Rot, Germany) following electrophoresis of 10 µl on a 1 % agarose gel.

Sequencing: PCR products of the D2-D3 and ITS containing region from three individual nematodes for each population were purified for sequencing using the protocol listed by manufacturer (High Pure PCR elution kit, Roche, Germany). Purified DNA fragments were cloned and sequenced in both directions. The sequences have been submitted to GenBank with the following accession numbers HG329722 – HG329724. A BLAST (Basic Local Alignment Search Tool) search at NCBI (National Center for Biotechnology Information) was performed using *X. vuittenezi* D2-D3 and ITS sequences as queries to confirm

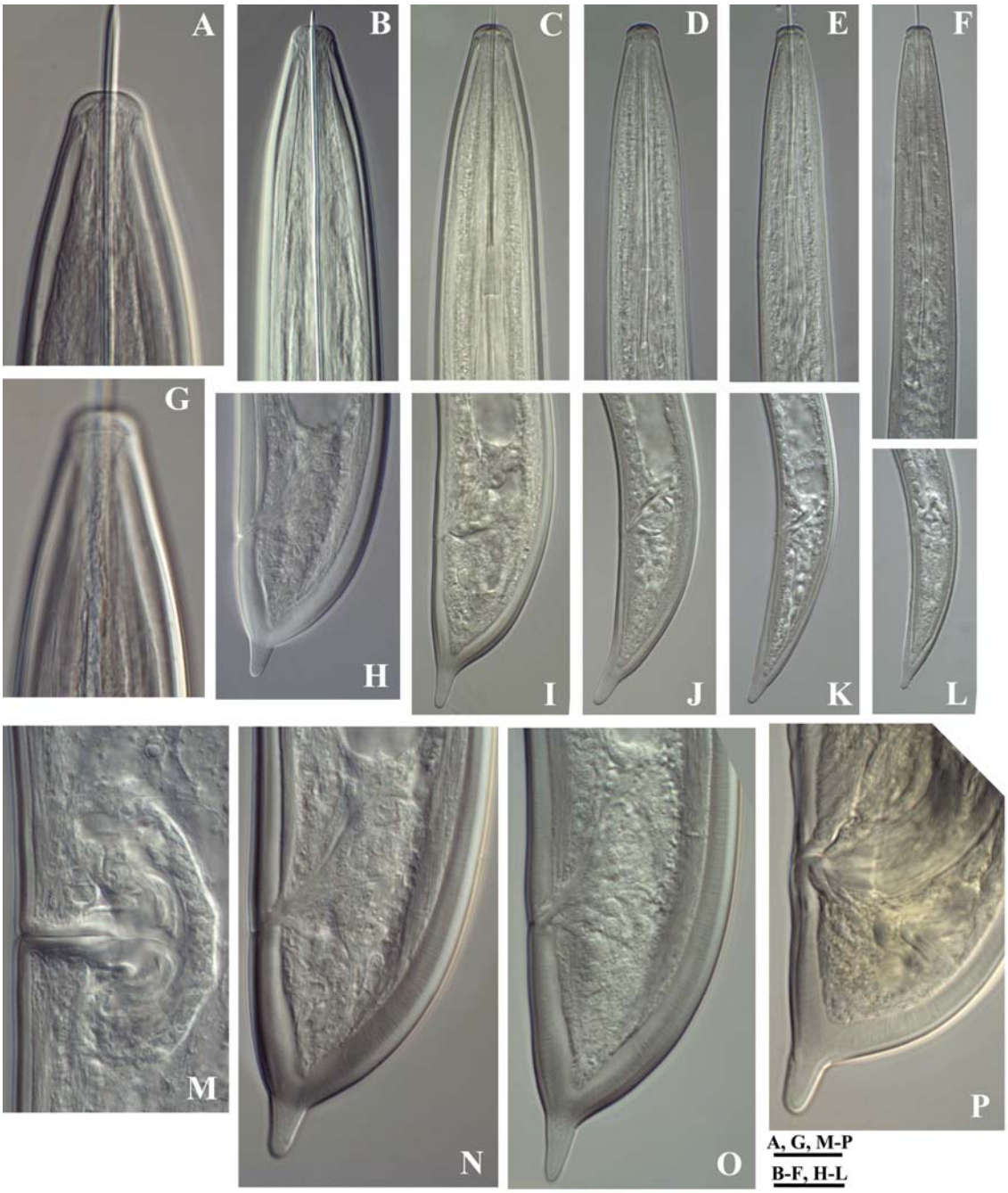


Fig. 3. *Xiphinema index. Females, male and juveniles.* A – G, Anterior ends of female (A, B), fourth to first juvenile stages (C – F); G, Amphid of female; H – L, Tails of female (H) and fourth- to first- juvenile stages (I – L); M, Vaginal region; N, O, Female tail variation; P, Male tail; Scale bars: A, G, M – P: 12 µm; B – F, H – L: 20 µm

their nematode origins (Altschul *et al*., 1997).

The acquired sequences and those available at NCBI were aligned using ClustalX 2.1 programme (Jeanmougin *et al.* 1998). Subsequently both ends were trimmed and edited in order to remove the amplification mistakes and sequence divergences were calculated using MEGA 5.0 (Tamura *et al.* 2011). *RFLP analysis:* Ten µl of each PCR product from two individual nematodes of both populations was digested with five units of the following restriction enzymes: *Bam*HI, *Dde*I, *Rsa*I, *Alu*I, *Hin*fI and *Xba*I (Roche Diagnostics, Manheim, Germany). Digested products were separated onto a 2.5 % agarose gel by electrophoresis, stained with ethidium bromide, visualised on a UV transilluminator and recorded by photography with a digital system.

Results and discussion

Taxonomy

Xiphinema diversicaudatum (Micoletzky, 1927) Thorne, 1939 **(**Figs. 1 and 2A)

Measurements

See Table 1.

Description

Females: Body assuming an open C-shape when heat relaxed; head region rounded, almost continuous with the rest of the body; amphidial aperture large, occupying c. 80 – 90 % of the corresponding labial diameter; cuticle at postlabial region 3 – 4 µm, at mid-body 4 µm, on tail 7 µm. Pharyngeal bulb measuring 78, 89 x 25, 26 µm. Uterus tripartite, Z differentiation in the form of irregular flower-like bodies and uterine spines. Rectum 30, 27 µm long; prerectum 469, 481 µm; tail convex conoid, dorsally rounded with a ventral peg 7 – 8.5 µm long, 5 caudal pores. Blind canal present.

Males: Common, morphologically similar to females, body more coiled in the posterior region; cuticle at postlabial region 3 µm, at mid body 5 – 6.5 µm, pharyngeal bulb measuring 81.5, 107 x 25, 21 µm; spicules massive 74, 75 µm long; ventromedian supplements 1 + 3 and 1 + 4, irregularly spaced; peg 11 µm long.

Table 2. Measurements of females and juvenile stages of *Xiphinema index* from Valea Călugărească (mean ± standard deviation, with range). All measurements in micrometers except L in mm.

Character	**Females**	**Males**	**J1**	**J2**	**J3**	**J4**
n	n = 4	n = 1	n = 5	n = 4	n = 3	n = 3
L	2.80 ± 0.10 2.70 – 2.925	3.59	0.81 ± 0.44 0.75 – 0.88	1.09 ± 0.24 1.06 – 1.11	1.66, 1.52, 1.51	2.33, 2.19, 2.40
a	55.0 ± 3.0 51.9-58.5	63.4	42.9 ± 1.6 40.7 – 44.9	43.8 ± 1.7 41.8 – 45.7	50.5, 47.5, 54.3	54.1, 56.3, 56.9
b	7.1 ± 1.0 6.1 – 8.3		3.7 ± 0.3 3.5 – 4.1	4.2 ± 0.2 4.0 – 4.5	4.9, 4.8, 4.0	6.2, 5.7, 5.2
c	70.9 ± 11.0 59.0 – 83.4	81.0	19.2 ± 1.3 17.7 – 21.0	22.6 ± 1.3 21.5 – 24.2	31.2, 29.9, 31.3	52.3, 46.0, 43.8
c'	1.2 ± 0.2 0.9 – 1.4	1.0	3.2 ± 0.2 2.9 – 3.5	2.7 ± 0.2 2.6 – 3.0	2.2, 2.2, 2.3	1.5, 1.5, 1.6
V (%)/Spicules length	42.9 ± 1.6 41.0 – 45.0	69				
G1 (%)	13.3 ± 1.7 12.3 – 15.8					
G2 (%)	10.7 ± 2.9 7.4 – 14.5					
d	9.4 ± 0.2 9.1 – 9.6	11.1	6.0 ± 0.4 5.6 – 6.5	6.4 ± 0.8 5.5 – 7.5	7.8, 5.8, 8.6	9.9, 7.8, 8.7
d'	3.0 ± 0.1 2.9 – 3.1	4.3	2.3 ± 0.1 2.2 – 2.4	2.6 ± 0.1 2.4 – 2.7	2.8, 2.7, 2.9	3.1, 3.1, 3.2
Anterior end to guide ring	109.2 ± 2.9 106 – 112	121	39.2 ± 1.9 37 – 41	47.8 ± 8.5 41 – 60	72, 52, 68	97, 76, 85
Odontostyle	127.3 ± 4.0 122 – 131	127	51.7 ± 1.9 49 – 54	57.3 ± 5.1 52 – 64	87, 69, 89	106, 101, 104
Replacement odontostyle			64.1 ± 2.6 60 – 67	85.3 ± 3.7 83 – 91	105, 97, 107	126, 124.5,
Odontophore	73.8 ± 2.1 72 – 76	76	33.0 ± 2.1 30 – 35	44.8 ± 1.8 43 – 47	56, 52, 51.5	69, 67, 63
Pharynx length	395.1 ± 47.5 343 – 447.5		219.4 ± 20.0 185 – 234	263.0 ± 11.6 250 – 277	341, 319, 376	375, 386, 460
Tail	39.8 ± 5.4 34 – 46	44	42.5 ± 2.0 41 – 45	48.4 ± 1.8 46 – 50	53, 51, 48	44.5, 48, 55
Hyaline part of tail	15.5 ± 1.6 14 – 18	17	7.6 ± 0.3 7 – 8	9.1 ± 1.0 8 – 10	11, 11, 11.5	16, 15, 17
Body diameter at: - lip region	11.7 ± 0.5 11 – 12	11	6.5 ± 0.2 6 – 7	7.5 ± 0.4 7 – 8	9, 9, 8	10, 10, 10
- guide ring	35.1 ± 1.5 34 – 37	47	15.1 ± 0.3 15 – 15.5	19.0 ± 1.4 18 – 21	26, 24, 23	30, 30, 31
- base of pharynx	44.0 ± 1.8 42 – 46		18.8 ± 0.7 18 – 20	23.5 ± 1.2 22 – 25	32, 30, 27	39, 38, 41
- mid-body/at vulva	50.6 ± 2.8 48.5 – 55	57	18.9 ± 0.8 18 – 20	25.0 ± 1.2 23 – 26	33, 32, 28	43, 39, 42
- anus	34.1 ± 1.8 32 – 36	43	13.3 ± 0.6 13 – 14	17.9 ± 1.1 16 – 19	24, 23, 21	30, 31, 34
- hyaline part	16.9 ± 2.0 15 – 20	20.5	5.5 ± 0.4 5 – 6	6.2 ± 0.8 6 – 7	7, 9, 6	13, 12, 13

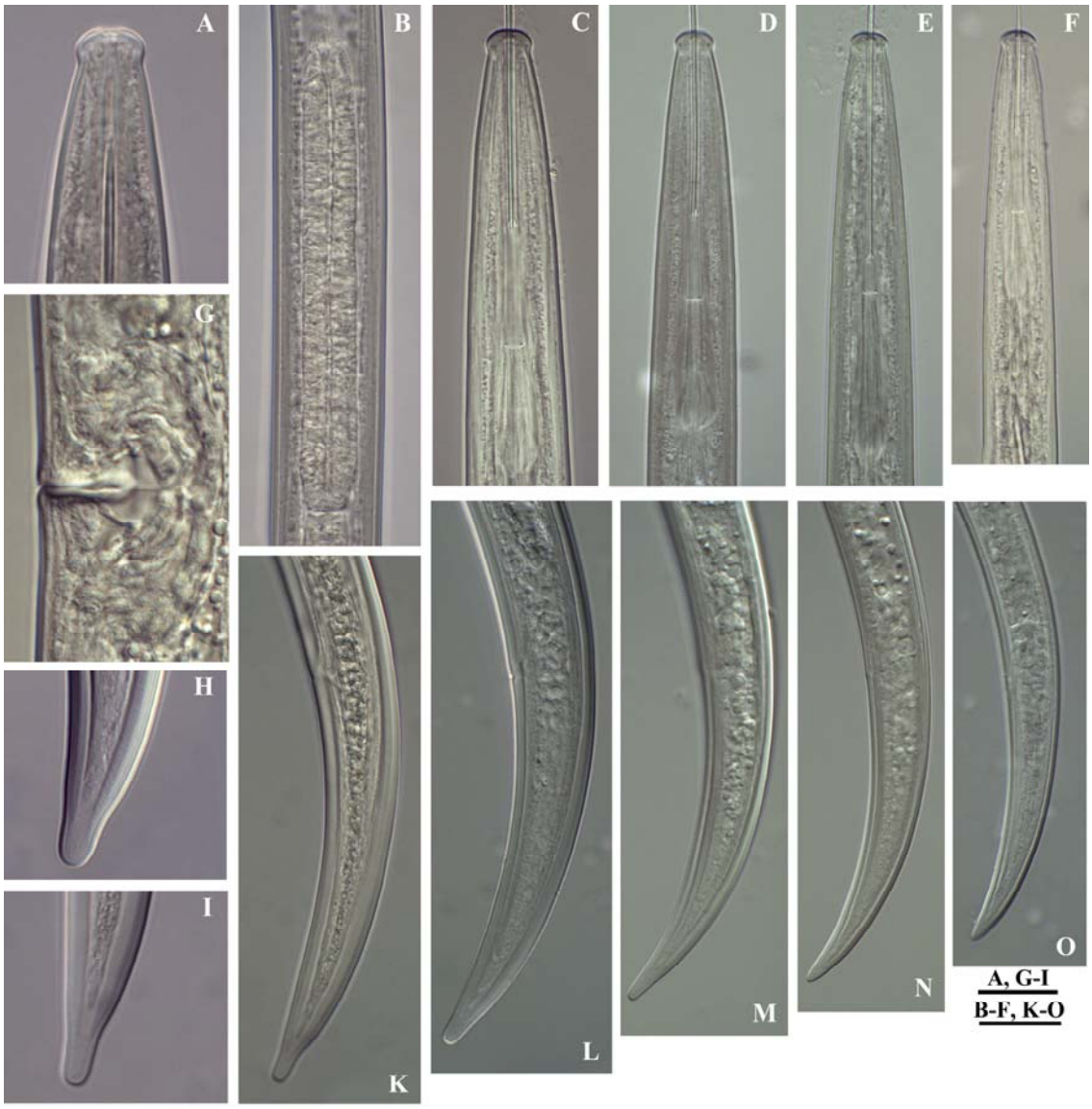


Fig. 4. *Xiphinema italiae. Females and juveniles.* A, Female head; B, Pharyngeal bulb; Anterior ends of female (C), fourth- to second-juvenile stages (D – F); G, Vagina; H, I, Tail tip variation; Tails of female (K, L), fourth- to second-juvenile stages (M – O); A, B, I, K – *Adamclisi*; C – H, L – O – *Bîrlad*; Scale bars: A – G, I: 12 µm; B – F, K – O: 20 µm.

Juveniles: The juveniles assigned to 4 stages (Fig. 2A), similar to the female in general appearance, smaller in size with the tail showing specific shape at different stages: in J1, J2 elongate conoid, ventrally arcuate; J3 dorsally convex conoid with subdigitate terminus; J4 dorsally convex conoid, digitate with ventral peg.

Locality and plant association

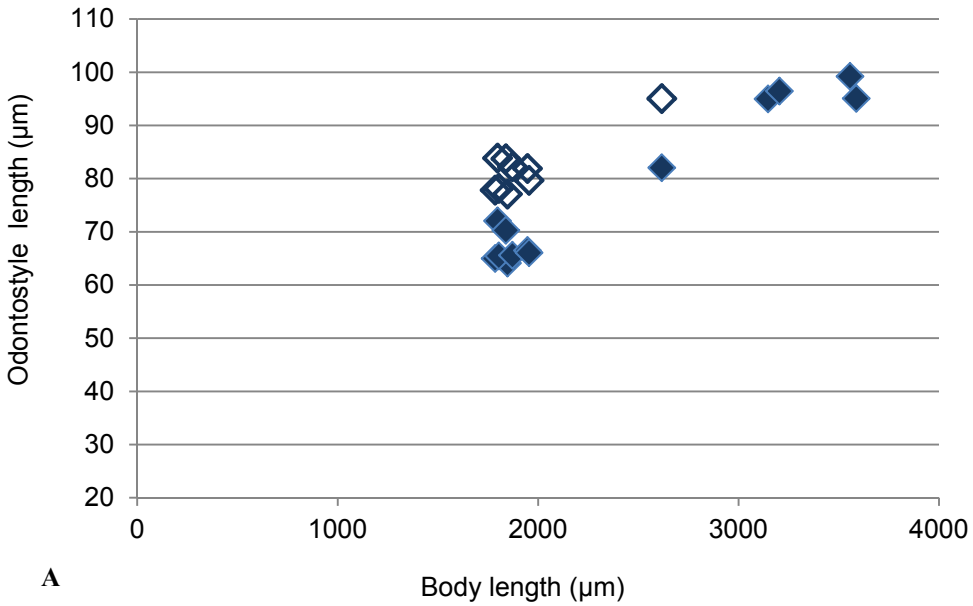
Soil around cherry trees from Voineşti locality (Dâmboviţa County).

Remarks

Morphometrics and morphology of this species have been in the focus of a great number of nematological studies during the years and it has been shown that *X. diversicaudatum* expressed a high morphological variability caused by its wide range and diversity of hosts and conditions it occurred (Brown & Topham, 1985; Roca & Bravo, 1997; Barsi & Lamberti, 2000a). The morphometrics of the population from Voineşti in general agrees with those reported earlier from Romania (Romaşcu, 1981), however specimens from Voineşti differ in the smaller body size, and in the lower values of **a** and **c** indices. The specimens in the present study are characterised by its rather small size (2.8, 3.6 mm). The odontostyle is shorter compared to the population of *X. diversicaudatum* from Britain (Goodey *et al*., 1960) and some Italian populations (Roca *et al*., 1988, Roca & Lamberti, 1993) and is more similar to the most populations reported from other European countries, South Africa and USA (Thorne, 1939; Sturhan, 1963; Heyns & Coomnas, 1984; Lamberti *et al*., 1999; Barsi & Lamberti, 2000b; Kumari, 2006). The spicules are in the ranges reported for this species except for the specimens from Bulgaria (Peneva & Choleva, 1992) characterised with the shortest spicules recorded so far. Our observations on the juvenile stages confirmed the findings of Barsi and Lamberti (2000a) concerning the tail shape of the second stage juvenile – dorsally convex conoid (following the

nomenclature by Coomans *et al*., 2001) in British population and elongate conoid in our population and previously reported data on juvenile stages from Serbia and Czech Republic (Barsi & Lamberti, 2000b, Kumari, 2006). It is worth mentioning that the second stage juvenile has longer tail compared to other stages and adults in above mentioned populations, not so in those from Britain (Goodey *et al*., 1960), which also is specific in its great body and odontostyle size.

Xiphinema diversicaudatum occurs both in cultivated soils and natural habitats and has been reported from different crops (Pitcher *et al*., 1974), in particular, vineyards (Lišková, 1997; Barsi & Lamberti, 2000b), orchards (Lišková, 1995), rhizosphere of walnut trees (Lišková & Brown, 1998) and forests (Lišková & Brown, 1999; Lišková & Sturhan, 2000), forest nurseries (Peneva & Choleva, 1992), grassland of fluvial plains and river banks (Lišková, 2001) and from wild growing grapevine (Tiefenbrunner & Tiefenbrunner, 2004). *Xiphinema diversicaudatum* is widespread in Europe, and has also been recorded from other temperate regions of the world, New Zealand, Australia, etc., most probably as a result of introduction (Pitcher *et al*., 1974, Brown & Topham, 1985; Sturhan *et al*., 1997; Taylor & Brown, 1997; Coomans *et al*., 2001, *CAB International* Distribution Maps of Plant Diseases, 2001).



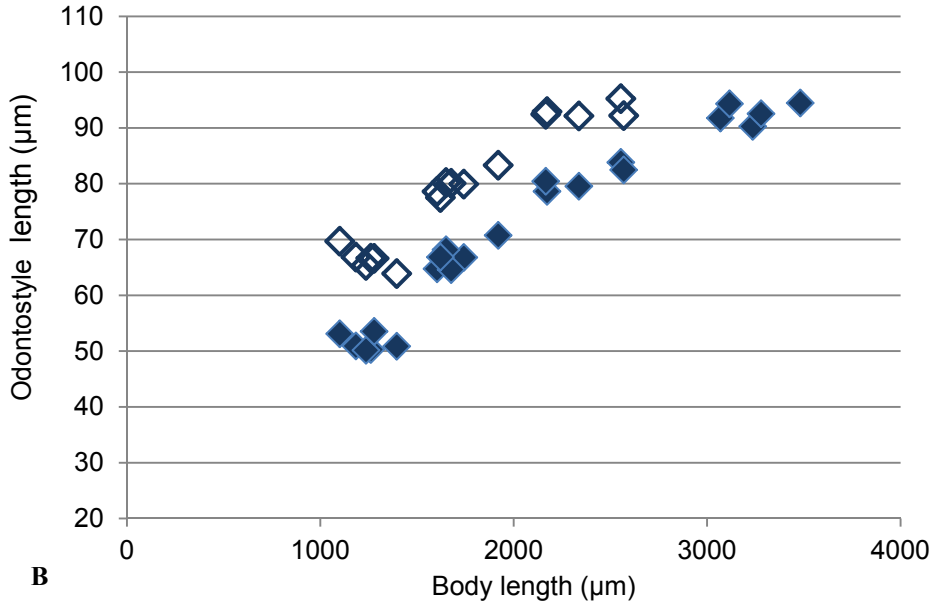


Fig. 5. *Xiphinema italiae.* Scatter plot of the functional (◆) and replacement (◇) odontostyle in relation to body length of the juvenile stages and adults. A, *Adamclisi* B, *Bîrlad*.

Xiphinema index Thorne & Allen 1950 (Figs 2B and 3)

Measurements

See Table. 2.

Description

Females. Body assuming an open C-shape when heat relaxed; lip region continuous with the neck contour or set off by a slight depression; amphidial apertures almost as broad as head; Cuticle at postlabial region 3 µm; at mid-body 3 µm, on tail posterior to anus 4.5 µm; uterus bipartite, pharyngeal bulb 105 – 116.5 µm long. Uteri bipartite without Z differentiation, prerectum 342 – 466 µm long; rectum 36.5 – 38 µm long, tail dorsally convex conoid with a ventral peg (9 – 10 µm) slightly variable in shape, caudal cuticle radially striated, 1 – 3 caudal pores present.

Male. Very rare. Cuticle at postlabial region 3 µm; at mid-body 4 µm on tail posterior to anus 5 µm, spicules strong, 69 µm long, arcuate; ventromedian supplements 1 + 3; ventral peg 11 µm long.

Table 3. Measurements of females and juvenile stages of *Xiphinema italiae* from Adamclisi and Bîrlad (mean ± standard deviation, with range). All measurements in micrometers except L in mm.

	Adamclisi			**Bîrlad**			
Character	**Females**	**J3**	**J4**	**Females**	**J2**	**J3**	**J4**
n	n = 4	n = 7	n = 1	n = 5	n = 5	n = 6	n = 5
L	3.37 ± 0.23 3.15 – 3.59	1.86 ± 0.65 1.79 – 1.96	2.62	3.24 ± 0.16 3.07 – 3.48	1.27 ± 0.78 1.18 – 1.40	1.70 ± 0.12 1.60 – 1.92	2.36 ± 0.20 2.17 – 2.57
a	102.8 ± 10.0 88.3 – 109.9	78.2 ± 6.8 69.6 – 89.0	95.2	95.9 ± 7.3 88.4 – 104.5	65.1 ± 2.2 62.8 – 68.4	79.1 ± 7.2 70.7 – 88.8	88.3 ± 5.3 83.3 – 97,1
b	8.3 ± 1.3 6.9 – 9.3	6.0 ± 0.3 5.6 – 6.4	7.3	9.1 ± 0.6 8.5 – 9.9	5.3 ± 0.6 4.7 – 6.3	5.6 ± 0.5 4.6 – 6.0	7.0 ± 0.5 6.5 – 7.5
c	35.8 ± 1.6 33.9 – 37.8	23.2 ± 1.9 20.6 – 26.2	28.0	34.8 ± 2.7 32.2 – 38.9	19.1 ± 1.2 17.5 – 20.6	20.9 ± 1.7 18.6 – 23.1	26.6 ± 2.1 24.3 – 29.2
c'	5.9 ± 1.3 4.4 – 7.4	5.0 ± 0.6 4.3 – 5.7	4.8	4.4 ± 0.5 3.8 – 5.0	5.1 ± 0.4 4.6 – 5.7	5.4 ± 0.5 4.6 – 6.0	4.9 ± 0.3 4.5 – 5.2
V (%)	46.8 ± 0.6 46.1 – 47.3			46.2 ± 1.7 44.7 – 48.1			
G1 (%)	8.5 ± 1.0 7.8 – 10.0			8.1 ± 0.3 7.7 – 8.5			
G2 (%)	9.1 ± 2.2 7.4 – 12.0			7.3 ± 0.8 6.4 – 8.3			
d	8.1 ± 0.3 7.8 – 8.6	6.8 ± 0.4 6.3 – 7.2	7.3	7.1 ± 0.2 6.8 – 7.2	5.4 ± 0.5 4.8 – 5.9	6.7 ± 0.4 6.3 – 7.5	7.1 ± 0.2 6.9 – 7.3
d'	2.4 ± 0.1 2.2 – 2.5	2.1 ± 0.1 2.1 – 2.2	2.2	2.2 ± 0.1 2.1 – 2.3	1.9 ± 0.1 1.9 – 2.0	2.1 ± 0.1 2.0 – 2.3	2.2 ± 0.0 2.1 – 2.2
Anterior end to guide ring	81.7 ± 4.0 78 – 87.5	58.1 ± 3.3 52 – 63	69	75.4 ± 2.6 73 – 78	41.9 ± 3.7 37 – 45	56.3 ± 4.1 51 – 64	65.6 ± 2.2 63 – 69
Odontostyle	96.4 ± 2.0 95 – 99	66.9 ± 2.8 64 – 72	82	92.7 ± 1.8 90 – 94.5	51.1 ± 1.4 50 – 53.5	67.0 ± 2.3 65 – 71	81.0 ± 2.1 79 – 84
Replacement odontostyle		80.6 ± 2.7 77 – 84	95		65.9 ± 1.3 64-67	79.9 ± 2.0 78-83	93.0 ± 1.3 92-95
Odontophore	61.4 ± 0.8 60 – 62	47.7 ± 1.8 45 – 50	55	60.1 ± 1.8 58 – 62	41.5 ± 0.6 40.5 – 42	47.5 ± 2.0 45 – 50	52.6 ± 1.3 51 – 55
Pharynx length	400.5 ± 48.5 363 – 455	310.5 ± 12.2 293.5 – 328	360	357.9 ± 9.9 344 – 366	241.6 ± 30.2 189 – 267	311.5 ± 34.1 274 – 362	337.8 ± 7.2 332 – 348
Tail	94.3 ± 7.8 88 – 105	80.6 ± 6.6 71 – 89	94	93.6 ± 9.3 84 – 108	66.8 ± 3.3 63 – 70.5	82.7 ± 5.4 75 – 89	88.6 ± 2.6 85 – 92
Hyaline part of tail	10.0 ± 1.3 9 – 11	7.2 ± 0.7 6 – 8	8	9.7 ± 0.8 8.5 – 11	5.6 ± 0.9 4 – 7	7.1 ± 1.2 6 – 9	7.6 ± 1,1 6.5 – 9
Body diameter at:	10.0 ± 0.6	8.6 ± 0.3	9	10.7 ± 0.3	7.9 ± 0.4	8.4 ± 0.2	9.2 ± 0.1
– lip region	9.5 – 11	8 – 9		10 – 11	7 – 8	8 – 9	9 – 9
– guide ring	24.2 ± 0.3 24 – 24.5	18.3 ± 0.6 17 – 19	21	23.4 ± 0.9 22 – 25	15.3 ± 0.5 14.5 – 16	17.8 ± 1.0 16 – 19	20.0 ± 0.6 19 – 20
– base of pharynx	29.2 ± 2.4 26 – 32	23.5 ± 2.4 20 – 27	27	30.5 ± 2.6 29 – 35	19.1 ± 0.6 18 – 20	22.0 ± 1.5 20 – 24	25.6 ± 1.4 24 – 28
– mid-body/at vulva	32.9 ± 2.0 31 – 36	24.0 ± 2.9 20 – 28	27.5	33.8 ± 1.7 31.5 – 36	19.5 ± 1.7 18 – 22	21.9 ± 2.1 19 – 24	26.8 ± 2.3 24 – 29.5
– anus	20.8 ± 0.8 20 – 22	16.1 ± 1.9 14 – 19	20	21.5 ± 0.8 21 – 22.5	13.2 ± 1.0 12 – 15	15.3 ± 0.8 14 – 16	18.1 ± 1.3 16 – 20
– hyaline part	6.7 ± 0.6 6 – 7.5	4.3 ± 0.4 4 – 5	6	6.8 ± 0.8 6 – 8	4.1 ± 0.3 3.5 – 4	4.1 ± 0.3 4 – 5	4.4 ± 0,6 4 – 5

Juveniles: All four stages present (Fig. 2B). Body posture less ventrally curved, than in adults, smaller in size with tail showing specific shape at different stages (elongate conoid more or less straight in first and second stages juveniles, dorsally convex conoid with subdigitate terminus in third stage and dorsally convex conoid, digitate with ventral peg in fourth stage juveniles).

Locality and plant association

Vineyard soil from Valea Călugărească (Prahova County).

Remarks

Compared to the type population (Siddiqi, 1974) Romanian specimens are charcterised by a shorter body (av. 2.8 (2.7 – 2.9) mm *vs* av. 3.1 (2.91 – 3.28 mm) and more posteriorly situated vulva (av. V = 42.9 (41 – 45) *vs* av. V = 39. 4 (38 – 40). The species has been reported mainly from vineyards but also in association with fig tree, roses, mulberry, citruses and riparian vegetation (Siddiqi, 1974; Lamberti *et al*., 1983, Ivezic *et al*., 2002; Magunacelaya *et al*., 2004; Jawhar *et al*., 2006; Tzortzakakis *et al*.; 2006, Lazarova *et al*., 2010) and there are numerous studies on its association with grapevine viruses (Avgelis & Tzortzakakis, 2001). The world distribution of *X. index* is closely related to that of its most important host, grapevine: Europe, Israel, Middle East, North and South Africa, USA, Mexico, South America (Siddiqi, 1974; Taylor & Brown, 1997; *CAB International* Distribution Maps of Plant Diseases, 2000, map 819; Coomans *et al*., 2001).

Xiphinema italiae Meyl, 1953 (Figs 4 and 5)

Measurements

See Table. 3.

Description

Females. Body slender. Lip region gently rounded, set off by constriction. Cuticle at postlabial region 1 – 1.5 µm, at

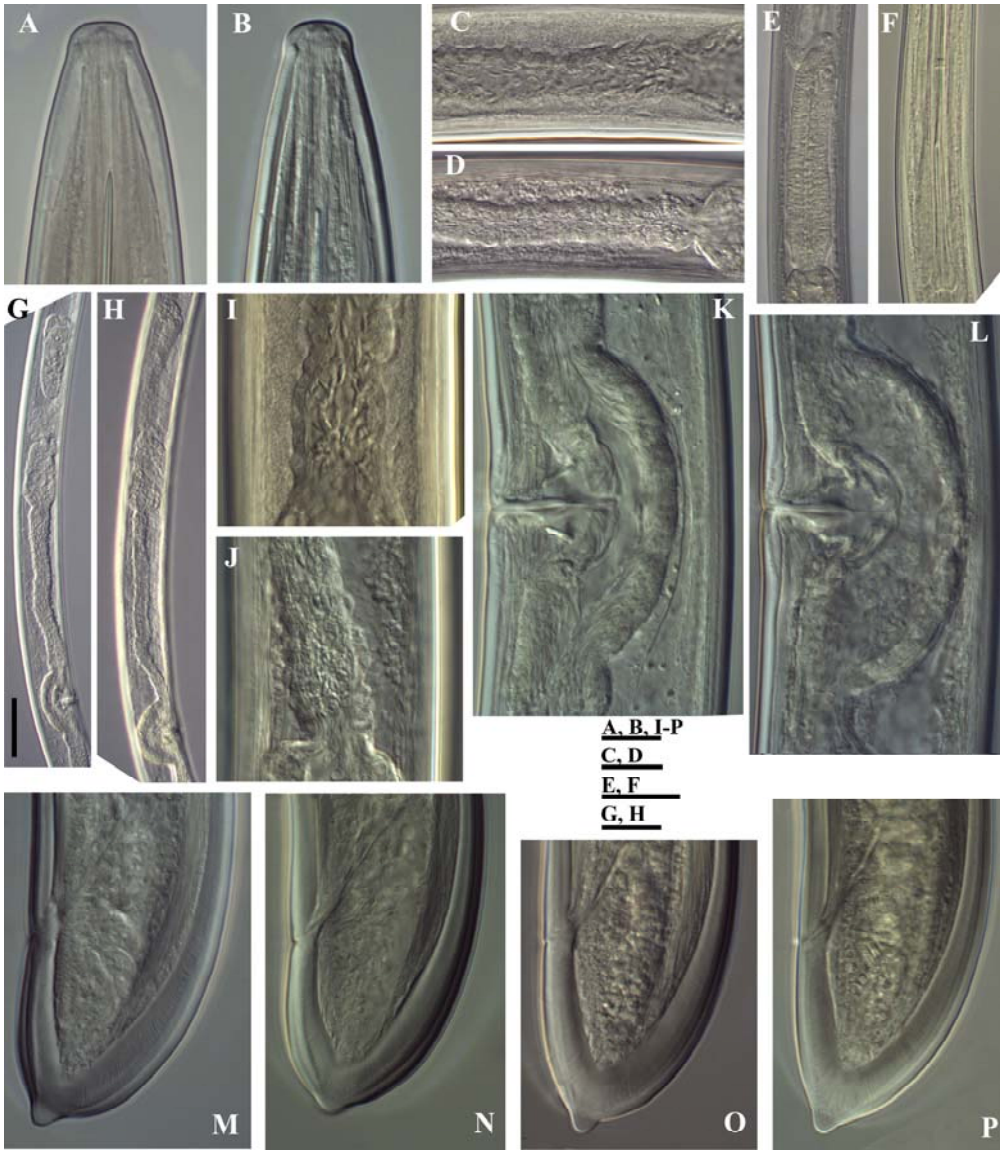


Fig. 6. *Xiphinema vuittenezi. Females*. A, B, Anterior end; C, D, tubular part of uterus with uterine spines; E, Pharyngeal bulb; F, Odontophore and part of odontostyle, G, H, Anterior genital branch; I, J, uterine spines; K, L, Vaginal region; M-P, Variations in tail shape. A, C, E, F, I, G, K, M, N – *Murfatlar*; B, D, H, J, L, O, P – *Ostrov.* Scale bars: A,B, I – P: 12µm; C, D: 20 µm; E, F: 40 µm; G, H: 60 µm.

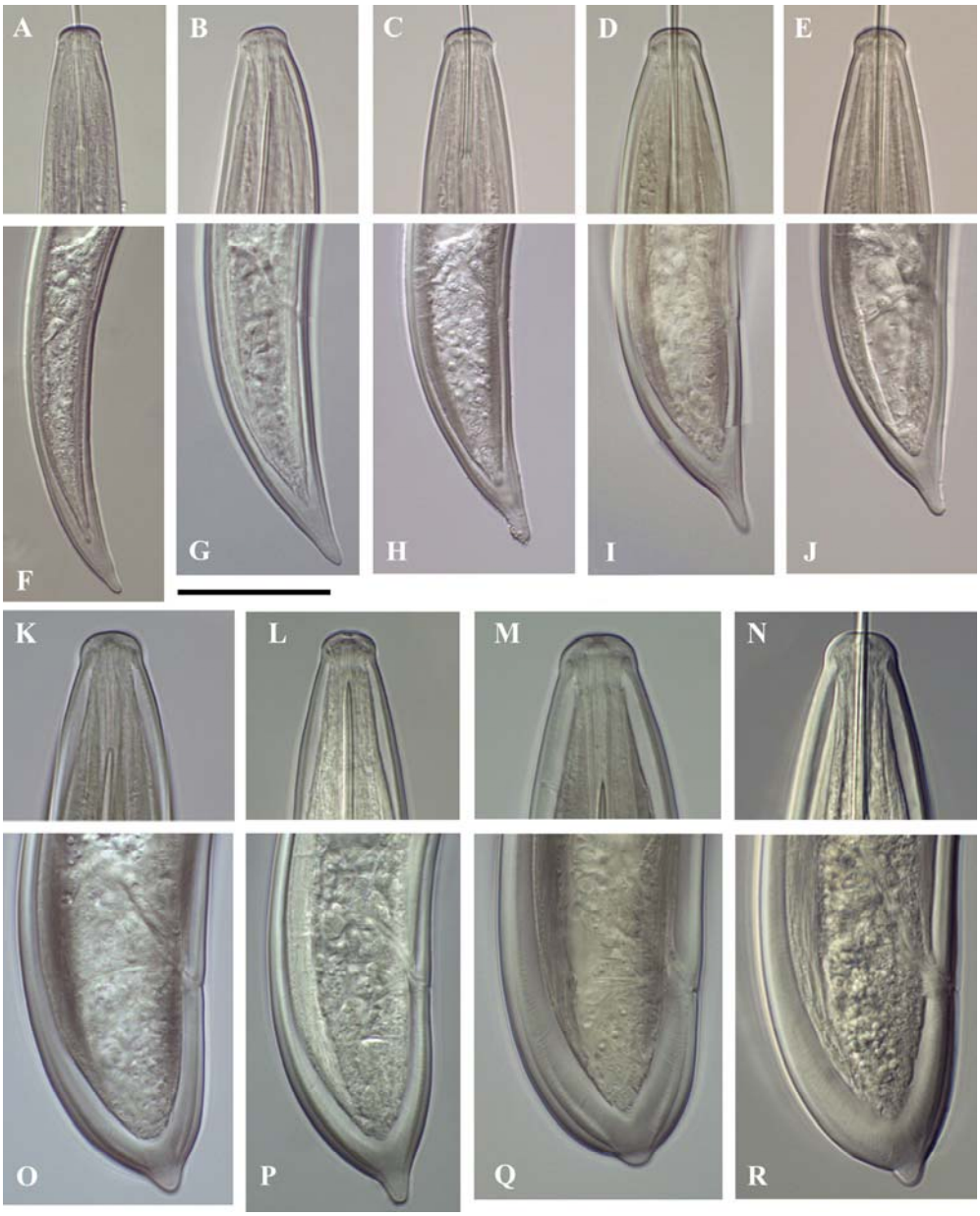


Fig. 7. *Xiphinema vuittenezi*. *Juvenles*. A – E, K – N, Head ends of first- to fourth-stages and females of both populations; F – J, O – R, Tails of first- to fourth- juvenile stages and females of both populations; A, B, D, F, G, I, K, M – *Murfatlar*; C, E, H, J, L, N – *Ostrov*. Scale-bar: 30 µm.

mid-body 1.5 – 2.5 µm, on tail posterior to anus 3 – 3.5µm. Genital system amphidelphic with equally developed gonads and reflexed ovaries, uteri bipartite, without Z-differentiation. Tail shape commonly elongate-conoid with slight dorsal or/and ventral constrictions towards the terminus, sometimes bluntly conoid, and even almost subdigitate.

Juveniles. Three juvenile stages were present for Bîrlad (first stage juvenile missing) and two for Adamclisi populations (the first two stages not found).

Locality and habitat

Vineyards in two region of the country: south-east (Adamclisi – Constanţa County) and east (around Bîrlad locality – Vaslui County).

Remarks

Xiphinema italiae is known as a Mediterranean dagger nematode and its range include Mediterranean and some Southeastern European countries, also Nigeria, South Africa, Seychelles, Cuba, Turkey, primarily associated with grapevine but also found in the rhizosphere of different fruit trees, olive, conifers, natural vegetation etc (Cohn, 1977; Coomans *et al*., 2001; CAB International, 2001).

Xiphinema vuittenezi Luc, Lima, Weischer & Flegg, 1964 (Figs 6 – 8)

Measurements

See Table 4 and 5.

Description

Females. Body arcuate, especially in posterior part, cuticle with distinct outer layer, lip region set off by a slight depression from the rest of the body, amphidial aperture almost as broad as lip region; genital tract with two well developed branches, uterus tripartite, Z differentiation in

the form of small globular bodies and spindle-like uterine spines, sometimes quadric in shape refractive structures observed at the proximal par of tubular region of uterus; tail rounded, ending in a short terminal peg 3 – 4 µm long, sometimes the peg has a reduced length.

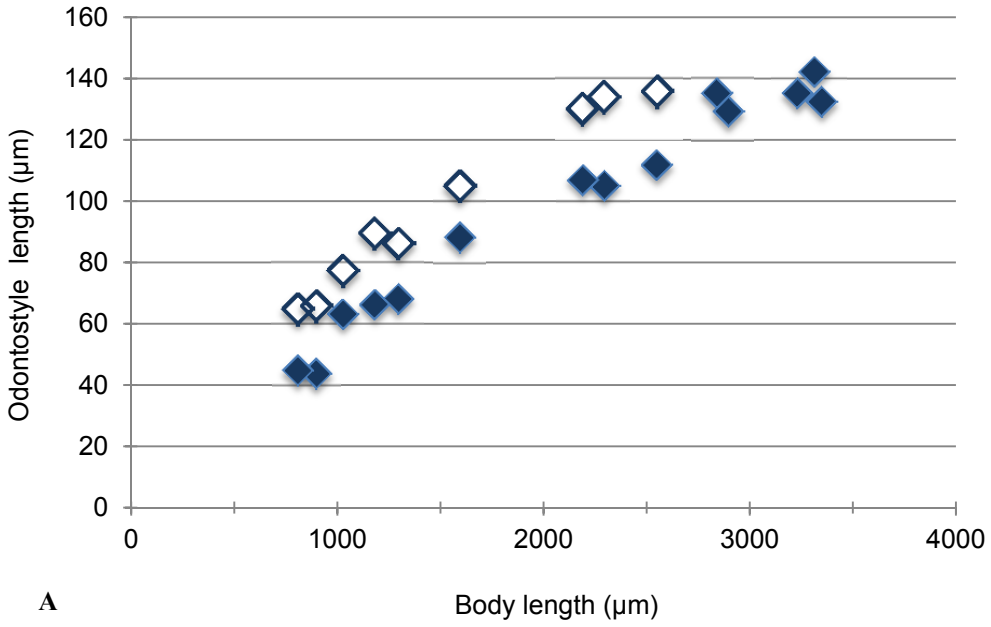
Juveniles Four stages present (Fig. 8). Similar to the female, body posture less ventrally curved, than in adults, smaller in size with tail showing specific shape at different stages (elongate-conoid in first stage juveniles and dorsaly convex conoid with digitated terminus in second, dorsaly convex conoid with central peg in third and fourth stages, the latter being most similar to female tail shape).

Locality and habitat

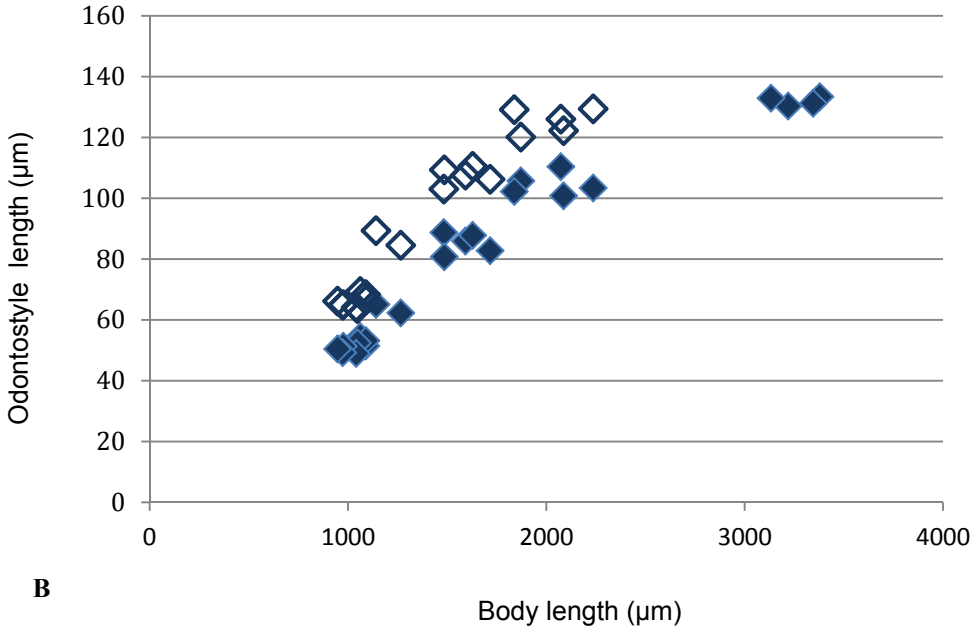
Murfatlar and Ostrov (Constanța County), rhizosphere of grapevines.

Remarks

Populations of *X. vuittenezi* studied exhibited interpopulation variability concerning some morphological characters,



A



B

Fig. 8. *Xiphinema vuttenezi.* Scatter plot of the functional (◆) and replacement (◇) odontostyle in relation to body length of the juvenile stages and adults. A, *Murfatlar* B, *Ostrov*

females from Ostrov have longer tail, lower **c** values, body width at beginning of hyaline part of tail smaller and more posteriorly situated vulva, compared with Murfatlar specimens. Further, the uterine spines are bigger and in a higher numbers in Murfatlar females, similar to those reported by Barsi and Lamberti (2000b), small globular bodies at the junction of pars dilalata and tubular part of uterus almost missing. In general *X. vuittenezi* females from two Romanian vineyards populations studied by Romaşcu and Zinca (1977) were similar to those reported here, only the recent specimens from Murfatlar had longer odontostyle and more antreiorly situated vulva; characters that are distinctive for this population; mean ranges for odontosyle of known *X. vuittenezi* populations from different regions and hosts varied between av. 120 and 132 µm with total range of 111 – 140 µm *vs* av. 135 (129.5 – 142) µm and vulva is situated around middle *vs* V = 46.4 (44.5 – 47) (Luc *et al*., 1964; Roca *et al*., 1988, 1989, 1991; Lamberti *et al*., 1997, 1999; Barsi & Lamberti, 2000b; Kumari, 2004). Only one population of *X. vuittenezi* originating from Italy (Coiro *et al*., 1989) has similar odontostyle length (135 µm (128 – 138) µm).

Table 4. Measurements of females and juvenile stages of *Xiphinema vuittenezi* from Murfatlar, (mean ± standard deviation, with range). All measurements in micrometers except L in mm.

Character	**Females**	**J1**	**J2**	**J3**	**J4**
n	n = 5	n = 2	n = 3	n = 1	n = 3
L	3.13 ± 0.24 2.84 – 3.35	896, 807	1290, 1176, 1024	1594	2290, 2547, 2187
a	61.1 ± 4.3 55.1 – 64.9	44.8, 40.8	41.3, 42.9, 42.5	46.3	54.9, 54.7, 58.3
b	7.0 ± 0.5 6.4 – 7.5	3.8, 3.5	4.5, 3.9, 3.7	4.9	5.4, 5.8, 5.2
c	98.6 ± 7.7 92.4 – 108.6	19.9, 16.5	27.3, 22.7, 22.9	36.2	56.2, 65.2, 53.4
c'	0.9 ± 0.1 0.8 – 1.0	3.8, 3.6	1.9, 2.5, 2.5	1.8	1.2, 1.1, 1.2
V (%)	46.3 ± 1.1 44.5 – 47.4				
G1 (%)	13.9 ± 1.2 13.0 – 15.6				
G2 (%)	13.6 ± 1.1 12.2 – 15.3				
d	9.8 ± 0.9 8.8 – 11.2	4.5	6.2, 6.3, 5.9	8.4	8.3, 8.1, 8.2
d'	2.9 ± 0.2 2.8 – 3.3	2.0	2.4, 2.4, 2.3	3.0	2.7, 2.8, 2.7
Anterior end to guide ring	128.3 ± 2.7 124 – 131	38, 37	59, 60, 54	75	102, 99, 99
Odontostyle	135.0 ± 5.4 129.5 – 142	44, 45	68, 66, 63	88.5	105, 112, 107
Replacement odontostyle		66, 65	86, 90, 77.5	105	134, 136, 130
Odontophore	80.6 ± 2.2 78 – 83	37, 38	50, 51, 47	57	70, 71, 68
Pharynx length	449.7 ± 20.3 429.5 – 477	235, 230	287, 303, 273	325.5	424, 440.5, 418
Tail	32.5 ± 2.6 30 – 36	45, 49	47, 52, 45	44	41, 39, 41
Hyaline part of tail	11.6 ± 0.9 10 – 13	10.2, 8.9	15, 13, 13	14	11.5, 15, 14
Body diameter at: – lip region	13.2 ± 1.4 11 – 15	8.4, 8.3	9.5, 10, 9	9	12, 12, 12
– guide ring	38.7 ± 1.9 37 – 41	17, 16.8	23, 23, 21	26	33, 34, 32
– base of pharynx	43.7 ± 0.9 42 – 44	19.2, 19	30, 26, 23	31	40, 42, 37
– mid-body/at vulva	51.1 ± 0.7 50 – 52	20, 19.8	31, 27, 24	34.5	42, 46.5, 37.5
– anus	37.0 ± 3.4 31 – 39	11.9, 13.5	25, 21, 18	25	35, 36, 33.5
– hyaline part	26.4 ± 1.8 25 – 29.5	5.5, 5.2	10, 8, 7	10	20, 24, 19

Table 5. Measurements of females and juvenile stages of *Xiphinema vuittenezi* from Ostrov, (mean ± standard deviation, with range). All measurements in micrometers except L in mm.

Character	Females	J1	J2	J3	J4
n	n = 4	n = 8	n = 2	n = 5	n = 5
L	3.24 ± 0.14 3.11 – 3.38	1.03 ± 0.56 0.95 – 1.09	1.27, 1.14	1.58 ± 0.99 1.48 – 1.72	2.02 ± 1.66 1.84 – 2.24
a	64.5 ± 1.0 63.5 – 65.5	46.1 ± 2.7 41.7 – 49.6	43.2, 43.8	48.6 ± 0.6 48.0 – 49.6	54.4 ± 3.5 51 – 59
b	6.8 ± 0.7 6.0 – 7.2	4.2 ± 0.2 3.8 – 4.5	4.5, 4.2	4.7 ± 0.4 4.2 – 5.2	5.2 ± 0.6 4.3 – 5.7
c	88.7 ± 2.5 85.2 – 91.0	17.9 ± 1.2 16.1 – 20.0	28.8, 26.8	35.6 ± 3.0 31.9 – 38.9	49.3 ± 5.8 44.5 – 56.1
c'	1.0 ± 0.0 1.0 – 1.0	3.8 ± 0.4 3.2 – 4.4	2.2, 2.2	1.8 ± 0.1 1.6 – 1.9	1.4 ± 0.1 1.3 – 1.4
V (%)/	49.6 ± 1.0 48.5 – 50.8				
G1 (%)	14.2 ± 2.6 12.5 – 18.0				
G2 (%)	14.1 ± 2.1 12.3 – 16.9				
d	8.8 ± 0.9 8.0 – 9.6	5.1 ± 0.3 4.6 – 5.6	5.5, 6.0	6.7 ± 0.8 5.6 – 7.4	7.0 ± 0.8 6.2 – 8.1
d'	2.8 ± 0.1 2.7 – 2.9	2.1 ± 0.1 2.0 – 2.3	2.4, 2.2	2.6 ± 0.2 2.4 – 2.9	2.7 ± 0.1 2.5 – 2.8
Anterior end to guide ring	115.8 ± 12.0 102 – 128	39.8 ± 1.9 37 – 42	47, 54	66.4 ± 8.9 55 – 78	73.6 ± 10.2 61 – 87
Odontostyle	132.0 ± 1.4 130 – 133	51.4 ± 1.9 49 – 54.5	62, 65	85.2 ± 3.4 81 – 89	104.5 ± 3.8 101 – 110
Replacement odontostyle		66.0 ± 2.0 64-69	85, 89	107.3 ± 2.9 103 – 110.5	125.4 ± 4.1 120 – 129
Odontophore	77.1 ± 4.4 72 – 82.5	37.5 ± 2.2 34 – 42	48, 50	57.9 ± 0.7 57 – 59	66.9 ± 2.2 64 – 69
Pharynx length	480.7 ± 51.0 438 – 537	244.3 ± 9.5 231 – 261	279, 274	337.7 ± 32.2 313 – 391	391.9 ± 28.6 366.5 – 439
Tail	36.8 ± 1.0 35 – 38	57.8 ± 4.5 52 – 66	44, 43	44.6 ± 2.7 41 – 48	41.2 ± 3.2 37 – 46
Hyaline part of tail	12.9 ± 1.0 11.5 – 14	10.2 ± 1.3 9-12	13, 11	13.0 ± 1.1 12 – 14.5	13.3 ± 1.1 12 – 14
Body diameter at:	13.1 ± 0.4	7.8 ± 0.2	8, 9	9.9 ± 0.5	11.4 ± 0.4
– lip region	13 – 14	7.5 – 8		9 – 10.5	11 – 12
– guide ring	37.3 ± 0.7 36 – 38	16.4 ± 1.0 15 – 18	20, 20	25.4 ± 1.0 24 – 27	30.7 ± 1.6 29 – 33
– base of pharynx	44.2 ± 0.5 43.5 – 45	21.6 ± 2.1 18 – 24	26, 24	31.2 ± 1.2 30 – 33	36.3 ± 3.2 34 – 42
– mid-body/at vulva	50.7 ± 1.8 49 – 53	22.4 ± 2.2 19.5 – 25	29, 26	32.5 ± 2.0 31 – 35.5	36.8 ± 1.5 35 – 38
– anus	37.3 ± 0.5 37 – 38	15.5 ± 1.7 13 – 18	20, 19	24.7 ± 0.6 24 – 25	30.3 ± 2.5 28 – 34
– hyaline part	23.8 ± 1.1 23 – 25	6.0 ± 0.3 5.5 – 6	9, 11	12.0 ± 0.4 11 – 12	17.4 ± 1.7 15 – 19

The present study of the Romanian populations is in agreement with the previous data for juvenile developmental stages as originally described by Luc *et al*. (1964) and confirmed by other authors (Barsi & Lamberti, 2000b; Kumari, 2004). *Xiphinema vuittenezi* is recorded in association with grapevine, stonefruits, pomerfruits; the species is widely distributed in Europe, especially Central Europe; reported also from Iran; its occurrence in USA and Australia is probably a result of introduction (Brown & Taylor, 1987; Coomans *et al.* 2001; Walker, 2004).

Diagnostics by multipex PCR

Overall, 24 multiplex PCR tests were performed for all studied populations (3 for *X. diversicaudatum*, 5 – *X. index*, 3 – *X. vuittenezi* from Murfatlar, 7 – *X. vuittenezi* from Ostrov, 2 – *X. italiae* from Adamclisi and 4 – *X. italiae* from Bîrlad). All tested populations were positive and the size of the fragments was constant within each *Xiphinema* species. Herein, a picture of one of the agarose gels with amplified PCR products from all four species is presented (Fig. 9). Single fragments of approximately 590, 340, 800 and 410 bp were amplified for *X. vuittenezi* (lanes 2–3,

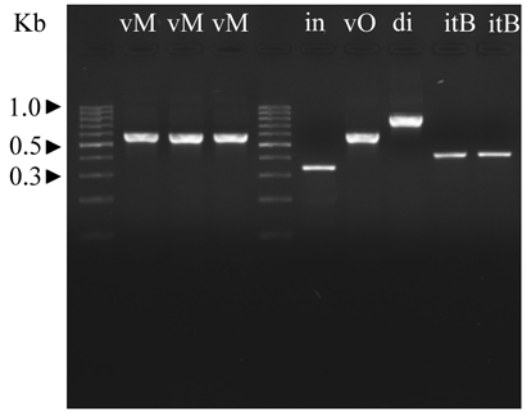


Fig. 9. Electrophoresis of the amplified DNA products in a multiplex test using sense and antisense species-specific primers designed by Wang et al. (2003). Lanes 1 and 5 – 1Kb DNA ladder; *X. vuittenezi*: lanes 2 – 4, *Murfatlar* (vM) and lane 7, Ostrov (vO); *X. index*: lane 5 (in); *X. diversicaudatum*: lane 8 (di); *X. italiae*: lanes 8 and 9, *Bîrlad* (itB).

Murfatlar and 7, Ostrov), *X. index* (lane 5), *X. diversicaudatum* (lane 8) and *X. italiae* (lane 8 and 9, Bîrlad), respectively, that are in accordance to the study by Wang *et al*. (2003). The PCR products for both studied populations of *X. vuittenez*i and *X. italiae* were of the same size (data not shown for Adamclisi and Bîrlad populations of *X. italiae*).

Sequencing and PCR-RFLP analyses

Due to the morphometrical differences found between the two *X. vuittenezi* populations additional DNA analyses have been done only for this species. Amplification, RFLP and sequencing of two ribosomal gene domains (D2-D3 28S rDNA and ITS) and the levels of genetic variation were discussed. The PCR products of the D2-D3 and ITS (including the 3′ end of the 18S and the 5′ end of the 28S) regions for both populations of *X. vuittenezi* were 840 bp and 1810 bp, respectively.

Four different nucleotides (i.e. 0.5 % dissimilarity) in D2-D3 sequences between the two Romanian populations were detected and one to three (or less than 0.3% divergence) with the corresponding sequences of *X. vuittenezi* from Hungary (AY601614) and Czech Republic (EF614266) deposited in GenBank (He *et al*. 2005; Kumari *et al*., 2009). Similar low level of interpopulation genetic diversity (<1.5 %) was revealed for other *Xiphinema* non-*americanum* group species such as *X. index* (0 – 0.3 %), *X. diversicaudatum* (0 – 0.8 % and *X. italiae* (0.3 – 1.4 % GenBank accession numbers AY601613, FJ713153, HM921350, HM921351) (He *et al*., 2005; Kumari & Lišková, 2009; Gutiérrez-Gutiérrez *et al*., 2011; Kumari & Di Cesare, 2013). The ITS-RFLP analysis for both populations produced identical species-specific patterns that clearly identified *X. vuittenezi* from Romania (Fig. 10). Furthermore, these profiles were identical to those obtained for *X. vuittenezi* from Hungary (De Luca, pers. comm.) revealing that these profiles could be used for species diagnostic.

The ITS sequence divergence between both Romanian populations was of 11 nucleotides and two indels (or 0.6 % dissimilarity). No complete ITS sequence of *X. vuittenezi* was found in the GenBank. However, 6 and 7 different nucleotides (or up to 0.6 % divergence) between the Romanian ITS1 and the corresponding region of *X. vuittenezi* from Germany was detected, whereas 1 and 5 different bases (i.e. 0.2/0.8 %) in the ITS2 region compared with that from Czech Republic (Wang *et al*., 2003; Kumari *et al*., 2009). These analyses revealed again a comparatively low level of ITS intra-specific/interpopulation genetic variability that has been shown also for other studies on *Xiphinema* non-*americanum* group species (*X. diversicaudatum* GenBank accession numbers JQ780353 – JQ780358 and *X. index* (AY584243, AY430175 JF437918 (Finetti-Sialer & Ciancio, 2005; Meza *et al*., 2012, Kumari & Di Cesare, 2013). However, the pairwise comparison of the entire ITS region of *X. vuittenezi* from Romania with the corresponding region of *X. vuittenezi* from Hungary (De Luca, pers. comm.) showed 23 different nucleotides and 12 gaps suggesting a much higher intra-specific nucleotide variability among these populations of *X. vuittenezi*.

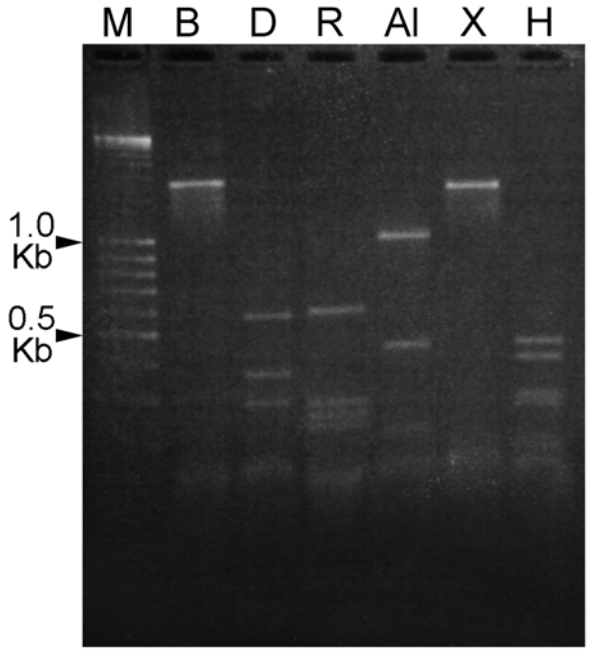


Fig. 10. Species-specific restriction ITS patterns of *Xiphinema vuttenezi* acquired with the following enzymes: B, *Bam*HI, D, *Dde*I, R, *Rsa*I, Al, *Alu*I, H, *Hin*fI, X, *Xba*I ; M, Molecular marker.

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