Phylogeny, diversity, and species delimitation in some species of the *Xiphinema americanum*-group complex (Nematoda: Longidoridae), as inferred from nuclear and mitochondrial DNA sequences and morphology

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Abstract During nematode surveys in southern Spain and Italy 14 populations of *Xiphinema* species tentatively identified as *Xiphinema americanum*-group were detected. Morphological and morphometrical studies identified three new species and six known *Xiphinema americanum*-group species, viz.: *Xiphinema parabrevicolle* n. sp., *Xiphinema parapachydermum* n. sp., *Xiphinema paratenuicutis* n. sp., *Xiphinema duriense, Xiphinema incertum, Xiphinema opisthohysterum, Xiphinema pachtaicum, Xiphinema rivesi*, and *Xiphinema santos*. The *Xiphinema americanum*-group is the most difficult *Xiphinema* species group for diagnosis since the morphology is very conservative and morphometric characters often overlap. This group includes vectors of several important plant pathogenic viruses that cause significant damage to a wide range of agricultural crops. Molecular characterisation of these species using D2-D3 expansion regions of 28S rRNA, 18S rRNA, ITS1-rRNA and the protein-coding mitochondrial gene, cytochrome oxidase c subunit 1 was carried out and maximum likelihood and Bayesian inference analysis were used to reconstruct phylogenetic relationships among these species and with other *Xiphinema americanum*-group species.

Keywords Bayesian inference · Cryptic species · Cytochrome c oxidase subunit 1 (*COI*) · Dagger nematodes · Maximum likelihood · rDNA · New geographic record · Virus vector

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Introduction

Dagger nematodes of the genus *Xiphinema* comprise phytopathogenic species that damage a wide range of wild and cultivated plants through direct feeding on root cells and transmission of several plant pathogenic viruses (Taylor and Brown 1997). This transmission is governed by a marked specificity between plant viruses and their *Xiphinema* spp. vectors. In fact, only nine out of the approximately 260 known species of *Xiphinema* have been shown to transmit nepoviruses (genus *Nepovirus*, family Secoviridae, Subfamily Comovirinae) (Decraemer and Robbins 2007). The *Xiphinema americanum*-group, which comprises a complex of about 50 species, many of them with a cosmopolitan distribution, appears to be the most difficult *Xiphinema* species group for species diagnosis based on morphology and morphometrics (Coomans et al. 2001) since their morphology is quite conserved and morphometrics overlap. This biases quarantine regulations and protection methods especially since the *Xiphinema americanum*-group includes the vectors of several important plant viruses that cause significant damage to a wide range of crops, including *Tobacco ringspot virus* (*TRSV*), *Tomato ringspot virus* (*TomRSV*), *Cherry rasp leaf virus* (*CRLV*), and *Peach rosette mosaic virus* (*PRMV*) (Taylor and Brown 1997). For this reason, accurate identification of the *X. americanum*-group species is essential, since several species of this group are listed as A1 (*Xiphinema americanum* Cobb 1913, *Xiphinema californicum* Lamberti and Bleve-Zacheo 1979, *Xiphinema bricolensis* Ebsary et al. 1989) and A2 (*Xiphinema rivesi* Dalmasso 1969) quarantine organisms by the European and Mediterranean Plant Protection Organisation (EPPO).

Tarjan (1969) introduced the notation '*Xiphinema americanum*-group' to stress the complexity of the problem and suggested that populations identified as *Xiphinema americanum* represented a complex of several species. This group was morphologically defined by Loof and Luc (1990) and Lamberti et al. (2000) with the following common characters: spiral or C-shaped small body, lip region more or less rounded and continuous or separated from the rest of the body by an incisure which can ranged from a depression to a constriction, female reproductive system with two equally developed genital branches, usually with short uteri without uterine differentiation, all features grouped per system vulva positioned at 40–60 % of the body length from anterior end and short conical to broadly convex-conoid tail. Even so, controversies exist concerning the nature of the 'true' *X. americanum*-group i.e. only grouping those species which show the presence of symbiotic bacteria with males absent or rare. In fact, Luc et al. (1998) proposed that morphologically the X. *pachydermum*-group including five species (*X. brevisicum* Lamberti et al. 1994, *X. longistilum* Lamberti et al. 1994, *X. mesostilum* Lamberti et al. 1994, *X. microstilum* Lamberti et al. 1994, and *X. pachydermum* Sturhan 1983) comprise a group outside the *X. americanum*-group, since males are frequent, females do not show symbiontic bacteria associated with the ovaries (1 exception *X. mesostilum*), oviduct with normal structure clearly separated from the uterus by a well developed sphincter muscle and unipartite uteri relatively long. The action by Luc et al. (1998) was confirmed by the morphological phylogeny carried out by Coomans et al. (2001) in contradiction to Lamberti and Ciancio (1993) and Lamberti et al. (2000) who considered the *X. americanum* species group in a large sense including the X. *pachydermum*-group. Thus, species differentiation remains difficult and species diversity and taxonomic validity of species controversial (Lamberti et al. 2000; Gozel et al. 2006). Lamberti and Ciancio (1993) analyzed the species diversity of the *X. americanum*-group using a hierarchical cluster analysis of morphometrics

The application of molecular methods to studies of nematode population structure and systematics has revealed that some long-assumed single species are in fact cryptic species that are morphologically indistinguishable but may be phylogenetically distant to one another (Gutiérrez-Gutiérrez et al. 2010; Barsi and De Luca 2008; Wu et al. 2007; Oliveira et al. 2005, 2006; Ye et al. 2004). During the last decade sequences of nuclear ribosomal DNA (rDNA) have been used for molecular characterisation and reconstruction of phylogenetic relationships within the Longidoridae and more particular within *Xiphinema* (Gutiérrez-Gutiérrez et al. 2011a; Ye et al. 2004; He et al. 2005). The D2-D3 expansion segments of the 28S rDNA gene provided rather homogeneous results for *Xiphinema* species and appeared a useful tool for species differentiation (He et al. 2005). Wu et al. (2007) clearly

distinguished *Xiphinema hunaniense* Wang and Wu 1992 from *Xiphinema radicicola* Goodey 1936 by D2-D3 rDNA sequences, and Oliveira et al. (2005) could differentiate *Xiphinema brevicolle* Lordello and Costa 1961 from *Xiphinema diffusum* Lamberti and Bleve-Zacheo 1979 by the internal transcribed spacer 1 (ITS1) region of rDNA. These species are very similar, showing only minor morphological differences e.g. in lip and tail region shape, and were previously synonymised by Loof et al. (1996) and Luc et al. (1998), respectively. Similarly, other species have been separated from almost identical species by molecular methods (Meza et al. 2011; Oliveira et al. 2006). The protein-coding mitochondrial gene, cytochrome oxidase c subunit 1 (*COI*), has also been shown to be applicable for genetic bar-coding (ECBOL, http://www.ecbol.org) and phylogeny of plant-parasitic nematodes (Lazarova et al. 2006; Palomares-Rius et al. 2009). Nevertheless, few studies have been carried out using mitochondrial DNA for *Xiphinema* species diagnostic since it revealed low intraspecific diversity and large interspecific divergences (Gutiérrez-Gutiérrez et al. 2011b; Kumari et al. 2010; Lazarova et al. 2006).

Consequently, current availability of molecular techniques may help to provide tools for differentiating *Xiphinema americanum*-group species and can significantly improve and facilitate the routine identification of these nematodes. Polyphasic identification, based on an integrative strategy of combining molecular techniques with morphology and measurements for species diagnosis have proven a tool beyond doubt in nematode identification within this group (Gutiérrez-Gutiérrez et al. 2010, 2011a; Palomares-Rius et al. 2009). For this reason, we have conducted an extensive nematode survey in cultivated and natural environments in southern Spain and one incidental sample in Italy, with the following objectives: *i*) to characterise morphologically and morphometrically species belonging to the *Xiphinema americanum*-species group and to compare them with previous records; *ii*) to molecularly characterise the sampled *Xiphinema americanum*-group populations using the D2-D3 expansion segments of 28S rRNA, ITS1, partial 18S rRNA, and *COI* gene sequences; and *iii*) to study the phylogenetic relationships of the identified *Xiphinema americanum*-group with other previously sequenced species.

Material and methods

Nematode population sampling

Nematode surveys were conducted from 2010 to 2011 during the spring season in cultivated and natural environments in southern Spain and one incidental sample in Italy, including carob tree (*Ceratonia siliqua* L.), cherry tree (*Prunus avium* L.), citrus (*Citrus aurantium* L.), grapevine (*Vitis vinifera* L.), grasses, lentisc (*Pistacia lentiscus* L.), marram grass [*Ammophila arenaria* (L.) Link], olive (*Olea europaea* L.) and Stone pine (*Pinus pinea* L.) (Table 1). Samples were collected with a shovel from the upper 50 cm of soil of four to five plants arbitrarily chosen in each locality. Nematodes were extracted from 500 cm^3 of soil by centrifugal flotation (Coolen 1979) and a modification of Cobb's decanting and sieving (Flegg 1967) methods. In some cases, additional soil samples were collected afterwards from the same locality for completing the necessary specimens for morphological and/or molecular identification.

Nematode morphological identification

Specimens for light microscopy were killed by gentle heat, fixed in a solution of 4 % formaldehyde + 1 % propionic acid and processed to pure glycerine using Seinhorst's method (1966). Specimens were examined using a Zeiss III compound microscope with Nomarski differential interference contrast at powers up to 1,000× magnification. Measurements were done using a drawing tube attached to a light microscope and, unless otherwise indicated in text. All measurements were expressed in micrometres (μm). For line drawing of the new species, light micrographs were imported to CorelDraw software version X5 and redrawn. All other abbreviations used are as defined in Jairajpuri and Ahmad (1992). In addition, a comparative morphological and morphometrical study on type specimens of some species were conducted with specimens kindly provided by Dr. A. Troccoli, from the nematode collection at the Istituto per la Protezione delle Piante, Sede di Bari, Consiglio Nazionale delle Ricerche, (C.N.R.), Bari, Italy (viz. *X. rivesi* from Portugal) and Dr Z.A. Handoo, from the USDA Nematode Collection, Beltsville, MD, USA (viz. *Xiphinema intermedium* Lamberti and Bleve-Zacheo 1979 T-3449p, *Xiphinema*

Table 1 Taxa sampled for *Xiphinema americanum* group complex species and sequences used in this study

Species	Locality	Host	GenBank accessions			
			D2-D3	ITS1	partial 18S	COI
Xiphinema parabrevicolle n. sp.	Pantanaggiani (Brindisi, Italy)	Lentisc and marram grass	JQ990042	JQ990043	JQ990050	JQ990059
Xiphinema parapachydermum n. sp.	Bollullos par del Condado (Huelva, Spain)	Grapevine	JQ990036	JQ990045	JQ990051	JQ990056
Xiphinema parapachydermum n. sp.	Hinojos (Huelva, Spain)	Olive	JQ990035	–	–	–
Xiphinema parapachydermum n. sp.	Chucena (Huelva, Spain)	Grapevine	JQ990034	–	–	–
Xiphinema paratenuicutis n. sp.	Andújar (Jaén, Spain)	Grasses	JQ990041	–	–	–
Xiphinema duriense	Almonte (Huelva, Spain)	Grapevine	JQ990032	–	–	JQ990053
Xiphinema incertum	Jerez de la Frontera (Cádiz, Spain)	Carob tree	JQ990031	JQ990044	–	JQ990058
Xiphinema opisthohysterum	Andújar (Jaén, Spain)	Grasses	JQ990040	–	–	JQ990054
Xiphinema pachtaicum	Jerez de la Frontera (Cádiz, Spain)	Grapevine	JQ990033	–	–	JQ990057
Xiphinema rivesi	Alcalá de Guadaira, (Sevilla province)	Grapevine	JQ990039	JQ990048	–	JQ990060
Xiphinema rivesi	Castillo de Locubín (Jaén, Spain)	Cherry tree	JQ990037	–	–	–
Xiphinema rivesi	Niebla (Huelva, Spain)	Citrus	JQ990038	JQ990049	–	–
Xiphinema santos	Rociana del Condado (Huelva, Spain)	Grapevine	JQ990030	JQ990047	JQ990052	JQ990055
Xiphinema santos	Rociana del Condado (Huelva, Spain)	Stone pine	JQ990029	JQ990046	–	–

(–) Not obtained

rivesi from France T-3440p, *Xiphinema tenuicutis* Lamberti and Bleve-Zacheo 1979 slides T-3447p, T-3448p, and T-3449p). Within the framework of the Synthesis project BE-TAF 1769, a collaboration between FERA and RBINS) type material of the *Xiphinema pachydermum* group was studied amongst them *Xiphinema santos* Lamberti et al. 1993 and *Xiphinema duriense* Lamberti et al. 1993 from nematode collection of Rothamsted Experimental Station, Harpenden, England.

Nematode molecular identification

For molecular analyses, two live nematodes from each sample were temporary mounted in a drop of 1 M NaCl containing glass beads and after taking measurements and photomicrographs of diagnostic characters the slides were dismantled and DNA extracted. Nematode DNA was extracted from single individuals and PCR assays were conducted as described by Castillo et al. (2003). The D2-D3 expansion segments of 28S rDNA was amplified using the D2A (5′-ACAAGTACCG TGAGGGAAAGTTG-3′) and D3B (5′-TCGGAA GGAACCAGCTACTA-3′) primers (Castillo et al. 2003; He et al. 2005; Palomares-Rius et al. 2008). The ITS1 region was amplified using forward primer 18S (5′TTGATTACGTCCCTGCCCTTT-3′) and reverse primer rDNA1 (5′-ACGAGCCGAGTGATC CACCG-3′) as described in Wang et al. (2003). Finally, the 18S rDNA gene was amplified using the SSU_F_07 (5′-AAAGATTAAGCCATGCATG-3′) and SSU_R_81 (5′- TGATCCWKCYGCAGGTTCAC-3′) primers (http://www.nematodes.org/barcoding/sourhope/nemo primers.html). The portion of the *COI* gene was amplified as described by Lazarova et al. (2006) using primers COIF (5′-GATTTTTTGGKCATCCWGARG-3′) and COIR (5′-CWACATAATAAGTATCATG-3′).

PCR products were purified after amplification using ExoSAP-IT (Affmetrix, USB products), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for direct sequencing in both directions using

the primers referred above. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyser; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA), at the SCAI, University of Córdoba sequencing facilities (Córdoba, Spain). The newly obtained sequences were submitted to the GenBank database under accession numbers indicated on the phylogenetic trees and Table 1.

Phylogenetic analyses

D2-D3 expansion segments of 28S rDNA and portion of the *COI* sequences of different *Xiphinema americanum*-group species from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were chosen according to previous published data (He et al. 2005; Lazarova et al. 2006). The newly obtained and published sequences for each gene were aligned using ClustalW (Thompson et al. 1994) with default parameters. Sequence alignments were manually edited using BioEdit (Hall 1999). Phylogenetic analyses of the sequence data sets were performed with maximum likelihood (ML) using PAUP * 4b10 (Swofford 2003) and Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). The best fitted model of DNA evolution was obtained using jModelTest v. 0.1.1 (Posada 2008) with the Akaike Information Criterion (AIC). The Akaike-supported model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then used in phylogenetic analyses. BI analysis under GTR + G model for D2-D3 expansion segment of 28S rDNA and TMP3uf + G for ITS1 region and TVM + I + G for *COI*, were run with four chains for 5.0×10^6, 2×10^6, and 1×10^6 generations, respectively. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50 % majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Trees were visualised using TreeView (Page 1996). In ML analysis the estimation of the support for each node was obtained by bootstrap analysis with 200 fast-step replicates.

Results

Morphology and morphometrics of species of the *Xiphinema americanum*-group

The morphological and morphometrical traits as well as molecular delineation of a Spanish population of *Xiphinema pachtaicum* (Tulaganov 1938) Kirjanova 1951 from Jerez de la Frontera (Cádiz province, southern Spain) infesting vineyards agreed completely with previous studies and was not repeated here (Gutiérrez-Gutiérrez et al., 2011a, b). This species is one of the most widespread and frequently occurring *Xiphinema* species in a wide variety of crops and natural habitats in the Iberian Peninsula and other Mediterranean basin countries (Taylor and Brown 1997; Téliz et al. 2007; Gutiérrez-Gutiérrez et al. 2011a, b).

Xiphinema parabrevicolle[1] n. sp. (Figs. 1, 2, and 3, Tables 2 and 3)

Female Body medium-sized, robust, forming a close C-shaped to open spiral when killed by heat. Cuticle appearing smooth, but with fine transverse striae on inner cuticle layer, 2.6±0.3 (2.0–3.0) μm thick along body but thicker at tail tip (Table 2). Lip region widely rounded, separated from the rest of the body by depression, 4.8±0.6 (4.0–5.5) μm high. Amphidial fovea large, stirrup-shaped, with wide aperture 12.3±0.3 (12.0–12.5) μm, as a straight transverse slit. Odontophore with basal flanges well developed basal flanges well developed 11.6±0.9 (10.0–12.5) μm wide. Pharyngeal basal bulb 86.2±9.1 (75–102) μm long and 21.7±0.1 (18.5–24.0) μm wide, occupying about 1/4 to 1/5 of the total pharyngeal length (Fig. 3). Glandularium 62.3±5.7 (51–65) μm long. Dorsal pharyngeal gland nucleus in anterior part of bulb, DN 28.3±2.0 (27.0–32.0)% (Fig. 2). Pharyngeal-intestinal valve inconspicuous, hemispherical, 7.9±0.7 (7.0–9.0) μm long. Reproductive system amphidelphic, both branches apparently equally developed, opposed and ovaries reflexed, uteri rather short (43–64 μm) without any differentiation. Ovaries with bacterial symbionts, oviducts relatively long, sphincter little developed. Vulva slightly post-equatorial, a transverse slit-like; vagina 8.0±0.7 (7.0–9.0) μm long with short distal

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[1] The species epithet refers to Gr. prep. para, alongside of, resembling; N.L. masc. n. *brevicolle*, because of its close resemblance to *Xiphinema brevicolle*

Fig. 1 Line drawings of *Xiphinema parabrevicolle* n. sp. (**a–d**), *Xiphinema parapachydermum* n. sp. (**e–h**), and *Xiphinema paratenuicutis* n. sp. (**i–l**). **a, e, i** Female lip regions. **b, f, j** Vulval region. **c, d, g, k** Female tail regions. **h, l** Male tail regions
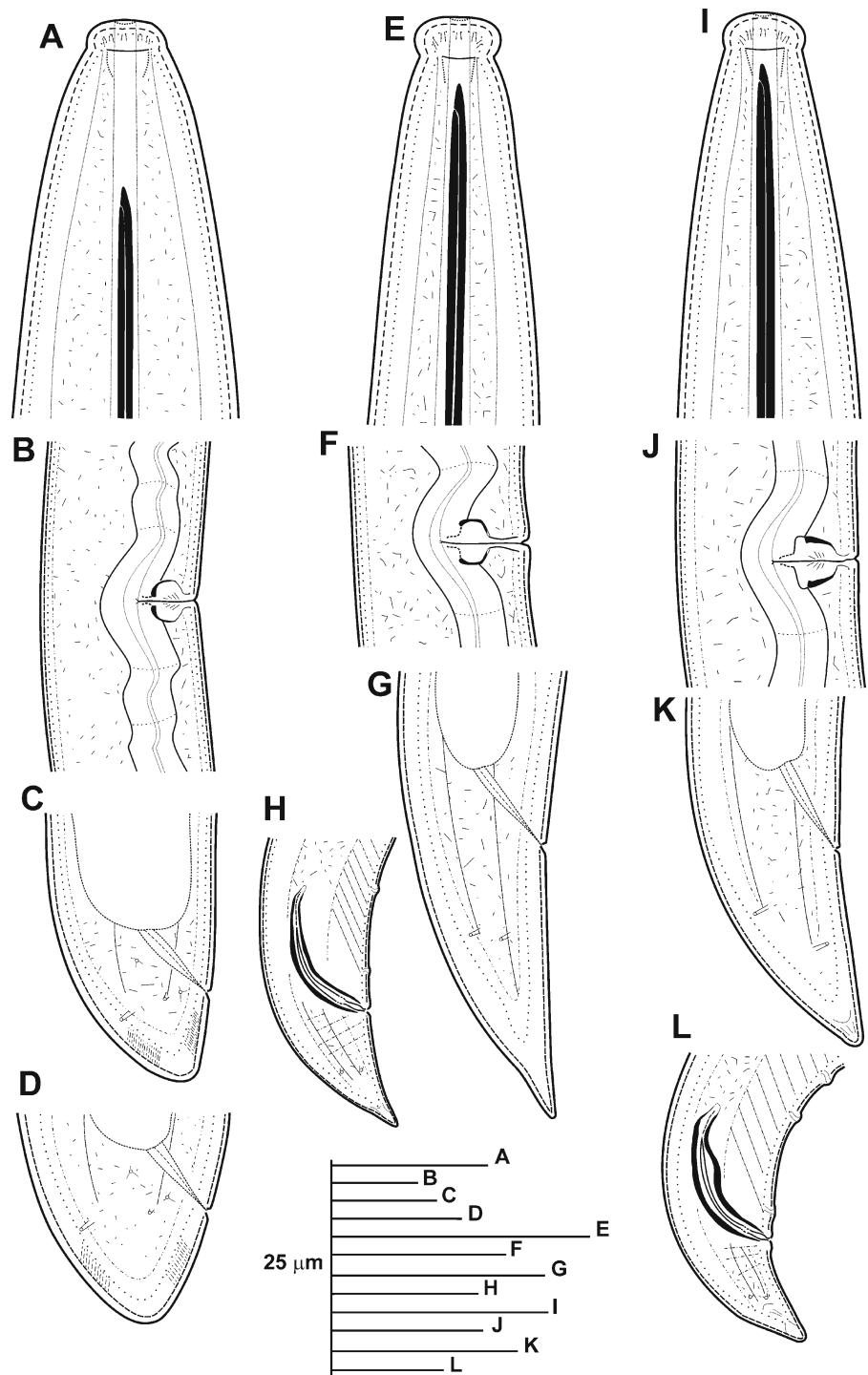

part and well-developed proximal. Prerectum often indistinct. Rectum 23.5±2.1 (20.0–27.0) μm long, or 0.4–0.5 times the anal body diameter. Tail short, dorsally convex-conoid, slightly concave ventrally with conoid-rounded terminus, bearing three pairs of caudal pores (Figs. 1c–d, 3k–n).

Male Not found.

Fig. 2 Line drawings of pharyngeal bulb and anterior genital branch of *Xiphinema parabrevicolle* n. sp. (**a**, **b**), *Xiphinema parapachydermum* n. sp. (**c**, **d**), and *Xiphinema paratenuicutis* n. sp. (**e**, **f**). (Scale bars: A, C, E=25 μm; B, D, F=50 μm)
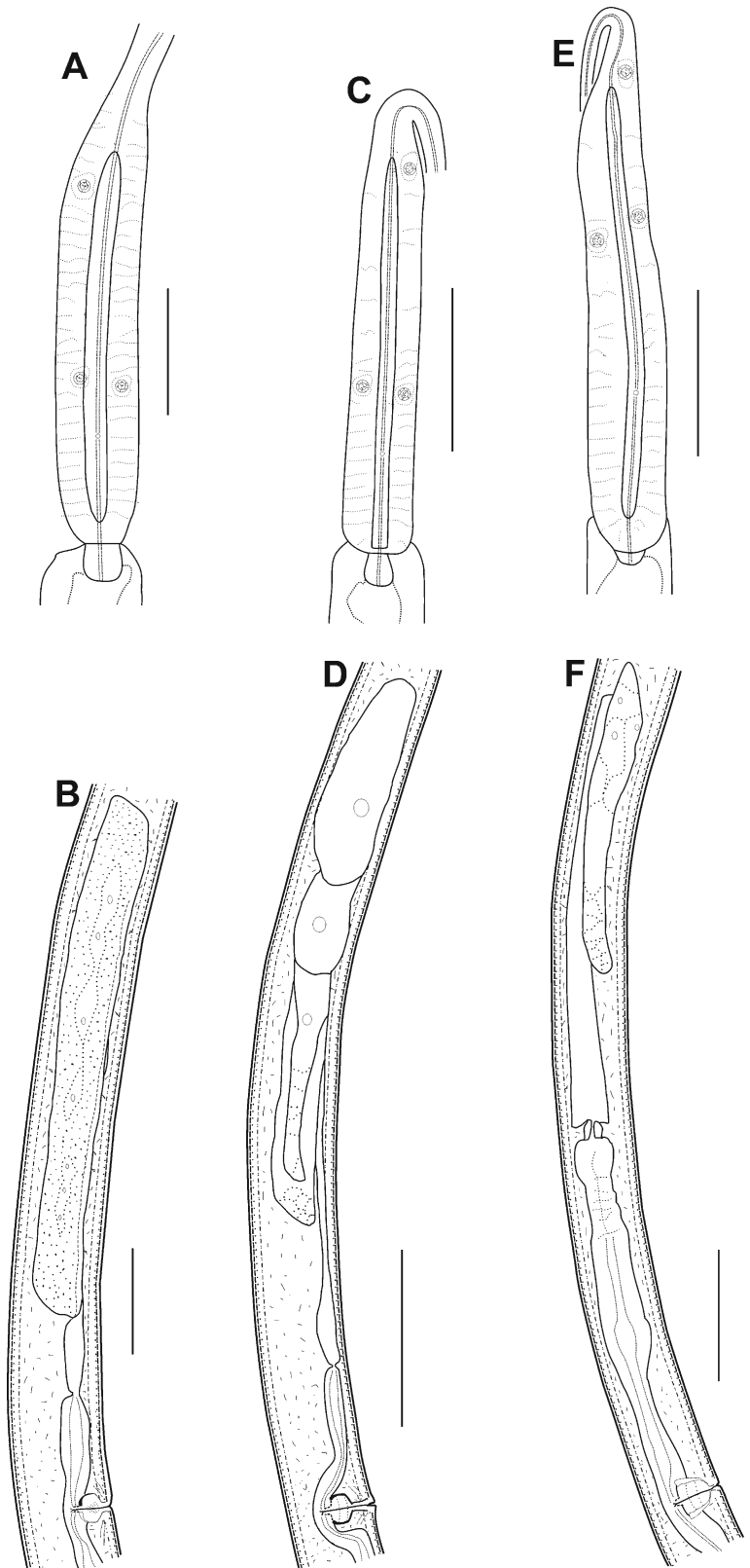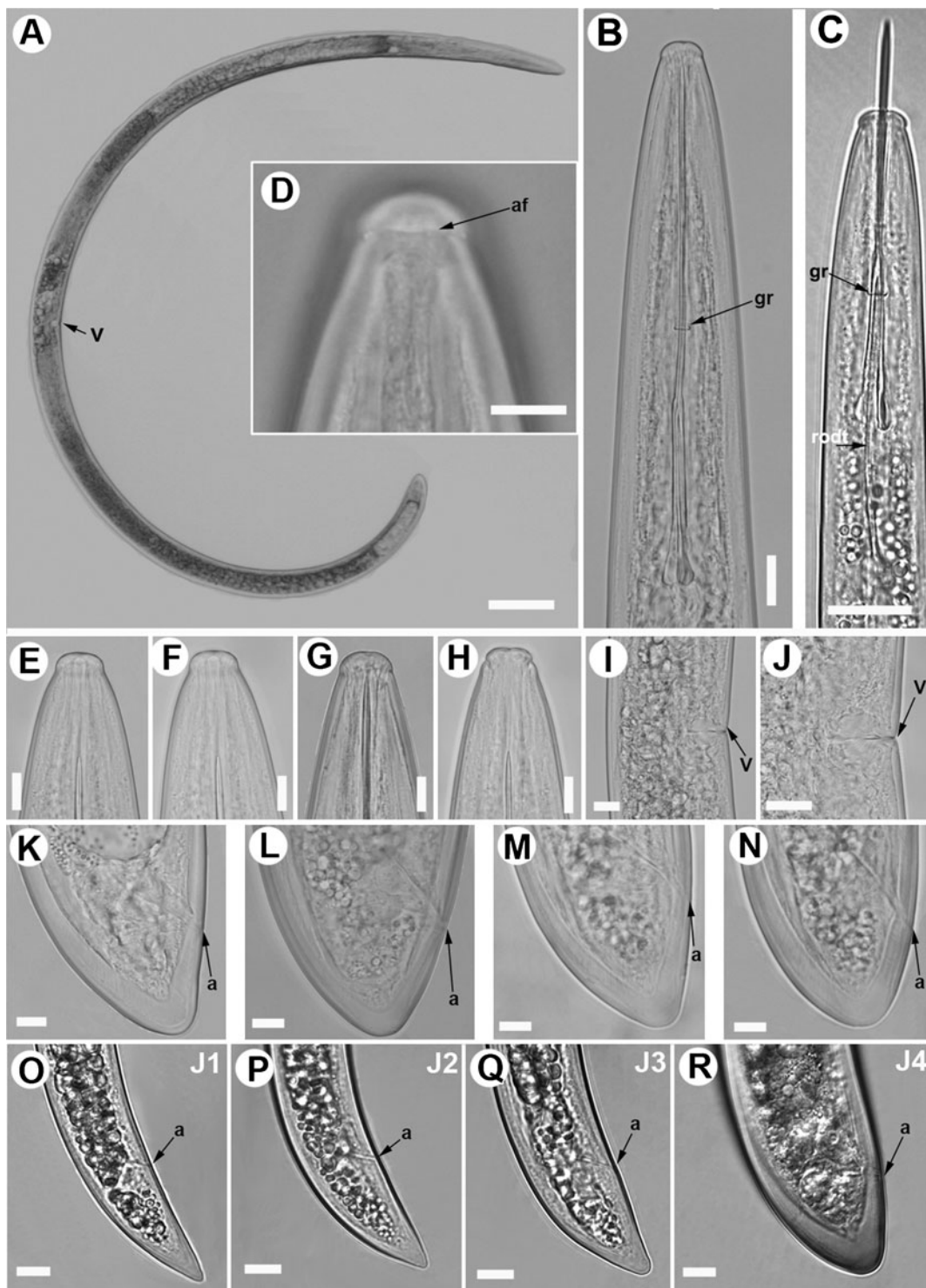
Fig. 3 Light micrographs of *Xiphinema parabrevicolle* n. sp. from southern Italy. **a** Whole female; **b** Female neck region; **c** First-stage juvenile neck region; **d–h** Anterior regions; **i–j** Vulval regions; **k–n** Female tail regions; **o–r** J1-, J2-, J3-, and J4- juvenile-stages tail, respectively. Abbreviations: *a* anus; *af* amphidial fovea; *gr* guiding ring; *rodt* replacement odontostyle; *V* vulva. (Scale bars: **a**=100 μm; **b–r**=10 μm)

Table 2 Morphometrics of *Xiphinema parabrevicolle* n. sp. from Pantanaggiani, Brindisi province, Italy. All measurements in μm and in the format: mean±s.d. (range)

Character	Holotype	Females	J1	J2	J3	J4
n	1	14	3	3	5	4
L	2,170	2,239±205 (1,716–2,540)	865±42.7 (816–894)	948±84 (900–1,045)	1,172±55 (1,100–1,205)	1,520±55 (1,444–1,561)
a	45.2	43.7±5.5 (34.3–50.8)	33.7±0.3 (33.5–34.0)	34.6±2.3 (33.3–37.3)	35.1±2.2 (32.4–38.5)	41.8±1.7 (39.9–43.8)
b	6.2	6.1±0.6 (5.1–6.9)	4.3±0.5 (4.0–4.8)	5.1±0.7 (4.3–5.5)	5.3±0.9 (4.5–6.8)	5.3±0.3 (5.1–5.8)
c	81.9	84.1±9.1 (68.6–95.5)	23.8±0.8 (23.0–24.3)	35.3±5.7 (29.0–40.2)	39.0±1.5 (36.7–40.2)	50.0±3.5 (47.4–54.8)
c'	0.8	0.7±0.03 (0.7–8)	1.9±0.2 (1.7–2.0)	1.6±0.1 (1.6–1.7)	1.3±0.1 (1.3–1.4)	1.1±0.0 (1.1–1.1)
V	53.5	53.8±1.2 (52–56)	–	–	–	–
G_1	12.4	11.0±1.3 (9.3–12.4)	–	–	–	–
G_2	12.4	10.2±2.1 (7.1–13.0)	–	–	–	–
Odontostyle length	112.0	105.8±5.1 (97.0–113.0)	49±1.4 (48.3–51.0)	57.3±2.3 (56.0–60.0)	71.6±2.2 (69.0–75.0)	89.8±2.2 (88.0–93.0)
Replacement odontostyle length	–	–	59±0.2 (58.7–59.0)	69.8±6.6 (66.0–77.5)	86.6±1.5 (85.0–89.0)	101.8±1.2 (101.0–103.0)
Odontophore length	59.0	58.3±2.2 (54.5–61.0)	37±1.6 (35.3–38.1)	36.5±4.3 (31.5–39.0)	44.0±2.4 (41.0–47.0)	49.0±2.6 (45.0–51.0)
Lip region width	13.5	13.5±0.4 (12.5–14.0)	9.3±0.3 (9.1–9.7)	10.5±0.5 (10.0–11.0)	10.4±0.2 (10.0–10.5)	11.3±0.5 (11.0–12.0)
Oral aperture-guiding ring	94.5	93.3±3.7 (87.0–98.5)	40±0.4 (39.2–39.9)	47.3±2.3 (46.0–50.0)	57.9±2.2 (54.0–60.0)	73.8±3.3 (70.0–78.0)
Tail length	26.5	26.6±1.4 (24.5–30.0)	36.4±2.6 (33.6–38.8)	32.0±1.7 (31.0–34.0)	30.1±1.4 (29.0–32.5)	30.5±1.4 (28.5–32.0)
J	7.5	7.5±1.0 (6.5–10.0)	7.5 ±1.5 (6.6–8.1)	7.2±0.3 (7.0–7.5)	6.7±0.3 (6.5–7.0)	6.8±0.3 (6.5–7.0)

Abbreviations are defined in Jairajpuri and Ahmad (1992)

Table 3 Differential morphometrics of *Xiphinema parabrevicolle* n. sp., *Xiphinema parapachydermum* n. sp., and *Xiphinema paratenuicutis* n. sp. from paratypes of *Xiphinema americanum*-group species complex. (All measurements in μm)

Characters/Ratio	parabrevicolle n. sp.	brevicolle[a]	diffusum	incognitum	luci	paramonovi	pseudoguirani	taylori
L	1,716–2,540	1,800–2,200	1,600–1,800	1,700–2,100	1,700–1,900	2,000–2,300	1,800–2,000	2,100–2,500
c'	0.7–0.8	0.9–1.1	0.8–1.1	0.9–1.3	1.1–1.4	0.9–1.2	0.7–0.8	0.9–1.0
V	52–56	51–54	47–52	48–53	49–52	51–55	54–56	48–52
Odontostyle length	97.0–113.0	85.0–108.0	84.0–89.0	82.0–93.0	93.0–99.0	88.5–120.0	107.0–117.0	90.0–97.0
Odontophore length	54.5–61.0	49.0–60.0	48.0–51.0	46.0–56.0	47.0–53.0	53.0–60.0	56.0–58.0	55.0–62.0
Lip region width	12.5–14.0	10.5–12.5	11.0–13.0	11.0–13.0	10.0–11.0	13.5–15.0	10.5–12.0	13.5–15.0
Oral aperture-guiding ring	87.0–98.5	72.0–92.5	60.0–64.0	67.0–78.0	68.0–85.0	66.0–103.0	84.0–93.0	75.0–82.0
Tail length	24.5–30.0	24.0–31.0	21.0–28.0	25.0–38.0	23.0–34.0	33.0–47.0	19.5–22.0	24.0–32.0
Tail shape	Dorsally convex-conoid, rounded tip	Short conoid	Conoid dorsally convex	Conoid slightly elongated	Conoid rounded tip	Bluntly conical	Conoid rounded tip	Conoid rounded tip
J	6.5–10.0	6.0–9.5	10.0.14.0	10.0–12.0	12.0–15.0	9	8.0–9.0	9.0–13.5

	parapachydermum n. sp.	pachydermum	brevisicum	duriense	intermedium	microstilum	opistholystherum	tarjanense
L	1,411–2,000	2,080–2,440	2,000–2,400	1,600–2,000	1,400–1,900	2,500–2,800	1,620–2,100	1,200–1,400
c'	1.5–2.3	1.3–1.6	2.4–2.7	1.9–2.3	1.3–1.7	1.6–2.0	1.25	1.5–1.7
V	55–66	57–60	54–57	56–63	50–57	55–60	56–61	53–56
Odontostyle length	70.0–87.5	79.0–87.0	57.0–62.0	68.0–72.0	68.0–80.0	68.0–77.0	62.0–72.0	78.0–88.0
Odontophore length	36.5–54.5	45.0–50.0	39.0–45.0	33.0–41.0	39.0–50.0	43.5–48.0	34.0–40.0	43.0–47.0
Lip region width	8.0–9.5	9	9.0–9.5	7.5–8.0	9.5–11.0	9.0–10.0	8.5	11.5–12.5
Oral aperture-guiding ring	59.5–75.5	64.0–72.0	52.0–56.0	56.0–64.0	58.0–67.0	57.0–68.0	60.0	54.0–65.0
Tail length	26.5–35.5	24.0–31.0	38.0–45.0	28.0–35.0	31.0–38.0	31.0–39.0	32.0	33.0–34.0
Tail shape	Dorsally convex-conoid, acute tip	Dorsally convex-conoid	Elongate slightly ventrally curved	Conoid, pointed tip	Conoid elongate, dorsally convex	Conoid elongate	Conoid, regularly tapering to tip	Conoid, with acute subdigital tip
J	7.0–12.5	8.5–14.0	7.0–10.0	6.0–8.0	7.0–112.5	7.0–12.0	–	11.5–13.5
Male	Present	Present	Present	Not found	Not found	Present	Not found	Not found

	paratenuicutis n. sp.	tenuicutis	bricolensis	californicum	mesostilum	oxycaudatum	parvum	peruvianum
L	1,800–2,205	1,600–1,900	1,700–2,300	1,800–2,200	2,400–2,800	1,500–1,700	1,400–1,800	1,400–1,900
c'	1.0–1.5	1.4–1.7	1.3–1.6	1.3–1.9	1.4–1.7	1.3–1.7	1.0–1.4	1.2–1.7
V	55–60	47–52	50–55	49–55	54–60	51–54	51–56	49–55
Odontostyle length	71.5–83.0	73.0–80.0	85.0–94.0	83.0–98.0	85.0–97.0	78.0–84.0	89.0–98.0	85.0–92.0
Odontophore length	42.0–46.5	38.0–46.0	47.0–56.0	44.0–53.0	45.0–53.5	43.0–46.0	45.0–53.0	46.0–52.0
Lip region width	9.0–10.0	9.0–10.0	11.0	10.0–11.0	9.0	9.0–10.0	9.5–15.0	9.0–10.5
Oral aperture-guiding ring	60.0–69.0	55.0–64.0	61.0–76.0	66.0–83.0	73.0–85.0	66.0–75.0	73.0–78.0	67.0–78.0

Table 3 (continued)

Tail length	25.0–34.5	26.0–32.0	31.0–41.0	27.0–36.0	23.5–28.0	27.0–35.0	22.0–27.0	26.0–35.0
Tail shape	Conoid, ventrally almost straight pointed tip	Elongated conoid, ventrally curved pointed tip	Dorsally convex, ventrally straight, rounded tip	Conoid elongate, dorsally convex	Conoid	Conoid elongate, dorsally convex, pointed tip	Conoid elongate, rounded tip	Conoid elongate, subdigital tip
J	6.5–10.0	7.0–9.0	8.5–9.5	5.0–8.5	8.0–11.0	7.5–11.0	4.0–6.0	7.5–12.0
Male	Present	Not found	Present	Present	Present	Not found	Not found	Present

[a] After Lamberti et al. (1991)

Juveniles All four juvenile stages (first-, second-, third- and fourth-stage) were found, and were basically similar to adults, except for their smaller size, longer tails and sexual characteristics. Tail becomes progressively shorter and stouter in each moult, being distinguishable by relative lengths of body and functional and replacement odontostyle (Fig. 3o–r, Table 2).

Type host and locality

Xiphinema parabrevicolle n. sp. was found in a sandy soil around the roots of lentisc (*Pistacia lentiscus* L.) and marram grass [*Ammophila arenaria* (L.) Link] at Pantanaggiani, Brindisi province, southern Italy (40°45′01.27″ N latitude, 17°43′13.65″ E longitude).

Type material

Holotype female and 14 female paratypes deposited in the Nematode Collection at the Institute for Sustainable Agriculture, CSIC, Córdoba, Spain. Another seven female paratypes deposited at Royal Belgian Institute of Natural Sciences, Brussels, Belgium (slides RIT808-809), and two female paratypes at the USDA Nematode Collection, Beltsville, MD, USA (collection number T-6125p). Specific D2-D3, ITS1, 18S-rRNA and *COI* sequences are deposited in GenBank with accession numbers JQ990042, JQ990043, JQ990050, and JQ990059, respectively.

Diagnosis and relationships

Xiphinema parabrevicolle n. sp. is characterized by a medium body size (1,716–2,540 μm), lip region widely rounded, separated from the rest of the body by a depression, odontostyle and odontophore 97.0–113.0 and 54.5–61.0 μm long respectively, vulva position at 52–56 %, female tail short (24.5–30.0 μm), dorsally convex-conoid, slightly concave ventrally with conoid-rounded terminus, c ratio (68.6–95.5), c' ratio (0.7–0.8), and a specific D2-D3, ITS1, 18S-rRNA and *COI* sequences.

Morphologically, *X. parabrevicolle* n. sp. can be distinguished from the most similar species by a number of particular characteristics resulting from its specific alpha-numeric codes (A 2, B 32, C 32, D 1, E 21, F 1, G 2, H 3, I 12, J 2) sensu Lamberti et al. (2000).

Using the polytomous key of Lamberti et al. (2000) and sorting on features A (lip region), C (odontostyle length), D (c' ratio) and J (tail end shape), *X. parabrevicolle* n. sp. groups with *X. brevicolle, X. diffusum, X. incognitum* Lamberti and Bleve-Zacheo 1979, *Xiphinema luci* Lamberti and Bleve-Zacheo 1979, *Xiphinema pseudoguirani* Lamberti et al. 1991, *Xiphinema paramonovi* Romanenko 1981, and *Xiphinema taylori* Lamberti et al. 1991. *Xiphinema parabrevicolle* n. sp. differs from topotypes of *X. brevicolle* by small differences in c' and a slightly wider lip region (Lamberti et al. 1991). Also, the new species showed some differences with other species in this group in measurements and ratios, including L, c', V ratio, odontostyle and odontophore length, lip region width, distance from guiding ring to anterior end, tail length and shape, and tail hyaline region (Table 3).

Remarks

Recently, Monteiro (2010) discussed the nomenclatorial disagreement between the original and widely used epithet *brevicolle* (=short neck) and the newly proposed by Luc et al. (1998) *brevicollum*. We concur with Monteiro (2010) that this is an "unjustified emendation", since the termination *colle* is used as an adjective (neuter gender), and the International Code of Zoological Nomenclature (ICZN) allows this use. Consequently, the correct original species name must be preserved unaltered as *Xiphinema brevicolle* Lordello and Costa 1961, as has also been recognized by Sakai et al. (2011).

Xiphinema parapachydermum[2] n. sp. (Figs. 1, 4, Tables 3, 4)

Female Body medium-sized, habitus coiled in a more or less closed C to open spiral when killed by heat. Body tapering very gradually towards the extremities. Cuticle smooth, inner finely striated, 1.9±0.2 (1.5–2.0) μm thick along body but thicker at tail tip (Table 4). Lip region flatly rounded, separated from the rest of the body by a constriction,

2.5±0.1 (2.0–2.5) μm high. Amphidial fovea funnel-shaped with slit-like aperture at constriction level, 7.9±0.3 (7.5–8.0) μm. Odontostyle 1.0–1.5 μm wide, with slightly furcate base. Odontophore with basal flanges weakly developed 6.5±0.0 (6.5–6.5) μm wide. Pharyngeal basal bulb 66.7±9.2 (52.0–85.5) μm long and 11.8±2.4 (8.5–15.5) μm wide, occupying about 1/3 to 1/4 of the total pharyngeal length (Fig. 4b). Glandularium 47.8±1.0 (47–49) μm long. Dorsal pharyngeal gland nucleus in anterior part of bulb, DN 30.6±2.8 (29.0–35.0) %. Pharyngeal-intestinal valve broadly rounded, rather indistinct. Reproductive system didelphic, ovaries reflexed, uteri rather short (45–56 μm) without differentiation; anterior genital branch slightly more developed than the posterior. Vulva a transverse slit, clearly posterior to mid-body; vagina 13.2±1.0 (12.0–15.0) μm long with short distal part and well-developed proximal. Ovaries without symbiontic bacteria and oocytes clearly separated; sphincter clearly visible; no sperm present. Prerectum often indistinct. Rectum 19.5±2.0 (17.0–21.5) μm long or 1–0.8 times the anal body diameter. Tail short, but twice longer than anal body diameter (1.5–2.3), dorsally convex-conoid, with acute rounded tip, and often with a dorso-ventral depression; two pairs of caudal pores present (Figs. 1g, 4f–g). Tail hyaline region about 1/3 of the tail length.

Male Almost as common as females. General morphology similar to that of female except for a rather coiled posterior region. Testes well developed, containing numerous large oval sperms. Spicules well sclerotized, ventrally curved (Fig. 4i,j). Lateral guiding pieces about 1/4 to 1/5 spicules length. A pre-anal pair of supplements 5 to 10 μm anterior to cloacal opening and a row of 5 to 6 single ventromedian supplements, located anterior to the spicule region.

Juveniles Two juvenile stages were detected (third- and fourth-stage), which were morphologically similar to females from which they differ by their size, longer and more tapering tails and development of reproductive system (Table 4).

Type host and locality

Xiphinema parapachydermum n. sp. was found in a sandy soil around the roots of grapevine (*Vitis vinifera*

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[2] The species epithet refers to Gr. prep. para, alongside of, resembling; N.L. masc. n. *pachydermum*, because of its close resemblance to *Xiphinema pachydermum*
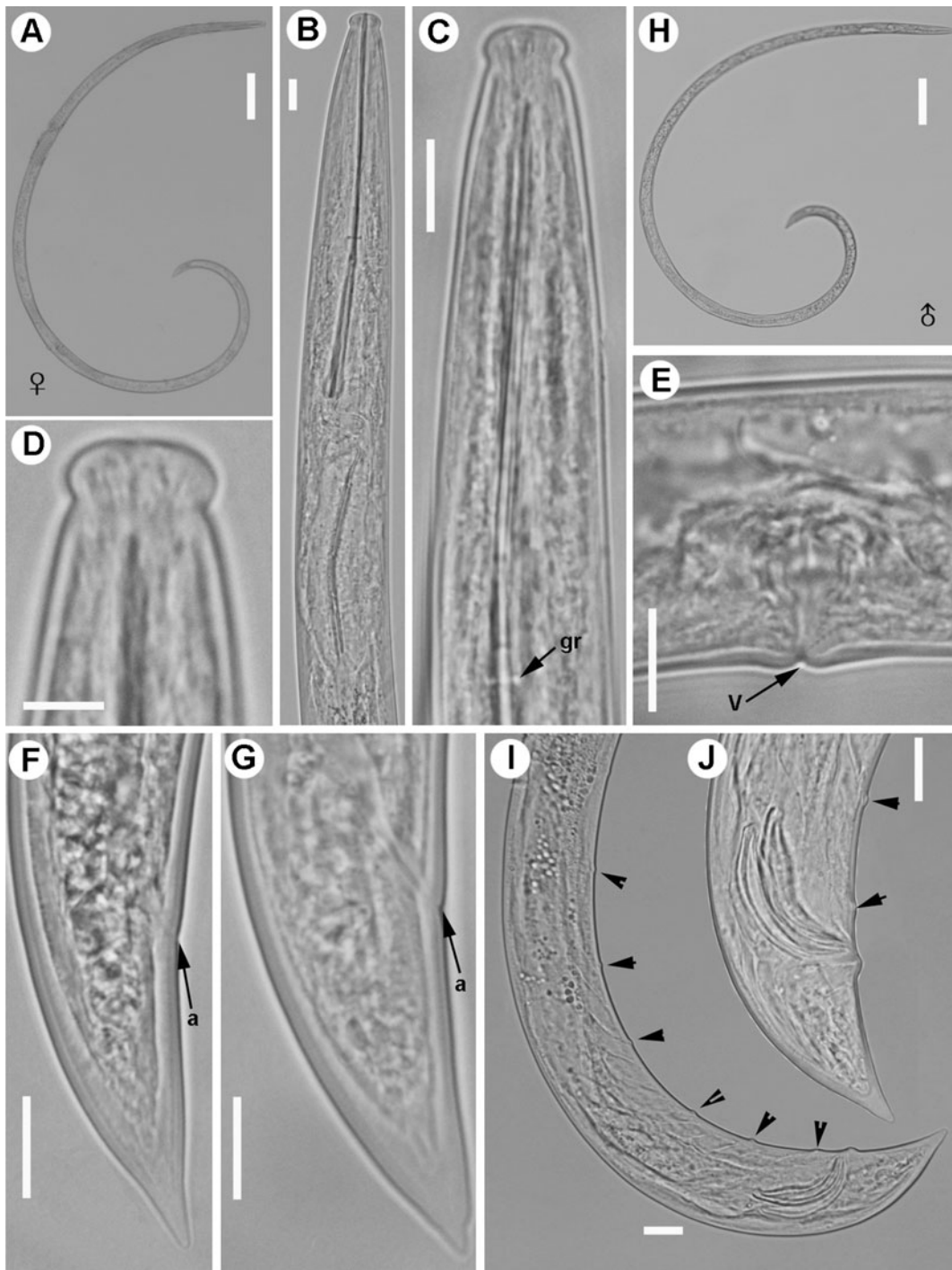
Fig. 4 Light micrographs of *Xiphinema parapachydermum* n. sp. from southern Spain. **a** Whole female; **b**, **c** Female neck regions; **d** Anterior region; **e** Vulval region; **f**, **g** Female tail regions; **h** Whole male; **i**, **j** Male tail regions, with ventromedian papillae arrowed. Abbreviations: *a* anus; *gr* guiding ring; *v* vulva. (Scale bars: **a**, **h**=100 μm; **b**, **c**, **e**–**j**=10 μm; **d**=5 μm)

Table 4 Morphometrics of *Xiphinema parapachydermum* n. sp. from several localities. All measurements in μm and in the format: mean±s.d. (range)

| Locality | Bollullos par del Condado (Paratypes) | | | | | Hinojos | | Chucena |
Character/life-stage	Holotype	Females	Males	J3	J4	Females	Males	Female
n	1	14	10	3	5	6	5	1
L	1,844	1,777±141 (1,411–2,000)	1,523±83 (1,383–1,622)	1,238±98 (11,140–1,335)	1,345±68 (1,230–1,400)	1,603±102 (1,450–1,750)	1,364±57 (1,300–1,450)	1,416
a	62.5	64.0±4.9 (51.3–73.1)	65.5±2.3 (62.9–69.0)	60.9±4.1 (57.0–65.1)	52.2±5.0 (47.2–57.7)	58.4±2.0 (55.8–60.6)	60.5±3.3 (55.3–64.3)	57.8
b	9.7	8.3±1.2 (6.1–10.3)	7.2±1.0 (5.9–8.9)	6.4±1.4 (5.0–7.9)	6.6±0.7 (6.2–7.8)	7.6±1.4 (5.8–9.6)	8.0±0.8 (6.8–9.0)	7.2
c	65.9	60.3±8.4 (46.3–75.5)	59.1±4.1 (51.2–62.9)	46.7±1.1 (45.6–47.7)	50.8±5.3 (45.4–58.3)	59.3±1.4 (57.7–61.7)	55.2±8.5 (47.3–69.5)	56.6
c'	1.6	1.8±0.3 (1.5–2.3)	1.5±0.1 (1.2–1.6)	1.8±0.2 (1.6–2.0)	1.7±0.2 (1.5–2.0)	1.6±0.1 (1.4–1.8)	1.4±0.2 (1.1–1.7)	1.9
V or T	57	59.0±3.4 (55–66)	30.8±13.1 (9–43)	–	–	58.9±1.7 (57–61)	34.0±10.8 (25–50)	58
G_1	5.5	9.1±3.1 (5.5–13.1)	–	–	–	10.4±4.5 (6.9–15.5)	–	7.8
G_2	5.2	8.8±2.2 (5.2–10.8)	–	–	–	9.8±3.6 (5.8–12.8)	–	6.4
Odontostyle length	86.0	81.0±5.5 (70.0–87.5)	70.5±4.0 (63.0–74.5)	59.0±3.6 (55.0–62.0)	66.8±2.0 (65.0–70.0)	76.8±7.6 (69.5–85.5)	65.1±3.3 (62.0–69.0)	71
Replacement odontostyle length	–	–	–	67.0±2.0 (65.0–69.0)	79.0±3.5 (74.5–83.0)	–	–	–
Odontophore length	45.5	46.5±5.8 (36.5–54.5)	42.4±1.3 (40.5–44.0)	34.0±3.6 (31.0–38.0)	40.5±0.7 (40.0–41.5)	41.7±3.0 (39.0–45.5)	34.2±3.8 (28.0–38.0)	35
Lip region width	8.5	8.8±0.5 (8.0–9.5)	8.0±0.3 (7.5–8.5)	8.0±0.0 (8.0–8.0)	8.0±0.4 (7.5–8.5)	7.9±0.2 (7.5–8.0)	7.8±0.3 (7.5–8.0)	8.5
Oral aperture-guiding ring	75.0	70.3±5.9 (59.5–75.5)	57.9±4.7 (52.5–63.5)	48.7±2.1 (47.0–51.0)	57.5±1.2 (56.0–59.0)	64.7±7.1 (55.5–72.5)	53.5±2.8 (50.0–57.0)	59.5
Tail length	28.0	29.8±2.9 (26.5–35.5)	25.9±2.5 (22.0–29.5)	26.5±1.8 (25.0–28.5)	26.6±2.4 (24.0–30.5)	27.0±2.0 (23.5–29.5)	25.1±3.3 (19.5–27.5)	25.0
J	8.0	9.3±1.4 (7.0–12.5)	8.1±0.7 (7.0–9.0)	6.0±1.5 (4.5–7.5)	7.4±0.5 (6.5–8.0)	8.8±0.8 (8.0–9.5)	6.4±2.3 (3.0–8.0)	5.5
Spicules	–	–	36.7±2.0 (34.5–40.0)	–	–	–	34.3±2.0 (32.0–37.0)	–
Lateral accessory piece	–	–	8.5±0.6 (7.5–9.0)	–	–	–	9.5±0.9 (8.5–10.0)	–

Abbreviations are defined in Jairajpuri and Ahmad (1992)

L.) at Bollullos par del Condado, Huelva province, southern Spain (37°18′02.71″ N latitude, 6°30′51.55″ W longitude).

Other localities

The species was also detected in sandy soils around the roots of olive (*Olea europaea* subsp. *europaea* L.) at Hinojos, and grapevine at Chucena, both in Huelva province, southern Spain.

Type material

Holotype female and 14 female and six male paratypes deposited in the Nematode Collection at the Institute for Sustainable Agriculture, CSIC, Córdoba, Spain. Another three female and one male paratypes deposited at Royal Belgian Institute of Natural Sciences, Brussels, Belgium (slides RIT 810, 811), and two female paratypes at the USDA Nematode Collection, Beltsville, MD, USA (collection number T-6126p). Specific D2-D3, ITS1, 18S-rRNA and *COI* sequences are deposited in GenBank with accession numbers JQ990034-JQ990036, JQ990045, JQ990051, and JQ990056, respectively.

Diagnosis and relationships

Xiphinema parapachydermum n. sp. is characterized by a medium body size (1,411–2,000 μm), lip region flatly rounded, separated from the body by a constriction, odontostyle and odontophore 70.0–87.5 and 36.5–54.5 μm, respectively, vulva position at 55–66 %, female tail short, about twice longer than anal body diameter (1.4–2.3), dorsally convex-conoid, with acute rounded tip, and often with a dorso-ventral depression (26.5–35.5 μm long), c ratio (46.3–75.5), c' ratio (1.5–2.3), and specific D2-D3, ITS1, 18S-rRNA and *COI* sequences.

Morphologically, *X. parapachydermum* n. sp. can be distinguished from the most similar species by a number of particular characteristics resulting from its specific alpha-numeric codes (A 2, B 21, C 12, D 34, E 32, F 21, G 12, H 2, I 23, J 1) sensu Lamberti et al. (2000).

Using the polytomous key of Lamberti et al. (2000) and sorting on features A (lip region), C (odontostyle length), D (c' ratio) and J (tail end shape), *X. parapachydermum* n. sp. groups with *X. pachydermum, X. brevisicum, X. duriense, X. intermedium, X. microstilum, X.*

opisthohysterum, and *X. tarjanense* Lamberti and Bleve-Zacheo 1979. *Xiphinema parapachydermum* n. sp. differs from *X. pachydermum* only by small differences in body length, female and male tail shape, and spicules shape (Table 3). Also, the new species showed some differences with other species in this group in measurements and ratios, including L, c', V ratio, odontostyle and odontophore length, lip region width, distance from guiding ring to anterior end, tail length and shape, tail hyaline region, and presence/absence of males (Table 3).

Xiphinema paratenuicutis[3] n. sp. (Figs. 1, 5, Tables 3, 5)

Female: Body medium to large sized, forming a close C-shape when killed by heat. Cuticle 2.4±0.3 (2.0–2.5) μm thick along body but thicker at tail tip (Table 5). Lip region expanded and flat at the anterior extremity, separated from body by constriction, 2.9±0.4 (2.5–3.5) μm high. Amphidial fovea aperture 8.5±0.5 (8.0–9.0) μm wide. Guiding apparatus with a double guiding ring. Odontophore with basal flanges moderately developed 7.8±0.3 (7.5–8.0) μm wide. Pharyngeal basal bulb 72.2±8.7 (60–89) μm long, occupying about 1/3 of the total pharyngeal length. Glandularium 63.2±8.1 (55–70) μm long. Dorsal pharyngeal gland nucleus in anterior part of bulb, DN 29.4±4.3 (25.0–34.0)%. Reproductive system amphidelphic, both branches apparently equally developed, opposed and ovaries reflexed; uteri rather long (76–100 μm) without any uterine differentiation but sperm present. Ovaries without symbiont bacteria. Vulva post-equatorial, a transverse slit-like; vagina 13.7±2.4 (7.5–16.5) μm long with short distal part and well-developed proximal (Fig. 5e,f). Prerectum often indistinct. Rectum 17.9±2.1 (15.0–19.5) μm long, or 1.6–2.1 times the anal body diameter. Tail short, conoid, ventrally almost straight with pointed terminus; two pairs of body pores (Fig. 5g–i).

Male Almost as common as females. General morphology similar to female except for a stronger coiled posterior region. Testes well developed, containing numerous large oval sperm. Spicules well sclerotized, ventrally

[3] The species epithet refers to Gr. prep. para, alongside of, resembling; N.L. masc. n. *tenuicutis*, because of its close resemblance to *Xiphinema tenuicutis*
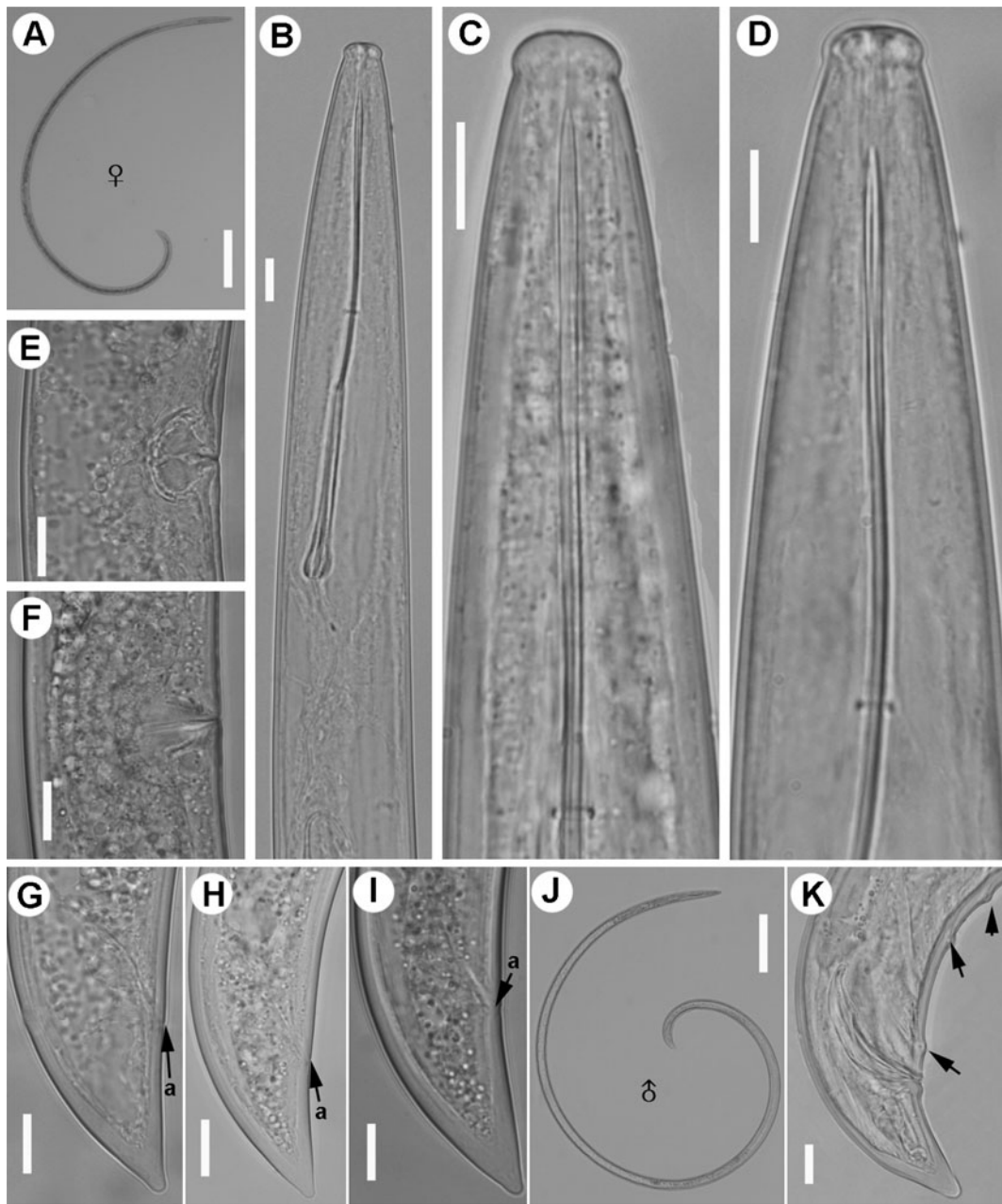
Fig. 5 Light micrographs of *Xiphinema paratenuicutis* n. sp. from southern Spain. **a** Whole female; **b–d** Female neck regions; **e**, **f** Vulval regions; **g–i** Female tail regions; **j** Whole male; **k** Male tail region, with ventromedian papillae arrowed. Abbreviations: *a* anus. (Scale bars: **a**, **j**=100 μm; **b–i**, **k**= 10 μm)

curved and lamina with clear ventral bulge (Fig. 5k). Lateral guiding pieces about 1/4 spicules length. A precloacal pair of supplements and a row of 5 single ventromedian supplements, located anterior to the retracted spicule.

Juveniles All four juvenile stages were detected (first-, second-, third- and fourth-stage), which were morphologically similar to females from which differ by their size and tails longer and more tapering (Table 5).

Table 5 Morphometrics of *Xiphinema paratenuicutis* n. sp. from Andújar, Jaén province, southern Spain. All measurements in µm and in the format: mean±s.d. (range)

Character/life-stage	Holotype	Females	Males	J1	J2	J3	J4
n	1	14	11	2	1	2	3
L	2,127	2,012±152 (1,740–2,205)	1,923±155 (1,690–2,144)	615±21 (600–630)	805	1,275±104 (1,100–1,350)	1,634±120 (1,500–1,730)
a	69.5	61.1±4.9 (51.9–69.7)	64.7±2.9 (58.5–69.2)	34.6±0.9 (34.0–35.5)	40.3	38.5±3.7 (33.8–42.5)	54.9±3.0 (51.4–56.7)
b	7.2	9.4±1.6 (7.1–11.9)	8.3±1.6 (6.0–11.6)	4.1±1.1 (3.3–4.9)	6.7	6.2±1.1 (4.8–7.5)	8.2±1.0 (7.6–9.4)
c	72.6	68.8±6.4 (58.8–79.9)	63.4±4.8 (55.4–71.7)	22.0±1.9 (20.7–23.3)	29.8	40.3±6.8 (30.6–46.2)	55.7±4.2 (50.8–58.6)
c'	1.5	1.4±0.1 (1.2–1.6)	1.4±0.1 (1.2–1.6)	2.4±0.3 (2.2–2.6)	2.3	1.6±0.1 (1.5–1.6)	1.4±0.02 (1.2–1.5)
V or T	55	56.8±1.6 (55–60)	38.5±7.9 (27–56)	–	–	–	–
G_1	5.6	6.6±2.2 (3.0–10.2)	–	–	–	–	–
G_2	7.5	6.8±1.8 (4.9–11.2)	–	–	–	–	–
Odontostyle length	80.5	75.2±3.7 (71.5–83.0)	76.1±2.9 (72.0–82.0)	39.3±0.4 (39.0–39.5)	45.0	52.5±2.6 (50.0–56.0)	61.0±1.0 (60.0–62.0)
Replacement odontostyle	–	–	–	44.0±1.4 (43.0–45.0)	51.5	60.6±1.7 (58.5–62.0)	72.3±3.2 (70.0–76.0)
Odontophore length	46.5	44.6±1.4 (42.0–47.0)	44.2±2.1 (40.0–47.0)	25.5±0.7 (25.0–26.0)	29.0	33.5±2.2 (30.5–35.5)	37.3±2.1 (35.0–39.0)
Lip region width	9.5	9.6±0.3 (9.0–10.0)	9.6±0.5 (8.5–10.0)	6.5±0.0 (6.5–6.5)	7.5	8.5±0.7 (8.0–9.5)	9.0±0.5 (8.5–9.5)
Oral aperture-guiding ring	68.5	63.2±3.4 (60.0–69.0)	63.5±3.4 (60.0–68.8)	30.8±2.5 (29.0–32.5)	36.5	48.8±5.6 (43.5–55.5)	53.3±2.1 (51.0–55.0)
Tail length	29.5	29.4±2.4 (25.0–34.5)	29.6±2.6 (25.3–34.5)	28.5±3.5 (26.0–31.0)	27.0	30.5±4.4 (26.0–36.0)	29.3±0.3 (29.0–29.5)
J	9.5	8.4±1.1 (6.5–10.0)	9.0±1.0 (7.0–10.0)	4.0±0.0 (4.0–4.0)	4.5	5.0±0.0 (5.0–5.0)	5.5±0.0 (5.5–5.5)
Spicules	–	–	39.2±4.1 (32.5–46.5)	–	–	–	–
Lateral accessory piece	–	–	9.1±0.5 (8.0–9.5)	–	–	–	–

Abbreviations are defined in Jairajpuri and Ahmad (1992)

Type host and locality

Xiphinema paratenuicutis n. sp. was found in a sandy soil around the roots of undetermined grasses (graminaceae) at Andújar, Jaén province, southern Spain (38°11′06.84″ N latitude, 4°02′18.96″ W longitude).

Type material

Holotype female and 14 female and 11 male paratypes deposited in the Nematode Collection at the Institute for Sustainable Agriculture, CSIC, Córdoba, Spain. Another three females and one male paratype deposited at Royal Belgian Institute of Natural Sciences (slide RIT 843), Brussels, Belgium, and two female paratypes at the USDA Nematode Collection, Beltsville, MD, USA (collection number T-6124p). Specific D2-D3 sequence is deposited in GenBank with accession number JQ990041.

Diagnosis and relationships

Xiphinema paratenuicutis n. sp. is characterized by a medium to large body size (1,888–2,205 μm), lip region expanded and anteriorly flat, separated from body by a constriction, odontostyle and odontophore 72–83 and 42–47 μm resp., vulva position at 55–60 %, female tail short, conoid, ventrally almost straight with pointed terminus (25–35 μm long), c ratio (56.7–79.9), c' ratio (1.0–1.7), and specific D2-D3 sequence.

Morphologically, X. paratenuicutis n. sp. can be distinguished from the most similar species by a number of particular characteristics resulting from its specific alpha-numeric codes (A 2, B 23, C 2, D 21, E 32, F 21, G 23, H 2, I 21, J 1) sensu Lamberti et al. (2000).

Using the polytomous key of Lamberti et al. (2000) and sorting on features A (lip region), C (odontostyle length), D (c' ratio) and J (tail end shape), X. paratenuicutis n. sp. groups with X. tenuicutis, X. bricolensis, X. californicum, Xiphinema mesostilum Lamberti et al. 1994, X. oxycaudatum, Xiphinema parvum Lamberti et al. 1991, and X. peruvianum. Xiphinema paratenuicutis n. sp. differs from X. tenuicutis only by small differences in V ratio and presence of males (Table 3). Also, the new species showed some differences with other species in this group in measurements and ratios, including L, c', V ratio, odontostyle and odontophore length, lip region width, distance from guiding ring to anterior end, tail

length and shape, and tail hyaline region (Table 3). Further detailed study of paratypes of X. tenuicutis (Fig. 6n–s) from USDA Nematode Collection (slides T-3447p, T-3448p, and T-3449p) showed the new species also differs by lip region (expanded and flat at the anterior extremity and separated from body by a constriction vs conoid-rounded and separated by a slight depression from the rest of the body), female tail shape (short, conoid, ventrally almost straight with pointed terminus vs elongated conoid with ventrally curved pointed terminus, Figs. 5 and 6), and some differences in measurements and ratios (Table 3).

Xiphinema duriense Lamberti et al. 1993 (Fig. 7, Table 6)

Female The Spanish population of this species is characterised by a coiled body habitus forming a more or less close C when killed with heat, a lip region almost elliptical, expanded and offset from the body by a constriction, female reproductive system amphidelphic with two equally developed genital branches, uterine differentiation absent, vulva slit-like and always posterior to mid-body, vagina occupying about 1/2 corresponding body width, tail conoid with pointed terminus, slightly curved ventrally and bearing two caudal pores on each side (Fig. 7). Male not found.

Examination of five female paratype specimens showed odontostyle with poorly developed basal collar and odontophore with weak flanges. Position of pharyngeal gland nuclei and their associated openings as follows (n=2): DO=9.4±1.9 (8.0–10.7); DN=10.1±0.3 (9.0–10.3); DO-DN = 0.7±0.4 (0.4–1.0); SN=56.9±1.7 (55.–58.14); SO=74.4±0.6 (73.9–74.8); Distance SN-SO=13.6±0.6 (13.1–14.0) μm or 16.8±0.7 (16.3–17.3) % of bulb length; SO=48.7±2.8 (46.7–50.6) μm anterior to end of platelet region. Neck 294.4±14.5 (284–305) μm long. Glandularium 65.1±3.1 (62–69) μm. Female reproductive system with relatively long unipartite uteri (50 μm) and without sperm; oviduct clearly differentiated, sphincter well-developed. Ovarial sac large, ovaries small and compact, not swollen, without symbiotic bacteria and developing oocytes clearly demarcated. Prerectum indistinct, 6.9–7.2 anal body widths long. Morphology and morphometrics of the Spanish population agree closely with the original description from Portugal by Lamberti et al. (1993) (Table 6), except for a lower L and a ratio in females (av. 1,436 μm, 61.8 vs av.1800 μm, 74.0, respectively), more posterior vulva position (av. 65.1 vs av. 60).
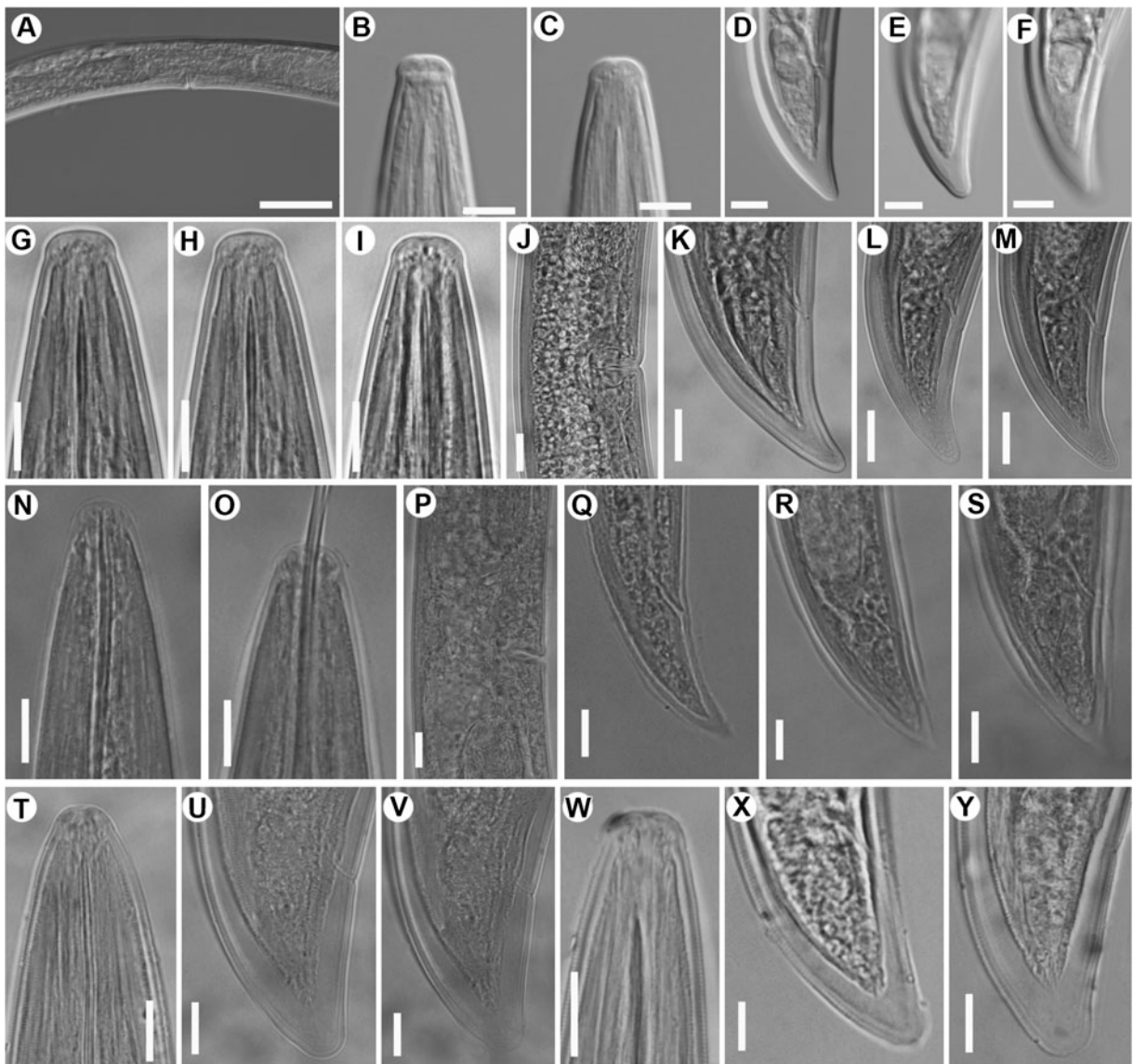
Fig. 6 Light micrographs of paratypes of *Xiphinema santos* Lamberti et al. 1993 (**a–f**) from Rothamsted; *Xiphinema intermedium* Lamberti and Bleve-Zacheo 1979 (**g–m**) from USDA-Nematode collection; paratypes of *Xiphinema tenuicutis* Lamberti and Bleve-Zacheo 1979 (**n–s**) from USDA-Nematode collection; paratypes of *Xiphinema rivesi* Dalmasso 1969 (**t–v**) from USDA-Nematode collection; and *X. rivesi* from Portugal (**w–y**) deposited in the Nematode Collection of Bari, Italy. **a, d, j** Vulval regions; **b–c, g–i, n–o, t, w** Anterior regions; **d–f, k–m, q–s, u–v, x–y** Female tail regions. (Scale bars: **a**=50 μm; **b–y**= 10 μm)

Nevertheless, these differences further expand but do not exceed intraspecific variation reported by Lamberti et al. (1994) for others *X. duriense* populations associated with grapevine and weeds in Central and Northern Portugal. Morphologically and morphometrically this species is close to *Xiphinema microstilum* Lamberti et al. 1994 and *Xiphinema opisthohysterum* Siddiqi 1961. It can be differentiated from *X. microstilum* in having a lower L, a, c and c′ ratios (av. 1,436 μm, 61.8, 47.2, 2.2 vs av. 2,600 μm, 86.0, 74.0, 1.8), and a shorter odontophore and tail (av. 36.6 μm, 30.6 μm vs av. 45.0 μm, 35.0 μm); and from *X. opisthohysterum* in a higher c′ and V ratios (2.0–2.6, 63–67 vs 1.9–2, 56–59, respectively), longer odontostyle and distance from guiding ring to anterior end (70–74 μm, 53–60 μm vs 62–72 μm, 49–51 μm, respectively). *Xiphinema duriense*
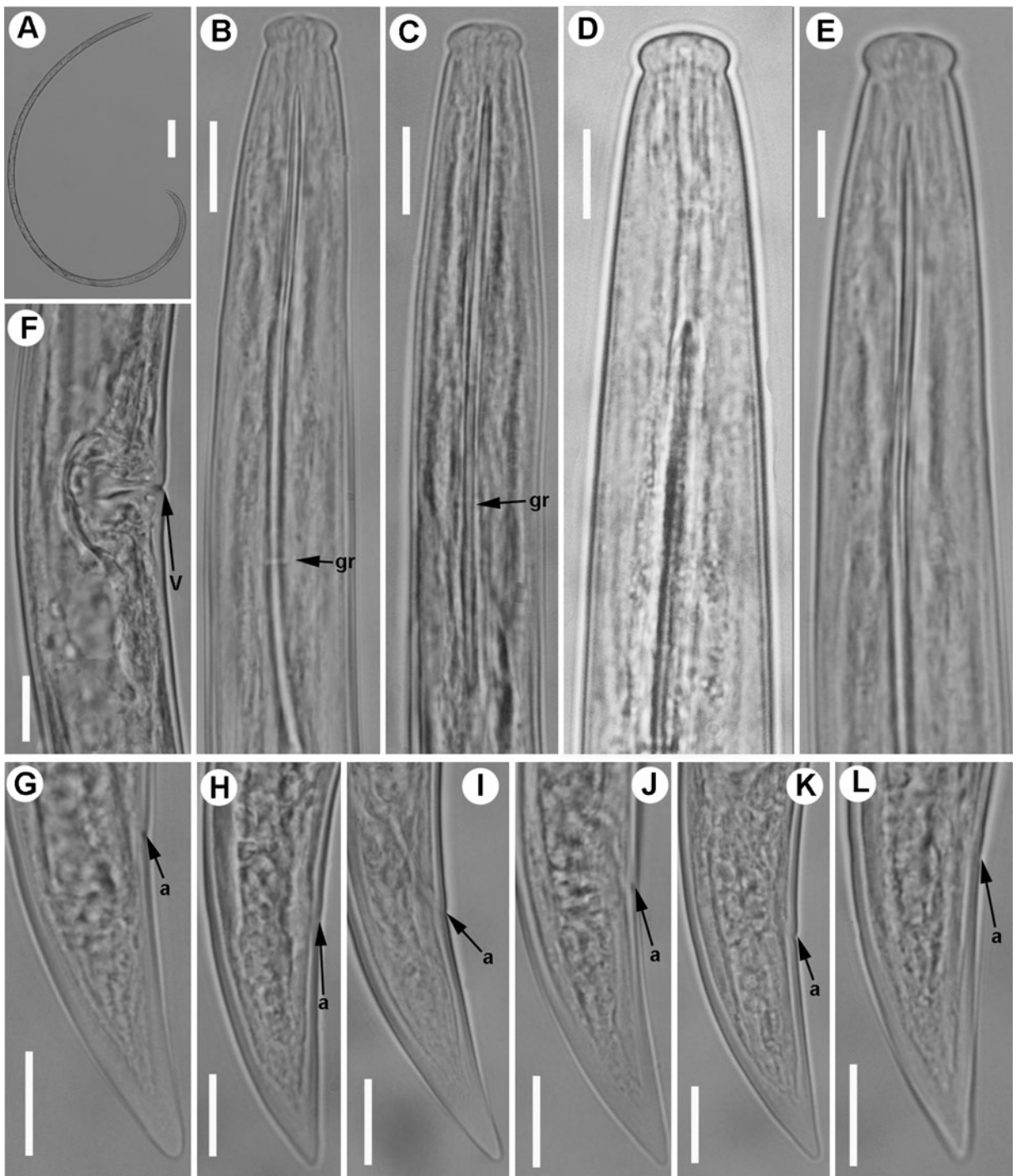
Fig. 7 Light micrographs of *Xiphinema duriense* Lamberti et al. 1993 from southern Spain. **a** Whole female; **b–e** Female neck regions; **f** Vulval region; **g–l** Female tail regions. Abbreviations: *a* anus; *gr* guiding ring; *V* vulva. (Scale bars: **a**=100 μm; **b–l**=10 μm)

is morphologically similar to other species in the *X. pachydermum*-group, namely *X. brevisicum*, *X. exile*, *X. lafoense*, *X. longistilum*, *X. mesostilum*, *X.* *microstilum* and *X. pachydermum*. It differs from these species by the smaller body size (1.5–2.0 mm compared to a range of 1.9–4.4 mm) and by the absence of males

Table 6 Morphometrics of *Xiphinema duriense* Lamberti et al. 1983, *Xiphinema incertum* Lamberti et al. 1983, and *Xiphinema opisthohysterum* from several localities. All measurements in μm and in the format: mean±s.d. (range)

Locality Character	*X. duriense* Almonte Females	*X. incertum* Jerez de la Frontera Females	*X. opisthohysterum* Andújar Females
n	10	6	10
L	1,436±122 (1,288–1,611)	1,844±52 (1,788–1,922)	1,514±104 (1,377–1,550)
a	61.8±4.4 (53.7–67.6)	49.7±3.0 (44.6–52.5)	59.2±4.4 (52.9–63.4)
b	7.4±1.1 (6.4–9.5)	9.0±1.4 (7.7–11.0)	7.6±1.4 (5.1–8.1)
c	47.2±4.8 (39.9–56.5)	64.5±2.7 (61.8–68.6)	57.6±4.2 (50.4–62.8)
c'	2.2±0.2 (2.0–2.6)	1.2±0.1 (0.9–1.3)	1.7±0.2 (1.5–1.9)
V	65.1±1.2 (63–67)	52.4±1.1 (51–54)	63.8±1.9 (60–67)
G_1	6.1±0.9 (4.5–7.1)	4.8±0.6 (4.3–5.5)	8.1±1.1 (7.1–10.0)
G_2	6.2±1.1 (4.1–7.2)	5.4±0.5 (5.1–6.0)	8.4±1.2 (6.8–9.4)
Odontostyle length	71.1±2.0 (70.0–74.0)	92.2±3.4 (88.0–97.0)	61.3±3.5 (54.0–65.5)
Odontophore length	36.6±0.9 (35.0–37.5)	49.7±3.0 (46.0–53.5)	36.9±2.7 (34.0–40.0)
Lip region width	8.0±0.3 (7.5–8.5)	9.5±0.5 (8.5–10.0)	8.1±0.6 (7.3–8.6)
Oral aperture-guiding ring	56.3±3.4 (53.5–60.0)	76.4±4.8 (70.0–82.0)	48.6±1.9 (47.5–51.5)
Tail length	30.6±3.1 (26.5–36.5)	28.6±1.2 (27.0–30.5)	26.3±1.5 (24.0–28.0)
J	7.3±1.3 (6.5–8.5)	6.5±1.0 (5.5–8.5)	7.9±0.8 (6.5–8.5)

Abbreviations are defined in Jairajpuri and Ahmad (1992)

and female gonoducts without sperm present. The presence of *X. duriense* is the first record for Spain, and the third after the original description from Portugal (Lamberti et al. 1993). These data indicate that this species may be an Iberian endemic species as suggested by Peña-Santiago et al. (2006).

The alpha-numeric codes for *X. duriense* to be applied to the polytomic identification key for the *Xiphinema americanum*-group species by Lamberti et al. (2000) are: A 2, B 12, C 1, D 34, E 3, F 21, G 1, H 1, I 23, J 1.

Xiphinema incertum Lamberti et al. 1983 (Fig. 8, Table 6)

Female The Spanish population of this species was characterised by a body forming a coiled spiral when killed with heat, a lip region slightly expanded and set off by a constriction, less pronounced in some specimens (Fig. 8) separated from the body by a depression, female reproductive system amphidelphic with two equally developed genital branches and absence of uterine differentiation, vulva a transverse slit located posterior to mid-body, vagina occupying about 1/2 of the corresponding body diameter, female tail conoid with narrowly rounded terminus and bearing two pairs

of caudal pores (Fig. 8). Males unknown. The morphology and morphometrics of this population closely agree with the original description from several localities in the Balkan region e.g. Bulgaria, Croatia, Serbia (Lamberti et al. 1983; Barsi and Lamberti 2002), except for lower a, c' and V ratios (49.7, 1.2, 52.4 vs 57, 1.5, 57, respectively) and a higher b ratio (9 vs 6.4). The observed differences extend the known range of the features in *X. incertum*. *Xiphinema incertum* in the Balkan region co-occurs with *X. pachtaicum* and is also morphologically very similar to it except for tail shape, and Barsi and Lamberti (2002) questioned the validity of *X. incertum*. To avoid inflation of the number of new species, we currently consider the few differences observed as intraspecific until molecular data are available from the populations of the Balkan region. This species is quite similar to *Xiphinema franci* Heyns and Coomans 1994, *X. intermedium* and *X. tenuicutis*, but can be differentiated from *X. franci* in L, a, b, c, and c' values (1,800 μm, 49.7, 9.0, 64.5, 1.2 vs 1,430 μm, 40.4, 4.7, 47.5, 1.4, respectively), a shorter hyaline region (6.5 μm vs 12.5 μm) and the tail shape (rounded terminus vs pointed terminus), from *X. intermedium* in a higher c ratio (64.5 vs 47.0), a longer odontostyle (92.2 μm vs
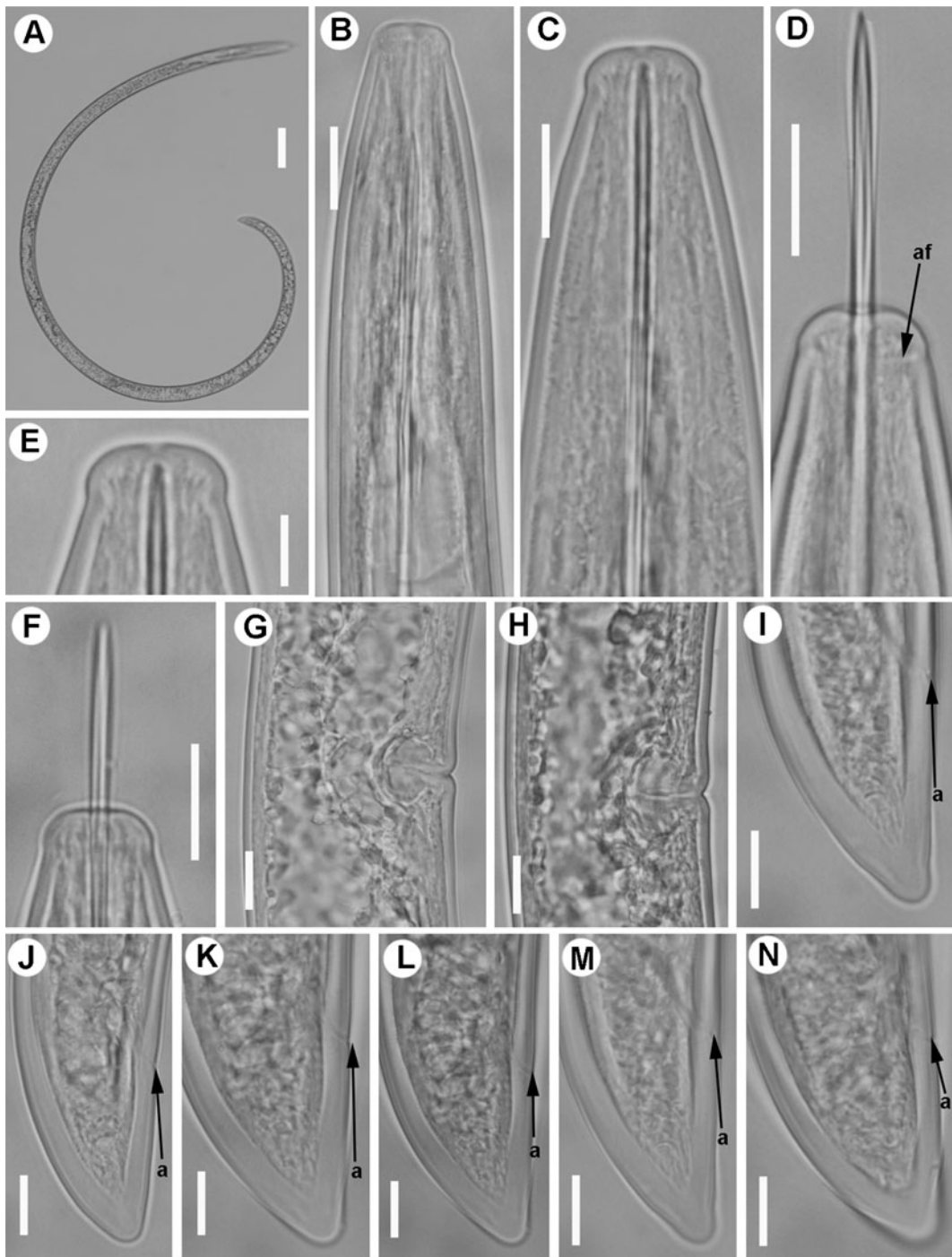
Fig. 8 Light micrographs of *Xiphinema incertum* Lamberti et al. 1983 from southern Spain. **a** Whole female; **b–f** Anterior regions; **g–h** Vulval regions; **i–n** Female tail regions. Abbreviations: *a* anus; *af* amphidial fovea. (Scale bars: **a**=100 μm; **b–d**, **f–n**=10 μm; **e**=5 μm)

76.0 μm), a shorter tail and hyaline region (28.6 μm, 6.5 μm vs 33.0 μm, 9.0 μm) and the tail shape (ventrally straight with narrowly rounded terminus vs

ventrally curved and concave with pointed terminus), and from *X. tenuicutis* by the shape of lip region (less expanded and separated from the rest of the body by

an obscure depression in *X. tenuicutis*) and a longer odontostyle and odontophore (92.2 μm, 49.7 μm vs 76.0 μm, 45.0 μm, respectively). The presence of *X. incertum* is the first record for the Iberian Peninsula and the third from Europe after the original description in Bulgaria and later in Croatia (Lamberti et al. 1983; Barsi 1989).

The alpha-numeric codes for *X. incertum* to be applied to the polytomic identification key for the *Xiphinema americanum*-group species by Lamberti et al. (2000) are (in parentheses are exceptions): A 2, B 2(3), C 2, D 2(13), E 3 (12), F 1(2), G 2(1), H 3(2), I 2(1), J 2.

Xiphinema opisthohysterum Siddiqi 1961 (Fig. 9, Table 6)

Female The Spanish population of this species was characterised by a body forming a coiled spiral when killed with heat, lip region widely expanded, of elliptical shape and clearly offset from the body by a constriction, female reproductive system with two equally developed genital branches and absent uterine differentiation, vulva clearly posterior to mid-body, vagina occupying about 1/2 of the corresponding body diameter, female tail conical and elongated with almost pointed terminus and bearing two pairs of caudal pores (Fig. 9). Males not found. Morphology and morphometrics of this population agree closely with the redescription by Lamberti and Bleve-Zacheo 1979 (Table 6), except for L, c' and V ratio (1,377–1,550 μm, 1.5–1.9, 60–67 vs 1,800–1,850 μm, 1.9–2.0, 56–59, respectively) and a shorter tail (24–28 μm vs 30–36 μm). Nevertheless, these differences may be due to the low number of specimens (two) studied by Lamberti and Bleve-Zacheo (1979), and do not exceed the intraspecific variation of species from the *X. americanum* group. Also, this species is quite similar to *X. duriense* and *X. tenuicutis*. From *X. duriense* it can be differentiated in lower c' (1.5–1.9 vs 1.9–2.3) and a shorter odontostyle and distance from guiding ring to anterior end (61.3 μm, 48.6 μm vs 70.0 μm, 61.0 μm, respectively), and from *X. tenuicutis* by the shape of lip region (separated from the rest of the body by a deep depression vs slight depression), a higher a and V ratio (59.2, 63.8 vs 46, 52.4, respectively), and a shorter odontostyle, odontophore and distance from guiding ring to anterior end (61.3 μm, 36.9 μm, 48.6 μm vs 76.0 μm,

45.0 μm, 70.0 μm, respectively). The presence of *X. opisthohysterum* it is the first record for Spain, and confirms its occurrence in the Iberian Peninsula, first recorded by Sturhan (1983) in North-East Portugal.

The alpha-numeric codes for *X. opisthohysterum* to be applied to the polytomic identification key for the *Xiphinema americanum*-group species by Lamberti et al. (2000) are: A 2, B 12, C 1, D 32, E 3, F 12, G 12, H 1, I 12, J 1.

Xiphinema rivesi Dalmasso 1969 (Figs. 6 and 10 Table 7)

Female Spanish populations of this species are recognisable by a rounded lip region, continuous with rest of the body, vulva slightly posterior to mid-body, female tail conoid with acute rounded tip (Fig. 10). The morphology and morphometrics of these populations are most similar among them (Table 7) and to other populations of this species from Southern Spain (Gutiérrez-Gutiérrez et al. 2011a), except for a slightly higher c' ratio and a more narrow lip region in the population from Niebla (Table 7), but do not exceed the intraspecific variations, as confirmed by molecular analyses (see below). Similarly, morphology and morphometrics traits of these three Spanish populations agree very well with the original description of this species (Dalmasso 1969) and detailed study of paratypes (Figs. 6, 10) from USDA Nematode Collection (slide T-3440p) (Fig. 6), except for a slightly higher b ratio in the population from Alcala de Guadaira (7.4 vs 6.2) and narrower lip region in the population from Niebla (7.9 μm vs 10 μm), and some differences in a, c, c' ratio, odontostyle, distance from guiding ring to anterior end, and tail length, which do not exceed the intraspecific variations as showed for these Spanish population. Also, morphology and morphometrics traits of these three Spanish populations agree very well with the other description of *X. rivesi* associated to grapevine from Central Portugal (Lamberti et al. 1994, Fig. 6w–y), except for some almost negligible differences in a, b ratio and hyaline region length, which do not exceed either the intraspecific variations.

Only one male was found in this investigation (Fig. 10, Table 7), which is similar to female except for reproductive system, and was mainly characterized by six ventromedian precloacal papillae and anterior to adanal pair, and spicules well developed (Fig. 10, Table 7).
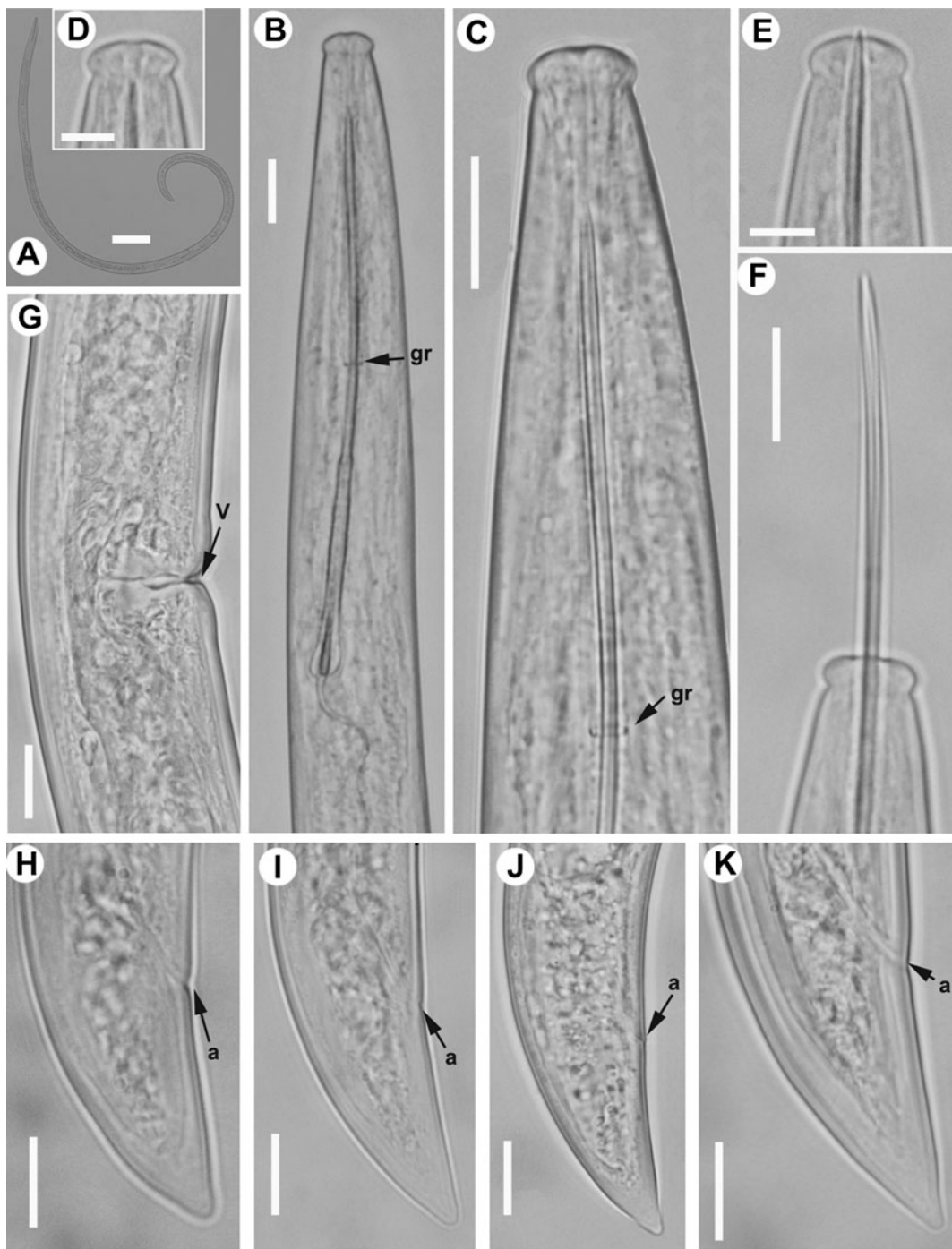
Fig. 9 Light micrographs of *Xiphinema opisthohystherum* Siddiqi 1961 from southern Spain. **a** Whole female; **b, c** Female neck regions; **d–f** Anterior regions; **g** Vulval regions; **h–k** Female tail regions. Abbreviations: *a* anus; *gr* guiding ring; *V* vulva. (Scale bars: **a**=100 μm; **b–c, f–k**=10 μm; **d, e**=5 μm)

General morphology and morphometrics agree very well with the original description of this species (Dalmasso 1969) and other reports (Urek et al. 2005). This species can be differentiated from *X. franci* by a longer body (1,886 μm vs 1,430 μm), a shorter hyaline tail region (8.6 μm vs 12.5 μm) and a different shape of the
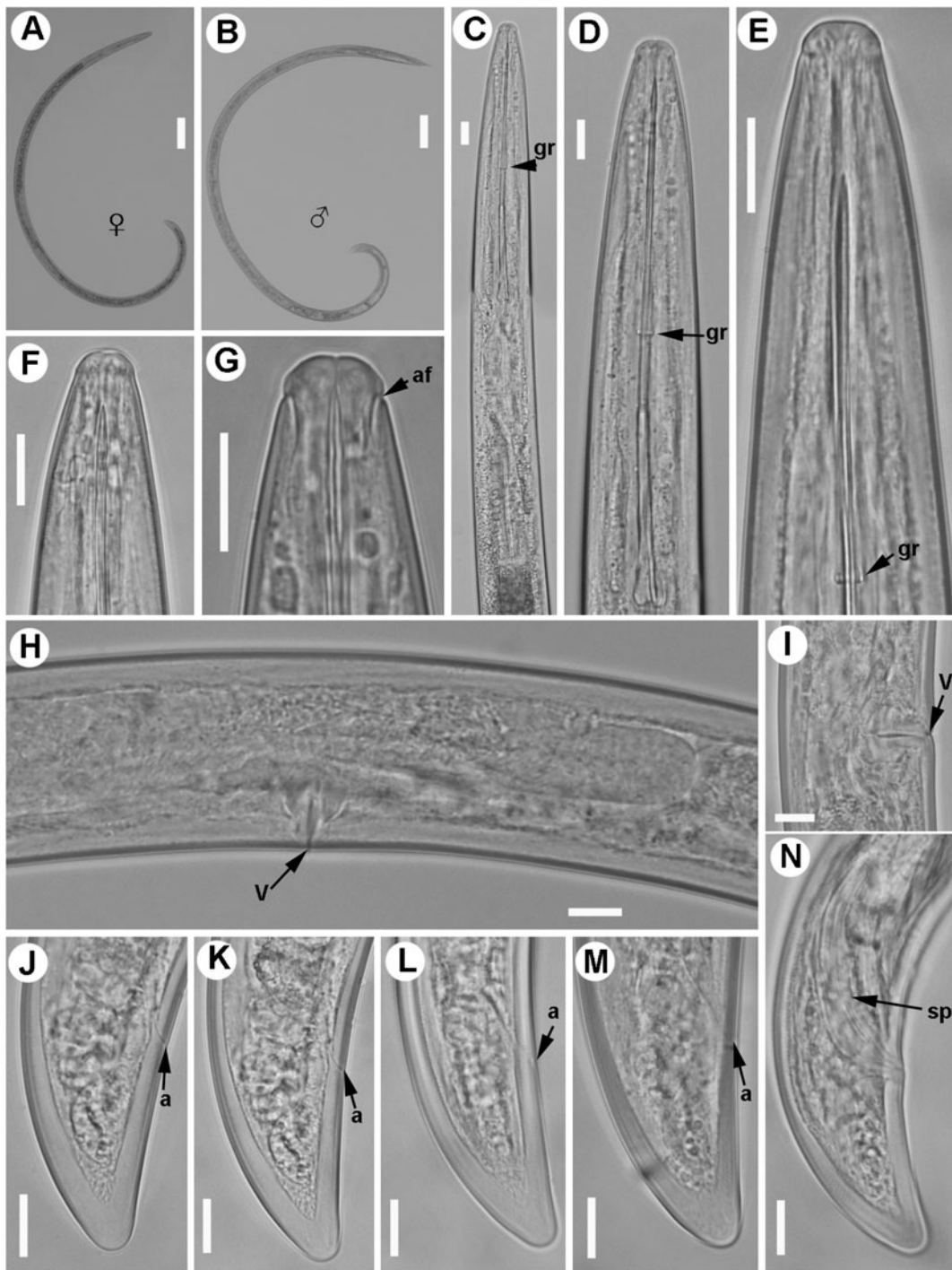
Fig. 10 Light micrographs of *Xiphinema rivesi* Dalmasso 1969 from southern Spain. **a** Whole female; **b** Whole male; **c** Female neck region; **d–g** Anterior regions; **h**, **i** Vulval regions; **j–m** Female tail regions; **n** Male region. Abbreviations: *a* anus; *af* amphidial fovea; *gr* guiding ring; *sp* spicules. (Scale bars: **a**, **b**= 100 μm; **c–n**=10 μm)

lip region (continuous with rest of the body vs offset from the rest of the body by a depression); from *X. luci*

by the tail shape (shorter and more rounded in *X. luci*) and the shape of the lip region (rounded vs flattened on

Table 7 Morphometrics of *Xiphinema rivesi* Dalmasso 1969 from several localities. All measurements in μm and in the format: mean± s.d. (range)

Locality	Alcalá de Guadaira	Castillo de Locubín	Niebla	
Character	Females	Females	Females	Male
n	10	10	10	1
L	1,886±127 (1,583–2,055)	1,831±124 (1,572–1,922)	1,875±107 (1,788–1,961)	1,605
a	45.7±3.3 (37.2–48.4)	48.6±4.2 (43.4–54.4)	50.8±1.8 (46.9–52.6)	52.6
b	7.4±0.9 (6.4–8.4)	8.0±1.9 (5.8–11.8)	7.6±1.1 (6.2–8.9)	7.6
c	53.4±3.3 (49.1–58.5)	60.3±5.8 (49.1–67.4)	53.4±4.3 (48.1–56.1)	50.2
c'	1.4±0.1 (1.2–1.5)	1.3±0.1 (1.1–1.5)	1.5±0.1 (1.4–1.7)	1.3
V/T	53.0±1.0 (52–55)	52.9±1.1 (51–54)	52.9±1.0 (51–54)	26.8
G_1	4.8±2.3 (3.4–8.2)	11.2±1.4 (11.1–12.6)	8.4±1.9 (5.2–10.1)	–
G_2	5.3±2.0 (3.9–8.2)	8.9±0.9 (7.8–10.4)	7.5±0.9 (6.5–9.1)	–
Odontostyle length	92.1±5.9 (79.0–98.5)	88.0±3.8 (83.0–95.5)	89.8±3.5 (81.5–94.5)	87.0
Odontophore length	51.7±3.8 (43.5–56.5)	48.5±2.9 (41.0–52.0)	49.8±2.3 (48.0–53.0)	48.5
Lip region width	9.9±0.9 (8.5–12.0)	8.9±0.7 (8.5–10.0)	7.9±0.3 (7.5–8.0)	8.5
Oral aperture-guiding ring	79.3±6.1 (65.5–87.0)	66.4±10.3 (50.0–80.5)	74.7±4.8 (65.0–80.5)	71.5
Tail length	35.4±2.5 (31.5–39.0)	30.5±2.0 (28.0–33.5)	35.2±2.3 (32.0–39.03)	32.0
J	8.6±0.7 (7.5–8.5)	7.5±1.0 (6.0–9.0)	8.2±1.2 (7.5–10.0)	7.0
Spicules	–	–	–	47
Lateral accessory piece	–	–	–	13.5

Abbreviations are defined in Jairajpuri and Ahmad (1992)

top); from *Xiphinema occiduum* Ebsary et al. 1984 by a lower L, b and c ratios (1,886 μm, 7.4, 53.4 vs 2,300 μm, 9, 70, respectively), a longer odontostyle (92.1 μm vs 75 μm), a different lip region shape (continuous with rest of the body vs offset from the rest of the body by a constriction) and a smaller number of ventromediam supplements (6 vs 9). And from *X. oxycaudatum* by a higher b ratio (7.4 vs 5.5), a slightly longer odontostyle (92.1 μm vs 82 μm), lip region shape (continuous with rest of the body vs offset from the rest of the body by constriction), and tail shape (with rounded terminus vs pointed terminus).

Xiphinema rivesi, originally described from South-East France (Dalmasso 1969), has been subsequently reported from a great number of localities of European countries including Portugal, Slovenia and Spain (Lamberti et al. 1994; Bello et al. 2005; Urek et al. 2005; Gutiérrez- Gutiérrez et al. 2011a) as well as from North America (Ebsary et al. 1984; Robbins 1993; Mekete et al. 2009), South and Central America (Doucet et al. 1998; Auger et al. 2009), Iran and Pakistan (Nasira and Maqbool 1994; Fadaei et al. 2003) and Western Australia (Sharma et al. 2003). The alpha-numeric codes

for *X. rivesi* to be applied to the polytomic identification key for the *Xiphinema americanum*-group species by Lamberti et al. (2000) are: A 1, B 2, C 21, D 23, E 23, F 1, G 12, H 23, I 32, J 2.

Although there is a general agreement about the morphology and intraspecific variability of this species, rDNA markers suggest an unresolved complex of cryptic species, since populations from Spain, Pennsylvania and Prosser, Washington (USA) were clearly separated in three different clades (see below). These findings did not support the hypothesis by Lamberti and Ciancio (1993) suggesting that *X. rivesi* may have been introduced from North America into Europe, since *X. rivesi* is known as a vector species of *CLRV, TomRSV*, and *TRSV*, in North America (Georgi 1988; Robbins and Brown 1991; Brown and Halbrendt 1992) and *TomRSV* and *TRSV* in Slovenia. (Širca et al. 2007).

Xiphinema santos Lamberti et al. 1993 (Figs. 6, 11, Table 8)

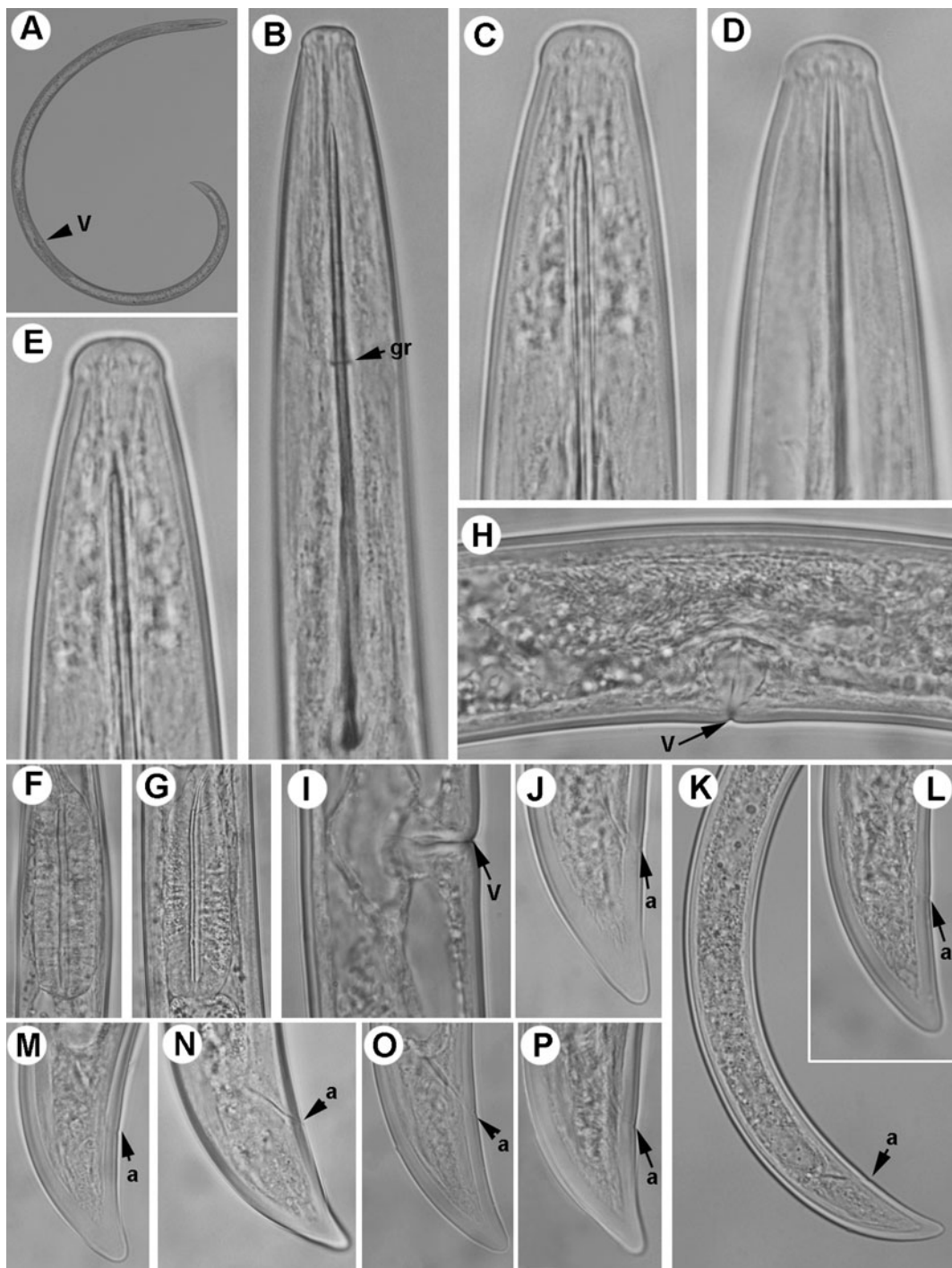*Female* The Spanish population of this species was characterised by a body forming a close C-shaped

Fig. 11 Light micrographs of *Xiphinema santos* Lamberti et al. 1993 from southern Spain. **a** Whole female; **b** Female neck region; **c–e** Anterior regions; **f**, **g** Detail of pharyngeal bulb; **h**, **i** Vulval regions; **j–p** Female tail regions. Abbreviations: *a* anus; *gr* guiding ring; *V* vulva. (Scale bars: **a**=100 μm; b–l=10 μm)

to coiled spiral when killed by heat. Cuticle smooth, 2.1±0.2 (2.0–2.5) μm thick along body

but thicker at tail tip (Table 8). Lip region rounded anteriorly, slightly expanded and separated from

Table 8 Morphometrics of *Xiphinema santos* Lamberti et al. 1983 from Rociana del Condado, Huelva province, southern Spain. All measurements in μm and in the format: mean±s.d. (range)

Character/life-stage	Females	J3	J4
n	9	1	4
L	1,504±75 (1,466–1,633)	844	1,152±104 (1,050–1,294)
a	44.3±2.1 (42.7–48.1)	37.5	41.6±6.0 (36.2–50.2)
b	6.2±0.9 (4.7–7.6)	8.1	4.9±0.9 (3.8–6.0)
c	53.3±6.2 (45.9–65.3)	26.0	34.8±2.9 (30.9–37.9)
c'	1.3±0.1 (1.0–1.4)	2.0	1.8±0.2 (1.5–2.0)
V	52,4±1.1 (52–55)	–	–
G_1	8.0±0.9 (6.8–8.5)	–	–
G_2	7.5±1.0 (6.4–8.9)	–	–
Odontostyle length	78.9±2.1 (76–82)	51	62.4±1.6 (61–64)
Replacement odontostyle length	–	64	78.1±2.8 (75–81.5)
Odontophore length	44.1±2.3 (41–49)	31	38.4±3.9 (35–43)
Lip region width	10.2±0.7 (9.5–11.5)	9.5	9.4±0.3 (9.0–9.5)
Oral aperture-guiding ring	64.4±1.8 (60–67)	43.0	51.4±1.5 (50–54)
Tail length	28.5±2.9 (25–34)	32.5	33.3±3.2 (31–38)
J	9.8±1.7 (6.5–12.0)	7.0	8.7±1.3 (7.0–10.0)

Abbreviations are defined in Jairajpuri and Ahmad (1992)

the body by a depression, 3.3±0.3 (3.0–3.5) μm high. Pharyngeal basal bulb 70.3±8.4 (61.5–84.0) μm long and 19.2±4.6 (15.5–29.5) μm wide, occupying about 1/3 to 1/4 of the total pharyngeal length (Fig. 11f,g). Pharyngeal-intestinal valve inconspicuous, hemispherical. Reproductive system amphidelphic, both branches apparently equally developed, opposed and reflexed, without any uterine differentiation. Ovaries with symbiont bacteria, oviducts relatively long. Vulva slightly post-equatorial, a transverse slit-like; vagina 15.3±1.1 (13.5–17.0) μm long with short distal part and well-developed proximal. Prerectum often indistinct. Rectum 18.8±2.6 (17.5–25.5) μm long, or 1.0–1.2 times the anal body diameter. Tail short, conoid, weakly curved ventrally with conoid-rounded terminus, bearing two pairs of caudal pores (Fig. 11j–p). Two juvenile stages were detected (third- and fourth-stage), which were morphologically similar to females apart from smaller body and longer, more tapered tails (Table 8). The present population of *X. santos* was found in a sandy soil around the roots of grapevine (*Vitis vinifera* L.) and Stone pine (*Pinus pinea* L.) at Rociana del Condado, Huelva province, southern Spain; it is the first record for Spain, and confirms its presence in the Mediterranean Basin, after several reports in Portugal and North Egypt (Lamberti et al. 1994, 1996).

Morphology and morphometrics of this population agree with original description by Lamberti et al. (1993), except for L, a, c' ratio, and tail length (1,466–1,633 μm, 42.7–48.1, 1.0–1.4, 25.0–34.0 μm vs 1,700–2,000 μm, 54.0–59.0, 1.5–1.8, 32.0–37.0 μm respectively). Nevertheless, these differences are within the range of intraspecific variation, as showed in different Portuguese populations of this species (Lamberti et al. 1994). This species is also close to *X. intermedium*. After detailed study of paratypes (Fig. 6) from Rothamsted and USDA Nematode Collection (T-3449p) the Spanish population differs from *X. intermedium* by female tail (25–34 μm long, short, conoid, weakly curved ventrally with conoid-rounded terminus vs 31–38 μm long, conoid elongated, dorsally convex and ventrally clearly curved and concave with pointed terminus, Fig. 11g–m), and some differences in measurements and ratios, including body length (1,466–1,633 μm vs 1,400–1,900 μm), odontostyle and odontophore length (76–82, 41–49 μm vs 68–80, 39–50 μm, respectively), V (52–55 vs 50–57), a, c, and c'

ratio (43–48, 46–65, 1.0–1.4 vs 38–51, 41–52, 1.3–1.7, respectively).

The alpha-numeric codes for *X. santos* to be applied to the polytomic identification key for the *Xiphinema americanum*-group species by Lamberti et al. (2000) are: (A 2, B 12, C 1, D 21, E 21, F 1, G 12, H 2, I 23, J 2).

Phylogenetic relationships of the Xiphinema americanum-*group*

The amplification of D2-D3 expansion segments of 28S rDNA, ITS1, partial 18S, and *COI* regions yielded a single fragment of approximately 800 bp, 1,030 bp, 1,600 bp, and 400 bp, respectively, based on gel electrophoresis. Sequences from other species of *Xiphinema americanum*-group obtained from NCBI were used for further phylogenetic studies. Sequences for *X. parabrevicolle* n. sp., *X. parapachydermum* n. sp., *X. paratenuicutis* n. sp., *X. duriense*, *X. incertum*, *X. opisthohysterum*, *X. pachtaicum*, *X. rivesi*, and *X. santos* were obtained for these species in this study. Sequences for *X. pachtaicum* and *X. rivesi* matched well with former sequences deposited in GenBank, except for one isolate of *X. rivesi* from Pensylvania, USA, extending the molecular diversity of these species to newly studied areas.

Xiphinema parabrevicolle n. sp. (JQ990042) matched well with the D2-D3 sequences of the *X. americanum*-group deposited in GenBank, being 98 % to 97 % similar to some of them, such as, *X. citricolum* (DQ299490-DQ299494 and DQ285668), *X. santos* (AY601587), *X. brevicolle* (HQ184473), and *X. diffusum* (AY601600). ITS1 sequence showed similarity with several sequences of *X. brevicolle* with 85 % similarity (HQ184474, FM211421-25, AY430190), and 84 % with *X. diffusum* (AY359858). Using partial 18S, many similar sequences were found differing in seven nucleotides with a 99 % similarity. *COI* sequences from *X. parabrevicolle* n. sp. did not show any similarity with other sequences from *Xiphinema americanum*-group or non *americanum*-group, but showed a 75 % similarity with *Longidorus helveticus* Lamberti et al. 2001 (EF538747).

Xiphinema parapachydermum n. sp. D2-D3 showed 96 % similarity with *X. pachtaicum* (HM921393-HM921395), and 88 % with *X. pachydermum* (AY601608). Intra-specific variation detected among the three studied populations was four nucleotides. Partial 18S also agree with results obtained from D2-D3, the maximum similarities were related to '*X.

pachtaicum' (AM086682) with 98 % similarity. No similarities in the GenBank were found for ITS1 and *COI* sequences in this species.

Xiphinema paratenuicutis n. sp. D2-D3 showed 85 % similarity with *X. brevisicum* (AY601610), and 84 % to 83 % with some species, including *X. simile*, *X. pachydermum*, *X. pachtaicum*, *Xiphinema utahense* Lamberti and Bleve-Zacheo 1979, *Xiphinema thornei* Lamberti and Golden 1986 (AY601609, AY601608, AY601607, AY601598, AY601595, respectively). Sequencing of the ITS1, partial 18S and partial *COI* sequences were not successful despite several attempts.

Xiphinema duriense D2-D3 (JQ990032) showed 89 % similarity with *X. brevisicum* (AY601610), and 88 % to 87 % with *X. pachydermum*, *X. pachtaicum*, *X. americanum* group sp. LZ-2011 (AY601608, AY601607, JN091972, respectively). Partial *COI* sequence did not show any similarity with sequences deposited in the GenBank. *Xiphinema incertum* (JQ990031) was closely related in D2-D3 sequence to other species of the *Xiphinema americanum*-group such as *X. pachtaicum* (HM921355 and HM921356) with 98 % similarity and '*X. pachtaicum*' (AY601607) with 93 % similarity, and ITS1 showed 94 % similarity with *X. pachtaicum* (AY430178, HM921337). However, no similarities in the GenBank were found for *COI* sequences in this species. D2-D3 region from *X. opisthohysterum* (JQ990040) showed similarity with *X. brevisicum* (AY601610) with similarity value of 88 to 87 % with *X. brevisicum*, *X. pachydermum*, X. *americanum* group sp. LZ-2011 (AY601610, AY601608, AY601607, JN091972, respectively); and partial *COI* sequence did not show any similarity with sequences deposited in the GenBank. D2-D3 and partial *COI* sequences from *X. pachtaicum* (JQ990033, JQ990057) matched well with sequences from *X. pachtaicum* deposited in GenBank, being 99 % similar with all of them (HM921393-HM921395, HM921369-HM921379, respectively). Likewise, *X. rivesi* D2-D3 region matched closely (99 % similarity) with other species of *X. americanum*-group such as *X. rivesi* (AY210845, HM921357 and HM921358) and *X. americanum* (AY580056). Intra-specific differences were not found among our populations. ITS1 showed even higher similarities with many *X. americanum*-group species as *X. rivesi* (HM921338, 100 %), *X. inaequale* (GQ231530, 98 %), *X. thornei* (AY430176, 97 %); intra-specific variability within our populations was low (99 %). Partial 18S also showed

high similarity with other *X. americanum*-group species such as *X. rivesi* (HM921344), 99 % similarity, differing in 5 nucleotides. Some similarity was found with *COI* sequence, 85 % to 84 % similarity with *X. americanum* (AY382608) and *X. peruvianum* (AM086692). Finally, *X. santos* D2-D3 matched well with the *X. americanum*-group deposited in GenBank, being identical to *X. santos* (AY601587), and 99 % similar to some other species, including *X. citricolum* (DQ299490-DQ299494 and DQ285668), *Xiphinema georgianum* Lamberti and Bleve-Zacheo 1979 (DQ299495-DQ299502). Similar results were detected for other markers such as ITS1 and partial 18S. ITS1 showed 97 % similarity with several *X. americanum*-group species such as *Xiphinema americanum* Cobb 1913 (AF511423, AY430189), *Xiphinema laevistriatum* Lamberti and Bleve-Zacheo 1979 (DQ299529), and *X. georgianum* (GQ299521). Also, partial 18S showed a high interspecific similarity, since many sequences differing in only 11 nucleotides (99 % similarity), indicating that this sequence is not adequate for species delimitation. Partial *COI* sequence showed 86 % similarity with *X. peruvianum* (AM086692) and 83 % with *X. brevicolle* (AM086707). Sequencing of the ITS1, partial 18S and partial *COI* sequences for some species and populations were not successful despite several attempts (Table 1).

Phylogenetic analysis (BI and ML) of *X. americanum*-group based on D2-D3 expansion segments of 28S rDNA of a multiple edited alignment including 52 sequences and 766 total characters showed two clades clearly separated and supported (Fig. 12). The phylogenetic analysis showed well supported groups at major and terminal clades in both analyses. The phylogenetic tree resolved two major clades: (I) with 37 sequences including *X. parabrevicolle* n. sp., *X. brevicolle*, *X. santos*, *X rivesi*, and other species, while clade (II) with 14 sequences, includes *X. parapachydermum* n. sp., *X. pachydermum*, *X. paratenuicutis* n. sp., *X. duriense, X. incertum, X. opisthohysterum*, and *X. pachtaicum* among others (Fig. 12). In group I, *Xiphinema parabrevicolle* n. sp. (JQ990042) formed a non well supported clade with *X. brevicolle*, *X. taylori, X. diffusum, X. inaequale, X. lambertii*, and *X. americanum* s. l. from Japan (AB635403) but within the group it is separated from *X. brevicolle* of which the three sequences (HM163209 HQ184473, and AB635401) do not cluster together. Further there is no support for the sister group composed of three

sequences of *X. santos* (one from Portugal and another from Spain), and two sequences of *X. citricolum* (from Florida) with no resolution on their relationship. Group I shows a basal group composed of 11 sequences of five species among them *X. rivesi* from Spain (5 sequences) form a non-supported group separated from *X. rivesi* (USA); also the two sequences of *X. bricolensis* do not group together. In group II, *Xiphinema parapachydermum* n. sp. (JQ990034-JQ990036) formed a well supported clade with *X. pachtaicum* and *X. incertum* (JQ990031), as well as with the sister group *X. pachydermum* (AY601608) (Fig. 12). Similarly, *X. duriense* (JQ990032) formed other a well supported clade with *X. opisthohysterum* (JQ990040) as well as *X. simile* with *X. parasimile*. The relationship of the three former clades and of *X. paratenuicutis* n. sp. (JQ990041) within group II is not resolved.

The ITS1 sequences were obtained from five species (Table 1). Phylogenetic analysis (BI and ML) of *X. americanum*-group based on ITS1 from sequences showing major similarities were included in the analysis, other sequences not included performed weak alignments. Multiple edited alignment including 34 sequences with 842 positions in length showed two clearly separated and supported clades (I and II, Fig. 13). Clade I included 30 sequences and 17 species, separated in two supported sub-clades with 11 and five species, respectively, a) *X. georgianum*, *X. citricolum*, *X. peruvianum*, *X. oxycaudatum*, *X. santos*, *X. laevistriatum*, *X. rivesi* (JQ990046, JQ990047, HM921338, AY430186, AY430185), *X. thornei*, *X. inaequale*, *X. californicum*, *X. brevicolle*, *X. floridae*, and *X. tarjanense*; and b) *X. brevicolle* (FM211425, AY359856), *X. americanum*, *X. diffusum*, *X. incognitum*, and *X. parabrevicolle* n. sp.; the latter subgroup is moderately supported and within it *X. brevicolle* is paraphyletic. For *X. rivesi*, the population found in USA (AY430185 and AY430186) is clearly not closely related phylogenetically with European populations. Clade II showed an unresolved relationship with group I. It comprises three species with *X. parabrevicolle* as a sister group of the well supported clade *X. incertum* and *X. pachtaicum*.

Phylogenetic analysis (BI and ML) of *X. americanum*-group based on the partial *COI* of a multiple edited alignment including 40 sequences with 383 positions in length showed six moderate to highly supported clades (Fig. 14). Clade I included 11 species
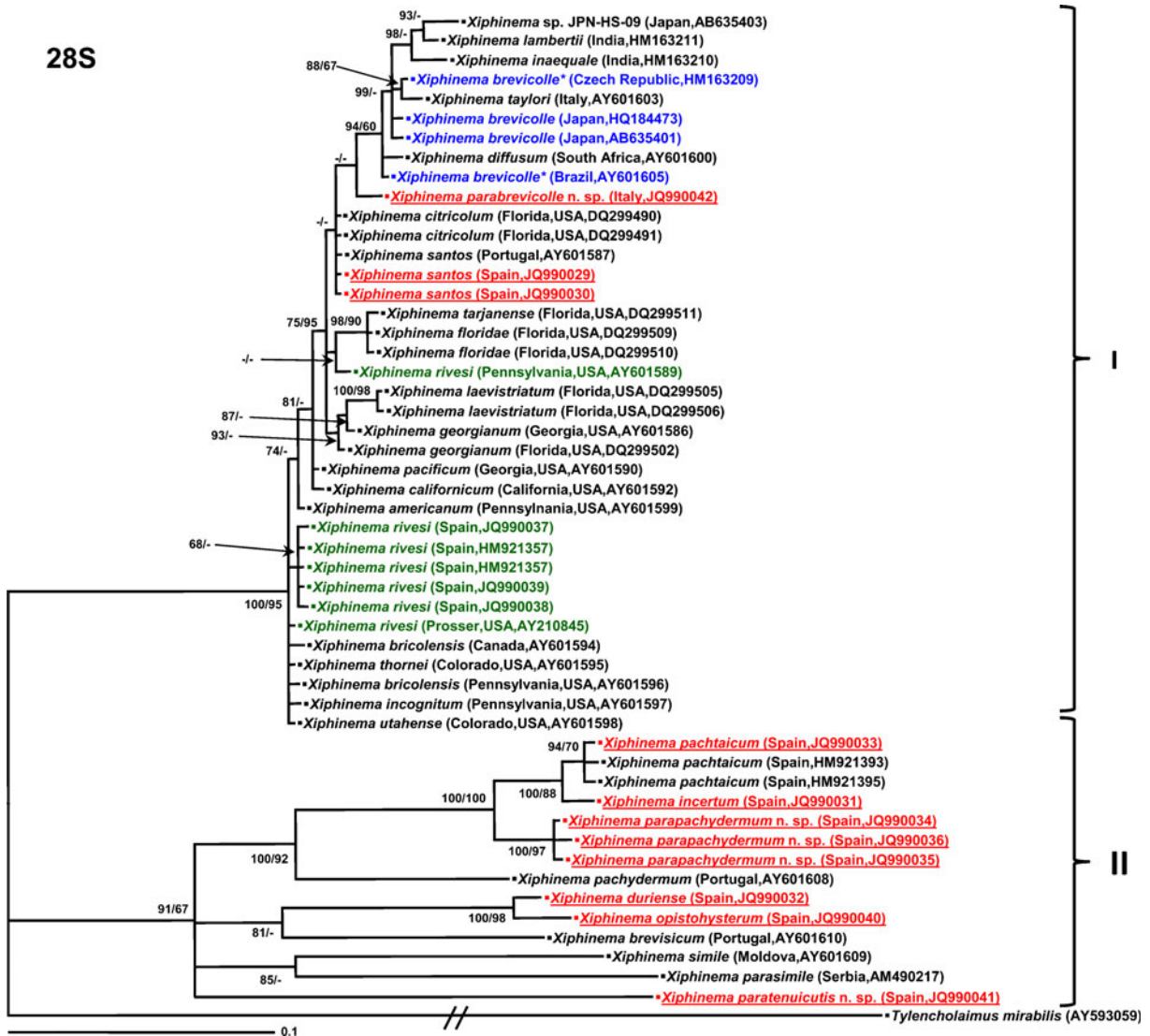
Fig. 12 Phylogenetic relationships within *Xiphinema americanum*-group complex. Bayesian 50 % majority rule consensus trees as inferred from D2 and D3 expansion segments of 28S rRNA sequences alignments under the GTR + G model. Posterior probabilities more than 65 % are given for appropriate clades; bootstrap values greater than 50 % are given on appropriate clades in ML analysis. Newly obtained sequences in this study are *underlined*. *: species name modified according to Monteiro (2010)

in two sub-clades, a) *X. santos*, *X. parabrevicolle* n. sp., *X. peruvianum* (USA, AM086692)*, X. citricolum*, *X.* cf. *americanum* RN-2005 (USA), and *X. americanum s.str.*; and b) *X. rivesi* (Arkansas,AM086697), *X. floridae*, *X. georgianum*, and *X. tarjanense*. Clade II included four species *X. duriense, X. opisthohysterum, X. peruvianum* (Brazil, AM086712), and *X. rivesi* (Spain, JQ990060). Clade III included 10 species in two sub-clades: a) *Xiphinema* sp. JPN-HS-09, *X. inaequale*, *X.* cf. *americanum* RN-2005 (Nepal), *X.*

lambertii, X. incognitum; and b) *X. diffusum, X. brevicolle* (Japan, AB604337), *X. taylori*, and *X. brevicolle* (Czech Republic, HM163206). Clade IV included three species *X. pachtaicum, X. parapachydermum* n. sp., and *X. incertum*. Clade V included two species *X. pachtaicum* (Czech Republic, GU222424) and *X. simile* (Slovakia, AM086708). Finally, clade VI included three species *X.* cf. *americanum* RN-2005 (Portugal), *X. simile* (Czech Republic, GU222427) and *X. simile* (Serbia, AM086711).
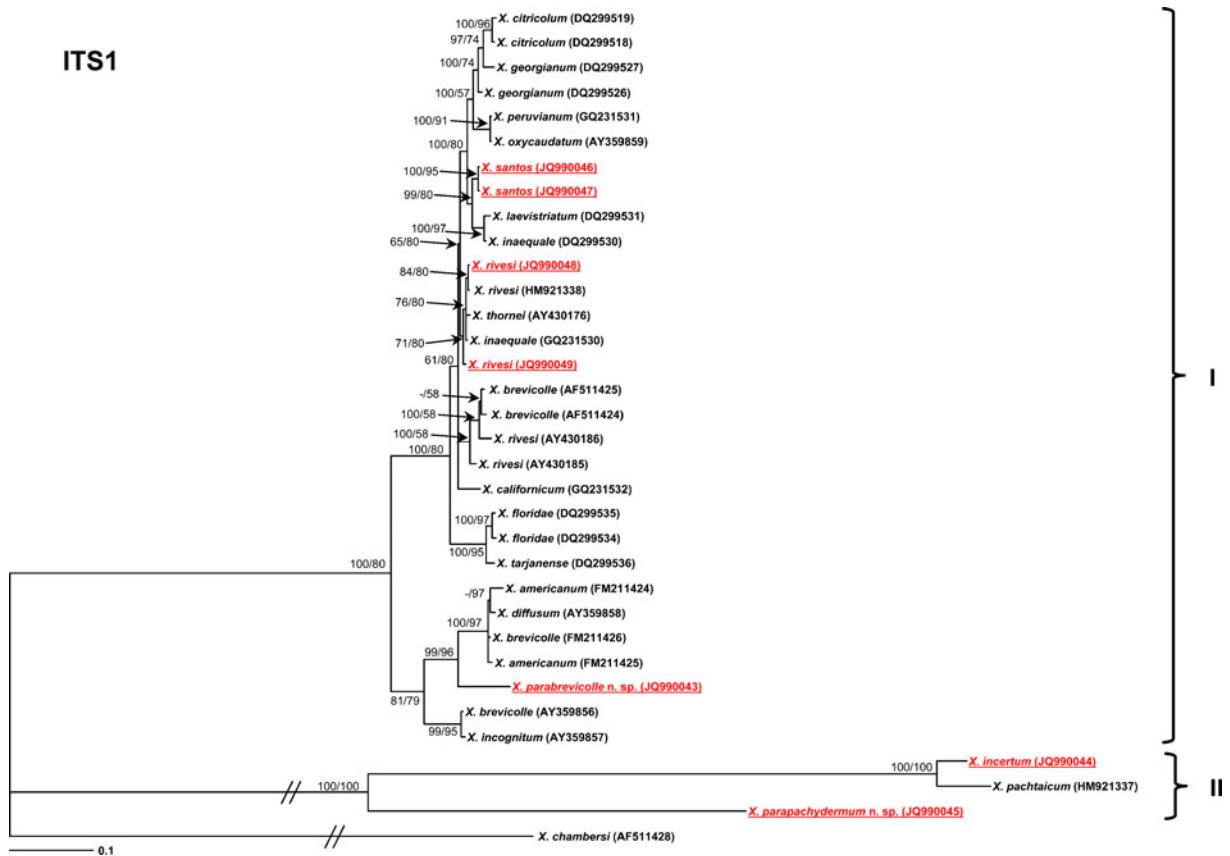
Fig. 13 Phylogenetic relationships within *Xiphinema americanum*-group complex. Bayesian 50 % majority rule consensus trees as inferred from ITS1 rRNA gene sequence alignment under the TMP3uf + G model. Posterior probabilities more than 65 % are given for appropriate clades (in *bold letters*); bootstrap values greater than 50 % are given on appropriate clades in ML analysis. Newly obtained sequences in this study are *underlined*

Discussion

The primary objective of this study was to identify and molecularly characterize species belonging to the *Xiphinema americanum*-group in cultivated and natural environments in southern Spain and one sample in Italy. Our results demonstrate that the application of rDNA and mtDNA molecular markers integrated with morphological studies revealed that some long-assumed single species are in fact cryptic species. Results of this study suggest that species from *Xiphinema americanum*-group show a high diversity in the southern part of Iberian Peninsula, and demonstrate that intensive surveys within a region may still increase substantially our knowledge of nematode diversity. However, the main contribution of this work in the *X. americanum*-group is the holistic approach of diagnosis, that also has critical phytopathological implications, since some species are virus vectors or are listed as A1 (*X. americanum*, *X. californicum*, *X. bricolensis*) and A2 (*X. rivesi*) quarantine organisms by the EPPO.

Phylogenetic analysis of D2-D3 of this and previous studies demonstrate that *X. brevicolle* comprises a complex of species with similar morphological traits but differing molecularly as shown in Figs. 12 and 13, implying the need for additional integrative studies for clarification. Current results agree with Lamberti et al. (2000) which discussed the synonymies proposed by Luc et al. (1998) within the *X. brevicolle* species complex (i.e. *X. diffusum*, *X. parvum*, *X. pseudoguirani*, *X. taylori*), as it was demonstrated later for some of these species by using ITS (Chen et al. 2005; Oliveira et al. 2005). Similarly, on the basis on D2-D3 and ITS1 phylogeny, *X. rivesi* appears to be also a species complex, since isolates from Spain were related to an isolate
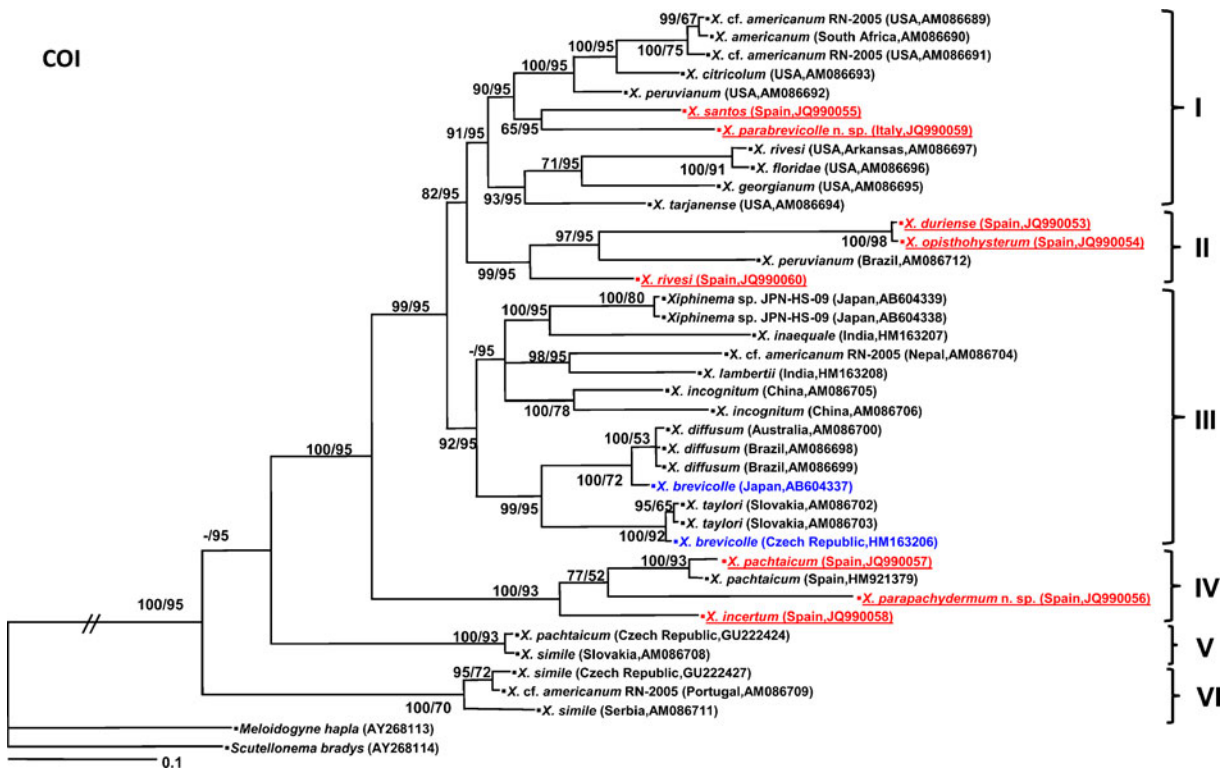
Fig. 14 Phylogenetic relationships within *Xiphinema americanum*-group complex. Bayesian 50 % majority rule consensus trees as inferred from *COI* gene sequence alignment under the TVM + I + G model. Posterior probabilities more than 65 % are given for appropriate clades (in *bold letters*); bootstrap values greater than 50 % are given on appropriate clades in ML analysis. Newly obtained sequences in this study are *underlined*

from Prosser, USA (AY210845), but separated from another isolate from Pennsylvania, USA (AY601589, AY430185, AY430186) (Figs. 12, 13). These differences were also confirmed in the phylogeny of partial *COI*, which clearly separate the Spanish isolates from an isolate from Arkansas, USA (AM086697) (Fig. 14). Similar differences were detected by Vrain (1993) studying several American isolates of *X. rivesi* using of restriction fragments of amplified internal transcribed spacer regions. Nevertheless, this situation needs to be clarified with additional integrative studies and using other molecular markers (i. e. ITS1 and *COI*) for those isolates, and also checking possible misidentification of some of these populations.

D2-D3 expansion segments of 28S rDNA and ITS1 showed congruent phylogenetic information. *Xiphinema parabrevicolle* n. sp. is well associated in the same clade as *X. brevicolle*, *X. diffusum* and *X. americanum*. Similar results were obtained for *X. rivesi*, in which one population found in USA (AY430185 and AY430186) is clearly not closely related phylogenetically with European

populations. *Xiphinema incertum* showed a congruent position as D2-D3 expansion segments of 28S rDNA with *X. pachtaicum* (HM921337). The position of *X. santos* is completely different, showing a well supported clade with *X. laevistratum* Lamberti and Bleve-Zacheo 1979 (DQ299531) and *X. inaequale* (DQ299530). However, the position of *X. santos* in D2-D3 analysis is not supported.

The phylogenetic relationships inferred in this study based on the D2-D3 of 28S rRNA, the ITS1 of rRNA gene, and partial *COI* sequences mostly agree with the morphological grouping of species obtained by the cluster analysis carried out by Coomans et al. (2001) based on key diagnostic characters of the genus *Xiphinema*. In comparison with a cluster analysis based on morphometrics that resulted in the subdivision of the *X. americanum*-group into several subgroups (Lamberti and Ciancio 1993), our results did not support these subgroups apart from the *X. pachtaicum* group (D2-D3 analysis), since species of these subgroups clustered in several clades (Figs. 12, 13, and 14). Nevertheless, as

stated by Coomans et al. (2001), molecular and morphological evolution may differ because of e.g. proceeding at different speed as a result of different mechanisms. The present study also rejects the hypothesis that *X. americanum*-group represents numerous morphotypes with large inter-and intra- population variability resulting from environmentally driven morphometric plasticity, based on the phylogeny of the 18S rRNA gene (Oliveira et al. 2004). Phylogenetic analyses of D2-D3 and partial *COI* regions in this study were congruent supporting the clade including *X. brevicolle*, *X. diffusum*, and *X. taylori* (Figs 12, 14), whereas *X. parabrevicolle* n. sp. clustered with *X. santos* and outside this group in the partial *COI* tree (Fig. 14). For ITS1, *X. parabrevicolle* n. sp. clustered in the sub-clade b) with *X. incognitum*, *X. diffusum*, *X. americanum*, and *X. brevicolle* ((AY359856, FM211426). Phylogenetic relationships based on the D2-D3 and ITS1 agree with those by He et al. (2005) and partially support the hypothesis by Luc et al. (1998) and Coomans et al. (2001), that the X. *pachydermum*-subgroup (including *X. brevisicum*, *X. exile*, *X. lafoense*, *X. longistilum*, *X. mesostilum*, *X. microstilum*, *X. pachydermum*) comprises a subgroup outside the *X. americanum*-group, as well as some other species belonging to other *X. americanum* subgroups, viz. *X. brevisicum*, *X. pachtaicum*, *X. incertum*, *X. duriense*, *X. opisthohysterum*, *X. simile* and *X. paratenuicutis* n. sp. (Figs. 12, 13). Nevertheless, the partial *COI* reject this hypothesis, since *X. parapachydermum* n. sp., *X. pachtaicum* from Spain and *X. incertum* clustered together and clearly within the *X. americanum*-group species (Fig. 14). These results agree with those by He et al. (2005) based on D2-D3 region which differentiate a group with *X. brevicolle* and *X. diffusum* (and other species), as well as the *X. pachtaicum* group including *X. pachydermum*, *X. brevisicum* and *X. simile*. Consequently the relationships of this subgroup remain unclear and need the availability of additional ITS sequences for a larger analysis and other molecular markers which may allow the construction of a more complete and precise phylogeny in this group. Surprisingly, the partial *COI* for *X. duriense* (JQ990053) and *X. opistohysterum* (JQ990053) showed 100 % similarity and 97 % alignment coverage from the query sequence, but were clearly separated by D2-D3 (96 % similarity and 99 % alignment coverage from the query sequence), which may suggests a recent speciation event in this gene and maybe only mutations

with amino acid changes occurred and they have been not kept in the species separation.

ITS1 diversity was higher than that for D2-D3 for differentiating species of the *X. americanum*-group and should be considered as a very useful marker for the identification of species in this group (Chen et al. 2005; Meza et al. 2011). Furthermore, our results on mtDNA did not support the hypothesis by Lazarova et al. (2006) on the apparent dichotomy existing among *X. americanum*-group populations from North America and those from Asia, South America and Oceania. On the contrary, as shown by the phylogenetic tree of partial *COI* sequence, the Italian and Spanish populations of the *X. americanum*-group clustered with populations from North America, South Africa, or Brazil (Fig. 14).

In summary, present study establishes the importance of using polyphasic identification highlighting the time consuming aspect and difficulty of a correct identification at species level within the *Xiphinema americanum* group. This study also provides molecular markers for precise and unequivocal diagnosis of some species of the X. *americanum*-group in order to differentiate virus vector or quarantine species, since the morphology is quite similar among them and mixed populations of the X. *americanum*-group in the same soil sample are quite frequent all over the world.

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