New insight into the identification and molecular phylogeny of dagger nematodes of the genus *Xiphinema* (Nematoda: Longidoridae) with description of two new species

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The genus *Xiphinema* constitutes a large group of about 260 species of plant-ectoparasitic nematodes, polyphagous and almost worldwide distributed. Some of the species of this genus damage agricultural crops by direct feeding on root cells as well as by transmitting nepoviruses. Species discrimination in *Xiphinema* is complicated by phenotypic plasticity leading to potential misidentification. We conducted nematode surveys in cultivated and natural environments in Spain from 2009 to 2012, in which we have identified 20 populations of *Xiphinema* species morphologically close to the virus-vector nematode species *X. diversicaudatum*, three apomictic

populations tentatively identified as species from complex *X. aceri-pyrenaicum* group, and one population morphologically different from all others characterised by a female tail elongate to conical and absence of uterine differentiation. We developed comparative multivariate analyses for these related species by using morphological and morphometrical features together with molecular data from nuclear ribosomal DNA genes (D2-D3 expansion segments of large ribosomal subunit 28S, internal transcribed spacer 1 or ITS1, and partial small ribosomal subunit or 18S). The results of multivariate, molecular and phylogenetic analysis confirmed the morphological hypotheses and allowed the delimitation and discrimination of two new species in the genus described herein as ***Xiphinema baetica* sp. nov.** and ***Xiphinema turdetanensis* sp. nov.**, and 10 known species including, *X. adenohystherum, X. belmontense*, *X. cohni*, *X. coxi europaeum*, *X. gersoni*, *X. hispidum*, *X. italiae*, *X. lupini, X. nuragicum* and *X. turcicum*. Multivariate analyses based on quantitative and qualitative characters and phylogenetic relationships of *Xiphinema* spp. based on the three molecular ribosomal markers resulted in a partial consensus of these species grouping, since nematode populations were maintained for the majority of morphospecies groups (i.e. morphospecies groups 5 and 6), but not in some others (i. e. position of *X. granatum*), demonstrating the usefulness of these analyses for helping in the diagnosis and identification of *Xiphinema* spp. The clade topology of phylogenetic trees of D2-D3 and partial 18S regions in this study were congruent supporting the polyphyletic status of some characters, such as the female tail shape and the degree of development of genital system in species with both genital branches equally developed. This is the most complete and with the higher number of species included in a phylogenetic study for *Xiphinema* non-*americanum*-group species. Agreement between phylogenetic trees and some morphological characters (uterine spines, pseudo-Z organ and tail shape) was tested by reconstruction of their histories on rDNA based trees using parsimony and Bayesian approaches. Thus, integrative taxonomy, based on combination of multivariate, molecular analyses with morphology constitutes a new insight in the identification *Xiphinema* species.

ADDITIONAL KEY WORDS: Bayesian inference, cryptic species, dagger nematodes, D2-D3, multivariate analysis, PCoA, rDNA

INTRODUCTION

The phylum Nematoda includes the genus *Xiphinema* Cobb, 1913, a large group of invertebrates that are polyphagous root-ectoparasites of many plants including various agricultural crops and trees. Damage is caused by direct feeding on root cells as well as by transmitting nepoviruses (Taylor & Brown 1997). This transmission is governed by a marked specificity between plant viruses and their *Xiphinema* spp. vectors. In fact, only nine of the approximately 260 known species of *Xiphinema* have been shown to transmit nepoviruses (genus *Nepovirus*, family *Comoviridae*) (Decraemer & Robbins 2007). Because of the large morphological diversity, the genus *Xiphinema* was divided into two differentiated species groups (Loof & Luc, 1990; Coomans *et al*., 2001): i) the *Xiphinema americanum*-group which comprises a complex of about 50 species, many of them with a cosmopolitan distribution, and characterized by spiral or C-shaped medium to small body, female reproductive system with two equally developed genital branches, usually with short uteri without uterine differentiation, and short conical to broadly convex-conoid tail; and ii) the *Xiphinema* non-*americanum*-group which comprises a complex of more than 200 species, characterized by a longer body and odontostyle, usually with long uteri and uterine differentiation (including the "Z-organ", spines or crystalloid structures in the tubular part of the uterus). Some species of both groups are vectors of several important plant viruses that cause significant damage to a wide range of crops. Species of the *X.* non-*americanum*-group are vectors of *Arabic mosaic virus* (*ArMV*), *Grapevine fanleaf virus* (*GFLV*), *Strawberry latent ringspot virus* (*SLRV*), or Cherry leaf roll virus (*CLRV*) (Taylor & Brown, 1997). The large number of species within the *X.* non-*americanum*-group complicates the identification process and has required the construction of polytomous and dichotomous keys, based on a combination of major diagnostic characters, to enable morphological identification (Loof & Luc, 1990; Loof, Luc & Baujard, 1996). Also for pragmatic diagnostic, this group was divided into eight morphospecies groups based on the structural diversity of the female reproductive system and female tail shape (Loof & Luc, 1990).

Multivariate analyses, including principal components, hierarchical cluster, and canonical discriminant analyses, had provided useful tools for species delimitation in the genus *Xiphinema* (Lamberti & Ciancio, 1993; Roca & Bravo, 1997; Ye, Szalanski & Robbins, 2004; He *et al*., 2005; Gutiérrez-Gutiérrez *et al*., 2011). Other multivariate analyses, such as Principal Coordinate Analysis (PCoA), enable taxonomically similar species to be compared with increased precision by considering simultaneously quantitative and qualitative features (Dufrêne & Legendre, 1997; Legendre & Legendre, 1998). Thus, PCoA has been used to resolve taxonomy of diverse groups of organisms, including vascular plants (Kelleher *et al.*, 2005; Lihová *et al.*, 2007), invertebrates (Nicholls, 2009) or vertebrates (Thompson *et al.*, 2011).

Up to date, a total of 75 *Xiphinema* species (about 30% of total nominal species) have been molecularly characterized, constituting a complementary useful tool to distinguish among *Xiphinema* spp. In fact, several recent taxonomic and systematic studies in the genus *Xiphinema* have revealed the existence of complex cryptic species, *i.e.*, species that are morphologically almost identical but genetically distant species by applying molecular analysis (Gutiérrez-Gutiérrez *et al*., 2010, 2012; Barsi & De Luca, 2008; Wu, Zheng & Robbins, 2007; Oliveira *et al*., 2005; Oliveira, Ferraz & Neilson, 2006; Ye, Szalanski & Robbins, 2004). Consequently, application of integrative taxonomic approaches provides a major approximation to species delimitation based on integration of different perspectives, e.g. morphology and DNA sequences (Dayrat, 2005). In fact, integrative taxonomy has been efficiently applied for other invertebrates (Schlick-Steiner *et al*., 2010), vertebrates (Wiens & Penkrot, 2002) or plants (Marcussen, 2003). Therefore, the rapid and accurate identification of this complex and homogeneous species group is essential for selection of appropriate measures for control against plant pathogenic or virus-vector species, as well as a reliable method allowing distinction between species under quarantine or regulatory strategies. Ribosomal RNA genes encoding small subunit (SSU) or 18S, large subunit (LSU) or 28S, and the internal transcribed spacer 1 (ITS1) region have been used as meaningful genetic markers for the molecular characterization of species and resolving phylogenetic relationships within Longidoridae (He *et al*., 2005; Ye, Szalanski & Robbins, 2004; Gutiérrez-Gutiérrez *et al*., 2010; 2011; 2012). Oliveira, Ferraz & Neilson (2006) clearly separated *Xiphinema radicicola* Goodey, 1936 from *Xiphinema hunaniense* Wang & Wu, 1992 two species showing only minor morphological differences (*e.g.* lip region and tail shape), by using D2-D3 expansion regions of 28S rDNA. Also, ITS1 rDNA region has been considered as very useful marker for the development of species specific primers (Oliveira *et al*., 2005; Wang *et al*., 2003). D2-D3 expansion segments of 28S rDNA and ITS1 rDNA region have been showed to be more useful for species identification compared to partial 18S. This later gene has been showed to be a suitable marker for establishing evolutionary relationships among taxa at higher taxonomic level than species (Gutiérrez-Gutiérrez *et al.,* 2010; Oliveira *et al*., 2004). Nevertheless, the partial 18S rDNA gene has also been shown to be useful for discriminating among some *Xiphinema americanum*-group species (Lazarova *et al*., 2006).

As an alternative of considering each identification method independently, an integrative taxonomic approach based on combination of morphological and morphometrical studies with molecular based phylogenetic inference, and sequence analysis for species diagnosis have proven to be a tool beyond doubt in nematode identification within this group (Gutiérrez-Gutiérrez *et al*., 2010; 2011; 2012; Palomares-Rius *et al*., 2008). Therefore, such integrative strategy, including multivariate analyses of quantitative and qualitative diagnostic features may help to provide new reliable and rapid tools for identifying of these plant-parasitic nematodes

allowing the distinction among virus vectors or non virus vectors *Xiphinema* spp. and assists in the exclusion of species under quarantine or regulatory strategies.

The objectives of this study were: *i*) to characterise morphologically and morphometrically species belonging to the *Xiphinema* non-*americanum*-species group and to compare them with previous records; *ii*) to conduct a morphometric study of related species of the *Xiphinema* non-*americanum* morphospecies groups 1, and 5 to 8 using multivariate Principal Coordinate (PCoA) and hierarchical clustering analyses; *iii*) to characterise molecularly the sampled *Xiphinema* spp. populations using the D2-D3 expansion segments of 28S rDNA, ITS1, and partial 18S rDNA gene sequences and *iv*) to study the phylogenetic relationships of the identified *Xiphinema* species with available sequenced species.

MATERIAL AND METHODS

NEMATODE POPULATIONS AND MORPHOLOGICAL STUDIES

Nematode surveys were conducted from 2009 to 2012 during the spring season in cultivated and natural environments in southern Spain and four supplementary samples in northern Spain, including carob tree (*Ceratonia siliqua* L.), chestnut (*Castanea sativa* Mill.), cork oak (*Quercus suber* L.), eucalyptus (*Eucalyptus globulus* Labill.), European holly (*Ilex aquifolium* L.), grapevine (*Vitis vinifera* L.), pedunculate oak (*Quercus robur* L.), stone pine (*Pinus pinea* L.), and undetermined grasses (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.

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 1,000x magnification. Morphometric study of each nematode population included classical diagnostic features in longidoridae (i.e. de Man body ratios, lip region and amphid shape, oral aperture-guiding ring, odontostyle and odontophore length) (Jairajpuri & Ahmad, 1992). All measurements were expressed in micrometers (μm), unless otherwise indicated in text. 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 of 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. cadavalense* and *X. belmontense* from Portugal); Dr J. Hallmann, from Julius Kühn-Institute, Institute for Epidemiology and Pathogen Diagnostics, Münster, Germany (*viz*. *X. pseudocoxi* from Germany); and Dr Z.A. Handoo, from the USDA Nematode Collection, Beltsville, MD, USA (*viz*. *Xiphinema capriviense* Hutsebaut, Heyns and Coomans, 1989).

MULTIVARIATE ANALYSES

Multivariate analyses were based upon the characters used in the polytomous key by Loof and Luc (1990) and character analysis by Coomans *et al*. (2001): uterine differentiation, female tail shape, body length (L), odontostyle length, lip region width and shape, oral aperture-guiding ring length, body habitus, female tail length, body anus width, presence or absence of males, and the ratios a (body length/maximum body width), b (body length/pharyngeal length), c (body length/tail length), c' (tail length/body width at anus), V [(distance from anterior end to vulva/body length) x 100]'. These 16 characters represented a mix of 11 quantitative and five qualitative (one binomial and four multistate) data types. To accommodate such a mixed data types, we performed a multivariate principal co-ordinate analysis (PCoA) on a set of 71 populations, including the type populations of 43 species of *Xiphinema,* belonging to morphospecies groups 1 and 5 to 8. The 43 species were selected based on the availability of molecular data. Analyses were made using R version 2.15.2 (R Foundation for Statistical Computing, http://www.R-project.org/). The PCoA analysis was performed by means of a distance matrix among populations in a Q-mode type analysis using the package labdsv (Roberts, 2012). The distance matrix was based on Gower's coefficient calculated using the gower algorithm of FD package (Laliberté and Legendre, 2010). Additionally, a hierarchical cluster analysis was performed to identify associated groupings of *Xiphinema* species. To find functional groupings of correlated species, an agglomerative clustering based on first two dimensions associated with the 16 characters mentioned above where used to characterize each population. The optimum number of clusters was estimated on the basis of the average silhouette width according to the Mantel statistic. Thus, the number of clusters in which the within-group mean intensity of the link of the objects (*Xiphinema* species) to their groups was highest (i.e. with the largest average silhouette width) indicated the optimum cluster number (Borcard *et al*., 2011). The identified groupings were then represented in the PCoA biplot. All calculations for cluster analyses were made using the cluster (Maechler *et al*., 2012) and vegan (Oksanen *et al*., 2013) packages. Quantitative morphometric characters were then subjected to a multivariate analysis of variance (MANOVA) with the functional groups identified in the cluster analysis as explanatory variable using the general linear model procedure of SAS (Statistical Analysis System v.9.3; SAS Institute, Cary, NC, USA).

DNA EXTRACTION, PCR AND SEQUENCING

For molecular analyses, two live nematodes from each sample were temporary mounted in a drop of 1M NaCl containing glass beads and after taking measurements and photomicrographs taken. 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'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-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´-ACGAGCCGAGTGATCCACCG-3´) as described in Wang *et al*. (2003). Finally, the 18S rDNA gene was amplified using the small subunit SSU_F_07 (5´-AAAGATTAAGCCATGCATG-3´) and SSU_R_81 (5´- TGATCCWKCYGCAGGTTCAC-3´) primers (http://www.nematodes.org/barcoding/sourhope/nemoprimers.html).

PCR cycle conditions were: one cycle of 94ºC for 2 min, followed by 35 cycles of 94ºC for 30 s, annealing temperature of 57ºC for 45 s, 72ºC for 3 min and finally one cycle of 72ºC for 10 min. Sequencing of some of the ITS1 and partial 18S rDNA genes of some known *Xiphinema* spp. identified herein were not successful despite several attempts (Table 1). 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 Servicio Central de Apoyo a la Investigación , 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 ANALYSIS

D2-D3 expansion segments of 28S, ITS1, and partial 18S rDNA sequences of different *X.* non-*americanum* group species from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were chosen according to previous published data (Cantalapiedra-Navarrete *et al*., 2011; Gutiérrez-Gutiérrez *et al*., 2011; He *et al.,* 2005). The newly obtained and published sequences for each gene were aligned using ClustalW (Thompson *et al.,* 1997) with default parameters. Sequence alignments were manually edited using BioEdit (Hall*,* 1999). Phylogenetic analyses of the sequence data sets were performed based on maximum likelihood

(ML) using PAUP * 4b10 (Swofford, 2003) and Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The best fitted model of DNA evolution was obtained using jModelTest v. 2.1.1 (Darriba *et al*., 2012) 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 a general time reversible of invariable sites and a gamma-shaped distribution (GTR + I+ G) model for D2-D3 expansion segment of 28S rDNA and 012340+G+F for ITS1 region and GTR + I+ G for 18S, were run with four chains for 2.0×10^6, 1×10^6, and 2×10^6 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.

MORPHOLOGICAL MATRIX AND MAPPING OF MORPHOLOGICAL CHARACTERS

Morphological characters used in morphospecies group delimitation were used for mapping them into the D2-D3 expansion segment of 28S rDNA phylogenetic tree. The three characters consisted in: i) presence/absence of pseudo-Z-organ; ii) presence/absence of spines in uterus and iii) different tail shapes. For morphological matrix the most representative value for each character was considered. A new phylogenetic tree using a Bayesian approach was constructed using only one sequence for each species. Two approaches were used to map morphological characters (Parsimony and Bayesian approaches). The criterion of parsimony was used to optimize character state evolution on the molecular consensus tree using Mesquite 2.73 (Madison & Madison, 2010). Ancestral character states were estimated according to their posterior probability distributions in a Bayesian approach using the program SIMMAP 1.5 (Bollback, 2006). This program uses prior in morphological data analyses (Schulz & Churchill, 1999). Morphology priors were calculated using a R script in the SIMMAP 1.5 program using R statistical package (www.r-project.org). In both parsimony and Bayesian character history analysis, all outgroup taxa have no pseudo-Z-organ differentiation and no spines in the uterus. While for tail shape they were assumed as a different character (0), for the reason that more different kind of tails are found in these groups.

RESULTS

SYSTEMATICS

GENUS *XIPHINEMA* COBB, 1913

XIPHINEMA BAETICA SP. NOV.

(FIGS. 1-3, TABLES 2-3)

Holotype. Female extracted from soil samples collected from grapevine in Manzanilla, Huelva province, Spain, (37º19'35.55'' N latitude, 6º27'53.12'' W longitude) by J. Martín Barbarroja and G. León Ropero, mounted in pure glycerine and deposited in the nematode collection at Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain (collection number H31-04).

Paratypes. Female, male and juvenile paratypes extracted from soil samples collected from grapevine in Manzanilla, Huelva province, Spain, and additional populations were collected in Hinojos, Huelva province, and Benalup-Casas Viejas, Cádiz province, associated with stone pine and undetermined grasses respectively, were deposited in the following nematode collections: IAS-CSIC (collection numbers H31-01-H31-13, H31-16-H31-20); one female and one male at Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (C.N.R.) (H31-014); two females at Royal Belgian Institute of Natural Sciences, Brussels, Belgium (H31-21); and one female and one male at USDA Nematode Collection, (H31-15).

Etymology. The species epithet refers to the Latin word *Baetica*, the Roman province of the Iberian Peninsula where the species was collected.

Description of female. Body cylindrical, narrowing very gradually towards anterior end, assuming an open C- to J-shaped when killed by heat. Cuticle with fine transverse striations more visible in the tail region, 3.5-4.0 µm thick at mid-body and 15.0-20.0 µm at tail tip (Table 2). Lip region broadly rounded, separated from the rest of the body by a weak depression, and 2.4-3.2 times as high as width. Amphidial fovea stirrup-shaped, aperture occupies about one-half of the lip region width and located just anterior to demarcation line. Body pores present between anterior end and guiding ring, three and two on the dorsal and ventral side, respectively. Odontostyle robust, 10.6 (9.9-11.7) times lip region width, or 1.8-2.2 odontophore lengths long. Odontophore with well developed basal flanges (11.0-12.5 µm wide). Guiding ring double, guiding sheath 14-24 µm long depending on degree of protraction/retraction of stylet. Pharynx consisting of an anterior slender narrow part, 366 (322-445) μm long, extending to a terminal pharyngeal bulb with three nuclei. Pharyngeal basal bulb 116.7 (95-127) µm long and 24.3 (20-32) µm wide, occupying about 1/4 to 1/5 of the total pharyngeal length. Glandularium 93.4 (82-110) µm long. Nucleus of dorsal pharyngeal gland (DN) located at the

beginning of bulb (6.8-12.0%), being larger than two subventrolateral nucleus (SVN) located around the middle of bulb (51.5-65.5%) (location of gland nuclei according to Loof & Coomans, 1972). Pharyngeal intestinal valve conoid-rounded, 9.5 (7.0-11.5) µm long. Intestine simple, prerectum 14.8-30.1 anal body diameter long, rectum 0.9-1.1 anal body diam. long. Female reproductive system didelphic-amphidelphic with branches about equally developed. Each branch composed of a 75-160 µm long ovary, a 135-220 µm long reflexed oviduct with well developed *pars dilatata oviductus*, a sphincter and a 305-469 µm long bipartite uterus composed of *pars dilatata uteri* followed by a tubular part containing in the proximal part a well developed pseudo-Z-organ, comprising irregular shape sclerotized bodies, variable in number (8-13) and size, and each body consisting of a central very large spherical and transparent portion surrounded by irregular shaped refractive pieces (Fig. 2D-F). Tubular region of uterus devoid of spiniform structures. Ovejector well developed, 37-43 μm wide, vagina (17-19 μm long) perpendicular to body axis, extending inwards for 31-35% of corresponding body diam., vulva slit-like and situated slightly anterior to mid body. Tail short, conoid, slightly rounded dorsally and almost straight ventrally, with distinctly digitate terminus (8-14 µm long) ventrally oriented in the caudal axis. Three or four body pores are visible on each side of the tail.

Male. Common (almost as frequent as female *ca*. 35%). Morphologically similar to female except for genital system, but with posterior part of the body more curved. Male genital tract diorchic with testes opposed, containing multiple rows of different stages of spermatogonia. Tail similar to that of female, with three caudal pores on each side. Spicules arcuate, robust, *ca* 2 times longer than tail length, lateral guiding pieces more or less straight or with curved proximal end. One pair of adanal supplements located at 25.3 (23.5-26.5) µm from cloacal aperture, and a series of 4 (exceptionally 3) midventral supplements.

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. 3, Table 2). First-juvenile stage was characterised by the replacement odontostyle tip close to base of functional odontostyle and located at level of odontophore, and an elongate-conoid tail 3.6 times as long as the anal body diameter (Fig. 2).

Diagnosis. Xiphinema baetica sp. nov. is a gonochoristic species characterized by a large-size body length (4909-6091 µm); lip region broadly rounded, separated from the rest of the body by a weak depression; a long odontostyle and odontophore 142-157 and 70-84 µm, respectively;

vulva position at 42-48%; well developed pseudo-Z-organ, comprising 8-13 sclerotized bodies of variable size, and spiniform structures absent in the uterus; female tail short, conoid, slightly rounded dorsally and almost straight ventrally, with distinctly digitate terminus ventrally oriented in the caudal axis, slightly longer than anal body diameter (1.1-1.6); c (body length/tail length) ratio (92.7-131.2); spicules medium-size (61-71 µm long); and specific D2-D3, ITS1, and 18S-rDNA sequences were deposited in GenBank with accession numbers KC567165-KC567169, KC567156-KC567157, and KC567148-KC567149, respectively. According to the polytomous key by Loof and Luc (1990) and the supplement by Loof, Luc & Baujard (1996), the new species belongs to the *X.* non-*americanum* group 5 and has the following specific alpha-numeric codes (codes in parentheses are exceptions): A 4, B 2, C 4(5), D 5 (4), E 5(4), F 5, G 3, H 2, I 3, J 4, K 2, L 2.

XIPHINEMA TURDETANENSIS SP. NOV.

(FIGS. 3-5, TABLE 4)

Holotype. Female extracted from soil samples collected from stone pine in Sanlúcar de Barrameda, Cádiz province, Spain, (36º51'14.78'' N latitude, 6º19'06.43'' W longitude) by J. Martín Barbarroja and G. León Ropero, mounted in pure glycerine and deposited in the nematode collection at IAS-CSIC (collection number J212-01).

Paratypes. Female, male and juvenile paratypes extracted from soil samples collected from stone pine in Sanlúcar de Barrameda, Cádiz province, Spain, and an additional population was collected in the same locality, associated with wild olive, were deposited in the following nematode collections: IAS-CSIC (collection numbers J212-02-J212-14, J212-16-J212-22, AR15-01, AR15-02); one female and one male at Istituto per la Protezione delle Piante (IPP) of Consiglio Nazionale delle Ricerche (C.N.R.) (J212-015); one female at Royal Belgian Institute of Natural Sciences(J212-23); and one female at USDA Nematode Collection (J212-24).

Etymology. The species epithet is derived from the Latin word *Turdetania*, the Tartesian province of the Iberian Peninsula where the species was collected.

Description of female. Body cylindrical, tapering towards anterior end, open C-shaped upon fixation. Cuticle with very fine transverse striations more visible in tail region, varying to 2.5-3.5 μm at mid-body, and 13.0-18.0 μm at tail tip, and marked by very fine superficial transverse striae mainly in tail region. Lip region rounded-hemispherical, separated from body contour by a shallow depression and 2.0-2.5 times as high as wide. Amphidial fovea stirrup-shaped; aperture

extending for 62.0-65.5% of lip region width and located slightly anterior to depression marking lip region. Three pairs of body pore present between anterior end and guiding ring. Odontostyle typical of genus, long and slender, 9.2 (7.8-10.0) times lip region diam. or 1.8 (1.5-1.9) times odontophore lengths long. Odontophore with well developed flanges. Guiding ring double, guiding sheath 12-18 μm long depending on degree of protraction/retraction of stylet. Pharynx consisting of an anterior slender narrow part, 339 (250-479) μm long, extending to a terminal pharyngeal bulb, 114 (99-145) μm long, with three nuclei. Nucleus of dorsal gland (DN) large, located at 10.1-18.2% of pharyngeal bulb length, being larger than the two ventrosublateral nuclei (S1N) located at 47.7-61.5% of terminal bulb length (location of gland nuclei according to Loof & Coomans, 1972). Cardia conoid-rounded, 6.0-8.0 μm long. Intestine simple, prerectum 7.7-18.0 anal body diam. long, rectum 0.8-1.2 anal body diam. long. Female reproductive system didelphic-amphidelphic with branches about equally developed, vulva slit-like, situated anteriorly to mid body. Each branch composed of a 81-148 μm long ovary, a 145-245 μm long reflexed oviduct with well developed *pars dilatata oviductus*, a sphincter and a 360-441 μm long tripartite uterus composed of *pars dilatata uteri* followed by a tubular part containing in the proximal part a well developed pseudo-Z-organ, comprising 6-8 sclerotized bodies of variable size, each body consisting of a central very large and almost rounded hyaline portion surrounded by irregularly shaped refractive granules of variable thickness (Fig. 5C) and spiniform structures, some of them very large, distributed over the entire length of the tube-like portion of uterus (Fig. 5D). Ovejector well developed, (64-72) × (32-44) μm, vagina perpendicular to body axis, extending for 59-76% of corresponding body diam., vulva a transverse slit. Tail short, conoid, slightly rounded dorsally and almost straight ventrally, with digitate or subdigitate terminus, generally directed ventrally with respect to the body axis.

Male. Common (less frequent than *ca*. 25%). Similar to female but with the posterior part of the body more curved. Morphology similar to that of female except for the genital apparatus and associated somatic structures. Spicules curved, not cephalated; lateral guiding pieces of the gubernaculum well sclerotized, slightly curved. Precloacal pair of supplements at 21-25 μm from the cloacal aperture, preceded by four medioventral supplements. Tail similar to that of female, with three caudal pores on each side.

Juveniles. Morphologically similar to adults, except for smaller size and a relatively longer tail. All four juvenile stages were found, being distinguishable by relative lengths of body and functional and replacement odontostyle (Table 4, Robbins *et al*., 1996). Lip region of all juvenile stages similar to that of adults. First stage-juveniles were characterised by a elongate-conoid tail with a c' ratio (tail length/body width at anus) 2.5-2.8 (Fig. 5L), an odontostyle

length 56-59 µm, and shorter distance from anterior end to stylet guiding ring than that in adult stages (Table 4).

Diagnosis. *Xiphinema turdetanensis* sp. nov. is a gonochoristic species characterized by a large body size (4066-5227 µm); lip region rounded-hemispherical; separated from the body by a shallow depression; odontostyle and odontophore 121-142 and 67-80 µm long, respectively; vulva position at 43-48%; well developed pseudo-Z-organ, comprising 6-8 globular bodies of variable size and spiniform structures; female tail short, conoid, slightly rounded dorsally and almost straight ventrally, with digitate or subdigitate terminus, slightly longer than anal body diameter (1.1-1.3); c ratio (94.4-116.2); and specific D2-D3, ITS1, and 18S-rDNA sequences are deposited in GenBank with accession numbers KC567186, KC567163, and KC567155, respectively. According to the polytomous key by Loof and Luc (1990) and the supplement by Loof, Luc & Baujard, (1996), the new species belongs to the *X.* non-*americanum* group 5 and has the following specific alpha-numeric codes (codes in parentheses are exceptions): A 4, B 2 3, C 5, D 5, 5 (4), F 5(4), G 3(2), H 2, I 3, J 5, K 2, L 2.

MORPHOLOGY AND MORPHOMETRICS OF SPANISH POPULATIONS OF *XIPHINEMA* SPECIES (FIGS. S1-S4, TABLES S1-S3)

The morphological and morphometrical data as well as molecular delineation of *Xiphinema hispidum* Roca & Bravo, 1994, *Xiphinema italiae* Meyl, 1953, *Xiphinema lupini* Roca & Pereira, 1993, *Xiphinema nuragicum* Lamberti, Castillo, Gómez-Barcina and Agostinelli, 1992, and *Xiphinema turcicum* Luc, 1963 were previously studied and compared with original descriptions and paratype specimens within preceding studies on the prevalence, polyphasic identification and molecular phylogeny of dagger and needle nematodes infesting vineyards in southern Spain (Gutiérrez-Gutiérrez *et al*., 2010; 2011). These *Xiphinema* spp. have been widely reported in the Iberian Peninsula and Europe (Brown & Taylor, 1987; Peña-Santiago *et al*., 2006; Gutiérrez-Gutiérrez *et al*., 2010; 2011). Consequently, only D2-D3 sequences had been reported here for these samples because the morphological and morphometrical data were identical to those of the previous works (Gutiérrez-Gutiérrez *et al*., 2010; 2011). For other known species studied, a brief description and a morphometric comparison with previous records is provided below.

XIPHINEMA ADENOHYSTHERUM LAMBERTI, CASTILLO, GÓMEZ-BARCINA AND AGOSTINELLI, 1992

(TABLE S1)

The *Xiphinema* non-*americanum* group population of this species from holly tree at Arévalo de la Sierra (Soria province) was morphologically and morphometrically (Table S1) coincident with original description of *X. adenohystherum* and the Spanish population from grapevine at Bollullos par del Condado (Huelva province) (Lamberti *et al*., 1992; Gutiérrez-Gutiérrez *et al*., 2010). This population was characterized by a lip region hemispherical and slightly offset from body, odontostyle and odontophore about 145 and 80 µm, respectively; two equally developed female genital branches, vulva slightly anterior to mid-body, uterus devoid of Z-differentiation but with small to large uterine spines in the tubular portion of the uterus, and female tail bluntly rounded with four caudal pores. Morphometrics are similar to those provided in the original description, except for some minor differences in c and V ratio [(distance from anterior end to vulva/body length) x 100], odontostyle, odontophore, and tail length, which may be due to geographical intraspecific variability (Lamberti *et al*., 1992; Gutiérrez-Gutiérrez *et al*., 2010). This is the second record for the Iberian Peninsula, and the first in the northern part of the country, confirming the extension and biodiversity of the *Xiphinema aceri-pyrenaicum* species complex group (Gutiérrez-Gutiérrez *et al*., 2010). According to the polytomous key Loof and Luc (1990), the species belongs to the *X.* non-*americanum* group 6 and has the following specific alpha-numeric codes: A 4, B 3, C 7, D 6, E 5, F 4, G 3, H 2, I 3, J 7, K ?, L 1.

XIPHINEMA BELMONTENSE ROCA AND PEREIRA, 1992

(FIG. S1, TABLE S1)

The three gonochoristic populations of *X.* non-*americanum* group from chestnut and pedunculate oak at Puebla de Sanabria (Zamora province), and Merza (Coruña province), respectively, were morphologically and morphometrically (Table S1) coincident with original description of *X. belmontense* and examined paratypes. Females were characterized by an almost hemispherical, broadly rounded lip region; two equally developed genital branches, vulva clearly anterior to mid-body, uterus with a pseudo-Z-organ consisting of 8-10 granular structures with a large central portion, rosette-shaped, and spines in the tubular portion of the uterus (Fig. S1); and tail conoid-rounded, slightly curved ventrally, and with a terminal peg (ventrally directed). In addition some female adult showed a few crystalline structures (10-17 µm long) of diamond shaped in the ovejector and the tubular portion of uterus, such as had been reported for others *Xiphinema* species (Kruger, 1988; Coomans *et al*., 2001). Males were almost as common as females and showed a habitus mostly similar to that of female (almost straight) but with posterior region curved ventrally, and were characterized by a genital tract diorchic with spicules curved, not cephalated; and precloacal pair of supplements located at 25-26 µm, preceded generally by four (exceptionally five) ventral supplements. Morphometrics of

chestnut and pedunculate oak populations were coincident with original description, except for shorter spicule length, *viz*. 70 (65-74) µm *vs* 95 (89-112) µm (Roca & Pereira, 1992). Although spicule size showed low variation in the genus *Xiphinema*, may be considerable for some species, such as occurs in *Xiphinema surinamense* Loof and Maas, 1972 varying from 48-92 µm (Loof & Maas, 1972; Loof & Sharma, 1979). The species has been reported in several localities in Portugal (Roca & Pereira, 1992; Roca & Bravo, 1997), and it is the second report in Spain, after that from Guadiamar River in the southern part of the country by Murillo-Navarro *et al*. (2005). According to the polytomous key Loof and Luc (1990), the species belongs to the *X.* non-*americanum* group 5 and has the following specific alpha-numeric codes: A 4, B 23, C 5, D 5, E 3, F 4, G 3, H 2, I 3, J 5, K 3, L 2.

XIPHINEMA COHNI LAMBERTI, CASTILLO, GÓMEZ-BARCINA AND AGOSTINELLI, 1992

(FIG. S2, TABLE S2)

The Spanish population of this species is characterised by a lip region hemispherical separated from body by a slight depression, two equally developed female genital branches, vulva equatorial, uterus containing numerous and large spines in the tubular portion and devoid of any Z-differentiation, female tail convex-conoid with rounded terminus, with inconspicuous terminal bulge, generally in line with the body axis (Fig. S2). Morphometrics of the stone pine population are similar with those provided in the original description, except for some differences in odontostyle length, c and c' ratio *viz*. 141 (133-155) µm *vs* 164 (149-174) µm, 116.2 (101.1-134.2) *vs* 95.7 (82.6-115.2), 1.1 (1.0-1.3) *vs* (0.9 (0.8-1.1), respectively, which may be due to few specimens originally studied or geographical intraspecific variability (Lamberti *et al*., 1992). This is the first report for Spain and confirms the wide biodiversity of the species from the complex *Xiphinema aceri-pyrenaicum* group in the Iberian Peninsula, characterised by a rounded tail with or without an inconspicuous terminal bulge and a uterus devoid of Z-differentiation but showing spiniform structures (Gutiérrez-Gutiérrez *et al*., 2010). According to the polytomous key Loof and Luc (1990), the species belongs to the *X.* non-*americanum* group 6 and has the following specific alpha-numeric codes (codes in parentheses are exceptions): A 4, B 3, C 6, D 5 (6), E 5(6), F 5(4), G 3, H 2, I 3, J ?, K ?, L 1. These data suggests that *X. cohni* is morphological and morphometrically a species of the *X. aceri-pyrenaicum* group (Gutiérrez-Gutiérrez *et al*., 2010), but clearly distinguishable as separate and valid species by phylogenetic analysis of ribosomal DNA genes such as D2-D3, ITS1and the partial 18S (see below). Therefore, molecular data confirms the cryptic speciation previously suggested in this species group (Gutiérrez-Gutiérrez *et al*., 2010; 2011), and hence, the status of this species which were previously synonymized with *X. pyrenaicum* (Baujard, Luc & Loof, 1996) must be rejected.

XIPHINEMA COXI EUROPAEUM STURHAN, 1984

(FIG. S3, TABLE S3)

The six parthenogenetic populations of *X. coxi europaeum* from carob tree, cork oak, and grapevine, at Almonte, Hinojos, and Manzanilla (Huelva province), respectively, and cork oak at Cortes de la Frontera (Cádiz province) in southern Spain (Tables 1, S3) were morphologically and morphometrically coincident with original description (Sturhan, 1984), and other European populations, including Spain (Arias, Navas & Andrés, 1987), Italy (Coiro *et al*., 2001) and Portugal (Bravo, Roca & Mota, 2001). The six studied populations were characterised by a medium body-size (3023-4360 μm); a lip region broadly rounded, separated from the rest of body by a slight depression; odontostyle 114-146 μm long; uterus with a pseudo-Z-organ consisting of 5-8 granular structures of variable size and irregularly shaped, sometimes with a pointed apophysis (Fig. S3); and tail conoid-rounded, with a terminal peg ventrally oriented (Fig. S3). The *X. coxi* complex belongs to the *X.* non-*americanum* group 5 and include populations from Florida, USA, which were recognized as distinct from those in Europe (Brown & Taylor, 1987) to the extent that Sturhan (1984) recognized two species, *X. pseudocoxi* and *X. coxi*, which divided latter into two sub-species: *X. coxi coxi* for American populations and *X. coxi europaeum* for European populations (Sturhan, 1984; Brown & Taylor, 1987). However, molecular data in this study suggest that both sub-species should be consider a complex of cryptic species almost morphological- and morphometrically undistinguishable (see below multivariate analysis) but phylogenetically distant to one another (see below molecular analysis). According to the polytomous key Loof and Luc (1990), the species belongs to the *X.* non-*americanum* group 5 and has the following specific alpha-numeric codes (codes in parentheses are exceptions): A 4, B 3, C 34, D 5, E 4 (3), F 45, G 23, H 2, I 3, J 3, K 2, L 1.

XIPHINEMA GERSONI ROCA AND BRAVO, 1993

(FIG. S4, TABLE S2)

The gonochoristic population of *X. gersoni* from eucalyptus in Almonte (Huelva province) (Table S2) agrees fairly well with original description (Roca & Bravo, 1993). This population was characterised by an open C-shaped habitus, a lip region rounded, separated from body contour by a slight depression; odontostyle 134-164 μm long; uterus with a pseudo-Z-organ consisting of 10-15 granular structures of variable size and a large central portion, rosette-shaped, and spiniform structures are present in the portion tubular of the uterus (Fig. S4); and tail conoid-rounded, with a digitate or subdigitate terminus (Fig. S4). Males were less common than females (about 30%) and showed a habitus mostly similar to that of female but with posterior region curved ventrally, and were characterized by a genital tract diorchic with spicules curved, not cephalated; and a precloacal pair of supplements preceded generally by

three (exceptionally four) ventral supplements. Morphometrics of the eucalyptus population was coincident with original description, except for shorter body and tail length (4545-5573, 42.0-53.5 *vs* 5400-7300, 51.5-69.0 μm, respectively), and lower a (body length/maximum body width) and c' ratios (75.5-96.2, 1.2-1.5 *vs* 101.5-136.5, 1.4-2.0) which should be consider intraspecific variability (Roca and Bravo, 1993). The species has been reported in southern Portugal (Roca & Bravo, 1993), and it is the second report in southern Spain, after that from Guadiamar River by Murillo-Navarro *et al*. (2005). According to the polytomous key Loof and Luc (1990), the species belongs to the *X.* non-*americanum* group 5 and has the following specific alpha-numeric codes: A 4, B 23, C 4, D 5, E 5 (4), F 5, G 3, H 2, I 3, J 4, K 3, L 2.

MOLECULAR CHARACTERISATION OF *XIPHINEMA BAETICA* SP. NOV., *XIPHINEMA TURDETANENSIS* SP. NOV. AND OTHER KNOWN *XIPHINEMA* SPECIES

Amplification of the D2-D3 expansion segments of 28S rDNA, ITS1, and the partial 18S rDNA from the two new and the previously known *Xiphinema* spp. yielded single fragments of approximately 800 bp, 1100 bp and 1500 bp, respectively, based on direct fragment sequencing. D2-D3, ITS1, and the partial 18S sequences of *Xiphinema baetica* sp. nov. and *Xiphinema turdetanensis* sp. nov. matched well with the *X.* non-*americanum* group spp. deposited in GenBank (Table 5). These sequences were related to *Xiphinema abrantinum* Roca and Pereira, 1991, *X. diversicaudatum*, *Xiphinema turdetanensis* sp. nov., *X. bakeri*, *Xiphinema globosum* Sturhan, 1978, and *X. coxi europaeum* and *X. vuittenezi* (Table 5). Intra-specific variation of D2-D3 segments detected among the five studied populations (three from stone pine, grapevine and grasses) of *Xiphinema baetica* sp. nov. consisted in 2 to 8 nucleotides (99% similarity) and 2 indels (0.26-0.28%); and variability was similar within the same locality and host-plant (1 to 5 nucleotides, 99% similarity, and no indels). However, no intra-specific variation of D2-D3 segments was detected among the two studied populations (stone pine and wild olive) of *Xiphinema turdetanensis* sp. nov.. Similarly, intra-specific variation of ITS1 detected between the two studied populations (grapevine and stone pine) of *Xiphinema baetica* sp. nov. was low (99% similarity with 11 nucleotide differences and 4 indels, 0.39%). Intra-specific variation of ITS1 detected among the three studied populations (two from chestnut and pedunculate oak) of *Xiphinema turdetanensis* sp. nov. was from 1 to 2 nucleotides (99% similarity) and no indels; and variability within the same locality and host-plant (1 nucleotide). Finally, no intraspecific variability of the partial 18S of *Xiphinema baetica* sp. nov. was detected between individuals from the two studied populations (grapevine and stone pine).

Molecular characterization of other known *Xiphinema* species sampled in this study can be found in Tables S4, S5. Intra-specific variation of D2-D3 detected among the three studied

populations (two from chestnut and pedunculate oak) of *X. belmontense* (KC567170-KC567172) was from 1 to 2 nucleotides (99% similarity and no indels). ITS1 of *X. cohni* (KC567159) did not show homology with ITS1 sequences in this study, and hence was not included in the phylogenetic analysis of ITS1. Intra-specific variation of D2-D3 detected among the six studied populations (three from cork oak, two from grapevine, and carob tree) of *X. coxi europaeum* (KC567174-KC567179) was from 1 to 2 nucleotides (99% similarity and no indels); variability was similar within cork oak (1 to 2 nucleotides, 99% similarity, and no indels), but within grapevine and within the same locality sequences were identical. Similarly, intra-specific variation of ITS1 detected between the three studied populations (two from grapevine and cork oak) of *X. coxi europaeum* (KC567160-KC567162) was also low (99% similarity with 1 to 3 nucleotide differences and 1 to 2 indels, 0.-10-0.19%).

MULTIVARIATE ANALYSIS OF THE GENUS *XIPHINEMA*

The variance explained by the first two dimensions was 87.31% of the total variance (Fig. 6a). The subsequent hierarchical cluster analysis using the first two dimensions of the PCoA as input variables showed the occurrence of four functional cluster groupings according to the Mantel statistic among the 43 *Xiphinema* species in the study. Functional cluster grouping are delimited on the scatter plots on Fig. 6b, and comprised 12, 10, 12 and nine species, respectively, that were not directly related to previously described morphospecies groups. All populations of a same species were included into a single functional group. Functional groups greatly differed in both, morphometric and morphological characters. Among quantitative morphometric characters, eigenvectors of characteristic roots in MANOVA analysis identified c', V and b ratios, and anal body diameter as the characters with the greatest influence on group separation, while c and a ratios, oral aperture-guiding ring and tail length showed intermediate weights and lip region width, body length and odontostyle length had the lowest weights (*data not shown*). Functional group 1 comprised 12 species, five belonging to morphospecies group 5 (*viz*. *X. cadavalense*, *X. globosum*, *X. hispanum*, *X. lusitanicus* and *X. turcicum*) and seven belonging to morphospecies group 6 (*viz*. *X. aceri*, *X. adenohystherum*, *X. cohni*, *X. nuragicum*, *X. pyrenaicum*, *X. sphaerocephalum* and *X. zagrosense*). Functional group 2 comprised 10 species, of which four were included in morphospecies group 1 (*viz*. *X. brasiliense*, *X. chambersi*, *X. hunaniense* and *X. naturale*) and six in morphospecies group 7 (*viz*. *X. elongatum*, *X. insigne*, *X. italiae*, *X. savanicola*, *X. setariae* and *X. vulgare*). These two functional groups showed opposite-extreme values for all morphometric characters. Thus, species in functional groups 1 and 2 characterized by the highest and lowest values, respectively for anal body diameter, oral aperture-guiding ring length, lip region width, odontostyle length and V and c ratios; but the opposite occurred for tail length and c' ratio. Concerning morphological characters, both groups

characterized by hook-shaped habitus and most of the species within these two groups had a lip region separated by a week depression or shallow constriction. However, while functional group 1 characterized by a uterus with a pseudo-Z-organ or with uterine spines and a wide range in female tail shape, functional group 2 was characterized by no uterine differentiation and a long female tail. Functional group 3 included 12 species: *X. abrantinum*, *X. baetica* sp. nov., *X. belmontense*, *X. dentatum*, *X. dissimile*, *X. diversicaudatum*, *X. gersoni*, *X. granatum*, *X. hispidum*, *X. lupini*, *X. silvesi* and *X. turdetanensis* sp. nov. All species in this group belonged to morphospecies group 5 except for *X. granatum* that was included in the morphospecies group 8. Overall, species within this group are characterized by the longest body and highest a and b ratios in the study, showing intermediate values for the rest of quantitative characters. Furthermore, these species are characterized by a uterus with a pseudo-Z-organ or pseudo-Z-organ with spines, a short conical to hemispherical female tail, a hook-shaped body habitus and are the only group in which males are common. Finally, functional group 4 included nine species, seven of them were included in morphospecies group 5 (*X. basiri*, *X. capense*, *X. coxi coxi*, *X. coxi europaeum*, *X. diversum*, *X. pseudocoxi* and *X. vuittenezi*), one in morphospecies group 7 (*X. bakeri*) and two in morphospecies group 8 (*X. index* and *X. vuittenezi*). Species within this group are characterized by intermediate values for all quantitative characters, uterus with a pseudo-Z-organ, female tail regularly short conical, lip region continuous with body contour and hook-shaped body habitus.

PHYLOGENETIC RELATIONSHIPS OF THE GENUS *XIPHINEMA*

Phylogenetic relationships among *X.* non-*americanum* group species inferred from analyses of D2-D3 expansion segments of 28S, ITS1 and the partial 18S rDNA gene sequences using BI and ML are given in Figures 7, 8, and 9, respectively. No significant differences in topology were obtained using the BI or ML approach and only a few species in some minor clades with low bootstrap support were not congruent with the general topology tree. The 50% majority rule consensus BI and ML trees of *Xiphinema* spp. based in a multiple edited alignment including 67 D2-D3 sequences and 783 bp showed three moderate supported major clades (Fig. 7). Clade (i) included twenty-three species [PP = 95%; bootstrap support (BS) = 88%] with broadly convex-conoid tail and belonging to morphospecies group 6, *viz*. *X. adenohystherum* (GU725075, KC567164), *X. cohni* (KC567173), *X. nuragicum* (GU725071, GU725072, KC567184), *X. pyrenaicum* (GU725073, France), *X. sphaerocephalum* (GU725076), and *X. zagrosense* (JN153101); morphospecies group 5 with pseudo-Z-organ and dorsally convex-conoid tail with subdigitate or digitate terminus *viz*. *X. hispidum* (HM924346, KC567181), *X. gersoni* (KC567180), *X. lupini* (HM921352, KC567183), and some with tail broadly convex-conoid to hemispherical *viz*. *X. hispanum* (GU725074), and *X. turcicum* (KC567185,

GU725077); morphospecies group 7 with no uterine differentiation and an elongate to conical tail *viz*. *X. elongatum* (AY601618, EF140790), *X. insigne* (AY601619), *X. italiae* (HM921350, AY601613, FJ713153, KC567182), *X. savanicola* (AY601620), *X. setariae* (AY601621 and = *vulgare,* DQ299514); morphospecies group 1 including four species characterized by no anterior genital branch and a long elongate to conical tail *viz*. *X. brasiliense* (AY601616), *X. chambersi* (AY601617), *X. hunaniense* (EF188839, EF188840) and *X. naturale* (DQ299515); and two species of mophospecies group 8 characterized by no uterine differentiation and a tail convex-conoid with a terminal peg or mucro [*X. granatum* (JQ240273) and *X. vuittenezi* (EF614266, AY601614)]. Clade (ii) included twelve species (PP = 98%; BS = 88%) mostly belonging to morphospecies group 5, and including the two new species from southern Spain [*X. baetica* sp. nov. (KC567165-KC567169) and *X. turdetanensis* sp. nov. (KC567186)], as well as other known species with a tail dorsally convex-conoid with subdigitate or digitate terminus *viz*. *X. abrantinum* (AY601625), *X. belmontense* (KC567170-KC567172), *X. coxi europaeum* (KC567174-KC567179), *X. diversicaudatum* (AY601624 from Portugal and EF538755 from Slovakia), and species with tail round to hemispherical [*X. globosum* (GU549474) and *X. dentatum* (AY621627, EF781538)]; one species identified belonging to groups 6, 7, and 8, *viz*. [*X. pyrenaicum* (AY601626 from Cyprus); *X. bakeri* (AY601623); and *X. index* (HM921404, HM921406), respectively]. Finally, clade (iii) occupied a basal and well defined position in the tree included two species (PP = 77%; BS = 72%) of group 5 characterized by a tail short conical distinctly digitate *viz*. *X. basiri* (AY601629, AY601630) and *X. coxi coxi* (AY601631, USA). *Xiphinema baetica* sp. nov. is related phylogenetically to a large number species characterized by a dorsally convex-conoid tail with digitate terminus belonging to *Xiphinema diversicaudatum*-complex (*viz*. *X. belmontense*, *X. coxi europaeum*, and *X. diversicaudatum*), but this clade is moderately supported in our analysis (PP = 92%; BS = 88%) (Fig. 7). However, the clade including the former species and *X. turdetanensis* sp. nov. and *X. globosum* is moderately supported by BI and ML (PP = 63%; BS = 88%).

Difficulties were experienced with alignment of the ITS1 sequences due to scant homology, and only related sequences were included in our study using *X. index* (AJ437026) as outgroup. The phylogenetic tree based on ITS1 sequences resolved two major clades (Fig. 8). Clade (i) included eight species with similar tail shape (PP = 98%; BS = 62%), divided in two subclades comprising mostly species belonging to morphospecies group 5, *viz*. *X. baetica* sp. nov. (KC567156-KC567157), *X. belmontense* (KC567158), *X. coxi europaeum* (KC567160-KC567162), *X. diversicaudatum* (AY430183, AJ437027), *X. turdetanensis* sp. nov. (KC567163), *X. globosum* (GU549475), and *Xiphinema* sp. JZ-2006 (DQ364686), and a species belonging to morphospecies group 7, *viz*. *X. bakeri* (AF511426-AF511427). And clade (ii) occupied a basal position in the tree and including only a species with a tail conoid-rounded

belonging to morphospecies group 4, *viz*. *X. bernardi* (EU375482-EU375484). The majority of the sub-clades in clade (i) were well supported by BI and ML analysis. *Xiphinema baetica* sp. nov. occupied a paraphyletic position distant from *X. turdetanensis* sp. nov. for this marker. *Xiphinema baetica* sp. nov. is related phylogenetically to a large number species characterized by a dorsally convex-conoid tail with subdigitate or digitate terminus including *X. coxi europaeum*, *X. diversicaudatum* and *X. belmontense*, but this clade is weakly supported in our analysis (Fig. 8). However, the clade including the former species and *X. turdetanensis* sp. nov. and *X. globosum* is well supported by BI and ML (PP = 100%; BS = 78%).

Phylogenetic analysis based on the partial 18S rDNA gene sequences separated clearly the lineage of *X.* non-*americanum*-group from the lineage of *X. americanum*-group, including *X. incognitum*, *X. peruvianum*, and *X. oxycaudatum* (Fig. 9). The 50% majority rule consensus BI and ML trees of *X.* non-*americanum*-group based in a multiple edited alignment including 51 partial 18S rDNA gene sequences and 1545 bp showed three low to moderate supported major clades (Fig. 9). Clade (i) included a large group of thirty-one species (PP = 100%; BS = 59%), divided in three subclades comprising the two new species described herein as well as other known species from morphospecies group 5 [13 species, such as *X. belmontense* (KC567150), *X. coxi europaeum* (KC567152- KC567153), *X. gersoni* (KC567154), *X. hispidum* (KC567152), *X. globosum* (GU549476), *X. turcicum* (GU725086) or *X. montenegrinum* (EU477382)] , group 6 [10 species, such as *X. adenohystherum* (GU725084), *X. cohni* (KC567151), *X. nuragicum* (GU725078-GU725081), *X. pyrenaicum* (GU725085)]; group 4 with a conoid-rounded tail [1 species, *X. ifacolum* (AY297826)]; group 7 [4 species, *X. bakeri* (AY283173), *X. elongatum* (AY297824), *X. italiae* (FJ713154, HM921343) and *Xiphinema* sp. (AY297840)]; group 8 [2 species, *X. index* (AY687997, EF207249, HM921342) and *X. vuittenezi* (AY552979, EF614267)]; and one species of group 1 with an elongate, ventrally, arcuate tail [1 species, *X. chambersi* (AY283174)]. Clade (ii) appeared as sister clade of (i) comprising two species of group 1 with elongate-conoid tail, *viz*. *X. brasiliense* (AY297836) and *X. ensiculiferum* (AY297834). Finally, clade (iii) occupied a basal position in the *X.* non-*americanum*-group lineage and comprised five species of group 2 characterized by a tail long, *viz*. *X. variegatum* (AY297828), *X. krugi* (AY297827, South Carolina), *X. longicaudatum* (AY297829), *X. krugi* (AY297828, Mississippi), and *X. surinamense* (AY297833). *Xiphinema baetica* sp. nov. also occupied a paraphyletic position from *X. turdetanensis* sp. nov. for this marker, showing a close relationship with some species including two species with similar tail shape *viz*. *X. belmontense* and *X. coxi europaeum*, and a species with rounded tail (*viz*. *X. globosum*) (Fig. 9).

Morphological characters evolution showed a feasible ancestral stage for pseudo-Z-organ and for the absence of spines. Both characters showed an appearance of the character in different

period of the evolutionary tree (Figs. 10-A & B). However, the character reconstruction for tail shape showed an equivocal reconstruction for the majority of the clades studied (Fig. 10 C).

DISCUSSION

The primary objective of this study was to identify and to characterize morphometrical- and molecularly species of dagger nematodes belonging to the *X.* non-*americanum* group in cultivated and natural environments in Spain, assigning molecular markers useful to distinguish virus vectors from non vector species, a fact which may have critical phytopathological implications. We described here two new species of the genus *Xiphinema*, belonging to the morphospecies group 5 of Loof and Luc (1990), based on integrative taxonomy and to understand the phylogenetic relationships among the new and known species of the genus *Xiphinema* spp. based on nuclear rDNA.

MORPHOLOGICAL COMPARISON OF *XIPHINEMA BAETICA* SP. NOV. AND *XIPHINEMA TURDETANENSIS* SP. NOV. WITH RELATED TAXA

Morphologically, *X. baetica* sp. nov. belongs to the *X.* non-*americanum* group 5 in Loof and Luc (1990). Based upon the diagnostic characters used in the polytomous key by Loof and Luc, (1990) and character analysis by Coomans *et al*. (2001), including body length (L), habitus, lip region shape, odontostyle length, oral aperture-guiding ring length, uterine differentiation, vulva position, female tail shape and length, presence or absence of males, and the ratios a, b (body length/pharyngeal length), c, c', it closely resembles *Xiphinema capense* Coomans and Heyns, 1985, *X. coxi europaeum*, *X. diversicaudatum*, *X. dissimile*, *X. gersoni*, and *X. turdetanensis* sp. nov. From *X. capense* it differs by a longer body and odontostyle (4909-6091, 141.5-157.0 vs 3660-4180, 110.0-122.0 µm, respectively), a higher a (body length/maximum body width), b (body length/pharyngeal length), and c ratio (82.2-114.9, 10.4-12.6, 92.7-131.2 *vs* 65.5-85.3, 8.3-9.9, 67.2-91.2, respectively), and presence *vs* absence of male. From *X. coxi europaeum* it differs by a longer body and odontostyle (4909-6091, 141.5-157.0 *vs* 3600-4000, 131.8-145.3 µm, respectively), and a higher a, b and c ratio (82.2-114.9, 10.4-12.6, 92.7-131.2 *vs* 70.2-85.3, 7.8-8.6, 71.8-77.0, respectively), and frequency of male (common *vs* rare). From *X. diversicaudatum* it differs by a higher a (body length/maximum body width), b (body length/pharyngeal length), c (body length/tail length) and V ratio (82.2-114.9, 10.4-12.6, 92.7-131.2, 42-48 *vs* 57.0-92.0, 6.6-11.4, 61.0-134.0, 39-46, respectively). From *X. gersoni* it differs by a lower body length, a (body length/maximum body width) and c´ (tail length/body width at anus) ratios (4909-6091 µm, 82.2-114.9, 1.1-1.6 *vs* 5400-7300 µm, 101.0-136.5, 1.4-2.0, respectively), and a shorter odontostyle (141.5-157.0 µm *vs* 146.0-164.5 µm). From *X. dissimile* it differs by a different lip region shape (separated from the rest of the body by a weak

depression *vs* offset from the rest of the body by a wide constriction), a longer odontostyle (141.5-157.0 µm *vs* 121.3-134.3 µm), and a larger distance from guiding ring to anterior end (112.0-147.0 µm *vs* 116.0-127.3 µm), a different female tail shape (peg ventrally oriented in the caudal axis *vs* peg with large base and located ventrally in line with body profile), and a lower c´ ratio of J1 (3.4-3.9 *vs* 5.3-5.9). From *X. turdetanensis* sp. nov. it differs by a larger body and odontostyle (4909-6091, 141.5-157.0 *vs* 4066-5227, 121.0-142.0 µm, respectively), a higher c' ratio (1.1-1.6 *vs* 1.1-1.3), and uterine differentiation (pseudo-Z-organ comprising 8-13 sclerotized bodies of variable size without uterine spines *vs* pseudo-Z-organ with 6-8 globular bodies plus uterine spines in tubular part of uterus).

Based upon the same diagnostic characters (Loof & Luc, 1990), *X. turdetanensis* sp. nov. also belongs to the *X.* non-*americanum* group 5 and it closely resembles *X. belmontense*, *X. diversicaudatum*, *X. gersoni*, and *Xiphinema silvesi* Roca and Bravo, 1998. From *X. belmontense* it differs by a higher a, V [(distance from anterior end to vulva/body length) x 100], and c ratio (70.0-99.8, 43-48, 94.4-116.2 *vs* 58.4-72.3, 36-42, 63.1-96.7, respectively), and shorter spicules (59-69 *vs* 89-112 µm). From *X. diversicaudatum* it differs by lip region shape (region rounded-hemispherical, separated from the body by a shallow depression *vs* low, smoothly rounded, continuous with body contour), a shorter odontostyle and odontophore length (121-142, 67-80 *vs* 130-157, 70-97 µm, respectively), and shorter spicules (59-69 *vs* 69-81 µm). From *X. gersoni* it differs by a shorter body, odontostyle and odontophore length (4066-5227, 121-142, 67-80 *vs* 5400-7300, 146-164, 76-89, µm, respectively), a lower a (body length/maximum body width), c (body length/tail length), and c' ratio (70.0-99.8, 94.4-116.2, 1.1-1.3 *vs* 101.5-136.5, 89.7-127.7, 1.4-2.0, respectively), and shorter spicules (59-69 *vs* 70-80 µm).

Delimiting closely related *X.* non-*americanum* group species is a particularly difficult issue. The comparative morphological and morphometrical studies based on PCoA and cluster analyses of the 43 species of *X.* non-*americanum* group belonging to the morphospecies groups 1, 5, 6, 7 and 8, and including the 23 Spanish populations, confirmed that diagnostic and identification of these species based solely on morphometric features is problematic since there is almost a continuous range of characters measurements among species in some characters and for this reason, polytomous keys are used. The current study has demonstrated that multivariate analyses performed upon quantitative and qualitative characters of these species was partially congruent with molecular differences based on rDNA, and in a lesser extend at phylogenetic level. Also, the fact that all populations within a species were included into a single functional group demonstrates the usefulness of these analyses for helping in the diagnosis and identification of *Xiphinema* spp. Nevertheless, multivariate analyses solely cannot confidently discriminate between species closely related (i.e. *X. gersoni* and *X. dissimile*). Indeed, similar

levels of intra-specific morphological variation can be found for *X. baetica* sp. nov. as exist between two different species (i.e. *X. granatum* and *X. silvesi*), which emphasizes the need for a integrative identification by combining molecular techniques with morphology and morphometry measurements, that showed essential for a correct *Xiphinema* identification because of the low inter-population variability found for some of these species at rDNA level (i.e. *X. baetica* sp. nov., *X. index*) (Gutiérrez-Gutiérrez *et al*., 2010; 2011). The present results (including new and known species) enlarge the biodiversity of *X.* non-*americanum* group in the Iberian Peninsula and species morphometrics and diagnosis agree with the results obtained by previous researchers (Arias, Navas & Andrés, 1987; Bravo, Roca & Mota, 2001; Murillo-Navarro *et al*., 2005; Peña-Santiago *et al*., 2006).

MOLECULAR AND PHYLOGENETIC RELATIONSHIPS IN *XIPHINEMA*

Sequences of nuclear rDNA genes, particularly D2-D3 and ITS1, have proven to be a powerful tool for providing accurate and molecular species identification in Longidoridae (He *et al*., 2005; Gutiérrez-Gutiérrez *et al*., 2010; 2011; 2012; 2013; Palomares-Rius *et al*., 2013). Our results confirm the usefulness of these markers in the *X.* non-*americanum* group, since nucleotide differences among species ranged from 22 to 142 nucleotides for D2-D3 and 76 to 208 nucleotides for ITS1 within related sequences. However, our findings also corroborate that partial 18S sequence showed a potential use to distinguish some species of morphospecies groups 1 or 2, but generally does not have enough resolution to distinguish the majority of species in the *X.* non-*americanum* group, since nucleotide differences among species were as low as 1-6 bp. The phylogenetic relationships inferred in this study based on the D2-D3, ITS1 and the partial 18S sequences mostly agree with the lineages obtained by Gutiérrez-Gutiérrez *et al*. (2010; 2011); Cantalapiedra-Navarrete *et al*. (2011), and Palomares-Rius *et al*. (2013) with the phylogeny of dagger and needle nematodes.

To confirm a correlation of the results obtained by conventional morphological and new molecular methods is important in order to understand the evolution of the *X.* non-*americanum* group. Phylogenetic analyses based on D2-D3, ITS1 and partial 18S using BI and ML (Figs. 7-9) resulted in a congruent position of the new sequenced species of *X.* non-*americanum* group from Spain, which grouped in separate clades the majority of species belonging to groups 5 and 6, except for some species such as *X. hispidum* (KC567181), *X. gersoni* (KC567180), *X. lupini* (KC567183), and *X. turcicum* (KC567185) in D2-D3, which belong to group 5 but clustered with species of morphospecies groups 6 and 7 (Fig. 7). The close morphological relationships in the morphospecies group 5 with some of the species studied here (*X. baetica* sp. nov., *X. turdetanensis* sp. nov., *X. belmontense*, and *X. coxi europaeum*) were also phylogenetically correlated in all three markers studied. Our results on phylogenetic relationships in the *X.* non-

americanum group inferred by D2-D3 and the partial 18S trees suggest that the huge morphospecies groups 5 and 6 may be considered paraphyletic, which agree with cladistic analysis by Coomans *et al*. (2001). The clade topology of phylogenetic trees of D2-D3 and partial 18S regions in this study were congruent supporting the polyphyletic status of some characters, such as the female tail shape and the degree of development of genital system in species with equally developed genital branches. Then, our findings confirmed that some characters should have a multiple origin, which agrees with cladistic analysis by Coomans *et al*. (2001). Nevertheless, the clade topology of phylogenetic trees of D2-D3 and 18S genes suggest that the tripartite condition of the uterus is the less derived state (plesiomorphy), which disagrees with the hypothesis developed by Coomans *et al*. (2001) about the uterus evolution in *Xiphinema*. In fact, the particular branching pattern of phylogenetic trees of D2-D3 and 18S genes suggests that the bipartite condition must have been originated many times from the tripartite condition. However, the lack of molecular data of species with bipartite uterus as well as their proportion, in term of number of abundant sequences, between species with bipartite and tripartite condition could have affected our results. Also, phylogeny inferred from D2-D3 and 18S sequences suggested that the status paraphyletic of some characters such as the number and degree of development of genital branches in morphospecies group 1 and 2. In fact, Coomans *et al*. (2001) revealed that the monodelphic and pseudomonodelphic forms must have been originated several times independently from didelphic ancestors. Then, due to high frequency of parallelism or reversal of states of some characters studied such as uterus structure and tail shape, these characters must be of limited value in establishing phylogenetic relationships in the genus *Xiphinema*. In our case, the presence of pseudo-Z-organ in tubular part of uterus was an ancestral stage which has been lost and regained again during evolution in this genus, similar conclusions could be drown for the presence of uterine spines in tubular part of uterus. It has been suggested a role for Z-organ and pseudo-Z-organ in keeping the sperm in the uterus, forcing the slow passage of eggs during the shell formation and add secretions to the egg shell (Grimaldi de Zio *et al*., 1979; Cho, Robbins & Kim, 2000). A similar role for uterine spines has been suggested in slowing the egg passage through the uterus (Grimaldi de Zio *et al*., 1979). The case of tail shape is more complex because a clear pattern of evolution could not be distinguished. Coomans *et al*. (2001) suggested that in Dorylaimida, including *Xiphinema*, long tail is a pleisomorphic character because their better adaptation for swimming. This hypothesis was corroborated by ontogenic tail studies in species description (Coomans *et al.* 2001). This lack of concordance between our character study and molecular phylogeny could be related to a fast diversification of the genus *Xiphinema* (more than 260 species) and/or the lack of molecular data of species with filiform tail. In addition, the partial agreement between taxonomy based on morphological characters and the new use of molecular markers has been observed in species

complexes and cryptic biodiversity within the *X.* non-*americanum* group. It has been demonstrated for several species within the genus *Xiphinema* (Oliveira, Ferraz & Neilson, 2006; Wu, Zheng & Robbins, 2007; Gutiérrez-Gutiérrez *et al*., 2010; 2013). Nevertheless, additional integrative taxonomic studies are needed to clarify and confirm these hypotheses.

In any case, the position of some species is difficult to assign with the sequences data deposited in GenBank. The case for *X. diversicaudatum* in D2-D3 and ITS1 trees (Figs. 7 and 8), and *X. vuittenezi* in 18S tree (Fig. 9) are paradigmatic examples. A D2-D3 sequence from Slovakia (EF538755) and another from Portugal (AY601624), and a ITS1 sequence from France (AJ437027) and another from unknown origin (AY439183), generated different positions in the phylogenetic trees obtained in this study (Figs. 7, 8). Similarly, *X. pyrenaicum* from Cyprus (AY601627) clustered quite separately from *X. pyrenaicum* (GU725073, France), but the former was identified on the basis of 'general morphology' (He *et al*., 2005) and the latter in a integrative study using morphological and molecular characterizations (Gutiérrez-Gutiérrez *et al*., 2010); as well as *X. elongatum* (EF140790, China) and *X. elongatum* (AY601618, Israel) (Fig. 7); or *X. vuittenezi* (AY552979) from Kenya and from Czech Republic (EF614267), Fig. 9. These occurrences are good examples demonstrating the difficulties for species identification in this complex genus due to character overlap (He *et al*., 2005), and may also suggest the presence of cryptic species within these species groups (Gutiérrez-Gutiérrez *et al*., 2010). In fact, these data suggest that population of *X. diversicaudatum* (AY601624) from Portugal, identified on the basis of 'general morphology' (He *et al*., 2005), most probably is a misidentification and should be considered conspecific with *X. coxi europaeum*, since D2-D3 sequences from the six Spanish studied populations matched closely (99% similarity) with D2-D3 from this population of *X. diversicaudatum* (AY601624, Portugal). These examples demands special attention in assigning molecular markers to *Xiphinema* spp. as some species are virus vectors, thus, may have critical phytopathological implications. In fact, *X. diversicaudatum* and *X. coxi coxi* had been reported as vectors of *ArMV*, *SLRV* and *CLRV* (Taylor & Brown, 1997).

CONCLUSIONS

In summary, the present study establishes the importance of using integrative taxonomic identification highlighting the time consuming aspect and difficulty of a correct identification at species level within the *X.* non-*americanum* group. This study also provides molecular markers for precise and unequivocal diagnosis of some species of the *X.* non-*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.* non-*americanum*-group in the same soil sample are frequent. Multivariate and phylogenetic analyses were partially congruent, since lineages were maintained for some of the morphospecies groups (i.e. morphospecies groups 5 and 6), except

for some species (*viz*. position of *X. hispidum*, *X. lupini*, *X. gersoni*, and *X. turcicum*). However, multivariate analyses allowed delimitating species groups. Consequently, our results strengthened that *Xiphinema* species delimitation should be the result of integrated studies based on morphology, morphometry and molecular taxonomic identification and phylogeny of D2-D3 region ITS1 of rDNA, and in a lesser extend of partial 18S-rDNA sequences. Future phylogenetic studies should include other additional genetic markers as mitochondrial DNA genes and nuclear protein coding genes such as cytochrome c oxidase subunit 1 (*COI*) or heat shock protein (*hsp90*) genes, in order to resolve the relationships within *Xiphinema* (Gutiérrez-Gutiérrez *et al*., 2012). Ecological requirements of the species could also help to differentiate them (Weischer & Almeida, 1995). However, the polyphagous character of the majority of these species makes this task more difficult and only based in edaphic and climatic parameters.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Light micrographs of *Xiphinema belmontense* Roca and Pereira, 1992. A, female neck region. B, C, lip regions. D, E, detail of pseudo-Z-organ. F-H, female tails. i male tail. Abbreviations: a = anus; psz = pseudo-Z-organ. (Scale bars = 20 μm).

Fig. S2. Light micrographs of *Xiphinema cohni* Lamberti, Castillo, Gómez-Barcina and Agostinelli, 1992. A, female lip region. B, detail of uterine spines. C-F, female tail regions. Abbreviations: a = anus; us = uterine spine. (Scale bars = 20 μm).

Fig. S3. Light micrographs of *Xiphinema coxi europaeum* Sturhan, 1985. A, B, female lip region. C, vulval region. D, E, detail of pseudo-Z-organ. F-J, female tail regions. Abbreviations: a = anus; psz = pseudo-Z-organ; V = vulva. (Scale bars = 20 μm).

Fig. S4. Light micrographs of *Xiphinema gersoni* Roca and Bravo, 1993. A, female lip region. B, detail of female anterior gonad. C, vulval region. D, detail of pseudo-Z-organ. E-G, female tail regions. H, I, male tail. Abbreviations: a = anus; psz = pseudo-Z-organ; V = vulva. (Scale bars: a, c-i = 20 μm; b = 100 μm).

Table S1. Morphometrics of *Xiphinema adenohystherum* and three populations of *Xiphinema belmontense* from Spain.

Table S2. Morphometrics of seven populations of *Xiphinema coxi europaeum* from southern Spain.

Table S3. Morphometrics of *Xiphinema cohni* and *Xiphinema gersoni* from Spain.

Table S4. Molecular similarity values (% and number of nucleotides) among the known *Xiphinema* species sampled in this study and those deposited in GenBank using the D2-D3 expansion segments of 28S rDNA.

Table S5. Molecular similarity values (% and number of nucleotides) among the known *Xiphinema* species sampled in this study and those deposited in GenBank using the D2-D3 expansion segments of 28S rDNA, ITS1, and partial 18S rDNA gene sequences.

Figure legends

Fig. 1. Line drawings of ***Xiphinema baetica* sp. nov.** A, female neck region. B, female lip region. C, anterior gonad. D, detail of pseudo-Z-organ. E-G, female tail regions. H, male tail region.

Fig. 2. Light micrographs of ***Xiphinema baetica* sp. nov.** A, B, female lip region. C, vulval region. D-F, detail of pseudo-Z-organ. G-K, female tail regions. L, first-stage juvenile neck region. M-P, first-, second-, third-, and fourth-stage juvenile tails (J1-J4), respectively. Q-R, male tail regions. Abbreviations: a = anus; psz = pseudo-Z-organ; rost = replacement odontostyle; V = vulva. (Scale bars = 20 μm).

Fig. 3. Relationship of body length to length of functional and replacement odontostyle (Ost and rOst, respectively) length in all developmental stages from first-stage juveniles (J1) to mature females of: (A) ***Xiphinema baetica* sp. nov.** and (B) ***Xiphinema turdetanensis* sp. nov.**

Fig. 4. Line drawings of ***Xiphinema turdetanensis* sp. nov.** A, female neck region. B, female lip region. C, pharyngeal bulb. D, vulval region. E, detail of pseudo-Z-organ. F-H, female tail regions. I, male tail region.

Fig. 5. Light micrographs of ***Xiphinema turdetanensis* sp. nov.** A, B, female lip region. C, detail of pseudo-Z-organ. D, detail of uterine spines. E, vulval region. F, detail of sperm cells. G-I, female tail regions. J, male tail. K, first-stage juvenile neck region. L-O, first-, second-, third-, and fourth-stage juvenile tails (J1-J4), respectively. Abbreviations: a = anus; psz = pseudo-Z-organ; rost = replacement odontostyle; sp = spine. (Scale bars: A-C = 20 μm; D = 10 μm; E-O = 20 μm).

Fig. 6. Principal coordinates analysis (PCoA) of 11 morphometric characters and five morphological characters used to characterize 43 species of *Xiphinema*-non *americanum* group. (A) Ordination plot for the first two dimensions of PCoA showing projection of the 43 species and populations within each species. Underline and font bold species indicated populations characterized in this study, whereas normal font are from original descriptions. (B) Functional cluster groupings of *Xiphinema* spp. and populations identified according to previously described morphospecies groups projected on the plane of the first two dimensions of PCoA.

Fig. 7. Phylogenetic relationships within *Xiphinema* non-*americanum*-group complex. Bayesian 50% majority rule consensus tree as inferred from D2 and D3 expansion segments of 28S rDNA sequence alignment under the general time reversible of invariable sites and gamma-shaped distribution (GTR + I+ 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 in bold letters. (*****) *Xiphinema* spp. which need diagnostic confirmation. Images in each subclade refer to female type-shape and uterine differentiation discussed in the main text. Colours or grey scale font refer to *Xiphinema* spp. groups discussed in the main text.

Fig. 8. Phylogenetic relationships within *Xiphinema* non-*americanum*-group complex. Bayesian 50% majority rule consensus tree as inferred from ITS1 rDNA sequence alignment under the 012340+G+F 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 in bold letters. (*****) *Xiphinema* spp. which need diagnostic confirmation. Colours or grey scale font refer to *Xiphinema* spp. groups discussed in the main text.

Fig. 9. Phylogenetic relationships within *Xiphinema* non-*americanum*-group complex. Bayesian 50% majority rule consensus tree as inferred from 18S rDNA gene sequence alignment under the general time reversible of invariable sites and gamma-shaped distribution (GTR + I+ 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 in bold letters. (*****) *Xiphinema* spp. which need diagnostic confirmation. Colours or grey scale font refer to *Xiphinema* spp. groups discussed in the main text.

Fig. 10. Morphological character history reconstruction for two uterine differentiation and tail shape characters using Bayesian simulations (trees on left) and parsimony (trees on right) on the consensus tree using only one population for each *Xiphinema* species. Charts on selected nodes show relative posterior probabilities of each stage of the character**.** (A) Uterine differentiation (pseudo-Z-organ), 0: absence, 1: presence. (B) Uterine differentiation (spines in tubular part of uterus), 0: absence, 1: presence. (C) Tail shape, 0: tail long (c' between 2.5 and 7.5), conical or with clavate terminus, 1: tail regularly short conical (c' at most 2.5), 2: tail short conical (c' at most 2.5), distinctly digitate, 3: Tail conical to hemispherical with a terminal peg, mucro or bulge, 4: tail broadly convex-conoid, 5: tail regularly hemispherical.

Table 1. Taxa sampled for *Xiphinema* species, morphospecies group, locality, and associated host sequenced in this study.

Species	Group[a]	Locality	Host-plant/reference population	D2-D3	ITS1	partial 18S
Xiphinema adenohystherum	6	Arévalo de la Sierra (Soria, Spain)	holly tree/SORI	KC567164	-	-
***Xiphinema baetica* sp. nov.**	5	Hinojos (Huelva, Spain)	stone pine/H016	KC567165	KC567156	KC567148
***Xiphinema baetica* sp. nov.**	5	Hinojos (Huelva, Spain)	stone pine/H023	KC567166	-	-
***Xiphinema baetica* sp. nov.**	5	Manzanilla (Huelva, Spain)	grapevine/H031	KC567167	KC567157	KC567149
***Xiphinema baetica* sp. nov.**	5	Benalup-Casas Viejas (Cádiz, Spain)	grasses/LOMA	KC567168	-	-
***Xiphinema baetica* sp. nov.**	5	Hinojos (Huelva, Spain)	stone pine/H001	KC567169	-	-
Xiphinema belmontense	5	Puebla de Sanabria (Zamora, Spain)	chestnut/CEFR	KC567170	-	-
Xiphinema belmontense	5	Merza (Coruña, Spain)	chestnut/MOUD	KC567171	-	-
Xiphinema belmontense	5	Merza (Coruña, Spain)	pedunculate oak/MOUB	KC567172	KC567158	KC567150
Xiphinema cohni	6	El Puerto de Santa María (Cádiz, Spain)	stone pine/J126	KC567173	KC567159	KC567151
Xiphinema coxi europaeum	5	Hinojos (Huelva, Spain)	cork oak/H027	KC567174	KC567160	KC567152
Xiphinema coxi europaeum	5	Hinojos (Huelva, Spain)	carob tree/H028	KC567175	-	-
Xiphinema coxi europaeum	5	Manzanilla (Huelva, Spain)	grapevine/H031	KC567176	KC567161	-
Xiphinema coxi europaeum	5	Almonte (Huelva, Spain)	cork oak/H048	KC567177	-	-
Xiphinema coxi europaeum	5	Hinojos (Huelva, Spain)	grapevine/H050	KC567178	KC567162	KC567153
Xiphinema coxi europaeum	5	Cortes de la Frontera (Cádiz, Spain)	cork oak/SAUC	KC567179	-	-
Xiphinema gersoni	5	Almonte (Huelva, Spain)	eucalyptus/H059	KC567180	-	KC567154
Xiphinema hispidum	5	Bollullos par del Condado (Huelva, Spain)	grapevine/H062	KC567181	-	-
Xiphinema italiae	7	Cabra (Córdoba, Spain)	grapevine/M044	KC567182	-	-
Xiphinema lupini	5	Hinojos (Huelva, Spain)	grapevine/H050	KC567183	-	-
Xiphinema nuragicum	6	Espejo (Córdoba, Spain)	grapevine/M054	KC567184	-	-
Xiphinema turcicum	5	Sanlúcar de Barrameda (Cádiz, Spain)	grapevine/J230	KC567185	-	-
***Xiphinema turdetanensis* sp. nov.**	5	Sanlúcar de Barrameda (Cádiz, Spain)	stone pine/J212	KC567186	KC567163	KC567155
***Xiphinema turdetanensis* sp. nov.**	5	Sanlúcar de Barrameda (Cádiz, Spain)	wild olive/AR15	*	-	-

[a] Morphospecies group according to Loof & Luc (1990)

(-) Not obtained or not performed.

(*) Sequenced population but not deposited in GenBank database, since was identical to KC567186

Table 2. Morphometrics of ***Xiphinema baetica* sp. nov.** from grapevine at Manzanilla (Huelva, Spain)[a].

Characters/ratios [b]	Holotype	Paratypes					
		Females	Males	J1	J2	J3	J4
n	1	21	16	4	3	6	5
L	5422	5532 ± 330 (4909-6091)	5467 ± 577 (4616-6227)	1024 ± 25.6 (994-1050)	2053 ± 186 (1839-2179)	2576 ± 243 (2295-2886)	3704 ± 253 (3386-4023)
a	100.4	103.4 ± 8.3 (82.2-114.9)	115.6 ± 9.7 (99.9-131.8)	42.1 ± 2.2 (40.4-45.2)	55.0 ± 8.8 (44.9-60.5)	72.7 ± 8.1 (64.9-87.3)	91.2 ± 5.2 (83.2-97.2)
b	10.7	11.7 ± 0.9 (10.4-10.6)	11.7 ± 1.6 (8.2-14.6)	6.5 ± 0.4 (6.1-7.1)	7.8 ± 1.0 (6.9-8.8)	7.4 ± 0.6 (6.7-8.3)	9.2 ± 1.3 (7.6-10.8)
c	92.7	111.3 ± 12.0 (92.7-131.2)	107.2 ± 12.9 (88.0-133.8)	18.8 ± 0.5 (18.4-19.4)	34.0 ± 2.5 (32.3-36.9)	43.2 ± 5.2 (35.9-48.9)	65.6 ± 4.8 (61.0-71.2)
c′	1.6	1.4 ± 0.1 (1.1-1.6)	1.4 ± 0.1 (1.2-1.6)	3.6 ± 0.2 (3.4-3.9)	2.6 ± 0.1 (2.6-2.7)	2.3 ± 0.1 (2.1-2.5)	1.9 ± 0.2 (1.7-2.1)
V or T	45.0	45.5 ± 1.8 (42-48)	51.1 ± 8.6 (40-74)	-	-	-	-
G_1	10.7	10.2 ± 1.2 (7.4-11.7)	-	-	-	-	-
G_2	10.6	10.1 ± 0.8 (7.9-11.4)	-	-	-	-	-
Odontostyle length	146.5	148.3 ± 4.1 (141.5-157.0)	146.3 ± 4.7 (141.0-158.0)	60.9 ± 2.7 (57.0-63.0)	86.0 ± 3.0 (83.0-89.0)	103.5 ± 1.6 (102.0-106.0)	126.1 ± 3.8 (122.0-131.5)
Replacement odontostyle length	-	-	-	73.1 ± 2.0 (71.5-76.0)	105.3 ± 4.0 (103.0-110.0)	125.4 ± 1.4 (123.0-126.5)	148.4 ± 5.6 (143.0-157.0)
Odontophore length	79.0	76.0 ± 3.3 (70.0-84.5)	75.3 ± 2.6 (72.0-80.0)	39.8 ± 1.7 (38.0-42.0)	50.7 ± 0.6 (50.0-51.0)	56.3 ± 1.2 (55.0-58.0)	71.0 ± 4.8 (63.0-76.0)
Lip region width	14.0	14.1 ± 0.5 (12.5-14.5)	13.9 ± 0.5 (13.0-14.5)	7.8 ± 0.3 (7.5-8.0)	9.3 ± 0.3 (9.0-9.5)	10.9 ± 0.5 (10.5-11.5)	12.8 ± 0.4 (12.0-13.0)
Oral aperture-guiding ring	116.0	127.5 ± 10.6 (112.0-147.0)	128.4 ± 7.3 (110.0-141.0)	46.9 ± 1.2 (46.0-48.5)	57.7 ± 0.6 (57.0-58.0)	77.2 ± 5.6 (70.0-86.0)	101.4 ± 17.2 (81.0-120.0)
Tail length	58.5	49.9 ± 3.3 (44.5-58.5)	51.2 ± 4.4 (45.0-60.0)	54.4± 0.5 (54.0-55.0)	60.3 ± 4.2 (57.0-65.0)	59.8 ± 2.6 (58.0-64.0)	56.6 ± 3.7 (51.0-60.0)
J	18.5	17.5 ± 1.7 (15.0-20.0)	18.5 ± 2.1 (16.5-23.0)	8.1 ± 0.3 (8.0-8.5)	21.0 ± 1.4 (20.0-22.0)	20.0 ± 2.4 (17.0-24.0)	19.5 ± 2.3 (17.0-22.0)
Spicules	-	-	64.7 ± 2.8 (61.0-70.5)	-	-	-	-
Lateral accessory piece	-	-	14.1 ± 0.9 (13.0-15.5)	-	-	-	-

[a] Measurements are in µm and in the form: mean ± standard deviation (range).

[b] a = body length/maximum body width; b = body length/pharyngeal length; c = body length/tail length; c' = tail length/body width at anus; V = (distance from anterior end to vulva/body length) x 100; J = hyaline tail region length.

Table 3. Morphometrics of ***Xiphinema baetica*** **sp. nov.** from several localities in southern Spain[a].

	Hinojos (Huelva province) stone pine/H016		Hinojos (Huelva province) stone pine/H023		Hinojos (Huelva province) stone pine/H001		Benalup-Casas Viejas (Cadiz province) grasses/LOMA
Characters/ratios[b]	Females	Male	Females	Males	Females	Males	Females
n	4	1	6	2	4	2	2
L	5279 ± 506 (4659-5878)	5318	5513 ± 383 (5045-5977)	(4955-5659)	5649 ± 610 (4811-6273)	(5818-5841)	(4705-5818)
a	101.4 ± 6.3 (95.1-109.9)	102.3	112.7 ± 6.1 (106.5-118.7)	(112.6-123.0)	103.1 ± 10.8 (91.6-114.1)	(118.7-124.3)	(94.1-105.8)
b	11.5 ± 1.1 (10.8-13.1)	11.4	13.2 ± 0.5 (12.9-13.8)	(10.4-11.3)	11.3 ± 1.6 (9.3-12.7)	(11.3-12.6)	(11.7-13.4)
c	102.7 ± 13.7 (84.7-117.6)	106.4	110.7 ± 8.3 (103.0-119.5)	(95.3-120.4)	102.9 ± 10.0 (88.1-110.4)	(95.8-97.0)	(84.0-118.7)
c´	1.5 ± 0.1 (1.4-1.7)	1.3	1.5 ± 0.1 (1.4-1.6)	(1.2-1.5)	1.4 ± 0.2 (1.2-1.5)	(1.6-1.6)	(1.5-1.8)
V or T	46.8 ± 1.3 (45-48)	58.6	46.0 ± 2.4 (44-50)	(47-52)	45.5 ± 1.7 (43-47)	(41-55)	-
G_1	11.6 ± 1.0 (10.9-12.7)	-	8.9 ± 0.4 (8.6-9.4)	-	9.8 ± 1.9 (7.8-11.5)	-	-
G_2	11.4 ± 1.6 (9.6-12.9)	-	9.3 ± 0.7 (8.7-10.0)	-	9.8 ± 2.0 (7.6-11.4)	-	-
Odontostyle length	148.0 ± 5.7 (142.0-155.0)	155.0	147.7 ± 4.0 (143.0-150.0)	(140.0-150.0)	151.1 ± 4.6 (146.5-156.0)	(14.0-146.5)	(140.0-145.0)
Odontophore length	74.3 ± 2.2 (71.0-76.0)	75.5	74.8 ± 0.8 (74.0-76.0)	(72.0-74.0)	78.5 ± 4.1 (75.0-83.0)	(75.0-76.0)	(76.0-78.0)
Lip region width	14.1 ± 0.3 (14.0-14.5)	14.0	14.4 ± 0.4 (14.0-15.0)	(14.0-14.5)	14.5 ± 0.4 (14.0-15.0)	(14.0-14.5)	(14.0-15.0)
Oral aperture-guiding ring	119.0 ± 7.7 (112.0-129.0)	123.0	129.5 ± 2.0 (128.0-133.0)	(123.0-133.0)	131.8 ± 8.7 (120.0-140.5)	(124.0-125.0)	(125.0-130.0)
Tail length	51.6 ± 2.3 (50.0-55.0)	50.0	50.3 ± 4.6 (45.0-56.5)	(47.0-52.0)	55.4 ± 8.8 (45.0-65.5)	(60.0-61.0)	(49.0-56.0)
J	18.0 ± 0.5 (17.5-18.5)	17.5	20.0 ± 1.4 (19.0-21.0)	(16.0-18.0)	17.8 ± 0.8 (17.0-18.5)	(20.0-23.0)	(20.0-21.0)
Spicules	-	60	-	(66.0-68.0)	-	(65.0-68.0)	-
Lateral accessory piece	-	12	-	(13.0-14.0)	-	(14.0-15.0)	-

[a] Measurements are in µm and in the form: mean ± standard deviation (range).

[b] a = body length/maximum body width; b = body length/pharyngeal length; c = body length/tail length; c' = tail length/body width at anus; V = (distance from anterior end to vulva/body length) x 100; J = hyaline tail region length.

Table 4. Morphometrics of ***Xiphinema turdetanensis* sp. nov.** from Sanlúcar de Barrameda (Cádiz, Spain)[a].

Characters/ratios[b]	Holotype	Paratypes (Stone pine)						Wild olive
		Females	Males	J1	J2	J3	J4	
n	1	21	10	3	5	5	4	3
L	4478	4630 ± 308 (4066-5227)	4738 ± 323 (4316-5295)	972 ± 31.3 (944-1006894)	1372 ± 91.4 (1256-1422)	2131 ± 269.4 (1727-2273)	3023 ± 434.2 (2432-3477)	4447 ± 466 (3932-4841)
a	85.3	84.0 ± 6.8 (70.0-99.8)	92.6 ± 6.8 (83.3-101.6)	33.9 ± 3.2 (30.2-35.9)	44.7 ± 1.6 (42.9-46.9)	51.2 ± 6.1 (42.1-55.6)	75.2 ± 11.0 (52.9-79.0)	86.1 ± 7.8 (80.2-94.9)
b	9.5	10.5 ± 0.8 (9.5-12.2)	11.3 ± 0.9 (10.2-13.3)	5.1 ± 0.3 (4.8-5.3)	8.6 ± 0.7 (7.9-9.5)	6.4 ± 0.9 (5.0-7.0)	9.8 ± 2.7 (6.3-12.9)	11.0 ± 0.5 (10.5-11.5)
c	97.3	103.0 ± 6.8 (90.4-116.2)	96.7 ± 6.3 (83.6-105.9)	18.6 ± 0.05 (18.6-18.7)	25.3 ± 0.9 (23.3-25.3)	45.2 ± 4.1 (39.3-48.4)	69.8 ± 13.0 (44.2-75.6)	94.3 ± 9.2 (83.7-99.8)
c′	1.3	1.2 ± 0.1 (1.1-1.3)	1.2 ± 0.05 (1.1-1.3)	2.7 ± 0.2 (2.5-2.8)	2.3 ± 0.05 (2.2-2.3)	1.6 ± 0.05 (1.6-1.7)	1.4 ± 0.1 (1.4-1.6)	1.26 ± 0.02 (1.2-1.3)
V or T	45.5	45.4 ± 1.6 (43-48)	53.1 ± 7.5 (41-63)	-	-	-	-	46.0 ± 1.0 (45-47)
G_1	10.9	11.2 ± 2.9 (7.9-16.2)	-	-	-	-	-	9.5 ± 0.3 (9.3-9.9)
G_2	10.7	11.0 ± 3.3 (7.7-18.3)	-	-	-	-	-	9.2 ± 0.2 (8.9-9.4)
Odontostyle length	141.0	135.2 ± 6.3 (121.0-142.0)	132.8 ± 6.6 (125.0-145.0)	57.3 ± 1.5 (56.0-59.0)	65.9 ± 1.3 (63.0-66.0)	86.9 ± 1.0 (85.5-88.0)	103.3 ± 4.6 (97.0-108.0)	134.7 ± 1.5 (133.0-136.0)
Replacement odontostyle length	-	-	-	67.3 ± 1.6 (65.5-68.5)	85.4 ± 1.0 (84.0-86.0)	103.6 ± 2.3 (101.0-106.5)	128.1 ± 6.5 (122.0-134.5)	-
Odontophore length	78.5	76.2 ± 4.0 (67.0-81.0)	77.0 ± 3.8 (71.0-84.0)	34.7 ± 2.5 (32.0-37.0)	35.6 ± 1.0 (34.0-36.0)	59.5 ± 0.8 (59.0-61.0)	60.0 ± 1.7 (60.0-64.0)	78.2 ± 1.9 (76.0-79.5)
Lip region width	14.5	14.8 ± 0.7 (14.0-16.0)	15.0 ± 0.5 (14.5-16.0)	8.2 ± 0.3 (8.0-8.5)	9.6 ± 0.4 (9.0-10.0)	12.0 ± 0.3 (11.5-12.0)	11.9 ± 0.5 (12.0-13.0)	14.5 ± 0.5 (14.0-15.0)
Oral aperture-guiding ring	133.0	121.9 ± 7.0 (110.0-134.0)	120.3 ± 10.2 (100.0-132.0)	44.7 ± 1.2 (44.0-46.0)	47.4 ± 1.8 (45.0-49.0)	69.0 ± 2.7 (67.0-73.0)	91.4 ± 6.4 (82.0-96.5)	121.7 ± 1.5 (120.0-123.0)
Tail length	46.0	45.2 ± 2.3 (40.5-50.0)	41.8 ± 1.2 (39.5-44.0)	52.2± 1.8 (50.5-54.0)	54.4 ± 2.2 (53.0-58.0)	47.0 ± 2.2 (44.0-49.0)	49.8 ± 3.9 (46.0-55.0)	47.2 ± 1.3 (46.0-48.5)
J	18.0	14.9 ± 1.3 (12.0-18.0)	14.1 ± 0.8 (13.0-15.0)	12.5 ± 0.5 (12.0-13.0)	13.6 ± 0.6 (13.0-14.0)	10.5 ± 0.6 (10.0-11.0)	15.3 ± 2.2 (13.0-18.0)	15.5 ± 0.5 (15.0-16.0)
Spicules	-	-	66.6 ± 3.3 (59.0-71.0)	-	-	-	-	-
Lateral accessory piece	-	-	16.3 ± 0.9 (15.0-18.0)	-	-	-	-	-

[a] Measurements are in µm and in the form: mean ± standard deviation (range).

[b] a = body length/maximum body width; b = body length/pharyngeal length; c = body length/tail length; c' = tail length/body width at anus; V = (distance from anterior end to vulva/body length) x 100; J = hyaline tail region length.

Table 5. Molecular similarity values (%, number of nucleotides and indels) among the new *Xiphinema* species sampled in this study and those deposited in GenBank using the D2-D3 expansion segments of 28S rDNA, ITS1, and partial 18S rDNA gene sequences.

	X. baetica sp. nov.				*X. turdetanensis* sp. nov.		
	KC567165-KC565169	KC567156-KC565157	KC567148-KC567149		KC567186	KC567163	KC567155
Species Accession numbers[a]	**D2-D3**	**ITS1**	**18S**	Species Accession numbers[a]	**D2-D3**	**ITS1**	**18S**
X. abrantinum (AY601625, -,-)	96%, 31, 3	-	-	*X. abrantinum* (AY601625)	96%, 29, 4	-	-
X. diversicaudatum (EF538755, AJ437027, EF538761)	96%, 34, 4	86%, 146, 39	99%, 11, 1	*X. diversicaudatum* (AY601624, AJ437027, EF538761)	96%, 27, 2	83%, 122, 46	99%, 4, 1
X. turdetanensis sp. nov. (KC567186)	96%, 34, 5	81%, 144, 22	99%, 7, 0	*X. globosum* (GU549474, GU549475, GU549476)	96%,, 27, 6	88%, 57, 18	99%, 6, 0
X. bakeri (AY601623, AF511426, AY283173)	95%, 36, 2	86%, 145, 40	99%, 12, 2	*X. coxi europaeum* (KC567176, KC567162)	95%, 34, 2	82%, 129, 33	99%, 5, 0
X. globosum (GU549474, GU549475, GU549476)	95%, 35, 5	83%, 184, 63	99%, 6, 0	*X. bakeri* (AY601623, AF511426, AY283173)	96%, 36, 1	83%, 118, 46	99%, 6, 1
X. coxi europaeum (KC567176, KC567162)	95%, 38, 0	85%, 162, 50	99%, 4, 0	*X. baetica* sp. nov. (KC567165, KC567157, KC567155)	96%, 34, 5	81%, 144, 22	99%, 7, 0
-	-	-	-	*X. vuittenezi* (AY601614, -, AY552979)	89%, 87, 23	-	99%, 5, 1

[a] Accession numbers for each *Xiphinema* spp. correspond with D2-D3, ITS1 and 18S sequences, respectively.

(-) Sequences not available.