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Molecular analysis and comparative morphology to resolve a complex of cryptic *Xiphinema* species

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Running Head:

Resolving a complex of cryptic *Xiphinema* species

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During nematode surveys in cultivated and natural environments in southern Spain nine populations of parthenogenic *Xiphinema* species tentatively identified as *Xiphinema cf. pyrenaicum* and one population morphologically close to *Xiphinema turcicum* were detected. Surveys in southern France also identified one population resembling *X. pyrenaicum*. We developed a comparative study among these related *Xiphinema* species, including topotypes of two species of this group previously synonymised, *viz*. *Xiphinema hispanum* and *Xiphinema sphaerocephalum*, by considering morphological and morphometrical features together with molecular data from nuclear ribosomal RNA genes (D2-D3 expansion segments of 28S, ITS1, and partial 18S). Morphological and morphometrical results identified eight of the Spanish populations as *Xiphinema nuragicum* (previously synonymised with *X. pyrenaicum*) whereas the ninth population was identified as *Xiphinema adenohystherum* (also synonymised with *X. pyrenaicum*). The species *X. adenohystherum*, *X. nuragicum, X. pyrenaicum*, and *X. sphaerocephalum* were shown to be morphologically almost indistinguishable but clearly separated by phylogenetic analyses, thus constituting a complex of cryptic species. Consequently, *X. adenohystherum*, *X. nuragicum,* and *X. sphaerocephalum* were re-established as valid species. Similarly, *X. hispanum* (morphologically similar to *X. aceri*) was also shown as a valid species. *Xiphinema turcicum,* morphologically related to *X. pyrenaicum* complex by its rounded tail, uterus with a pseudo-Z differentiation and small spines, was phylogenetically distant to these species based on D2-D3 expansion segments of 28S and ITS1, which suggests a morphological convergence in their evolution.

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Introduction

The genus *Xiphinema* Cobb, 1913 tends to be greatly conserved in gross morphology which makes species identification a very difficult task. Although morphometric variation is common in *Xiphinema* spp., identification of the species is extremely difficult within the two species groups (*X. americanum* and non-americanum) because of overlapping morphology and morphometry and, consequently, the use of sets of character combinations as opposed to unambiguous autapomorphies (Gozel *et al*., 2006). In fact, *Xiphinema* may be considered as an oligomorphic genus because of the rather large number of diagnostic features that are available at the species level (Loof & Luc, 1990) but with a rather high intraspecific variability of some of these characters (Gozel *et al*., 2006). As a result, taxonomic difficulties often arise from under- or over-estimation of intraspecific variability of certain morphological characters that are currently being used for species diagnosis. Juvenile stages, particularly first-stage juveniles (J1) of Dorylaimida, including Longidoridae, have practical significance when distinguishing closely related species (Hunt, 1995). In Longidoridae, J1 can be positively identified by the position of the replacement odontostyle which lies mostly within the odontophore, with the anterior tip near the base of the functional odontostyle (Hunt, 1995; Robbins *et al*., 1996). Species identification of *Xiphinema* has traditionally been based on the morphological or typological species concept (Chen *et al*., 1997; Coomans *et al*., 2001). However, the application of molecular methods to studies of nematode population structure and systematics has revealed that some long-assumed single species are in fact cryptic; *i.e.*, species that are morphologically indistinguishable and may be phylogenetically distant to one another (Wu *et al*., 2007; Oliveira *et al*., 2005; 2006; Ye *et al*., 2004).

Sequences of nuclear ribosomal DNA (rDNA) have been recently used for molecular characterisation and reconstruction of phylogenetic relationships of nematodes from *Xiphinema* and related genera (Ye *et al*., 2004; He *et al*., 2005). Thus, the nucleotide composition of the D2-D3 expansion segments of the 28S rDNA gene has resulted rather homogeneous within *Xiphinema* species and useful for distinguishing them (Ye *et al*., 2005). Wu *et al*. (2007) clearly distinguished *Xiphinema hunaniense* Wang & Wu, 1992 from *Xiphinema radicicola* Goodey, 1936 by D2-D3 rDNA sequences, and Oliveira *et al*. (2005) also distinguished two species of the *Xiphinema americanum* group *Xiphinema brevicollum* Lordello & Costa, 1961 from *Xiphinema diffusum* Lamberti & Bleve-Zacheo, 1979 by their internal transcribed spacer 1 (ITS1) region of rDNA. These species have identical morphological characters and very similar morphometrics within them, and were previously synonymised by Loof *et al*. (1996) and Luc *et*

al. (1998), respectively. Also, Oliveira *et al*. (2006) provided evidence that *Xiphinema krugi* Lordello, 1955 is a species complex comprised of at least four distinct genotypes, some of which may be cryptic species.

Xiphinema aceri Chizhov, Tiev & Turkina, 1986 and *Xiphinema pyrenaicum* Dalmasso, 1969, have been widely reported in the Mediterranean region, including several areas of the Iberian Peninsula (Arias *et al*., 2005; Jiménez-Guirado *et al*., 1995; Lamberti *et al*., 1992; Peña Santiago *et al*., 2003; Roca & Bravo, 1993), while *Xiphinema turcicum* Luc & Dalmasso, 1964 has been recorded as discrete and widely separated populations (Arias & Navacerrada, 1973; Jiménez-Guirado *et al*., 1995; Peña Santiago & Jiménez-Millán, 1986). In the Mediterranean region, Lamberti *et al.* (1992) described five new didelphic *Xiphinema* species closely related to *X. pyrenaicum,* associated with perennial plants, such as *Quercus faginea* Lam., *Vitis vinifera* L., *Prunus amygdalus* Batsch. and *Cistus albidus L.* They were characterised by a rounded tail with or without an inconspicuous bulge projecting slightly ventrally and an uterus devoid of Z-differentiation but showing spiniform structures. These five species, quite similar morphologically either by the measurements or by the diagnostic characters, were: *Xiphinema adenohystherum* Lamberti *et al.,* 1992, *Xiphinema cohni* Lamberti *et al.,* 1992, *Xiphinema macrogastrum* Lamberti *et al.,* 1992, *Xiphinema nuragicum* Lamberti *et al.,* 1992 (Cagliari, Italy), and *Xiphinema sphaerocephalum* Lamberti *et al.,* 1992. Two of them (*X. nuragicum* and *X. cohni*), based on populations previously attributed to *X. turcicum,* could be differentiated by the structure of the uterus which has no pseudo-Z-organ and large spines, whereas this organ is present and spines are small in *X. turcicum* (Radivojevic & Baujard, 1998). Later on, Baujard *et al*. (1996) examined the paratypes of *X. pyrenaicum* and those of the five species described by Lamberti *et al.* (1992) and concluded that there were not enough morphological differences allowing differentiating those species from *X. pyrenaicum* and, hence, they were proposed as junior synonyms of *X. pyrenaicum*. Simultaneously, Loof *et al*. (1996) examined the paratypes of *Xiphinema hispanum* Lamberti *et al*., 1992 and compared it with the original description of *X. aceri*, concluding that both species do not show consistent morphological differences and consequently, the former was considered a junior synonym of the later. These actions were accepted by Arias *et al*. (2005) in their study of more than 50 populations of *X. pyrenaicum* and one population of *X. aceri* from central and northern Spain, confirming that both species are regarded as typical of Mediterranean environments. Recently, Pedram *et al*. (2009) described *Xiphinema iranicum* Pedram, Nikham, Robbins, Ye & Karegar, 2009 with a uterus devoid of Z-differentiation but showing sclerotised spines and being morphologically close to *X. aceri*, but clearly distinguishable by their rDNA genes (Pedram *et al*., 2009).

During nematode surveys conducted in cultivated and natural environments in southern Spain, nine populations of a parthenogenic *Xiphinema* species characterized by a rounded tail and uterus devoid of Z-differentiation but showing large spines appeared to be morphologically related to *X. pyrenaicum* and another population also with rounded tail but uterus with a pseudo-Z differentiation and small spines appeared to be morphologically related to *X. turcicum.* This prompted us to carry out a comparative study among those related species. Likewise, topotype populations of *X. hispanum* and *X. sphaerocephalum* were collected from the type localities in order to characterise them molecularly and to compare them with the gene sequences of *X. aceri* and *X. pyrenaicum* available in GenBank database, as well as with a new population of *X. pyrenaicum* collected from southern France. These comparative morphological and molecular analyses would allow unravelling this taxonomic conundrum based on an open view and without *a priori* prejudice and considered the maximum number of potential species notwithstanding synonymies. Therefore, the objectives of this work were: i) to identify and compare morphologically and morphometrically the nine Spanish populations resembling *X. pyrenaicum*; ii) to characterise these Spanish populations and the molecularly topotype populations of *X. hispanum* and *X. sphaerocephalum*, based on D2-D3 expansion segments of 28S, the ITS1, and partial 18S rDNA gene sequences; and iii) to study their phylogenetic relationships along with other *Xiphinema* species, allowing to confirm their synonymy to *X. aceri* and *X. pyrenaicum* or their re-establishment as valid species.

Material and methods

Nematode populations

Topotype specimens of *X. hispanum* and *X. sphaerocephalum* were collected at type localities, in Las Viñas, Sierra Morena, Andújar, and in Coto Rios, Sierra de Cazorla (Jaén Province), respectively. Spanish nematode populations resembling *X. pyrenaicum* were obtained from several host and localities in southern Spain and France (Table 1). Samples were collected with a shovel from the upper 50 cm of soil of host plants chosen arbitrarily in each sample site. Nematodes were extracted from soil samples by magnesium sulphate centrifugal flotation (Coolen, 1979) and by the sieving method described by Flegg (1967). Specimens for light microscopy (LM) were killed by gentle heat, fixed in a solution of 4% formaldehyde + 1% propionic acid, and processed to pure glycerin using Seinhorst's (1966) method. Specimens were examined using a Zeiss III compound microscope with Nomarski differential interference contrast at powers up to ×1000 magnification. Measurements were made using a drawing tube

attached to a light microscope and, unless indicated otherwise in the text, all measurements were made in relation to the nematode body and expressed in micrometers (μm). All other abbreviations used are defined in Jairajpuri & Ahmad (1992). In addition, a comparative morphological and morphometrical study on type specimens of *X. nuragicum* and *X. adenohystherum*, kindly provided by Mrs. A. Agostinelli, from the nematode collection at the Istituto per la Protezione delle Piante, Sezione di Bari, Consiglio Nazionale delle Ricerche, (C.N.R.), Bari, Italy, was conducted.

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 the slides were dismantled and DNA extracted. Nematode DNA was extracted from single individuals and protocols for PCR 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*., (2002) Finally, the 18S rDNA gene was amplified using the SSU_F_07 (5´-AAAGATTAAGCCATGCATG-3´) and SSU_R_81 (5´- TGATCCWKCYGCAGGTTCAC-3´) primers (http://www.nematodes.org/barcoding/sourhope/nemoprimers.html). All PCR used the following conditions: 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. PCR products were purified after amplification with a gel extraction kit (Geneclean turbo; Q-BIOgene SA, Illkirch Cedex, France), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for direct DNA sequencing. For the 18S gene sequencing the internal primer SSU_R_13R (5´-GGGCATCACAGACCTGTTA-3´) (http://www.nematodes.org/barcoding/sourhope/nemoprimers.html) was also used. DNA fragments from two independent PCR amplifications from two different nematodes were sequenced in both directions using the same primers with a terminator cycle sequencing ready reaction kit (BigDye; Perkin-Elmer Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyzer; Applied Biosystems, Foster City,

CA, USA) at the University of Córdoba sequencing facilities. Sequences were deposited in GenBank (Table 1).

Table 1

Statistical analysis

Morphometric data were processed using Statistix 9.0 (NH Analytical Software, Roseville, MN, USA) and expressed as mean ± standard deviation (minimum to maximum). Morphometric values and ratios of the eight Spanish nematode populations resembling *X. pyrenaicum* and the new French population of *X. pyrenaicum* from Cahors were subjected to analysis of variance (ANOVA) and means were compared using Tukey honestly significant difference test (HSD) at $P = 0.05$.

Phylogenetic analysis

D2-D3 expansion segments of 28S, ITS1 and partial 18S rDNA sequences of different *Xiphinema* spp. from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were chosen according to previous published data (Pedram *et al*., 2009). 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 analysis of the sequence data sets were performed with maximum likelihood (ML) using PAUP * 4b10 (Swofford, 2003) and Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Permutation tail probability test (PTP) for the existence of phylogenetic structure was conducted using PAUP* with 1,000 repetitions. The best fit model of DNA evolution was obtained using the program ModelTest server (Posada, 2006) with the Akaike Information Criterion in conjunction with PAUP*. The Akaike-supported model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the Akaike information criterion (AIC) were used in phylogenetic analyses. BI analysis under GTR + G model for ITS1 region and D2-D3 expansion segment of 28S rDNA and GTR + I + G model for partial 18S rDNA was initiated with a random starting tree and was run with four chains for 2.0×10^6 generations. 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 analysis. 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 program (Page, 1996). In ML analysis the estimation of the support for each node was made using a bootstrap analysis with 1,000 fast-step replicates.

Results

Comparative morphology and morphometrics

Morphology and morphometrics of Spanish populations resembling X. pyrenaicum

Adult females of eight (out of nine) Spanish dagger nematode populations resembling *X. pyrenaicum* from several host (grapevine, wild and cultivated olive, and Spanish fir) and localities were morphologically identical among themselves and were identified as *X. nuragicum*. These populations were characterised by the following traits (Fig. 1 E-X, Table S1): a body length of 3.8 (3.1-4.3) mm; 14 µm wide lip region, continuous or slightly offset from body (Fig. 1E, I, M, Q and U); large, stirrup-shaped amphidial fovea; odontostyle and odontophore of 159.3 (155.2-167.7) and 85.5 (80.1-89.1) µm, respectively; five to six lateral body pores in the region of the odontostyle; vulva located at mid-body, uterus devoid of Z-differentiation but with large (7-10 µm long) uterine spines in the tubular portion of the uterus (Fig. 1F, G, J, K, N, O, R, S, and V, W), but neither observed in the *pars dilatata uteri* nor in the vicinity of the ovejector; large muscular ovejector, occupying nearly the complete body diameter at vulval level; and tail about 40 µm long, rounded without or with (normally) an inconspicuous bulge projecting slightly ventrally (Fig. 1H, L, P, T, and X), with four caudal pores in each side and frequently blind canal. These characters agree well with the original description of *X. nuragicum* (Fig. 1A-D) by Lamberti *et al*. (1992) or are within its range, except for a slightly longer odontostyle (Table S1). Males were absent in all the eight Spanish populations, which also agree well with the original description by Lamberti *et al*. (1992). In fact, detailed study of paratypes of *X. nuragicum* showed identical structure and distribution of spines in the uterus as in the Spanish populations, including a characteristic accumulation of spines in the distal area of the tubular part of the uterus (Fig. 1 B, C, F, G, J, K, N, O, R, S, and V, W).

Fig. 1

Analysis of variance of morphometric characters and ratios of females showed that body length (L), odontostyle, odontophore, and tail length, as well as ratios a, b, c, c', and V were significantly ($P < 0.05$) different among the Spanish populations of *X. nuragicum*, irrespective of geographic origin or host plant (Table S1), as well as with *X. pyrenaicum*. Also, the odontostyle length of all the Spanish populations of *X. nuragicum* was significantly higher than that of *X. pyrenaicum* from Cahors, France (Table S1). Nonetheless, no significant ($P \geq 0.05$)

differences were detected in other measurements such as diameter of lip region and distance from oral aperture to guiding ring (Table S1).

Four juvenile stages were detected in *X. nuragicum*. Nevertheless, since scarce specimens from second-, third- and fourth-stage juveniles (J2, J3, J4, respectively) were detected in the majority of the studied populations, these life stages were detailedly studied only in the olive population from Marchena (sample 47) (Table S2). Conversely, J1 were detected in most of the Spanish populations including grapevine (Puente Genil), olive (Castro del Río, sample 9; Marchena, samples 47 and 57); and Spanish fir (Ronda), allowing the comparative morphological and morphometrical study (Table S2, Fig. 2 A-F). All these J1 were positively identified by the replacement odontostyle which lied mostly within the odontophore (Fig. 2A). J1 were characterised by a J-shaped body; lip region, amphid, and pharynx similar to that of female but smaller; tail elongate conoid with characteristic digitate rounded terminus (Fig. 2 B-F), bearing two caudal pores on each side. Morphological and morphometric characteristics of J1 from the Spanish populations of *X. nuragicum* (Table S2) are coincident with those of the type population of *X. pyrenaicum*, except for a larger odontostyle, 70 µm (64-76) vs. 57 µm (50-62), a shorter tail length, 55 µm (51-59) vs. 68 µm (62-74), and a lower c' ratio, 3.1 (2.6-3.6) vs. 4.0 (3.6-4.9). J2, J3 and J4 in the olive population from Marchena (sample 47) were identified by comparing body length, functional and replacement odontostyle (ost and rost, respectively) according to Robbins *et al*. (1996) (Fig. 3), and were identical to other occasional juvenile life-stages detected in the other populations. Tail of J2 is slightly shorter than J1 with a mucro (Fig. 2 G). J3 and J4 showed almost similar tails, *i.e.* short, broadly convex-conoid with a mammiform terminal peg, in J4 more rounded dorsally with a less well developed peg (Fig. 2 H-K). Replacement odontostyle in J2-J4 is located far from base of functional odontostyle. Blind terminal canal is observed in some individuals. The alpha-numeric codes for *X. nuragicum* to be applied to the polytomic identification key for *Xiphinema* species by Loof & Luc (1990) are: A4-B3-C7-D6-E56-F345-G3-H2-I3-J7-K2-L1.

Fig, 2. Fig. 3.

Xiphinema pyrenaicum *Dalmasso, 1969*

Females from the population from Cahors, France were characterised by a body length of about 3 mm; lip region almost hemispherical and slightly offset from body, about 13 µm wide (Fig. 4A,B, Table S1); large, stirrup shaped amphid; odontostyle and odontophore about 135 and 84 µm, respectively; six to seven lateral body pores in the region of the odontostyle; vulva located at mid-body, uterus devoid of Z-differentiation but with medium to large 6.5 (4-8) µm long uterine spines in the tubular portion of the uterus (usually abundant and rarely scarce) (Fig. 4C),

but neither observed in the *pars dilatata uteri* nor in the vicinity of the ovejector; ovejector large muscular, occupying nearly the complete body diameter at vulval level (Fig. 4D); tail conoid-rounded of about 38 µm long (Fig. 4E-G); and males absent. These characters agree well with the original description of *X. pyrenaicum* by Dalmasso (1969), as well as observations of paratypes indicated by Baujard *et al*. (1996), including uterine spines, except for slight differences in body length, c and c' ratios which are common in this genus and do not exceed the intraspecific variations. No juvenile stages were detected. The alpha-numeric codes for *X. pyrenaicum* to be applied to the polytomic identification key for *Xiphinema* species by Loof & Luc (1990) are: A4-B3-C6-D6-E56-F345-G3-H2-I3-J6-K2-L1.

Fig. 4

Xiphinema adenohystherum *Lamberti* et al., *1992*

The *Xiphinema* population from grapevine in Bollullos par del Condado (Huelva) was morphologically and morphometrically coincident with *X. adenohystherum* (Table S3, Fig. 5). This population was characterised by a body length of about 5 mm; lip region hemispherical and slightly offset from body, 14 µm wide (Fig. 5A); large, stirrup shaped amphid; odontostyle and odontophore about 140 and 82 µm, respectively; seven lateral body pores in the region of the odontostyle; vulva located at mid-body, uterus devoid of Z-differentiation but with small to large (4-8 µm long) uterine spines in the tubular portion of the uterus (Fig. 5B,C); large muscular ovejector, occupying nearly the complete body diameter at vulval level (Fig. 5B); tail almost rounded of about 40 µm long (Fig. 5D-F); and males absent. These characters agree well with the original description of *X. adenohystherum* by Lamberti *et al*. (1992), except for a slight difference in the tail shape. Four stage juveniles present, but only J3 and J4 were detected (Fig. 5G, H), with similar morphology to that of female, except for tail shape. J3 tail long, conoid, with a rounded digitate terminus. J4 tail broadly conoid with a mammiform terminal peg. The alpha-numeric codes for *X. adenohystherum* to be applied to the polytomic identification key for *Xiphinema* species by Loof & Luc (1990) are: A4-B3-C7-D6-E56-F45-G3-H2-I3-J7-K?-L1.

Fig. 5

Xiphinema sphaerocephalum *Lamberti* et al., *1992*

Topotypes (females and males) of this species studied under LM were identical with the type population (Fig. 6) described by Lamberti *et al*. (1992). Females showed small granules (Fig. 6B) in the lumen of tubular portion near the *pars dilatata uteri*, mixed with large (7-12 µm long) spines, which were abundantly distributed along the tubular portion of the uterus, and absent in the *pars dilatata uteri* or in the vicinity of the ovejector, as also occurred in the Portuguese population (Roca & Bravo 1993). Since all four juvenile stages were described by Roca &

Bravo (1993) in Portugal, we present here only morphology and measurements (Table S3) of J1 (the only juvenile stage detected in this survey). J1 is morphologically similar to female (Roca & Bravo, 1993) except for the tail which is elongate conoid with digitate terminus (Fig. 6E). Morphometrics of J1 from the topotype population are similar to those of the population from Portugal, except for a slightly shorter odontostyle, 70 µm (68-73) vs. 82 µm (69-90), and a shorter tail, 58.5 µm (58-59) vs. 69 µm (59-76), respectively (Roca & Bravo, 1993). The alpha-numeric codes for *X. sphaerocephalum* to be applied to the polytomic identification key for *Xiphinema* species by Loof & Luc (1990) are: A4-B3-C5-D6-E56-F34-G3-H2-I3-J5-K2-L1.

Fig. 6

Xiphinema hispanum *Lamberti* et al., *1992*

Female topotypes of this species studied under LM were identical with the type population (Fig. 7A-F) described by Lamberti *et al*. (1992). The uterus showed an indistinct pseudo-Z-organ identifiable as small granules located in the lumen of the tubular portion of the uterus close to the *pars dilatata uteri* mixed with medium to large (6-8 µm long) spines which were numerous in the vicinity of the ovejector (Fig. 7B-C), as already reported by Roca & Bravo (1993) in the population from Portugal. No spines were observed in the *pars dilatata uteri,* in which the widened part of the uterus is distinguishable without sperms inside. Males were absent, and the females do not contain sperm in the genital tracts; consequently they can be assumed to be parthenogenic. Also, in this new survey in the type locality we have detected the first-stage juvenile, clearly distinguished by the unique position of the replacement odontostyle (i.e., the tip of the replacement odontostyle overlapping the base of the odontophore). J1 were characterised by an elongate-conoid tail (Fig. 7D; Table S3), an odontostyle length *ca* 58 *µ*m. J4 showed similar morphology to that of female, except for genital tract and tail broadly convex-conoid with a short mammiform terminal peg (Fig. 7E). Other juvenile stages were not found. The alpha-numeric codes for *X. hispanum* to be applied to the polytomic identification key for *Xiphinema* species by Loof & Luc (1990) are: A4-B23-C5-D56-E56-F45-G3-H2-I3-J5-K2-L1.

Fig. 7

Xiphinema turcicum *Luc and Dalmasso, 1964*

Females from the population from grapevine in Moriles (Córdoba Province) were characterised by a lip region almost continuous with body contour (Fig. 7G), the uterus showed a pseudo-Z-organ clearly identifiable as small numerous irregular granules and numerous and minute spines (3-5 *µ*m long) located in the lumen of the tubular portion of the uterus (Fig. 7H-I). Female tail regularly hemispherical, with ventral and dorsal curvatures equal (Fig. 7J). Males were absent, and the females did not contain sperm in the genital tracts; consequently can be assumed to be

parthenogenic. First-stage juvenile tail was characterised by an elongate-conoid tail with almost clavate terminus (Fig. 7L; Table S3). The alpha-numeric codes for *X. turcicum* to be applied to the polytomic identification key for *Xiphinema* species by Loof & Luc (1990) are: A4-B23-C7-D6-E56-F45-G34-H2-I3-J7-K2-L1.

***Molecular characterisation of* X. adenohystherum, X. hispanum, X. nuragicum, X. pyrenaicum, X. sphaerocephalum, X. turcicum *and phylogenetic position within the genus* Xiphinema**

Amplification of the D2-D3 expansion segments of 28S rDNA, partial 18S rDNA and ITS1 from *X. adenohystherum*, *X. hispanum, X. nuragicum, X. pyrenaicum* and *X. sphaerocephalum* yielded single fragments of approximately 800 bp, 1,600 bp and 1,200 bp, respectively, based on direct fragment sequencing. *Xiphinema turcicum* yielded similar size of PCR products, except for ITS1 which yielded a fragment of approximately 1,500 bp. Level of nucleotide differences were related with the analysed rDNA regions. Minimum differences were obtained with partial 18S rDNA gene and maximum differences were obtained with ITS1 region. In species morphologically almost indistinguishable to *X. pyrenaicum* (*X. adenohystherum*, *X. nuragicum, X. pyrenaicum*, and *X. sphaerocephalum*) differences based on D2-D3 and ITS1 (Table 2), and partial 18S regions were as follows: i) for ITS1 region the differences ranged from 274 bp between *X. adenohystherum* and *X. nuragicum* to 334 between *X. sphaerocephalum* and *X. pyrenaicum* (Table 2); ii) for D2-D3 region, differences ranged from of 42 bp between *X. adenohystherum* and *X. pyrenaicum* to 55 bp between *X. nuragicum* and *X. pyrenaicum* (Table 2); and iii) for the partial 18S, differences ranged from 3 bp between *X. adenohystherum* and *X. sphaerocephalum* to 15 bp between *X. adenohystherum* and *X. nuragicum*. However, higher levels of differences were detected among *X. turcicum* compared with the other species from the *X. pyrenaicum* group included in this study. Comparing the partial 18S and ITS1 regions *X. aceri* showed remarkable differences with *X. hispanum*, *viz*. 104 bp and 297 bp; but unfortunately no available D2-D3 sequence for *X. aceri* was deposited in GenBank database for comparison. BLAST search from all sequences from our study against those from the GenBank database showed close relationship with several *Xiphinema* spp. that were used for further phylogenetic analyses. Similarity of sequences for D2-D3, partial 18S and ITS1 regions studied were mainly coincident with related *Xiphinema* species, such as *X. aceri* (EU477381, EU477385), *X. italiae* Meyl, 1953 (FJ713154, AY601613, AJ437029), *X. vuittenezi* Luc, Lima, Weischer & Flegg, 1964 (EF614267, EF614266, AJ437028), *X. iranicum* (EU477384, EU477386), *X. montenegrinum* Barsi, Lamberti & Agostinelli, 1998 (EU477382)

and unidentified *Xiphinema* species (YH-2004-AY601615). Nevertheless, *X. turcicum* showed different similarities with regards to the other sampled taxa, the most similar species based on D2-D3 region being *X. vuittenezi* (EF614266), *X. pyrenaicum* (AY601626), *X. hunaniense* (EF188841) and *X. naturale* Lamberti, De Luca, Molinari, Duncan, Agostinelli, Coiro, Dunn & Radici, 2002, while ITS1 from *X. turcicum* did not find homologies with sequences deposited in GenBank. No differences were detected in D2-D3 region or partial 18S sequences among the studied *X. nuragicum* populations, while in the ITS1 region only 2 nucleotides were found inserted in the population from wild olive in Vejer de la Frontera (Cádiz province).

Fig. 8. Table 2

Difficulties were experienced with alignment of the ITS1 sequences and only related sequences were included in our study. The phylogenetic tree based on ITS1 sequences resolved two major clades (Fig. 8A): i) *X. vuittenezi* (AJ437028) and *X. iranicum* (EU477386); and ii) other species comprising the *X. pyrenaicum* group. However, *X. turcicum* (GU725064) presented a scarce relationship with species of the *X. pyrenaicum* group, and its position is close to used outgroup (*L. diadecturus* Eveleigh & Allen, 1982, AF511415). Some of the clades of ITS1 tree were well supported to the species level, while clades at higher levels showed good posterior probabilities in the BI analysis, while they were not well supported by boostrap values in ML analysis.

Figure 8B represents the phylogenetic analysis using the D2-D3 expansion segments of 28S rDNA. This tree clearly shows the lineage of *X. americanum*-group and the rest of *Xiphinema* species. Non-americanum group included our species of the *X. pyrenaicum* group and within it two main clades were detected: i) species morphologically related to *X. pyrenaicum* and *X. aceri*; however, *X. brasiliense* Lordello, 1951 is monodelphic opistodelphic and female tail presenting a peg was also included in this clade; and ii) species with a rather diverse morphology involving *X. diversicaudatum* (Mico1etzky, 1927) Thorne, 1939 (EF538755 and AY601624) and *X. index* Thorne & Allen, 1950 (AY601628) related species, and a sister clade formed by *X. basiri* Siddiqi, 1959 (AY601630) and *X. coxi* Tarjan, 1964 (AY601619). Other subclades were formed by *X. elongatum* Schuurmans Stekhoven & Teunissen, 1938 (AY601618), *X. insigne* Loos, 1949 (AY601619), *X. savanicola* Luc & Southey, 1980 (AY601620) and *X. chambersi* Thorne, 1939 (DQ299512) with *X. naturale* (DQ299515). Also with this gene, *X. turcicum* (GU725077), *X. hunaniense* (EF188841) and *X. radicicola* (AY601622) occupied a basal and not well defined position in this second main clade. However, the bootstrap values using ML for the clade (ii) is low, but the node support

which separates the two main clades in non-americanum group was well supported (values of 100/75 for posterior probabilities and bootstrap values, respectively).

Finally, Figure 8C represents the phylogenetic analysis using the partial 18S rDNA. This tree also clearly separated the lineage of *X. americanum*-group from the rest of species. For non-americanum group several interrelated clades were formed: i) clade comprising the main number of species, including all the species of the *X. pyrenaicum* group studied in this research; ii) a sister clade with *X. brasiliense* (AY297836) and *X. ensiculiferum* (AY297825); iii) another clade with *X. longicaudatum* (AY297829) and *X. variegatum* (AY297834) and iv) another one including *X. chambersi* (AY283174) and *X. surinamense* (AY297833). ML bootstrap values did not well support clades (i) and (ii); however, higher clades including (iii) and (iv) were well supported.

Discussion

Comparative morphology and morphometry

The present comparative morphological and morphometrical studies confirmed that eight out of nine Spanish populations resembling *X. pyrenaicum* considered in this study can be identified as *X. nuragicum*, from which they differ mainly by a slightly larger odontostyle which should be considered as an intraspecific variability as previously reported also in other species of this group (Baujard *et al*., 1996; Lamberti *et al*., 1992; Roca & Bravo, 1993). In fact, the original population of *X. nuragicum (= X. turcicum sensu* Prota *et al*., 1971) based on 30 females enlarged the range of the odontostyle length up to 161 µm (Prota *et al*., 1971). Additionally, the Spanish populations of *X. nuragicum* (which were genetically identical based on rDNA genes) were morphologically and morphometrically almost indistinguishable from the new population of *X. pyrenaicum* from Cahors (France) (genetically different from all the Spanish populations based on rDNA genes), except for a significantly lower odontostyle length (Table S1). Nevertheless, also in this case, the original description, with a higher number of females (15), enlarged the range of the odontostyle length to 149 µm (Dalmasso, 1969), which was not very different to the lowest range of the Spanish populations (Table S1), as was also reported by Baujard *et al*. (1996). Similarly, our data on the morphological and morphometrical characteristics of *X. sphaerocephalum* and *X. adenohystherum* showed that both species were quite close to those of *X. pyrenaicum* and *X. nuragicum* as also previously reported (Baujard *et al*., 1996; Lamberti *et al*., 1992; Prota *et al*., 1971; Roca & Bravo, 1993), including the presence of uterine spines in *X. pyrenaicum* as also detected in the present French population. As suggested by Baujard *et al*. (1996) and Arias *et al*. (2005) the slight differences observed among

these species do not exceed the intraspecific variations, which are commonly detected within the genus *Xiphinema*. But again, molecular analysis showed that all these taxa were clearly separated among them, and the only remarkable morpho-biological difference among this group of species is the presence of males in *X. sphaerocephalum* (Lamberti *et al*., 1992; Roca & Bravo, 1993). Also, the comparison of J1 among the Spanish populations of *X. nuragicum*, *X. sphaerocephalum* and *X. pyrenaicum* showed that odontostyle of the two former species was clearly larger than that of the latter (Dalmasso, 1969). These data confirm that J1 of *X. nuragicum, X. sphaerocephalum,* and X. *pyrenaicum* have a practical significance when distinguishing species closely related (Hunt, 1995). And finally, comparative morphometrics of J1 topotypes from *X. hispanum* showed a shorter body, odontostyle and tail length than those reported for the northern Spanish population of *X. aceri* by Arias *et al*. (2005).

Comparative morphological and morphometrical studies of the population infesting grapevines in Bollullos par del Condado (Huelva province) confirmed that this species can be identified as *X. adenohystherum*, from which it mainly differs by a slightly intraspecific variability, including larger body length (5.0 vs. 4.4 mm), tail (40 vs. 32 µm), a, b, and c' ratios (83.4, 9.6, and 0.9 vs. 68.5, 7.6, and 0.8, respectively); and slightly shorter odontostyle and odontophore length (140, 82 vs. 149, 81 µm, respectively), and c ratio (124 vs. 136), which should be considered as an intraspecific variability as previously reported also in other species of this group (Baujard *et al*., 1996; Lamberti *et al*., 1992; Roca & Bravo, 1993). Similarly, morphological and morphometrical traits of females and juvenile-stages of *X. turcicum* from Moriles (Córdoba, Province) (Table S3) agree with previous descriptions of *X. turcicum* (Luc & Dalmasso, 1963; Dalmasso, 1969), except for minor differences which should be considered as an intraspecific variability. Also, our data confirm the presence of minute and numerous spines in the uteri of *X. turcicum* as also reported by Radivojevic & Baujard (1998).

Molecular and phylogenetic relationships

Phylogenetic analysis of sequences of nuclear ribosomal DNA were similar for *X. pyrenaicum* group (except for the partial 18S) and analogous to previous studies of this genus (Pedram *et al*., 2009; Oliveira *et al*., 2004 and He *et al*., 2004). Analyses of the rDNA features in other plant-parasitic nematodes have previously shown that partial 18S is a rather poorly evolved marker comparing with the D2-D3 region and ITS1 (Lazarova *et al*., 2006; Vovlas *et al*., 2008). *X. americanum*-group had a lineage well differentiated from the other species using D2-D3 region and partial 18S trees, as previously demonstrated in other studies (Pedram *et al*., 2009; Oliveira *et al*., 2004, Ye *et al*., 2004). The tree topology analysis by Shimodaira-Hasegawa test of D2-D3

region including other genera from Longidoridae did not refute the monophyly of genus *Xiphinema* (He *et al*., 2005).

Phylogeny based on the partial 18S rDNA showed a poor resolution at clade species level even though it seemed to be a good molecular marker for major lineage group resolution between non-americanum and *X. americanum*-group. This low resolution may be caused by the scarce number of mutations between species. On the contrary, D2-D3 and ITS1 regions produce well defined phylogenies and they may be useful for species identification. PTP tests constraining several groups revealed phylogenetic signal for D2-D3 region and the partial 18S alignments.

The close morphological relationships of *X. aceri*, *X. adenohystherum*, *X. hispanum, X. iranicum*, *X. nuragicum*, *X. pyrenaicum*, *X. sphaerocephalum*, and *X. vuittenezi*, were supported by a common species origin in their clade; however, the position of *X. brasiliense* is this clade for D2-D3 region is difficult to explain, but this GenBank accession was supported upon specimens identified on the basis of general morphology (He *et al*., 2005). However, *X. turcicum* comprises an opposite case, in which phylogenetic relationship is not consistent with the general morphology of adult female and could be considered as a case of morphological convergence from a different ancestor. These different origins could explain the polyphyletic status of "round-tailed species" which has been previously reported by Coomans *et al*. (2001) using cladistic analysis based on morphological data. Consequently, close morphological *Xiphinema* species could be resolved by using DNA sequences indicated in this and previous studies (Wu *et al*., 2007; Oliveira *et al*., 2005, 2006). Nevertheless, relationships of tree clades derived from phylogeny inferred with rDNA sequences with some morphological characters are difficult to assign. It has been also indicated from a molecular phylogenetic approach to Longidoridae conducted by He *et al*. (2005), in which, only the amphid shape for the genus *Longidorus* was well correlated with phylogenetic relationships. In our case, taking into account the pattern of tail type into the ontogeny process was well related with the different clade position observed in *X. turcicum*, since this species presents first-stage juveniles with an elongate-conoid tail with almost clavate terminus. This approach has been used in preliminary phylogenetic cladistic of *Xiphinema* and then was subjected to further analysis by including all taxa with similar adult tail shape and by employing all phylogenetically informative characters (Coomans *et al*., 2001). Conversely, *X. pyrenaicum* (AY601626) from Cyprus identified on the basis of 'general morphology' (He *et al*., 2005) did not agree with our phylogenetic study. Consequently, one plausible explanation is that species resembling *X. pyrenaicum* other than those considered in this study probably exist.

Conclusions

This study suggests that *X. aceri*, *X. adenohystherum*, *X. hispanum*, *X. nuragicum*, *X. pyrenaicum* and *X. sphaerocephalum* comprise a complex group with a highly convergent morphology but that they are clearly distinguishable as separate and valid species by phylogenetic analysis of ribosomal DNA genes such as the partial 18S, ITS1 and D2-D3 expansion segments of 28S rDNA. Nonetheless, on the basis of molecular analyses, these species must be considered as a group of cryptic species, and the status of these species which were previously synonymised with *X. aceri* and *X. pyrenaicum* (Baujard *et al*., 1996) must be rejected. Consequently, although the polytomous keys aid in the identification of species of *Xiphinema* (Loof & Luc, 1990), those working on *Xiphinema pyrenaicum-aceri* group taxonomy should pay close attention to identification of these morphologically similar species when it is based only on morphology. Thus an accurate identification of species within this group requires integrative taxonomy based on molecular data combined with morphological characters, as well as ecological and host-plant data.

In summary, more extensive molecular phylogenetic investigations should help to clarify the identity and phylogenetic relationships of this complex *Xiphinema* group from the Mediterranean basin and Middle East, including populations of *Xiphinema* previously identified as *X. aceri*, *X. nuragicum*, *X. pyrenaicum, X. robbinsi* Pedram, Niknam & Decraemer, 2008, and *X. turcicum*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Morphometrics of females from eight Spanish populations of *Xiphinema nuragicum* Lamberti *et al*., 1992 and females of *Xiphinema pyrenaicum* Dalmasso, 1969 from southern France.

Table S2. Morphometrics of four developmental juvenile stages of *Xiphinema nuragicum* Lamberti *et al*., 1992 from southern Spain.

Table S3. Morphometrics of first-stage juvenile topotypes of *Xiphinema sphaerocephalum* Lamberti *et al*., 1992, females and third- and fourth-stage juveniles of *X. adenohystherum* Lamberti *et al*., 1992, first- and fourth-stage juvenile topotypes of *X. hispanum* Lamberti *et al*.,

1992 and all developmental juvenile stages and females of *X turcicum* Luc and Dalmasso, 1964 from southern Spain.

Figure legends

Fig. 1. Comparative morphology of anterior region (A, E, I, M, Q, U), uterus (B, C, F, G, J, K, N, O, R, S, V, W) and female tail (D, H, L, P, T, X) of Spanish populations of *Xiphinema nuragicum* Lamberti *et al*., 1992 with paratypes. A-D, paratypes from grapevine, Guspini, Italy. E-H, population from grapevine, Puente Genil, Córdoba Province, Spain. I-L, population from olive (sample 6), Castro del Río, Córdoba Province, Spain. M-P, population from Spanish fir, Ronda, Málaga Province, Spain. Q-T, population from olive (sample 57), Marchena, Seville Province, Spain. U-X, population from olive, Alcalá la Real, Jaén Province, Spain. Abbreviations: a = anus; gr = guiding ring; pdu = *pars dilatata uteri*; sp = spines. (Scale bars: A, E, I, M, Q, U = 50 µm; B, F, J, N, R, V = 20 µm; C, D, G, H, K, L, O, P, S, T, W, X = 10 µm).

Fig. 2. Odontostyle region and comparative morphology of tail in J1 of *Xiphinema nuragicum* Lamberti *et al*., 1992 and J2, J3 and J4. A, B, population from grapevine, Puente Genil, Córdoba Province, Spain. C, population from olive (sample 9), Castro del Río, Córdoba Province, Spain. D, population from olive (sample 47), Marchena, Seville Province, Spain. E, population from olive, (sample 57), Marchena, Seville Province, Spain. F, population from Spanish fir, Ronda, Málaga Province, Spain. G, J2 tail from population from olive (sample 47), Marchena, Seville Province. H-I, J3 tail from population from olive (sample 47), Marchena, Seville Province; and (J-K) J4 tails from population from olive (sample 47), Marchena, Seville Province. Abbreviations: a = anus; gr = guiding ring; ost = odontostyle; odt= odontophore; rost = replacement odontostyle. (Scale bars: A = 50 µm; B-F = 10 µm; G-K = 20 µm).

Fig. 3. Relation of body length with length of functional and replacement odontostyle (ost and rost, respectively) length in all developmental stages from J1 to mature females of: (A) *X. nuragicum* Lamberti *et al*., 1992 population from olive (sample 47), Marchena, Seville Province, Spain; and (B) *X. turcicum* from grapevine, Moriles, Córdoba Province, Spain.

Fig. 4. Light micrographs of anterior region (A, B), uterus (C), vulval region (D) and tail regions (E-G) of *Xiphinema pyrenaicum* Dalmasso, 1969 from southern France. Abbreviations: a = anus; gr = guiding ring; pdu = *pars dilatata uteri*; ost = odontostyle; odt = odontophore; ovj = ovejector; sp = spines. (Scale bars: A = 50 µm; B = 10 µm; C-G = 20 µm).

Fig. 5. Light micrographs of *Xiphinema adenohystherum* Lamberti *et al*., 1992 from grapevine in Bollullos par del Condado (Huelva, Spain). A, anterior region. B-C, vagina region with ovejector and part of uteri. D-F, female tails. G-H, second- and third-stage juvenile tails, respectively. Abbreviations: a = anus; gr = guiding ring; ovj = ovejector; pdu = *pars dilatata uteri*; sp = spines. (Scale bars: A = 50 µm; B-F = 20 µm).

Fig. 6. Light micrographs of anterior region (A), uterus (B), male (C), female (D), and first-stage juvenile (E) tails of topotypes of *Xiphinema sphaerocephalum* Lamberti *et al*., 1992. Abbreviations: a = anus; gr = guiding ring; pdu = *pars dilatata uteri*; sp = spines; spc = spicules. (Scale bars: A = 50 µm; B = 20 µm; C, D, E = 10 µm).

Fig. 7. Light micrographs of anterior regions (A, G), uterus (C, H, I), female tails (F, J) and first-stage juvenile anterior regions (K) and tails (D, L) of topotypes of *Xiphinema hispanum* Lamberti *et al*., 1992 and *Xiphinema turcicum* Luc and Dalmasso, 1964 from grapevine in Moriles, southern Spain. Abbreviations: a = anus; gr = guiding ring; ost = odontostyle; ovj = ovejector; pdu = *pars dilatata uteri*; p-Z = pseudo-Z-organ; sp = spines; V = vulva. (Scale bars: A-C, E = 20 µm; D, F, G, I = 10 µm; H, J-L = 50 µm).

Fig. 8. Phylogenetic relationships within the species studied and some *Xiphinema* spp. Bayesian 50% majority rule consensus trees as inferred from (A) ITS1 (B) D2 and D3 expansion segments of 28S rRNA and (C) 18S rRNA gene sequence alignments under the model selected (GTR+G for ITS1 and D2-D3 region and GTR+I+G for partial 18S). Posterior probabilities more than 65% are given for appropriate clades (in bold letters); bootstrap values greater than 50% are given on appropriate clades in ML analysis. Newly obtained sequences are indicated in bold. *: populations identified on the basis of general morphology (He *et al*., 2005).

Table 1. *Xiphinema* species studied and sequences used.

Nematode species	Locality of sample	Host	GenBank accession		
			D2-D3	ITS1	partial 18S
X. adenohystherum Lamberti *et al.,* 1992	Bollullos par del Condado (Huelva, Spain)	*Vitis vinifera* L.	GU725075	GU725063	GU725084
X. hispanum Lamberti *et al*., 1992	Andujar (Jaén, Spain)	*Cistus albidus* L.	GU725074	GU725061	GU725083
X. nuragicum Lamberti *et al*., 1992	Ronda, (Málaga, Spain)	*Abies pinsapo* Boiss.	GU725066	GU725059	GU725081
	Puente Genil, (Córdoba, Spain)	*Vitis vinifera* L.	GU725067	GU725056	GU725079
	Alcalá la Real, (Jaén, Spain)	*Olea europaea sp. europaea* L.	GU725072	-----	-----
	Castro del Río, (Córdoba, Spain) sample 6	*Olea europaea sp. europaea* L.	GU725068	-----	-----
	Castro del Río, (Córdoba, Spain) sample 9	*Olea europaea sp. europaea* L.	GU725070	-----	-----
	Marchena (Seville, Spain), sample 47	*Olea europaea sp. europaea* L.	GU725069	GU725057	GU725078
	Marchena (Seville, Spain), sample 57	*Olea europaea sp. europaea* L.	GU725071	-----	-----
	Vejer de la Frontera, (Cádiz, Spain)	*Olea europaea* sp. *sylvestris* L.	GU725065	GU725058	GU725080
X. pyrenaicum Dalmasso, 1969	Cahors (Midi-Pyrenees, France)	*Vitis vinifera* L.	GU725073	GU725060	GU725085
X. sphaerocephalum Lamberti *et al*., 1992	Coto Ríos (Jaén, Spain)	*Quercus faginea* Lam.	GU725076	GU725062	GU725082
X. turcicum Luc and Dalmasso, 1964	Moriles, (Córdoba, Spain)	*Vitis vinifera* L.	GU725077	GU725064	GU725086

(-----) Not performed.

Table 2. Number of fixed nucleotide differences among species of the *Xiphinema pyrenaicum* group. Above diagonal (in bold letters): D2-D3 expansion segments of 28S rDNA; below diagonal: ITS1 region.

	X. adenohystherum	*X. nuragicum*	*X. pyrenaicum*	*X. sphaerocephalum*
X. adenohystherum		**49**	**42**	**45**
X. nuragicum	274		**55**	**49**
X. pyrenaicum	281	303		**46**
X. sphaerocephalum	310	282	334	