Integrative taxonomy of Xiphinema histriae and Xiphinema lapidosum from Spain

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The genus Xiphinema Cobb, 1913 is a large and morphologically diverse group of plant-parasitic nematodes comprising more than 275 species (Archidona-Yuste et al., 2016a, 2016b; Peraza-Padilla et al., 2018). The economic importance of this group of nematodes is not only because of its extensive range of host plants and worldwide distribution, but for the transmission of several important plant viruses (genus Nepovirus, family Comoviridae) that cause direct damage to a wide variety of crops (Taylor and Brown, 1997; Decraemer and Robbins, 2007). Due to their economic importance, complex identification because of the sharing of a variety of morphological characters and existence of cryptic species, it is essential to identify species accurately and developing integrative taxonomy methods to control such plant pathogenic species (Archidona-Yuste et al., 2016a, 2016b). Species identification in this group is complex because of the sharing of a variety of morphological characters and the existence of cryptic species (Archidona-Yuste et al., 2016a, 2016b). According to the key for species of Xiphinema established by Loof and Luc (1990), the genus Xiphinema consists of X. americanum-group and X. non-americanum species. Later, non-americanum group was divided into eight morphospecies groups (Loof and 1990). Several authors have highlighted the great diversity of Xiphinema spp. detected in the Iberian Peninsula, in particular, around 40 species of the

Abstract

Three populations of Xiphinema non-americanum group species were detected in agricultural and natural ecosystems, during routine surveys for plant-parasitic nematodes in Spain. Based on morphological and molecular analyses, the species were identified as Xiphinema histriae and Xiphinema lapidosum, being this the first record and molecular characterization of both species in Spain. The morphometrics and morphology of the Spanish populations agree with those of the original description and paratype specimens and the present study provided a first description of the second to fourth juvenile stages of both species. A detailed study on the morphology in the Spanish populations of X. histriae, as well as in paratypes, showed a pseudo-Z-organ with weakly muscularized wall and containing numerous small dense granular bodies, which was different to the original description by Lamberti et al. (1993). This new finding suggests that X. histriae must be considered a member of the morphospecies Group 5 of X. non-americanum. Phylogenetic analysis based on D2 to D3 expansion segments of 28S gene, ITS1 and partial CoxI gene indicated that X. histriae and X. lapidosum are phylogenetically related with other Xiphinema non-americanum group spp. reported from Spain. Considering the pathological and economic importance of this group of nematodes, the combination of morphological characters, measurements, and molecular analysis is crucial for accurate identification of these species.

Key words

First record, Juvenile stages, Molecular, Morphology, Morphometrics, Phylogeny, Xiphinema, X. histriae, X. lapidosum.
genus *Xiphinema* have been reported in Spain, mainly associated with woody, ornamental, and vegetable plant species (Gutiérrez-Gutiérrez et al., 2010, 2013, 2016; Archidona-Yuste et al., 2016a, 2016b).

Routine nematological surveys in agricultural and natural ecosystems in Spain yielded three populations of *Xiphinema non-americanum* group species, which were typologically different to previous reported species in Spain. Two populations of *Xiphinema histriae* were isolated from Quercus faginea Lam. and Pinus nigra Arnold, whereas one population of *Xiphinema lapidosum* was identified in association with Olea europaea subsp. europaea L. Lamberti et al. (1993a, 1993b) and Roca and Bravo (1993) described female and male stages of *X. histriae* and *X. lapidosum*, respectively, but in both species no juvenile stages were detected and described. The objectives of this study were: (i) to provide updated morphological descriptions of juvenile stages of *X. histriae* and *X. lapidosum*, (ii) to characterize the molecular data of both species using the D2 to D3 segments, ITS1 and partial Cox1 gene sequences, and (iii) to determine the phylogenetic relationships of both species within the *X. non-americanum* group species.

**Materials and methods**

**Nematode sampling, extraction, and morphological study**

Nematodes were surveyed from 2017 to 2018 during the spring season in natural ecosystems and olive growing area in Andalucía, southern Spain (Table 1). Soil samples were collected for nematode analysis with a shovel from four to five cores randomly selected in each sampling site. Nematodes were extracted from a 500-cm³ sub-sample of soil by a modification of Cobb’s decanting and sieving method (Flegg, 1967). Specimens were killed and fixed with hot formalin (4% with 1% glycerol), and processed in glycerin (Seinhorst, 1959) as modified by De Grisse (1969). The measurements and light micrographs of nematodes were performed using a Zeiss III compound microscope.

A comparative morphological and morphometrical study of type specimens of *X. histriae* were conducted with specimens kindly provided by Dr A. Troccoli, from the nematode collection at the Istituto per la Protezione Sostenibile delle Plante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy; and paratypes of *X. lapidosum* kindly provided by Dr Z.A. Handoo from USDA Nematode Collection, Beltsville, MD, USA (T-4406p; T4407p). Spanish nematode populations of both *Xiphinema* species in this study are proposed as standard and reference populations for each species given until topotype material becomes available and molecularly characterized. Voucher specimens of these described species have been deposited in the nematode collection of Institute for Sustainable Agriculture, IAS-CSIC, Córdoba, Spain.

**Molecular analyses**

For molecular analyses, in order to avoid mistakes in the case of mixed populations, two live nematodes from each sample were temporary mounted in a drop of 1M NaCl containing glass beads (to avoid nematode crushing/damaging specimens) to ensure specimens conformed to the unidentified populations of *Xiphinema*. Following morphological confirmation, the specimens were removed from the slides and DNA extracted. DNA was extracted from single specimens as described by Archidona-Yuste et al. (2016a, 2016b). The D2 to D3 segments were amplified using the D2A (5′-ACAAGTACCGTGAGGAAAGTTG-3′) and D3B (5′-TGGAAGTGGAAACCAGCTACTA-3′) primers (De Ley et al., 1999). The ITS1 region was amplified using forward primer 18S (5′-GTATTACGTCCTGGCCCTTT-3′) (Vrain et al., 1992) and reverse primer rDNA1 5.8S (5′-ACGAGGCGGTGATCCACC-3′) (Cherry et al., 1997). And Cox1 gene was amplified as described by Lazarova et al. (2006) using the primers COIF (5′-GATTGGTCGCAACCCATT-3′) and COIR (5′-CWACATAATAAGTATCATG-3′). The newly obtained sequences were submitted to the GenBank database under accession numbers indicated on the phylogenetic trees and in Table 1.

**Phylogenetic analysis**

D2 to D3 segments, partial ITS1 rDNA, and partial Coxl sequences of different *Xiphinema* species belonging to the *X. non-americanum* group were obtained from GenBank and used for phylogenetic reconstruction. Outgroup taxa for each data set were chosen following previous published studies: *Longidorus oleae* (KT308871); *Xiphinema america num* (KX263175); *Longidorus caespiticola* (KJ567469); *Xiphinema duriense* (KX244935); *Xiphinema pachtaic um* (HM921337); *Scutellonema bradys* (AY268114); *Meloidogyne hapla* (AY268113) (He et al., 2005; Holtermann et al., 2006; Gutiérrez-Gutiérrez et al., 2013; Tzortzakakis et al., 2015; Archidona-Yuste et al., 2016a, 2016b; Susulovska et al. 2018; Varela-Benavides et al., 2018). Multiple sequence alignments of the different genes were made using the Q-INS-i algorithm of MAFFT V.7.205 (Kato9 and Standley, 2013), which accounts for secondary RNA structure. Sequence alignments were visualized and their percentage of
Table 1. Taxa sampled for *Xiphinema* species and sequences from NCBI used in this study.

| Species | Sampling code | Locality | Host-plant | D2 to D3 | ITS1 | CoxI |
|---------|---------------|----------|------------|----------|------|------|
| 1. *X. histriae* | QUECAZ<sup>a</sup> | Arroyo Frio, Cazorla, Cazorla, Jaén province, Spain | Portuguese oak | MK801302 | MK801298 | MK796911 |
| | NAVSAP | Navas de San Pedro, Cazorla, Jaén province, Spain | Black pine | MK801303 |
| 2. *X. lapidosum* | JAO132 | Aroche, Huelva province, Spain | Cultivated olive | MK801304 | MK801299 | MK796912 |
| 3. *X. andalusiense* | 419b | Andújar, Jaén province, Spain | Wild olive | MK801305 | MK801306 | MK801300 |
| | AR108 | Villaviciosa, Córdoba province, Spain | Wild olive | MK801307 | MK801308 | MK801309 |
| 4. *X. abruntinum* | CAN223 | Portugal | – | KY816595 |
| 5. *X. aceri* | M13 | Maragheh city, Iran | Wild rose | KY816596 |
| 6. *X. adenohystherum* | –<sup>b</sup> | Bollullos par del Condado, Huelva province, Spain | Grapevine | KY816597 |
| | AR086 | Prado del Rey, Cádiz province, Spain | Wild olive | KY816598 |
| 7. *X. baetica* | H001 | Hinojos, Huelva province, Spain | Stone pine | KY816599 |
| | LOMAS | Hinojos, Huelva province, Spain | Stone pine | KY816600 |
| 8. *X. bakeri* | CD947 | Olympic Peninsula, Washington, USA | Unknown | KY816601 |
| | CD852 | Point Reyes, Marin county, California, USA | Unknown | KY816602 |
| 9. *X. barense* | CNR1 | Brindisi province, Italy | Wild olive | KY816603 |
| | itsLUC | Brindisi province, Italy | Wild olive | KY816604 |
| 10. *X. basiri* | EU126 | San Jose, Cuba | – | KY816605 |
| 11. *X. belmontense* | MOUB | Merza, Coruna, Spain | Pedunculate oak | KY816606 |
| | MOUCH | Merza, Pontevedra province, Spain | Chestnut | KY816607 |
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| No.  | Species         | Accession Numbers | Hosts                                                                 |
|------|-----------------|-------------------|----------------------------------------------------------------------|
| 12.  | *X. brasiliense* | SZX1305           | Shenzhen, Guangdong province, China                                   |
| 13.  | *X. cadavalense*| ST77              | Espiel, Córdoba province, Spain                                       |
| 14.  | *X. castilloi*   | –                 | Gilan province, Iran                                                    |
| 15.  | *X. celtiense*   | AR83              | Penafiel, Sevilla province, Spain                                      |
|      |                 | AR82              | Adamuz, Córdoba province, Spain                                        |
| 16.  | *X. chambersi*   | 1,602             | Jekyll Island, Georgia, USA                                            |
|      |                 | 3,357             | Jekyll Island, Georgia, USA                                            |
| 17.  | *X. cohnii*      | AR16              | Sanlucar de Barrameda, Cádiz province, Spain                           |
|      |                 | J126-2            | El Puerto de Santa Maria, Cádiz, Spain                                |
|      |                 | J0126             | El Puerto de Santa Maria, Cádiz, Spain                                |
| 18.  | *X. conurum*     | ST45              | Uleila del Campo, Almería province, Spain                             |
|      |                 | ST45V             | Sorbas, Almería province, Spain                                       |
| 19.  | *X. costaricense*| ACC61             | La Suiza de Turrialba, Cartago province, Costa Rica                   |
|      |                 | ACC46             | Santa Rosa, Limon province, Costa Rica                                |
| 20.  | *X. coxi*        | GG10              | Glynn County, Georgia, USA                                            |
| 21.  | *X. coxi*        | AR92              | Alcolea, Córdoba province, Spain                                      |
|      |                  | AR020             | Hinojos, Huelva province, Spain                                        |
| 22.  | *X. cretense*    | OLI40             | Hersonisos province, Greece                                           |
|      |                  | AR039             | Hersonisos province, Greece                                           |
| 23.  | *X. dentatum*    | –                 | Silnicna, Czech Republic                                              |
| 24.  | *X. diversicaudatum* | KOS              | Klucovec, Slovakia                                                     |
|      |                  | AUS               | Marchegg, Austria                                                     |
| 25.  | *X. elongatun*   | CD426             | Brisbane, Australia                                                    |
| 26.  | *X. gersoni*     | H0059             | Almonte, Huelva province, Spain                                        |

Continued
| No. | Species | Accession | Location | Host | Accession1 | Accession2 | Accession3 |
|-----|---------|-----------|----------|------|------------|------------|------------|
| 27. | *X. globosum* | – | Alcalá de los Gazules, Cádiz Province, southern Spain | Unknown | GU549474 | – | – |
| 28. | *X. granatum* | Xmar1 | Markazi province, Iran | Pomegranate | JQ240273 | – | – |
| 29. | *X. hangzhouense* | – | Hangzhou, Zhejiang province, China | Bull Bay | MF538772 | – | MF706262 |
| 30. | *X. herakliense* | OLE18 | Agiofarago, Crete Island, Greece | Wild olive | KM586349 | – | KY816613 |
| | | OLE17 | Agiofarago, Crete Island, Greece | Wild olive | – | KM586355 | – |
| | | OLE16 | Agiofarago, Crete Island, Greece | Wild olive | – | KM586354 | – |
| 31. | *X. hispanum* | – | Andújar, Jaén province, Spain | Estepa blanca | GU725074 | GU725061 | – |
| | | 00419 | Andújar, Jaén province, Spain | Wild olive | – | – | KY816614 |
| 32. | *X. hispidum* | AR004 | Medina Sidonia, Cádiz province, Spain | Wild olive | KX244906 | – | – |
| | | Xhi426 | Bollullos par del Condado, Huelva province, Spain | Grapevine | – | HM921367 | – |
| | | H026b | Rociana par del Condado, Huelva province, Spain | Grapevine | – | – | KY816616 |
| 33. | *X. hunaniense* | CD2465 | Thailand | – | MF510432 | – | MF510427 |
| 34. | *X. ifacolum* | AE90 | Kalutara province, Sri Lanka | Grasses | MH012181 | – | MH013396 |
| 35. | *X. index* | XinTre | Trexenta, Cagliari province, Italy | Grapevine | HM921406 | – | HM921388 |
| 36. | *X. ingens* | Ps | Kermanshah province, Iran | Unknown | KJ956388 | – | – |
| 37. | *X. insigne* | CD1238 | Fresno County, California, USA | Grasses | MF510430 | – | MF510425 |
| 38. | *X. iranicum* | M46 | Maragheh city, Iran | wild rose | – | EU477386 | – |
| 39. | *X. israeliae* | OLI34 | Voutes province, Greece | Olive | KJ802886 | – | – |
| | | OLI13 | Roufas province, Greece | Olive | – | KJ802896 | – |
| | | AR013 | Roufas province, Greece | Wild olive | – | – | KY816618 |
| 40. | *X. italae* | AR91 | Puerto Real, Cádiz province, Spain | Wild olive | KX244912 | KX244937 | – |
| | | XIP12 | Sbiba, Kasserine province, Tunisia | Cultivated olive | – | KX062698 | – |
| | | APUL | Bari, Bari province, Italy | Grapevine | – | – | KY816623 |
| 41. | *X. iznajarense* | JAO25 | Iznajar, Córdoba province, Spain | Cultivated olive | KX244892 | KX244928 | KY816624 |

Continued
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| No. | *Xiphinema*          | Accession(s) | Host(s)                                      | Location(s)                                           | Reference(s) |
|-----|----------------------|---------------|---------------------------------------------|-------------------------------------------------------|--------------|
| 42  | *X. japonicum*       | JH-2017       | Arhat pine                                 | Japan                                                 |              |
| 43  | *X. krugi*           | CD1827        | Rubber Plant                               | Matapalo, Puntaneras province, Costa Rica             |              |
|     |                      |               | Sand Gertrudis,                            | Santa Gertrudis, Alajuela province, Costa Rica        |              |
| 44  | *X. lupini*          | H050          | Grapevine                                  | Hinojos, Huelva, Spain                                 |              |
|     |                      | 388GD         | Grapevine                                  | Bollulos par del Condado, Huelva, Spain               |              |
| 45  | *X. macroacanthum*   | individual    | Olive                                       | Adriatic Sea coast, Italy                             |              |
|     |                      | 70            |                                             | Brìdisi province, Italy                               |              |
|     |                      | ITAL          | Cultivated olive                           |                                                       |              |
| 46  | *X. macrodora*       | JAO6          | Wild olive                                 | La Granjuela, Córdoba province, Spain                |              |
|     |                      | AR097         |                                             | Santa Mª de Trassierra, Córdoba province, Spain        |              |
| 47  | *X. meridianum*      | XMP11         | Cultivated olive                           | Sbitla, Kasserine province, Tunisia                   |              |
|     |                      | 11R16         | Common buckthorn                           |                                                       |              |
| 48  | *X. mengibarense*    | OC3C4         | Cultivated olive                           | Mengibar, Jaén province, Spain                        |              |
|     |                      | O3V05         |                                             | Mengibar, Jaén province, Spain                        |              |
| 49  | *X. naturale*        | N1            | Oak                                        | Fort Pierce, Florida, USA                             |              |
| 50  | *X. nuragicum*       | JAO36         | Wild olive                                 | Casarabanola, Malaga province, Spain                  |              |
|     |                      | RONDA         |                                             | Ronda, Malaga province, Spain                         |              |
|     |                      | AR113         | Wild olive                                 | Alcolea, Córdoba province, Spain                      |              |
| 51  | *X. oleae*           | AR35          | Wild olive                                 | Tarifa, Cádiz province, Spain                         |              |
| 52  | *X. parachambersi*   | –             | Cape jasmine, Spindle                      | Japan                                                 |              |
| 53  | *X. paracentrum*     | 17-82         | Meadow                                     | Tara Mountain, Serbia                                 |              |
| 54  | *X. poasense*        | AJ74          | Eucalyptus, Cypress and Fountain grass     | San Carlos, Alajuela province, Costa Rica             |              |
|     |                      | AP99          |                                             | San Carlos, Alajuela province, Costa Rica             |              |

Continued
|   | Species             | GenBank Accession Numbers | Host | Province/Country                        |
|---|---------------------|---------------------------|------|-----------------------------------------|
| 55. | *X. pseudocoxi*    | AR95                      | Wild olive | Alcaracejos, Córdoba province, Spain |
| 56. | *X. pyrenaicum*  | PYRDJ                     | Grapevine | Cahors, Midi-Pyrenees, France          |
| 57. | *X. radicicola*  | V1273                     | –     | Chu’momray, Viet Nam                   |
| 58. | *X. robbinsi* Topotype  | TypPop                   | Common buckthorn | Goshayesh village, East Azarbaijan, Iran |
| 59. | *X. robbinsi* Tunisia  | 12R28                    | Cultivated olive | Sbitla, Kasserine province, Tunisia |
| 60. | *X. savanica*     | CAN72                     | –     | Dakar, Senegal                         |
| 61. | *X. setariae*     | AC009                     | Banana | Pueblo Nuevo de Duacari, Limón, Costa Rica |
| 62. | *X. sphaerocephalum* | AR73                     | Wild olive | Castillo de Locubín, Jaén province, Spain |
| 63. | *X. tica*         | ACC81                     | Coffeae | Sabanillas, San Jose province, Costa Rica |
| 64. | *X. turcicum*     | ST149                     | Wild olive | Prado del Rey, Cádiz province, Spain |
| 65. | *X. turdetanense* | J212                      | Stone pine | Sanlucar de Barrameda, Cádiz Province, Spain |
| 66. | *X. vuittenezi*   | ~                          | Apple | Slany, Czech Republic                  |
| 67. | *X. zagrosense*   | Yasooj                    | Grass  | Zagros Mountains, Iran                 |
|   |                     |                           |       |                                         |

Note: New species in bold font; (–) data not provided or sequence not used.

Similarity calculated using the sequences identity matrix using BioEdit (Hall, 1999) and manually edited by Gblocks ver. 0.91b (Castresana, 2000) in Castresana Laboratory server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) using options for a less stringent selection (minimum number of sequences for a conserved or a flanking position: 50% of the number of sequences + 1; maximum number of contiguous non-conserved positions: 8; minimum length of a block: 5; allowed gap positions: with half).

Phylogenetic analyses of the sequence data sets were based on Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). The best-fit model of DNA evolution was obtained using JModel-
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eLTest V.2.1.7 (Darriba et al., 2012) with the Akaike Information Criterion (AIC). The best-fit model, the base frequency, the proportion of invariant sites, the gamma distribution shape parameters, and substitution rates in the AIC were then given to MrBayes for the phylogenetic analyses. BI analyses were performed under the general time-reversible model with invariant sites and a gamma-shaped distribution (GTR + I + G) for the D2 to D3 segments of 28S, rRNA ITS1 and partial CoxI gene. These BI analyses were run separately per data set using four chains for $2 \times 10^6$ generations for all of molecular markers. The Markov chains were sampled at intervals of 100 generations. Two runs were conducted 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 from all analyses were visualized using FigTree software V.1.42 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Systematics

*Xiphinema histriae* Lamberti et al. (1993a, 1993b). (Figs. 1–3; Table 2).

![Figure 1. Light micrographs of Xiphinema histriae (Lamberti et al., 1993a, 1993b). Females: A, Pharynx; B to D, Lip regions; E to G, I, J, Tail region; H, Gonad; K, L, Details of pseudo-Z-organ; Juveniles: M to O, Tail region of 2nd, 3rd and 4th stage juveniles; Males: P, Tail region of male. Abbreviations: a, anus; cb, crystalloid bodies; gr, guiding ring; psZ, pseudo-Z-organ; sp, spicule; spl, supplements; v, vulva. (Scale bars: A = 40 μm; B-D = 15 μm; E-G, I, J = 20 μm; H = 65 μm; K = 10 μm; L = 20 μm; M-O = 10 μm; P = 20 μm.]

Description

Female

The description of female body of *Xiphinema histriae* is as follows: body is cylindrical with an open C-shaped when heat relaxed; cuticle is 3.1 (2.5–3.5) μm thick at mid-body; Lip region is flatly rounded, separated from body by a slight depression, 15.4 (14.0–17.0) μm wide and 8.0 (7.5–9.0) μm high; amphids are stirrup shaped and amphidial fovea aperture extending for ca 60.7 to 70.5% of lip region diam; odontostyle long and narrow, 1.6 times longer than odontophore; odontophore with well-developed flanges 15.6 (14.0–17.0) μm wide; pharynx extending to a terminal pharyngeal bulb with three nuclei: one dorsal gland nucleus (DN) located at the beginning of basal bulb (9.0–12.7%) and two ventro-sublateral nuclei (SVN) are located near to the middle of bulb (51.9–57.5%); glandularium is 152.8 (129.5–170.5) μm long; reproductive system didelphic-amphidelphic is with equally developed branches, and vulva slit-like and situated slightly posterior to mid-body; each branch comprises a reflexed ovary and a tubular oviduct with a developed *pars dilatata oviductus* separated from uterus by a sphincter; uteri tripartite with a long tubular part, consisting of a developed *pars dilatata uteri* link with a narrower, muscular tube-like portion containing crystalloid bodies distributed over the entire length, pseudo-Z-organ with weakly muscularized wall with numerous small dense granular bodies; ovejector is well developed, 22.2 (16.0–32.5) μm wide, and vagina is 30.4 (20.0–40.0) μm long or 47.4% (34.5–57.1%) of corresponding body width; pre-rectum is reaching around 8.9 to 11.5% of nematode body from the anus to anterior part; rectum is extending more or less than the body width at anus; and tail is short and hemispherical with a peg 6.0 to 9.5 μm long.

Male

Very rare, only one male specimen was found in both Spanish populations. It is morphologically similar to female except for the genital system. Male genital tract is diorchic with testes with multiple rows of spermatogonia. Spicules are moderately long, curved ventrally, and lateral guiding pieces 21.0 μm long. Tail is short and hemispherical with a peg 3.5 μm long. One pair is of adanal supplements and seven of mid-ventral supplements.
Table 2. Comparative morphometrics of females and males of *Xiphinema histriae* (Lamberti et al., 1993a, 1993b) from different localities. All measurements are in µm and in the form: mean ± sd (range)\(^a\).

| Locality/host-plant | Arroyo Frío, Cazorla Jaén, Spain, Portuguese oak | Nava de San Pedro, Cazorla Jaén, Spain, pine | Paratypes, (Lamberti et al., 1993a) Gorizia, Italy, *Vitis* sp. | (Lamberti et al., 1993b) Trieste, Italy, *Vitis* sp. |
|---------------------|--------------------------------------------------|--------------------------------------------|-------------------------------------------------|------------------------------------------------|
| **Characters/ratios\(^b\)** | **Females** | **J2** | **J3** | **J4** | **Females** | **Male** | **Females** | **Males** | **Females** | **Male** | **Females** | **Male** | **Females** | **Male** |
| **n** | 20 | 5 | 3 | 3 | 11 | 1 | 5 | 3 | 4 | 1 |
| **L\(^b\)** | 4,959.0 ± 253.7 (4,477.0–5,455.0) | 1,715.0 ± 83.9 (1,583.0–1,795.0) | 2,629.0 ± 271.4 (2,409.0–2,932.0) | 4,000.0 ± 300.7 (3,669.0–4,227.0) | 4,762.4 ± 322.4 (4,364.0–5,364.0) | 4,773.0 | 4.3 ± 0.19 (4.0–4.5) | 4.2 ± 0.21 (4.0–4.3) | 4.2 | 4.5 |
| **a** | 77.6 ± 5.4 (68.2–86.4) | 41.5 ± 4.0 (37.4–46.7) | 57.4 ± 2.7 (54.8–60.2) | 68.2 ± 1.4 (67.1–69.7) | 73.0 ± 3.9 (67.1–79.2) | 68.2 | 67.9 ± 2.2 (64.1–69.4) | 65.9 ± 0.8 (65.4–66.5) | 68.4 | 78.8 |
| **b** | 8.7 ± 0.6 (7.4–10.2) | 4.3 ± 0.4 (3.7–4.7) | 5.8 ± 0.5 (5.4–6.3) | 7.1 ± 0.1 (7.1–7.3) | 8.9 ± 0.8 (8.1–10.6) | 7.6 | 8.3 ± 0.5 (7.7–8.9) | 8.0 ± 0.4 (7.7–8.3) | 8.1 | 9.1 |
| **c** | 111.4 ± 8.4 (98.4–130.2) | 32.6 ± 1.5 (31.0–34.5) | 45.3 ± 4.4 (40.4–48.9) | 73.8 ± 3.4 (71.0–77.6) | 114.1 ± 7.7 (99.7–128.1) | 116.4 | 103.7 ± 8.5 (96.6–117.8) | 82.5 ± 4.9 (79.0–86.0) | 100.0 | 103.4 |
| **c’** | 1.0 ± 0.1 (0.8–1.1) | 1.8 ± 0.1 (1.7–2.0) | 1.9 ± 0.2 (1.8–2.1) | 1.3 ± 0.1 (1.2–1.4) | 1.0 ± 0.05 (0.9–1.0) | 0.9 | 0.9 ± 0.04 (0.9–1.0) | 1.0 ± 0.7 (0.9–1.1) | 1.0 | 1.0 |
| **V or T (%)** | 53.8 ± 1.5 (51.5–57.0) | – | – | – | 53.7 ± 1.5 (52.0–57.5) | 36.7 | 44.5 ± 0.5 (44.0–45.0) | – | 44.0 | – |
| **Odontostyle** | 144.1 ± 4.2 (135.5–151.5) | 81.6 ± 3.8 (77.5–86.5) | 95.5 ± 3.5 (93.0–99.5) | 116.7 ± 5.5 (110.0–122.0) | 142.3 ± 6.6 (132.5–150.5) | 133.5 | 148.5 ± 4.9 (142.9–156.5) | 147.3 ± 1.2 (146.5–148.2) | 151.9 | 132.3 |
| **Odontophore** | 89.4 ± 2.7 (85.0–95.0) | 59.8 ± 2.0 (56.5–61.5) | 65.5 ± 2.6 (64.0–68.5) | 79.0 ± 2.6 (76.0–81.0) | 85.9 ± 4.5 (80.5–93.0) | 88.0 | 86.7 ± 2.3 (83.5–89.4) | 89.4 | 86.4 | 74.7 |
| **Total stylet** | 233.5 ± 6.4 (223.5–245.0) | 141.4 ± 4.9 (134.0–146.5) | 161.0 ± 6.1 (157.0–168.0) | 195.7 ± 7.8 (187.0–202.0) | 228.2 ± 8.7 (213.5–241.0) | 221.5 | – | – | – | – |
| **Replacement** | – | 100.7 ± 3.1 (95.5–104.0) | 123.0 ± 0.9 (122.0–123.5) | 143.0 ± 5.7 (136.5–147.0) | – | – | – | – | – | – |
| **Odontostyle** | – | – | – | – | – | – | – | – | – | – |
| **Continued** | | | | | | | | | | | |
| Locality/host-plant | Arroyo Frío, Cazorla Jaén, Spain, Portuguese oak | Nava de San Pedro, Cazorla Jaén, Spain, pine | Paratypes, (Lamberti et al., 1993a) Gorizia, Italy, Vitis sp. | (Lamberti et al., 1993b) Trieste, Italy, Vitis sp. |
|---------------------|-----------------------------------------------|-----------------------------------------------|-------------------------------------------------|-----------------------------------------------|
| **Characters/ratios** | **Females** | **J2** | **J3** | **J4** | **Females** | **Male** | **Females** | **Males** | **Females** | **Male** |
| Lip width | 15.4 ± 0.9 (14.0–17) | 10.4 ± 0.2 (10.0–10.5) | 11.8 ± 0.3 (11.5–12.0) | 13.8 ± 0.4 (13.5–14.0) | 15.9 ± 0.6 (14.5–16.5) | 15.0 ± 0.3 (14.7–15.3) | 15.0 | 14.0 | 13.5 |
| Oa-guiding ring | 128.0 ± 6.8 (116.0–139.0) | 75.4 ± 2.4 (71.5–78.0) | 88.0 ± 8.9 (78.0–95.0) | 109.7 ± 15.5 (93.5–124.5) | 117.9 ± 7.4 (108.5–129.5) | 126.5 | 136.4 ± 6.1 (129.4–144.7) | 135.0 ± 7.1 (130.0–140.0) | 137.0 | 123.5 |
| Tail length | 45.3 ± 3.1 (40.5–60.0) | 52.6 ± 1.8 (50.5–54.5) | 58.2 ± 6.0 (51.5–63.0) | 54.2 ± 2.5 (51.5–56.5) | 41.8 ± 2.0 (38.5–44.5) | 41.0 | 41.4 ± 2.3 (38.2–43.5) | 50.0 | 42.6 | 43.5 |
| J | 16.0 ± 1.8 (13.0–19.5) | 16.2 ± 2.9 (12.5–20.5) | 17.3 ± 2.8 (15.5–20.5) | 18.0 ± 1.3 (16.5–19.0) | 17.3 ± 2.8 (13.5–23.5) | 5.0 | 13.4 ± 0.8 (12.9–14.7) | 14.4 ± 0.4 (14.1–14.7) | 16.3 | 9.4 |
| Spicules | – | – | – | – | – | 74.0 | – | 84.0 ± 2.1 (82.3–85.2) | – | 82.0 |
| Lateral accessory piece | – | – | – | – | – | 21.0 | – | – | – | – |
| Suppl. 1-anus | – | – | – | – | – | 24.5 | – | – | – | – |

Note: *Measurements are in µm and in the form: mean ± standard deviation (range); **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) × 100; T = (distance from cloacal aperture to anterior end of testis/body length) × 100; J = hyaline tail region length.*
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Juveniles

Three juvenile stages (J2, J3, and J4) were found and they were basically similar to adults, except for their smaller size, shorter tails, and sexual characteristics (Figs. 1, 2). The tails of juvenile stages become progressively wider after each moult. All of the stages are distinguishable by relative body lengths, functional, and replacement odontostyle (Robbins et al., 1996).

Locality and habitat

Spanish populations of Xiphinema histriae were collected in the rhizosphere of Portuguese oak (Quercus faginea Lam.) and black pine (Pinus nigra Arnold) at Arroyo Frío and Nava de San Pedro, Cazorla, Jaén Province, Spain.

Remarks

The two amphimictic populations of X. histriae agree fairly with studied paratypes (Fig. 3) and original description of X. histriae by Lamberti et al. (1993a, 1993b). According to the polytomous key (Loof and Luc, 1990), these populations belong to the X. non-americanum Group 5 and has the following specific α-numeric codes: A4, B23, C5a, D56, E6, F45, G3, H2, I3, J5, K?, L1, which fits with the original description of X. histriae, except in having bigger values of V (51.5–57.0 vs 44.0–44.5), shorter oral aperture-guiding ring length (116.0–139.0 μm vs 129.4–144.7 μm), and spicule length (74.0 μm vs 82.3–85.2 μm). No juvenile stages were described in the original description. This is the first time that J2 to J4 juvenile stages were detected and described, being similar to adults, except in body length, tail morphology, and sexual characteristics. Additionally, females of the Spanish populations of X. histriae, a pseudo-Z-organ with weakly muscularized wall, containing numerous small dense granular bodies was observed, which differ from the original description by Lamberti et al. (1993a, 1993b). This pseudo-Z-organ was also confirmed in detailed examination of paratypes (Fig. 3). Therefore, X. histriae should be placed in morphospecies Group 5. To our knowledge, this is the first report of this species in Spain.

Xiphinema lapidosum Roca and Bravo (1993). (Figs. 4–6; Table 3).

Description

Female

The female body of Xiphinema lapidosum is as follows: body is cylindrical, slightly tapering anteriorly and posteriorly and assuming a hook-shape upon fixation; cuticle appearing smooth, 5.0 (3.5–6.5) μm thick at the middle body; lip region is flatly rounded, separated by a weak depression; odontostyle is robust, and odontophore is with well-developed basal flanges (10.5–14 μm wide); guiding ring is double; pharynx is extending to a terminal pharyngeal bulb with three nuclei with one dorsal gland nucleus located at the beginning of pharyngeal bulb (SN12 = 55–60%), while two subventrolateral nuclei located at middle of bulb (SN12 = 56–60%); pharyngeal basal bulb 127 to 148 μm long and 24.5 to 35 μm diam; glandularium is 111.5 (106.5–115) μm long; female reproductive system is didelphic, with two complete genital branches equally developed, each 541 (465–580) μm long; the length of ovaries is variable, and a pars dilatata oviductus separated from the uterus by a conspicuous sphincter muscle, tripartite uterus consisting of a pars dilatata uteri followed by a tubular portion, a pseudo-Z-organ, a dilated part and an ovejector; pseudo-Z-organ well developed with a thick wall and longitudinal folding is easy to observe, comprising 15 to 20 sclerotized bodies of large size, but all of them of variable size; no spines or different structures are observed in the uterus; vulva is a transverse slit, vagina 33.0 (30.5–37.5) μm wide and perpendicular to body-axis, ovejector well developed, 50.5 (40.5–58.5) μm wide, extending inwards more than half of corresponding body diam; and tail short, convex dorsally and ending with bulge.

Male

Males are common but less frequent (50%) than female. They are morphologically similar to female except for the genital system; spicules are curved, lateral guiding pieces well sclerotized; tail is conoid with one pair of adanal supplements and five mid-ventral supplements (Table 3, Fig. 4).

Juveniles

Three juvenile stages (J2, J3, and J4) were found and they were basically similar to adults, except for their smaller size, shorter tails, and sexual characteristics (Table 3, Fig. 4). Tail becomes progressively wider and shorter after each moult.

Locality and habitat

The population was collected from the rhizosphere of cultivated olive (Olea europaea subsp. europaea L.) at Aroche, Huelva province, Spain.
Figure 4: Light micrographs of *Xiphinema lapidosum* (Roca and Bravo, 1993). Females: A, Pharynx; B to C, Lip regions; D, Gonad; E, F, Details of pseudo-Z-organ; G, H, Tail regions; Males: I, Tail region of male; Juveniles: J to L, Tail region of 2nd, 3rd and 4th stage juveniles. Abbreviations: a, anus; gr, guiding ring; psZ, pseudo-Z-organ; sb, sclerotized bodies; sp, spicule; spl, supplements; v, vulva. (Scale bars: A = 40 μm; B, C = 15 μm; D = 65 μm; E–I = 20 μm; J–L = 10 μm.)
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Figure 5: Relationship of body length to length of functional and replacement odontostyle (△ = Odontostyle and ● = Replacement odontostyle); length in three developmental stages and mature females of *Xiphinema lapidosum*.

Figure 6: Light micrographs of *Xiphinema lapidosum* paratypes (Roca and Bravo, 1993). Female: A, Pharynx; B, Lip region; C, D, Details of pseudo-Z-organ; E, F, Tail regions; Males: G, Tail region of male. Abbreviations: a, anus; gr, guiding ring; psZ, pseudo-Z-organ; sp, spicule; spl, supplements. (Scale bars: A = 40 μm; B = 15 μm; C-G = 20 μm.)
Table 3. Morphometrics of *Xiphinema lapidosum* (Roca and Bravo, 1993) from cultivated olive at Aroche (Huelva, Spain). All measurements are in µm and in the form: mean ± s.d. (range).a.

| Characters/ ratios | Females | Males | J2 | J3 | J4 | Females | Males |
|-------------------|---------|-------|----|----|----|---------|-------|
| n                 | 10      | 5     | 2  | 5  | 7  | 14      | 7     |
| Lb                | 4,600±271.5 (4,250–5,023) | 4,618±415.4 (4,091–4,977) | 2,292±270.7 (2,068–2,704) | 3,101±178.9 (2,864–3,318) | 4,300±344 (3,700–4,600) | 4,300±310 (3,900–4,800) |
| a                 | 71.5±4.9 (64.9–75.5) | 70.9±6.7 (63.4–79.6) | 63.4±3.5 (59.6–68.5) | 67.0±6.3 (59.3–77.6) | 80.0±5.3 (70.4–88.2) | 85.0±6.5 (75.5–95.5) |
| b                 | 9.8±0.6 (8.8–10.9) | 10.9±1.5 (9.1–12.3) | 6.9±0.9 (6.1–7.8) | 7.4±0.6 (6.4–8.1) | 9.5±0.8 (7.8–10.7) | 9.0±0.8 (7.5–10.0) |
| c                 | 113.2±8.2 (98.8–123.6) | 118.4±9 (109–129.8) | 40.5±5.5 (35.3–49.2) | 67.7±4.3 (62.9–74.5) | 110.0±12.16 (92.0–132.0) | 113.2±9.73 (101.5–130.0) |
| c'                | 0.9±0.07 (0.8–1.0) | 0.9±0.08 (0.8–1.0) | 2.2±0.2 (2.0–2.5) | 1.3±0.1 (1.2–1.5) | 1.04±0.07 (0.92–1.13) | 0.99±0.07 (0.92–1.09) |
| V or T (%)        | 46.0±2.8 (40.6–51.0) | 50.3±5.6 (45.2–56.3) | – | – | – | 43.4±1.28 (41.0–46.0) |
| Odontostyle       | 133.0±2.5 (128.5–136.5) | 133.3±5.5 (127.0–139.0) | 94.6±3.8 (90.5–100.0) | 115.6±2.8 (111.5–120.0) | 134.5±3.71 (124.5–139.5) | 136.0±3.88 (130.5–142.5) |
| Replacement odontostyle | – | – | 100.0 | 116.1±5.5 (109.5–124.0) | 136.6±4.7 (129.0–142.0) | – | – |
| Odontophore       | 82.4±4.6 (72.0–88.5) | 78.0±3.9 (73.0–83.5) | 58.2±3.5 (54.0–63.0) | 70.9±3.1 (66.5–76.0) | 71.5±1.39 (69.0–73.5) | 71.5±2.67 (67.5–74.5) |
| Lip region width  | 14.6±0.5 (14.0–15.5) | 14.7±0.8 (13.5–15.5) | 10.0 | 11.5±0.6 (10.5–12.0) | 12.6±1.3 (10.5–14.0) | 15.0±0.60 (14.0–16.0) | 15.0±0.31 (14.5–15.5) |
| Oral aperture-guiding ring | 122.5±6.9 (111.5–135.0) | 117.9±3.0 (113.0–120.5) | 81.5±4.2 (76.5–86.0) | 98.2±7.3 (84.0–106.0) | 116.5±7.03 (97.5–127.5) | 121.5±5.94 (115.5–130.5) |
| Tail length       | 40.8±2.8 (36.5–44.0) | 39.0±2.0 (36.5–42.0) | 57.0±3.5 (51.5–59.5) | 46.5±3.1 (43.0–52.0) | 39.0±2.04 (34.0–41.0) | 38.5±2.77 (35.4–43.0) |
| Hyaline tail      | 11.2±1.5 (9.5–14.0) | 9.3±1.4 (7.5–10.5) | 14.5 | 13.7±3.4 (9.0–18.5) | 13.3±0.8 (12.0–14.0) | 11.5±1.8 (9.0–15.5) | 11.0±1.59 (8.5–13.0) |
| Spicules          | – | 66.2±3.1 (63.0–71.0) | – | – | – | – | – |
| Lateral accessory piece | – | 18.0±1.5 (16.5–19.5) | – | – | – | 15.5±1.55 (13.5–18.0) |

Note: aMeasurements are in µm and in the form: mean ± standard deviation (range); 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)×100; T = (distance from cloacal aperture to anterior end of testis/body length)×100; J = hyaline tail region length.
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Similarity value of 87% with *X. lapidosum* (MK801300). Scarcely similarity was found with the rest of *Xiphinema* spp. deposited in GenBank, showing coverage values below 30% with all of them. Finally, three new Coxl from *X. lapidosum* (MK796913-MK796915) were obtained in this study, being clearly different to the other accession from *X. non-americanum* group species deposited in GenBank and showing similarity values from 82 to 73% with all of them, being *X. lupini* the closet species (82% similar, 66 nucleotides and no indels) as in the D2 to D3 and ITS1 regions. No intra-specific variation was found between D2 and other regions.

Figure 7: Phylogenetic relationships within the *Xiphinema non-americanum* group complex. Bayesian 50% majority rule consensus tree as inferred from D2 to D3 expansion segments of 28S rRNA sequence alignment under the general time-reversible model of sequence evolution with correction for invariable sites and a gamma-shaped distribution (GTR + I + G) (lnL = 11,543.7822; AIC = 23,383.5644; freq A = 0.2502; freq C = 0.2298; freq G = 0.2995; freq T = 0.2205; R(a) = 0.9908; R(b) = 2.7656; R(c) = 2.4778; R(d) = 0.4894; R(e) = 4.2554; R(f) = 1.0000). Posterior probabilities greater than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in bold. Scale bar = expected changes per site.
D3 and CoxI sequences from *X. lapidosum* obtained in this study (MK801306-MK801307, MK796913-MK796915).

Phylogenetic relationships among *Xiphinema* non-americanum group species inferred from analyses of D2 to D3 expansion segments of 28S, ITS1, and the partial CoxI gene sequences using BI are given in Figures 7 to 9, respectively. Poorly supported clusters were not explicitly labelled. The 50% majority rule consensus 28S rRNA gene BI tree of *X. non-americanum* group
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Figure 9: Phylogenetic relationships within the *Xiphinema non-americanum* group complex. Bayesian 50% majority rule consensus tree as inferred from partial cytochrome c oxidase subunit I (CoxI) gene sequence alignment under the general time-reversible model of sequence evolution with correction for invariable sites and a gamma-shaped distribution (GTR + I + G), (lnL = -8,561.5874; AIC = 17,347.1747; freq A = 0.3687; freq C = 0.1301; freq G = 0.1382; freq T = 0.3630; R(a) = 3.3231; R(b) = 22.8405; R(c) = 2.0144; R(d) = 10.5400; R(e) = 75.4228; R(f) = 1.0000). Posterior probabilities greater than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in bold. Scale bar = expected changes per site.

spp. based in a multiple edited alignment including 70 sequences and 771 total characters showed two clearly separated (PP = 1.00) major clades (Fig. 7). Clade I grouped species from all morphospecies groups, including the new accessions obtained in this study of *X. histriae* and *X. lapidosum*. Clade II was not well supported (PP = 0.84) and was mostly composed by species from the morphospecies Group 5, except for *X. tica*, *X. bakeri*, and *X. index* which belong to Groups 4, 7, and 8, respectively. *Xiphinema histriae* (MK301302-
MK801305) occupies a superior position within this major clade I clustering with X. hispanum, X. celtiense, and X. cohni in a well-supported subclade (PP = 0.97). On the contrary, X. lapidosum (MK801306-MK801307) occupied a basal position and seemed to be related with X. lupini, X. turcicum, and X. oleae since all of them formed a well-supported subclade (PP = 0.99). The low similarity and small coverage between the ITS1 region from X. lapidosum and the rest of the ITS1 sequences available in GenBank made it impossible to perform a phylogenetic analysis for this region. For X. histriae, only ITS1-related sequences were used; the edited alignment generated for the 29 sequences of ITS1 was of 1,104 characters after discarding ambiguously aligned regions. This ITS phylogenetic tree (Fig. 8) showed two major clades (PP = 1.00), similar to those obtained for D2 to D3 region. Xiphinema histriae (MK801298-MK801299) appeared in the basal major clade but their phylogenetic position was not well resolved for this marker (Fig. 8). The CoxI region using a multiple alignment of 52 sequences and 390 characters after editing was used to obtain the 50% majority rule BI tree (Fig. 9). The position of X. histriae (MK796911-MK796912) was not well-defined, but clustering with X. hispanum, X. hispidum, X. cohni, and X. celtiense. By contrast, the relationship among X. lapidosum (MK796913-MK796915) and X. lupini was maintained.

Discussion

This study aimed to provide and to characterize morphometrically and molecularly two Xiphinema species belonging to Xiphinema non-americanum Group 5 from Spain, and to carry out an updated phylogenetic study of both species within the X. non-americanum group species. To date, this is the first record of the occurrence of X. histriae and X. lapidosum in Spain and the first time that describes the molecular characterization and the juvenile stages of both species.

Xiphinema histriae was originally described from Italy associated with grapevine (Lamberti et al., 1993a, 1993b, and later on, reported from the rhizosphere of wild growing grape (Vitis vinifera ssp. silvestris) in Austria (Tiefenbrunner and Tiefenbrunner, 2004). Based on the detailed study of paratypes and both Spanish populations described here, we detected that this species is characterized by having a pseudo-Z-organ with weakly muscularized wall with numerous small dense granular bodies against that initially described by Lamberti et al. (1993a, 1993b). Therefore, X. histriae must be transferred to morphospecies Group 5 (Loof and Luc, 1990). This study illustrates the importance of paratypes deposited in different official collections and reference nematology laboratories of nematodes, which are provided as a useful tool in the accurate identification and revision of nematodes species. On the other hand, X. lapidosum was, first, described from the rhizosphere of broad bean and pea in the south of Portugal (Roca and Bravo, 1993) and now it is reported from cultivated olive at Huévela, southwestern Spain. These data suggest that X. histriae may have a wider distribution than that described until now (including agricultural and natural ecosystems), and X. lapidosum may be an Iberian endemism, also associated with cultivated hosts.

The use of different ribosomal and mitochondrial markers in this study, D2 to D3, ITS1, and partial CoxI, provides a precise and unequivocal tool for the identification of X. histriae and X. lapidosum. Phylogenetic analyses based on D2 to D3, ITS1, and CoxI gene using BI resulted in a consistent position for X. histriae and X. lapidosum. Xiphinema histriae clustered with Xiphinema species from morphospecies Group 5, such as X. hispanum, X. cohni, X. celtiense, and X. hispidum, while X. lapidosum seems to be related with X. lupini because of both species clustered together in all the analyses carried out in this study. The present study on the phylogeny based on D2 to D3 segments supported a very weak correlation in the phylogenetic relationships among the different morphospecies groups within Xiphinema, a finding already reported by several authors namely, Gutiérrez-Gutiérrez et al., 2013; De Luca et al., 2014; Tzortzakakis et al., 2014, 2015; Archidona-Yuste et al., 2016a, 2016b, 2016c).

In summary, this study highlighted the diagnosis of Xiphinema non-americanum group species because a large number of species and the lack of good diagnostic characteristics among the X. non-americanum group (Loof and Luc, 1990; Loof et al., 1996). For this reason, we recommend the use of integrative taxonomy that are crucial for accurately identify species and better understanding of the present geographical distribution and host range of X. non-americanum group species. In this case, we provide new morphological and molecular data for the precise identification of these species, the first reports of these species in Spain, new hosts, and their phylogenetic position in the Xiphinema genus.

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