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First reports of *Xiphinema rivesi* and *Xiphinema incertum* (Nematoda: Longidoridae) in Bosnia-Herzegovina

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Summary. A nematode survey was carried out (in 2020) in apple and poplar orchards in Banja Luka, Bosnia-Herzegovina detected two nematode species belonging to the *Xiphinema americanum*-group. Polyphasic identification, combining morphological, molecular and phylogenetic analyses identified the nematodes as *X. rivesi* in apple orchards and *X. incertum* in poplar trees. Sequence and phylogenetic analyses used two rRNA genes (D2-D3 expansion segments of 28S rRNA, and ITS regions), and partial mitochondrial COI region detected high intra- and inter-specific variability within *X. rivesi*. These are the first reports of *X. rivesi* and *X. incertum* in Bosnia-Herzegovina, which extend the geographical distribution of these species in Europe.

Keywords. Integrative taxonomy, Longidorids, *Malus domestica*, mitochondrial COI, *Populus* sp., rRNA genes.

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INTRODUCTION

Xiphinema is a cosmopolitan nematode genus within the Longidoridae, which includes more than 290 described species (Archidona-Yuste *et al*., 2020; Ali *et al*., 2024; Kornobis *et al*., 2025). Nematodes in the *X. americanum* group (more than 60 species) are studied because they have well-conserved and overlapping morphometrics, and some species transmit by economically important nepoviruses (genus Nepovirus, family Comoviridae) (Taylor and Brown, 1997; Decraemer and Robbins, 2007).

Polyphasic identification of members of the *X. americanum* group provides the most efficient discrimination between virus vector or quarantine species, and to develop effective control strategies (Širca *et al*., 2007; Archidona-Yuste *et al*., 2016; Troccoli *et al*., 2024).

A survey carried out in Bosnia-Herzegovina, in 2020, of *Malus domestica* Borkh., 1803 (apple) and *Populus* sp. (poplar) plantations revealed the pres-

ence of two *Xiphinema* populations belonging to the *X. americanum* group. Initial hypotheses based on morphological observations showed that the two matched with *X. rivesi* and *X. pachtaicum* subgroup within the *X. americanum* group.

Xiphinema rivesi Dalmasso 1969 is widespread in Europe, and has been recorded in France, Germany (Sturhan, 2014), Italy (De Luca and Agostinelli, 2011; Troccoli *et al.,* 2024), Moldova (Poiras, 2012), Portugal (Gutiérrez-Gutiérrez *et al*., 2016), Slovenia (Urek *et al*., 2003), and Spain (Bello *et al*., 2005; Gutiérrez-Gutiérrez *et al*., 2011). The *X. pachtaicum* subgroup includes eight species (*X. fortuitum* Roca, Lamberti & Agostinelli, 1988, *X. incertum* Lamberti, Choleva & Agostinelli, 1983, *X. madeirense* Brown, Faria, Lamberti, Halbrendt, Agostinelli & Jones, 1993, *X. opisthohysterum* Siddiqi, 1961, *X. pachtaicum* (Tulaganov, 1938) Kirjanova, 1951, *X. pachydermum* Sturhan, 1984, *X. simile* Lamberti, Choleva & Agostinelli, 1983, and *X. utahense* Lamberti & Bleve-Zacheo, 1979), which have similar morphologies but different molecular and phylogenetic characteristics (Archidona-Yuste *et al*., 2016; Lazarova *et al*., 2016; Troccoli *et al*., 2024). Lazarova *et al*., 2016 suggested the occurrence of the *X. simile*-subgroup including *X. simile*, *X. parasimile*, *X. browni* and *X. vallense*. *Xiphinema incertum* can be misidentified as *X. pachtaicum* due to conserved gross morphology. *Xiphinema incertum* has been reported from Bulgaria (Lamberti *et al*., 1983), Croatia (Barsi, 1989), Slovenia (Barsi, 1994), Serbia (Barsi and Lamberti, 2002), Spain (Gutiérrez-Gutiérrez *et al*., 2012; Archidona-Yuste *et al*., 2016) and Italy (Troccoli *et al*., 2024), and *X. simile*, *X. densispinatum* Barsi, Lamberti & Agostinelli, 1998 and *X. pachtaicum* have been recorded in Bosnia-Herzegovina (Barsi *et al*., 1998; Barsi and Lamberti, 2004; Milašin *et al.,* 2024).

The objectives of the present study were to provide accurate identification of the two nematodes found in the Bosnia-Herzegovina survey, using an integrative approach, combining morphological, molecular, phylogenetic, and multivariate analyses. Phylogenetic relationships of the nematodes were inferred by their D2-D3 expansion segments of the 28S rRNA gene, the ITS, and the partial mitochondrial COI.

MATERIALS AND METHODS

Nematode samples and their morphologies

Soil samples were obtained from rhizospheres of apple and poplar trees in the Botanical Garden of the University of Banja Luka, Bosnia-Herzegovina. The samples were taken with an auger from 30 cm depth. Nematodes were extracted from 500 cm^3 of soil from each sample using the Flegg and Cobb technique (Flegg, 1967). *Xiphinema* specimens were killed by gentle heat, then fixed in a solution of 4% formaldehyde + 1% propionic acid, and then processed in pure glycerine using Seinhorst's (1962) method. Light micrographs were captured, and measurements were made using a Leica compound microscope equipped with a Leica DFC7000 T digital camera, and with Leica Las® version 2.6 software. All abbreviations used are as defined by Jairajpuri and Ahmad (1992).

Multivariate morphometric analyses

Principal Component Analysis (PCA) of the morphological traits within the *X. americanum* group and the *X. pachtaicum* subgroup, according to the subgroups indicated by Lamberti and Ciancio, 1993, were assessed with particular attention to *X. rivesi*, *X. incertum*, and *X. penevi* Lazarova, Peneva & Kumari, 2016. Two distinct PCA analyses were conducted for *X. rivesi* and for *X. pacthtaicum*-subgroup in XLSTAT (Addinsoft, 2007). Statistical analyses were carried out using PAST v. 4.03 (Hammer and Harper, 2001). Measurements obtained from literature used the mean value for each population (Supplementary Table 1). Measurements were normalized through PAST software before analyses. PCA for the *X. americanum* group was carried out using 13 diagnostic characters, including: body length (L), 'de Man's indices' (a, b, c, c′), percentage distance from anterior end to vulva/body length (V), odontostyle (ODS) and odontophore (ODP) lengths, oral aperture to guided ring distance (OA/gr), tail length (T), body diameters at lip region (LRD) and mid-body (MDB), and J tail length (JTA). PCA for the *X. pachtaicum*-subgroup analysed fourteen features, adding body diameter (ABD). Scores for the first three components (PC1, PC2 and PC3) were determined to form a two-dimensional plot for each nematode population, based on default parameters of the software.

DNA extractions, PCR and sequencing

For molecular analyses, and to exclude the cases of mixed populations in the same sample, total DNA was extracted from individual juvenile specimens, as described by De Luca *et al*. (2004). The ITS1-5.8S-ITS2 regions were amplified using the forward primer 18S (5′-GTTTCCGTAGGTGAACCTGC-3′) and the reverse primer 26S (5′-ATATGCTTAAGTTCAGCGGGT-3′) (Vrain *et al*., 1992). The D2-D3 expansion segments

Figure 1. Photomicrographs of *Xiphenema rivesi* Dalmasso, 1969 (A B and C), and *X. incertum* Lamberti, Choleva & Agostinelli, 1983 (D and E), from Bosnia-Herzegovina. A, female anterior region; B, detail of a female lip region; C, female tail region; D, female anterior region; E, female tail region. (Scale bars: A = 50 µm; B to E = 20 µm).

of the 28S rRNA gene were amplified using the primers D2A (5′-ACAAGTACCGTGGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) (Nunn, 1992). The portion of the mitochondrial cytochrome oxidase c subunit 1 (*mt*COI) gene was amplified with this primer set COI-F1 (5′-CCTACTATGATTGGTGGTTTTGGTAATTG-3′) and COI-R2 (5′-GTAGCAGCAGTAAAATAAGCACG-3′) (Kanzaki and Futai, 2002). The PCR cycling conditions used for amplifications were an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation each at 94°C for 50 s, annealing at 55°C for 50 s and extension at 72°C for 1 min, and a final step at 72°C for 7 min. PCR products of the ITS, D2-D3 expansion domains of 28S rRNA gene regions, and the partial mitochondrial COI from three individual nematodes were purified using the protocol suggested by the manufacturer (Nucleospin Gel and PCR Clean-up, Macherey-Nagel). Purified D2-D3 amplicons were directly sequenced using specific primers, while ITS and COI purified DNA fragments were cloned in pGEM-T Vector System II kit (Promega). Positive clones were sent for sequencing, in both directions, to MWG-Eurofins (Germany). The obtained sequences were submitted to GenBank database under the accession numbers: PV397451, PV397455-PV397458, PV461875-PV461878.

Evolutionary divergence between sequences

The pairwise distances within the D2-D3 expansion domains and mitochondrial COI sequences of *X. rivesi* populations were determined using the MEGA-X software package (Kumar *et al*., 2018). All positions with gaps and missing data were excluded. The D2-D3 expansion domains involved 32 nucleotide sequences, and the COI analyses involved 21 sequences.

Phylogenetic analyses

To assess the genetic variability within the *X. americanum* group and *X. pachtaicum*-subgroup nematodes, sequences from different geographical populations were included in a phylogenetic analysis. The newly obtained sequences of *X. rivesi* and *X. incertum* were aligned with the corresponding sequences present in GenBank, using MAFFT software 7 with default parameters (Katoh and Standley, 2013). Sequence alignments were manually edited using BioEdit 7.2.5 (Hall *et al*., 1999). Phylogenetic analyses of the sequence datasets were carried out with Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). The best-fit model of DNA evolution was obtained using JModelTest V.2.1.7 (Darriba *et al*., 2012) with the Akaike information criterion (AIC). The best-fit model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then used in MrBayes for the phylogenetic analyses. The Markov chains were sampled at intervals of 100 generations and two runs were conducted for each analysis. After discarding burn-in samples of 25% and evaluating convergence, the remaining samples were retained for in-depth analyses. The topologies were used to generate a 50% majority rule consensus tree. Posterior prob-

abilities (PP) were given on appropriate clades. Trees from all analyses were visualized using FigTree software version v.1.42 (Rambaut *et al*., 2014). D2-D3 alignment of sequences belonging to *X. americanum* group included 101 sequences, and *Tylencholaimus mirabilis* (Bütschli, 1873) De Man, 1876 was included as the outgroup. COI alignment of corresponding sequences belonging to the *X. americanum* group included 45 sequences and *X. chambersi*, and *X. browni* Lazarova, Peneva & Kumari, 2016 were used as the outgroups.

RESULTS

Survey for occurrence of Xiphinema *species in Bosnia-Herzegovina*

In 2020, apple and poplar trees in the Botanical Garden of the University of Banja Luka, Banja Luka, Bosnia-Herzegovina, were sampled to ascertain the occurrence of dagger nematodes. *Xiphinema rivesi* was associated with apple trees, and *X. incertum* with poplar trees. These are the first reports of the two nematodes for Bosnia-Herzegovina, extending the geographical distribution of these species in Europe.

Bosnia-Herzegovina (BH) populations of Xiphinema rivesi *and* X. incertum

Morphometrics of *X. rivesi* (Table 1) and *X. incertum* (Table 2) from Bosnia-Herzegovina were compared with the closest reported populations for both species. Morphometrics of BH *X. rivesi* aligned with the descriptions of Italian and Slovenian populations, showed small variations in comparisons with other populations of *X. rivesi*. The BH and Slovenian populations had similar V ratio (respectively, 53.9 *vs* 53.5%) (Urek *et al*., 2005), while the BH population had more posterior vulva compared to *X. rivesi* from Italy (respectively, 53.9 *vs* 52.6%) (Troccoli *et al*., 2024) or from Portugal (respectively 53.9 *vs* 52.0%) (Lamberti *et al*., 1994). The BH population of *X. rivesi* had longer odontostyle (95.7 µm) and tail (36.7 µm) compared to all other previously described populations (Fadaei *et al*., 2003; Urek *et al*., 2005; De Luca and Agostinelli, 2011; Gutiérrez-Gutiérrez *et al*., 2012; Handoo *et al*., 2015; Troccoli *et al*., 2024). Furthermore, Table 1 clearly showed that *X. rivesi* from Egypt is the most different population compared to the others reported in this study.

Morphology and morphometrics of the BH *X. incertum* population agreed with the type population described by Lamberti *et al*. (1983) (Table 2). The lip regions were slightly expanded and set off by constrictions and separated from the bodies by depressions. Female tails were conoid, each with a narrowly rounded terminus, as has been previously described for European populations (Barsi, 1994; Barsi and Lamberti, 2002; Gutiérrez-Gutiérrez *et al*., 2012). The main differences between the BH population and the type population of *X. incertum* from Bulgaria were: slightly longer body length (1952 *vs* 1900 µm), slightly lower V ratio (respectively, 55.4 *vs* 57.0%), and moderately lower c value (57 *vs* 69.0%). There was a lower a and c ratio for the BH nematodes when compared with *X. incertum* from Croatia and Serbia (Table 2). BH *X. incertum* had more anterior vulva (55.4%) compared to other populations, except for that from Spain (52.4%). The BH population had shorter odontostyles (mean = 75.2 µm) compared with specimens from Spain (92.2 µm) and the type population from Bulgaria (92.0 µm). The BH *X. incertum* also had similarities with *X. penevi*, *X. parasimile*, and *X. pachtaicum*, but can be distinguished from *X. penevi* (Lazarova *et al*., 2016) by body length (L = 1952.2 µm *cf*. 1687 µm), a (54.3 *cf*. 55.4%), V values (61 *cf*. 57.1%), and tail shape (rounded *cf*. pointed termini). The BH *X. incertum* can be distinguished from *X. parasimile* (Barsi and Lamberti, 2004) by smaller a and c' values (54.3 and 57.6 *vs* 70.5 and 59.9), and longer odontostyles (means = 75.2 *vs* 69.7 µm). When compared to *X. pachtaicum*, several characters showed overlapping ranges, with stylet lengths being the most significant differentiating feature for *X. pachtaicum* from Serbia (75.2 *vs* 88.3 µm) and for *X. pachtaicum* from Italy (75.2 *vs* 87.3 µm).

Multivariate morphometric analyses

In the PCA analyses built with species of the *X. americanum* group belonging to clade I, an accumulated variability of 68.34% of the total variance was measured for *X. rivesi* compared with other populations of this species (Figure 2). The contributions of PC1 were 32.47%, for PC2 was 21.84% and for PC3 was 14.02%. Kaiser Meyer Olkin's (KMO) measure of sampling indicated a KMO value of 0.608. The loading factors for each character were used to interpret biological meaning of the factors. The c ratio (r = 0.418), odontophore length (r = 0.40), body (r = 0.396) and oral aperture to guided ring length (r = 0.378), had greatest coefficients of correlation, and were responsible for the significant variability of the F1. For the F2, almost all characters showed positive correlations except for body and odontostyle lengths, and all the de Man indices. This component is associated with the general nematode size. F2 was also dominated by the greatest coefficient of correlation for lip region

Table 1. Morphometrics comparisons in *Xiphinema rivesi* Dalmasso, 1969 populations and locations where they were detected. Measurements of females obtained in the present study versus measurements reported from other previous studies. All measurements in µm, except body length in mm and in the form: mean±sd (range).

Character	*X. rivesi*	*X. rivesi*	*X. rivesi*	*X. rivesi*	*X. rivesi*	*X. rivesi*	*X. rivesi*	*X. rivesi*
	Bosnia-Herzegovina	Italy	Italy	Spain	Slovenia	Egypt	Iran	Portugal
		Troccoli *et al.*, 2024	De Luca and Agostinelli, 2011	Gutiérrez-Gutiérrez *et al.*, 2012	Urek *et al.*, 2005	Handoo *et al.*, 2015	Fadaei *et al.*, 2003	Lamberti *et al.*, 1994
n	11	18	10	10	11	10	13	5
L	1874.4 ± 50.7 (1808.1-1967.7)	1892.8 ± 133.4 (1607-2166.7)	2000 ± 0.12 (1900-2200)	1886 ± 127 (1583-2055)	1960 (1780-1070)	1600.6 ± 75.5 (1480-1660)	1700 ± 0.08 (1500-1800)	2000 (1900-2000)
a	52.2 ± 2.5 (47.0-55.7)	49.4 ± 2.1 (45.3-53.5)	55 ± 2.39 (50-58)	45.7 ± 3.3 (37.2-48.4)	45.39 (36.44-53.34)	35.1 ± 1.3 (32.7-37)	44.7 ± 2.9 (38.5-50)	53 (50-55)
b	-	8.4 ± 0.7 (7.2-9.7)	6.4 ± 0.61 (5.5-7.5)	7.4 ± 0.9 (6.4-8.4)	6.00 (5.08-7.05)	6.6 ± 0.7 (5.5-7.4)	6.4 ± 0.5 (5.5-7)	7.0 (5.5-7.0)
c	51.2 ± 4.0 (43.6-56.5)	58.8 ± 5.5 (50.2-67.7)	63.3 ± 4.99 (56.9-69.8)	53.4 ± 3.3 (49.1-58.5)	57.95 (51.12-66.82)	56.0 ± 3.4 (50.2-59.5)	55.2 ± 3.0 (49.5-60)	61 (59-63)
c’	1.6 ± 0.1 (1.5-1.8)	1.4 ± 0.1 (1.2-1.7)	1.4 ± 0.09 (1.2-1.5)	1.4 ± 0.1 (1.2-1.5)	1.44 (1.27-1.59)	1.0 ± 0.1 (1.0-1.1)	1.3 ± 0.07 (1.2-1.4)	1.5 (1.4-1.6)
V (%)	53.9 ± 0.8 (53.1-55.1)	52.6 ± 0.9 (50.9-54.2)	52 ± 0.85 (50-53)	53.0 ± 1.0 (52-55)	53.5 (52.6-54.4)	52.1 ± 1.4 (50.4-54)	52.2 ± 0.7 (51-53.5)	52 (50-55)
Odontostyle length	95.8 ± 0.9 (94.5-96.9)	90.8 ± 2.3 (86.4-94.1)	93.6 ± 1.60 (90.8-94.8)	92.1 ± 5.9 (79.0-98.5)	91.9 (84.9-96.3)	87.5 ± 2.0 (85-90)	81.5 ± 2.8 (73.5-86)	91 (88-95)
Odontophore length	46.4 ± 1.0 (44.0-47.2)	51.2 ± 2.3 (48.4-56.1)	53.3 ± 1.80 (50.6-55.7)	51.7 ± 3.8 (43.5-56.5)	51.8 (48.1-55.4)	52.8 ± 2.9 (50-57)	46.2 ± 1.8 (42-48.5)	50 (47-53)
Oral aperture – guide ring	73.2 ± 1.2 (70.6-74.6)	76.8 ± 2.6 (72.2-84.5)	75.4 ± 1.14 (73.6-77.6)	79.3 ± 6.1 (65.5-87.0)	78.6 (74.4-84.2)	72.2 ± 4.0 (65-75)	66.2 ± 2.0 (63-70)	73 (72-73.5)
Tail length	36.7 ± 2.9 (32.8-42.9)	32.4 ± 2.8 (28.4-38.9)	32.2 ± 1.25 (30.5-35.0)	35.4 ± 2.5 (31.5-39.0)	34.0 (30.4-37.9)	28.6 ± 1.9 (25-30)	30.8 ± 1.9 (28-33.5)	33 (31-34)
J tail	-	8.3 ± 1.2 (5.8-11.6)	7.4 ± 0.83 (6.3-8.6)	8.6 ± 0.7 (7.5-8.5)	-	9.1 ± 1.5 (8-12)	9.4 ± 0.8 (8-11)	9 (8-10)
Body diam. at lip region	-	8.9 ± 0.5 (8-9.9)	10.3 ± 0.00 (10-10)	9.9 ± 0.9 (8.5-12.0)	-	-	11.1 ± 0.4 (10.5-12)	9 (9-9)
Body diam. at guide ring	-	27.5 ± 1.3 (25.6-30.5)	-	-	-	-	25.6 ± 0.9 (25-27)	
Body diam. at mid-body	22.9 ± 1.0 (21.4-24.4)	38.3 ± 3.1 (33.8-43.5)	36.9 ± 1.58 (34.5-38.5)	-	43.8 (34.4-52.7)	45.7 ± 3.3 (40-50)	38.2 ± 3.3 (33.5-46.5)	
Body diam. at anus	-	23.8 ± 1.9 (21.1-28.4)	23.6 ± 0.81 (22.4-25.3)	-	-	-	23.2 ± 1.1 (21.5-25)	
Body diam. at beginning of J	-	10.4 ± 1.0 (8.8-11.8)	10.2 ± 0.47 (9.2-10.9)	-	-	12.6 ± 1.2 (11.5-15)	10.9 ± 0.8 (10-12.5)	

Table 2. Morphometrics comparisons in *Xiphinema incertum* Lamberti, Choleva & Agostinelli, 1983, *X. penevi* Lazarova, Peneva & Kumari, 2016, *X. parasimile* Barsi & Lamberti, 2004, and *X. pachtaicum* (Tulaganov, 1938) Kirjanova, 1951 populations and locations where they were detected. Measurements of females obtained in the present study versus measurements reported from other previous studies. All measurements in µm, except body length in mm and in the form: mean ± sd (range).

Character	*X. incertum*	*X. incertum*	*X. incertum*	*X. incertum*	*X. incertum*	*X. penevi*	*X. parasimile*	*X. pachtaicum*	*X. pachtaicum*
	Bosnia – Herzegovina	Spain	Bulgaria	Croatia	Serbia	Morocco	Serbia	Serbia	Italy
		Gutiérrez-Gutiérrez *et al.*, 2012	Lamberti *et al.*, 1983	Barsi, 1994	Barsi and Lamberti, 2002	Lazarova *et al.*, 2016	Barsi and Lamberti, 2004	Barsi and Lamberti, 2002	Troccoli *et al.*, 2024
n	8	6	4	2	2	6	53	30	10
L	1952.2 ± 89.3 (1858–2126.4)	1844 ± 52 (1788–1922)	1900 (1800–2000)	1910–1960	1920–2050	1687 ± 100 (1532–1846)	1990 ± 130 (1750-2260)	1990 ± 100 (1810-2190)	1926.9 ± 113.5 (1789 - 2091)
a	54.3 ± 1.7 (52.5–58.3)	49.7 ± 3.0 (44.6–52.5)	57 (54–64)	65.3–60.6	63.8–65.6	61.0 ± 2.6 (57.2–65.0)	70.5 ± 3.81 (61.0–76.1)	66.7 ± 3.70 (60.7-75.1)	61.9 ± 2.8 (57.8 - 66.3)
b	–	9.0 ± 1.4 (7.7–11.0)	6.4 (5.9–6.8)	6.2–6.4	6.0–5.9	6.1 ± 1.1 (5.0–7.0)	7.0 ± 0.49 (6.1–8.1)	5.8 ± 0.43 (4.8-7.0)	6.4 ± 0.5 (5.8 - 7.2)
c	57.6 ± 1.9 (55.5–61.2)	64.5 ± 2.7 (61.8–68.6)	69 (62–78)	67.6–78.2	72.5–81.3	57.7 ± 3.9 (50.8–61.5)	59.9 ± 4.51 (50.9–69.8)	61.5 ± 5.50 (51.6-71.8)	61.6 ± 5.1 (54.0 - 70.2)
c’	1.7 ± 0.1 (1.6–1.9)	1.2 ± 0.1 (0.9–1.3)	1.5 (1.4–1.7)	1.61–1.42	1.51–1.33	1.8 ± 0.1 (1.6–1.9)	2.02 ± 0.12 (1.79–2.28)	1.77 ± 0.14 (1.58-2.00)	1.7 ± 0.1 (1.6 - 1.9)
V	55.4 ± 2.1 (51.4–58.8)	52.4 ± 1.1 (51–54)	57 (56–58)	58.8–58.6	58.0–58.1	57.1 ± 0.6 (55.9–58.1)	55.5 ± 1.38 (52.2–58.7)	57.0 ± 1.33 (54.8-59.2)	56.8 ± 1.2 (55.1 - 59.4)
Odontostyle lenght	75.2 ± 2.5 (71.6–79.2)	92.2 ± 3.4 (88.0–97.0)	92 (87–97)	90.5–90.5	88.7–90.0	76.7 ± 2.1 (72–79)	69.7 ± 2.22 (64.4–73.7)	88.3 ± 2.58 (83.7 -93.7)	87.3 ± 2.7 (83.5 - 90.9)
Odontophore lenght	53.4 ± 4.1 (45.0–57.9)	49.7 ± 3.0 (46.0–53.5)	51 (50–54)	50.3–49	46.3–47.5	47.7 ± 1.8 (44–50)	41.6 ± 1.21 (38.8–43.8)	48.9 ± 1.34 (46.3-51.3)	51.2 ± 1.5 (48.4 - 53.3)
Oral aperture–guiding ring	70.0 ± 1.2 (68.8–72.4)	76.4 ± 4.8 (70.0–82.0)	71 (64–82)	81.7 –84.2	80.6–82.5	68.0 ± 0.6 (66–71)	62.6 ± 1.72 (59.4–66.3)	80.5 ± 2.39 (77.3-85.6)	80.1 ± 3.0 (75.8 - 83.8)
Tail lenght	33.9 ± 1.9 (31.2–36.5)	28.6 ± 1.2 (27.0–30.5)	28 (26–32)	28.3–25.1	26.4–25.0	29.3 ± 1.9 (26–32)	33.3 ± 1.62 (30.3 -37.1)	32.6 ± 2.60 (28.6-38.2)	31.4 ± 1.5 (29.8 - 33.7)
J (hyaline portion of tail)	–	6.5 ± 1.0 (5.5–8.5)	7 (6–9)	7.5–8.2	8.8–6.3	8.4 ± 0.7 (8–10)	8.2 ± 0.88 (6.3 -10.0)	10.1 ± 0.94 (8.1-12.5)	9.3 ± 0.4 (8.4 - 9.8)
Body diam. at lip region	–	9.5 ± 0.5 (8.5–10.0)	9 (8–9)	8.8–8.8	8.8–8.8	8.3 ± 0.3 (8–9)	9.0 ± 0.24 (8.4–9.7)	9.1 ± 030 (8.4-9.7)	8.7 ± 0.5 (8.3 - 9.7)
Body diam. at mid–body	35.9 ± 1.2 (34.8–38.7)	–	34 (29–37)	29.3–32.4	30–31.3	27.6 ± 1.4 (25–31)	28.3 ± 1.26 (24.7–30.6)	29.9 ± 1.45 (27.5-33.8)	31.2 ± 2.0 (28.0 - 34.8)
Body diam. at anus	19.7 ± 1.1 (18.4–22.2)	–	19 (18–19)	17.6–17.6	17.5–18.8	16.2 ± 0.7 (15–17)	16.5 ± 0.65 (15.0 -17.7)	18.4 ± 0.71 (16.9-20.0)	18.0 ± 0.5 (17.2 - 18.7)
Body diam. at be beginning of J	–	–	10 (9–10)	10–9.7	11.7–10.0	7.1 ± 0.4 (7–8)	7.1 ± 0.51 (6.3 -8.1)	8.5 ± 0.79 (6.9-103)	–

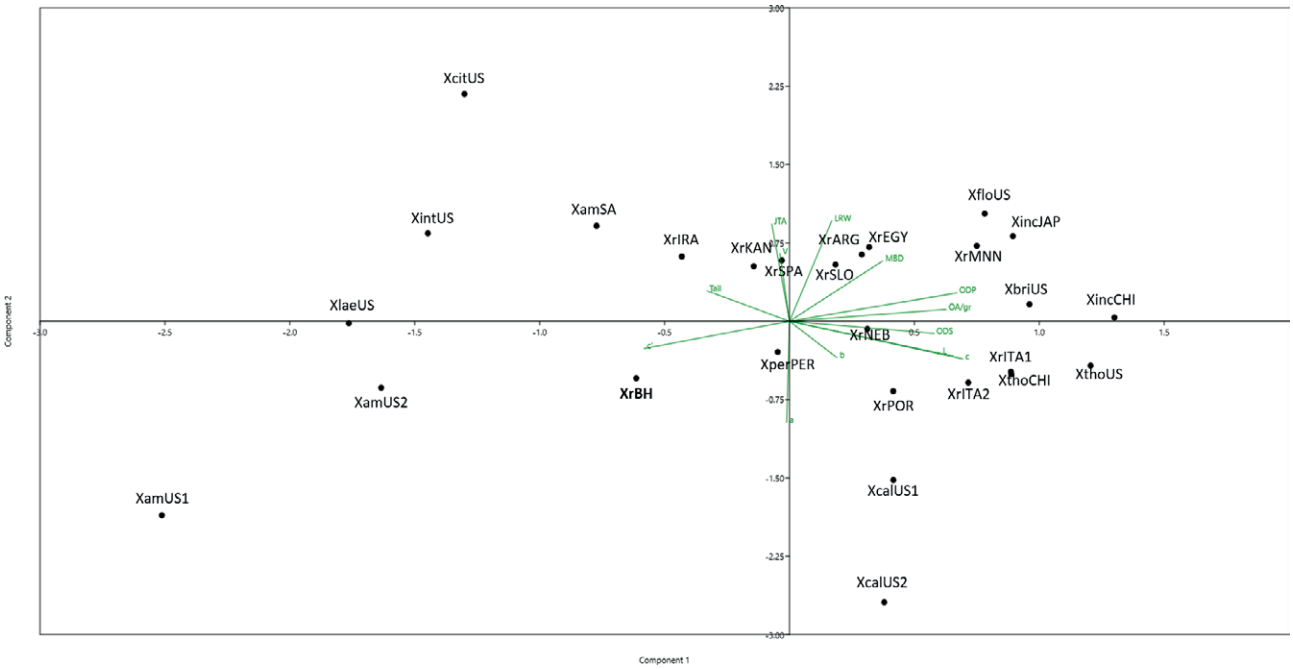

Figure 2. Principal component based on morphometric parameters of *Xiphinema rivesi* Dalmasso, 1969 from Bosnia-Herzegovina and other *X. americanum*-subgroup species from all over the world. Correlation biplot based on a PCA of the morphometric characters of *X. rivesi* from Bosnia-Herzegovina (XrBH) compared with population previous descripted in literature: XrARG (*X. rivesi*, Argentina; Chaves and Mondino, 2013); XrEGY (*X. rivesi*, Egypt; Handoo *et al*., 2015); XrIRA (*X. rivesi*, Iran; Fadaei *et al*., 2003); XrITA1 (*X. rivesi*, Italy; De Luca and Agostinelli, 2011); XrITA2 (*X. rivesi*, Italy; Troccoli *et al*., 2024); XrKAN (*X. rivesi*, USA; Lamberti and Bleve, 1979); XrMNN (*X. rivesi*, USA; Orlando *et al*., 2016); XrNEB (*X. rivesi*, USA; Lamberti and Bleve, 1979); XrPOR (*X. rivesi*, Portugal; Lamberti *et al*., 1994); XrSLO (*X. rivesi*, Slovenia; Urek *et al*., 2005); XrSPA (*X. rivesi*, Spain; Gutierrez-Gutierrez *et al.* 2012); XthoUS (*X. thornei*, USA; Lamberti and Golden 1986); XthoCHI (*X. thornei*, China; Wang *et al*., 1996); XincJAP (*X. incertum*, Japan, Lamberti and Bleve, 1979): XincCHI (*X. thornei*, China; Wang *et al*., 1996); XfloUS (*X. floridae*, USA; Lamberti and Bleve, 1979); XperPER (*X. peruvianum*, USA; Lamberti and Bleve, 1979); XlaeUS (*X. laevistriatum,* USA; Lamberti and Bleve, 1979); Xcal1US (*X. californicum,* USA; Lamberti and Bleve, 1979); Xcal2US (*X. californicum,* USA; Orlando *et al*., 2016); XintUS (*X. intermedium,* USA; Lamberti and Bleve, 1979); XcitUS (*X. citricolum,* USA; Lamberti and Bleve, 1979); XbriUS (*X. bricolense,* Canada*;* Ebsary *et al*., 1989); XamUSA1 (*X. americanum,* USA; Cobb, 1913); XamUSA2 (*X. americanum,* USA; Lamberti and Golden, 1984); XamSA (*X. americanum,* South Africa; Loots and Heyns, 1984).

diameter (r = 0.474) and J tail length (r = 0.459), as well as a high negative correlation for a ratio (r = -0.478). F3 was mainly dominated by a high positive correlation for tail length ratio (r = 0.493) and V ratio (r = 0.489). Specimens of species were not casually situated, and a widespread spatial separation was observed among the *Xiphinema* populations. *Xiphinema rivesi* populations clustered together in the middle of the diagram confirming the morphological similarity among populations. The values of vulva position, oral aperture to guided ring distance and the *a* ratio increased along y-axis (PC2) contributing to the variability among *X. rivesi* populations.

The PCA, built on the *X. pachtaicum*-subgroup species, showed an accumulated variability of 68.50% for total variance, with the contribution of PC1 of 31.21%, for PC2 of 22.18% and for PC3 of 15.11%. The KMO value was 0.583, indicating that the sample size was suitable for the analysis. The loading factors for each character were used to interpret their biological validities. For PC1, almost all the characters had positive correlations, except for tail length (r = - 0.008), a (r = - 0.113) and c′ ratio (r = - 0.301). PC2 was positively correlated with body length L (r = 0.464), c (r = 0.462) and b ratio (r = 0.431), with a gradual increase in values associated with body dimensions and general nematode size. For PC3, a strong positive correlation was found for tail length (r = 0.613) and c′ ratio (r = 0.389), but negative correlations to V (r = -0.432). *Xiphinema pachtaicum* and *X. incertum* grouped on the right side of the diagram, forming a closely related morphocluster, while *X. simile* and *X. parasimile* clustered together on the left side of the diagram clearly separated from the *X. pachtaicum*-subgroup. This spatial separation was mainly dominated by the PC1, grouping the species according to body width, distance of oral aperture to guided ring, odontostyle, and odontophore length.

Molecular characterization

The length of the amplified products of the D2-D3 expansion segments, ITS region and of the partial *mt*COI gene are reported in Table 3.

Low intra-population variability of D2-D3 region of *X. rivesi* sequences included in the present study was observed (2 to 12 nt). Blast NCBI searches using D2-D3 domains of BH *X. rivesi* showed 100% similarity with *X. rivesi* isolates from Italy, Chile (JX912150, OR683648) and the USA (KU680972), and 99% similarity with the remaining sequences of *X. rivesi* present in the Genbank database. Pairwise distances among D2-D3 sequences of *X. rivesi* ranged from 0 to 32 nucleotides, only *X. inaequale* (HM163210) differed between 3 to 40 bp (Supplementary Table S3). Blast search revealed that ITS sequences of *X. rivesi* from BH apple samples had 100% similarity with *X. rivesi* from Chile (OR698922) and *X. inaequale* Khan & Ahmad, 1975 (GQ231530), 99% similarity with *X. rivesi* from Italy (OR698913-OR698921; FR878063-66; 8-16 nt differences; 2-11 gaps), 98% similarity with *X. thornei* Lamberti & Morgan Golden, 1986 (27 nt differences; 21 gaps) and *X. rivesi* from USA (MT524488), and 94 to 96% similarity with all the remaining Genbank sequences for *X. rivesi*. The three individuals of the BH *X. rivesi* population that were amplified produced 435 bp COI fragments. Amplified products were cloned and two clones for each specimen were sequenced. Sequence analyses showed greatest similarity with Italian *X. rivesi*, differing by 1 to 5 bp (99 to 100% similarity). Pairwise distances of all *Xiphinema* COI present in the subgroup A ranged from 0 to 21% (from 0 to 70 nucleotides) (Supplementary Table S4).

D2-D3 sequences of the BH population of *X. incertum* showed 100% similarity with *X. penevi* from Morocco (KU250157), and *X. incertum* from Spain (KX244908) ; 99% similarity with Italian and other Spanish *X. incertum* (1-7 nt differences), and 96 to 98% similarity with corresponding sequences of *X. pachtaicum* and other species belonging to the *X. americanum*-group, confirming the considerable sequence similarity within the *Xiphinema americanum*-group.

Phylogenetic analyses

The D2-D3 phylogenetic tree produced two main clades: clade I containing all *X. rivesi* sequences and the closest species of the *X. americanum* group; and clade II containing all sequences of the *X. pachtaicum*-subgroup including *X. incertum* sequences (Figure 3). Clade I, as previously reported by Troccoli *et al*. (2024), showed different subgrouping for *X. rivesi*. Sequences of *X. rivesi* from Spain and the USA grouped together and separated from other subgroupings. The BH sequences formed a separated subgrouping due to nucleotide variability within *X. rivesi* populations. Clade II contained all sequences belonging to the *X. pachtaicum*-subgroup including *X. incertum* sequences. The subgrouping of *X. incertum* sequences also included *X. penevi* from Morocco, with high support. This indicates that *X. incertum* and *X. penevi* requires further investigation to clarify whether they are distinct or the same species.

The COI phylogenetic tree showed different subgroupings within the *X. americanum* group and within *X. rivesi* populations, separating according to geographical origins (Figure 4; A-D subgroupings). COI sequences of *X. rivesi* formed a well-supported subgroup (97% similarity), in which Italian and BH *X. rivesi* subgrouped together (Figure 4 A).

DISCUSSION

The present study reports previously unrecorded occurrences of *X. rivesi* and *X. incertum* nematodes in

Table 3. Populations of *Xiphinema* characterized in the present study.

Species	Location	Host	Sample code	Genbank accession number			Source
				D2D3	ITS	COI	
X. rivesi	Banja Luka, Bosnia and Herzegovina	*Malus domestica*	N 6_10	-	PV397451	-	B. Nježić
X. rivesi	Banja Luka, Bosnia and Herzegovina	*Malus domestica*	10_11BH	PV397456	-	-	B. Nježić
X. rivesi	Banja Luka, Bosnia and Herzegovina	*Malus domestica*	17_D2	PV397457	-	-	B. Nježić
X. rivesi	Banja Luka, Bosnia and Herzegovina	*Malus domestica*	21_BH	PV397458	-	-	B. Nježić
X. rivesi	Banja Luka, Bosnia and Herzegovina	*Malus domestica*	47_COIF	-	-	PV461875	B. Nježić
X. rivesi	Banja Luka, Bosnia and Herzegovina	*Malus domestica*	51_COIF	-	-	PV461876	B. Nježić
X. rivesi	Banja Luka, Bosnia and Herzegovina	*Malus domestica*	52_COIF	-	-	PV461877	B. Nježić
X. rivesi	Banja Luka, Bosnia and Herzegovina	*Malus domestica*	53_COIF	-	-	PV461878	B. Nježić
X. incertum	Banja Luka, Bosnia and Herzegovina	*Populus* sp.	XI_2_BH	PV397455	-	-	B. Nježić

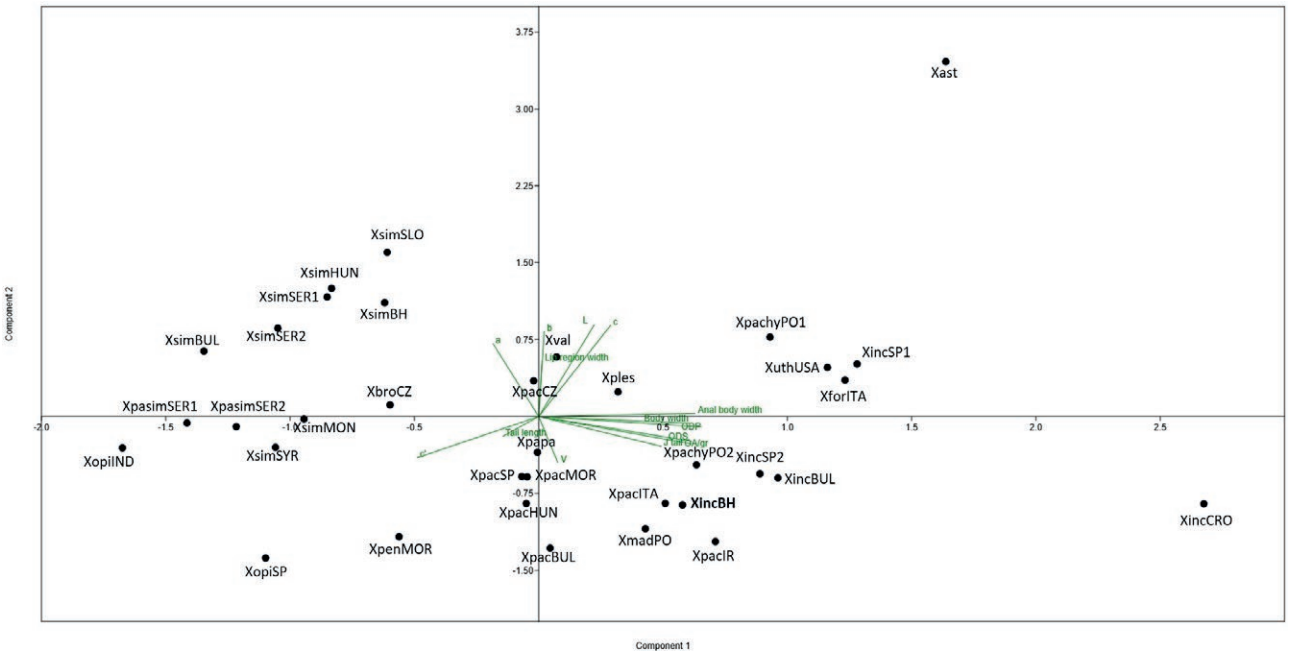

Figure 3. Principal component analysis based on morphometric parameters of *Xiphinema incertum* Lamberti, Choleva & Agostinelli, 1983 from Bosnia-Herzegovina and other *X. pachtaicum*-subgroup species from other geographic regions. The Correlation biplot based on a PCA of the morphometric characters of *X. incertum* from Bosnia-Herzegovina (XincBH) compared with previously described populations: XforITA (*X. fortuitum*, Italy; Roca *et al*., 1987); XuthUSA (*X. utahense*, United States of America; Lamberti and Bleve, 1979); XincBUL (*X. incertum*, Bulgaria; Lamberti *et al*., 1983); XincCRO (*X. incertum*, Croatia; Barsi, 1994); XincSP1 (*X. incertum*, Spain; Gutiérrez-Gutiérrez *et al*., 2012); XincSP2 (*X. incertum*, Spain; Archidona-Yuste *et al*., 2016); XmadPO (*X. madeirense,* Portugal; Lamberti *et al*., 1993); XpachyPO1- XpachyPO2 (*X. pachydermum,* Portugal; Lamberti *et al*., 1994); XopiIND (*X. opisthohysterum,* India; Siddiqi, 1961) XopiSPA (*X. opisthohysterum,* Spain; Archidona-Yuste *et al*., 2016); XsimBUL (*X. simile,* Bulgaria; Lamberti *et al*., 1993); XsimMON (*X. simile,* Montenegro; Barsi, 1994); XsimSER1 (*X. simile,* Serbia; Barsi, 1994); XsimSER2 (*X. simile,* Serbia; Barsi and De Luca, 2008); XsimBH (*X. simile,* Bosnia and Herzegovina; Barsi and Lamberti, 2004); XsimSLO (*X. simile*, Slovakia; Lamberti *et al*., 1999); XsimHUN (*X. simile,* Hungary; Repasi *et al*., 2008); XsimSYR (*X. simile*, Syria; Ali *et al*., 2024); XpasimSER1 (*X. parasimile*, Serbia; Barsi and Lamberti 2004); XpasimSER2 (*X. parasimile*, Serbia; Barsi and De Luca, 2008); XpenMOR (*X. penevi,* Morocco; Lazarova *et al*., 2016); XbroCZR (*X. browni*, Czech Republic; Lazarova *et al*., 2016); Xpapa (*X. parapachydermum,* Spain; Gutiérrez-Gutiérrez *et al*., 2012); Xast (*X. astaregiense,* Spain; Archidona-Yuste *et al*., 2016); Xples (*X. plesiopachtaicum,* Spain; Archidona-Yuste *et al*., 2016); Xval (*X. vallense,* Spain; Archidona-Yuste *et al*., 2016); XpacHUN (*X. pachtaicum,* Hungary; Repasi *et al*., 2008); XpacBUL (*X. pachtaicum,* Bulgaria; Lazarova *et al*., 2016); XpacMOR (*X. pachtaicum,* Morocco; Mokrini and Dababat, 2019); XpacIR (*X. pachtaicum,* Iran; Mobasseri *et al*., 2019); XpacSP (*X. pachtaicum,* Spain; Archidona-Yuste *et al.,* 2016); XpacITA (*X. pachtaicum,* Italy; Troccoli *et al*., 2024); XpacCZ (*X.pachtaicum,* Czech Republic; Kumari, 2006).

Bosnia-Herzegovina (BH), extending incidences of these nematodes in Europe. Morphometric comparisons of the two nematodes from this country with the type and other descriptions of these species confirmed the high intraspecific variability in both species (Tables 1 and 2). Multivariate analyses of *X. rivesi* populations, including the BH population, shared some overlapping characters along with morphometric differences, which may be due to their geographical origins and phenotypic plasticity (Figure 2). PCA of *X. incertum* showed that BH *X. incertum* shared overlapping characters with *X. incertum* populations from Bulgaria and Spain, and several species of the *X. pachtaicum* subgroup for size and body length. The Croatian population of *X. incertum* was closely related but separated from the BH and Bulgarian populations for posterior vulva position and having longer hyaline tails. *Xiphinema penevi* from Morocco was distant from BH *X. incertum* (Figure 3). These results have confirmed the existence of the *X. pachtaicum*-subgroup (Gutiérrez-Gutiérrez *et al*., 2012; Palomares-Rius *et al*., 2014; 2017), and the difficulty to correctly identify these nematode species. Sequence analyses of the BH *X. rivesi* markers confirmed the high interspecific variability within the species (Troccoli *et al*., 2024). For BH *X. incertum*, the D2-D3 sequence had low intra- and inter-specific variability among *X. incertum, X. penevi* (0-7 nt differences) and *X. pachtaicum* populations.

The phylogenetic trees based on D2-D3 showed two main clades, in agreement with previous reports (Archidona-Yuste *et al*., 2016; 2020; Orlando *et al*., 2016; Mobasseri *et al*., 2019; Troccoli *et al*., 2024).

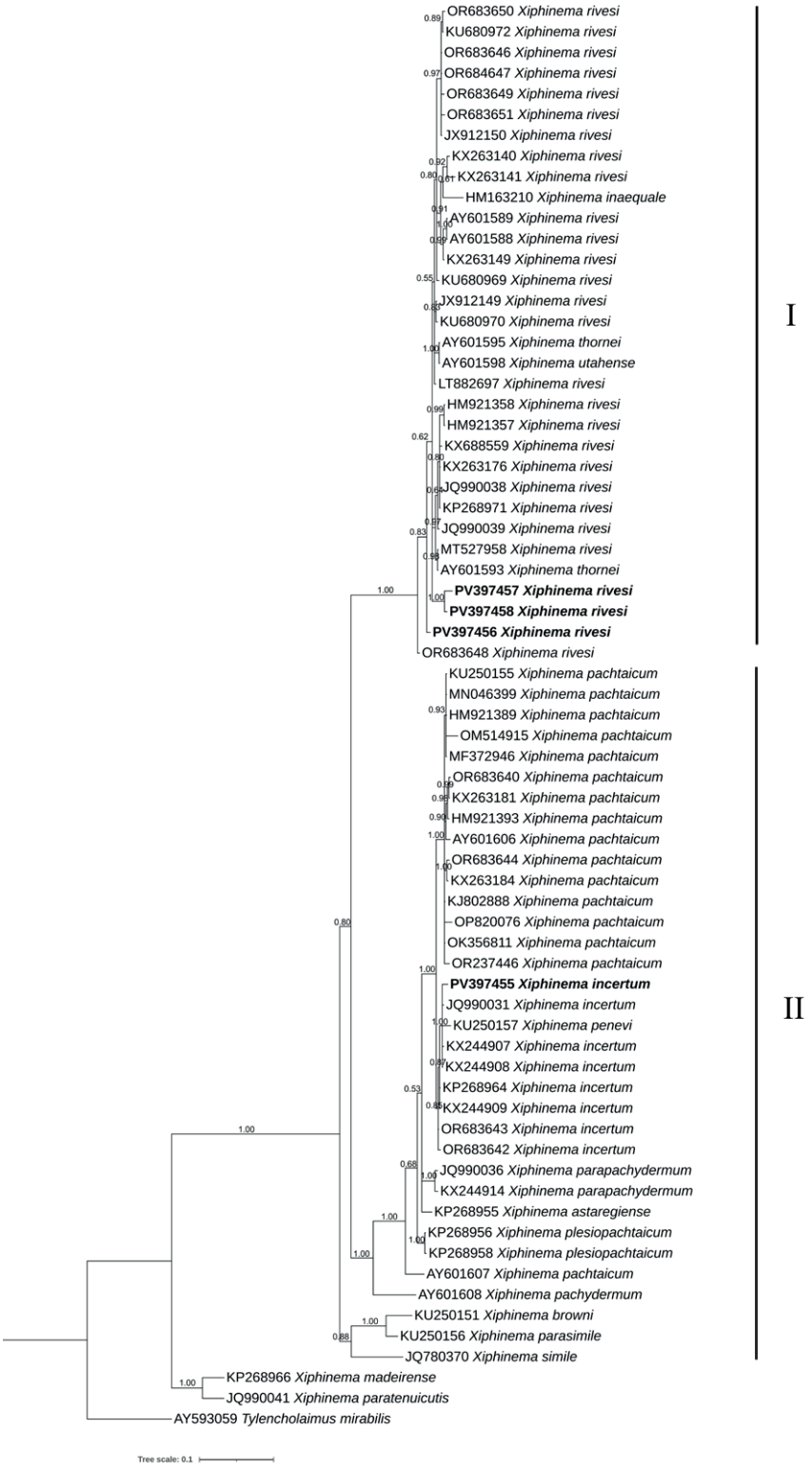

Figure 4. Phylogenetic relationships among *Xiphinema rivesi* Dalmasso, 1969 and *Xiphinema incertum* Lamberti, Choleva & Agostinelli, 1983 populations, indicated from the Bayesian 50% majority rule consensus tree as inferred from D2-D3 expansion domains of the 28S rRNA sequence alignment under a transversional, with correction for invariable sites and a gamma-shaped distribution model (GTR+I+G). Posterior probabilities of more than 0.50 are given for appropriate clades. Newly obtained sequences in the present study are shown in bold font. The scale bar indicates expected changes per site.

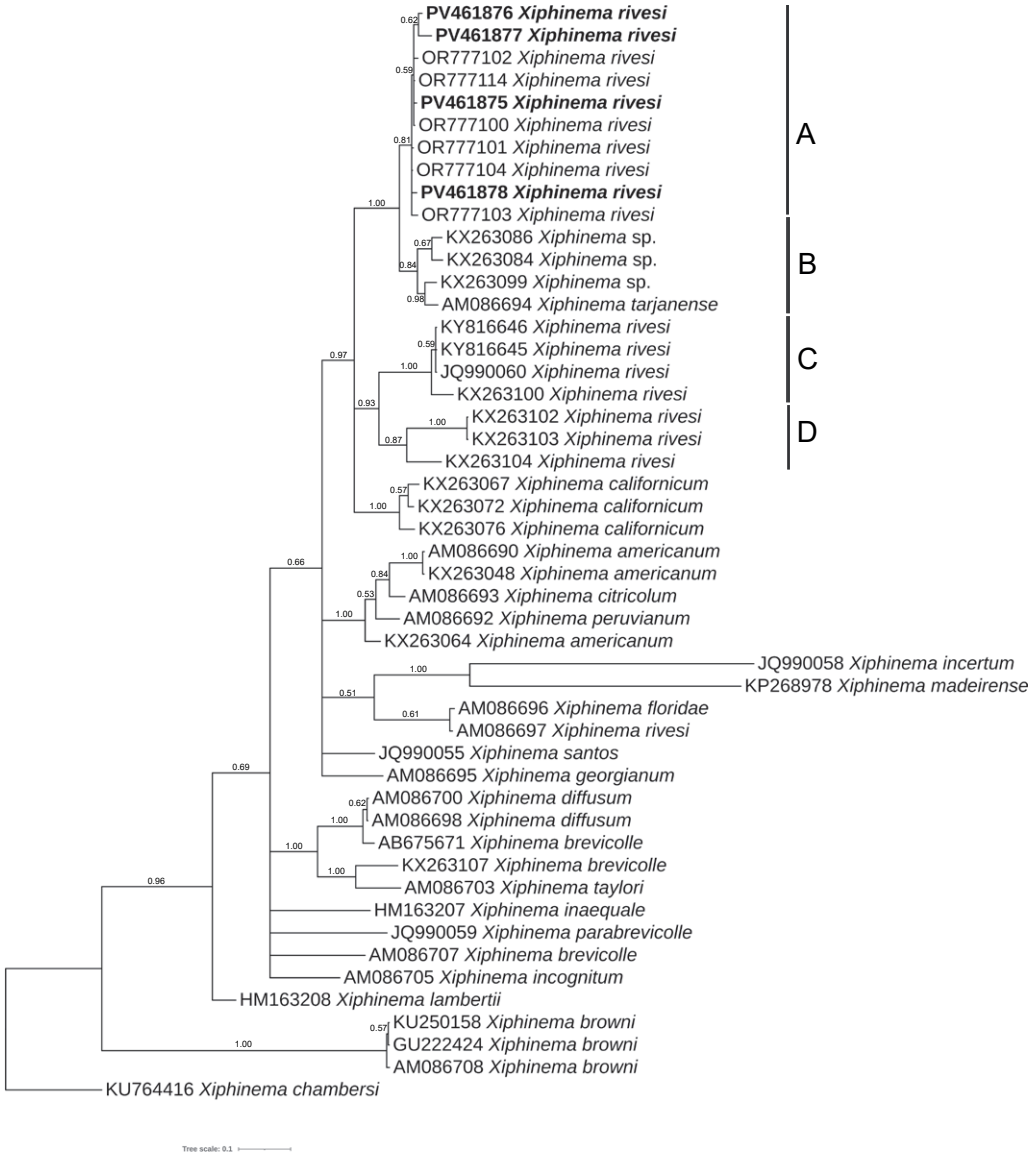

Figure 5. Phylogenetic relationships among *Xiphinema rivesi* Dalmasso, 1969 and *Xiphinema incertum* Lamberti, Choleva & Agostinelli, 1983 populations, indicated from Bayesian 50% majority rule consensus tree as inferred from mitochondrial COI sequence alignment under a transversional, with correction for invariable sites and a gamma-shaped distribution model (GTR+I+G). Posterior probabilities of more than 0.50 are given for appropriate clades. Newly obtained sequences in this study are shown in bold font. The scale bar indicates expected changes per site.

Clade I consisted of several subgroupings of *X. rivesi*, in particular the BH *X. rivesi* had high variability. Clade II grouped all sequences belonging to the *X. pachtaicum*-subgroup (Figure 4). *Xiphinema incertum* populations, including the BH population, and X. *penevi* formed a well-supported subgrouping, despite the PCA results placing *X. penevi* distant from *X. incertum* populations. This result confirms existence of different evolutionary rates of molecular and morphological mechanisms and indicates that *X. penevi* may represent a recent speciation event, as the D2-D3 sequences showed 100% similarity with those of *X. incertum*.

In the phylogenetic tree of COI sequences, different subgroupings of *X. rivesi* populations occurred (Figure 5). BH and Italian *X. rivesi* subgrouped together (Figure 5A), while *Xiphinema* sp. 1 and *X. tarjanense* were in a separate subgroup (Figure 5B) showing sister relationships with subgroup A. *Xiphinema rivesi* from Spain and United States of America were in separate subgroupings (Figure 5, C and D). These results demonstrate

the occurrence of different haplotypes within *X. rivesi*, probably resulting from different mutation rates in the mitochondrial genomes among the different geographical populations. Therefore, the COI marker was useful for population and intraspecific genetic variation studies among *X. americanum* group populations.

In conclusion, the present study shows new occurrence of *X. rivesi* and *X. incertum* in Bosnia-Herzegovina, extending their distribution in Europe. Particular attention is required when identifying *X. pachtaicum*, because of the existence of species complexes. Regarding to *X. incertum* and *X. penevi*, more molecular data are required to verify the occurrence of cryptic species or variability within *X. incertum*, as *X penevi* has only been described from Morocco. Knowledge of intra- and inter-specific molecular variability is important to detect misidentification or cryptic speciation within the *X. americanum* group. Use of COI for integrative nematode taxonomy can delimit species within *Nematoda*, but the high nucleotide variability of mitochondrial COI within the *X. americanum* group suggests high mutation rates, that can represent potential for development of cryptic species or the ability of these species to adapt to climate and environmental changes.

AUTHOR CONTRIBUTIONS

EF and AV equally contributed to molecular and phylogenetic analyses; AT and BN carried out morphological measurements; AV and RD contributed to sampling and nematode recovery; AV, AT, EF and BN reviewed and edited the paper manuscript; FDL contributed to the study design, writing of the original draft, and review and editing; FDL contributed to fund acquisition. The first draft of the manuscript was commented on by all authors, and they all approved the final manuscript.

DATA AVAILABILITY

All sequences described in this paper are freely available from the GenBank database.

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