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Occurrence of *Xiphinema santos* Lamberti, Lemos, Agostinelli & D'Addabo 1993 (Nematoda: Longidoridae), a *X. americanum*-group member in Iran

Farahnaz Jahanshahi Afshar · Fatemeh Shahryari · Ali Mokhtassi-Bidgoli · Pablo Castillo · Zeinab Mirzaie Fouladvand · Majid Pedram

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Abstract A population of *Xiphinema americanum*-group was recovered in association with stone fruit trees in Isfahan province, center of Iran. A reverse taxonomic approach based upon the large subunit ribosomal DNA (LSU rDNA D2-D3) and the mitochondrial cytochrome c oxidase subunit I (*COI* mtDNA) gene sequences in integration with morphological studies, revealed that the recovered population belongs to *Xiphinema santos.* The Iranian population was mainly characterized by 1240–1868 μm long females with 60–84 μm long odontostyle, a = 37.2–51.9 and c = 42.8–54.6. It is further characterized by a lip region having a depression in junction with the body, presence of visible endosymbiont bacteria in ovaries under light microscope, dorsally convex and ventrally slightly concave conical tail with a blunt tip and three juvenile developmental stages. This population was similar to the type population in its morphology; however overlapped and extended morphometric data ranges, as well as differences in some indexes were observed. Compared to a Spanish population of this species, the Iranian population had a close morphology, similar morphometric data ranges and identical LSU and *COI* sequences. In LSU phylogeny, the relationship between the present and some previously sequenced isolates of the species and some isolates of three species *X. georgianum, X. laevistriatum* and *X. citricolum* was not resolved. In *COI* phylogeny, the clade of the Iranian and Spanish populations appeared as an independent lineage inside an unsupported clade including several species. The comparison with other populations of the species was reported and discussed. A second species, *X. primum,* that is native to Iran, was recovered from a new locality and characterized molecularly.

Keywords *COI*mtDNA · First report · Isfahan province · LSU rDNA D2-D3 · Phylogeny · Stone fruit trees · Taxonomy · *Xiphinema primum*

F. Jahanshahi Afshar
Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

F. Shahryari
Department of Plant Protection, Faculty of Agriculture, University of Zanjan, Zanjan 45371-38791, Iran

A. Mokhtassi-Bidgoli
Department of Agronomy, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran

P. Castillo
Instituto de Agricultura Sostenible (IAS), Consejo Superior de Investigaciones Científicas (CSIC), Córdoba, Spain

Z. Mirzaie Fouladvand · M. Pedram (✉)
Department of Plant Pathology, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran
e-mail: majid.pedram@modares.ac.ir

Introduction

The *Xiphinema americanum*-group currently includes 60 species (Mobasseri et al. 2019). The group is known with 10 representatives in Iran (Pedram 2018; Mobasseri et al. 2019). *Xiphinema santos* Lamberti et al. 1993 was

originally described from Portugal in association with *Vitis* sp. (Lamberti et al. 1993) and was later reported from Portugal, Egypt and Spain in association with hop, pine, peach, grapevine and stone pine (Lamberti et al. 1993, 1994, 1996; Gutiérrez-Gutiérrez et al. 2012). In the present study, a population of the species was recovered from Isfahan province in association with stone fruit trees following our samplings to determine the infestation of pomegranate and fruit tree gardens with plant parasitic nematodes, and studied based upon morphological and molecular approaches. The species *X. primum* Mobasseri et al. 2019 that was originally described from Iran (Mobasseri et al. 2019) was also recovered from a new location in north of Iran and characterized molecularly.

Materials and methods

Sampling and morphological studies

Several soil samples were collected from fruit tree gardens in Isfahan, Tehran and Qom provinces. The samples were especially collected from the rhizosphere of trees showing general weakness symptoms similar to those of nutrient deficiency. Furthermore, 15 soil samples were collected from forests of Golestan province, north of Iran. Nematodes were extracted from soil samples using the tray method (Whitehead and Hemming 1965). A series of 20 and 60 mesh sieves (US standard mesh nos. equal to 850 and 250 μm sized openings, respectively) were used to extract tentative longidorid species. After extraction, the specimens of interest were hand-picked under a Nikon SMZ1000 stereomicroscope, heat-killed by adding boiling 4% formaldehyde solution, and transferred to anhydrous glycerin according to De Grisse (1969). Permanent slides were prepared and morphological characters were studied using an Olympus BX51 light microscope equipped with differential interference contrast (DIC). The light microphotographs were taken using an Olympus DP72 digital camera attached to the microscope. Morphometric values were obtained using a drawing tube attached to a Nikon E600 light microscope. The juvenile developmental stages were identified according to Robbins et al. (1996).

Molecular studies

For DNA extraction, two live *Xiphinema americanum*-group nematode specimens from the both populations recovered from the both provinces were picked out, washed in distilled water and photographed in temporary slides. Each selected nematode specimen was then transferred to a small drop of Tris-EDTA (TE) buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0, QIAGEN Inc., Valencia CA, USA) on a clean slide and squashed using a clean slide cover glass with the aid of a plastic material. Each DNA sample was taken from one individual. The suspension was collected by adding 20 μl of TE buffer. DNA samples were stored at −20 °C until used as polymerase chain reaction (PCR) templates. To amplify the near-full-length fragment of the LSU rDNA D2-D3 and partial *COI* mtDNA, several primer pairs were used in the PCR reactions. Primers used for the amplification of the D2–D3 expansion domains of the LSU rDNA were forward D2A (5′-ACAAGTACCGTGAGGGAAAGT-3′) and reverse D3B (5′-TGCGAAGGAACCAGCTACTA-3′) (Nunn 1992) primers. In some cases, the reverse primer was replaced by the reverse primer KK28S-4 (5′-GCGGTATTTGCTACTACCAYYAMG ATCTGC-3′) (Kiontke et al. 2004). The used primers for amplification of the partial *COI* mtDNA were: forward primer JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and reverse primer JB4.5 (5′-TAAAGAAAGAACAT AATGAAAATG-3′) (Bowles et al. 1992), and forward primer COIF (5′-GATTTTTTGGKCATCCWGARG-3′) and reverse primer COIR (5′-CWACATAATAAGTATC ATG-3′) (Lazarova et al. 2006). The PCRs for amplification of the genomic and mitochondrial fragments were done according to Jahanshahi Afshar et al. (2019). The newly obtained sequences were submitted to the GenBank database under the accession numbers given in LSU and *COI* trees.

Phylogenetic analyses

The recently obtained LSU rDNA D2-D3 and *COI* mtDNA sequences were manually checked, edited and assembled. These sequences were compared with those of other relevant sequences available in the GenBank database using the BLAST homology search program. The BLAST search using one of the LSU sequences of the Isfahan province population (MN602712) revealed it has maximal identity with several sequences of *Xiphinema santos* (JQ990029, JQ990030, AY601587 and some others) and the BLAST search using its *COI* sequence (MN627267) revealed it has maximal identity with a sequence of *X. santos* (JQ990055). The BLAST search using the LSU sequence of the Golestan province

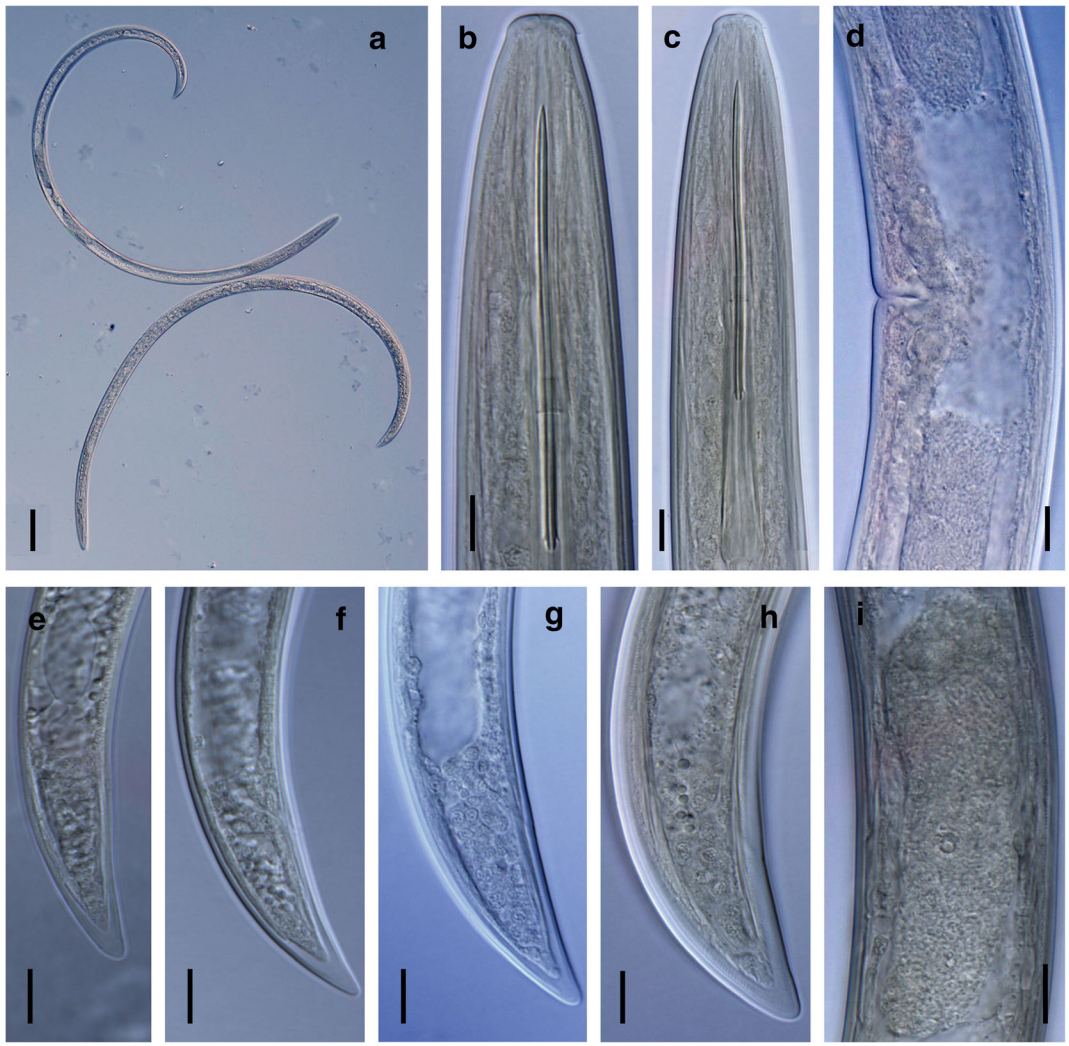

Fig. 1 Light microphotographs of Iranian population of *Xiphinema santos* Lamberti et al. 1993. **a**: Entire of female, **b** & **c**: Female anterior body region, **d**: Vulval region, **e**-**h**: Tail of the first, second and third stage juveniles and female, respectively, **i**: Anterior ovary and endosymbiont bacteria. Scale bars: a = 100 μm, b-i = 10 μm)

population (MN602714) revealed it has 99.55–99.89% identity with several sequences of *X. primum* (MF372941–43, MF372947) and the BLAST search using its *COI* sequence (MN627266) revealed it has 93.07% identity with two sequences of *X. primum* (MK202795, MK202796). The DNA sequences for reconstructing the phylogenetic trees were selected according to recent studies (Archidona-Yuste et al. 2016; Mobasseri et al. 2019).

Multiple alignments of the LSU and *COI* datasets were performed using the Q-INS-i algorithm of the online version of MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standley 2013). The Gblocks program (version 0.91b) (Castresana 2000) with all three less stringent parameters, a server tool at the Castresana Lab (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), was used for post-editing of the LSU alignment, i.e., to eliminate poorly aligned regions or divergent positions. The best-fitting substitution model for each of the two datasets was selected according to the Akaike information criterion (AIC) by using PAUP*/MrModeltest v2.2 (Nylander et al. 2004). A general time reversible model, including among-site rate heterogeneity and estimates of invariant sites (GTR + G + I) was selected and used for both LSU rDNA D2-D3 and *COI* mtDNA datasets analyses.

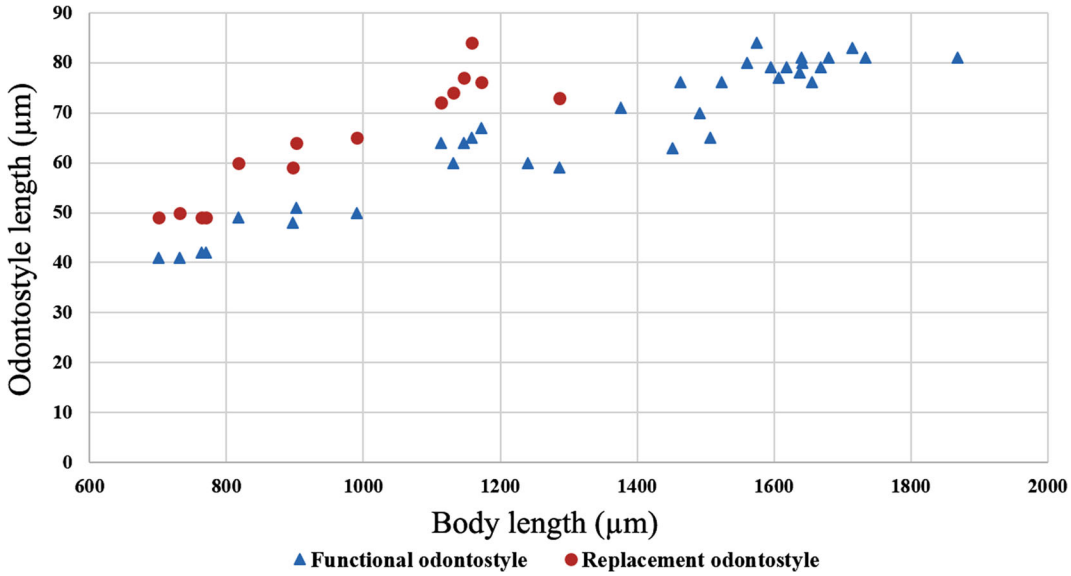

Fig. 2 Correlation of functional and replacement odontostyle to body length in juvenile developmental stages and females of Iranian population of *Xiphinema santos* Lamberti et al. 1993. The replacement odontostyle in each juvenile stage is equal to the functional odontostyle in the next stage, and in J3, is equal to the functional odontostyle length in females

Bayesian analysis was performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) with a starting random tree and running the chains for 5×10^6 generations for the both datasets. The Markov chains were sampled every 100 generations for estimating the posterior probabilities of the phylogenetic trees (Larget and Simon 1999) using the 50% majority rule. Of the converged runs, 25% were regarded as 'burn-in'. Convergence of model parameters and topology were assessed based on the average standard deviation of split frequencies and potential scale reduction factor values. Adequacy of the posterior sample size was evaluated using autocorrelation statistics as implemented in Tracer v.1.6 (Rambaut and Drummond 2009). The output files of the phylogenetic programs used herein were visualized using Dendroscope V.3.2.8 (Huson and Scornavacca 2012) and re-drawn by CorelDRAW software version 17. The Bayesian posterior probability (BPP) values exceeding 0.50 are given on appropriate clades.

Results and description

Xiphinema santos Lamberti, Lemos, Agostinelli & D'Addabo 1993 (Figures 1 & 2, Tables 1 & 2)

Female Body cylindrical, tapering gradually towards the extremities, ventrally curved, varying from an open to closed C, the posterior end more ventrally bent when heat-killed. Cuticle 2.0–3.5 µm thick along the body, 1.5–2.0 µm thick in post labial region, 2–4 µm thick at mid-body, 3–4 µm thick just anterior to anus and 5–12 µm thick at tail tip. Lateral chord 10–16 µm wide at mid-body, occupying 27–38% of corresponding body diam. Lip region anteriorly rounded, having a depression in junction with the body, slightly expanded, 11–12 µm wide and 4–5 µm high. Odontostyle 1.4–2.1 times longer than the odontophore, the latter with well-developed flanges. The guiding sheath 1–9 µm long. The pharyngeal bulb 55–76 µm long and 13–23 µm wide, its glandularium 51–67 µm long, occupying about 1/3 to 1/5 of the total pharynx length, with three pharyngeal nuclei, the larger dorsal gland nucleus (DN) located at 18.9–25.0%, the two smaller ventrosublateral nuclei (S1N) located at 58–63% of pharyngeal bulb (location of glands' nuclei according to Loof and Coomans 1972). Cardia rounded, 4–8 × 6–11 µm in size, intestine simple. Prerectum often indistinct, with great range in length (138–227 µm long) and rectum 13–22 µm long, or 0.6–1.1 times the anal body diameter. Reproductive system didelphic-amphidelphic with two seemingly equally developed branches, each branch ca. 145 µm long, including a ca. 50 µm long tubular uterus and a ca. 100 µm long ovary with visible endosymbiont bacteria. Vulva a transverse slit, vagina 16–19 µm long with short *pars distalis vaginae*, *pars proximalis vaginae* and ovejector ca.

Table 1 Morphometric data for females of *Xiphinema santos* Lamberti et al. 1993. All measurements in μm and in the format: mean ± s.d. (range)

Origin	Iranian population	Lamberti et al. (1993) (Type population)	Lamberti et al. (1994) (Total range of three Portuguese populations from Azores, Madeira and Braga)*	Lamberti et al. (1996) (Egypt population)	Gutiérrez-Gutiérrez et al. (2012) (Spain population)
Characters	Females	Females	Females	Females	Females
n	22	20	15	22	9
L	1578.2 ± 134.0 (1240–1868)	1800 ± 800 (1700–2000)	(1700–2000)	1900 ± 100 (1700–2000)	1504 ± 75 (1466–1633)
a	44.3 ± 3.8 (37.2–51.9)	54.0 ± 2.3 (54–59)	(41–49)	51.0 ± 2.3 (47–54)	44.3 ± 2.1 (42.7–48.1)
b	5.7 ± 0.4 (4.9–6.3)	6.0 ± 0.4 (5.1–6.7)	(5.7–7.5)	6.5 ± 0.4 (5.7–7.1)	6.2 ± 0.9 (4.7–7.6)
c	48.4 ± 3.2 (42.8–54.6)	54.0 ± 3.1 (51–65)	(51–62)	53.0 ± 2.7 (48–57)	53.3 ± 6.2 (45.9–65.3)
c’	1.6 ± 0.1 (1.3–1.8)	1.7 ± 0.8 (1.5–1.8)	(1.3–1.7)	1.6 ± 0.1 (1.5–1.8)	1.3 ± 0.1 (1.0–1.4)
V%	52.3 ± 1.1 (50.1–54.0)	51.0 ± 1.7 (48–53)	(47–53)	52.0 ± 1.3 (49–53)	52.4 ± 1.1 (52–55)
Lip region width	11.3 ± 0.4 (11–12)	9–11	(10–11)	10–11	10.2 ± 0.7 (9.5–11.5)
Lip region height	4.2 ± 0.4 (4–5)	–	–	–	–
Odontostyle length	76.2 ± 6.6 (60–84)	83.0 ± 2.3 (79–88)	(79–88)	82.0 ± 1.8 (77–85)	78.9 ± 2.1 (76–82)
Odontophore length	42.7 ± 2.8 (36–47)	50.0 ± 1.4 (48–53)	(46–52)	47.0 ± 1.4 (44–49)	44.1 ± 2.3 (41–49)
Total stylet length	118.9 ± 8.1 (102–128)	–	–	–	–
Anterior end to guiding ring	65.0 ± 4.6 (56–73)	67.0 ± 1.8 (65–72)	(60–70)	68.04 ± 1.8 (63–71)	64.4 ± 1.8 (60–67)
Pharynx	279.8 ± 21.6 (238–319)	–	–	–	–
G1	9.2 ± 0.8 (7.8–10.5)	–	–	–	8.0 ± 0.9 (6.8–8.5)
G2	9 ± 2 (7.5–14.5)	–	–	–	7.5 ± 1.0 (6.4–8.9)
Lip region-vulva distance	827.3 ± 64.4 (666–981)	–	–	–	–
Body width at mid-body	36 ± 4 (29–44)	35.0 ± 2.2 (31–39)	(35–45)	37.0 ± 1.6 (35–40)	–
- at anus	20.8 ± 1.6 (19–24)	20.0 ± 1.2 (18–23)	(19.0–23.5)	22 ± 1 (20–24)	–
- at base of pharynx	31.1 ± 1.7 (28–33)	32.0 ± 1.3 (29–34)	(32–42)	33 ± 1 (31–35)	–
Tail	32.8 ± 2.5 (29–38)	34.0 ± 1.5 (32–37)	(28–38)	35.0 ± 1.2 (33–38)	28.5 ± 2.9 (25–34)

*All three populations had similar morphometric and ratio ranges

Table 2 Morphometric data of juveniles of Iranian population of *Xiphinema santos* Lamberti et al. 1993. All measurements are in μm and in the format: mean ± s.d. (range)

	J1	J2	J3
n	4	4	6
L	741.3 ± 31.8 (701–770)	901.8 ± 70.3 (818–990)	1168 ± 61.5 (1113–1286)
a	35.3 ± 1.6 (33.2–36.7)	40.2 ± 4.0 (35.6–45.1)	44.1 ± 2.1 (41.2–46.9)
b	4.4 ± 0.5 (3.8–4.8)	4.2 ± 0.3 (3.9–4.6)	4.7 ± 0.6 (4.1–5.5)
c	24.3 ± 1.1 (23.3–24.5)	26.5 ± 0.7 (25.6–27.3)	33.6 ± 1.8 (31.7–35.8)
c’	2.4 ± 0.2 (2.1–2.5)	2.3 ± 0.0 (2.3–2.4)	2.0 ± 0.1 (1.9–2.1)
Lip region width	8.0 ± 0.0 (8–8)	9.3 ± 1.0 (8–10)	10.0 ± 0.0 (10–10)
Odontostyle length	41.5 ± 0.6 (41–42)	49.5 ± 1.3 (48–51)	63.2 ± 3.1 (59–67)
Odontophore length	29.5 ± 1.0 (28.5–30.5)	33.5 ± 2.5 (31–37)	37.8 ± 3.3 (34–42)
Replacement odontostyle length	49.3 ± 0.5 (49–50)	62.0 ± 2.9 (59–65)	76.0 ± 4.3 (72–84)
Anterior end to guiding ring	33.5 ± 1.0 (32–34)	41.8 ± 2.2 (39–44)	50.8 ± 2.0 (49–53)
Pharynx length	171.5 ± 13.3 (160–183)	213.8 ± 25.3 (189–238)	249.7 ± 28.1 (210–280)
Body width at mid-body	21.0 ± 0.8 (20–22)	22.5 ± 1.7 (20–24)	26.5 ± 1.5 (25–29)
- at anus	13 ± 1 (12–14)	14.5 ± 1.3 (13–16)	17.0 ± 0.6 (16–18)
Tail	30.7 ± 2.1 (29–33)	34.0 ± 2.6 (31–37)	34.8 ± 1.8 (32–37)

35 μm wide and 6 μm high. Tail short, conoid, weakly curved ventrally with a rounded terminus, bearing two pairs of caudal pores.

Male Not found.

Juveniles Morphologically similar to females except for smaller body and narrower tail. Three juvenile developmental stages were detected. The scatter diagram separating juveniles and females is given in Fig. 2. All three juvenile developmental stages were separated from each other using body length and length of replacement and functional odontostyle. In the first juvenile developmental stage, the tip of the replacement odontostyle is close to the base of the functional odontostyle, and in two rest stages, it is distantly located. All three juvenile stages have conical dorsally convex and ventrally concave tail.

Habitat and locality

The Iranian population of *Xiphinema santos* was recovered in association with stone fruit trees in Aran va Bidgol, Isfahan province (GPS coordinates are N 34°02′30.173″, E 51°28′49.193″, altitude: 907 m.a.s.l.).

Xiphinema primum Mobasseri, Hutchinson, Jahanshahi Afshar & Pedram 2019

A population of this species was recovered in the present study from Golestan province (GPS coordinates are N 36°46′9″, E 54°34′34.079″) in association with forest trees and sequenced for its LSU D2-D3 and *COI* mtDNA regions. The recovered population was in accordance with the type population and the newly generated sequences for this population are presented in corresponding trees. This finding shows the species could be more prevalent in the country than already assumed.

Molecular characterization and phylogenetic relationships

To determinate the phylogenetic affinities of the Iranian population of *Xiphinema santos* and the newly recovered population of *X. primum* with other species in the Longidoridae Thorne 1935 (and mostly with those species belonging to the *X. americanum*-group), two separate LSU rDNA D2-D3 and *COI* mtDNA datasets were prepared for Bayesian inference (BI). For LSU phylogeny (Bayesian tree), a total number of 81 sequences, including the newly generated sequences and sequences of *Xiphinema* non-

Fig. 3 Bayesian 0.50 majority rule consensus tree inferred using LSU rDNA D2-D3 sequences of Iranian population of *Xiphinema santos* Lamberti et al. 1993. The Bayesian posterior probability values are given for the clades. The newly generated sequences are in bold

americanum group (*X. index* Thorne and Allen 1950 and *X. diversicaudatum* (Micoletzky 1927) Thorne 1939), a Tylencholaimidae Filipjev 1934 member (*Tylencholaimus teres* Thorne 1939) and a Qudsianematidae Jairajpuri 1965 member (*Ecumenicus monohystera* (de Man 1880) Thorne 1974), as outgroups (accession numbers in Fig. 3), were selected. The LSU dataset was composed of 722 characters of which 308 were variable. The average nucleotide composition was: A: 23.5%, T: 18.7%, C: 25.8%, G: 32.0%. Figure 3 represents the Bayesian phylogenetic tree inferred using the above-mentioned dataset. In this tree, the two sequences of the Iranian isolate of *X. santos* (MN602712, MN602713) and some sequences from the previously sequenced isolates of the species as well as some isolates of three

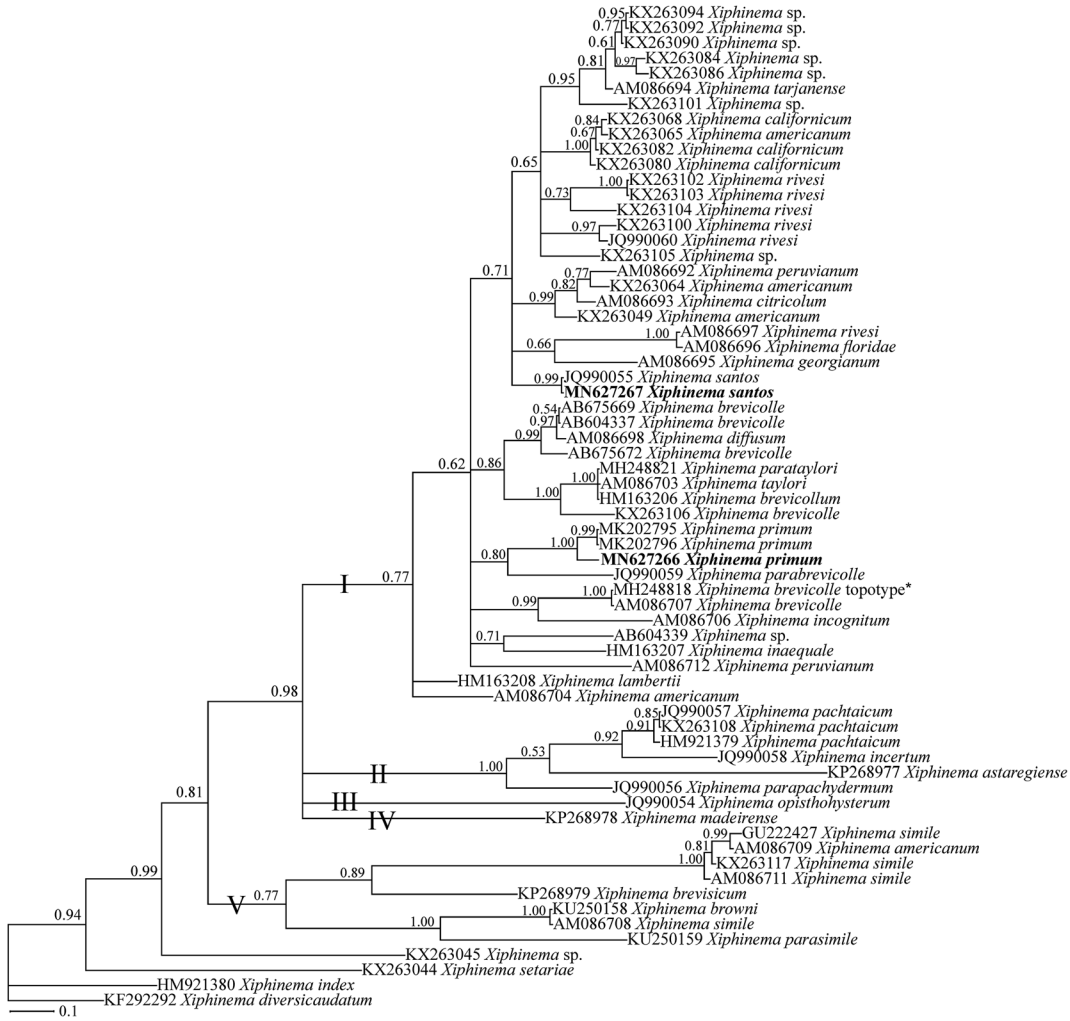

Fig. 4 Bayesian 0.50 majority rule consensus tree inferred using *COI* mtDNA sequences of Iranian population of *Xiphinema santos* Lamberti et al. 1993. The Bayesian posterior probability values are given for the clades. The newly generated sequences are in bold

species *X. georgianum* Lamberti and Bleve-Zacheo 1979, *X. laevistriatum* Lamberti and Bleve-Zacheo 1979 and *X. citricolum* Lamberti and Bleve-Zacheo 1979 formed a highly supported clade (0.94 BPP). The phylogenetic relationships of these species were not resolved due to polytomy. The newly generated LSU sequence of *X. primum* also fell into the clade including original sequences of the species.

A total number of 66 sequences, including the newly generated sequences, plus three sequences of *Xiphinema* non-*americanum* group spp. (*X. index, X. diversicaudatum* and *X. setariae* Luc 1958) as outgroups, were selected for *COI* mtDNA Bayesian phylogeny (accession numbers in Fig. 4). The *COI* dataset was composed of 391 characters of which 222 were variable. The average nucleotide composition was as follows: A: 25.4%, T: 37.1%, C: 15.1%, G: 22.4%. Figure 4 represents the Bayesian phylogenetic tree inferred using this dataset. Based upon this tree, the newly generated sequence of the Iranian population of *X. santos* (MN627267) formed a highly supported clade (0.99 BPP) with the Spanish isolate (JQ990055). All currently available *COI* sequences of *X. primum* formed a clade too.

Discussion

A relatively high density population (around 50 individuals/200 g soil) of *X. santos* was recovered

from the rhizosphere of stone fruit trees in a garden in Aran va Bidgol. Compared to the type population, the Iranian population has a close morphology, but some differences in morphometric data ranges i.e. shorter body (1.6 (1.2–1.9) vs. 1.8 (1.7–2.0) mm) and smaller a (44.3 (37.2–51.9) vs. 54 (54–59)) were observed. For most other indexes, the ranges were overlapped; however, present study extended the morphometric data ranges for the species (Table 1). Compared to other populations reported from Portugal (Lamberti et al. 1994) and Egypt (Lamberti et al. 1996), again extension in indexes was observed (Table 1). Compared to the Spanish population, more similarity in some indexes like body and odontophore length and c ratio was observed. In summary, *X. santos* has a wide intraspecies ranges for body length of females (1.2–2.0 mm). Such a wide range for body length was also observed for the third juvenile developmental stage of the Egyptian population of the species (Lamberti et al. 1997). The wide range for body length has already been reported for some species like *Xiphinema* sp.1 (isolate CD47) by Orlando et al. (2016) (L = 1193–2268 or 1.2–2.3 mm), *X. plesiopachtaicum* Archidona-Yuste et al. 2016 (L = 1.5–2.1 mm) and *X. parabrevicolle* Gutiérrez-Gutiérrez et al. 2012 (L = 1.4–2.0 mm). The present study showed that *X. santos* also belongs to the group of species having a wide body length range. The species has three juvenile developmental stages (Lamberti et al. 1997) and the reported four juvenile developmental stages by Gutiérrez-Gutiérrez et al. (2012) is amended herein.

In molecular phylogenetic analyses, similar to our previous study (Mobasseri et al. 2019), the relationships of *X. santos* with three species *X. georgianum*, *X. laevistriatum* and *X. citricolum* were not resolved in LSU tree due to polytomy. Although *X. citricolum* is shown to be the most closely related species to *X. santos* in most LSU analyses (He et al. 2005; Gutiérrez-Gutiérrez et al. 2012; Lazorava et al. 2016; Orlando et al. 2016; Mobasseri et al. 2019), the effect of the alignment/postediting methods on the resulted topology should not be ruled out. The topology of the present LSU tree and dividing of the species into two subclades I and II are similar to the resolved topology by Mobasseri et al. (2019).

The *COI* mtDNA of *Xiphinema americanum*-group species has an acceptable interspecies variation (Lazarova et al. 2016; Gutiérrez-Gutiérrez et al. 2012; Orlando et al. 2016; Archidona-Yuste et al. 2016) and could yield better cladogenesis in the corresponding tree. In our *COI* phylogeny, *X. santos* has appeared as an independent lineage in an unsupported clade including several other species. The general topology and the subclades resolved in the present *COI* tree are similar to the *COI* tree presented by Mobasseri et al. (2019).

This is the first record of *X. santos* from Iran, corroborating its occurrence out of the Mediterranean Basin, after its several reports from Portugal, North Egypt and Spain (Lamberti et al. 1993, 1994, 1996; Gutiérrez-Gutiérrez et al. 2012).

Conclusion

In this study, *Xiphinema santos,* a member of *X. americanum*-group was reported for the first time from Iran, extending its morphometric data ranges like body length and its geographical distribution areas.

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Compliance with ethical standards

Conflict of interest All the authors certify that they do not have any conflict of interest, the presented data are original and the presented data are not published, and are not under evaluation for publication elsewhere, all prevailing local, national and international regulations and conventions, and normal scientific ethical practices, have been respected. They also certify that the final version of this manuscript has been reviewed by all authors.

Research involving human participants and/or animals The soil samples analyzed in this study were collected under the direct monitoring of the garden owner or from the natural public regions that are not under protection against soil samplings.

Informed consent The authors certify that they have accepted the principles of ethical standards and that guidelines of professional conduct have been followed. The funders had no role in designing of this study, collecting and analyzing of the data, preparing the manuscript and are not involved in evaluating or publishing process of this research.

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