

Xiphinema japonicum n. sp. (Nematoda: Longidorinae) from the Rhizosphere of Japanese *Podocarpus macrophyllus* (Thunb.), a Cryptic Species Related to *Xiphinema bakeri* Williams, 1961

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Abstract: *Xiphinema japonicum* n. sp., isolated in Ningbo, China, from the rhizosphere of *Podocarpus macrophyllus* (Thunb.) imported from Japan is described. The new species belongs to *Xiphinema non-americanum* group 7 and is characterized by medium body length (3.0–3.7 mm), total stylet length 190–201 µm, vulva located anteriorly (V = 30.5%–35.3%), two equally developed female genital branches without uterine differentiation (no Z or pseudo-Z organ and/or spines in the uteri), short tail, convex-conoid with subdigitate peg in terminus, and absence of males. The species has four juvenile developmental stages (J1 was not found). The polytomous identification codes of the new species are (codes in parentheses are exceptions) A4-B4-C4-D5(4)-E2(3)-F3(4)-G2(3)-H2-I3-J4-K2-L1. Morphologically, the new species is mainly characterized by combination of the codes C4 and E2(3), making the species unique and different from other species in the genus. It is most similar to the North American species *Xiphinema bakeri*, herein considered as its cryptic species by the nature of high morphological similarity, but with significant differences in DNA sequences in nearly full length 18S, ITS1, 28S D2/D3, and cytochrome c oxidase subunit 1 sequences. The second morphologically close species is *Xiphinema setariae* from which the new species could be separated based on morphological and molecular characters. Morphological comparisons with phylogenetically related species are also discussed.

Key words: molecular, morphology, morphometrics, new species, phylogeny, taxonomy, *Xiphinema bakeri*, *X. diversicaudatum*, *X. setariae*, *Xiphinema non-americanum* group.

Members of the genus *Xiphinema* Cobb, 1913 are worldwide distributed and economically important nematodes that cause damage to an extensive range of plants by feeding on plant root cells and in some cases, by transmitting viruses to fruit and vegetable crops (Taylor and Brown, 1997). This transmission is governed by a marked specificity between plant viruses and their *Xiphinema* spp. vectors. In fact, only nine of the approximately 260 known species of *Xiphinema* have been shown to transmit nepoviruses (Decraemer and Robbins, 2007).

Species identification in genus *Xiphinema* is very difficult because of conservative and overlapping morphological and morphometric characters. These identification procedures are further complicated because of the morphological intraspecific variation and the presence of cryptic species that are morphologically indistinguishable but may be phylogenetically distant to one another (Brown and Topham, 1985; Gutiérrez-Gutiérrez et al., 2010, 2012; Archidona-Yuste et al., 2016b). Because of the diversity of diagnostic/morphological

characters, *Xiphinema* species are divided into two groups *viz* *X. americanum* group which contains more than 55 species and a second bigger group namely *X. non-americanum* group (Ghaemi et al., 2012; Gutiérrez-Gutiérrez et al., 2013). As the *Xiphinema* species have phytosanitary significance, reliable identification allows distinction between virus vector and nonvirus vector nematodes and it can assist in the exclusion of species under quarantine or regulatory strategies (Tzortzakakis et al., 2014).

In China, arhat pine (*Podocarpus macrophyllus* (Thunb.)) is regarded as an important cultural and ornamental tree. Since 2012, about 20,000 arhat pine trees with a worth of 70 million USD have been imported from Japan. During two routine quarantine inspections in February and June 2015, an unknown *Xiphinema* species was detected from the soil samples of imported arhat pines from Japan. Morphological and molecular characterization revealed that this species is new to science and herein described as *X. japonicum* n. sp.

MATERIALS AND METHODS

Nematode samplings, extraction, and morphological study: Arhat pine trees are permitted to have small amount of rhizospheric soil for tree survival during transportation and handling procedures when imported. Rhizospheric soil samples were obtained by making 2–3 holes, 20–30 cm depth, and about one kilogram soil with some roots were collected. Nematodes were extracted by a modified Baermann funnel technique for 24–48 hr. These dagger nematodes were detected from two shipments whose registered numbers are 0535 and 0662, respectively. Measurements were made on specimens fixed in TAF and processed to glycerin following the method of Seinhorst (1959). Only

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population number 0662 was enough to perform morphometrics. The nematodes were measured using AxioVs40 (v4.6.3.0) of Zeiss company. All the abbreviations used are as defined in Decraemer and Hunt (2006).

Light micrographs were made using a Zeiss Imager Z1 microscope equipped with a Zeiss AxioCam MRm CCD camera.

Molecular analyses: For DNA extraction, a single nematode was transferred to worm lysis buffer (WLB: 20 mM Tris-HCl pH 8.0, 100 mM KCl, 3.0 mM Mg₂Cl, 2.0 mM DTT, and 0.9% Tween) and crushed with a sterilized pipette tip. The crushed nematode was pipetted into 8 µl ddH₂O with 2 µl proteinase K (60 µg/ml) in an Eppendorf tube, which was then briefly spun and stored at -70°C for at least 10 min. Subsequently, the Eppendorf tube was incubated at 65°C for 1–2 hr and the proteinase K was denatured at 95°C for 10 min. Finally, the DNA suspension was cooled to 4°C and used for conducting PCR (Li et al., 2008).

Three single females were prepared as three DNA samples each from 0535 and 0662, respectively. Four sets of primers (synthesized by Invitrogen, Shanghai, China) were used in the PCR analyses to amplify the partial 18S, ITS1, the 28S rDNA D2/D3 regions, and a portion of the mitochondrial cytochrome oxidase c subunit I gene (*COXI*). Primers for amplification of 18S rDNA were forward primer K4f and reverse primer K1r (Penas et al., 2006). Primers for amplification of ITS1 rDNA were forward primer V1 (Ferris et al., 1993) and reverse primer 5.8S (Cherry et al., 1997). Primers for amplification of 28S D2/D3 were forward primer D2A and reverse primer D3B (De Ley et al., 1999). Primers for amplification of *COXI* were JB3 and JB5 (Hu et al., 2002). The 25-µl PCR was performed using Apex Taq Red Master Mix DNA polymerase (Genesee Scientific Corporation, San Diego, CA) according to the manufacturer's protocol in an Applied Biosystems Veriti® thermocycler. The thermal cycler program for 28S and *COXI* was as follows: denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C (28S) or 51°C (*COXI*) for 45 sec, and extension at 72°C for 2 min. A final extension was performed at 72°C for 10 min. The thermal cycler program for 18S and ITS1 was as follows: denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 60 sec, annealing at 55°C for 60 sec, and extension at 72°C for 2 min. A final extension was performed at 72°C for 5 min as described by Ye et al. (2007) and Li et al. (2008). PCR products were separated and visualized on 1% agarose gels and stained with ethidium bromide. PCR products of sufficiently high quality were cleaned using ExoSap-IT (Affymetrix, Inc., Santa Clara, CA) according to the manufacturer's protocol and were sequenced by Invitrogen (Shanghai, China).

Phylogenetic analysis: DNA sequences were edited with ChromasPro1.5 (© 2003–2009 Technelysium Pty Ltd) and aligned using ClustalW (<http://workbench.sdsc.edu>,

Bioinformatics and Computational Biology group, Dept. Bioengineering, UC San Diego, CA). The model of base substitution in the 18S, ITS1, 28S, and *COXI* was selected using MODELTEST version 3.06 (Posada and Criandall, 1998). The Akaike-supported model, the proportion of invariable sites, and the gamma distribution shape parameters were used in Bayesian inference using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) running the chains for 1,000,000 generations and setting the “burnin” at 2,500. We used MCMC (Markov chain Monte Carlo) method within a Bayesian framework to estimate the posterior probabilities (PP) of the phylogenetic trees (Larget and Simon, 1999) using the 50% majority rule. The Bayesian PP higher than 50% are given on appropriate clades. Some outgroup species and other species from GenBank were also used for phylogenetic analysis as shown in phylogenetic trees.

RESULTS

SYSTEMATICS

*Xiphinema japonicum**n. sp. (Figs. 1–3; Table 1)

Description

Female: Body cylindrical, narrowing gradually toward anterior and tail region, assuming an open C- to J-shaped when killed by heat. Cuticle comprises two optically different layers with fine transverse striations more visible in the tail region, 3.5–4.0 µm thick at midbody and thicker (7.0–8.0 µm) at the dorsal side of the tail. The lip region anteriorly rounded, separated from the rest of the body by a weak depression and 2.4–3.2 times as high as width. Amphids with funnel-shaped fovea and wide slit-like aperture, slightly narrower than lip width. Five to six body pores present between anterior end and guiding ring on the dorsal and ventral side, respectively. Odontostyle long, 9.8–12.1 times lip region width, or 1.7–2.1 times odontophore length long. Odontophore with well-developed basal flanges (10–12 µm wide). Guiding ring double and guiding sheath 14–21 µm long depending on the degree of protraction/retraction of odontostyle. Pharynx consisting of an anterior slender narrow part, 431–470 µm long, extending to a terminal pharyngeal bulb with three nuclei, the dorsal gland nucleus (DN) located at the beginning of bulb, i.e., 14%–22% of the pharyngeal bulb length, while the other two subventro-lateral nuclei (S1N) are located at 41.2%–53%. Pharyngeal bulb 100–110 µm long and 22–28 µm wide, occupying about 1/4 to 1/5 of the total pharynx length. Pharyngo-intestinal valve conoid-oblonged and 7.2–9.3 µm long. Intestine simple, prerectum 16.5–20.4 times, and rectum 0.9–1.1 times anal body diameter long. Reproductive system didelphic-amphidelphic with both branches about equally developed. Each branch composed of a long (85–163 µm) reflexed ovary, long (73–95 µm) oviduct with enlarged *pars dilatata oviductus* separated from the

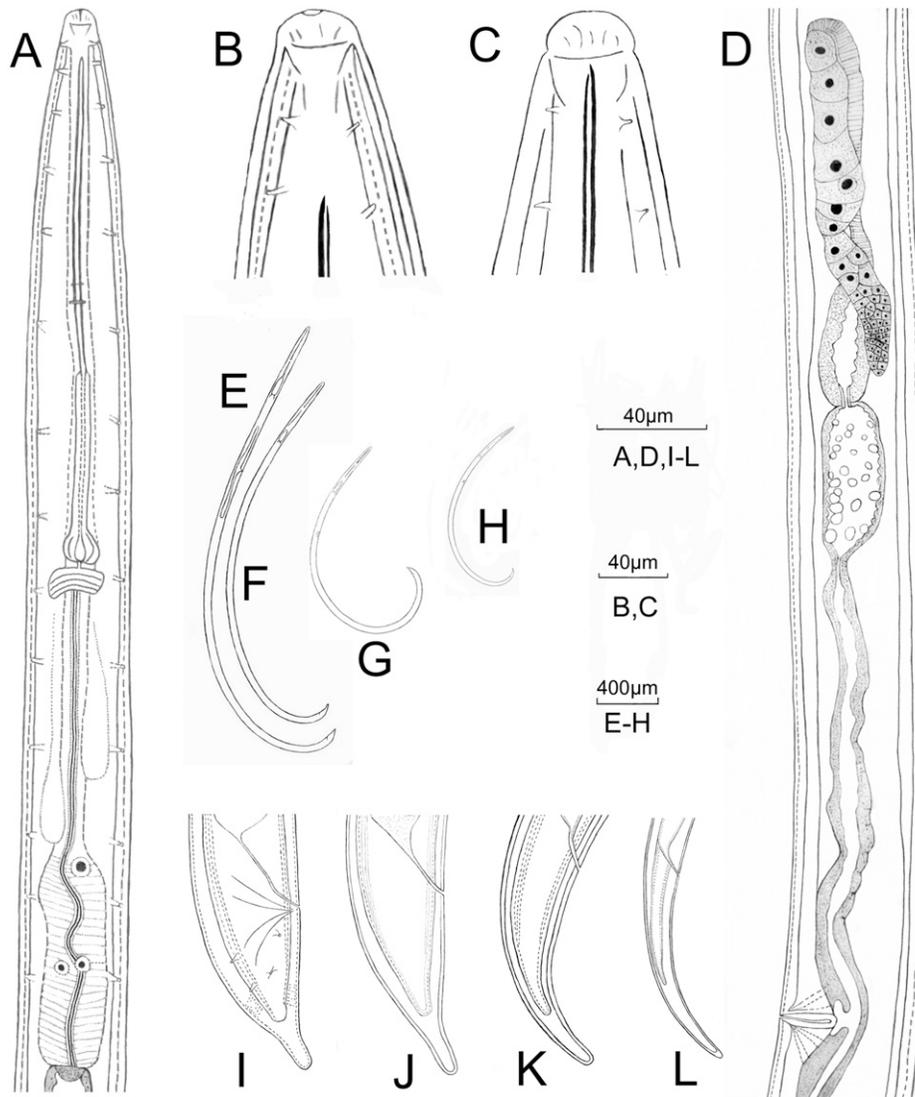


FIG. 1. *Xiphinema japonicum* n. sp. A. Female pharyngeal region; B, C. Lip region; D. Anterior genital branch; E–H: Entire body of female, 4th, 3rd, and 2nd stage juveniles; and I–L: Tail of female, 4th, 3rd, and 2nd stage juveniles (Scale bars = A–D, I–L 40 μ m; E–H 400 μ m).

uterus by a well-developed sphincter, long (172–304 μ m) bipartite uterus composed of a well-developed *pars dilatata uteri* and tubular part, well-developed ovejector, 24–31 μ m wide, vagina (15–24 μ m long) perpendicular to the body axis, extending inward for 35%–43% of corresponding body diameter, and the vulva slit-like and situated well anterior to the midbody ($V = 30.5$ – 35.3). Tail short, conoid, convex in the dorsal side, and flat in the ventral side with a subdigitate peg-like terminus.

Male: Not found.

Juveniles: Three juvenile developmental stages (J2, J3, and J4) were found. Juveniles are similar to adults in gross morphology, except for their smaller size, longer tail, and undeveloped reproductive organs. Tail becomes progressively shorter and stouter in each moult, being distinguishable by relative lengths of body and functional and replacement odontostyle.

Type locality and habitat: The new species was detected in the rhizosphere of arhat pine tree (*Podocarpus macrophyllus*)

imported from Japan on February and June, 2015, at Ningbo Port, P. R. China.

Type material: Holotype female and nine female paratypes (slide numbers 0535-1 to 0535-9 and 0662-1 to 0662-6) deposited in the nematode collection of Ningbo Entry–Exit Inspection and Quarantine Bureau, China. Three paratype females (slide numbers T532) deposited in the Canadian National Collection of Nematodes, Ottawa, Canada.

Differential diagnosis: *Xiphinema japonicum* n. sp. is a gonochoristic species characterized mainly by having two equally developed genital tracts without any type of differentiations and more anteriorly located vulva at 30.5%–35.3% of the total body length. It is further characterized by having a medium body length (3.0–3.7 mm), stylet length 190.0–201.4 μ m, composed of a 119–126 μ m long odontostyle and 64.4–77.0 μ m long odontophore, short conoid female tail, dorsally convex and ventrally flat with a subdigitate peg, four juvenile

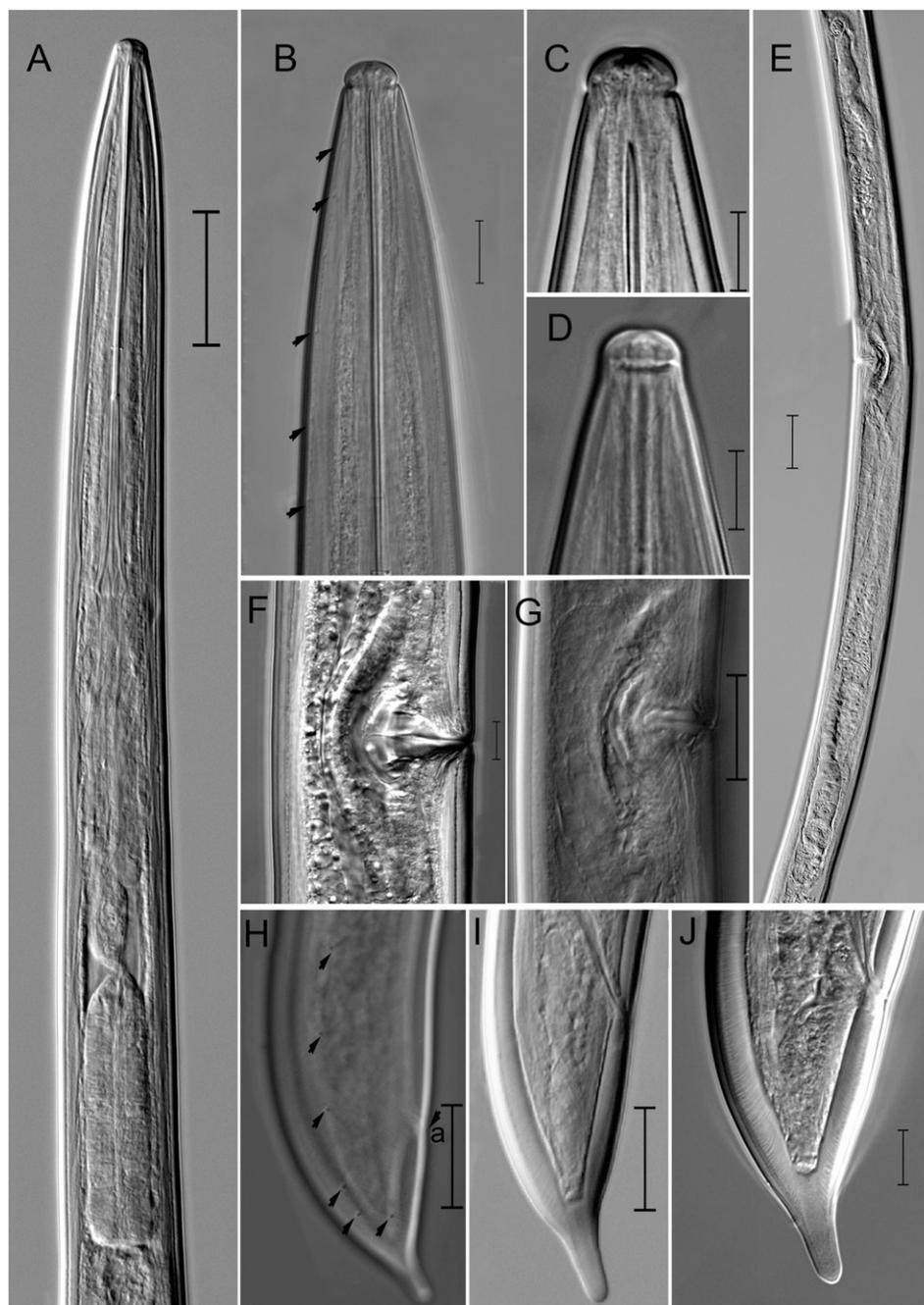


FIG. 2. *Xiphinema japonicum* n. sp. A. Female pharyngeal region; B–D. Lip region (arrows showing anterior body pores); E. Genital branches; F, G. Vulval region; and H–J. Tail of female (Scale bars = 10 μ m).

developmental stages, and males were absent. Based on the polytomous key presented by Loof & Luc (1990) and Loof et al. (1996), the new species belongs to *Xiphinema non-americanum* group 7 and has the following specific alphanumeric codes (codes in parentheses are exceptions): A4-B4-C4-D5(4)-E2(3)-F3(4)-G2(3)-H2-I3-J4-K?-L1. Males were not observed for *X. japonicum* n. sp., thus the comparisons were provided only for female characters and morphometrics.

Etymology: The species epithet is derived from the location where the species was found.

Morphologically, *X. japonicum* n. sp. largely resembles *X. bakeri*. Compared with the original population of *X. bakeri* described from British Columbia (Williams, 1961), the new species has slightly shorter body (2,965–3,757 vs 3,700–4,740 μ m) and shorter odontostyle (119–126 vs 142 μ m), but the new species is very similar to populations of *X. bakeri* reported from Arkansas, California, Florida, Iowa, Illinois, Indiana, North Carolina, Kentucky, Oregon, Tennessee, and Washington (Ye and Robbins, 2010). However, compared to *X. bakeri*, the new species has differences in the morphology of tail

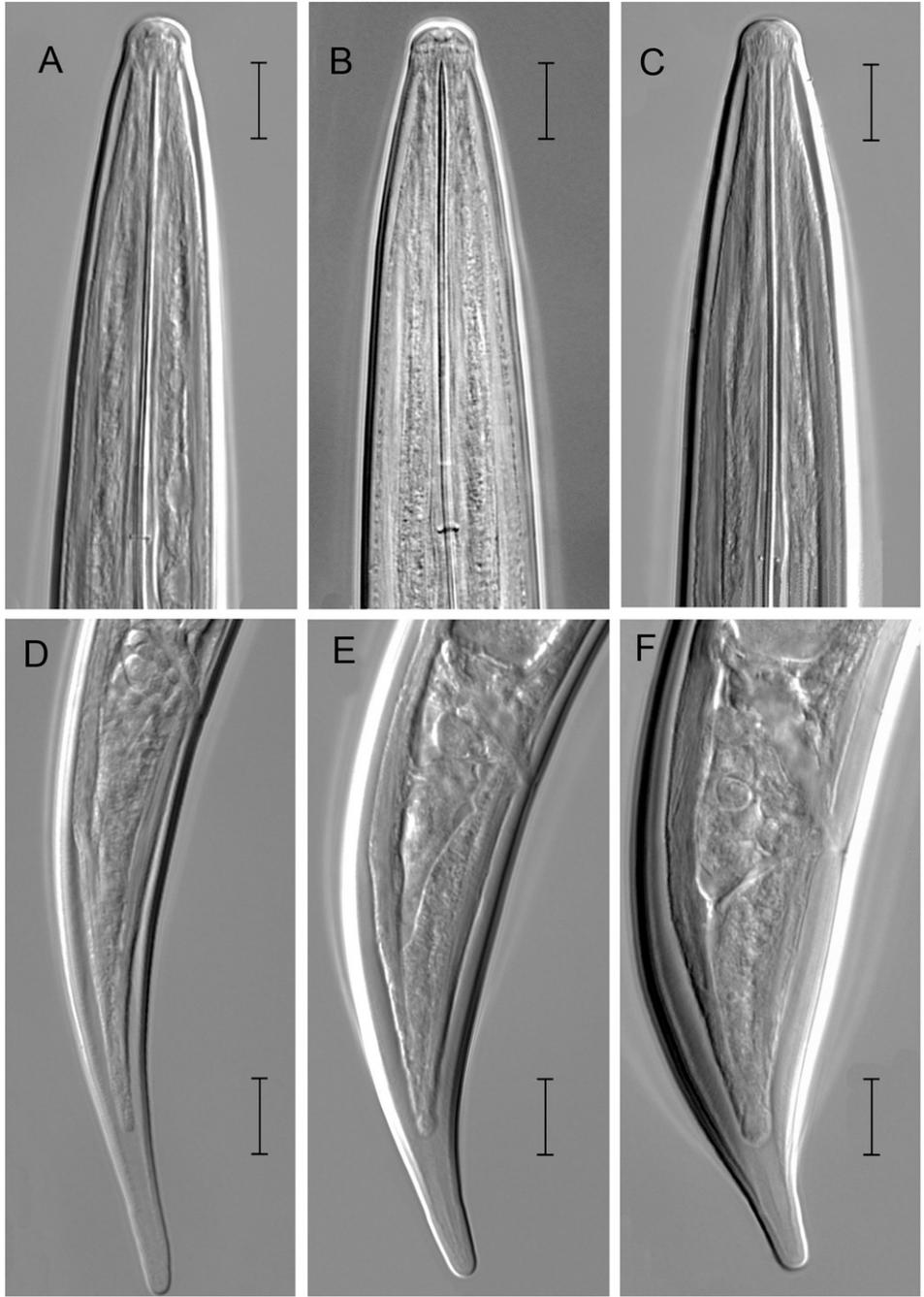


FIG. 3. *Xiphinema japonicum* n. sp. A–C. Lip region of 2nd, 3rd, and 4th stages juveniles and D–F. Tail region of 2nd, 3rd, and 4th stages juveniles (Scale bars = 10 μm).

in the third and second juvenile developmental stages, i.e., the new species has a ventrally curved uniformly narrowing conical tail in J3 (*vs* conical, slightly ventrally bent, as illustrated by Coomans et al., 2001) and narrow, uniformly narrowing conical tail in J2 (*vs* dorsally convex and ventrally slightly concave as illustrated by Coomans et al., 2001).

The new species is also very similar to *X. setariae* Luc, 1958 from group 7 based on the new morphometric data provided by Peraza-Pallida et al. (2017) and differs from it by having a slightly longer body (2,965–3,757 *vs*

2,305–3,089 μm), posteriorly located guiding ring (100.5–127 *vs* 73–104 μm), anteriorly located vulva ($V = 30.5\text{--}35.3$ *vs* 38–42%), slimmer body ($a = 56.2\text{--}75.8$ *vs* 52.3–84.5), greater *c* value (55–69 *vs* 44.7–64.4), and on the morphology of the terminal peg in the tail end (sharp separation from the rest of the body in new species *vs* uniformly narrowing to the terminal peg).

The new species forms a clade with *X. diversicaudatum* (Micoletzky, 1927) Thorne, 1939 from group 5 in our phylogenetic trees. It is different from *X. diversicaudatum* by an anteriorly located vulva ($V = 30.5\text{--}35.3$ *vs*

TABLE 1. Morphometrics data for *Xiphinema japonicum* n. sp. (measurements μm ; mean \pm s.d. (range) for paratype).

Character/ratios	Female			J2			J3			J4		
	Holotype	Paratypes		Paratypes		Paratypes		Paratypes		Paratypes		
n	1	9		2		3		7		7		
L	3,289	3,272 \pm 198.4 (2,965–3,757)		1,217 \pm 8.9 (1,211–1,224)		1,846 \pm 46.4 (1,779–1,889)		2,509.6 \pm 224.6 (2,262–2,813)		54.2 \pm 5.4 (45.9–62.5)		
a	72.2	67.1 \pm 6.2 (56.2–75.8)		39.2 \pm 4.3 (36.1–42.2)		47.2 \pm 5.9 (42.6–53.8)		54.2 \pm 5.4 (45.9–62.5)		5.7 \pm 0.6 (4.8–6.6)		
b	7.0	7.2 \pm 0.4 (6.6–7.7)		4.4 \pm 0.07 (4.3–4.4)		5.0 \pm 0.4 (4.6–5.4)		5.7 \pm 0.6 (4.8–6.6)		41.8 \pm 3.8 (38.5–48.4)		
c	62.1	62 \pm 3.6 (55–69)		18.0 \pm 0.5 (17.6–18.3)		26.0 \pm 1.6 (24.6–27.8)		41.8 \pm 3.8 (38.5–48.4)		1.9 \pm 0.2 (1.7–2.2)		
c'	1.5	1.5 \pm 0.1 (1.3–1.7)		3.3 \pm 0.4 (3.0–3.5)		2.8 \pm 0.3 (2.5–3.1)		1.9 \pm 0.2 (1.7–2.2)		-		
V	31.1	32.2 \pm 1.4 (30.5–35.3)		-		-		-		-		
Lip diam.	12.1	12.0 \pm 0.5 (10.6–12.4)		8.3 \pm 0.3 (8.1–8.5)		9.7 \pm 0.8 (9.0–10.6)		10.7 \pm 0.2 (10.4–10.9)		4.7 \pm 0.3 (4.3–5.0)		
Lip height	4.5	4.7 \pm 0.6 (4.0–5.6)		3.3 \pm 0.07 (3.2–3.3)		4.3 \pm 0.1 (4.3–4.4)		4.7 \pm 0.3 (4.3–5.0)		104.3 \pm 3.5 (101.2–109.2)		
Odontostyle	126.1	122.6 \pm 2.3 (119–126)		61.1 \pm 1.5 (60.0–62.1)		84.0 \pm 1.9 (82.6–86.0)		62.9 \pm 3.5 (56.4–66.2)		167.3 \pm 3.0 (164.5–172.6)		
Odontophore	74.5	72.6 \pm 3.5 (64.4–77)		44.6 \pm 1.0 (43.9–45.3)		51.5 \pm 0.8 (50.8–52.3)		123.5 \pm 4.5 (117.4–128.3)		89.3 \pm 7.7 (78.9–99.6)		
Total stylet	201.4	195.2 \pm 3.6 (190–201.4)		106 \pm 0.1 (105.3–106.7)		135.3 \pm 2.6 (133.1–138.3)		167.3 \pm 3.0 (164.5–172.6)		442.8 \pm 28.6 (414.0–489.8)		
Replacement odontostyle	121.0	115.4 \pm 6.2 (100.5–121.7)		86.1 \pm 3.5 (83.6–88.6)		105.3 \pm 3.2 (101.8–108.1)		123.5 \pm 4.5 (117.4–128.3)		91.9 \pm 12.4 (74.4–104.5)		
Distance from anterior end to guide ring	468.8	452.3 \pm 13.5 (431–469.6)		57.6 \pm 1.0 (56.9–58.3)		74.7 \pm 7.4 (67.0–81.7)		89.3 \pm 7.7 (78.9–99.6)		24.0 \pm 2.4 (20.4–26.8)		
Pharynx	109.1	104.5 \pm 3.4 (100.1–110.2)		278.7 \pm 1.6 (277.5–279.8)		370.3 \pm 20.8 (348.6–390)		442.8 \pm 28.6 (414.0–489.8)		46.7 \pm 5.1 (37.7–51.4)		
Pharyngeal bulb length	25.1	24.1 \pm 1.7 (22.1–27.7)		62.9 \pm 7.1 (57.8–67.9)		83.4 \pm 7.1 (75.4–88.6)		91.9 \pm 12.4 (74.4–104.5)		35.5 \pm 3.0 (32.2–39.5)		
Pharyngeal bulb diam.	45.5	49.2 \pm 6.2 (41–58.2)		18.2 \pm 0.5 (17.8–18.5)		23.0 \pm 0.7 (22.2–23.5)		24.0 \pm 2.4 (20.4–26.8)		43.8 \pm 4.1 (37–47.8)		
Body diam.	37.5	38 \pm 1.2 (36.4–40.3)		31.3 \pm 3.2 (29–33.6)		39.8 \pm 4.2 (35.1–43.3)		46.7 \pm 5.1 (37.7–51.4)		-		
Body diam. at guide ring	46.1	46.3 \pm 3.7 (40–51.4)		20.4 \pm 1.1 (19.6–21.2)		29.6 \pm 3.3 (26.7–33.1)		35.5 \pm 3.0 (32.2–39.5)		-		
Body diam. at base of pharynx	48.6	49.3 \pm 5.1 (42.2–55.6)		30.3 \pm 1.8 (29–31.5)		37.2 \pm 4.2 (32.4–40.3)		43.8 \pm 4.1 (37–47.8)		-		
Anterior genital branch length	328.0	328.5 \pm 24.7 (286–363.0)		-		-		-		-		
Posterior genital branch length	335.0	380.6 \pm 62.8 (299–497)		-		-		-		-		
Distance from anterior end to vulva	1,024	1,054.2 \pm 70.1 (938–1,157)		-		-		-		-		
Anal diam.	34.6	34.6 \pm 2.7 (31.6–41.6)		20.9 \pm 2.7 (19–22.8)		26.7 \pm 3.4 (24.5–30.7)		31.2 \pm 2.3 (28.2–33.8)		-		
Tail	52.9	53.0 \pm 2.0 (47.0–56.9)		67.9 \pm 1.3 (67–68.8)		71.2 \pm 6.1 (64.7–76.7)		58.8 \pm 4.9 (55.7–65.0)		-		
Hyaline tail part	16.1	19.3 \pm 2.2 (15.5–23)		16.7 \pm 0.4 (16.4–16.9)		22.1 \pm 2.1 (20.1–24.3)		21.2 \pm 2.2 (18.9–24.7)		-		

39–46%), uterus characters (lacking any type of differentiation *vs* presence of irregular globular-shaped pseudo-Z organ), and tail shape (short conoid *vs* hemispherical).

Furthermore, the new species also showed close phylogenetic relationships (Figs. 4–7) with *X. coxi europaeum* Sturhan, 1985; *X. globosum* Sturhan, 1978; *X. belmontense* Roca and Pereira, 1992; *X. turdetanense* Gutiérrez-Gutiérrez et al., 2013; *X. abrantinum* Roca and Pereira, 1991; and *X. baetica* Gutiérrez-Gutiérrez et al. 2013, which are all in morphospecies group 5. However, it differs from these species by uterus differentiation (absent *vs* presence of variable-shaped

pseudo-Z organ [i.e. irregular globules, granular structures, and irregular weakly sclerotized bodies] and some with pseudo-Z organ plus uterine spines).

In the ITS tree, the new specie is in close affinity with an unidentified *Xiphinema* population deposited in GenBank with the accession number DQ364686. The new species differs from this population reported in a dissertation by Wu (2007), from Shenyang City, Liaoning Province, China, mainly by its anteriorly located vulva ($V = 30.5\text{--}35.3$ *vs* $35.3\text{--}38.4\%$), tail shape (having a subdigitate peg *vs* having a slightly digitate terminus not forming a peg), and by uterus characters (lacking of differentiation *vs* having pseudo-Z organ).



FIG. 4. Bayesian consensus tree of *Xiphinema japonicum* n. sp. inferred from 18S under GTR+I+G model ($-\ln L = 3,332.0762$; AIC = 6,682.1523; $\text{freqA} = 0.1821$; $\text{freqC} = 0.209$; $\text{freqG} = 0.3285$; $\text{freqT} = 0.2804$; $R(a) = 1.4406$; $R(b) = 5.2581$; $R(c) = 2.6656$; $R(d) = 0.7382$; $R(e) = 5.2581$; $R(f) = 1$; $\text{Pinva} = 0.2316$; and $\text{Shape} = 0.4544$). Posterior probability values exceeding 50% are given on appropriate clades.

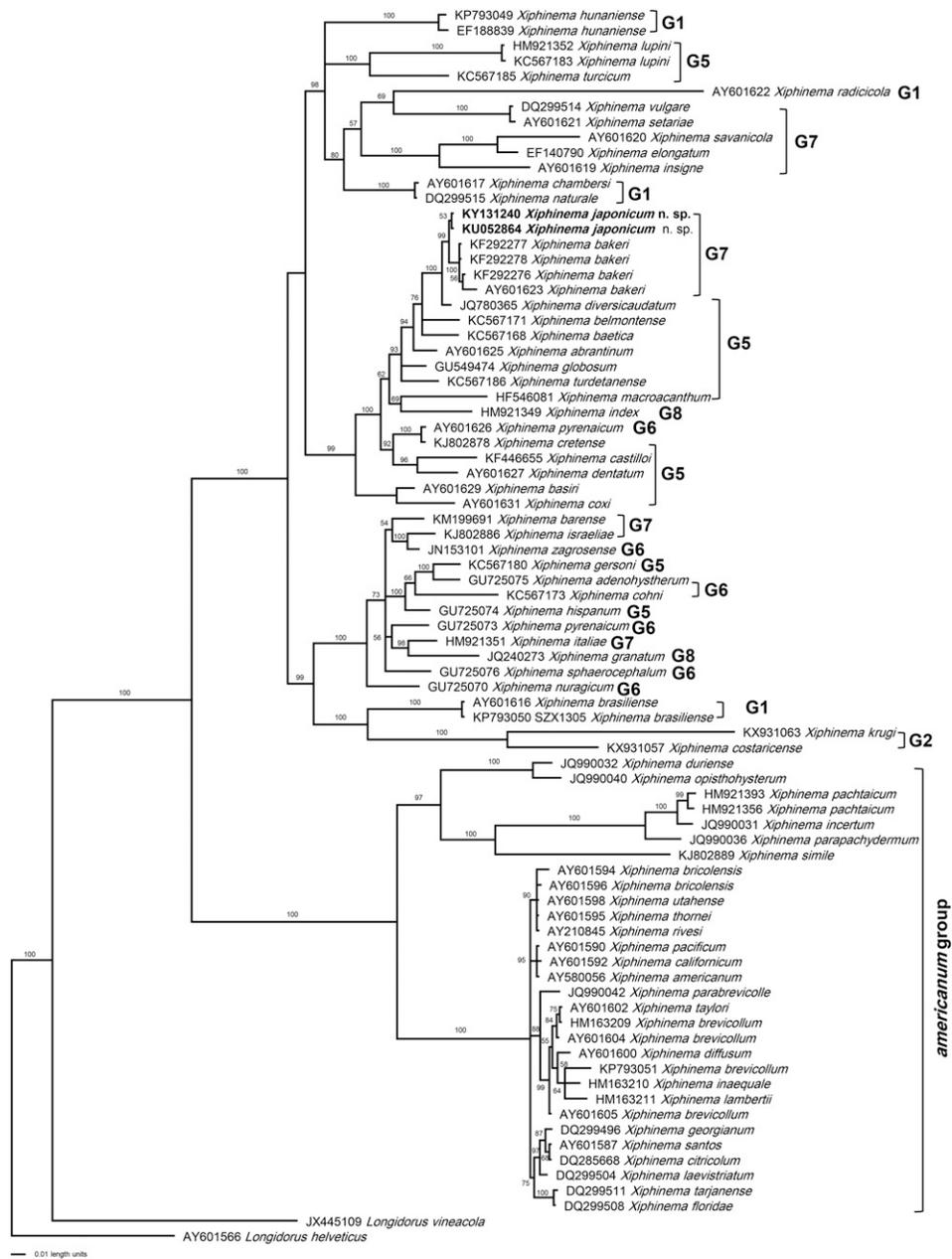


FIG. 5. Bayesian consensus tree of *Xiphinema japonicum* n. sp. inferred from 28S D2/D3 under GTR+I+G model ($-\ln L = 1,0912.0693$; AIC = 21,844.1387; freqA = 0.2463; freqC = 0.2277; freqG = 0.2969; freqT = 0.2291; R(a) = 0.9039; R(b) = 2.4909; R(c) = 2.4092; R(d) = 0.4557; R(e) = 3.9274; R(f) = 1; Pinva = 0.3185; Shape = 0.782). Posterior probability values exceeding 50% are given on appropriate clades.

Molecular profiles and phylogenetic status: Sequences of *Xiphinema japonicum* n. sp. were deposited in GenBank with the accession numbers KY131241-KY131243 (1704 bp for 18S), KY131244-KY131246 (1089 bp for ITS1), KY131240 and MF511179 (851 bp for 28S D2/D3), and KY628214 (447 bp for *COXI*), respectively. No sequence variation was observed among three individuals and between two populations (0535 and 0662). Phylogenetic analyses of *X. japonicum* n. sp. and other related species are presented in Figs. 4–7. The full length 18S sequences identities between the new species and *X. bakeri* (AY283173) from USA, *X. baetica* (KC567149), *X.*

belmontense (KC567150), *X. coxi europaeum* (KC567152), *X. diversicaudatum* (JQ780348), *X. globosum* (GU549476), *X. turdetanense* (KC567155) are 99.4%, 99.2%, 99.6%, 99.6%, 99.4%, 99.2%, and 99.7%, respectively. ITS1 sequences identities between *X. japonicum* n. sp. and *X. bakeri* (KF292281) from USA, *Xiphinema* sp. (DQ364686), *X. baetica* (KC567157), *X. belmontense* (KC767158), *X. coxi europaeum* (KC567162), *X. diversicaudatum* (KF292282), *X. setariae* (AY430179), *X. globosum* (GU549475), *X. turdetanense* (KC567163) are 94.1%, 96%, 84.6%, 82.6%, 85.2%, 92.2%, 87%, 84.7%, and 71.4%, respectively. The 28S D2/D3 sequences identities between the new species

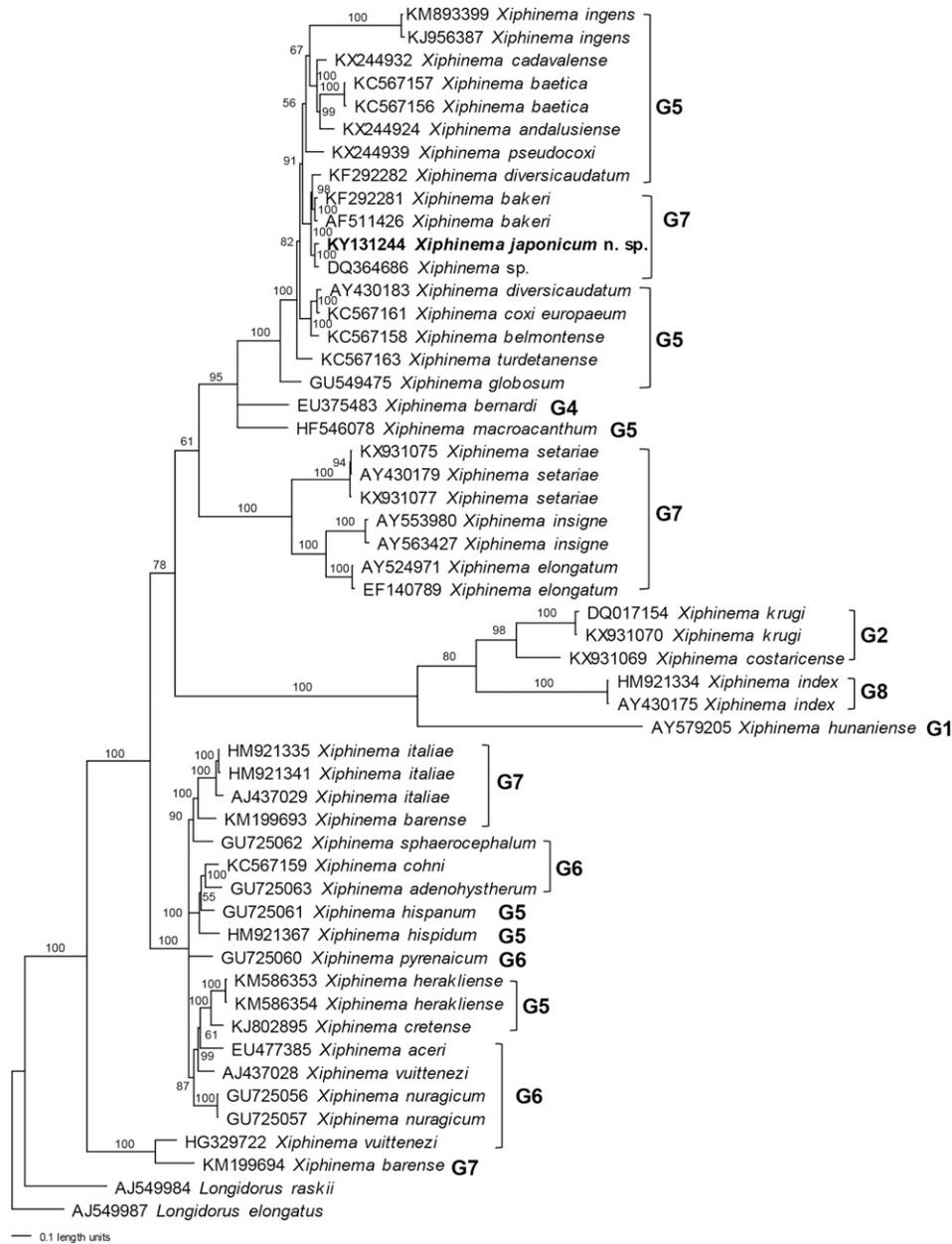


FIG. 6. Bayesian consensus tree of *Xiphinema japonicum* n. sp. inferred from ITS1 under GTR+I+G model ($-\ln L = 28,666.7617$; AIC = 57,353.5234; freqA = 0.2666; freqC = 0.2103; freqG = 0.2581; freqT = 0.2649; R(a) = 0.8907; R(b) = 2.1925; R(c) = 1.3381; R(d) = 0.8292; R(e) = 2.6455; R(f) = 1; Pinva = 0.0888; and Shape = 2.1461). Posterior probability values exceeding 50% are given on appropriate clades.

and *X. bakeri* (AY601623), *Xiphinema* sp. (DQ364685), *X. abrantinum* (AY601625), *X. baetica* (KC567168), *X. belmontense* (KC567171), *X. diversicaudatum* (JQ780365), *X. globosum* (GU549474), and *X. turdetanense* (KC567186) are 98.5%, 99.3%, 96.8%, 95.9%, 95.4%, 98.9%, 96.5%, and 95.8%, respectively. A BLAST search using the 447 bp DNA fragment of *COXI* gene of *X. japonicum* n. sp. revealed that this species is most close to *X. bakeri* (KF292305 and KF292306) from the USA and *X. diversicaudatum* (GU222421) with 38–48 bp differences in aligned sequences and 87%–89% identity. In 140 aligned amino acid sequences of *COXI* encoded protein, *X. japonicum* n. sp. had 2 amino acid differences

from *X. bakeri* and 3 amino acid differences from *X. diversicaudatum*. This large sequence divergence further supported *X. japonicum* n. sp. as a distinct species.

The 18S tree (Fig. 4) reconstructed from 64 sequences out of which 57 sequences belong to species of *Xiphinema* non-americanum-group, four from americanum group, and *Xiphididorus yepesara yepesara* Monteiro, 1976, *X. balcarceanus* Chaves and Coomans, 1984, and *Longidorus ferrisi* Robbins et al., 2009, sequences as out group taxa. The *X. americanum* group species appear distinct from species in *Xiphinema* non-americanum group. In this tree, the new species formed a moderately supported clade (PP = 71)



FIG. 7. Bayesian consensus tree of *Xiphinema japonicum* n. sp. inferred from *COXI* under GTR+I+G model ($-\ln L = 13,814.9082$; AIC = 27,649.8164; $\text{freqA} = 0.3355$; $\text{freqC} = 0.1459$; $\text{freqG} = 0.1281$; $\text{freqT} = 0.3905$; $R(a) = 0.796$; $R(b) = 9.4411$; $R(c) = 0.4133$; $R(d) = 1.205$; $R(e) = 12.7477$; $R(f) = 1$; $\text{Pinva} = 0.3913$; and $\text{Shape} = 0.5091$). Posterior probability values exceeding 50% are given on appropriate clades.

with *X. bakeri* belonging to group 7. This clade is in the same fully supported clade with *X. diversicaudatum* and *X. turdetanense* in group 5 with 100% support. In general, the morphospecies groups are not monophyletic in this

phylogenetic tree and species belonging to group 7 are clustered in three different clades.

The 28S D2/D3 tree (Fig. 5) reconstructed from 81 sequences out of which 49 sequences belong to species

of *Xiphinema non-americanum* group, 30 from *X. americanum* group, and *Longidorus vineicola* Sturhan and Weischer, 1964 and *L. helveticus* Lamberti et al. 2001 sequences as out group taxa. In this tree, the *Xiphinema americanum* group species are in a highly supported clade. *X. japonicum* n. sp. is in a monophyletic group with *X. bakeri* with 99% support. This clade is close to some group 5 species including *X. diversicaudatum*, *X. belmontense*, *X. baetica*, *X. abrantinum*, *X. globosum*, *X. turdetanense*, and *X. macroacanthum*. As in 18S tree, the morphospecies groups are not monophyletic and species in group 7 are in four different clades. The 28S D2/D3 sequences of the new species are identical to a species recently intercepted in Shanghai from *Acorus gramineus* from Japan originally identified as *X. bakeri* (KU052864). Through personal communication with the submitter, this is the same species as *X. japonicum* n. sp.

The ITS1 tree (Fig. 6) was reconstructed from 53 sequences of *Xiphinema non-americanum* group species and *Longidorus raskii* Lamberti and Agostinelli, 1993, and *L. elongatus* (de Man, 1876) Micoletzky, 1922, sequences as out groups. Species in *Xiphinema americanum* group are not included in this analysis because their large dissimilarity results in numerous ambiguous sites in multiple alignments. The ITS tree placed *X. japonicum* n. sp. in the same clade with *X. bakeri* and *X. diversicaudatum* with 100% support. As in 18S tree and 28S tree, the morphospecies groups are not monophyletic and species belonging to group 7 are in four different clades. In this tree, the new species is in a clade with an unidentified *Xiphinema* species (DQ364686). However, ITS1 sequence identity of both species is 1056/1097(96%) with 17/1097(1%) gaps.

The COXI tree (Fig. 7) was reconstructed from 113 sequences, out of which 65 sequences belong to species of *Xiphinema non americanum* group. All species of *Xiphinema non-americanum* group are in a monophyletic group with 77% support, clearly separated from species in the *americanum* group. *Xiphinema japonicum* n. sp. is most close to *X. bakeri* and *X. diversicaudatum*. They are in a monophyletic group with 99% support. As in 18S, 28S, and ITS trees, the morphospecies groups are not monophyletic and species belonging to group 7 are in four different clades.

The *Xiphinema non-americanum* species included in the analysis represent all morphogroups except for group 3 as defined by Loof and Luc (1990). In all four phylogenetic trees, *X. japonicum* n. sp. is clustered with *X. bakeri* and species from group 5 which have V value more than 39%, amphidelphic reproductive system, and dorsally convex-conoid tail with subdigitate or digitate terminus.

Xiphinema bakeri was described from British Columbia, Canada, by Williams (1961) and has been reported from Arkansas, California, Florida, Iowa Illinois, Indiana, North Carolina, Kentucky, Oregon, Tennessee,

Washington (Ye and Robbins, 2010), Canada (Sutherland and Sluggett, 1974), Japan (Yokoo, 1970; Hirata and Yuhara, 1986), and Korea (Lee and Han, 1976). It appears that *X. bakeri* is a native species to North America, the species recorded from Japan and Korea are very likely *X. japonicum* n. sp. and need reevaluation because no DNA sequences were available at the time.

DISCUSSION

Nematodes comprise the most abundant and diverse metazoans on earth, with estimated number of species over a million (Lamshead, 1993), but only over 25,000 have been described (Hodda, 2011; Zhang, 2013). Nematodes tend to be highly conserved in gross morphology and cryptic species are common because of a lack of conspicuous differences in both the external appearance and internal structure (Palomares-Rius et al., 2014). Many cryptic species have been discovered in plant-parasitic nematodes, such as *Globodera roschochiensis* (Wollenweber, 1923) Behrens, 1975, *G. pallida* (Stone, 1973) Behrens, 1975, *Meloidogyne chitwoodi* Golden, O'Bannon, Santo & Finley, 1980, *M. fallax* Karsen, 1996, *Radopholus similis* (Cobb, 1893) Thorne, 1949, *R. citrophilus* Huettel, Dickson & Kaplan, 1984, *Nacobbus aberrans sensu lato* (Thorne, 1935) Thorne & Allen, 1944, *Pratylenchus* Filipjev, 1936, *Rotylenchus* Filipjev, 1936, *Helicotylenchus* Steiner, 1945, *Longidorus* Micoletzky, 1922, and *Xiphinema* Cobb, 1913 (Golden and Ellington, 1972; Karsen, 1996; Lax et al., 2014). Recently, cryptic species is also reported for tylenchids (Soleymanzadeh et al., 2016). In the genus *Xiphinema*, *X. americanum* group comprised of more than 55 species and are a good example of the cryptic species. They are all quite similar in morphology and often overlap in morphometric data. However, their species boundary was supported by molecular data and thus more species were described or re-established as valid species (Gutiérrez-Gutiérrez, 2010; 2012; Zasada et al., 2014; Archidona-Yuste et al., 2016a). The present study provided further evidence of cryptic species nature in *Xiphinema*.

Based on our nuclear and mitochondrial DNA sequences, *X. japonicum* n. sp. is most close to, but distinct from *X. bakeri*. It forms a well-supported monophyletic group with *X. bakeri* in morphospecies group 7 and *X. diversicaudatum* in group 5. The morphological and phylogenetic relationship between *X. japonicum* n. sp. and *X. bakeri* is congruent with morphospecies scheme provided by Loof and Luc (1990). Species in morphospecies group 7 and group 5 have very similar gross morphology, but can be differentiated by the absence (group 7)/presence (group 5) of pseudo-Z organ in female reproductive system. The Z organ is probably a primitive state which has been lost and regained during the evolution in this genus (Gutiérrez-Gutiérrez et al., 2013). The function of the Z organ has been suggested in keeping the egg in the uterus, forcing the

slow passage of egg during shell formation and adding secretions to the egg shell (Grimaldi de Zio et al., 1979; Cho et al., 2000).

Xiphinema diversicaudatum was described from Saratov, Russia by Micoletzky (1927) and it has been reported from Canada (Mulvey, 1961), North America (Norton et al., 1984), New Zealand (Sturhan et al., 1997), Asia (Acharya et al., 1988; Mulawarman, 2008; Talezari et al., 2010), and Africa (Morkini et al., 2014). Earlier reports of this species suggested it was probably imported with plant material as this species is polyphagous and parasitizes various woody plants.

Roshan-Bakhsh et al. (2014) considered that the species of morphospecies group 5 are polyphyletic which is supported by the phylogenetic studies by Chizhov et al. (2014), who demonstrated that *X. diversicaudatum*, *X. bakeri*, *X. abrantinum*, *X. baetica*, and several other species from group 5 are related to each other and possibly originated from a common ancestor. *Xiphinema japonicum* n. sp. is grouped with several morphospecies from group 5 species all of which having similar tail shape and anteriorly located vulva. Hence, we agree with several other studies (Gutiérrez-Gutiérrez et al., 2013; Roshan-Bakhsh et al., 2014; Tzortzakakis et al., 2014) that the morphospecies groups were made for the convenience of identification but showed close relationship in evolutionary history.

In conclusion, this study provided morphological and molecular characterization of a dagger nematode intercepted from imported arhat pine from Japan, herein described as *X. japonicum* n. sp. belonging to morphospecies group 7. This Japanese species is mostly related to North American species *X. bakeri* with high similarity in gross morphology and morphometrics, but with significant differences in DNA sequences on ribosomal 18S, ITS1, 28S D2/D3, and mitochondrial *COXI* genes.

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