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Molecular and Morphological Characterization of *Xiphinema chambersi* Population from Live Oak in Jekyll Island, Georgia, with Comments on Morphometric Variations

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Abstract: A population of *Xiphinema chambersi* from the root zone around live oak (*Quercus virginiana* Mill.) trees on Jekyll Island, GA, is described using both morphological and molecular tools and compared with descriptions of type specimens. Initially, because of a few morphological differences, this nematode was thought to represent an undescribed species. However, on further examination, the morphometrics of the nematodes from live oak tend to agree with most of the morphometrics in the original description and redescription of *X. chambersi* except for few minor differences in V% relative to body length, slightly shorter stylet length, different c value, and the number of caudal pores. We consider these differences to be part of the normal variation within this species and accordingly image this new population of *X. chambersi* and redescribe the species. The new population is characterized by having females with a body length of 2.1 to 2.5 mm; lip region slightly rounded and set off from head; total stylet length 170 to 193 μm; vulva at 20.4% to 21.8% of body length; a monodelphic, posterior reproductive system; elongate, conoid tail with a blunt terminus and four pairs of caudal pores, of which two pairs are subdorsal and two subventral. Sequence data from the D2–D3 region of the 28S rRNA molecule subjected to GenBank sequence comparison using BLAST showed that the sequence had 96% and 99% similarity with *X. chambersi* from Alabama and Florida, respectively. Phylogenetic relationships of *X. chambersi* with other xiphinematids based on analysis of this DNA fragment are presented. This finding represents a new location of *X. chambersi* in Georgia on live oak for this species.

Key words: 28S rRNA, live oak, morphology, morphometrics, phylogeny, redescription, taxonomy, tree, *Xiphinema chambersi*.

The genus *Xiphinema* Cobb, 1913, includes more than 265 species of plant-ectoparasitic nematodes that are polyphagous and distributed throughout the world. Dagger nematodes of the genus *Xiphinema* comprise phytopathogenic species that damage a wide range of wild and cultivated plants through direct feeding on root cells and transmission of several plant pathogenic viruses (Taylor and Brown, 1997). During an October 2002 visit to Jekyll Island, GA, one of us recovered a limited number of specimens of species of *Xiphinema* and sent them to the senior author for identification. Later in November 2002, and again in May 2015, additional samples were obtained in the area where the species was originally found, and several additional females and a few juveniles were recovered from soil around the roots of live oak (*Q. virginiana*). Initially, this nematode was thought to represent an undescribed species because of several morphological differences from known species of this genus. However, on further examination, the morphometrics of the specimens from live oak agreed with most of the morphometrics in the original description (Thorne, 1939) and redescription (Cohn and Sher, 1972) of *X. chambersi*, except for few minor differences in V% relative to body length, slightly shorter stylet length, different c value, and the number of caudal pores. We consider these differences to be part of the normal variation within this species, and accordingly image this new population of *X. chambersi* from soil around roots of live oak, redescribe the original species, and assess the diagnostic value of both morphological and molecular characters.

Materials and Methods

Morphological characterization: Soil samples (2.5-cm-diam. to a 20-cm-depth) were collected from the root zone around live oak (*Q. virginiana*) trees on Jekyll Island, GA. Nematodes were extracted from a 200-cm^3 composite soil sample that was thoroughly but gently mixed, using the technique of Flegg (1967) with modifications by Fraedrich and Cram (2002). Nematodes were removed from Baermann funnels, and juveniles and females were fixed in warm 3% formaldehyde fixative and processed to glycerine by the formalin–glycerine method (Hooper, 1970; Golden, 1990). Light microscopy images of fixed nematodes were taken on a Leica WILD MPS48 Leitz DMBR compound microscope (Beltsville, MD) fitted with an ocular micrometer for image measurement.

DNA extraction, PCR assays, and sequencing: DNA was extracted from individual nematodes. Several specimens were analyzed for a population. Protocols for DNA extraction, PCR, and sequencing were described by Tanha Maafi et al. (2003). The forward D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and the reverse D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) (Rubtsova et al., 2001) primers were used for amplification of the D2–D3 expansion segments of *28S rRNA* gene, the forward TW81 (GTT TCC GTA GGT GAA CCT GC) and the reverse Xip5.8S (GAC CGC TTA GAA TGG AAT CGC)

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(Chizhov et al., 2014) primers were used for amplification of the *ITS1 rRNA* gene, or forward TW81 was paired with the reverse primer AB28 (ATA TGC TTA AGT TCA GCG GGT) for amplification of the *ITS1-5.8S-ITS2 rRNA* gene fragment. The partial *cytochrome c oxidase subunit 1* gene was amplified with the forward primer COIF (GAT TTT TTG GKC ATC CWG ARG) and the reverse primer XIPHR2 (5′-GTA CAT AAT GAA AAT GTG CCA C) (Lazarova, et al., 2006).

PCR products were purified after with QIAquick (Qiagen, Valencia, CA) Gel or PCR extraction kit. The same primers were used for direct sequencing. Some ITS and COI PCR amplicons were cloned using the Strataclone PCR Cloning Kit (Agilent, Santa Clara, CA) according to manufacturer's instructions. Plasmid clone DNA was prepared with the QiaPrep Spin Miniprep Kit (Qiagen) and digested with *Eco*RI to verify the presence of the insert. Cloned amplicons were sequenced by Macrogen Inc. (Rockville, MD). Several clones of each sample were isolated using blue/white selection and submitted to PCR with same primers. PCR products from each clone were sequenced. Sequences were submitted to the GenBank database under accession numbers: KU660075, KU764405-KU764419, and KT698205-KT698208.

Sequence and phylogenetic analysis: Sequences of the D2–D3 of *28S rRNA*, *18S rRNA*, and *coxI mtDNA* genes obtained from several specimens of *X. chambersi* were aligned by ClustalX 1.83 (Thompson et al., 1997) using default parameters with corresponding published sequences of genes of *X. chambersi* (He et al., 2005; Gozel et al., 2006) and/or other *Xiphinema* species belonging to the Clade I of non-*Xiphinema americanum* group according to Gutiérrez-Gutiérrez et al. (2013). Sequence of the *ITS1 rRNA* gene was aligned with those of other *X. chambersi* (Ye et al., 2004; Yu et al., 2010; Zeng et al., 2015) and two species outgroup species. All sequence datasets were analyzed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) under the GTR + I + G model. BI analysis for each aligned dataset was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating

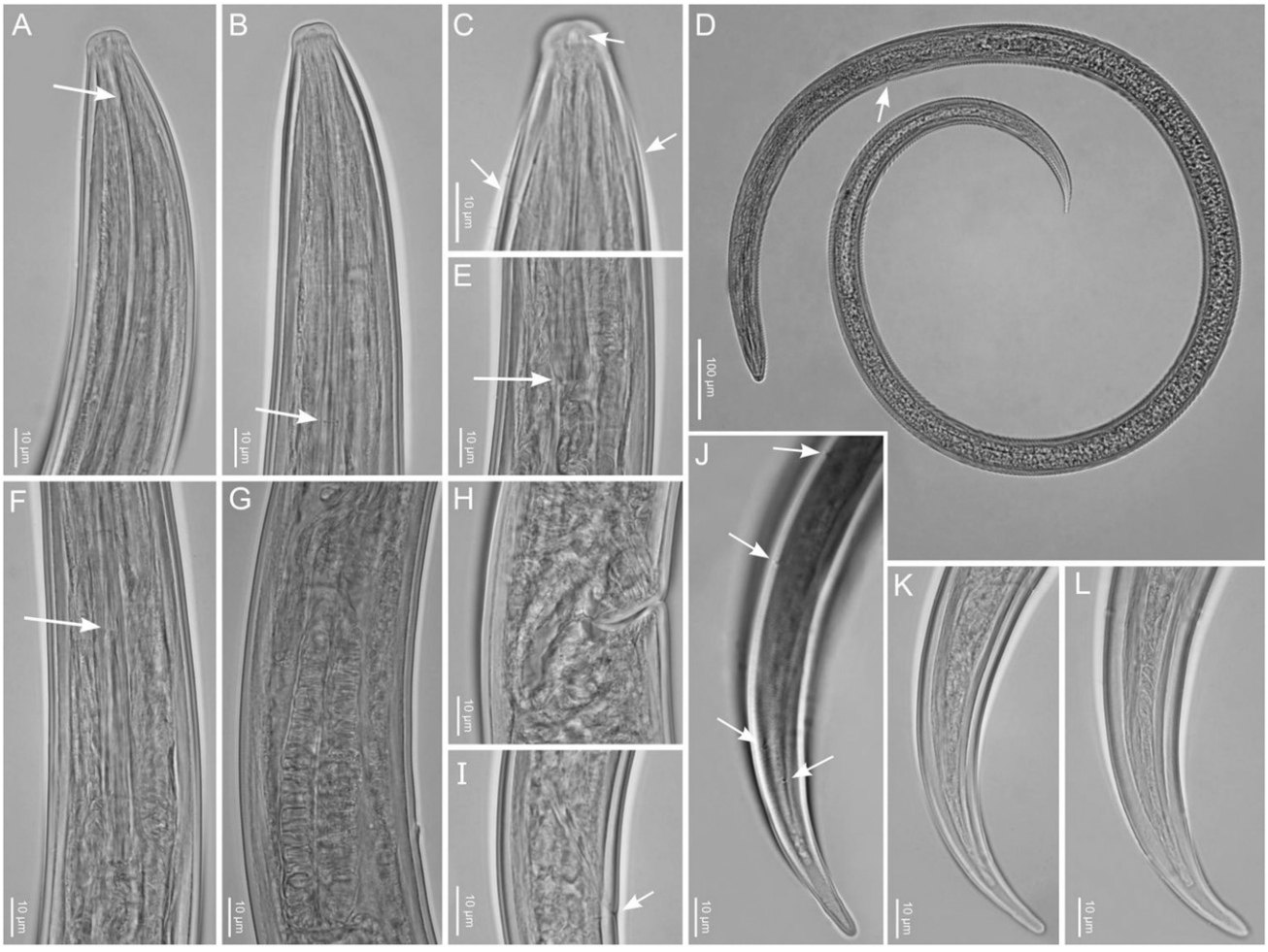

Fig. 1. Photomicrographs of *Xiphinema chambersi* on live oak from Jekyll Island, GA. A–C. Anterior regions with arrows indicating in A) tip of odontostyle, B) guiding ring, C) amphid and lateral pores. D. Whole body. E–G. Anterior regions with arrows showing in E) flanges of odontophore, F) nerve ring. H. Vulval region. I–L. Tail with arrows indicating in I) anus and J) caudal pores.

TABLE 1. Morphometrics of *Xiphinema chambersi* population on live oak in Jekyll Island, GA, and data from the original and redescriptions of the species. All measurements are in μm (except for body length) and in the form mean ± SD (min–max).

	Location and host						
Characters	Jekyll Island, GA, live oak	Arlington Farm, VA, soil, Thorne, 1939	Arlington Farm, VA, pine woods, Cohn and Sher, 1972	Bartow, FL, live oak, Lamberti et al., 2002	Merrit Island, FL, live oak, Lamberti et al., 2002	Ontario, Canada, red oak, Yu et al., 2010	Arkansas, hardwood/ maple/shrub, Ye et al., 2010[a]
N	10 ♀♀	-	6 ♀♀	10 ♀♀	8 ♀♀	9 ♀♀	49 ♀♀
Body length (mm)	2.314 ± 0.1214 (2.125–2.500)	2.5	2.4 (2.2–2.5)	2.5 ± 0.09 (2.4–2.7)	2.5 ± 0.15 (2.3–2.7)	2.22 ± 0.1 (2.1–2.4)	2.46 ± 0.15 (2.2–2.7)
Odontostyle length	118.1 ± 6.7 (110.0–130.0)	100	-	117 ± 2.70 (111.8–122.3)	114.7 ± 1.56 (111.8–116.5)	114.7 ± 1.9 (110.5–118.1)	114.4 ± 6.1 (98.7 ± 122.0)
Odontophore length	60.1 ± 2.1 (57.5–62.2)	69	-	65.3 ± 2.00 (63–70)	64 ± 1.55 (61.8–66)	65.9 ± 2.4 (62.5–70.2)	65.4 ± 4.6 (56.3–75)
Total stylet length	178.2 ± 7.8 (167.5–192.5)	169	192 (187–198)	182.3 ± 4.70 (174.8–192.3)	178.7 ± 3.11 (174.3–182.5)	180.5 ± 3.3 (173.0–185.1)	179.5 ± 7.4 (160–187.7)
Distance anterior to nerve ring	103.9 ± 3.7 (98.0–110.0)	-	-	104.3 ± 3.10 (101.2–111.8)	102 ± 4.72 (92–108.8)	110 ± 3.3 (105.4–115.0)	98.4 ± 8.4 (75.3–110.7)
Maximum body width	44.2 ± 2.7 (40.0–49.0)	-	-	-	-	42.9 ± 2.4 (39.9–47.8)	49.9 ± 8.1 (34.7–60.3)
Width at vulva	42.7 ± 2.7 (39.0–47.5)	-	-	-	-	-	-
Width at base of esophagus	39.2 ± 3.9 (33.1–45.0)	-	-	36.2 ± 1.41 (33.5–38.2)	37.8 ± 1.30 (36–40)	-	-
Head height	4.8 ± 0.5 (4.0–6.0)	-	-	-	-	5.3 ± 0.8 (4.5–7.2)	-
Head width	11.2 ± 0.6 (10.5–12.0)	-	-	-	-	10.9 ± 0.6 (10.0–12.0)	11.1 ± 0.9 (8.7–12.3)
Esophageal length from anterior end	349.3 ± 19.3 (322.0–380.0)	-	-	-	-	384.9 ± 13.5 (359.4–403.0)	-
a	52.4 ± 3.9 (45.5–59.7)	55	-	63.7 ± 2.50 (60–68.5)	61.4 ± 2.90 (57.5–66.5)	51.9 ± 2.5 (47.7–55.7)	50.9 ± 7.8 (40.8–68.7)
b	6.6 ± 0.5 (6.3–7.7)	6	-	6.4 ± 0.39 (5.6–7)	6.9 ± 0.19 (6.5–7.1)	5.8 ± 0.3 (5.4–6.2)	6.4 ± 0.53 (5.7–7.5)
c	27.1 ± 1.8 (25.0–29.6)	20	22 (21–22)	23.6 ± 1.20 (22–25)	26.5 ± 1.33 (25–28.5)	17.0 ± 1.9 (12.5–19.7)	23.0 ± 1.7 (21.9–27.6)
c′	3.4 ± 0.3 (3.1–4.1)	-	4.4 (4.3–4.7)	4.3 ± 0.20 (4.0–4.6)	3.7 ± 0.23 (3.3–4)	5.3 ± 0.7 (4.4–7.0)	3.9 ± 0.43 (3.1–4.7)
Tail length	85.3 ± 5.9 (75.0–90.0)	-	-	105.5 ± 4.40 (100–111.8)	95 ± 4.89 (88.3–100)	132.1 ± 17.6 (110.2–177.3)	90.1 ± 6.5 (65.3–87.7)
Vulva %	21.2 ± 0.4 (20.4–21.8)	23	24 (23–25)	23 ± 0.79 (22–24)	21 ± 1.06 (20–23)	24.4 ± 0.9 (23.1–26.4)	22.9 ± 0.96 (21.6–25.4)
Body width at anus	24.9 ± 1.1 (23.0–27.0)	-	-	25.2 ± 1.79 (23.5–29.4)	26.0 ± 0.95 (24.7–27)	24.9 ± 1.6 (23.4–28.9)	29.6 ± 4.3 (21.1–36.1)
j[b]	21.4 ± 1.6 (20.0–25.0)	-	-	20.4 ± 2.14 (17.6–23)	20.5 ± 2.17 (17.6–23.5)	37.4 ± 6.1 (22.0–43.4)	-
j width at beginning	7.5 ± 0.4 (7.0–8.5)	-	-	7.4 ± 0.66 (6.5–8.2)	8.8 ± 0.79 (8.2–10)	-	-
j width 5 μm from tail terminus	4.3 ± 0.4 (4.0–5.0)	-	-	-	-	-	-

[a] Mean measurements taken from four *Xiphinema chambersi* populations (Ye et al., 2010).
[b] j = length of hyaline portion of tail (μm).

convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities are given on appropriate clades.

SYSTEMATICS

Xiphinema chambersi Thorne, 1939
(Fig. 1, Table 1)

Females: Body ventrally curved, open C shaped to spiral form with posterior region of the body more strongly curved. Cuticle with fine transverse striations, comprises two optically different layers, 2.5 to 5.0 μm wide at midbody, but thicker at tail. Lip region slightly rounded and set off from rest of the body. Amphids stirrup shaped. Basal portion of spear with strongly developed flanges 12 μm wide. Esophagus typical of the genus. Anterior part slender, with an "S" bend near its junction with the posterior part, when spear is not extended. Basal portion of esophageal bulb with three prominent nuclei. Gonad single, extending posteriorly. Vagina directed slightly posteriorly; ovary reflexed. Tail arcuate, elongate conoid terminating in a cylindroid nonprotoplasmic bluntly rounded tip; tail with four

pairs of caudal pores, of which two pairs are subdorsal and two subventral.

Males: Not found, although Thorne (1939) described and illustrated a male, no males were seen on the syntype slides (Yu et al., 2010). Only two males were found in Arkansas (Ye, 2002).

Results and Discussion

This population of *Xiphinema* showed females with a body length of 2.1 to 2.5 mm; lip region slightly rounded and set off from head; total stylet length 170 to 193 μm; vulva quite anteriorly located at 20.4% to 21.8% of body length; a monodelphic, posterior reproductive system; elongate, conoid tail with a blunt terminus and four pairs of caudal pores, of which two pairs are subdorsal and two subventral. Morphologically, it resembled *X. chambersi* Thorne, 1939; *Xiphinema monohysterum* Brown, 1968; *Xiphinema insigne* Loos, 1949; and *Xiphinema mali* Ganguly et al., 2002. These facts led us to undertake a detailed morphological and molecular comparative study with previously reported data combined with molecular analyses to help in its species identification and to clarify the phylogeny of some of the closely related species of the genus. These studies showed that the live oak population differed from all these *Xiphinema* species either in the body length, vulva position, stylet length, c and c′, or in the tail shape. However, the population was almost identical to that published in the original description and redescription of *X. chambersi*. Minor morphometric differences of this population from the

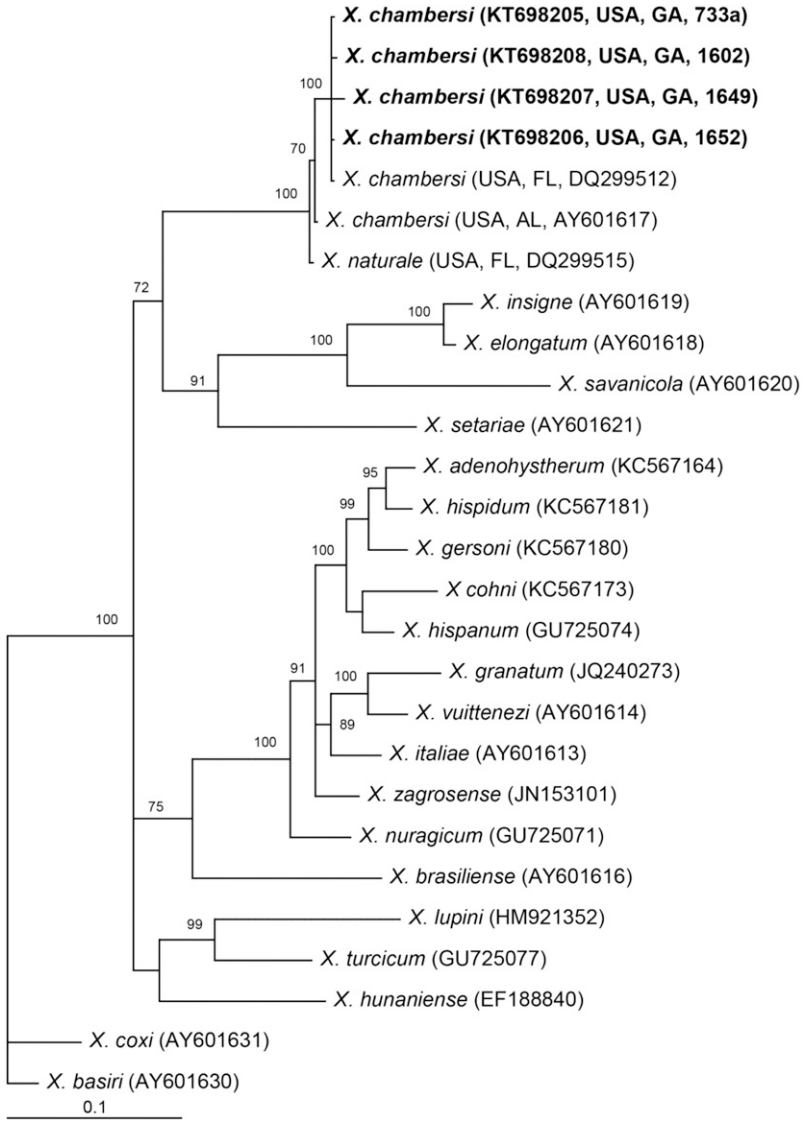

Fig. 2. Phylogenetic relationships within *Xiphinema* species belonging to the Clade I of non-*Xiphinema americanum* group as it has been defined by Gutiérrez-Gutiérrez et al. (2013). Bayesian 50% majority rule consensus tree from two runs as inferred from the D2–D3 of *28S rRNA* gene sequence alignment under the GTR + I + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are indicated by bold letters.

redescription include total stylet length (168–193 vs. 187–198 μm), V (20–22 vs. 23–25%), b = (5.1–6.4 vs. 6.3–7.7), c = (21–22 vs. 25–30), c′ = (4.3–4.7 vs. 3.1–4.1) and caudal pores (4 vs. 2) (Cohn and Sher, 1972). We consider these differences to be part of the normal variation within this species. These measurements for *X. chambersi* extend the recorded variation (Thorne, 1939; Loof and Yassin, 1970; Cohn and Sher, 1972; Ye and Robbins, 2010; and Yu et al., 2010) in stylet length, a, b, c and c′ ratios, a smaller V value, and increase in the number of caudal pores. The discovery of this new population on live oak in Jekyll Island constitutes a new location and a new host for Georgia.

Yu et al. (2010) provided morphological and molecular details about *X. chambersi*, along with updated distribution. Regarding the molecular analysis, four new sequences of the D2–D3 of *28S rRNA* gene were obtained from the sample of *X. chambersi* in the present study. Intraspecific variability for *X. chambersi* sequences was 0% to 1.1% (0–9 bp). The alignment used for phylogenetic reconstruction included 27 sequences belonging to 22 *Xiphinema* species. Phylogenetic relationships of *X. chambersi* with other *Xiphinema* species from the Clade I of non-*X. americanum* group (Gutiérrez-Gutiérrez et al., 2013) as inferred from the D2–D3 of *28S rRNA* gene sequences are presented on the BI tree (Fig. 2). This species formed a highly supported clade with *Xiphinema naturale*.

Seven new sequences the *ITS rRNA* gene were obtained from studied sample. Intraspecific variability for *X. chambersi* ITS1 rRNA sequences was 0% to 8.2% (0–48 bp). Phylogenetic relationships within *X. chambersi* based on the partial *ITS1 rRNA* gene sequences are given in Fig. 3.

One new *18S rRNA* gene sequence was obtained from a sample. Intraspecific variability for *X. chambersi* 18S rRNA sequences was 0% to 0.6% (0–10 bp). Phylogenetic relationships of *X. chambersi* with other *Xiphinema* species from the Clade I of non-*X. americanum* group (Gutiérrez-Gutiérrez et al., 2013) as inferred from the *18S rRNA* gene sequences are presented on the BI tree (Fig. 4). Relationships of *X. chambersi* with other *Xiphinema* remain unresolved.

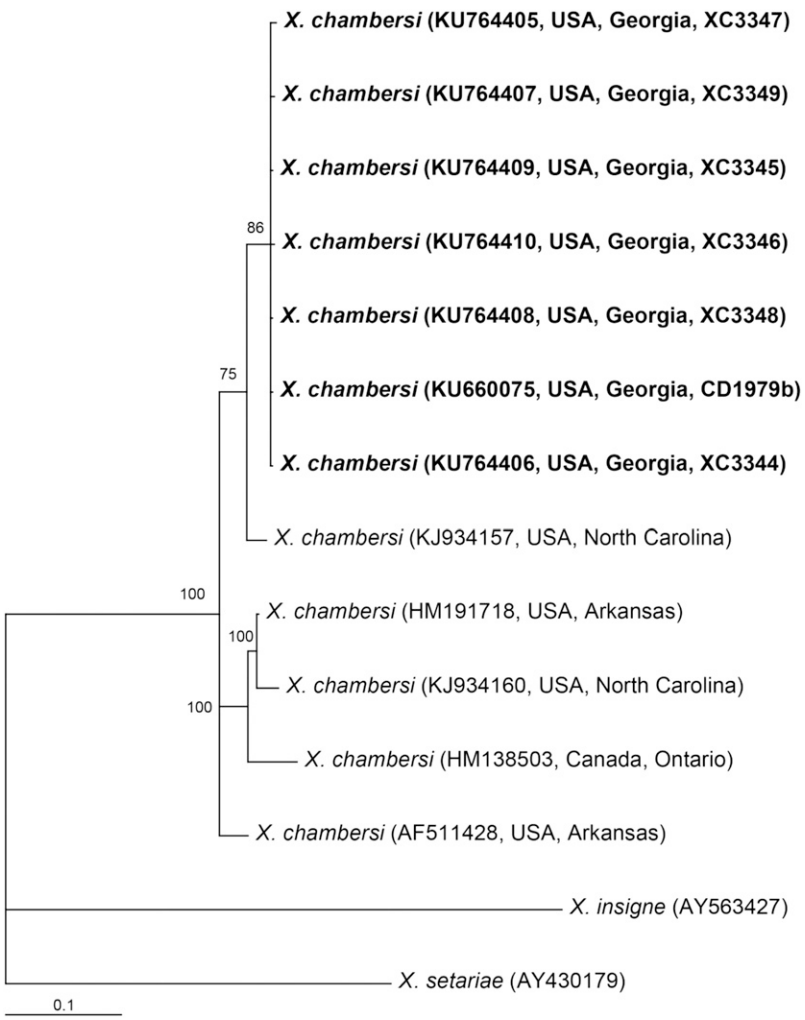

Fig. 3. Phylogenetic relationships within *Xiphinema chambersi*. Bayesian 50% majority rule consensus tree from two runs as inferred from the partial ITS1 sequence alignment under the GTR + I + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequences are indicated by bold letters.

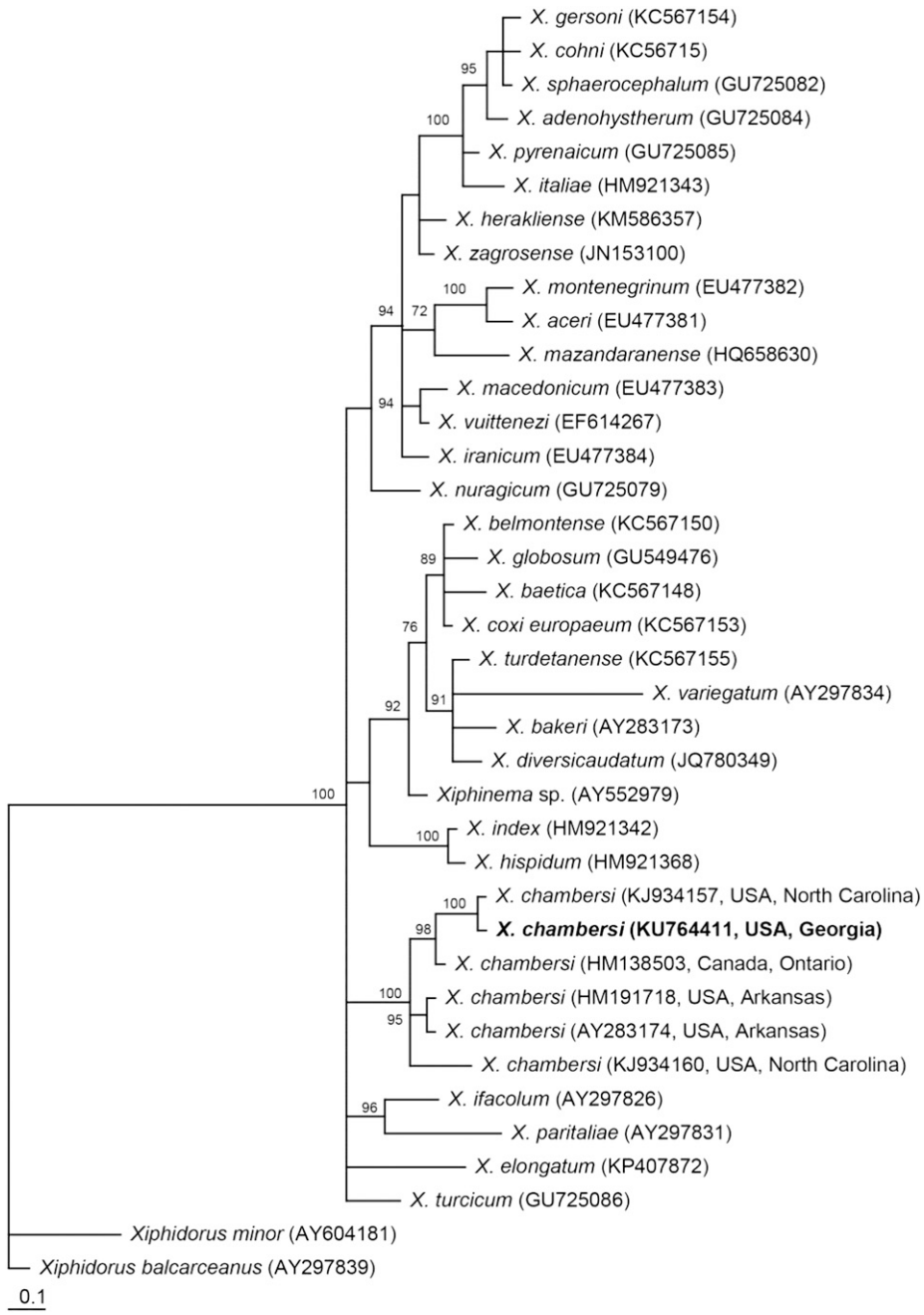

FIG. 4. Phylogenetic relationships within *Xiphinema* species belonging to the Clade I of non-*Xiphinema americanum* group as it has been defined by Gutiérrez-Gutiérrez et al. (2013). Bayesian 50% majority rule consensus tree from two runs as inferred from the *18S rRNA* gene sequence alignment under the GTR + I + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequence is indicated by bold letters.

Variation between obtained coxI mtDNA sequences was 0% to 0.7% (0–3 bp). Phylogenetic relationships of *X. chambersi* with other *Xiphinema* as inferred from the partial sequences of *coxI mtDNA* gene is given in Fig. 5. With respect to tree hosts, Ruehle (1968) recovered *X. chambersi* in soil around roots of several 25-year-old sweetgum (*Liquidambar styraciflua*) trees growing on a streambank in a natural forest near Athens, GA, and it was subsequently determined that the nematode was capable of causing moderate-to-severe damage to sweetgum roots (Ruehle, 1972). During 2013 and 2014, one of us (Stephen Fraedrich) observed occasional dieback and mortality of sweetgum in the Athens, GA, although the possible role of *X. chambersi* or other plant-parasitic nematodes in this damage has not been investigated. In woodland areas in New Jersey, Springer (1964) found *X. chambersi* associated with American beech (*Fagus grandifolia*), white ash (*Fraxinus americana*), chokecherry (*Prunus virginiana*), and pin oak (*Quercus palustris*). This nematode was also found in a pine nursery in Florida by Hopper (1958), associated

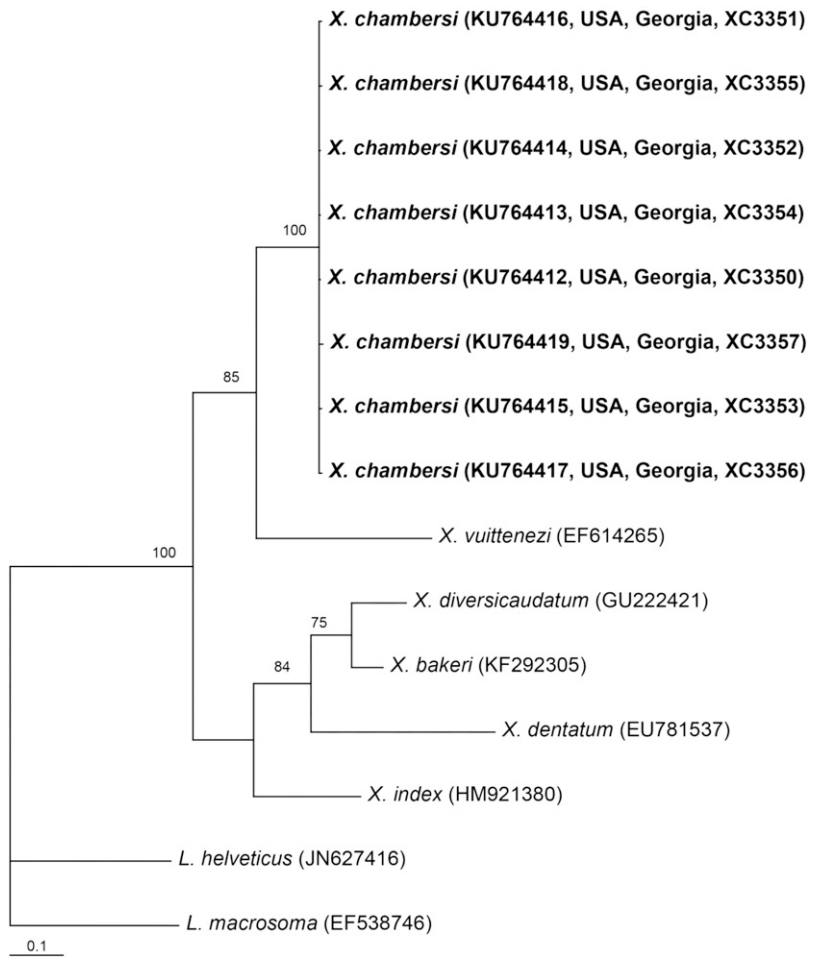

Fig. 5. Phylogenetic relationships within *Xiphinema* species. Bayesian 50% majority rule consensus tree from two runs as inferred from the partial *coxI* mtDNA sequence alignment under the GTR + I + G model. Posterior probabilities more than 70% are given for appropriate clades. Newly obtained sequence is indicated by bold letters.

with roots of pine in Louisiana, and recovered from soil under eastern hemlock (*Tsuga canadensis*) in mountains of Georgia (Ruehle, 1968). In addition, this species was reported on northern red oak (*Quercus rubra*) (Yu et al., 2010), live oak (Lamberti et al., 2002), and ubame oak (*Quercus phillyraeoides*) (Shishida, 1983).

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