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Molecular characterization of pseudomonodelphic dagger nematodes of the genus *Xiphinema* Cobb, 1913 (Nematoda: Longidoridae) in Costa Rica, with notes on *Xiphinema setariae* Tarjan, 1964

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Abstract Pseudomonodelphic dagger nematodes of the genus *Xiphinema* are characterized by having one of the genital branches reduced and lacking an ovary. They are usually reported from tropical regions. Nematode surveys conducted during rainy seasons in Costa Rica resulted in detection of several *Xiphinema costaricense* populations, but also other pseudomonodelphic and didelphic species of *Xiphinema.* We undertook detailed integrative morphometric and molecular studies using D2-D3 expansion segments of 28S rDNA, and ITS1-rDNA. From those studies, we also identified several populations of *Xiphinema krugi* and two populations of *Xiphinema setariae* with characteristics in agreement with those of the original and later descriptions of these species. The phylogenetic analyses of these species with other representatives of *Xiphinema* spp. indicated that pseudomonodelphic species are phylogenetically related (*X. costaricense* and *X. krugi*). On the basis of ITS1 sequences of *X. costaricense* and *X. variegatum* from Brazil, as well as similar morphology and morphometrics of both species, the latter is proposed here as a junior synonym of the former. Also, our morphometric data showed some intraspecific variability within *X. setariae*, which in combination with the molecular evidence, suggests that *X. setariae* and *X. vulgare* need to be considered as a single taxon.

Keywords Bayesian inference · D2-D3 · ITS1 · Longidorids · Phylogeny · rDNA · Taxonomy

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Pseudomonodelphic dagger nematodes of the genus *Xiphinema* Cobb 1913, belonging to morphospecies Group 2, are usually reported from ropical regions (Cohn and Sher 1972; Luc 1981; Loof and Luc 1990). This morphospecies group comprises nematodes with the anterior genital branch reduced and incomplete,

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anterior ovary absent and the oviduct drastically reduced to a mass of cells in which it is difficult to identify any functional structure; the sphincter and uterus are usually recognizable but reduced (Loof and Luc 1990). One species of this group is *Xiphinema costaricense* Lamberti and Tarjan 1974 which was originally described from specimens collected from the rhizosphere of plantain at Guayabo de Turrialba, Costa Rica, and was also found in association with banana, citrus, coffee, and sugar-cane (Lamberti and Tarjan 1974). This species has been also reported from other Central and South American countries (Lamberti and Tarjan 1974; Hunt and Singh 1984; Germani 1989; Alkemade and Loof 1990; Swart and Quénéhervé 1998). Nevertheless, to our knowledge, no molecular data is available for *X. costaricense*. Consequently, nematological surveys of several regions of Costa Rica, including the type locality, were conducted in 2015 to 2016 to provide specimens for molecular characterization. The surveys detected several *X. costaricense* populations, but also one pseudomonodelphic and one didelphic species of *Xiphinema*. That prompted us to undertake detailed morphological and molecular studies for comparison with previously reported data.

Molecular approaches, using ribosomal DNA (rDNA) sequences including the D2-D3 expansion segments of 28S and ITS rRNA region, are useful diagnostic markers for revealing phylogenetic relationships within the Longidoridae, especially in cases where morphological characters may lead to ambiguous interpretation (De Luca et al. 2004; He et al. 2005; Gutiérrez-Gutiérrez et al. 2013; Subbotin et al. 2014; Archidona-Yuste et al. 2016).

Therefore, the objectives of the present study were to: (i) provide an accurate identification of pseudomonodelphic and didelphic *Xiphinema* species detected in Costa Rica through an integrative morphological and molecular characterization using the D2-D3 expansion segments of 28S rRNA, and ITS1 rRNA gene sequences; and (ii) explore the phylogenetic relationships of these species within the genus *Xiphinema*.

Nematode surveys were conducted during the rainy seasons in 2015 and 2016 in cultivated, ornamental and wild plants in a wide range of areas in Costa Rica (Table 1). Soil samples from agricultural sites were a composite of 20–25 soil cores arbitrarily chosen from the same field to a depth of 25–40 cm with an Oakfield tube of 2.5-cm diameter. Samples from areas of wild plants were composites of one to three subsamples of soil directly under an individual plant. Samples were placed in labelled plastic bags, sealed and brought back to the nematology laboratory where they were stored at 4 °C until processed for nematode extraction. For most of the samples (ACC prefix, Table 1), nematodes were extracted from 500 cm^3 of soil by centrifugal flotation (Coolen 1979) and a modification of Cobb's decanting and sieving method (Flegg 1967). For other samples (prefix HF code, Table 1), nematodes were extracted by a combination of decanting and sieving and a modified Baermann tray method (Barker 1985). Females were processed and mounted in glycerine for diagnostic studies. Specimens for study by light microscopy were killed by gentle heat, fixed in a solution of 4% formalin +2% glycerol and processed to pure glycerine using Seinhorst's method (Seinhorst 1966). Nematodes were measured using a drawing tube attached to a light microscope and expressed in micrometers (μm). All abbreviations used are as defined in Jairajpuri and Ahmad (1992).

DNA extraction and PCR assays were conducted as described by Castillo et al. (2003). The D2-D3 expansion segments of 28S rRNA was amplified using the D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) primers (De Ley et al. 1999). The ITS1 region was amplified using forward primer 18S (5'TTGATTACGTCCCTGCCCTT T-3´) (Vrain et al. 1992) and reverse primer rDNA1 (5´-ACGAGCCGAGTGATCCACCG-3´) (Cherry et al. 1997). PCR products were purified, quantified and used for direct sequencing as described by Peraza-Padilla et al. (2016). The newly obtained sequences were submitted to the GenBank database under accession numbers KX931056-KX931077 (Table 1).

D2-D3 and ITS1 rDNA and sequences of different *Xiphinema* spp. from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were chosen according to previous published data (Archidona-Yuste et al. 2016). The newly obtained and published sequences for each gene were aligned using MAFFT v. 7.205 (Katoh and Standley 2013), strategy FFT-NS-1 with default parameters. Sequence alignments were visualized using BioEdit (Hall 1999) and edited by Gblocks v0.91b (Castresana 2000) in Castresana Lab server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) using options for a less stringent selection (minimum number of sequences for a conserved or a flanking position: 50% of the number of sequences +1; maximum number of

Table 1 *Xiphinema costarricense* Lamberti and Tarjan 1974, *Xiphinema krugi* Lordello 1955 and *Xiphinema setariae* Luc 1958 sampled and sequenced from Costa Rica in this study

Xiphinema spp.	Locality, province	Host-plant	Sample code	GenBank accession	
				D2-D3	ITS1
X. costarricense	Guayabo, Turrialba, Cartago (Type locality)	Forest	ACC86 (topotypes)	KX931056	KX931067
X. costarricense	Pueblo Nuevo, Palmira, Zarcero. Alajuela	Strawberry	ACC36	*	-
X. costarricense	Santa Rosa, Limón, Limón	Cocoa	ACC46	KX931057	KX931068
X. costarricense	Pueblo Nuevo de Pilas, Buenos Aires, Puntarenas	Beans	ACC55	KX931058	KX931069
X. costarricense	San Vito de Coto Brus, Puntarenas	Forest (unknown plant)	ACC50	*	*
X. costarricense	Pacayitas, La Suiza de Turrialba, Cartago	Sugarcane	ACC61	KX931059	*
X. krugi	Pacayas de Alvarado, Cartago	Sugarcane	ACC13	KX931060	KX931070 KX931071
X. krugi	Sucre, Ciudad Quesada, Alajuela	Robust star grass	ACC47	KX931061	KX931072
X. krugi	Rancho Redondo, Goicoechea, San José	Fallow	ACC68	*	-
X. krugi	Matapalo. Savegre, Quepos, Puntarenas	Rubber plant	HF001	KX931062 KX931063	KX931073 KX931074
X. krugi	Santa Gertrudis, Grecia, Alajuela	Sugarcane	ACC33	*	*
X. setariae	Matapalo, Savegre, Quepos. Puntarenas	Mango	HF002	KX931064 KX931065	KX931075 KX931076
X. setariae	Pueblo Nuevo de Duacarí, Guácimo, Limón	Banana	ACC09	KX931066	KX931077

(−) Not obtained

(*) Sequenced population but not deposited in GenBank database because of their similarity with others (see discussion section)

contiguous nonconserved positions: 8; minimum length of a block: 5; allowed gap positions: with half [positions where 50% or more of the sequences have a gap are treated as a gap position]). Phylogenetic analyses of the sequence data sets were performed as described by Archidona-Yuste et al. (2016). BI analysis for D2-D3 and ITS1 rDNA regions under the GTR + I + G model were initiated with a random starting tree and run with the four Metropolis-coupled Markov chain Monte Carlo (MCMC) for 1×10^6 generations. The MCMC were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Trees were visualised using TreeView (Page 1996).

Detailed observations, using light microscopy, of several diagnostic morphological and morphometric characters indicated that the topotype and other pseudomonodelphic populations of *X. costaricense* (Lamberti and Tarjan 1974) closely resembled the original description, including juvenile stages (Fig. 1a-h). There were minor differences in odontostyle length, c' ratio, and tail hyaline region, which may be due to the low number of specimens originally studied, or to geographical intraspecific variability (Fig. 1a-h, Table 2).

Similarly, morphological and morphometric traits of two other pseudomonodelphic and didelphic species closely resembled *Xiphinema krugi* Lordello 1955 and *Xiphinema setariae* Luc 1958, respectively (Fig. 1i-y, Table 2). The Costa Rican populations of *X. krugi* were characterized by a partially atrophied anterior gonad, tail subconoid with a slight bulge more or less developed but never a distinct peg (Fig. 1i-o), which agrees with previous descriptions (Coomans et al. 2001). The morphology and morphometrics agree well with original and other descriptions of the species (Lordello 1955; Heyns 1977; Loof and Sharma 1979; Razak and Loof 1999), except for minor differences in body and odontostyle length, c and c' ratios (Table 2), which may be due to geographical intraspecific variability. *Xiphinema krugi* is one of the most frequently occurring species in tropical regions of the world (Cohn and Sher

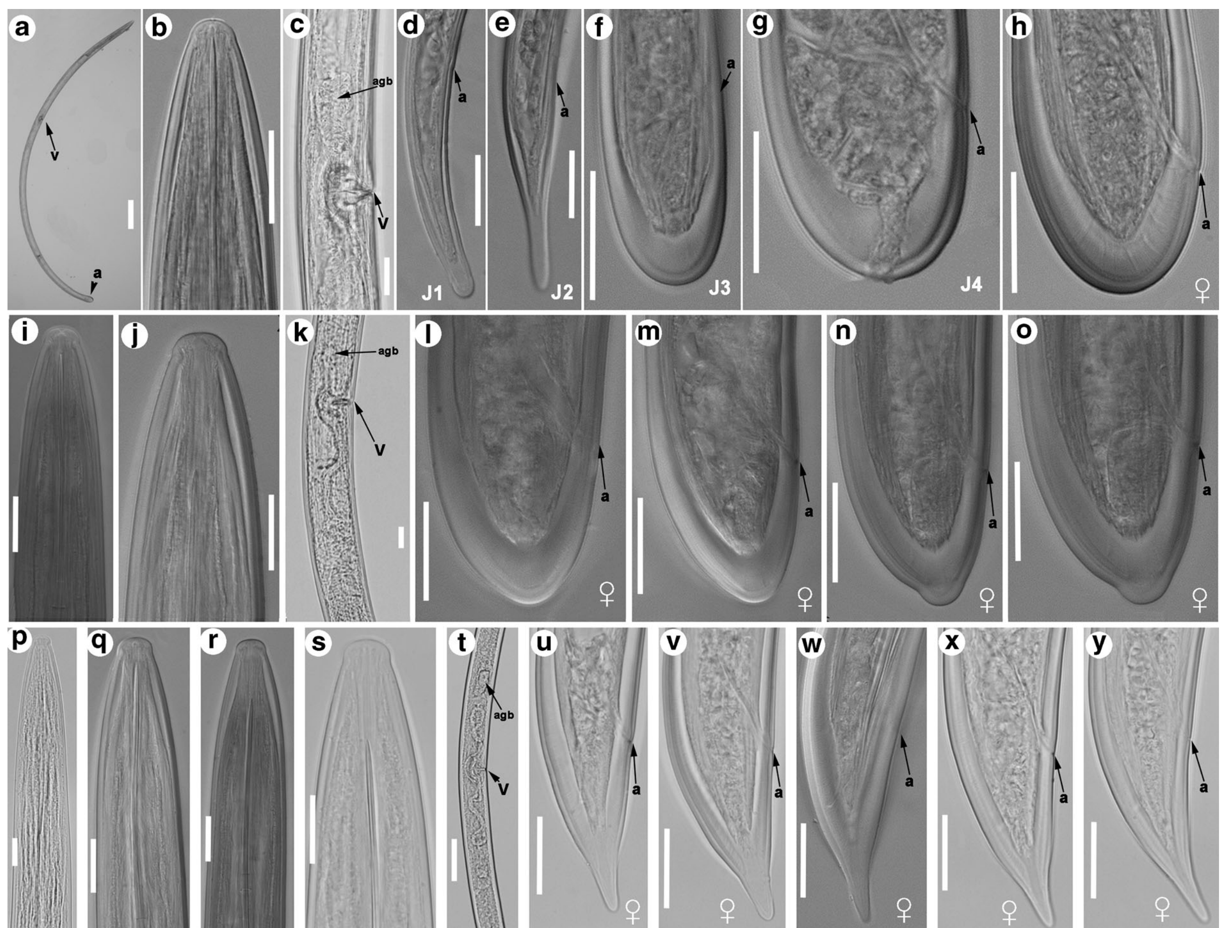

Fig. 1 Light micrographs of *Xiphinema costaricense* Lamberti and Tarjan 1974 (**a-h**), *Xiphinema krugi* Lordello 1955 (**i-o**), and *Xiphinema setariae* Luc 1958 (**p-y**). **a** Female whole body. **b,i,j,p-s** Female anterior regions. **c,k,t** Detail of anterior genital branch. **d,e,f,g** First-, second-, third- and fourth-stage juvenile tail, respectively. **h,l-o,u-y** Female tail regions. Abbreviations: a = anus; agb = anterior genital branch; V = vulva. (Scale bars: **a** = 200 μm; **b-y** = 20 μm)

1972; Luc and Hunt 1978). It has been reported in the USA (Frederick and Tarjan 1974; Robbins and Brown 1991), South America and Caribbean Islands (Lordello 1955, Loof and Maas 1972, Luc and Hunt 1978, Loof & Sharma 1979, Bala 1984, Luc and Doucet 1990, Volcy 1990, Luc and Coomans 1992, Decraemer et al. 1998, Crozzoli et al. 2001), South Africa (Heyns 1977),), and Malaysia (Razak and Loof 1999), (). The two Costa Rican populations identified as *X. setariae* were characterized by a lip region separated from the rest of the body by a depression, an amphidelphic reproductive system with equally developed genital branches, uteri devoid of any "Z" differentiation, and tail conoid with digitate terminus, slightly bent ventrally. Morphology and morphometrics of these populations agree well with previous descriptions of the *X. setariae*/*X. vulgare* complex (Lamberti et al. 1995). Since 1972 when Cohn and Sher (1972) proposed the junior synonymy of *X. vulgare* with *X. setariae*, there has been an ongoing taxonomic controversy regarding acceptance or rejection of this synonymy (Loof and Luc 1990; Lamberti et al. 1995). Both species were synonymized mainly on the basis of overlaps in morphometric characters among populations of the two species (Cohn and Sher 1972; Loof and Luc 1990).

Although there are some molecular markers for both species (*X. vulgare* and *X. setariae*) in NCBI, originating from populations from Brazil and Florida (USA), 28S sequences of both species (AY601621 and DQ299513-DQ299514, respectively) were 100–99% identical, and differed only in 0 to 2 nucleotides. Unfortunately, the only ITS sequence

Table 2 Morphometrics of *Xiphinema* spp. from the rhizosphere of several crops and wild plants from Costa Rica. All measurements in µm and in the format: mean ± s.d. (range)[a]

	Xiphinema costarricense			*Xiphinema krugi*				*Xiphinema setariae*	
Locality (sample code) Character	Guayabo de Turrialba. Cartago[b] (ACC86)	San Vito de Coto Brus. Puntarenas (ACC50)	Santa Rosa. Limón (ACC46)	Pacayas de Alvarado, Cartago (ACC13)	Pueblo Nuevo. Palmira. Zarcero. Alajuela (ACC36)	Sucre, Ciudad Quesada. Alajuela (ACC47)	Matapalo. Savegre. Quepos. Puntarenas (HF001)	Matapalo. Savegre. Quepos. Puntarenas (HF002)	Pueblo Nuevo de Duacarí. Guácimo. Limón(ACC09)
n	3	3	1	6	3	3	4	4	4
L	2470 ± 26.0	2319 ± 132	2270 ± 43.2	2320 ± 65.5	2135 ± 24.5	2143 ± 24.5	2259 ± 232.5	2874 ± 132.9	2366 ± 62.9
	(2455–2500)	(2182–2446)	(2239–2332)	(2244–2385)	(2116–2163)	(2116–2163)	(1982–2625)	(2703–3089)	(2305–2441)
a	46.6 ± 2.8	48.7 ± 7.2	53.8 ± 1.7	45.7 ± 5.4	48.3 ± 3.9	44.1 ± 2.9	46.8 ± 1.7	69.1 ± 11.9	55.9 ± 3.7
	(45.0–50.0)	(43.0–56.8)	(52.8–56.3)	(41.3–52.7)	(44.1–51.9)	(40.9–44.1)	(44.0–48.6)	(57.1–84.5)	(52.3–61.0)
b	6.0 ± 0.6	5.3 ± 0.2	5.9 ± 0.8	5.1 ± 0.7	5.0 ± 0.4	5.0 ± 0.4	5.7 ± 0.5	7.3 ± 0.7	5.5 ± 0.5
	(5.5–6.7)	(5.1–5.5)	(5.0–7.0)	(4.3–5.7)	(4.6–5.3)	(4.6–5.0)	(5.2–6.6)	(6.6–8.2)	(5.1–6.1)
c	118.4 ± 22.5	87.9 ± 5.0	105.2 ± 12.4	71.3 ± 8.9	62.7 ± 4.0	59.8 ± 4.0	69.2 ± 3.4	58.7 ± 3.1	47.9 ± 4.5
	(104.5–144.4)	(83.0–93.0)	(90.7–118.7)	(62.4–80.4)	(59.3–66.9)	(59.6–64.4)	(64.7–72.9)	(55.4–64.4)	(44.7–54.4)
c´	0.7 ± 0.0	0.8 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	1.0 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	1.9 ± 0.2	1.8 ± 0.2
	(0.7–0.7)	(0.7–.08)	(0.5–0.7)	(0.9–1.0)	(1.0–1.0)	(0.9–0.9)	(0.8–1.0)	(1.6–2.1)	(1.6–2.0)
V	38.5 ± 4.3	38.8 ± 1.2	38.1 ± 0.2	34.0 ± 1.6	35.4 ± 0.9	34.1 ± 0.5	34.6 ± 2.6	40.2 ± 1.2	39.1 ± 0.8
	(33.5–41.0)	(38.0–40.0)	(37.9–38.3)	(32.6–36.8)	(34.5–36.3)	(33.5–34.5)	(33.0–39.0)	(39.0–42.0)	(38.0–40.0)
Odontostyle length	130.2 ± 2.7	132.7 ± 2.7	138.1 ± 0.9	128.5 ± 2.2	121.8 ± 3.5	126.2 ± 6.1	116.2 ± 3.1	121.5 ± 9.2	110.8 ± 1.0
	(128.0–135.0)	(130.5–135.5)	(137.5–139.5)	(125.0–129.5)	(118.0–123.5)	(120.0–134.0)	(113.0–120.0)	(114.0–136.0)	(110.0–112.0)
Odontophore length	76.7 ± 5.8	82.4 ± 2.4	76.9 ± 3.2	73.8 ± 2.3	71.7 ± 1.7	73.9 ± 4.0	68.2 ± 2.4	71.2 ± 8.5	66.9 ± 0.8
	(65.0–80.5)	(80.5–85.0)	(73.8–80.5)	(72.0–76.5)	(70.0–74.0)	(70.0–78.0)	(65.0–71.0)	(63.0–87.0)	(66.0–68.0)
Lip region width	8.2 ± 1.5	10.1 ± 0.8	9.7 ± 0.5	14.6 ± 0.8	12.6 ± 0.6	12.1 ± 0.9	13.8 ± 0.8	12.5 ± 0.5	13.0 ± 0.4
	(7.0–10.0)	(9.0–10.5)	(9.0–10.5)	(14.0–16.0)	(12.0–13.0)	(11.0–13.0)	(13.0–15.0)	(12.0–13.0)	(12.5–13.5)
Oral aperture-guiding ring	131.0 ± 14.7	126.1 ± 3.4	128.4 ± 3.3	113.9 ± 17.1	115.6 ± 6.3	118.4 ± 3.6	111.8 ± 5.1	92.8 ± 11.4	101.5 ± 1.9
	(122.5–148.0)	(122.5–129.0)	(126.0–133.0)	(83.0–119.5)	(110.0–122.0)	(115.5–118.5)	(107.0–120.0)	(73.0–103.0)	(100.0–104.0)
Tail length	21.3 ± 3.8	26.4 ± 0.2	21.8 ± 2.9	32.8 ± 3.2	33.7 ± 1.2	33.6 ± 2.0	30.8 ± 1.6	49.0 ± 1.7	49.6 ± 3.8
	(17.0–23.5)	(26.0–26.5)	(19.0–26.0)	(29.0–36.0)	(32.5–35.0)	(31.5–35.5)	(29.0–33.0)	(47.0–52.0)	(44.0–52.0)
J	9.5 ± 1.3	9.5 ± 2.1	9.5 ± 1.3	12.6 ± 1.2	13.0 ± 1.3	11.4 ± 0.5	11.4 ± 1.3	20.2 ± 2.7	18.4 ± 1.3
	(8.0–10.5)	(7.0–11.0)	(8.0–10.5)	(11.0–13.5)	(12.0–14.5)	(11.0–11.5)	(9.0–12.0)	(16.0–23.0)	(17.0–20.0)

[a] Abbreviations are defined in Jairajpuri and Ahmad (1992)

[b] Type locality

available is from *X. setariae* (AY430179); there are no ITS data for *X. vulgare*. Our morphometric data suggest that there is some intraspecific variability in body and odontostyle length, and in maximum body width. Furthermore, our data agree with Loof and Luc (1990) showing that no significant differences can be detected between *X. setariae* and *X. vulgare* in tail and hyaline region length, as well as c' ratio (Table 2). In combination, the molecular and morphometric evidence suggests that both species need to be considered as a single taxon. Since our populations of *X. setariae* from Costa Rica have morphological and morphometric characters that overlap the data ranges from both species, and since ITS sequences from these populations were 99% similar to that of *X. setariae* from Brazil (AY430179), we consider that Costa Rican populations should be identified as *X. setariae*. The occurrence of *X. setariae* is widespread in Central and South America (Kermarrec and Scotto La Massese 1972; Loof and Maas 1972; Hunt and Towle 1979; Chaves 1984; Lamberti et al. 1987; Alkemade and Loof 1990; Luc and Doucet 1990; Luc and Coomans 1992; Costa Manso et al. 1994; Bala and Rosein 1996; Doucet et al. 1998).

The amplification of D2-D3 expansion segments of 28S rDNA and ITS1 regions each yielded single fragments of approximately 900 bp and 1100 bp, respectively, based on gel electrophoresis. D2-D3 expansion segments of 28S rDNA of *X. costaricense* (KX931056-KX931059) and *X. krugi* (KX931060-KX931063) and ITS1for *X. costaricense* (KX931067-KX931069) were obtained for the first time in this study. D2-D3 sequences from *X. costaricense* (KX931056-KX931059) showed similarity values of 87% (differed in 90 to 100 nucleotides) with several *Xiphinema* spp., including *Xiphinema pyrenaicum* (GU725073), *Xiphinema meridianum* (KX062679), and *Xiphinema hispidum* (KC567181). The new ITS1 sequences obtained from *X. costaricense* (KX931067-KX931069) were not similar to other *Xiphinema* spp. deposited in GenBank. Only one accession with coverage value above 50% was found, namely, *Xiphinema variegatum* (DQ017144), which had a similarity value of 94% (36 nucleotides). The closest species in relation to D2-D3 segments of *X. krugi* (KX931060-KX931063) were *X. costaricense* (KX931056-KX931059), *X. pyrenaicum* (GU725073), *X. meridianum* (KX062679), and *Xiphinema hispanum* (KC062679), all with similarity values of 84% (122–130 nucleotides). Five new ITS1 sequences of *X. krugi* were obtained in this study and all with 99% similarity; however, these sequences differed from other ITS1 sequences of *X. krugi* deposited in GenBank; the similarity values varied from 95 to 97% (23–35 different nucleotides) with accessions DQ017152-DQ017154 (profile C), and to 66% (more than 400 different nucleotides) with accessions DQ017149-DQ017151 (profile A, B and D). Finally, D2-D3 and ITS1 sequences from *X. setariae* (KX931064-KX931066, KX931075-KX931077) matched well, 99% similarity (0–8 nucleotides), with the other accessions from *X. setariae* and *X. vulgare* deposited in GenBank (AY601621, DQ299514, AY430179, respectively).

In phylogenetic analyses of D2-D3 region using BI, *X. costaricense* (KX931056-KX931059) and *X. krugi* (KX931060-KX931063) were clustered in a well-supported clade (PP = 0.99) with the monodelphic *X. brasiliense* (AY6016016); however, no molecular data are available for other species of morphospecies Group 2 (Fig. 2a). However, the phylogenetic analysis using partial ITS1 clustered all species from the morphospecies Group 2, *X. costaricense*, *X. krugi*, and *X. longicaudatum,* within a non-supported (PP = 0.52) (Fig. 2b). ITS1 sequences of *X. costaricense* (KX931067-KX931069) clustered together in a well-supported clade (PP = 1.00) with *X. variegatum* (DQ017144) from Brazil (Fig. 2b). Since morphology and morphometrics of *X. variegatum* from original description (Siddiqi 2000) and descriptions of other populations from Brazil (Oliveira et al. 2003) fits very well with *X. costaricense*, both species are proposed here as junior synonyms. The different accessions of ITS1 sequences from *X. krugi* clustered in four different well-supported clades (PP = 0.99), one of them formed by Costa Rican populations (KX931070-KX931074) and three Brazilian populations belonging to RFLP profile C (Oliveira et al. 2006), and the other three subclades were formed only by populations from Brazil populations belonging to RFLP profiles A, B, and D (Oliveira et al. 2006) (Fig. 2b). These results supported the possibility that *X. krugi* is a species complex comprising at least four cryptic species with distance genotypes and/or cryptic species (Oliveira et al. 2006). However, additional molecular data (i. e. D2-D3 sequences) about Brazilian populations, will be necessary in order to resolve

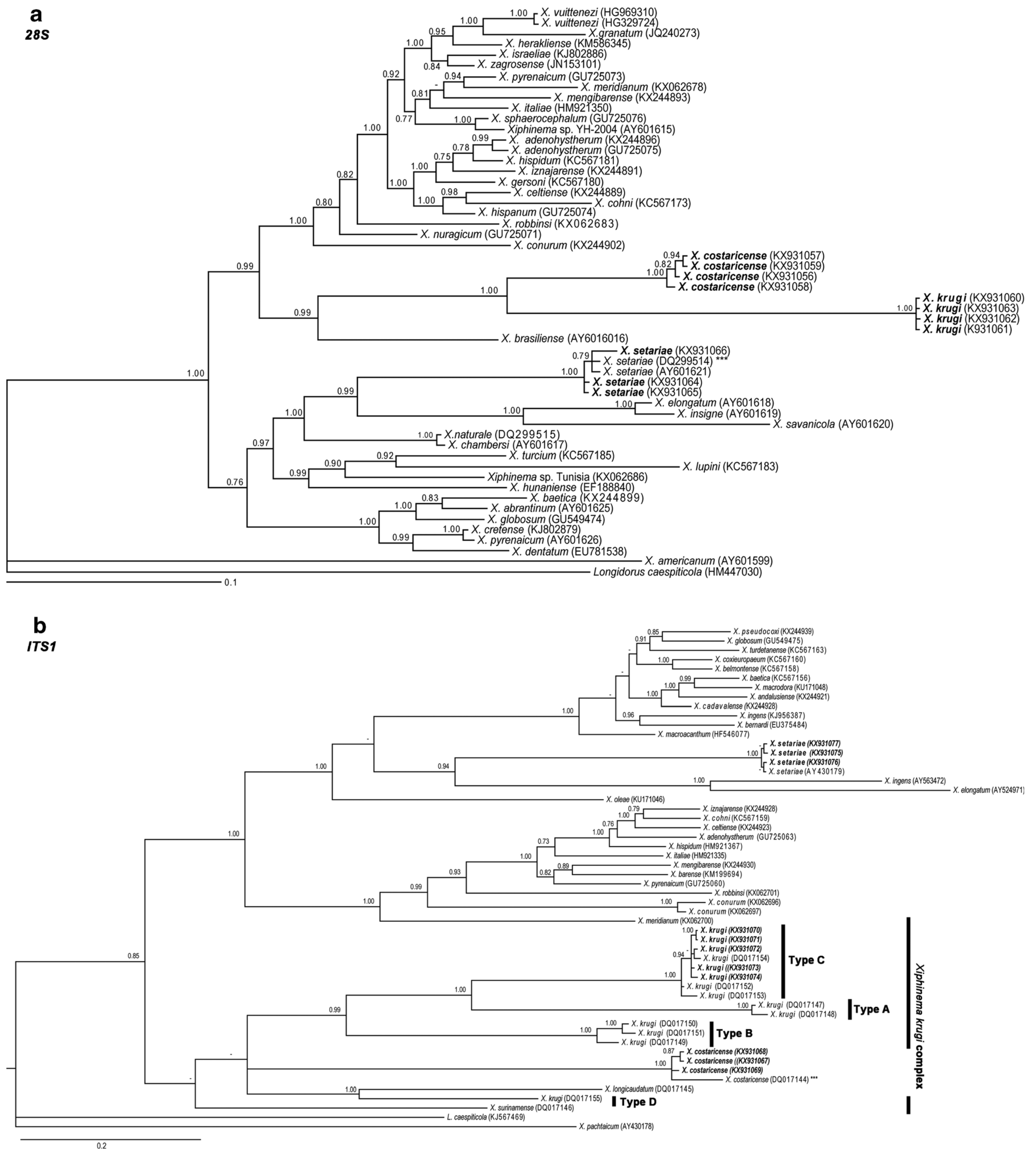

Fig. 2 Phylogenetic relationships of *Xiphinema costaricense* Lamberti and Tarjan 1974, *Xiphinema krugi* Lordello 1955, and *Xiphinema setariae* Luc 1958. Bayesian 50% majority rule consensus trees as inferred from D2-D3 expansion segments of 28S **a** and **b** ITS1 rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities more than 0.70 are given for appropriate clades. Newly obtained sequences in this study are in bold letters. (***) identified as *Xiphinema vulgare* in the GenBank by Oliveira et al. (2006)

this matter. Finally, *X. setariae* was phylogenetically related with other species from the morphospecies Group 7, including *X. vulgare, X. elongatum, X. insigne* and *X. savanicola,* all of which clustered in the same well-supported subclade in the D2-D3 and in the ITS1 trees (Fig. 2).

In summary, the present study provides new sequences for molecular markers (D2-D3 expansion segments of the 28S rRNA and ITS1) for precise and unequivocal diagnosis of *X. costaricense*, *X. krugi* and *X. setariae*. However, *X. krugi* needs more research associated with different populations in order to clarify this species complex. It clarifies their phylogenetic relationships with other *Xiphinema* spp.; that may help to facilitate quarantine regulations regarding the movement of plant material and soil.

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