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A new dagger nematode, *Xiphinema tica* n. sp. (Nematoda: Longidoridae), from Costa Rica with updating of the polytomous key of Loof and Luc (1990)

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Abstract A new dagger nematode, *Xiphinema tica* n. sp., is described and illustrated from several populations extracted from soil associated with several crops and wild plants in Costa Rica. The new dagger nematode is characterised by a moderate body size (3276–4240 μm), a rounded lip region, *ca* 13.5 μm wide, separated from body contour by a shallow depression, amphidial fovea large, stirrup-shaped, a moderately long odontostyle *ca* 135 μm long, stylet guiding ring located at *ca* 122 μm from anterior end, vulva almost equatorial (50–54%), well-developed Z-organ, with heavy muscularised wall containing in the most of specimens observed two moderately refractive inclusions variable in shape (from round to star-shaped), with uterine spines and crystalloid bodies; female tail short, dorsally convex-conoid, with rounded end and a small peg, with a c' ratio *ca* 0.8, bearing two or three pairs of caudal pores and male absent. The unique and novel uterine differentiation based on the coexistence of a well-developed Z-organ mixed with uterine spines and crystalloid bodies in *Xiphinema* prompted us to update and include this combination of characters in the polytomous key of Loof and Luc (1990). Integrative diagnosis was completed with molecular data obtained, using D2-D3 expansion segments of 28S rDNA, ITS1-rDNA, partial 18S–rDNA and the partial mitochondrial gene cytochrome c oxidase subunit 1 (*coxI*). The phylogenetic relationships of this species with other *Xiphinema* spp. indicated that *X. tica* n. sp. was monophyletic to the other species from the morphospecies Group 4, *Xiphinema oleae*.

Keywords Bayesian inference · *coxI* · D2-D3 · *ITS1* · *18S* · Longidorids · Phylogeny · rDNA · Taxonomy

Introduction

Dagger nematodes of the genus *Xiphinema* include a large number of ectoparasitic species with long life cycles that cause damage to an extensive range of wild and cultivated plants by their direct parasitism on root cells and by transmitting nepoviruses to a wide range of fruit and vegetable crops (Taylor and Brown 1997; Decraemer and Robbins 2007). *Xiphinema* was divided into two species groups due to the large morphological diversity, (i) the *Xiphinema americanum*-group, which comprises a complex of ~60 species; and the *Xiphinema* non-*americanum* group comprises a complex of ~220 species (Loof and Luc 1990; Luc et al. 1998; Lamberti et al. 2000; Coomans et al. 2001; Archidona-Yuste et al. 2016a). *Xiphinema* non-*americanum* group is mainly characterised by species with a long body and

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odontostyle length, the presence of different types of female genital tract resulting in a variable development of the two genital branches as well as different types of structures into the female genital branches, usually constituted with long uteri and uterine differentiation (including the 'Z-organ', spines and/or crystalloid structures in the tubular part of the uterus), and a wide diversity of tail shapes (Loof and Luc 1990; Archidona-Yuste et al. 2016a, b). However, although this species group comprise a wide morphological diversity, the high number of species with similar morphology makes species identification a difficult task (Loof and Luc 1990, 1993; Loof et al. 1996). In fact, no other genera belonging to the order Dorylaimida can compete with the *X.* non *americanum*-group in the diversity of the female reproductive system (Coomans et al. 2001), which, based on type of female reproductive system and tail shape, was divided into eight morphospecies groups in order to perform a correct identification species based on pragmatic diagnosis (Loof and Luc 1990). However, several recent taxonomic studies have revealed the existence of complex cryptic species within *X.* non *americanum*-group (Palomares-Rius et al. 2014), which creates the need for the application of integrative taxonomy based on the integrative use of different datasets, e.g. morphology and DNA sequences. In fact, the integrated use of different taxonomy approaches has been efficiently applied to the accurate identification of this complex species group (Oliveira et al. 2006; Barsi and De Luca 2008; Gutiérrez-Gutiérrez et al. 2010, 2012; Palomares-Rius et al. 2014; Archidona-Yuste et al. 2016a; Peraza-Padilla et al. 2016b). Molecular approaches using ribosomal DNA (rDNA) sequences including 18S, ITS regions, the D2 and D3 expansion segments of 28S rRNA, and the mitochondrial gene cytochrome c oxidase subunit 1 (*coxI*) have been shown to constitute a useful tool for molecular species identification, revealing phylogenetic relationships within Longidoridae (De Luca et al. 2004; He et al. 2005; Subbotin et al. 2014; Zasada et al. 2014; Archidona-Yuste et al. 2016a, b, c). Moreover, D2-D3 expansion segments of 28S rRNA and ITS rRNA have proved more efficient in species identification compared to partial 18S, as both these markers show more species variability with respect to partial 18S (Gutiérrez-Gutiérrez et al. 2013a, b; Archidona-Yuste et al. 2016a, b).

Nematological surveys of several regions of Costa Rica, conducted in 2015 and 2016, including both wild and cultivated crops, revealed a moderate to high soil infestations (15–105 nematodes per 500 cm^3 of soil) of five unidentified populations of *Xiphinema* fitting to the *X.* non-*americanum*-species group. Initial morphological observations showed that these populations seemed morphologically to match morphospecies Group 4 (with equal female genital branches and presence of a Z-organ) (Loof and Luc 1990). In addition, in all female specimens observed in these populations, high number of several structures including spines and crystalloid bodies were detected on the tubular part of both genital branches. Although the coexistence of spiniform structures and uterine differentiation including pseudo-Z-organ has been described and established within the *X.* non *americanum*-group (morphospecies Group 5), *Xiphinema* species characterized by the presence of uterine differentiation comprising a Z-organ plus uterine spines have not been detected to date (Loof and Luc 1990, 1993; Loof et al. 1996; Coomans et al. 2001; Archidona-Yuste et al. 2016a, b). That prompted us to undertake detailed observations using light microscopy and molecular characterisation indicating that these populations are conspecific and should be assigned to a new species. In the present study, we describe using integrative taxonomy this new species as *Xiphinema tica* n. sp.

The objectives of this study were: (1) to characterize morphologically and morphometrically the new *Xiphinema* non-*americanum*-species and compare with previous records; (2) to characterize molecularly the sampled populations using the D2-D3 expansion segments of the 28S rRNA, ITS1, partial 18S rRNA, and partial *coxI* gene sequences; and (3) to study the phylogenetic relationships of this *Xiphinema* species with available sequenced species.

Material and methods

Nematode population sampling and extraction

Nematode surveys were carried out during the rainy seasons in 2016 in cultivated and wild plants 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. Then samples were placed in labelled plastic bags, sealed and brought

Table 1 *Xiphinema tica* n. sp. sampled and sequenced from Costa Rica in this study

Locality, province	Host-plant	Sample code	GenBank accession			
			D2-D3	*ITS1*	*18S*	*coxI*
Chirraca, San Ignacio de Acosta, San José	Grapevine	ACC04	KY623485 KY623486	KY623493	KY623497	KY623500
Bajos de Jorco, San Ignacio de Acosta, San José	Citrus	ACC19	KY623487	–	–	–
Lagunilla, Santa Cruz, Guanacaste	Soursop	ACC32	KY623488 KY623489	KY623494	KY623498	KY623501
Sucre, Ciudad Quesada, Alajuela	Robust star grass	ACC47	KY623490	KY623495	–	KY623502
Sabanillas, San Ignacio de Acosta, San José	Coffeae	ACC81	KY623491 KY623492	KY623496	KY623499	KY623503

(−) Not obtained

back to the nematology laboratory where they were stored at 4 °C until processed. For each sample, 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).

Nematode morphological identification

Specimens for examination by light microscopy were killed by gentle heat, fixed in a solution of 8% formaldehyde +2% glycerol and processed to pure glycerine using Seinhorst's method (Seinhorst 1966). Specimens were examined using a Zeiss III compound microscope with Nomarski differential interference contrast up to a magnification of 1000X. Morphometric study of each nematode population included classical diagnostic features of the Longidoridae (i.e. de Man body ratios, lip region and amphid shape, oral aperture-guiding ring length, odontostyle and odontophore shape and length; Jairajpuri and Ahmad 1992). All measurements were expressed in micrometres (μm), unless otherwise indicated in the text. For line drawings of the new species, light micrographs were imported to CorelDraw Graphics Suite software version X8 and redrawn. All other abbreviations used are as defined in Jairajpuri and Ahmad (1992).

Nematode molecular identification

For molecular analyses and in order to avoid mistakes in the case of mixed populations in the same sample, two live nematodes from each sample were temporary mounted in a drop of 1 M NaCl containing glass beads (to avoid nematode crushing/damaging specimens) to ensure specimens conformed in form to the unidentified populations of *Xiphinema*. Morphometrics and photomicrographs recorded during this initial study were not used as part of the morphological study or analyses. Following morphological confirmation, the specimens were removed from the slides and DNA extracted (Archidona-Yuste et al. 2016a, b). Nematode DNA was extracted from single individuals and PCR assays were conducted as described by Castillo et al. (2003) and Archidona-Yuste et al. (2016c). The D2-D3 expansion segments of 28S rRNA was amplified using the D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) primers (De Ley et al. 1999). The ITS1 region was amplified using forward primer 18S (5′TTG ATT ACG TCC CTG CCC TTT-3′) (Vrain et al. 1992) and reverse primer rDNA1 (5′-ACG AGC CGA GTG ATC CAC CG-3′) (Cherry et al. 1997). The portion of 18S rRNA was amplified using primers 988F (5′-CTC AAA GAT TAA GCC ATG C-3′), 1912R (5′-TTT ACG GTC AGA ACT AGG G-3′), 1813F (5′-CTG CGT GAG AGG TGA AAT-3′) and 2646R (5′-GCT ACC TTG TTA CGA CTT TT-3′) (Holterman et al. 2006). Finally, the portion of the *coxI* gene was amplified as described by Lazarova et al. (2006) using the primers COIF (5′-GATTTTTTGGKCATCCWGARG-3′) and COIR (5′-CWACATAATAAGTATCATG-3′).

PCR cycle conditions for the ribosomal DNA markers were: one cycle of 94 °C for 2 min, followed by 35 cycles, of 94 °C for 30 s, an annealing temperature of 55 °C for 45 s, 72 °C for 1 min, and finally one cycle of 72 °C for 10 min. The cycle for mtDNA was as described by He et al. (2005): 95 °C for 10 min, five cycles at 94 °C for 30 s, 45 °C for 40 s, and 72 °C for

1 min, and a further 35 cycles at 94 °C for 30 s, 37 °C for 30 s, and 72 °C for 1 min, followed by an extension at 72 °C for10 min. PCR products were purified after amplification using ExoSAP-IT (Affmetrix, USB products), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for direct sequencing in both directions using the primers referred to above. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyzer; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Sequencing Kit V.3.1 (Applied Biosystems, Foster City, CA, USA), at the Stab Vida sequencing facilities (Caparica, Portugal). The newly obtained sequences were submitted to the GenBank database under accession numbers indicated on the phylogenetic trees and in Table 1.

Phylogenetic analyses

D2-D3 expansion segments of 28S rRNA, ITS1, partial 18S rRNA, and partial *coxI* sequences of different *Xiphinema* spp. belonging to *X.* non *americanum*-group from GenBank were used for phylogenetic reconstruction. Outgroup taxa for each dataset were chosen following previous published studies (He et al. 2005; Holterman et al. 2006; Gutiérrez-Gutiérrez et al. 2013b; Tzortzakakis et al. 2015; Archidona-Yuste et al. 2016a, b, c). Multiple sequence alignments of the different genes were made using the Q-INS-i algorithm of MAFFT V.7.205 (Katoh and Standley 2013), which accounts for secondary RNA structure. Sequence alignments were visualised using BioEdit (Hall 1999) and edited by Gblocks ver. 0.91b (Castresana 2000) in Castresana Laboratory 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 contiguous non-conserved positions: 8; minimum length of a block: 5; allowed gap positions: with half). Percentage similarity between sequences was calculated using a sequence identity matrix using BioEdit. For that, the score for each pair of sequences was compared directly and all gap or place-holding characters were treated as a gap. When the same position for both sequences had a gap it was not treated as a difference. Phylogenetic analyses of the sequence datasets were based on Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The best-fit model of DNA evolution was obtained using JModelTest V.2.1.7 (Darriba et al. 2012) with the Akaike Information Criterion (AIC). The best-fit model, the base frequency, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates in the AIC were then given to MrBayes for the phylogenetic analyses. Unlinked general time-reversible model with invariable sites and a gamma-shaped distribution (GTR + I + G) for the D2-D3 expansion segments of 28S rRNA and 18S rRNA and unlinked general time-reversible model with a gamma-shaped distribution (GTR + G) for the ITS1 region and the partial *coxI* gene were used. These BI analyses were run separately per dataset using four chains for 2×10^6 generations for the D2-D3, and 1×10^6 generations for the rest of molecular markers. A combined analysis of the three genes was not undertaken due to some sequences not being available for all species. The Markov Chains were sampled at intervals of 100 generations. Two runs were conducted 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 from all analyses were visualised using TreeView (Page 1996) and FigTree software V.1.42 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Xiphinema tica[1] **n. sp.** (Figs. 1, 2, 3 and 4, Table 2)

Female Body cylindrical, tapering towards anterior end, and open spiral-shaped upon fixation. Cuticle 2.0–3.0 μm at mid-body, and 8.5–14.0 μm at tail tip, marked by very fine superficial transverse striae mainly in tail region. Lip region rounded, separated from body contour by a shallow depression and 1.5–2.2 times as high as wide. Amphidial fovea stirrup-shaped; aperture extending for 65.2–84.6% of lip region width and located slightly anterior to depression marking lip region. Two pairs of body pores present between anterior end and guiding ring. Odontostyle typical of genus, long and

[1] The species epithet refers to Costa Rica, the geographic origin of the nematode.

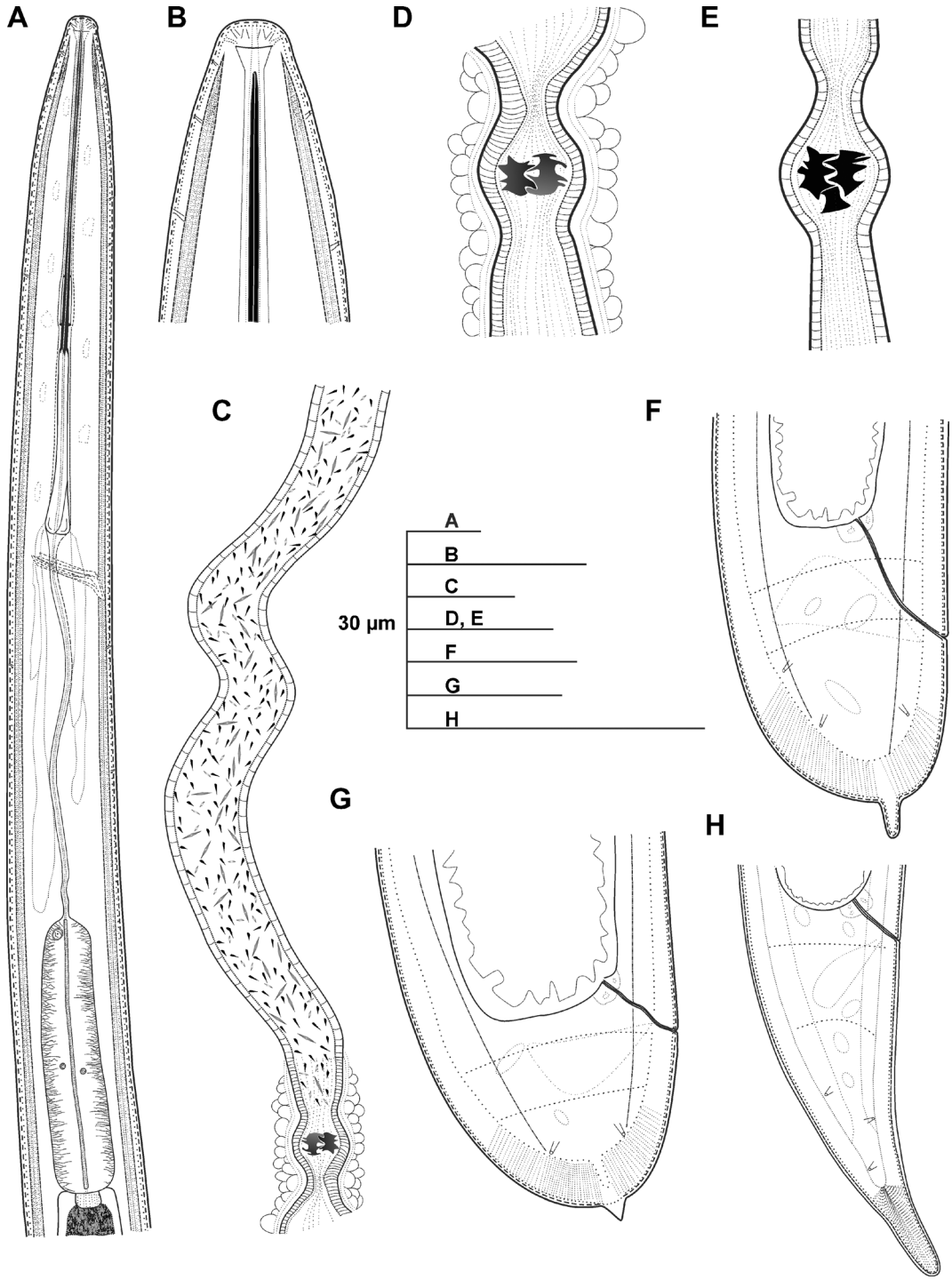


Fig. 1 Line drawings of *Xiphinema tica* n. sp. (**a**) Female neck region. (**b**) Female lip region. (**c**) Detail of genital tracks showing Z-organ, uterine spines and crystalloids bodies. (**D-E**) Detail of Z-organ. (**f-g**) Female tail regions. (**h**) First-stage juvenile tail region

slender, 10.1 (8.7–11.2) times lip region diameter or 5.5 (4.9–6.5) times odontophore length long. Odontophore with moderately developed flanges, 12.5 (12.0–13.0) µm wide. Guiding ring double, and guiding sheath 11–14 µm long depending on degree of protraction/retraction of stylet. Pharynx consisting of an anterior

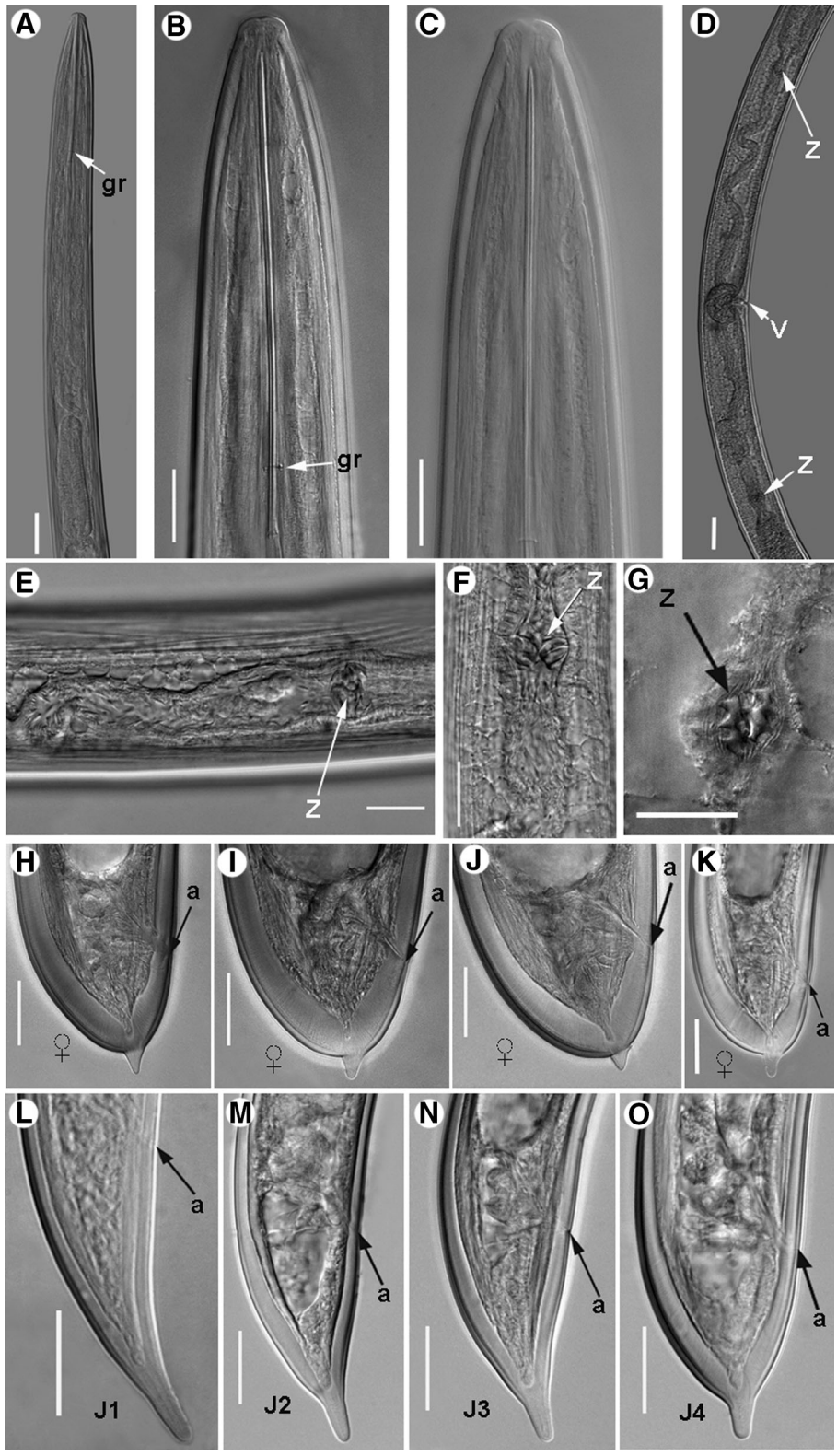


Fig. 2 Light micrographs of *Xiphinema tica* n. sp. (**a**) female neck region. (**b**-**c**) Female lip region. (**d**) Detail of genital tracks showing Z-organ. (**e**-**g**) Detail of Z-organ, uterine spines and crystalloids bodies. (**h**-**k**) Female tail regions. (**l**-**o**) First-, second-, third-, and fourth-stage juvenile tails (J1-J4), respectively. Abbreviations: a = anus; gr = guiding ring; v = vulva; z = Z-organ. Scale bars: **a, d** = 40 μm; **b, c, e**-**o** = 20 μm

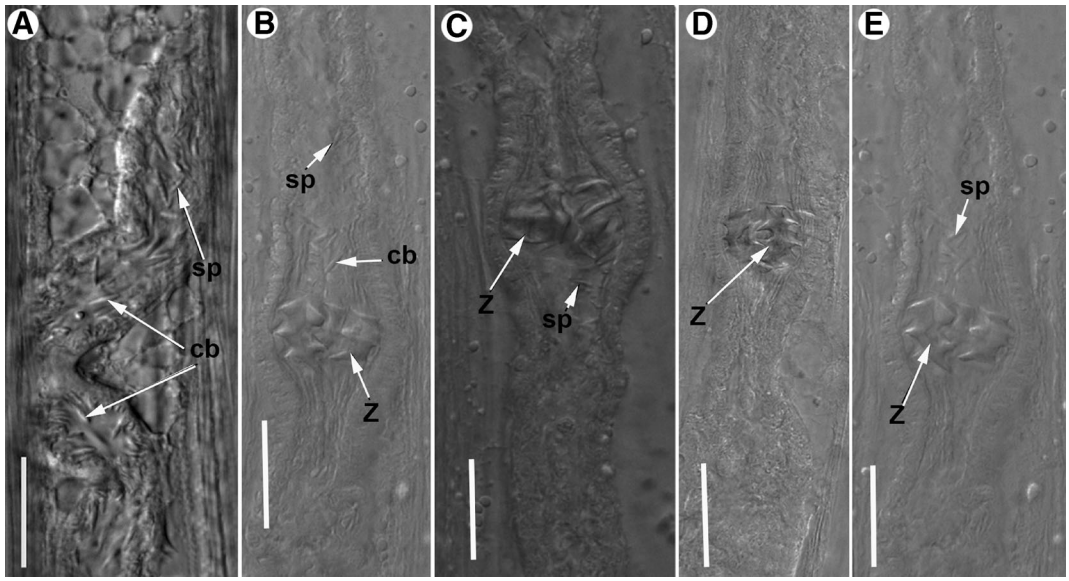


Fig. 3 Light micrographs of *Xiphinema tica* n. sp. (**a**-**e**) Details uterine structures showing Z-organ, uterine spines and crystalloids bodies. Abbreviations: cb = crystalloid body; sp. = spines; Z = Z-organ. Scale bars: **a**-**e** = 20 μm

slender narrow part, 327 (317–328) μm long, extending to a terminal pharyngeal bulb, 112 (110–115) μm long, comprising three nuclei typical of genus. Nucleus of dorsal gland (DN) large, located at 15.8% (14.7–19.0%) of pharyngeal bulb length, being larger than the two ventrosublateral nuclei (S1 N), which located at 53.8% (53.0–54.5%) of terminal bulb length (location of gland nuclei according to Loof and Coomans 1972). Cardia conoid-rounded, 5.0–6.0 μm long. Intestine simple, prerectum of variable length, 10.2–16.4 times anal body diameter long, and rectum 0.6–0.8 times anal body diameter long. Female reproductive system didelphic–amphidelphic with branches about equally developed, with vulva slit-like situated slightly posterior to mid body. Each branch composed of an ovary 54–69 μm long ovary, a reflexed oviduct 102–140 μm long with well-developed *pars dilatata oviductus* separated from uterus by a well distinguished sphincter. Uterus tripartite, 250–332 μm long, composed of *pars dilatata uteri* followed by a tubular part containing in the proximal

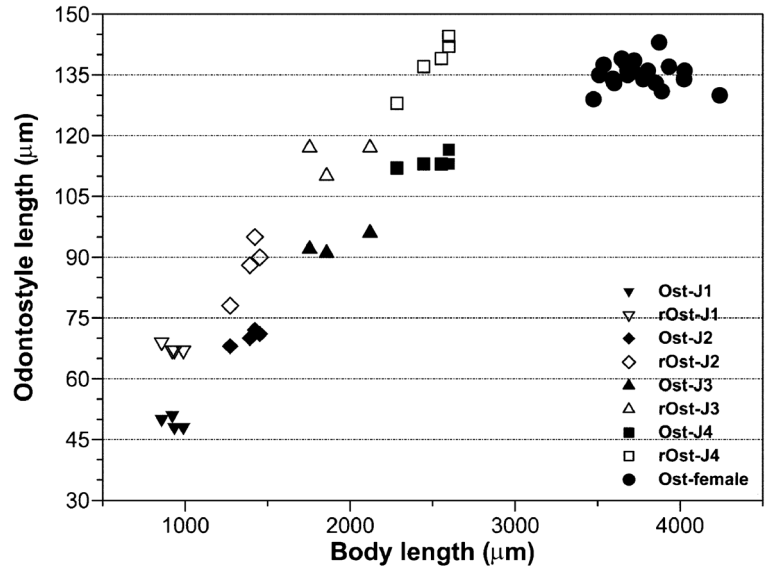


Fig. 4 Relationship of body length to length of functional and replacement odontostyle (Ost and rOst, respectively); length in all developmental stages from first-stage juveniles (J1) to mature females of *Xiphinema tica* n. sp.

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

Locality (sample code)	Chirraca. San Ignacio de Acosta, San José. *Paratypes* (ACC04)						Bajos de Jorco, San Ignacio de Acosta, San José (ACC19) Females	Lagunilla, Santa Cruz, Guanacaste (ACC32) Female	Sucre, Ciudad Quesada, Alajuela (ACC47) Females	Sabanillas, San Ignacio de Acosta, San José (ACC81) Females
Character	Holotype	Females	J1	J2	J3	J4				
n	1	21	4	4	2	5	2	2	3	3
L	3682	3750 ± 198 (3475–4240)	926 ± 54 (858–989)	1385 ± 79 (1273–1452)	(1755, 2121)	2496 ± 134 (2284–2599)	(3276, 3471)	(3687, 3760)	3609 ± 232 (3354–3807)	3756 ± 151 (3445–3928)
a	57.0	58.7 ± 7.4 (47.2–74.8)	42.2 ± 3.3 (38.0–45.6)	42.9 ± 7.3 (31.9–47.7)	(41.9, 51.2)	62.9 ± 7.3 (54.1–71.4)	(47.6, 62.0)	(63.1, 65.6)	51.8 ± 4.0 (48.2–56.1)	59.8 ± 5.2 (56.3–65.8)
b	7.8	8.5 ± 1.1 (6.6–11.1)	3.8 ± 0.2 (3.6–4.1)	4.7 ± 0.4 (4.2–5.0)	(4.7, 6.4)	6.2 ± 0.6 (5.8–7.2)	(7.3, 8.1)	(7.6, 8.1)	7.8 ± 0.9 (6.8–8.5)	8.6 ± 1.2 (7.5–9.9)
c	105.2	112.3 ± 13.6 (87.6–141.4)	24.0 ± 4.8 (18.6–28.3)	36.5 ± 5.4 (32.3–43.5)	(34.1, 53.7)	59.1 ± 2.7 (56.0–62.0)	(102.1, 104.0)	(91.4, 103.3)	102.1 ± 11.1 (89.4–110.0)	105.2 ± 6.4 (99.6–112.2)
c′	0.8	0.8 ± 0.1 (0.7–1.0)	3.2 ± 0.6 (2.7–3.8)	2.2 ± 0.2 (1.8–2.3)	(1.4, 1.9)	1.1 ± 0.2 (0.8–1.3)	(0.8, 0.8)	(0.8, 0.8)	0.7 ± 0.1 (0.6–0.8)	0.8 ± 0.01 (0.8–0.9)
V	53.0	52.3 ± 1.2 (50.0–54.5)	-	-	-	-	(52.0, 54.0)	(51.6, 53.6)	52.6 ± 1.2 (51.0–53.0)	51.6 ± 0.6 (51.0–52.0)
Odontostyle length	135.0	135.3 ± 3.1 (129.0–143.0)	49.2 ± 1.5 (47.5–51.0)	70.3 ± 1.4 (68.0–71.5)	(90.5, 96.0)	113.3 ± 1.9 (112.0–116.5)	(132.5, 134.0)	(134.0, 135.0)	129.9 ± 1.4 (128.0–131.0)	137.1 ± 3.5 (134.0–141.0)
Odontophore length	74.0	74.6 ± 1.9 (72.0–79.0)	36.5 ± 1.6 (35.0–39.0)	43.6 ± 2.6 (41.0–46.0)	(56.5, 61.0)	65.0 ± 3.8 (59.0–68.0)	(72.0, 72.5)	(76.0, 79.3)	78.1 ± 1.8 (76.0–79.0)	71.5 ± 3.9 (68.0–76.0)
Total stylet length	209	209.9 ± 3.0 (206.0–218.0)	-	-	-	-	(204.5, 206.5)	(206.5, 214.5)	208 ± 1.3 (207.0–209.5)	208.6 ± 5.4 (202.5–212.0)
Replacement odontostyle length	-	-	67.6 ± 0.8 (67.0–69.0)	87.7 ± 6.9 (78.0–94.5)	(110.0, 117.0)	138.3 ± 6.2 (128.0–144.5)	-	-	-	-
Lip region width	12.0	13.5 ± 0.9 (12.0–15.0)	7.8 ± 0.2 (7.5–8.0)	8.8 ± 0.2 (8.5–9.0)	(9.0, 10.0)	11.3 ± 0.5 (11.0–12.0)	(13.0, 14.0)	(12.0, 13.0)	12.8 ± 0.5 (12.0–13.0)	13.6 ± 0.5 (13.0–14.0)
Oral aperture-guiding ring	123.0	122.3 ± 7.2 (105.0–132.0)	41.1 ± 1.2 (40.0–43.0)	61.2 ± 2.4 (59.0–64.5)	(76.8, 84.9)	104.5 ± 1.7 (102.0–106.0)	(81.0, 82.5)	(117.5, 125.3)	121.5 ± 3.9 (117.5–125.0)	125.4 ± 2.3 (123.0–128.0)
Tail length	35.0	33.8 ± 3.5 (37.0–50.5)	39.5 ± 6.4 (33.0–46.0)	38.6 ± 6.4 (33.0–45.0)	(39.5, 42.0)	42.2 ± 1.9 (41.0–45.5)	(31.5, 34.0)	(34.0, 37.5)	35.5 ± 1.8 (34.0–37.5)	35.7 ± 1.2 (35.0–37.0)
J	11.0	10.6 ± 1.5 (8.5–14.0)	10.7 ± 0.5 (10.0–11.0)	15.9 ± 1.0 (15.0–17.0)	(13.0, 14.5)	12.1 ± 1.1 (10.5–13.5)	(10.0, 11.5)	(9.5, 13.5)	11.7 ± 2.1 (9.5–13.5)	11.1 ± 1.7 (9.0–12.5)

* Abbreviations are defined in Jairajpuri and Ahmad (1992)

part a well-developed Z-organ made by strong circular wall heavily muscularised and enlarged at the centre where are found two, but three in some specimens observed, cuticular and refractive apophyses attached to the wall, variable in shape (from round to star-shaped) which could fit among them (Figs. 1, 2, 3). Numerous small spiniform structures mixed with crystalloid bodies, variable in length but the most of them of small size, distributed over the entire length of the tube-like portion of the uterus (Figs. 1, 2, 3). No sperm observed in the female genital tract. Ovejector well developed, (22–32) μm wide, vagina perpendicular to body-axis, extending for 41–43% of corresponding body diameter, and vulva as a transverse slit. Tail short, almost as long as anal body diameter, hemispherical to dorsally convex-conoid, with a present blind canal and bearing two or three caudal pores. Tail contour rounded but ending in a small peg, 5.0–6.5 μm long, displaced to ventral side.

Male: not detected.

Juveniles All four juvenile stages (first-, second-, third- and fourth-stage) were identified using morphological characters such as body length, length of replacement and functional odontostyle (Robbins et al. 1996). Juveniles similar to adults apart from developed reproductive system, shorter body length, tail shape and presence of replacement odontostyle. Tail becomes progressively shorter and stouter in each moult (Figs. 2, 3, Table 2). First-juvenile stage was characterised by the replacement odontostyle tip close to base of functional odontostyle and located at level of odontophore. In J2-J4, replacement odontostyle located at some distance from odontophore base. J1 tail conoid and around 3 times as long as the anal body diameter (Fig. 4, Table 2). J2 and J3 tail broadly conoid, and J4 tail more rounded and comparable to that of female in shape but longer peg (Fig. 4, Table 2).

Type host and locality

Xiphinema tica n. sp. was found in the rhizosphere of grapevine (*Vitis vinifera* L.) at Chirraca, San Ignacio de Acosta, San José province, Costa Rica (9°06′42.91″ N latitude, 84°10′36.33″W longitude, altitude 940 m a.s.l). The species was also detected in the rhizosphere of citrus, soursop, robust star grass, and coffee plants, at San Ignacio de Acosta, San José; Lagunilla, Santa Cruz, Guanacaste; Sucre, Ciudad Quesada, Alajuela; and Sabanillas, San Ignacio de Acosta, San José, in Costa Rica, respectively.

Type material

Holotype female and 16 female paratypes deposited in the Nematode Collection at the Laboratorio de Nematología, Escuela de Ciencias Agrarias, Universidad Nacional, Heredia, Costa Rica. Two female paratypes were deposited in each Nematode collection situated in the Institute for Sustainable Agriculture, CSIC, Córdoba, Spain; Royal Belgian Institute of Natural Sciences, Brussels, Belgium; USDA Nematode Collection, Beltsville, MD, USA; and the Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Bari, Italy.

Diagnosis and relationships

Xiphinema tica n. sp. is an apparently parthenogenetic species characterised by a lip region rounded-hemispherical, separated from body contour by a shallow depression; odontostyle and odontophore 128–143 and 68–79 μm long, respectively; vulva position at 50–54%; well-developed Z-organ, with heavy muscularised wall, and two moderately refractive inclusions variable in shape in the most of specimens observed, with uterine spines and crystalloids bodies; female tail short, dorsally convex-conoid, with rounded end and a small peg. Specific D2-D3, ITS1, 18S rRNA and *cox1* sequences were deposited in GenBank with accession numbers KY623485-KY623492, KY623493-KY623496, KY623497-KY623499, and KY623500-KY623503, respectively.

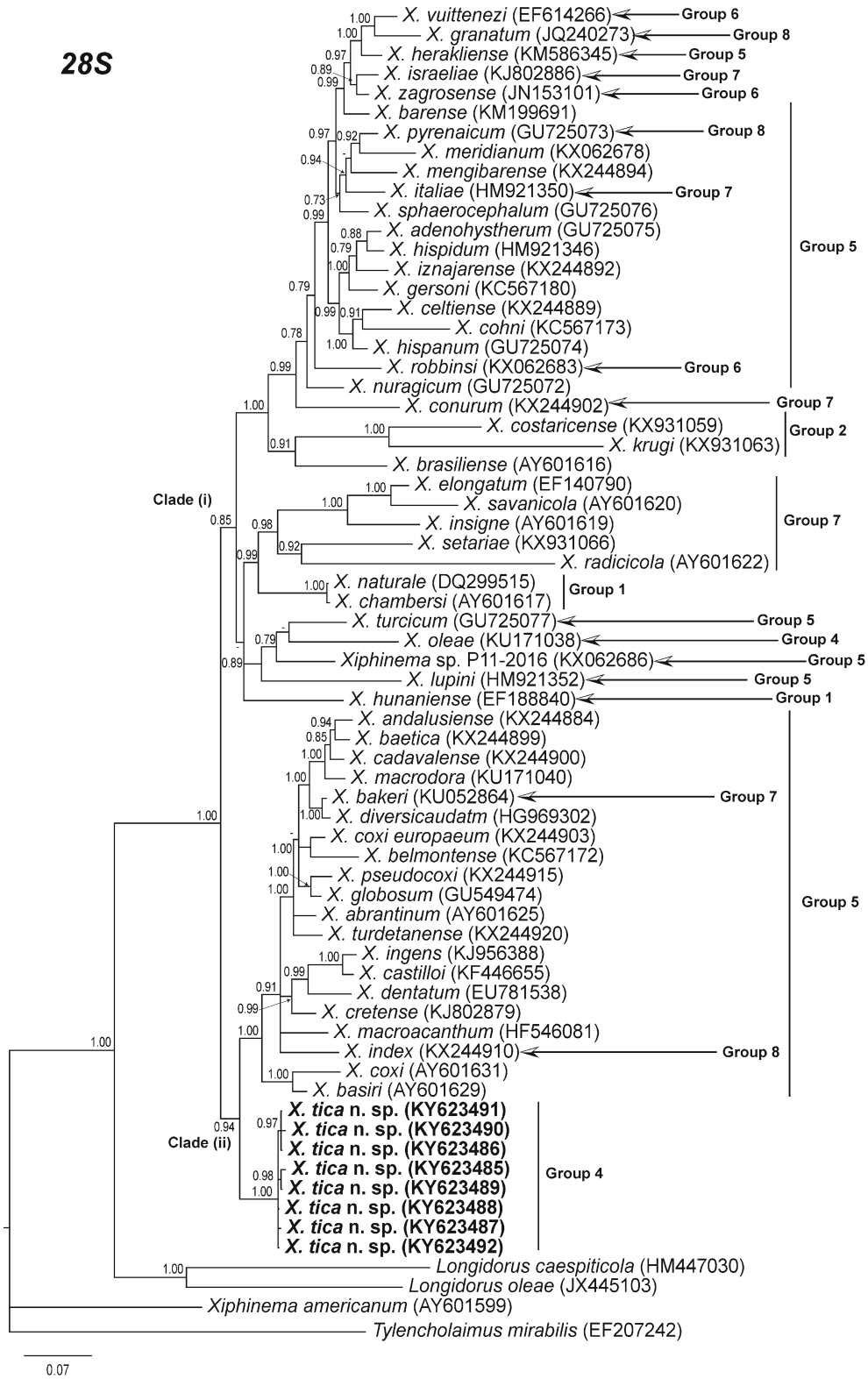
In order to clarify the species diagnosis within *X.* non *americanum*-group, Loof and Luc (1990) established a polytomous key composed by a total of 12 codes (A-L) corresponding to morphological diagnostic characters including type of female reproductive system, uterine differentiation, nematode body and stylet length or/and female and juvenile tail shape. Due to the exceptional coexistence of "true" Z-organ and spiniform structures observed in the female reproductive system of *Xiphinema tica* n. sp., the code corresponding to uterine differentiation characteristic should be updated and restructured. Then, the new Code B (uterine differentiation) will be composed by the following categories: 1) Z-organ present; **1 + 3) Z-organ plus spines present**; 2)

Z-pseudo-organ present; 2 + 3) Z-pseudo-organ plus spines present; 3) uterine spines present; and 4) no uterine differentiation (in bold new sub-code). Therefore, according to the polytomous key by Loof and Luc (1990), the supplements by Loof and Luc (1993) and Loof et al. (1996), and the comments above described, the new species belongs to the *X.* non-*americanum* Group 4 and has the following specific α-numeric codes: A4, B1 + 3, C5a, D6, E6, F4, G3, H2, I4, J5, K3, L1. Morphologically, *X. tica* n. sp. belongs to the *X.* non-*americanum* Group 4 according to Loof and Luc (1990), but can be differentiated from all know species of this morphotype group as well as within the genus by a combination of morphological characters, but particularly by the coexistence of a well-developed Z-organ, uterine spines and crystalloids bodies. However, based on the combination of morphological diagnosis characters including female tail shape and/or nematode body length, within species of *X.* non-*americanum* Group 4, *X. tica* n. sp. closely resembles with *Xiphinema phoenicis* Loof 1982, *Xiphinema pirinense* Mincheva et al. 2008, and *Xiphinema tropicale* Zullini 1973. Firstly, *X. tica* n. sp. differs from *X. phoenicis* in having a slightly shorter odontostyle length (128–143 μm vs 142–172 μm), female tail shape (hemispherical/convex-conoid with rounded end and a small peg vs dorsally convex-conoid with clearly offset terminal long peg), and lower c′ ratio (0.6–1.0 vs 1.1–1.6) (Loof 1982). On the other hand, the new species differs mainly from *X. pirinense* by having posterior vulva position (50.0–54.5 vs 41.8–47.5%), a slightly longer odontostyle length (129.0–143.0 vs 116.0–134.5 μm), lower c′ ratio (0.7–1.0 vs 1.0–1.3), slightly higher c ratio (87.6–141.4 vs 64.9–99.5), and female tail shape (hemispherical/convex-conoid vs dorsally convex-conoid) (Mincheva et al. 2008). Finally, it differs mainly from *X. tropicale* by having a longer body and odontostyle length (3.3–4.2 mm, 128–143 μm versus 2.2–2.6 mm, 110–122 μm; respectively), posterior vulva position (50–55% versus 37–39%), and higher a ratio (47.2–74.8 versus 34–45) (Zullini 1973). In addition, the new species also resembles in some characters to *Xiphinema oleae* Archidona-Yuste, Navas-Cortés, Cantalapiedra-Navarrete, Palomares-Rius & Castillo 2016 from which differs by a shorter body and odontostyle length (3.3–4.2 mm, 128–143 μm versus 4.3–5.3 mm, 136–149 μm; respectively), female tail shape in lacking peg, and lower a ratio (47.2–74.8 versus 80.9–106.6) (Archidona-Yuste et al. 2016b).

Molecular characterization and phylogenetic relationships of *Xiphinema tica* n. sp. with other *Xiphinema* species

The amplification of D2-D3 expansion segments of 28S rRNA, ITS1 region, the partial 18S rRNA and partial *coxI* regions yielded single fragments of ~800 bp, 1030 bp, 1600 bp, and 400 bp, respectively, based on gel electrophoresis. Sequences from *X. tica* n. sp. matched well with the *X.* non-*americanum* group spp. sequences deposited in GenBank being all of them clearly different. Eight new D2-D3 of 28S rRNA gene sequences were obtained in the present study. D2-D3 expansion segments of 28S rRNA sequences of *X. tica* n. sp. (KY623485-KY623492) showed a 93–92% similarity values (differed in a range from 57 to 66 nucleotides) with several *Xiphinema* spp. such as *X. index* Thorne and Allen 1950 (HM921348), *X. cretense* Tzortzakakis et al. 2014 (KJ802879), *X. diversicaudatum* (Micolezky 1927) Thorne 1939 (HG969302) and *X. basiri* Siddiqi 1959 (AY601629). ITS1 showed some similarity, 70%, with *X. bakeri* Williams 1961 (AF511426), *X. ingens* Luc and Dalmasso 1963 (KJ956387), *X. diversicaudatum* (AY430183) and *X. globosum* Sturhan 1978 (GU549475). The partial 18S rRNA sequences for *X. tica* n. sp. (KY623497-KY623499) showed high similarity (99% similar) with several *X.* non-*americanum*-group spp. deposited in GenBank, including *X. oleae* (KU171051) and *X. ifacolum* (Luc & de Guiran 1960) Luc 1961 (AY297826) from the morphospecies Group 4. Finally, the most related species for the new four *coxI* sequences from *X. tica* n. sp. obtained in this study were *X. index* (HM921388), *X. chambersi* Lamberti et al. 2002 (KU764419) and *X. diversicaudatum* (KF292302) which showed a similarity values of 80–79% (differed from 68 to 74 nucleotides). Intraspecific sequence diversity (uncorrected p-distance) of ribosomal markers among the studied populations was small. In fact, D2-D3 and ITS1 varied from 0 to 0.5%, and even no intraspecific sequence diversity for the 18S rRNA. However, higher diversity values were detected for the partial *coxI* than D2-D3 and

Fig. 5 Phylogenetic relationships within the *Xiphinema* non-*americanum*-group complex. Bayesian 50% majority rule consensus tree as inferred from D2 and D3 expansion segments of 28S rRNA sequence alignment under the general time-reversible model with correction for invariable sites and a gamma-shaped distribution (GTR + I + G). Posterior probabilities greater than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in bold. Scale bar = expected changes per site


28S
X. vuittenezi (EF614266)
Group 6
X. granatum (JQ240273)
Group 8
X. herakliense (KM586345)
Group 5
X. israeliae (KJ802886)
Group 7
X. zagrosense (JN153101)
Group 6
X. barense (KM199691)
X. pyrenaicum (GU725073)
Group 8
X. meridianum (KX062678)
X. mengibarense (KX244894)
X. italiae (HM921350)
Group 7
X. sphaerocephalum (GU725076)
X. adenohystherum (GU725075)
X. hispidum (HM921346)
X. iznajarense (KX244892)
X. gersoni (KC567180)
X. celtiense (KX244889)
X. cohni (KC567173)
X. hispanum (GU725074)
X. robbinsi (KX062683)
Group 6
X. nuragicum (GU725072)
X. conurum (KX244902)
Group 7
Group 5
X. costaricense (KX931059)
X. krugi (KX931063)
Group 2
X. brasiliense (AY601616)
Clade (i)
X. elongatum (EF140790)
X. savanicola (AY601620)
X. insigne (AY601619)
X. setariae (KX931066)
X. radicicola (AY601622)
Group 7
X. naturale (DQ299515)
X. chambersi (AY601617)
Group 1
X. turcicum (GU725077)
Group 5
X. oleae (KU171038)
Group 4
Xiphinema sp. P11-2016 (KX062686)
Group 5
X. lupini (HM921352)
Group 5
X. hunaniense (EF188840)
Group 1
X. andalusiense (KX244884)
X. baetica (KX244899)
X. cadavalense (KX244900)
X. macrodora (KU171040)
X. bakeri (KU052864)
Group 7
X. diversicaudatm (HG969302)
X. coxi europaeum (KX244903)
X. belmontense (KC567172)
X. pseudocoxi (KX244915)
X. globosum (GU549474)
X. abrantinum (AY601625)
X. turdetanense (KX244920)
X. ingens (KJ956388)
X. castilloi (KF446655)
X. dentatum (EU781538)
X. cretense (KJ802879)
X. macroacanthum (HF546081)
X. index (KX244910)
Group 8
Group 5
X. coxi (AY601631)
X. basiri (AY601629)
Clade (ii)
X. tica n. sp. (KY623491)
X. tica n. sp. (KY623490)
X. tica n. sp. (KY623486)
X. tica n. sp. (KY623485)
X. tica n. sp. (KY623489)
X. tica n. sp. (KY623488)
X. tica n. sp. (KY623487)
X. tica n. sp. (KY623492)
Group 4
Longidorus caespiticola (HM447030)
Longidorus oleae (JX445103)
Xiphinema americanum (AY601599)
Tylencholaimus mirabilis (EF207242)
0.07

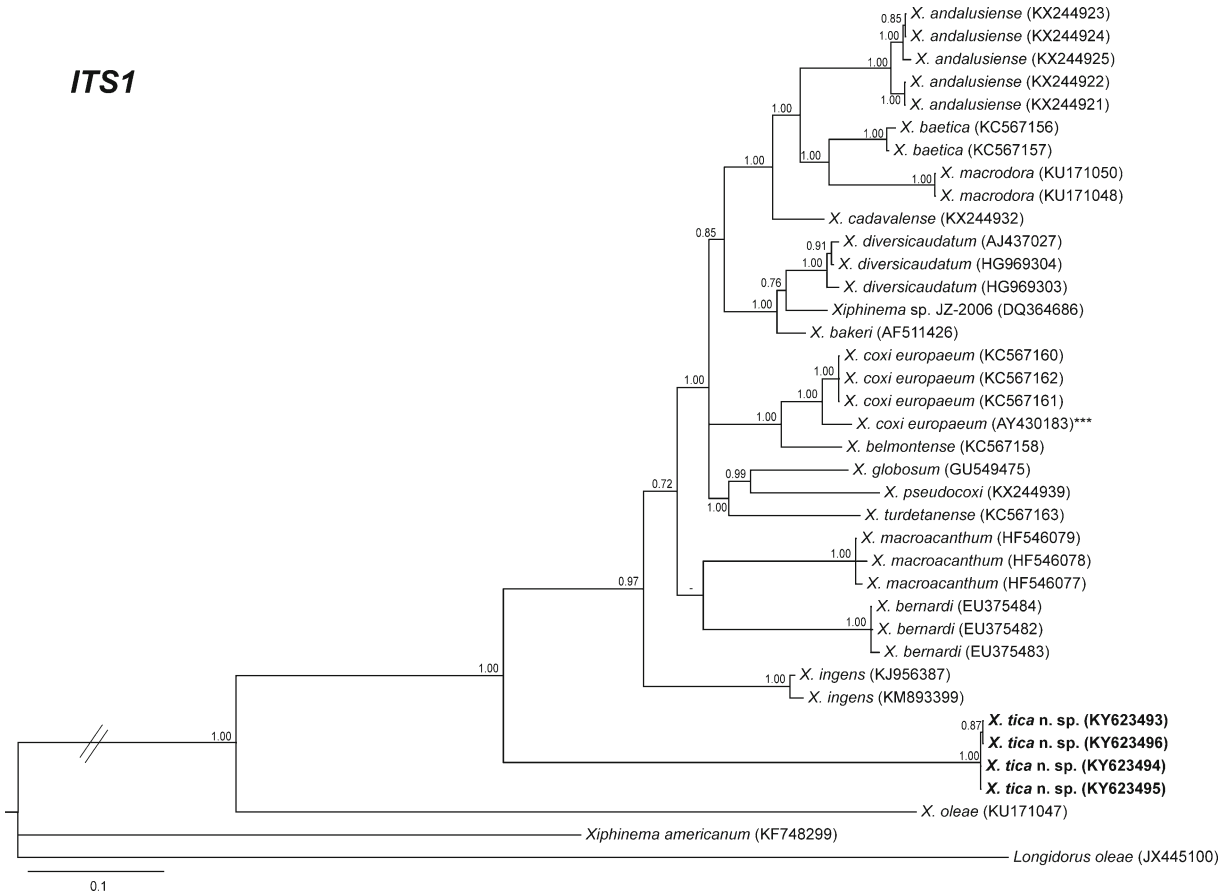


Fig. 6 Phylogenetic relationships within the *Xiphinema* non-*americanum*-group complex. Bayesian 50% majority rule consensus tree as inferred from ITS1 rRNA sequence alignment under the unlinked general time-reversible model with a gamma-shaped distribution (GTR + G). Posterior probabilities greater than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in bold. Scale bar = expected changes per site(***) Originally identified as *X. diversicaudatum*, but considered as *X. coxi europaeum* by Gutiérrez-Gutiérrez et al. (2013b)

ITS1 sequences, which ranged from 0 to 1.1%, being 0% within the population from San José, and then, 1.1% among this one with the rest of populations. Only two species from morphospecies Group 4 were found in GenBank, namely *X. oleae,* with D2-D3, ITS1 and 18S available data, and *X. ifacolum* only with 18S available data. Sequence similarity values among these species using different markers were low, 88% (differed in 93 nucleotides and 19 indels) for the D2-D3 and 80% (with a low coverage value, 60%) for the ITS1. 18S rRNA showed 99% of sequence similarity (differing in 13 nucleotides and 2 indels, and 25 nucleotides and 0 indels for *X. oleae* and *X. ifacolum*; respectively). Unfortunately, no data from *coxI* respect to *X. oleae* or *X. ifacolum* was available.

Phylogenetic relationships among *X.* non-*americanum*-group species inferred from analyses of D2-D3 expansion segments of 28S rRNA, ITS1, the partial 18S rRNA and *coxI* gene sequences using BI are given in Figs 5, 6, 7, and 8, respectively. Figure 5 shows the phylogenetic position of *X. tica* n. sp. based on D2-D3 expansion segments of 28S rRNA gene of a multiple-edited alignment (68 sequences) of 760 total characters. The 50% majority rule BI consensus tree of *Xiphinema* spp. showed two moderately supported major clades (PP = 0.85 and 0.94) (Fig. 5). Clade (i) was formed by 26 species, including species from all morphospecies Groups established by Loof and Luc (1990) except to morphospecies Group 3, since no molecular data were available. Clade (ii) grouped 16 species, 13 belonging to morphospecies Group 5, one species from Group 7, *X. bakeri* (KU052864), one from Group 8, *X. index* (KX244910), and finally, one species from the

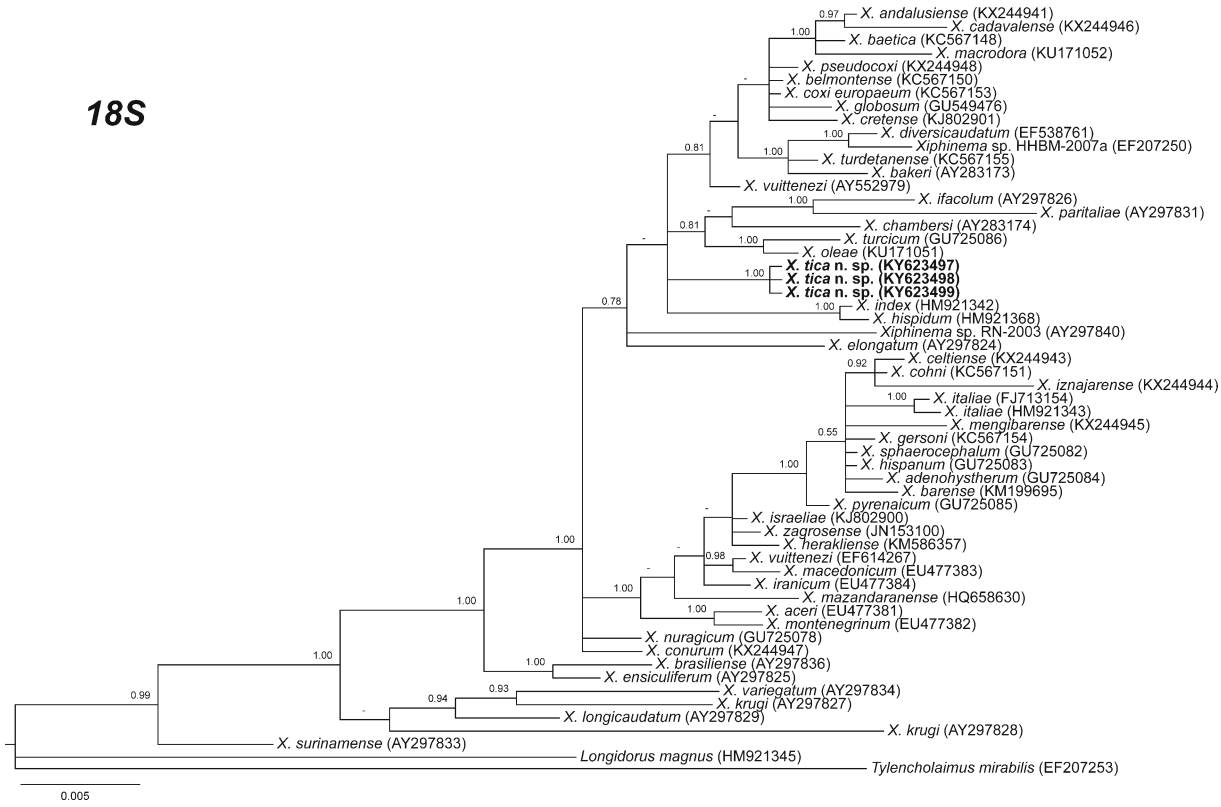


Fig. 7 Phylogenetic relationships within the *Xiphinema* non-*americanum*-group complex. Bayesian 50% majority rule consensus tree as inferred from 18S rRNA gene sequence alignment under the general time-reversible model of sequence evolution with correction for invariable sites and a gamma-shaped distribution (GTR + I + G). Posterior probabilities greater than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in *bold*. Scale bar = expected changes per site

morphospecies Group 4, which correspond to the new species described in this study *X. tica* n. sp. (KY623485-KY623492). *Xiphinema tica* n. sp. was positioned in a basal position in the tree and clearly separated from the rest of species from *X.* non-*americanum*-group. Difficulties were experienced with alignment of the ITS1 sequences due to low similarity. Thus, only related sequences were used in our study, all of them from morphospecies Group 5 and the two unique species from the Group 4 with available molecular data, *X. oleae* (KU171047) and *X. tica* n. sp. (KY623493-KY623496) (Fig. 6). The alignment generated for the 38 ITS1 sequences from 19 different species of *X.* non-*americanum*-group was 1036 bp in length after discarding ambiguously aligned regions. In the 50% majority-rule BI consensus ITS1 tree of *Xiphinema* spp. all accessions from morphospecies Group 5 were grouped within a well-supported clade (PP = 0.97) (Fig. 6), while, as well as in the D2-D3 tree, *X. tica* n. sp. position was clearly separated in a basal position of the tree.

The 50% majority-rule BI tree of a multiple sequence alignment including 58 partial 18S rRNA sequences of 1602 bp was similar to that obtained in previous studies (Gutiérrez-Gutiérrez et al. 2013b; Tzortzakakis et al. 2015; Archidona-Yuste et al. 2016a, b). Only for this region, *X. tica* n. sp. (KY623497-KY623499) (Fig. 7) clustered in a low supported clade (PP = 0.78) within the same clade with the other species from the morphospecies Group 4, e.g. *X. oleae* (KU171051) and *X. ifacolum* (AY297826). However, *X. tica* n. sp. was clearly separated phylogenetically from the other *Xiphinema* spp.

Only 24 *coxI* sequences from eight different *X.* non-*americanum*-group spp. were found in GenBank. The phylogenetic tree based on the *coxI* gene resolved several well supported clades. However, the phylogenetic relationships for *X. tica* n. sp. with other *X.* non-*americanum*-group spp. was not well resolved, since our sequences (KY623500-KY623503) clustered with *X. index* (HM921387, HM921388, HM921380 and HM921382) in a not well-supported sub-clade (Fig. 8).

Fig. 8 Phylogenetic relationships within the *Xiphinema* non-*americanum*-group complex. Bayesian 50% majority rule consensus tree as inferred from partial cytochrome c oxidase subunit I (*coxI*) sequence alignment under the unlinked general time-reversible model with a gamma-shaped distribution (GTR + G). Posterior probabilities greater than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in *bold*. Scale bar = expected changes per site

Discussion

We describe here a new species of *Xiphinema*, belonging to the morphospecies Group 4 of Loof and Luc (1990), based on integrative taxonomy and their phylogenetic relationships based on nuclear rRNA. In fact, D2-D3 expansion segments of 28S rDNA, ITS1 and *coxI* were useful for species identification, since they showed enough variability among species, confirming *Xiphinema tica* n. sp. as a new species, increasing the variability within the genus *Xiphinema*. No significant molecular differences among *X. tica* n. sp. populations from different host or localities were found. The present result expands the know diversity of the family Longidoridae, particularly of the *X.* non-*americanum*-group, in Costa Rica where several species of this group have been recently cited and/described in previous studies (Lamberti and Tarjan 1974; Doucet et al. 1998; Peraza-Padilla et al. 2016a, b). In addition, the present data give more information about the phylogenetic relationships among morphospecies within the genus *Xiphinem*a confirming a weak correlation between them using molecular markers, a finding already reported by Tzortzakakis et al. (2015), Gutiérrez-Gutiérrez et al. (2013b), Roshan-Bakhsh et al. (2014), De Luca et al. (2014), and Archidona-Yuste et al. (2016a). *Xiphinema tica* n. sp. did not cluster with other group 4 species with molecular markers (i. e. *X. oleae* and *X. ifacolum*) in the phylogenies obtained with nuclear markers, so these data support that probably morphospecies Group 4 is paraphyletic as well as the rest of the morphospecies Groups studied

until now. On the other hand, in the same way that the exceptional morphology observed in this new species within the *X.* non-*americanum*-group, regarding to the novelty uterine differentiation discussed above, phylogenetic relationships analyses among species from this complex group showed a clearly separated position for *X. tica* n. sp. or low values of similarity (Figs 5, 6, 7 and 8). In any case, more studies about other molecular markers (i.e. mitochondrial markers such as *coxI*) are necessary in order to resolve the phylogenetic relationships within the genus *Xiphinema*.

To date, the morphospecies Group 4 (Loof and Luc 1990) comprises 17 nominal species (including the description of *X. tica* n. sp.), which are mostly distributed in tropical and subtropical regions, with the exception of some species reported from regions with Mediterranean and Arid climates (Coomans et al. 2001; Archidona-Yuste et al. 2016b). Thus, this new species reported from Costa Rica consolidates the hypothesis that morphospecies Group 4 is associated usually with warm humid climate conditions (Archidona-Yuste et al. 2016b).

The knowledge about uterine differentiation (i.e. Z-organ, pseudo-Z-organ, spines and/or crystalloid bodies) has increased substantially over the past of few years (Coomans 1964; Luc 1973; Bleve-Zacheo et al. 1976; Geraert et al. 1980; Kruger 1988; Van de Velde et al. 1990a, b; Coomans et al. 2001; De Luca et al. 2014; Archidona-Yuste et al. 2016a, b), since a Z-organ was firstly described and illustrated (Luc 1958), as well as for pseudo-Z-organ (Siddiqi 1959) and spiniform structures (Luc 1973), being present in about 50% of all described *X.* non-*americanum* group species. As *X. tica* n. sp., *Xiphinema* spp. containing a Z-organ are characterized by didelphic female reproductive system with long uterus forming a tripartite uterus, not having been detected in species with a bipartite and unipartite uterus, as well as in monodelphic and/or pseudomonodelphic species (Coomans 1964; Kruger 1988; Coomans et al. 2001). However, spines or crystalloid bodies may occur in *X.* non-*americanum* group species with bipartite as well as in tripartite uterus, combined or not with Z-differentiation, particularly with pseudo-Z-organ (Loof and Luc 1990). In this sense, *X. tica* n. sp. is a novelty in *Xiphinema* spp. due to the exceptional coexistence of a "true" Z-organ mixed with spiniform structures and/or crystalloid bodies, not described to date in any *X.* non-*americanum* group species (Coomans 1964; Loof and Luc 1990; Coomans et al. 2001). On the other hand, the biological function of the Z-differentiation is still a matter of debate, which has remained unknown during a long time. However, as hypothesised by Coomans et al. (2001), the elongation of the uterus may be correlated with increased egg production, as well as Z-differentiation and/or spines may slow down the passage of eggs through the uterus acting as a mechanism preventing obstruction of eggs. The hypothesis of evolutionary process comprised by reducing complexity of uterine differentiation to more simply structured uterus in conjunction with the development of monodelphism from didelphic species was described Coomans et al. (2001). However, this point was not validated with our phylogenetic relationship of *X. tica* n. sp. using several molecular markers with other *Xiphinema* spp. and further research will be required in order to elucidate of the evolutionary process of this character in the genus *Xiphinema*.

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Compliance with ethical standards

Conflict of interest All the authors certify that 1) do not have any actual or potential conflict of interest, 2) the study described is original and has not been published previously, and is not under consideration for publication elsewhere, 3) all prevailing local, national and international regulations and conventions, and normal scientific ethical practices, have been respected. We also certify that all authors have reviewed the manuscript and approved the final version of manuscript before submission.

Research involving human participants and/or animals No specific permits were required for the described fieldwork studies. Permission for sampling the crop orchards was granted by the landowner. The samples from wild plants were obtained in public areas, forests, and other natural areas studied and do not involve any species endangered or protected in Costa Rica. The sites are not protected in any way.

Informed consent All the authors certify that the work carried out in this research followed the principles of ethical and professional conduct have been followed. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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