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A new ring nematode, *Xenocriconemella costaricense* sp. nov., (Nematoda: Criconematidae) from Costa Rica

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Research Paper

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Abstract

During nematode surveys of natural vegetation in forests of La Cima de Copey de Dota, San José, San José province, Costa Rica, a *Xenocriconemella* species closely resembling *X. macrodora* and related species was found. Integrative taxonomical approaches demonstrated that it is a new species described herein as *X. costaricense* sp. nov. The new species is parthenogenetic (only females have been detected) and characterised by a short body (276–404 µm); lip region with two annuli, not offset, not separated from body contour; first lip annulus partially covering the second lip annulus. Stylet thin, very long (113–133 µm) and flexible, occupying 30.5–47.8% of body length. Excretory pore located from one or two annuli anterior to one or two annuli posterior to level of stylet knobs, at 42 (37–45) µm from anterior end. Female genital tract monodelphic, prodelphic, outstretched, and occupying 35–45% of body length, with vagina slightly ventrally curved (14–18 µm long). Anus located 6–11 annuli from the tail terminus. Tail conoid and bluntly rounded terminus, the last 2–3 annuli oriented dorsally. Results of molecular characterisation and phylogenetic analyses of D2-D3 expansion segments of 28S rRNA, ITS, and partial 18S rRNA, as well as cytochrome oxidase c subunit 1 gene sequences further characterised the new species and clearly separated it from *X. macrodora* and other related species (*X. iberica*, *X. paraiberica*, and *X. pradense*).

Introduction

The ring nematode genus *Xenocriconemella* De Grisse and Loof, 1965 (De Grisse and Loof 1965) comprises obligate ectoparasite nematodes characterised by a short body length (*ca.* 250–400 µm), a long and flexible stylet (*ca.* 100–110 µm and up to 40% of body length), body annuli smooth without anastomosis, vulva closed, and juveniles similar to females (Geraert 2010). Until recently, *X. macrodora* (Taylor, 1936) De Grisse and Loof, 1965 (Taylor 1936; De Grisse and Loof 1965) was the only nominal species within the genus. The taxonomic validity of the genus *Xenocriconemella* has been a scientific controversy in the nematological literature over recent decades (Loof and De Grisse 1989; Siddiqi 2000; Geraert 2010). Lübbers and Zell (1989) morphologically studied several populations of *X. macrodora* from Germany and asserted that German populations differed morphologically from those of the USA and several European countries in a higher number of annuli (R = 147 *vs.* R = 99–120), concluding that these populations belong to a new species, *X. degrissei* Lübbers and Zell, 1989. Later, Ganguly *et al.* (2008) also studied *X. macrodora* populations from peach and blue pine in India and concluded that these populations belong to two new species, *X. pruni* Ganguly *et al.* 2008 and *X. pini* Ganguly *et al.* 2008. However, Sturhan (2013) compared the morphometry of all three species and concluded that they all overlap with populations of *X. macrodora*, and the presence of a filled spermatheca described and/or figured for *X. pruni* and *X. pini* suggested the presence of males in these species (as in some *X. macrodora* populations). Consequently, Sturhan (2013) proposed the synonymisation of all three species with *X. macrodora*, but no molecular markers were provided to confirm this action. Nevertheless, recent integrative taxonomical studies on more than 25 Iberian populations of *Xenocriconemella* supported the validity and monophyly of this genus, as well as the description of three new species including *X. iberica* Archidona-Yuste *et al.* 2024, *X. paraiberica* Archidona-Yuste *et al.* 2024, and *X. pradense* Archidona-Yuste *et al.* 2024 (Archidona-Yuste *et al.* 2024). This study also indicated that this genus is widespread, especially in North America and Europe, and undoubtedly has a close association with woodland forests (Archidona-Yuste et al. 2024). In Central America, the genus *Xenocriconemella* has been observed in Mexico (Cid del Prado 1988) and in protected areas of Costa Rica (Esquivel 2003). However, no morphological or molecular data on this nematode are available from Costa Rica.

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Integrative-based taxonomy studies (using a combination of morphology and morphometry with molecular data) have proved to be accurate and useful tools for assessing populations and species boundaries within Criconematidae spp. as well as in the genus *Xenocriconemella* (Subbotin et al. 2005; Etongwe et al. 2020; Powers et al. 2021; Nguyen et al. 2022; Clavero-Camacho et al. 2022; Archidona-Yuste et al. 2023). A Costa Rican population of *Xenocriconemella* associated with forests composed of wild avocado (*Persea caerulea*) and Ecuador laurel (*Cordia alliodora* (Ruiz and Pav.) Oken were found in La Cima de Copey de Dota, San José, showing a different cytochrome c oxidase subunit 1 (COI) and ribosomal sequences to the molecular markers deposited in the GenBank for this genus. This prompted us to study this *Xenocriconemella* population in more detail to clarify its taxonomic position. Hence, the main objectives of this study were to (i) characterise the newly recovered Costa Rican population of *Xenocriconemella* morphologically and morphometrically and compare it with *Xenocriconemella* spp. belonging to the *X. macrodora*-species complex; (ii) provide molecular characterisation of this *Xenocriconemella* population using ribosomal (D2-D3) expansion segments of 28S rRNA, Internal Transcribed Spacer region (ITS), partial 18S rRNA), and COI markers; and (iii) study phylogenetic relationships within Criconematidae spp. and this species of the *X. macrodora*-species complex.

Materials and methods

Nematode population and morphological characterisation

In winter 2023, a nematological survey was conducted in a forest area in La Cima de Copey de Dota, San José, Costa Rica, and in one of them, low population densities of a *Xenocriconemella* species were detected. Soil samples for nematode analysis were collected with a shovel from randomly selected trees and mixed to constitute a soil sample from each sampling site; samples came from the upper 5–40 cm of soil. Subsequently, nematodes were extracted from a 500 cm^3 subsample of soil by centrifugal flotation and modification of Cobb´s decanting and sieving methods (Flegg 1967; Coolen 1979).

Specimens for light microscopy (LM) and morphometric studies were killed at 70–75°C and fixed in an aqueous solution of 4% formaldehyde + 1% glycerin, dehydrated using an alcohol-saturated chamber, and processed to pure glycerin using Seinhorst's method (Seinhorst 1966) as modified by De Grisse (1969). Light micrographs and measurements of the nematode population, including important diagnostic characteristics were conducted using a Leica DM6 compound microscope with a Leica DFC7000 T digital camera (Leica, Wetzlar, Germany). Measurements and ratios included: L, (total body length); a = body length/maximal body width; b = body length/pharyngeal length; c = body length/tail length; c' = tail length/body width at anus; O = distance between stylet base and orifice of dorsal pharyngeal gland as percentage of stylet length; R = total number of body annuli; Roes = number of annuli in pharyngeal region; Rex = number of annuli between anterior end of body and excretory pore; Rst = number of body annuli between labial disc and stylet knobs; RV = number of annuli between posterior end of body and vulva; Rvan = number of annuli between vulva and anus; Ran = number of annuli on tail; V = (distance from anterior end to vulva/body length) × 100; VL/VB = distance between vulva and posterior end of body divided by body width at vulva; T = (distance from cloacal aperture to anterior end of testis/body length) × 100 (Archidona-Yuste *et al.* 2023; 2024). The raw photographs were edited using Adobe Photoshop SC6 v 24.7.2 (San Francisco, CA, USA).

DNA extraction and molecular characterisation

To avoid mistakes in case of mixed populations in the sample, single specimens were temporarily mounted in a drop of 1 M NaCl containing glass beads (to avoid nematode crushing/damaging specimens) to ensure specimens conformed with the target population. Genomic DNA extraction from single specimens was conducted as described by Archidona-Yuste *et al.* (2023). Briefly, an individual nematode was cut using a scalpel in a drop of polymerase chain reaction (PCR) buffer (ThermoPol®, Biolabs, New England, USA) (20 µL), and 2 µL proteinase K (600 µg/mL) was added. Tubes were frozen at -80°C (15 min) and then incubated at 65°C (1 h) and 95°C (10 min) consecutively. Tubes were centrifuged (1 min, 16,000 × *g*) and kept at −20°C until use in PCR; more importantly, all three molecular markers for the population of *Xenocriconemella* were extracted from the same single individual in each PCR tube without any exception.

The D2-D3 expansion domains of the 28S rRNA were amplified using the D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (De Ley *et al.* 1999). The ITS region was amplified using forward primer TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and reverse primer AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) (Subbotin *et al.* 2001). The partial 18S rRNA was amplified using the primers 988 (5′-CTCAAAGATTAAGCCATGC-3′), 1912R (5′-TTTACGGTCAGAACTAGGG-3′), 1813F (5´- CTGCGTGAGAGGTGAAAT -3´), and 2646R (5´- GCTACCTTGTTACGACTTTT -3´) (Holterman *et al.* 2006). The COI gene was amplified using the primers JB3 (5´-TTTTTTGGGCATCCTGAGGTTTAT-3`) and JB5 (5'- AGCACCTAAACTTAAAACATAATGAAAATG -3´) (Hu *et al.* 2002; Derycke *et al.* 2005). The PCR cycling conditions for the 28S rRNA, ITS, and 18S rRNA were as follows: 95°C for 15 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 one final cycle of 72°C for 10 min. The PCR cycling for COI primers was as follows: 95°C for 15 min, 39 cycles at 94°C for 30 s, 53° C for 30 s, and 68°C for 1 min, followed by a final extension at 72°C for 7 min. The PCR volumes were adapted to 20 µL for each reaction, and primer concentrations were as described in De Ley *et al.* (1999), Subbotin *et al.* (2005), Holterman *et al.* (2006), and Powers *et al.* (2021). We used 5x HOT FIREpol Blend Master Mix (Solis Biodyne, Tartu, Estonia) in all PCRs. The PCR products were purified using ExoSAP-IT (Affimetrix, USB products, Kandel, Germany) and used for direct sequencing in both directions with the corresponding primers. The resulting products were analysed in 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) at the Stab Vida sequencing facility (Caparica, Portugal). The sequence chromatograms of the four markers (18S rRNA, ITS, COI, and D2-D3 expansion segments of 28S rRNA) were analysed using DNASTAR Lasergene SeqMan v. 7.1.0. The Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) was used to confirm the species identity of the DNA sequences obtained in this study (Altschul *et al.* 1990). The newly obtained sequences were deposited in the GenBank database under accession numbers indicated on the phylogenetic trees and in Table 1.

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Table 1. Morphometrics of *Xenocriconemella costaricense* sp. nov. from the rhizosphere of wild avocado (*Persea caerulea*) and Ecuador laurel (*Cordia alliodora* (Ruiz & Pav.) Oken in La Cima de Dota, San José Province, Costa Rica

Character	Holotype	Paratype females
n	1	23
L	347	348.3 ± 31.9
		(276–404)
R	124	123.9 ± 3.7
		(117–130)
Rst	46	45.6 ± 1.8
		(41–49)
Roes	60	57.2 ± 2.1
		(54–61)
Rex	42	42.3 ± 1.7
		(37–45)
RV	14	13.3 ± 0.8
		(12–15)
Rvan	4	5.1 ± 1.2
		(3–7)
Ran	10	8.2 ± 1.3
		(6–11)
O	6.1	5.7 ± 0.7
		(3.8–7.0)
a	10.8	11.3 ± 1.6
		(8.9–15.3)
b	2.4	2.1 ± 0.2
		(1.8–2.5)
c	23.1	22.8 ± 3.1
		(16.0–28.2)
c’	0.8	0.8 ± 0.1
		(0.7–1.1)
V	91.1	91.8 ± 0.8
		(89.2–93.1)
VL/VB	1.1	1.1 ± 0.1
		(0.9–1.3)
Stylet	129.5	124.8 ± 6.0
		(113.0–133.0)
Pharynx	170	164.6 ± 6.8
		(154–177)
Maximum body width	32	31.1 ± 3.6
		(22.0–38.0)
Anal body width	20	18.8 ± 1.8
		(16.0–23.0)
Vulva to anus distance	11	14.2 ± 1.6
		(11.0–16.0)
Tail	15	15.6 ± 2.4
		(12.0–21.0)

Note: All measurements are in μm and in the form: mean ± s.d. (range). Abbreviations: a, body length/maximal body width; b, body length/pharyngeal length; c, body length/tail length; c’, tail length/body width at anus; L, total body length; n, number; O, distance between stylet base and orifice of dorsal pharyngeal gland as percentage of stylet length; R, total number of body annuli; Roes, number of annuli in pharyngeal region; Rex, number of annuli between anterior end of body and excretory pore; Rst, number of body annuli between labial disc and stylet knobs; RV, number of annuli between posterior end of body and vulva; Rvan, number of annuli between vulva and anus; Ran, number of annuli on tail; V, (distance from anterior end to vulva/body length) × 100; VL/VB, distance between vulva and posterior end of body divided by body width at vulva.

Phylogenetic analyses

D2-D3 expansion domains of the 28S, ITS, 18S rRNA, and COI mtDNA sequences of the recovered unidentified *Xenocriconemella* species were obtained in this study. These sequences and other sequences of Criconematidae spp. from GenBank were used for phylogenetic analyses. The selection of outgroup taxa for each dataset was based on previously published studies (Etongwe *et al.* 2020; Nguyen *et al.* 2022; Archidona-Yuste *et al.* 2024). Multiple sequence alignments of the different genes were completed using the FFT-NS-2 algorithm of MAFFT V.7.450 (Katoh *et al.* 2019). The BioEdit program V.7.2.5 (Hall 1999) was used for sequence alignment visualisation and manually edited and trimmed of the poorly aligned positions using a light filtering strategy (up to 20% of alignment positions), which has little impact on tree accuracy and may save computation time, as suggested by Tan *et al.* (2015), because methods for automated filtering of multiple sequence alignments frequently worsen single-gene phylogenetic inference (Tan *et al.* 2015).

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 achieved using JModelTest V.2.1.7 (Darriba *et al.* 2012) with the Akaike information criterion (AIC). The best-fit model, base frequency, proportion of invariable sites, and gamma distribution shape parameters and substitution rates in the AIC were then used in MrBayes for phylogenetic analyses. The 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 the partial 18S rRNA gene, the TPM2uf model with a gamma-shaped distribution (TPM2uf + G) for the ITS region, and the Hasegawa, Kishino, and Yano model with invariable sites and a gamma-shaped distribution (HKY + I + G) for the COI gene were run with four chains for 4×10^6 generations. A combined analysis of the three ribosomal genes was not undertaken because some sequences were not available for all species. The sampling for Markov chains was conducted at intervals of 100 generations. For each analysis, two runs were conducted. After discarding burn-in samples of 30% and evaluating convergence, the remaining samples were retained for more in-depth analyses. The topologies were used to generate a 50% majority-rule consensus tree. For each appropriate clade, posterior probabilities (PP) were given. FigTree software version v.1.4.4 (Rambaut 2018) was used for visualising trees from all analyses.

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Results

The presently studied population of *Xenocriconemella* was detected at low density (31 nematodes per 500 cm^3 of soil) in one of two soil samples collected from a forest area in La Cima de Copey de Dota, San José province, Costa Rica. Detailed morphological, morphometrical, and molecular information about this species is provided below, confirming its identity as a new species of *Xenocriconemella* described herein.

Taxonomy

Phylum: Nematoda Rudolphi, 1808
Class: Chromadorea Inglis, 1983
Order: Rhabditida Chitwood, 1933
Suborder: Tylenchina Chitwood, 1950
Superfamily: Criconematoidea Khan and Ahmad, 1975
Family: Criconematidae Taylor, 1936
Genus: *Xenocriconemella* De Grisse and Loof, 1965
***Xenocriconemella costaricense* sp. nov.**
urn:lsid:zoobank.org:act: 2B48A4F0-2DBE-48A9-B0C1-69905E1563CC

Description

See Figures 1–6 and Table 1.

Females

Body ventrally arcuate, slightly narrowing anteriorly and posteriorly. Body annuli smooth and retrorse, 2.5 (2.0–3.0) µm wide, with anastomosis (2–8). Lip region with two annuli, not offset, not separated from body contour, first lip annulus partially covering the second lip annulus (Figure 2), second lip annulus retrorse and slightly wider than first annulus 10 (9–12) µm wide. Stylet thin, very long, and flexible, occupying 36.0 (30.5-47.8) % of body length, with short basal portion 6 (5–8) µm long, and knobs slightly rounded 6.4 (5.0–7.5) µm wide. Pharynx typical criconematoid, with a cylindroid procorpus widening to a large muscular oval median bulb containing well developed valves (8–10 µm long), isthmus slender and amalgamated with basal bulb. Excretory pore located from one or two annuli anterior to one or two annuli posterior to level of stylet knobs, at 42 (37–45) µm from anterior end. Nerve ring located at level of isthmus, 16 (14–17) µm from anterior end. Vagina slightly ventrally curved (14–18 µm long). Female genital tract monodelphic, prodelphic, outstretched, and occupying 52 (50–54) % of the body length, spermatheca developed, but sperm absent. Anus located at 7 (6–9) annuli from the terminus. Tail conoid and bluntly rounded terminus, the last 2–3 annuli oriented dorsally.

Males

Not found.

Juveniles

Body similar to females, including tail shape, but shorter. Edge of body annuli without appendages, marked with delicate irregular punctations.

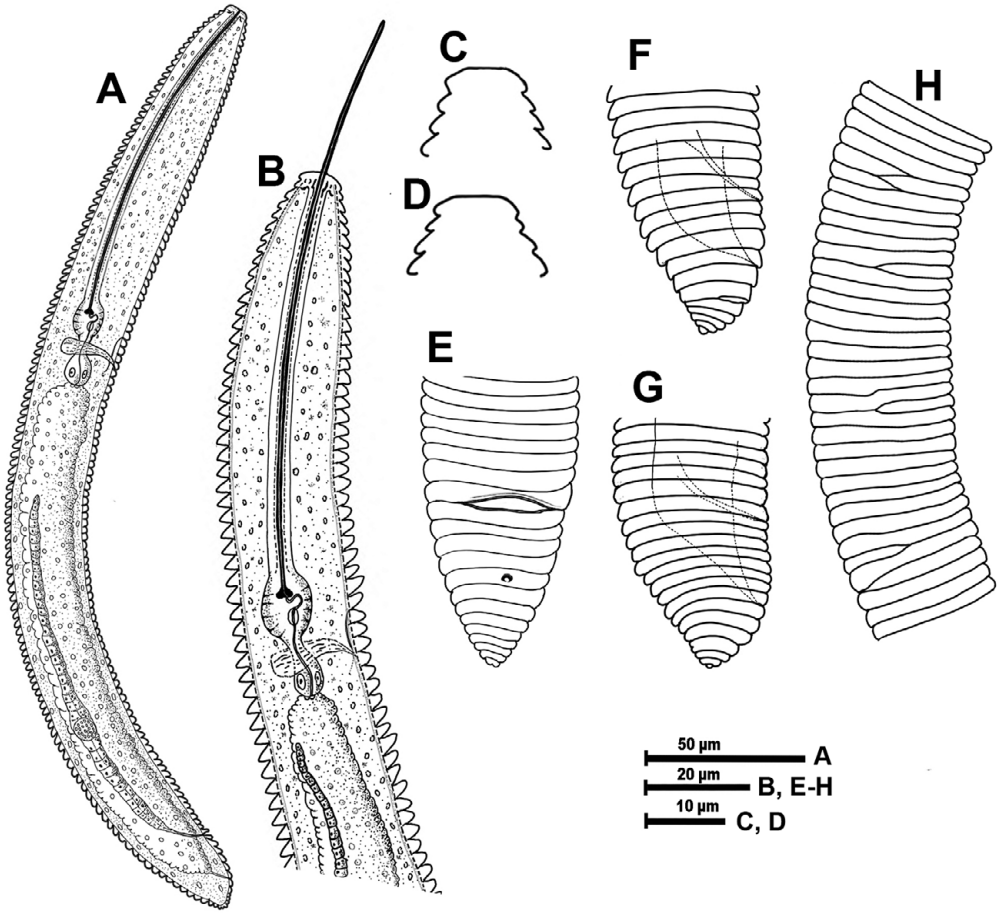

Figure 1. *Xenocriconemella costaricense* sp. nov. (drawings). (A) whole female; (B) female neck region; (C, D) female anterior region; (E) posterior female region in frontal view; (F, G) female tail; (H) female at mid-body showing anastomosis.

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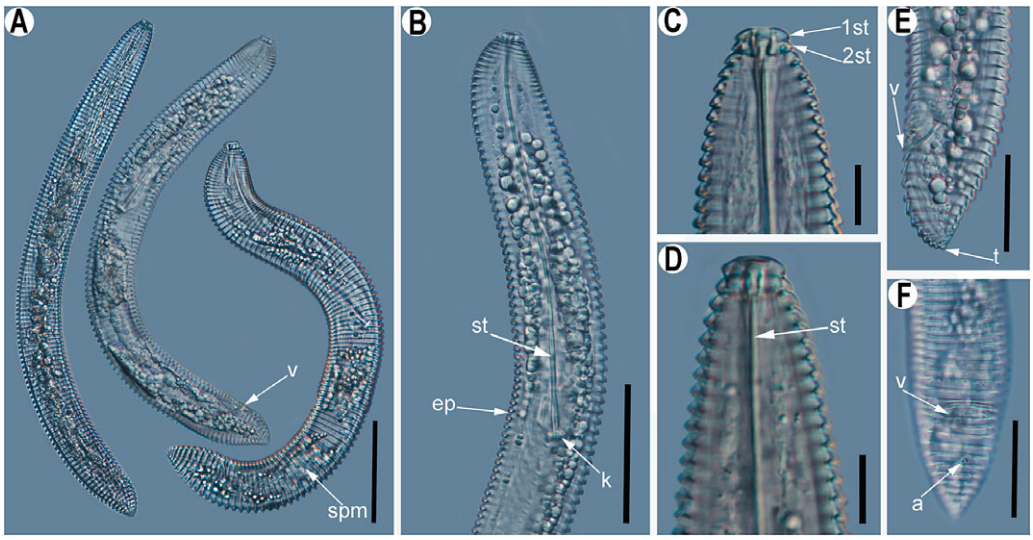

Figure 2. Light micrographs of *Xenocriconemella costaricense* sp. nov. (A) entire females; (B) female anterior body region showing stylet, knobs, and excretory pore (arrowed); (C, D) female lip region showing first and second annuli; (E, F) female tail in lateral and frontal view, respectively. Abbreviations: a, anus; ep, excretory pore; k, stylet basal knobs; spm, spermatheca; st, stylet; V, vulva. Scale bars: A = 50 µm; B = 40 µm; C, D = 10 µm; E, F = 30 µm.

Diagnosis and relationships

Xenocriconemella costaricense sp. nov. is characterised by the following measurements and ratios: a short-sized female body 276–404 µm, a long and flexible stylet = 113.0–133.0 µm long, V = 89.2–93.1, a = 8.9–15.5, b = 1.8–2.5, c = 16.0–28.8, c' = 0.7–1.1, R = 117–130, RV = 12–15, Ran = 6–11, VL/VB = 0.9–1.3. Morphologically and morphometrically, *X. costaricense* sp. nov. resembles members of the *X. macrodora*-species complex (including *X. macrodora*, *X. iberica, X. paraiberica*, and *X. pradense*) from which it can be differentiated by several morphometric traits and ratios. From *X. macrodora,* it differs by a longer body length 349 (276–404) µm *vs.* 273 (224–331) µm, a longer stylet length 125 (113.0–133.0) µm *vs.* 96.3 (83.0–113.0) µm, a slightly higher number of body annuli (R) 124 (117–130) *vs.* 109 (101–141), and a slightly higher c ratio 22.8 (16.0–28.8) *vs.* 19.6 (12.8–25.3). From *X. iberica,* it differs by a slightly longer body length 349 (276–404) µm *vs.* 294 (246–350) µm, a longer stylet length 125 (113.0–133.0) µm *vs.* 93.1 (80.0–103.0) µm, a higher number of R 124 (117–130) *vs.* 107 (97–119), a slightly shorter tail length 16 (12.0–21.0) µm *vs.* 16.4 (11.0–24.5) µm, and a slightly higher c ratio 22.8 (16.0–28.8) *vs.* 18.3 (12.1–27.3). From *X. paraiberica*, it differs by a slightly longer body length 349 (276–404) µm *vs.* 298 (221–386) µm, a longer stylet length 125 (113.0–133.0) µm *vs.* 89.6 (80.0–100.0) µm, a higher number of R 124 (117–130) *vs.* 104 (95–116), and a slightly higher c ratio 22.8 (16.0–28.8) *vs.* 20.2 (13.0–28.6). From *X. pradense*, it differs by a slightly longer body length 349 (276–404) µm *vs.* 333 (249–383) µm, a longer stylet length 125 (113.0–133.0) µm *vs.* 101.1 (92.0–110.0) µm, a slightly lower VL/VB ratio 1.1 (0.9–1.3) *vs.* 1.4 (1.1–1.5), a slightly higher number of body annuli from vulva to posterior end (RV) 13 (12–15) *vs.* 16 (14–18), a slightly shorter tail length 16 (12.0–21.0) µm *vs.* 20.2 (15.5–25.0) µm, a slightly higher c ratio 22.8 (16.0–28.8) *vs.* 16.6 (13.7–21.3), and a slightly lower c' ratio 0.8 (0.7–1.1) *vs.* 0.9 (0.8–1.2).

Etymology

The species epithet refers to the country where the species was detected, Costa Rica.

Type host and locality

The new species was recovered from a rainforest in the rhizosphere of wild avocado (*Persea caerulea*) and Ecuador laurel (*Cordia alliodora* (Ruiz and Pav.) Oken from a forest in La Cima de Copey de Dota, San José, San José Province, Costa Rica (coordinates 9° 42'41.2" N, 83°56'59.2" W).

Type material

Holotype female and 19 female paratypes were deposited at Laboratorio de Nematología, Escuela de Ciencias Agrarias, Universidad Nacional, Heredia, Costa Rica; two females are at Institute for Sustainable Agriculture (IAS) of Spanish National Research Council (CSIC), Córdoba, Spain; and two females are at the USDA Nematode Collection (T-8028p).

Molecular characterisation

Xenocriconemella costaricense sp. nov. was molecularly characterised by the sequences of three ribosomal genes, D2-D3 expansion segments of 28S rRNA, ITS, partial 18S rRNA, and the mitochondrial gene COI. The amplification of these regions yielded single fragments of approximately 900, 800, 1800, and 400 bp, respectively, based on gel electrophoresis. Four D2-D3 of 28S rRNA sequences from 687 to 718 bp (PP209388–PP209391), two ITS rRNA sequences of 754 bp (PP209392-PP209393), five 18S rRNA sequences from 1703 to 1713 bp (PP209394–PP209397), and four COI sequences of 377 bp (PP210897–PP210900) were generated for this new species. No intraspecific sequence variations in D2-D3 expansion segments of 28S rRNA, ITS, and COI, and low variations within 18S rRNA (99.6–100.0%, 1–6 bp, and 0–1 indel) were detected. D2-D3 expansion segments of 28S rRNA of *X. costaricense* sp. nov. (PP209388–PP209391) were 93.9–93.6% similar (differing by 42–45 bp, 0–2 indels) to *X. paraiberica* from Spain (OR880152–OR880200), 93.0–90.7% similar (differing by 65–66 bp, 7 indels) to *X. iberica* from Spain and Portugal (OR880112–OR880149), 89.8–89.9% similar (differing by 71 bp, 1 indel) to *X. pradense* from Spain (OR880203–OR880217), and 89.6% similar

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(differing by 56 bp, 1 indel) to *X. macrodora* from Italy (AY780960). ITS of *X. costaricense* sp. nov. (PP209392–PP209393) was 83.3–83.5% similar (differing by 134–135 bp, 69–70 indels) to *X. paraiberica* (OR878338–OR878349), 81.5% similar (differing by 144 bp, 46 indels) to *X. pradense* (OR878350), 79.8–79.3% similar (differing by 150–162 bp, 45–49 indels) to *X. iberica* (OR878332–OR878336), and 76.5% similar (differing by 100 bp, 56 indels) to *X. macrodora* from USA (JQ708139), but with a low coverage (54%). Partial 18S rRNA of *X. costaricense* sp. nov. (PP209394–PP209397) was 99.5–99.4% similar (differing by 9–11 bp, 0 indel) to *X. macrodora* (MF094906, MF094973, MF095001), 99.2% similar (differing by 13 bp, 0 indel) to *X. paraiberica* (OR878358), 98.2% similar (differing by 11–28 bp, 4 indels) to *X. pradense* (OR878360–OR878361), and 97.9% similar (differing by 35 bp, 4 indels) to *X. iberica* (OR878356). Finally, COI of *X. costaricense* sp. nov. (PP210897–PP210900) was 91.6–90.5% similar (differing by 30–35 bp, 0 indel) to *X. paraiberica* from Spain (OR885983–OR886017), 91.1–90.3% similar (differing by 31–36 bp, 0 indels) to *X. iberica* from Spain and Portugal (OR885936–OR885976), 91.4–90.5% similar (differing by 29–40 bp, 0 indel) to *X. macrodora* from USA (MF770894–MF770950, MN711386–MN711444), and 88.2–87.6% similar (differing by 36–39 bp, 0 indel) to *X. pradense* from Spain (OR886020–OR886029).

Phylogenetic relationships of *Xenocriconemella costaricense* sp. nov. with other Criconematidae spp

Phylogenetic relationships among *Xenocriconemella* species, as inferred from analyses of D2-D3 expansion domains of 28S rRNA, ITS, the partial 18S rRNA, and the partial COI mtDNA gene sequences using BI, are shown in Figures 3, 4, 5, and 6, respectively. The phylogenetic trees generated with the ribosomal and

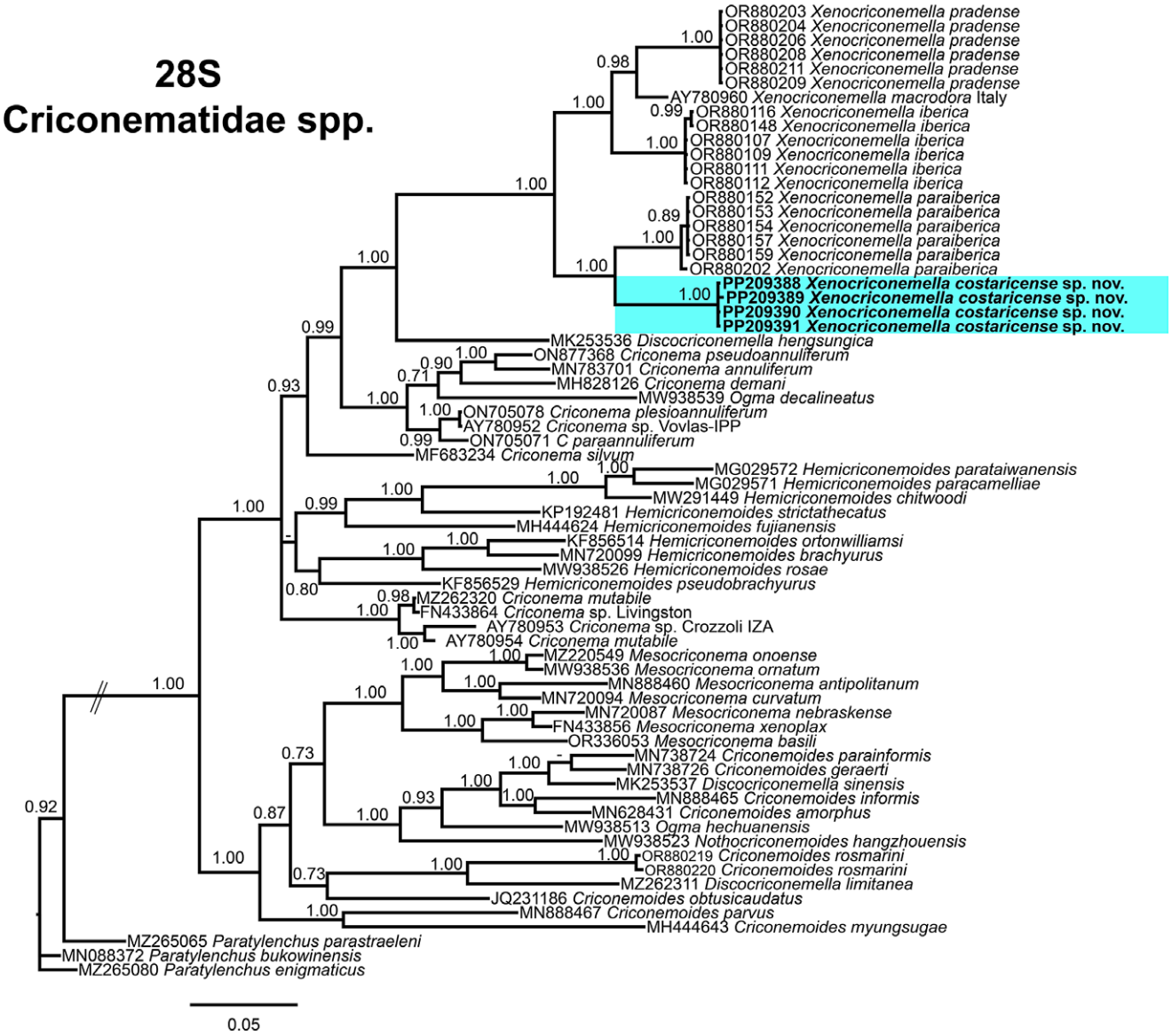

Figure 3. Phylogenetic relationships of *Xenocriconemella costaricense* sp. nov. with Criconematidae spp. Bayesian 50% majority rule consensus tree as inferred from D2 and D3 expansion domains of 28S rRNA sequence alignment under the under the GTR + I+ G model (-lnL = 8599.1303; AIC = 17490.260700; freqA = 0.1886; freqC = 0.2383; freqG = 0.3326; freqT = 0.2405; R(a) = 0.4937; R(b) = 1.5257; R(c) = 0.9666; R(d) = 0.4738; R(e) = 4.1298; R(f) = 1.0000; Pinva = 0.4220; and Shape = 0.9630). Posterior probabilities more than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in bold, and coloured box indicate clade association of the new species. Scale bar = expected changes per site.

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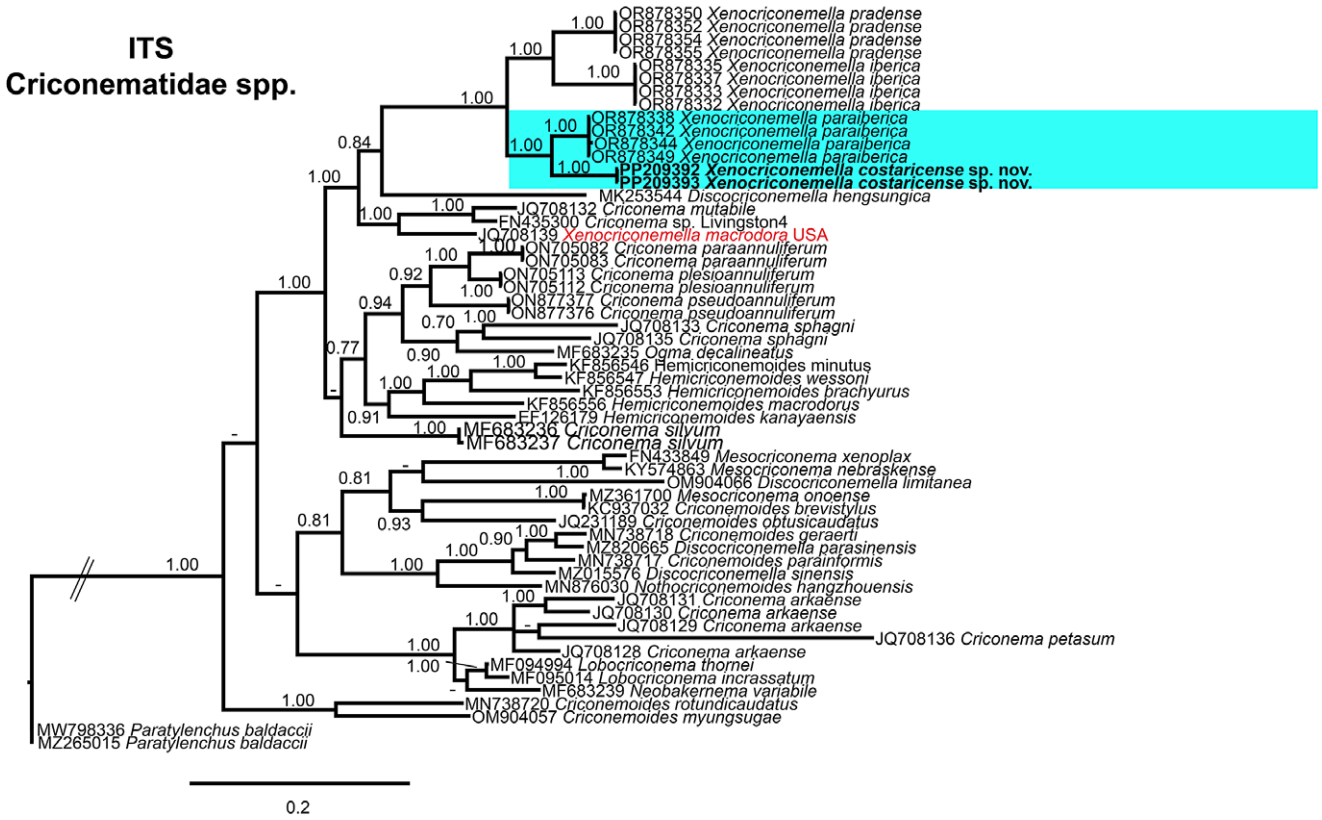

Figure 4. Phylogenetic relationships of *Xenocriconemella costaricense* sp. nov. with Criconematidae spp. Bayesian 50% majority rule consensus tree as inferred from ITS rRNA (sequence alignment under the TPM2uf + G model (-lnL = 11273.5464; AIC = 22783.092780; freqA = 0.2088; freqC = 0.2469; freqG = 0.2555; freqT = 0.2888; R(a) = 1.9265; R(b) = 3.3727; R (c) = 1.9265; R(d) = 1.0000; R(e) = 3.3727; R(f) = 1.0000; Pinva = 0.0000; and Shape = 0.5900). Posterior probabilities more than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in bold, and coloured box indicate clade association of the new species. Scale bar = expected changes per site.

mitochondrial DNA markers included 69, 57, 88, and 169 sequences, and their alignment had 706, 715, 162, and 326 characters, respectively. The Bayesian 50% majority rule consensus tree inferred from the D2-D3 expansion domains of 28S rRNA alignment is given in Figure 3. For this region, all species that belong to the *X. macrodora* species-complex clustered together in a well-supported clade (PP = 1.00), which was subdivided into two subclades, one of them (PP = 1.00) formed by *X. costaricense* sp. nov. (PP209388–PP209391) and *X. paraiberica* (OR880152–OR880202), and the other one (PP = 1.00) by *X. iberica* (OR880107–OR880151), *X. pradense* (OR880203–OR880218), and *X. macrodora* from Italy (AY780960).

The phylogenetic position of *X. costaricense* sp. nov. in the ITS region tree is shown in Figure 4. Phylogenetic relationships observed for this region were very similar to D2-D3 expansion segments of the 28S rRNA gene. In this analysis, *X. costaricense* sp. nov (PP209392–PP209393) and *X. paraiberica* clustered together, although clearly separated, in a well-supported subclade (PP = 1.00), whereas *X. iberica* and *X. pradense* clustered with another well-supported subclade (PP = 1.00). For this region *X. macrodora* from USA (JQ708139) clustered separately from the rest of *Xenocriconemella* spp. accessions, forming a well-supported subclade with *Criconema mutabile* (JQ708132) and *Criconema* sp. 4 Livingston (FN435300).

In 18S rRNA phylogeny, all accessions belonging to the *Xenocriconemella* genus clustered together in a well-supported clade (PP = 1.00), occupying the top of the tree. However, the phylogenetic relationships between the different *Xenocriconemella* spp. were not well resolved, as many of the internal clades were poorly supported.

Finally, the phylogenetic position of *X. costaricense* sp. nov. (PP210897–PP210900) and the other species, using COI gene sequences, are shown in Figure 6. The position of *X. costaricense* sp. nov. was well-supported (PP = 1.00), clustering alone in a basal position of the tree. Accessions from *X. macrodora* from USA appeared together in a well-supported (PP = 1.00) subclade. On the contrary, relationships of *Xenocriconemella* spp. from the Iberian Peninsula—*X. pradense*, *X, iberica*, and *X. paraiberica*—were not well resolved for this gene (Figure 6).

Discussion

The recent integrative taxonomic study on numerous *X. macrodora* populations from the Iberian Peninsula defined a new species complex within *X. macrodora* including at least four species, viz. *X. iberica*, *X. macrodora, X. paraiberica*, and *X. pradense* (Archidona-Yuste *et al.* 2024). The main objective of this study was to identify and describe, morphologically and molecularly, a new population of *Xenocriconemella* detected in a natural environment in a forest of Dota, San José province, Costa Rica, as well as clarify the phylogenetic relationships within the genus *Xenocriconemella.* All results confirmed that the unknown *Xenocriconemella* population is morphologically and morphometrically close to the *X. macrodora*-species complex, except for differences in body size,

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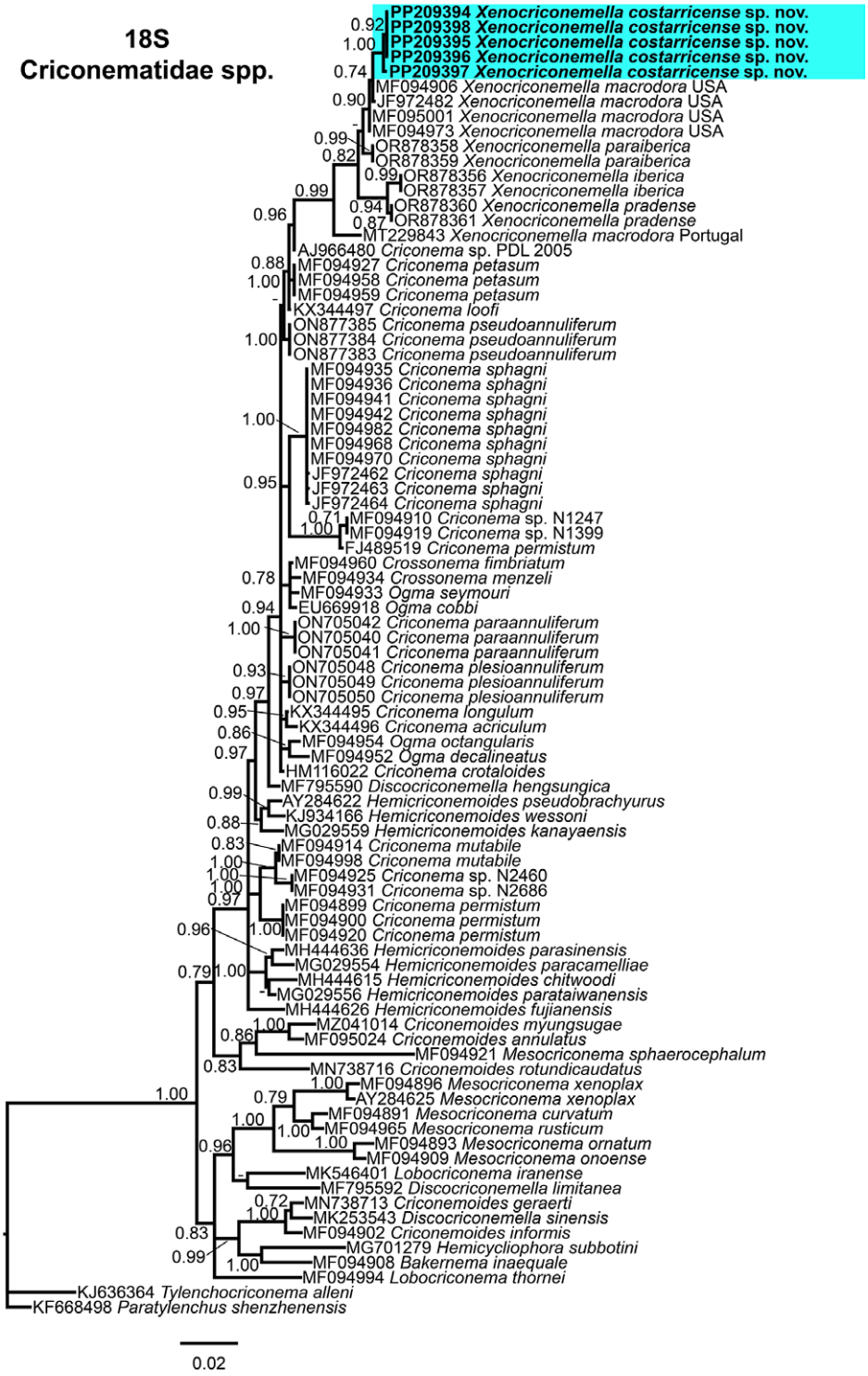

Figure 5. Phylogenetic relationships of *Xenocriconemella costaricense* sp. nov. with Criconematidae spp. Bayesian 50% majority rule consensus tree as inferred from 18S rRNA sequence alignment under the GTR + I+ G model (−lnL = 7729.8698; AIC = 15827.739680; freqA = 0.2442; freqC = 0.2388; freqG = 0.2780; freqT = 0.2391; R(a) = 1.4400; R(b) = 2.0892; R(c) = 1.0082; R(d) = 0.6719; R(e) = 5.7912; R(f) = 1.0000; Pinva = 0.6450; and Shape = 0.5370). Posterior probabilities more than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in bold, and coloured box indicate clade association of the new species. Scale bar = expected changes per site.

stylet length, and other minor morphometric differences. Nevertheless, all molecular markers clearly separated the new Costa Rican population from all other species, confirming that the new population is a new valid species of the genus *Xenocriconemella*. These data provide clear evidence that the worldwide biodiversity within this genus may be higher than previously understood, as recently suggested by Archidona-Yuste et al. (2024). Interestingly, although more studies are needed to confirm this hypothesis, our results suggest that additional taxa can be included within the widely reported populations of *X. macrodora* s.l. in those regions where

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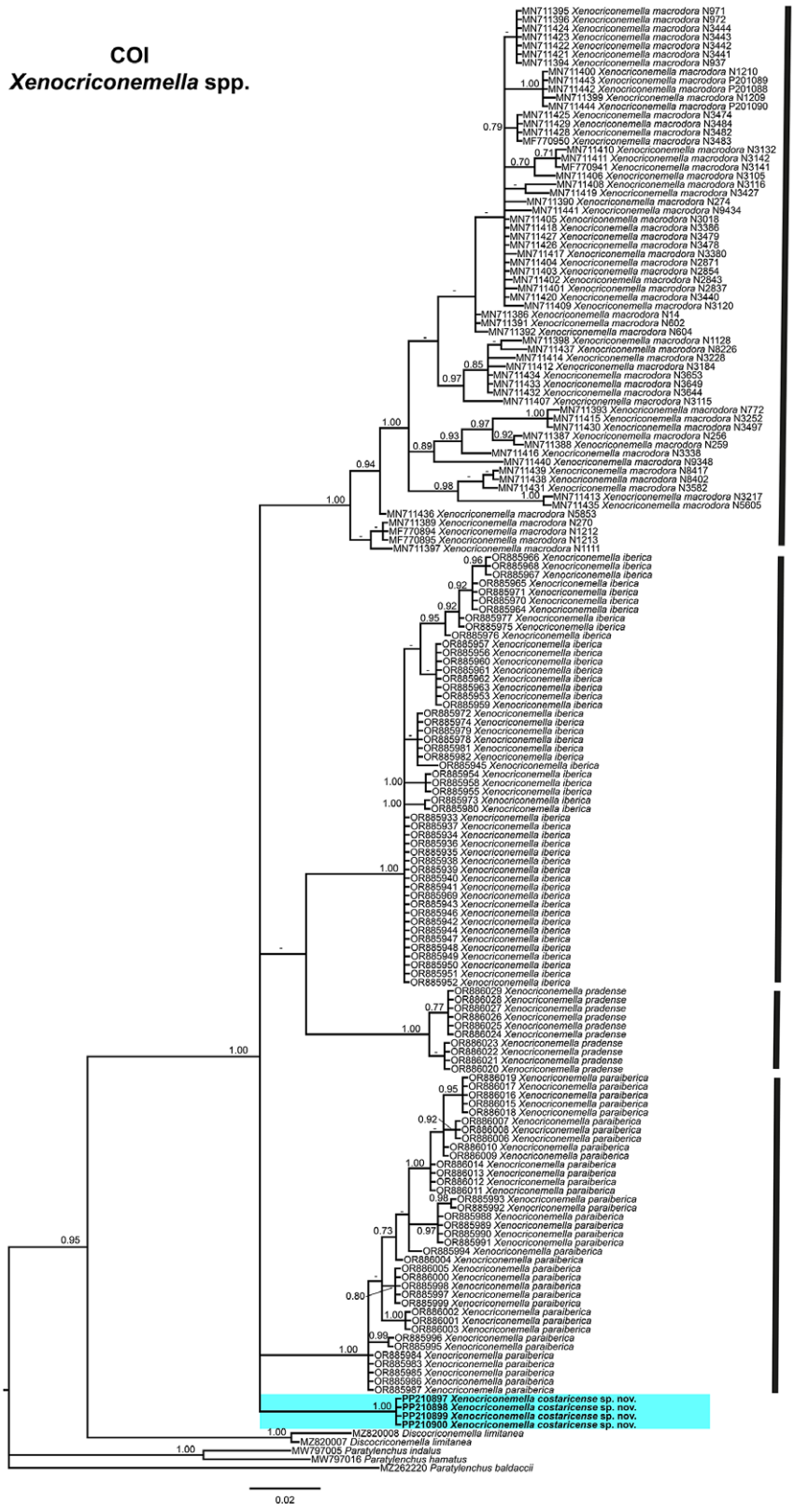

Figure 6. Phylogenetic relationships of *Xenocriconemella costaricense* sp. nov. with other *Xenocriconemella* spp. Bayesian 50% majority-rule consensus trees as inferred from cytochrome c oxidase subunit I (COI) mtDNA gene sequence alignments under the HKY + I + G model (-lnL = 2290.0099; AIC = 5264.019760; freqA = 0.3729; freqC = 0.0571; freqG = 0.0820; freqT = 0.4880; Kappa = 8.0975; Pinva = 0.3350; and Shape = 0.4540). Posterior probabilities more than 0.70 are given for appropriate clades. Newly obtained sequences in this study are shown in bold, and coloured box indicate clade association of the new species. Scale bar = expected changes per site.

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the species complex has been reported (Archidona-Yuste *et al.* 2024).

Again, ribosomal and mitochondrial markers (D2-D3 expansion domains of the 28S rRNA and ITS rRNA, and the mtDNA gene COI) are important tools for accurate identification of *Xenocriconemella* and remain essential for accurate diagnosis of ring nematodes (Subbotin *et al.* 2005; Etongwe *et al.* 2020; Powers *et al.* 2021; Nguyen *et al.* 2022; Archidona-Yuste *et al.* 2024). However, the low nucleotide variability found in partial 18S rRNA makes it difficult to identify individuals at the species level, but this molecular marker clearly separated the genus from other genera of the family Criconematidae. Phylogenetic analyses based on ribosomal genes resulted in a general consensus of species' phylogenetic positions for the majority of species and was congruent with those given by previous phylogenetic analyses (Subbotin *et al.* 2005; Etongwe *et al.* 2020; Nguyen *et al.* 2022; Archidona-Yuste *et al.* 2024). In particular, in all three ribosomal gene trees, *X. costaricense* sp. nov. clustered with all morphologically related species, including *X. iberica*, *X. macrodora*, *X. paraiberica*, and *X. pradense* (Figures 3–5).

In summary, the present study confirms the usefulness of an integrative approach based on the combination of morphometric and morphological traits and genotyping rRNA and mtDNA markers for correct discrimination among *Xenocriconemella* species, suggesting the need for continuing nematode surveys in natural environments to complete the unexplored worldwide biodiversity of this genus.

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Competing interest. None.

Ethical standard. This work has not been published previously and is not under consideration elsewhere. The research does not involve human or animal use.

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