RESEARCH/INVESTIGACIÓN

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF RING NEMATODES (NEMATODA: CRICONEMATIDAE) FROM COSTA RICA: TWO NEW SPECIES DESCRIBED

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ABSTRACT

Aráuz-Badilla, J., R. Artavia-Carmona, I. Hilje-Rodríguez, P. Castillo, and W. Peraza-Padilla. 2024. Morphological and molecular characterization of ring nematodes (Nematoda: Criconematidae) from Costa Rica: two new species described. Nematropica 54:49-70.

Plant-parasitic nematodes cause significant economic impact on agricultural crops. Among these, the members of the Criconematidae, ring nematodes, are considered as potential pests due to a strong stylet and worldwide distribution. In Costa Rica, there are several reports of ring nematodes associated with different plant species, however, there are no molecular studies of the species present in the country. The aim of this study was to identify nematodes of the Criconematidae family, using morphological and molecular methods to contribute to the knowledge of these organisms. Soil samples were collected from cocoa, sugar cane, grass, and natural areas from five provinces in Costa Rica. A morphological study was conducted using a set of measurements, percentages, and ratios to differentiate ring nematodes species. Molecular analysis included amplification of 28S D2D3 domain, 18S, ITS, and COI regions, which allowed phylogenetic analysis between species identified in this study and those deposited in GenBank. Four species of ring nematodes were identified, including two new species, which are described herein, for the first time in this study. *Mesocriconema onoense* and *Mesocriconema costarricense* n. sp. were identified in natural areas, *Mesocriconema paraonoense* n. sp. in cocoa and sugar cane plantations, and *Mesocriconema sphaerocephalum* in grass. This research reports the first morphological and molecular characterization of nematodes belonging to the Criconematidae in Costa Rica.

Key words: 18S, COI, D2D3, ITS, morphology, phylogenetic analysis, plant-parasitic nematodes, sequencing

RESUMEN

Aráuz-Badilla, J., R. Artavia-Carmona, I. Hilje-Rodríguez, P. Castillo y W. Peraza-Padilla. 2024. Caracterización morfológica y molecular de nematodos anillados (Nematoda: Criconematidae) de Costa Rica: dos nuevas especies descritas. Nematropica 54:49-70.

Los nematodos fitoparásitos son organismos que causan un impacto económico significativo en los cultivos agrícolas. Entre estos, miembros de la familia Criconematidae, los nematodos anillados, se

consideran una plaga potencial debido a su amplia distribución mundial y que poseen un fuerte estilete en su aparato bucal. En Costa Rica existen varios reportes de nematodos anillados asociados a diferentes especies de plantas, sin embargo, no existen estudios moleculares de las especies presentes en el país. El objetivo de este estudio fue identificar nematodos de la familia Criconematidae utilizando métodos morfológicos y moleculares, para contribuir al conocimiento de estos organismos. Se recolectaron muestras de suelo de cacao, caña de azúcar, pastos y áreas naturales de cinco provincias de Costa Rica. Se realizó un estudio morfológico a partir de una serie de medidas, porcentajes y proporciones para diferenciar entre especies de nematodos anillados. El análisis molecular incluyó la amplificación de las regiones D2D3, 18S, ITS y COI, las cuales permitieron establecer relaciones filogenéticas entre las especies identificadas en este estudio y las especies disponibles en la base de datos del GenBank. Se identificaron en total cuatro especies de nematodos anillados, que incluyendos especies nuevas descritas por primera vez en este estudio. Se identificó *Mesocriconema onoense* y *Mesocriconema costarricense* n. sp. en áreas naturales, *Mesocriconema paraonoense* n. sp. en plantaciones de cacao y caña de azúcar, y *Mesocriconema sphaerocephalum* en pastos. Esta investigación representa el primer estudio morfológico y molecular de nematodos pertenecientes a la familia Criconematidae en Costa Rica.

Palabras clave: 18S, COI, D2D3, ITS, morfología, análisis filogenético, nematodos fitoparásitos, secuenciación

INTRODUCTION

Plant-parasitic nematodes are tiny organisms with a typical "worm" shape and represent the most abundant group of multicellular organisms on Earth (Castro *et al*., 2011). They are characterized by the presence of a stylet in their mouthparts and stand out for their importance for agriculture. Currently, there are more than 4,100 described species (Poveda *et al*., 2020; Phani *et al*., 2021), and it is estimated that plant-parasitic nematodes cause worldwide agricultural losses between $125 and $173 billion annually (Watkins *et al*., 2012; Elling, 2013; Ravichandra, 2014; Kim, 2015).

Within the plant-parasitic nematodes is the family Criconematidae, ring nematodes, which have a cuticle with characteristic transverse wide annulations. In Costa Rica, several genera and species of ring nematodes have been reported in annual and perennial crops, including grasses, shrubs, vegetable, and fruit crops. *Criconemoides* spp. (González, 1978; Guzmán *et al*., 2011), *Criconemella onoensis* (Sancho and Salazar, 1985), *C. ornata* and *C. palustris* (Salazar and López, 1987) were reported in rice and corn. Also, Wingching *et al*. (2008) mentioned the presence of *Criconemella* spp. in different species of grasses. *Criconemella sphaerocephala* (López and Salazar, 1988), *Mesocriconema sphaerocephalum*, *M. anastomoides* (Peraza, 2014), *Crossonema civellae*, *Criconema neopacificum*, *C. graminicola* and *Criconemoides lizarbus* (Peraza and Orozco, 2018) were also morphologically identified parasitizing fruit-tree species.

There are gaps in knowledge of the identification of nematode species in Costa Rica, mainly due to the lack of specialists in nematology. Most of the research carried out in this area is based mainly on the observation of morphological and morphometric characters for species identification. More recently, nematode identification with molecular tools has been implemented. Wide intraspecific variability and little interspecific heterogeneity between species are the main reasons for using molecular identification tools (Barsalote *et al*., 2017; Palomares-Rius *et al*., 2018; Karaca *et al*., 2021). DNA-based characterization methods are now more common and necessary to differentiate cryptic species, including species within the Criconematidae (Powers *et al*., 2017; Clavero-Camacho *et al*., 2022; Archidona *et al*., 2023).

The correct and accurate identification of plant-parasitic nematodes in cultivated areas is extremely important because frequently the symptoms they cause in plants are confused with those caused by other phytopathogens or abiotic factors. Therefore, the level of damage nematodes cause is underestimated (Gandarilla *et al*., 2014). Proper diagnosis can also contribute to decision-making and generate a positive impact in reducing the use of agrochemicals, and economic losses caused by plant-parasitic nematodes. Therefore, the aim of this research was to identify species of

the Criconematidae in Costa Rica through morphological and molecular methods.

MATERIALS AND METHODS

Sample collection

Seven soil samples were collected from agricultural crops, grasses, and natural areas located in the provinces of San José, Cartago, Alajuela, Puntarenas, and Limón. Each sample consisted of approximately 1 kg of soil composed of 15-20 subsamples, taken by systematic sampling to a depth of 5-30 cm. Samples were stored in polyethylene plastic bags and transported in thermally insulated containers to the Laboratorio de Nematología at the Universidad Nacional (Heredia, Costa Rica) for further processing.

Extraction, mounting, and fixation of nematodes

Extraction was carried out according to Jenkins (1964) sugar solution centrifugation-flotation method. Extracted nematodes were placed in counting boxes for observation and subsequent selection for DNA extraction. Remaining specimens were fixed in 4% formaldehyde at 70°C using the "modified rapid Seinhorst" method (Seinhorst, 1959) and preserved on fixed slides following the paraffin method (de Maeseneer and D'Herde, 1963). Slides were labeled and used for morphometric analysis and morphological identification. All specimens fixed on Cobb slides were incorporated into the nematode collection of the Criconematidae of the Laboratorio de Nematología, Escuela de Ciencias Agrarias, Universidad Nacional, Heredia, Costa Rica.

Morphological and morphometric identification

Female nematodes were measured and imaged using a high-resolution microscope connected to a Nikon DS-Fi1 camera (Nikon Eclipse 80, Japan). Measurements, percentages, and ratios commonly used for morphometric identification of Criconematidae (Siddiqi, 2000) were: body length (L), stylet length (St), first cephalic annulus diameter (FAD), annulus width (AW), stylet knob width (KW), pharynx length (Pha), medium body diameter (MBD), vulval body diameter (VBD), distance from stylet base to dorsal oesophageal gland (DGO), number of annulus (R), number of annulus from head to excretory pore (Rex), number of annulus from vulva to posterior end (RV), distance from vulva to posterior end (VL), V% (((L–VL)/L)*100), St%L (St/L*100), St%Pha (St/Pha*100), a (L/MBD), b (L/Pha), and VL/VBD. For morphological species identification, original descriptions of species from the Criconematidae were used (Geraert, 2010). Microphotographs were edited with Photoshop CS6 (Adobe, San Jose, CA).

Molecular identification

DNA extraction, Polymerase Chain Reaction (PCR) and sequencing: Total genomic DNA was extracted from individual nematodes according to Solano *et al*. (2013) with some modifications. Specimens were placed in 0.2 ml tubes with 47 µl Tris-HCL, pH 8.0 (2.0 M). Three µl of proteinase K (20 mg/ml) were added to each tube and then placed in an ultrasonic bath for 10 min at 60°C to activate the enzyme. Subsequently, samples were incubated in a thermocycler for 30 min at 60°C, mixed in a vortex, and subjected to cold incubation for 15 min at -20°C for cellular lysis and hot incubation for 10 min at 90°C to inactivate proteinase K. After mixing, hot and cold incubations were repeated. Once finished, samples were vortexed for 30 sec, centrifuged at 2,000 rpm for 2 min, and stored at -20°C for later use in PCR.

For PCR analyses, four gene regions were amplified. The D2D3 region of 28S rDNA gene was amplified with primers D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) (De Ley *et al*., 1999). Primers TW81 (5′-GTT TCC GTA GGT GAA CCT GC-3′) and AB28 (5′-ATA TGC TTA AGT TCA GCG GGT-3′) were used to amplified ITS1 region, 5.8S gene and ITS2 region (Kaplan *et al*., 2000; Subbotin *et al*., 2001). The 18S gene was partially amplified using primers MN18F (′5-CGC GAA TRG CTC ATT ACA ACA GC-3′) and Nem_18S_R (5′-GGG CGG TAT CTG ATC GCC -3′) (Nunn, 1992). For partial amplification of the mitochondrial COI gene, primers COI-F5 (5′-AAT WTW GGT GTT GGA ACT TCT TGA AC-3′) and COI-R9 (5′-CTT AAA ACA TAA TGR AAA TGW GCW ACW ACA TAA TAA GTA TC-3′) were used (Powers *et al*., 2014).

PCR conditions for amplification of 28S, ITS, and 18S regions were based on Cordero *et al*.

(2012a). Reaction mix was adjusted to 25 µl, with 5 µl of DNA solution, 12.5 µl of DreamTaq™ PCR Master Mix (2X) (Sigma-Aldrich, Burlington, MA), 1 µl of each primer (10 µM) and 5.5 µl of nuclease-free water. Temperature profile consisted of an initial cycle of 94°C for 2 min, followed by 45 cycles of 94°C for 45 sec, 55°C for 45 sec (annealing), 72°C for 1 min and a final cycle of 72°C for 5 min. For the COI region, the Powers *et al*. (2014) protocol was used. A 30 µl reaction mix included 9 µl of DNA solution, 15 µl DreamTaq™ PCR Master Mix (2X), 2.4 µl of each primer (20 µM), and 1.2 µl of nuclease-free water. PCR parameters consisted of an initial cycle of 94°C for 5 min, continuing with 50 cycles of 94°C for 30 sec, 48°C for 30 sec (annealing), 72°C for 1.5 min with a ramping rate of 0.5°C per sec, and a final cycle of 72°C for 5 min.

PCR products were analyzed on a 1% (w/v) TopVision™ agarose gel (Thermo Fisher Scientific Inc., Rochester, NY) (100 volts, 1 hr). Four µl of PCR product were loaded with 2 µl of 6X DNA loading dye stained with 1X GelRedT™ (Thermo Fisher Scientific Inc., Rochester, NY). DNA bands were observed under UV light. GeneRuler™ (Thermo Fisher Scientific Inc.) and 100 bp Plus DNA ladder was used to estimate amplicon sizes. PCR products were sent to Macrogen, Inc. (South Korea) for purification and bi-directional Sanger sequencing. Once sequences were obtained, they were manually edited and assembled using BioEdit v.7.2.5 (Hall, 1999).

Phylogenetic analysis

Analyses were performed using the best sequences obtained in this study and GenBank accessions from other species of the Criconematidae. Outgroup taxa for each region analyzed was chosen as reference from previous publications (Clavero-Camacho *et al*., 2022; Hosseinvand *et al*., 2022). Multiple alignments were performed with FFT-NS-2 algorithm of MAFFT v.7.450 (Katoh *et al*., 2019). Phylogenetic analysis of each sequence dataset was performed by Bayesian Inference (BI) with MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003). jModelTest v.2.1.10 was used to find the best-fit nucleotide substitution model based on Akaike Information Criterion (AIC) (Darriba *et al*., 2012). Analyses were performed under the TIM3+I+G model for D2D3 region, TVM+I+G for ITS region, and GTR+I+G for 18S and COI regions. FigTree v.1.4.3 (Rambaut, 2016) was used to generate phylogenetic trees.

RESULTS

Morphological, morphometric, and molecular analyses allowed for the identification of four Criconematidae species. Among these were two known species, *Mesocriconema onoense* in a natural area and *Mesocriconema sphaerocephalum* in grass. Two new species were isolated and described, *Mesocriconema paraonoense* n. sp., a cryptic species discovered in cocoa and sugar cane plantations, and *Mesocriconema costarricense* n. sp. in a natural area.

Description and morphometry

Mesocriconema onoense (Luc, 1959) Loof and De Grisse, 1989 (Fig. 1; Table 1)

This nematode species was found in soil samples collected from a natural grass area in the margin of a lake at Alajuela province (n = 35) and in the rhizosphere of herbaceous plants from a forest edge at San José province (n = 16) provinces. Females have a robust body (range 356.0-541.1 µm long) with medium body diameter (39.6-58.2 µm) that narrows at the anterior and posterior ends. Annulations are small (3.5-5.4 µm wide), with smooth edges, projected towards the posterior region with occasional anastomosis. The number of rings varied from 105 to 122 along the body. The cephalic region shows small, rounded submedian lobes and a narrow first ring with a diameter between 9.4 and 14.4 µm. Stylet is moderately long (50.9-62.6 µm), straight, and robust, representing 50.3-51.8% pharynx length, and 12.3-12.7% body length. Knobs are broad and anchor-shaped (7.6-12.8 µm wide). The pharynx (97.1-130.6 µm) presents a characteristic criconematoid shape (fused procorpus and metacorpus). The excretory pore is located posterior to the pharynx base, between annulation 27 and 36 from the anterior end. Vulva is open, with 7-11 annulations. Conical tail with a terminal multi-shaped ring (simple, multilobed or truncated). Juveniles are like females, but smaller in size and with narrower annulations. No males were found in this population.

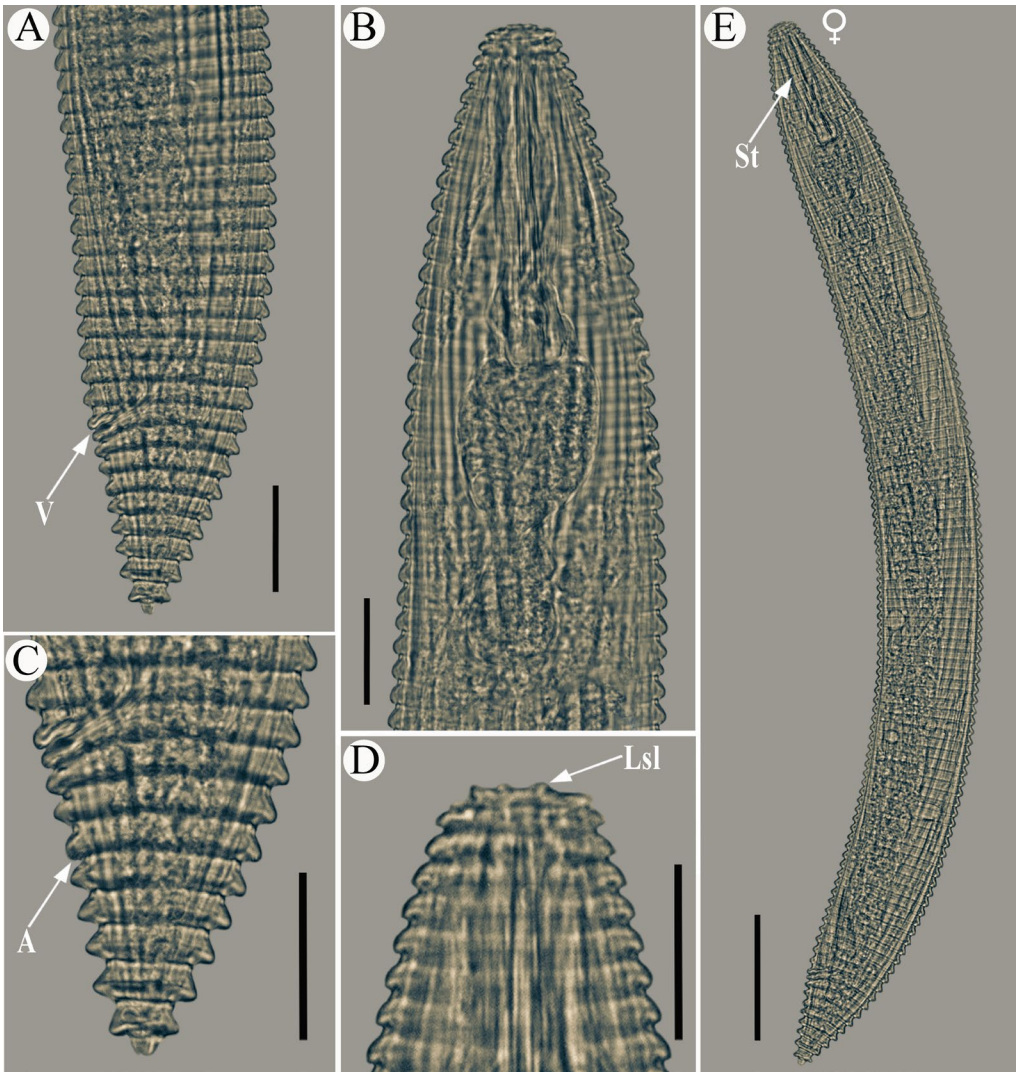

Figure 1. Photomicrographs of *Mesocriconema onoense* females: (A) posterior region showing vulva position, (B) anterior region, (C) tail shape and anus position, (D) cephalic region showing labial submedian lobes, and (E) whole female. Abbreviations: V = vulva, A = anus, Lsl = labial submedian lobe, St = stylet. Scales: A, B, C, D = 20 µm; E = 50 µm.

Mesocriconema paraonoense n. sp. (Fig. 2; Table 1)

This nematode species was found in cocoa plantations from Puntarenas province (n = 32; type population), Costa Rica (coordinates 8°39'55.0"N; 83°01'16.2"W). Additional populations were found in a cocoa plantation in Alajuela province (n = 30) and in a sugar cane plantation in Cartago province (n = 4). Females are similar to *M. onoense*, with a robust body (357.8-591.8 µm long) with a medium body diameter (44.0-61.5 µm), which acquires a ventrally curved position when heat-fixed in formaldehyde. Body with 109-150 annulations (3.0-5.1 µm wide), projecting towards the posterior region, with smooth edges and occasional anastomosis. The head region is typically flattened, with small submedian lobes and a first ring with slightly forward edges, ranging from 9.6 to 14.7 µm in diameter. Stylet is short (44.1-53.9 µm) relative to body length, but it is robust and strong, and represents 9.6-11.7% body length and 45.2-52.2% pharynx length. Knobs are anchor-shaped and varied from 8.5 to 11.7 µm. The pharynx with the typical criconematid shape varied between 80.9 and 126.1 µm. The excretory pore is found posterior to the pharynx. Vulva is clearly open, with a diameter between 32.5 and 48.3 µm and 7-12 rings towards the end of the tail. The tail shows morphological diversity, simple, multilobed,

truncated or flattened endings. A conical shape is mostly observed, although in some cases it can be rounded. Juveniles are like females, although smaller. No males were found in this population.

Etymology

The species epithet *paraonoense* is composed of the Greek preposition para (meaning alongside of and resembling) and onoense for its similitude to *Mesocriconema onoense*.

Type material

Holotype female, 76 paratypes females and 31 juveniles were deposited into the nematode collection of the Criconematidae family (identification codes 185C-1 to 185C-7) of the Laboratorio de Nematología, Escuela de Ciencias Agrarias, Universidad Nacional (UNA), Heredia, Costa Rica.

Diagnosis and relationships

Mesocriconema paraonoense n. sp. females have a moderate body length = 357.8-591.8 µm, stylet length = 44.1-53.9 µm, V = 89.7-93.7%, a = 7.3-10.6, b = 3.8-5.2, R = 109-150, RV = 7-12, Rex = 30-41, and VL/VB = 0.8-1.3, conical tail with multi-shaped terminal annulus.

The dichotomic key developed by Geraert (2010) for *Mesocriconema* species, suggests that *M. paraonoense* n. sp. should be compared with species that share characteristics such as occasional anastomosis, anterior bilobed vulva lip, simple unilayered cuticle, stylet length (35-93 µm), conical-rounded tail with bigger multilobed terminal ring, and R (110-132). All populations of the new species were morphologically and morphometrically similar to *M. onoense* (Luc, 1959; Loof and De Grisse, 1989), however, they were clearly separated through the molecular characterization of ribosomal and mitochondrial genes. *M. paraonoense* n. sp. also resembles related species such as *M. brevistylus* (Singh and Khera, 1976; Loof and De Grisse, 1989) and *M. onostre* (Phukan and Sanwal, 1981; Loof and De Grisse, 1989), however, they are differentiated by having a shorter stylet (44.1-53.9 µm vs. 56-60 µm and 54-61 µm) and a wider range in the number of body rings (109-150 vs. 140-156 and 133-147), RV (7-12 vs. 9- 10 and 7-9), and Rex (30-41 vs. 35-41 and 36-38).

Mesocriconema sphaerocephalum (Taylor, 1936) Loof and De Grisse, 1989 (Fig. 3; Table 2)

This nematode species was isolated from grass samples from San José province. Females (n = 36) have small body (256.2-389.9 µm) with

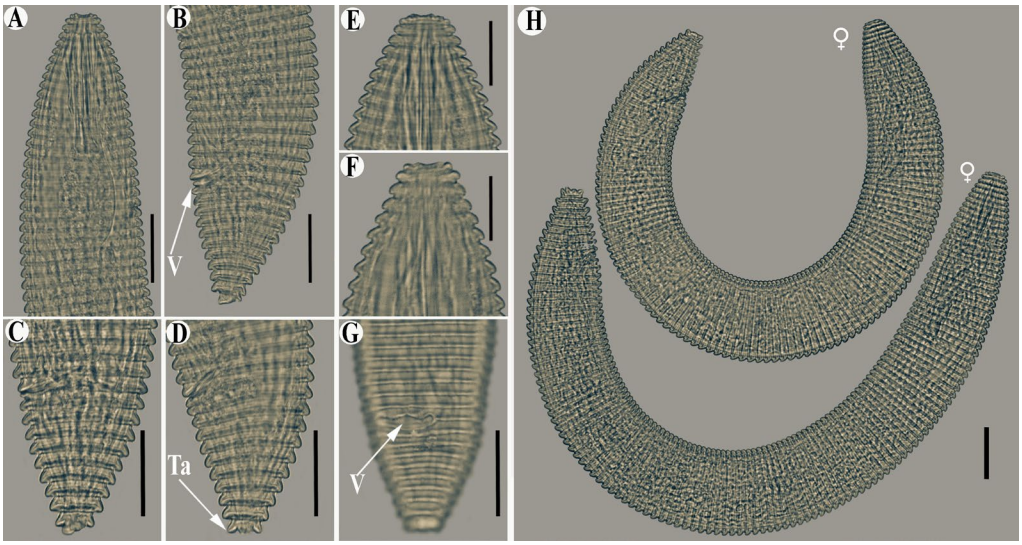

Figure 2. Photomicrographs of *Mesocriconema paraonoense* n. sp. females: (A) anterior region, (B) posterior region showing vulva position, (C, D) tail shape and terminal annulus, (E, F) cephalic region, (G) vulva, and (H) whole females. Abbreviations: V = vulva, Ta = terminal annulus. Scales: A, B, C, D, G = 20 µm; E, F = 10 µm; H = 30 µm.

Table 1. *Mesocriconema onoense* and *Mesocriconema paraonoense* n. sp.: Morphometry of females found in natural areas, cocoa and sugar cane plantations in Costa Rica. All measurements in micrometers and in format: mean ± standard deviation (range).

	Mesocriconema onoense		*Mesocriconema paraonoense* n. sp.			
Host	Natural area		Cocoa			Sugar cane
Location	Rio Cuarto, Alajuela	Vázquez de Coronado, San José	Ciudad Neily, Puntarenas		Guatuso, Alajuela	Jiménez, Cartago
			Holotype	Paratypes		
n	35 ♀♀	16 ♀♀	1♀	32 ♀♀	30 ♀♀	4 ♀♀
L	459.8 ± 37.3 (356.0-513.5)	464.6 ± 47.1 (368.4-541.1)	540.1	508.4 ± 38.1 (427.0-567.5)	505.8 ± 48.9 (424.3-591.8)	431.9 ± 75.4 (357.8-520.3)
a	9.5 ± 0.9 (7.5-11.3)	9.5 ± 0.4 (8.9-10.2)	9.0	9.3 ± 0.5 (8.2-10.3)	9.5 ± 0.6 (8.3-10.6)	8.5 ± 1.0 (7.3-9.7)
b	4.1 ± 0.3 (3.6-4.8)	4.1 ± 0.2 (3.8-4.4)	4.7	4.6 ± 0.3 (3.9-5.2)	4.5 ± 0.3 (4.0-5.1)	4.5 ± 0.5 (3.8-4.9)
V%	92.6 ± 0.7 (90.8-94.2)	92.4 ± 0.7 (91.6-94.3)	92.2	92.0 ± 0.7 (89.9-93.0)	92.1 ± 0.8 (90.7-93.7)	91.5 ± 0.6 (90.6-91.9)
St	58.0 ± 2.5 (52.9-62.6)	56.7 ± 2.9 (50.9-61.5)	51.3	50.2 ± 2.3 (44.1-53.9)	48.1 ± 1.5 (44.6-51.2)	49.3 ± 2.1 (46.3-51.1)
FAD	12.5 ± 1.2 (9.4-14.4)	11.9 ± 0.9 (10.2-13.7)	12.5	12.1 ± 0.7 (10.9-13.9)	11.9 ± 1.3 (9.6-14.2)	13.5 ± 1.1 (12.3-14.7)
AW	4.2 ± 0.4 (3.5-5.0)	4.3 ± 0.5 (3.5-5.4)	4.3	4.2 ± 0.4 (3.3-4.9)	4.3 ± 0.5 (3.4-5.1)	4.0 ± 0.5 (3.6-4.6)
KW	10.2 ± 1.2 (7.6-12.8)	10.6 ± 1.1 (8.8-12.8)	10.9	10.5 ± 0.7 (9.1-11.9)	9.9 ± 0.8 (8.5-11.7)	10.4 ± 1.1 (8.9-11.3)
Pha	112.2 ± 6.1 (99.3-129.6)	112.9 ± 9.2 (97.1-130.6)	115.6	111.4 ± 6.9 (98.5-125.5)	112.2 ± 7.5 (97.6-126.1)	95.5 ± 12.0 (80.9-110.1)
MBD	48.8 ± 3.8 (41.7-58.2)	48.8 ± 5.4 (39.6-57.2)	60.3	54.5 ± 3.9 (46.5-61.5)	53.1 ± 4.6 (44.0-60.6)	50.6 ± 2.8 (47.7-53.9)
VBD	32.5 ± 2.9 (27.0-38.0)	32.5 ± 4.1 (27.0-39.5)	45.4	39.7 ± 3.5 (33.3-48.3)	38.2 ± 4.1 (32.5-45.5)	36.0 ± 1.7 (34.0-38.1)
DGO	4.5 ± 0.6 (3.5-5.5)	4.5 ± 0.5 (3.6-5.6)	4.0	4.1 ± 0.5 (3.1-5.1)	3.8 ± 0.5 (3.0-5.1)	4.1 ± 0.2 (4.0-4.2)
R	115.9 ± 3.3 (108-122)	113.8 ± 4.5 (105-120)	135	129.1 ± 5.4 (122-150)	127.0 ± 4.4 (109-131)	123.5 ± 1.9 (121-125)
Rex	32.9 ± 2.1 (27-36)	33.8 ± 1.6 (32-36)	37	35.3 ± 2.8 (31-41)	35.2 ± 1.3 (33-38)	31.5 ± 1.3 (30-33)
RV	8.5 ± 0.7 (7-10)	9.3 ± 0.9 (7-11)	11	10.2 ± 0.8 (9-12)	10.1 ± 1.0 (7-12)	9.8 ± 0.5 (9-10)
St%L	12.7 ± 1.0 (11.2-15.2)	12.3 ± 0.9 (10.6-13.8)	9.5	9.9 ± 0.8 (9.0-12.6)	9.6 ± 0.8 (8.4-11.3)	11.6 ± 1.7 (9.5-13.2)
St%Pha	51.8 ± 2.5 (45.7-57.9)	50.3 ± 2.7 (45.9-54.3)	44.3	45.2 ± 2.9 (39.8-53.6)	43.0 ± 2.4 (38.7-49.4)	52.2 ± 7.4 (45.0-62.2)
VL	34.0 ± 3.6 (26.8-43.0)	35.3 ± 4.1 (25.3-44.3)	42.3	40.7 ± 4.4 (32.1-48.1)	39.9 ± 5.2 (29.8-49.6)	36.6 ± 4.8 (31.8-42.2)
VL/VBD	1.1 ± 0.1 (0.8-1.3)	1.1 ± 0.1 (0.9-1.3)	0.9	1.0 ± 0.1 (0.8-1.2)	1.0 ± 0.1 (0.8-1.3)	1.0 ± 0.1 (0.9-1.1)

Abbreviations: n (number of specimens measured), L (body length), a (body length / medium body diameter), b (body length / pharyngeal length), V% (((body length - distance from vulva to posterior end) / body length) x 100), St (stylet length), FAD (first cephalic annulus diameter), AW (annulus width), KW (stylet knob width), Pha (pharynx length), MBD (medium body diameter), VBD (vulval body diameter), DGO (distance from stylet base to dorsal oesophageal gland), R (number of body annulus), Rex (number of annulus from anterior end to excretory pore), RV (number of annulus from vulva to posterior end), St%L (stylet length / body length x 100), St%Pha (stylet length / pharynx length x 100), VL (distance from vulva to posterior end) and VL/VBD (distance from vulva to posterior end / vulval body diameter).

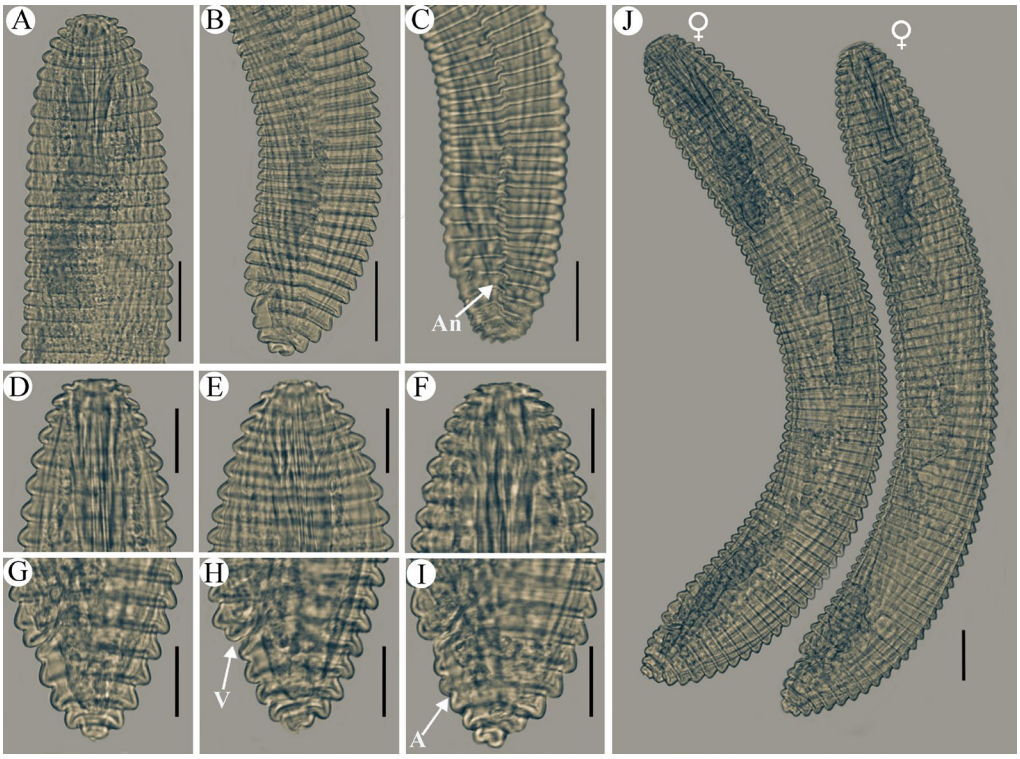

Figure 3. Photomicrographs of *Mesocriconema sphaerocephalum* females: (A) anterior region, (B) posterior region, (C) zigzag pattern anastomosis, (D, E, F) cephalic region, (G) tail shape, (H) vulva position, (I) anus position, and (J) whole females. Abbreviations: An = anastomosis, V = vulva, A = anus. Scales: A, B, C, G, H, I = 20 µm; D, E, F, = 10 µm; J = 30 µm.

uniform body diameter (29.1-53.6 µm). They have 65 to 76 rounded annulations (3.9-5.8 µm wide) with smooth edges slightly projecting backwards. Anastomosis is frequent in their rings, forming zigzag lateral lines. The cephalic region is typically flattened, with rounded submedian lobes. The first two cephalic annulations are narrower and the edges of the first one projects slightly forward. Stylet is straight and strong (41.1-62.1 µm long) representing 52.2% and 15.8% of the pharynx and body length, respectively. It presents anchor-shaped knobs (9.2-13.3 µm wide) and a typical criconematoid pharynx (77.9-109.2 µm long). The excretory pore is found posterior to the pharynx base. Vulva is open and found near the posterior end of the body, with 4-8 rings. The tail is rounded with a commonly bilobed terminal ring. Juveniles have a body like females, but they are smaller, with fine annulations, projected backwards and with abundant anastomosis. No males were found in this population.

Mesocriconema costarricense n. sp. (Fig. 4; Table 2)

This nematode species was discovered in soil samples from a natural area with herbaceous plants growing on a forest margin in Limón province, Costa Rica (coordinates 9°46'37.5"N; 82°54'28.3"W). Females (n = 38) have small body (300.4-396.1 µm long) with a medium body diameter (29.4-47.3 µm) that narrows towards the anterior and posterior ends. Anastomosis is infrequent, and its rings (3.3-4.7 µm in the middle of the body) are narrow and dotted, with smooth edges, clearly projected backwards. Their number varied between 95 and 105. The cephalic region presents well-developed submedian lobes projected forward. Stylet is long (65.0-76.9 µm), straight, and strong, representing 63.6% and 20.5% of the pharynx and body length, respectively. Knobs are anchor-shaped (7.8-10.9 µm wide). The pharynx (97.3-120.6 µm long) is typical of criconematids. The excretory pore is found near the

Table 2. *Mesocriconema sphaerocephalum* and *Mesocriconema costarricense* n. sp.: Morphometry of females found in pastures and natural areas in Costa Rica. All measurements in micrometers and in format: mean ± S.D. (range).

	Mesocriconema sphaerocephalum	*Mesocriconema costarricense* n. sp.	
Host	Grass	Natural area	
Location	Aserrí, San José	Talamanca, Limón	
		Holotype	Paratypes
n	36 ♀♀	1♀	38 ♀♀
L	320.8 ± 33.2 (256.2-389.9)	352.3	340.1 ± 26.4 (300.4-396.1)
a	7.9 ± 1.0 (6.0-10.1)	10.5	8.8 ± 0.9 (7.5-11.1)
b	3.3 ± 0.3 (2.7-3.8)	3.1	3.1 ± 0.2 (2.5-3.5)
V%	93.7 ± 0.9 (91.3-95.8)	91.8	91.6 ± 0.9 (90.0-93.3)
St	50.4 ± 4.5 (41.1-62.1)	72.2	69.5 ± 3.0 (65.0-76.9)
FAD	15.5 ± 1.5 (13.3-18.9)	10.6	10.1 ± 0.9 (8.2-11.6)
AW	5.0 ± 0.5 (3.9-5.8)	4.2	3.9 ± 0.4 (3.3-4.7)
KW	10.6 ± 0.9 (9.2-13.3)	9.5	8.9 ± 0.8 (7.8-10.9)
Pha	96.6 ± 6.9 (77.9-109.2)	115.3	109.5 ± 6.2 (97.3-120.6)
MBD	41.4 ± 6.7 (29.1-53.6)	33.7	39.0 ± 4.1 (29.4-47.3)
VBD	27.6 ± 4.0 (20.9-37.5)	24.1	25.9 ± 2.6 (21.2-33.3)
DGO	4.1 ± 1.2 (2.2-5.6)	4.3	3.9 ± 0.7 (2.9-5.7)
R	68.2 ± 2.4 (65-76)	102	101. 3 ± 2.2 (95-105)
Rex	20.0 ± 1.0 (19-21)	34	33.7 ± 1.2 (33-35)
RV	5.1 ± 0.8 (4-8)	8	8.0 ± 0.6 (7-10)
St%L	15.8 ± 1.6 (13.2-20.0)	20.5	20.5 ± 1.4 (18.0-25.2)
St%Pha	52.2 ± 4.0 (44.7-61.2)	62.6	63.6 ± 2.2 (58.8-69.3)
VL	20.3 ± 3.7 (13.2-27.6)	28.8	28.6 ± 3.6 (21.5-38.8)
VL/VBD	0.7 ± 0.1 (0.5-1.0)	1.2	1.1 ± 0.1 (0.8-1.3)

Abbreviations: n (number of specimens measured), L (body length), a (body length / medium body diameter), b (body length / pharyngeal length), V% (((body length - distance from vulva to posterior end) / body length) x 100), St (stylet length), FAD (first cephalic annulus diameter), AW (annulus width), KW (stylet knob width), Pha (pharynx length), MBD (medium body diameter), VBD (vulval body diameter), DGO (distance from stylet base to dorsal oesophageal gland), R (number of body annulus), Rex (number of annulus from anterior end to excretory pore), RV (number of annulus from vulva to posterior end), St%L (stylet length / body length x 100), St%Pha (stylet length / pharynx length x 100), VL (distance from vulva to posterior end) and VL/VBD (distance from vulva to posterior end / vulval body diameter).

middle of the basal bulb. Vulva is open, with 7-10 rings. Conoid-like tail has a simple terminal dotted or bifurcated ring. Juveniles have a body shape like females, but they are smaller, with annulations that project towards the posterior region, and prominent lobes in the cephalic region. No males were found in this population.

Etymology

The species epithet, *costarricense*, refers to the gentilic of inhabitants of the country where this new species was found (Costa Rica).

Type material

Holotype female, 56 paratypes females and 21 juveniles were deposited into the nematode collection of the Criconematidae family (identification codes 175C-1 to 175C-5) of the Laboratorio de Nematología, Escuela de Ciencias Agrarias Universidad Nacional (UNA), Heredia, Costa Rica.

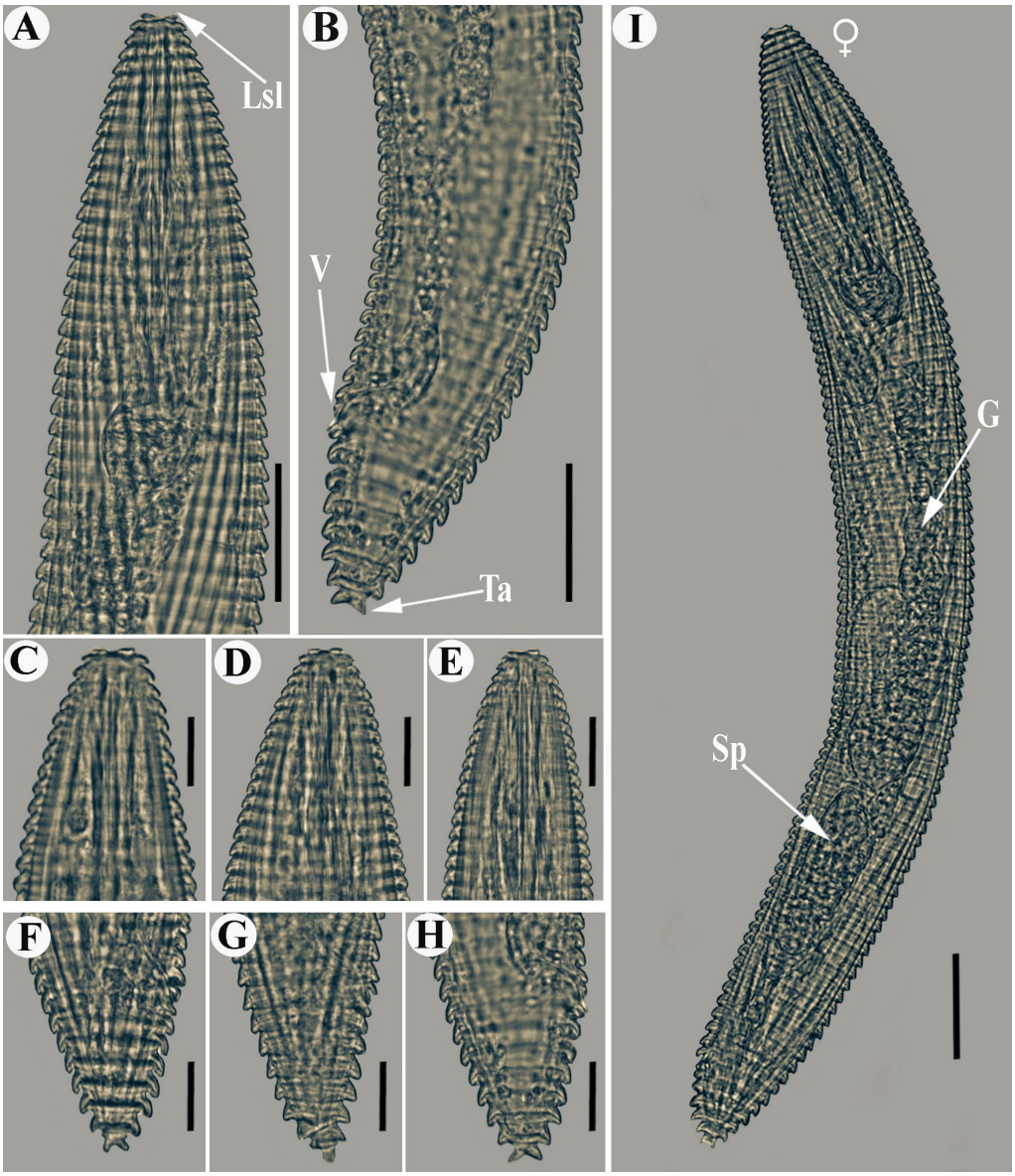

Figure 4. Photomicrographs of *Mesocriconema costarricense* n. sp. females: (A) anterior region showing labial submedian lobes, (B) posterior region showing vulva position and terminal annulus, (C, D, E) cephalic region, (F, G, H) tail shape, and (I) whole female showing the gonad and spermatheca. Abbreviation: Lsl = labial submedian lobe, V = vulva, Ta = terminal annulus, G = gonad, Sp = spermatheca. Scales: A, B = 20 µm; C, D, E, F, G, H = 10 µm; I = 30 µm.

Diagnosis and relationships

Mesocriconema costarricense n. sp. females have a small body length = 300.4-396.1 µm, stylet length = 65.0-76.9 µm, V = 90.0-93.3%, a = 7.5-11.1, b = 2.5-3.5, R = 95-105, RV = 7-10, Rex = 33-35, and VL/VB = 0.8-1.3.

This new species shares morphological similarities with *M. discus* (Thorne and Malek, 1968; Loof and De Grisse, 1989) and *M. rusticum* (Micoletzky, 1915; Loof and De Grisse, 1989) in vulva position (90.0-93.3% vs. 94-95% and 92-95%), R (95-105 vs. 94-106 and 81-107) and RV (7-10 vs. 7 and 7-10). However, they can be differentiated by body length (300.4-396.1 µm vs. 450-650 µm and 340-520 µm) and stylet length (65.0-76.9 µm vs 65-72 µm and 50-60 µm). Furthermore, *M. discus* and *M. rusticum* have rounded tail, well-developed submedian lobes and excretory pore posterior to the basal bulb, while *M. costarricense* n. sp. has less developed submedian lobes, conoid-like tail with a simple terminal dotted

or bifurcated ring and excretory pore in the middle of the basal bulb. Besides these morphological differences, molecular characterization based on ribosomal and mitochondrial genes allowed these species to be separated.

Molecular characterization

Amplification of 28S D2D3, ITS, 18S and COI regions generated fragments of 750, 770, 870, and 740 bp, respectively. Fifteen D2D3, 11 ITS, five 18S, and twelve COI sequences were obtained. Each sequence was compared with GenBank accessions by means of BLAST tool (Basic Local Alignment Search Tool) of the NCBI (National Center for Biotechnology Information) and deposited in GenBank (Table 3). Sequences of the 28S D2D3 rDNA gene of *M. onoense* (OR077113 to OR077115) showed high similarity (99.1-99.4%) with *M. onoense* from Vietnam (MZ220549) (Nguyen *et al*., 2021) and *Criconemoides brevistylus* (98.8-99.1%) from South Africa (JQ231185) (Van den Berg *et al*., 2012). *Mesocriconema paraonoense* n. sp. D2D3 sequences (OR077106 to OR077112) showed low similarity (94.3%) with *M. onoense* from Vietnam (MZ220549) (Nguyen *et al*., 2021) and high similarity (100%) with unidentified *Mesocriconema* species from India (OQ400911) (Sorokhaibam, 2023) and China (MW938501) (Zhao and Zeng, 2021). These two unidentified species may correspond to *M. paraononese* n. sp. discovered in this study; however, there is no morphological and morphometric data supporting those studies. *Mesocriconema sphaerocephalum* D2D3 sequences (OR077116 to OR077118) exhibited high similarity (99.5%) with *M. sphaerocephalum* from South Africa (MN262453) (Lamula *et al*., 2019) and *M. sphaerocephalum* (98.8-99.1%) from Vietnam (MK026628) (Nguyen *et al*., 2019). *Mesocriconema. costarricense* n. sp. D2D3 sequences (OR077119 and OR077120) showed low similarity (95.3-95.5%) with *M. xenoplax* from China (OQ674275) (Zhang *et al*., 2023) and *Mesocriconema nebraskense* (94.1-94.3%) from USA (MH013430) (Yan *et al*., 2018).

The obtained ITS sequences from *M. onoense* (OR077139 and OR077140) also showed high similarity (97.9-98.1%) with *M. onoense* from Vietnam (MZ361701) (Nguyen *et al*., 2021) and *C. brevistylus* (98.0-98.3%) from South Africa (JQ231188) (Van den Berg *et al*., 2012).

Table 3. Criconematidae populations found in this study with corresponding GenBank accession numbers for each sequenced region.

Species	ID[y]	Location	Host	D2D3	ITS	18S	COI
Mesocrionema onoense	153C	Río Cuarto, Alajuela	Natural area	OR077113 OR077114	OR077139 OR077140	OR077127	OR167994 OR167995
	173C	Vázquez de Coronado, San José	Natural area	OR077115	-[z]	OR077128	OR167992 OR167993
M. paraonoense n. sp.	185C	Ciudad Neily, Puntarenas	Cocoa	OR077109 OR077110	OR077136 OR077137 OR077138	OR077130	OR167986 OR167987 OR167988
	188C	Guatuso, Alajuela	Cocoa	OR077106 OR077107 OR077108	OR077132 OR077133	-	OR167989
	174C	Jiménez, Cartago	Sugar cane	OR077111 OR077112	OR077134 OR077135	OR077129	OR167990 OR167991
M. sphaerocephalum	171C	Aserrí, San José	Grass	OR077116 OR077117 OR077118	-	-	-
M. costarricense n. sp.	175C	Talamanca, Limón	Natural area	OR077119 OR077120	OR077141 OR077142	OR077126	OR168958 OR168959

[y]ID = Identification code from Laboratorio de Nematología, Escuela de Ciencias Agrarias, Universidad Nacional.

[z](-) = No sequence obtained.

Mesocriconema paraonoense n. sp. ITS sequences (OR077132 to OR077138) displayed low identity (84.7-84.9%) with *M. onoense* from Vietnam (MZ361701) (Nguyen *et al*., 2021) and *C. brevistylus* from South Africa (JQ231188) (Van den Berg *et al*., 2012). *Mesocriconema. costarricense* n. sp. ITS sequences (OR077141 and OR077142) showed low similarity (86.2-86.3%) with *M. xenoplax* from China (ON024313) (Zhang *et al*., 2022) and *M. nebraskense* (85.2-85.4%) from USA (MH013431) (Yan *et al*., 2018).

The obtained 18S gene sequences from *M. onoense* (OR077127 and OR077128) displayed high similarity (99.2-99.3%) with *M. onoense* from USA (MF094909) (Powers *et al*., 2017) and *M. onoense* (99.1-99.2%) from Vietnam (MZ361704) (Nguyen *et al*., 2021). *Mesocriconema paraonoense* n. sp. 18S sequences (OR077129 and OR077130) showed low similarity (96.2%) with *M. onoense* (MF094909) and *M. xenoplax* (95.7%) from USA (MF095022) (Powers *et al*., 2017). *Mesocriconema costarricense* n. sp. (OR077126) 18S sequences showed high similarity (99.0%) with *Mesocriconema* sp. (MF094974) from USA (Powers *et al*., 2017) in this genomic region.

The COI sequences from *M. onoense* (OR167992 to OR167995) and *M. paraonoense* n. sp. (OR167986 to OR167991) showed low identity (90.4% and 87.1-87.4% respectively) with *M. onoense* from USA (KJ787834) (Powers *et al*., 2014). Also, COI sequences of *M. costarricense* n. sp. (OR168958andOR168959) showed low similarity (90.4%) with *M. rusticum* from USA (KJ787855) (Powers *et al*., 2014).

Phylogenetic trees from 28S D2D3, ITS, 18S and COI regions (Figs. 5, 6, 7, and 8, respectively), generated by Bayesian Inference (BI), showed phylogenetic relationships between criconematids from *Mesocriconema* and *Criconemoides* genera. Nucleotide substitution probabilities were greater than 70% for all regions analyzed. The two new species described in this study were clearly separated from other *Mesocriconema* species. *M. paraonoense* n. sp. grouped into separate clades and differed phylogenetically from related species such as *M. onoense* in all genomic regions analyzed. However, sequences from the D2D3 region (Fig. 5) were identical to D2D3 sequences from unidentified species of *Mesocriconema* from India and China. *Mesocriconema costarricense* n. sp. was well separated from other species, however, was more closely related to *M. xenoplax* and *M. nebraskense* based on the D2D3 and ITS regions (Figs. 5 and 6, respectively). *Mesocriconema onoense* from this study grouped with *M. onoense* from other countries and with *C.*

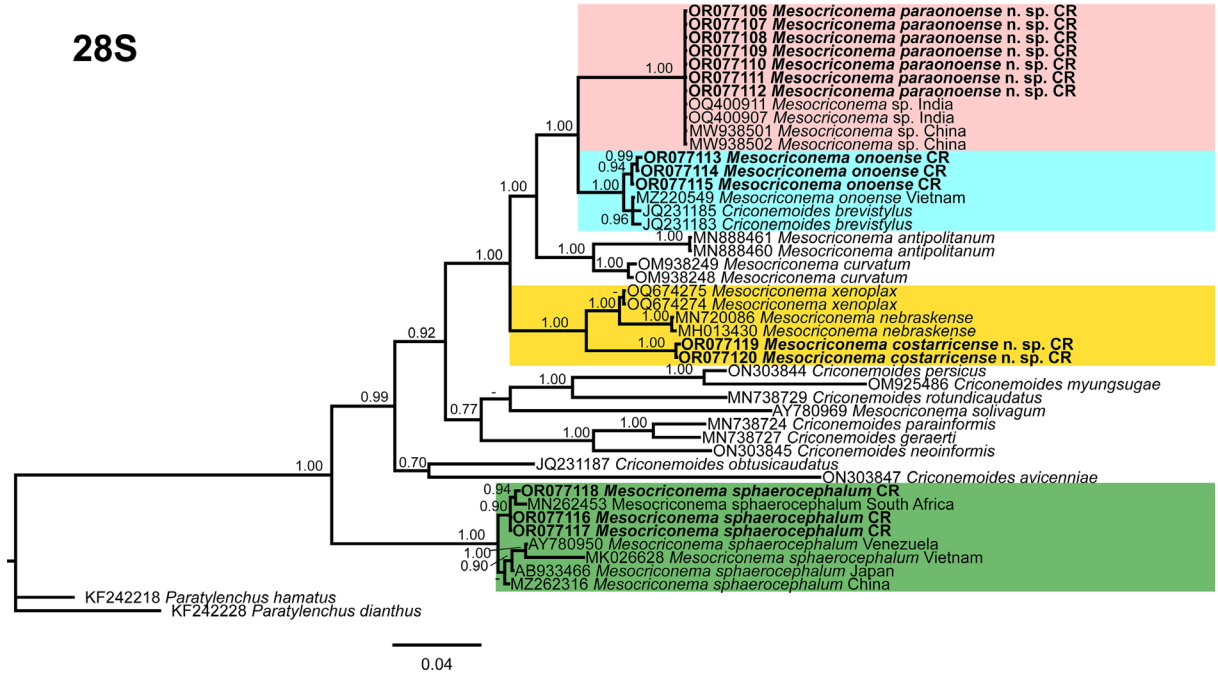

Figure 5. Phylogenetic tree generated from D2D3 sequences of the 28S gene by Bayesian Inference (BI) under TIM3+I+G model. Probabilities for appropriate clades were greater than 0.70. Sequences from this study are marked in bold. Colored boxes indicate related species in the clades.

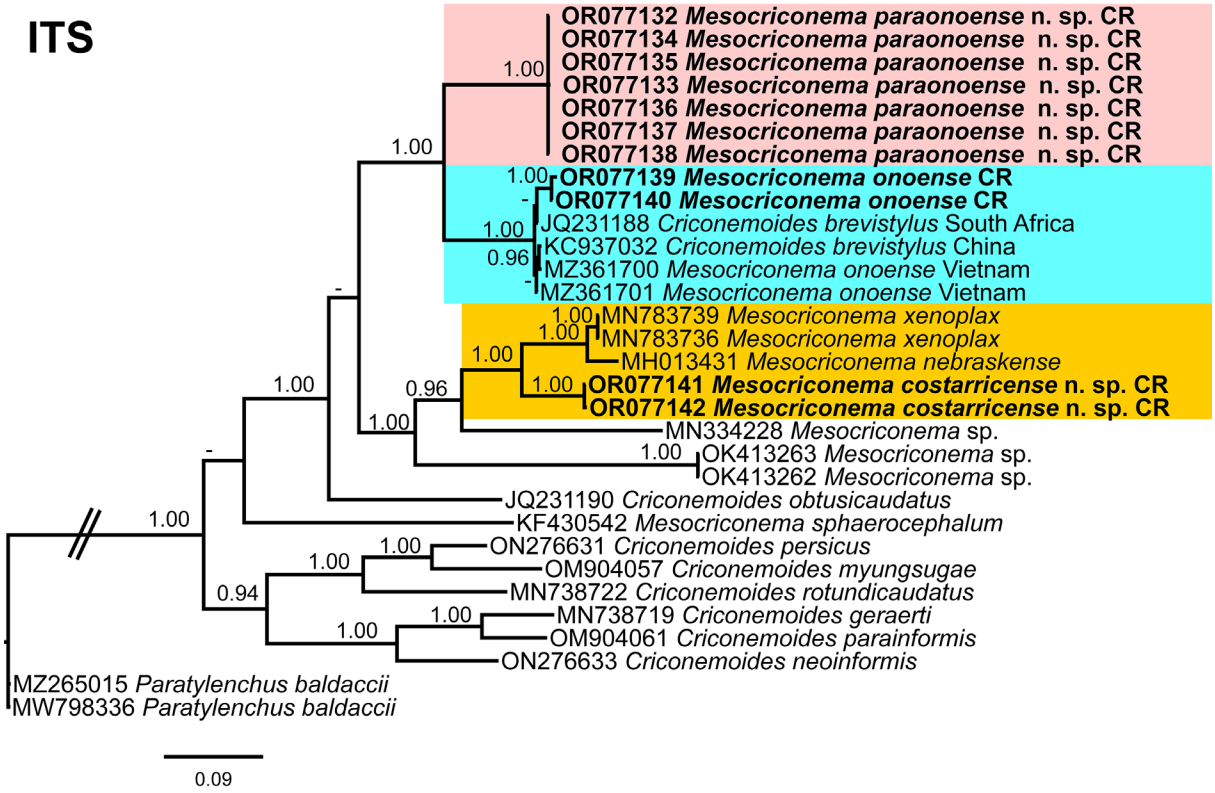

Figure 6. Phylogenetic tree generated from ITS sequences by Bayesian Inference (BI) under TVM+I+G model. The probabilities for appropriate clades were greater than 0.70. Sequences from this study are marked in bold. Colored boxes indicate related species in the clades.

brevistylus (D2D3, ITS, and 18S regions) (Figs. 5, 6, and 7). The *M. sphaerocephalum* D2D3 sequences (Fig. 5) from this study clustered in a separate clade with *M. sphaerocephalum* from other countries. It was not possible to obtain good quality ITS, 18S and COI sequences from *M. sphaerocephalum* (Table 3).

DISCUSSION

In Costa Rica, *M. onoense* was previously reported in rice (Sancho and Salazar, 1985); however, worldwide it has been found in the rhizosphere of crops such as avocado, rice, coffee, sugar cane, mango, citrus, grasses, pineapple, and tomato in Colombia (Volcy, 1998); medicinal plants in Vietnam (Nguyen *et al*., 2021); grasses, maple (Cordero *et al*., 2012b), turf (Powers *et al*., 2014), and pineapple (Zurbano *et al*., 2023) in North America; citrus (Crozzoli, 1998), sugar cane (Perichi *et al*., 2002), and rice in Venezuela (Medina *et al*., 2009); and, cotton, corn, and soybean in Brazil (Inomoto *et al*., 2011).

Mesocriconema sphaerocephalum was previously identified morphologically in avocado, peach (López and Salazar, 1988), and blackberry (Peraza, 2014) in Costa Rica. Worldwide this species is reported in corn (Powers *et al*., 2016a), turf (Munawar *et al*., 2018), and bermudagrass (Zeng *et al*., 2015) in North America; hybrid cane (Subbotin *et al*., 2005), sugar cane (Perichi *et al*., 2002), citrus (Crozzoli, 1998), and vegetables in Venezuela (Lugo *et al*., 2010); wheat in South Africa (Lamula, 2020); pepper in Egypt (Handoo *et al*., 2020); wild grass in Botswana (Shokoohi, 2021); sugar cane in Japan (Kawanobe *et al*., 2014); carrot in Vietnam (Nguyen *et al*., 2019); and, tomato in Spain (Gómez-Barcina *et al*., 1991). In Costa Rica, there are few official records of the presence of *M. onoense* and *M. sphaerocephalum*. However, these nematodes can be found in various ecosystems throughout the country in both cultivated areas and natural environments (W. Peraza, personal communication) According to Powers *et al*. (2014) *M. sphaerocephalum* is distributed worldwide and hosts include a wide variety of crops and native plant species.

Criconematids, being ectoparasites, are

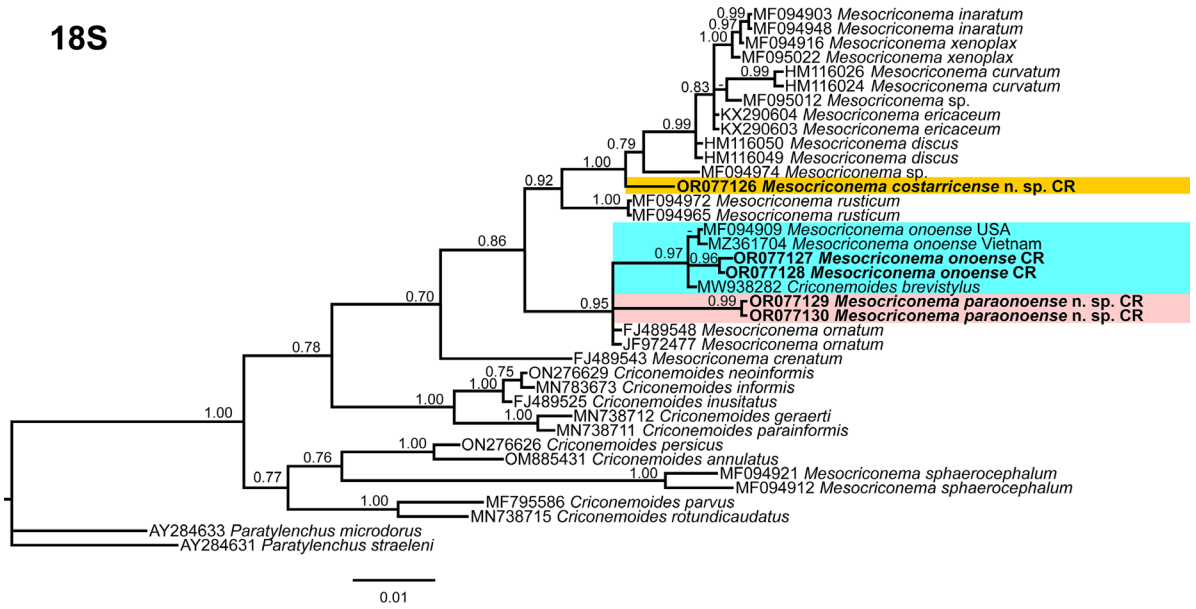

Figure 7. Phylogenetic tree generated from partial 18S gene sequences by Bayesian Inference (BI) under GTR+I+G model. The probabilities for appropriate clades were greater than 0.70. Sequences from this study are marked in bold. Colored boxes indicate related species in the clades.

characterized by having a well-developed stylets and an infective capacity in all their stages of development (Guzmán *et al*., 2012). These two characteristics, along with their wide variety of hosts and distribution, make these organisms highly relevant species for Costa Rican agricultural systems. Additionally, it is important to highlight that approximately 43.4% of the farm surface in Costa Rica is used for pasture production (INEC, 2015). This extensive presence of pastures could favor the distribution of these nematodes, since it has been observed that they are mainly associated with grasses such as rice, sugar cane, corn, turf, as well as other types of grasses all over the world.

Mesocriconema paraonoense n. sp. was found in the rhizosphere of sugar cane in the province of Cartago, as well as in cocoa at Alajuela and Puntarenas provinces. Based on morphology of this new described species, and considering other soil samples analyzed in the past by the Nematology Laboratory, we suggest that this species has a wide distribution throughout the country (W. Peraza, personal communication), since they coincide morphologically with other ring nematodes. Likewise, it is important to indicate that studies on nematodes associated with crops in Costa Rica are limited and relationship between criconematids and plant species with which they may be associated is not known with certainty.

Mesocriconema costarricense n. sp. was identified in a natural area in Limón province. Similar pristine sites in Costa Rica and other locations around the world harbor Criconematidae (Powers *et al*., 2009; Powers *et al*., 2016a; Powers *et al*., 2016b; Jahanshahi *et al*., 2020; Varela *et al*., 2022). Costa Rica stands out as one of the countries with the greatest biodiversity worldwide due to the variety of microclimates and environments, which is why plants host many nematode species (Peraza *et al*., 2017; Varela *et al*., 2018; Gamboa *et al*., 2023; Sandoval *et al*., 2023). Therefore, we do not rule out the presence of new species of nematodes from the Criconematidae such as those described in this study.

The two populations of *M. onoense* identified in this study showed remarkable similarity in their morphology and morphometry. Both populations are within the morphometric parameters established for the original populations described by Luc (1959, 1970) and Loof and De Grisse (1989). The populations collected in this study coincide morphometrically with the population of *M. onoense* from the USA (Cordero *et al*., 2012b) in terms of vulva position (92.4-92.6% vs. 93.9%), stylet length (56.7-58.0 µm vs. 58.7 µm), pharynx length (112.2-112.9 µm vs. 112.3 µm), DGO (4.5 µm vs. 4.3 µm), RV (8.5-9.3 vs. 9.9), and VL (34.0-35.3 µm vs. 35.5 µm). Furthermore, similarities were observed with the population of *M. onoense* from Vietnam, described by Nguyen *et al*. (2021)

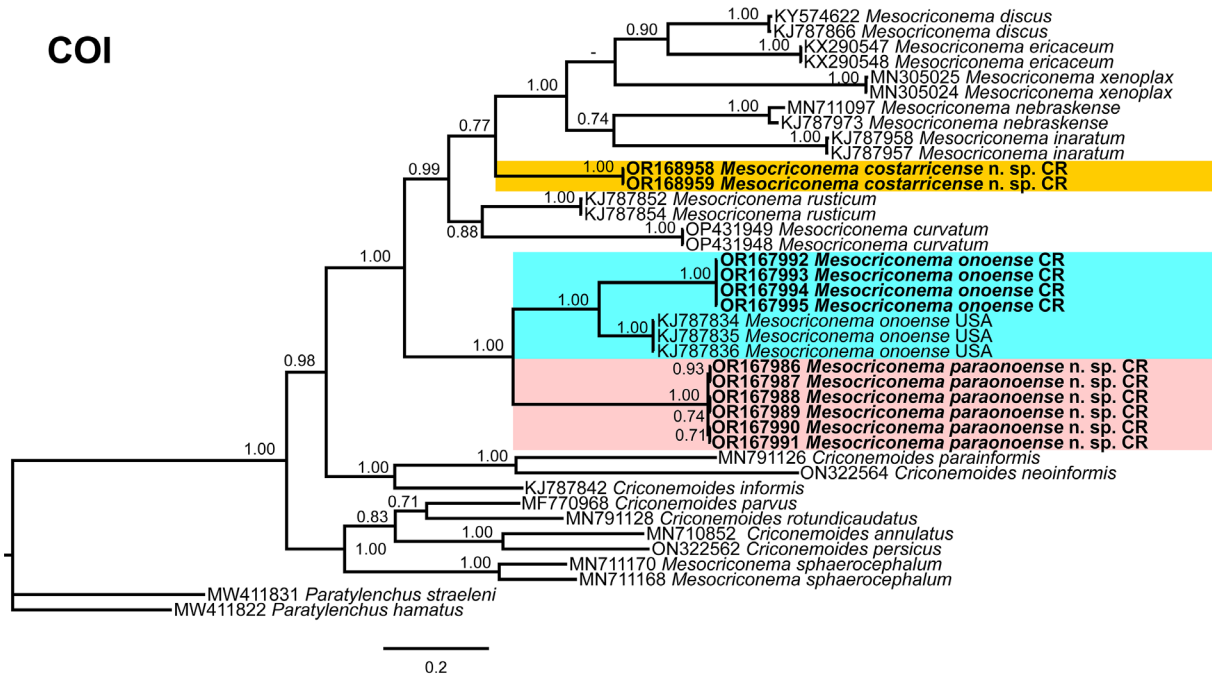

Figure 8. Phylogenetic tree generated from mitochondrial COI sequences by Bayesian Inference (BI) under GTR+I+G model. The probabilities of the appropriate clades are greater than 0.70. Sequences from this study are marked in bold. Colored boxes indicate related in the clades.

in the position of the vulva (92.4-92.6% vs. 93.0%), stylet length (56.7-58.0 µm vs. 59.0 µm), MBD (48.8 µm vs. 52.0 µm), VBD (32.9-33.8 µm vs. 34.0 µm), and RV (8.5-9.3 vs. 9.2).

Regarding *M. sphaerocephalum* in this study, a strong morphological relationship was found with the parameters originally described by Taylor (1936) and Loof and De Grisse (1989). In addition, this species showed a wide morphometric similarity with a previously described population in Costa Rica (Peraza, 2014), with similar values in vulva position (93.7% vs. 92.0%), stylet length (50.4 µm vs. 51.2 µm), pharynx length (96.6 µm vs. 96.6 µm), R (68.2 vs. 66.1), Rex (20.0 vs. 22.8), and RV (5.1 vs. 4.9). However, similarity with *M. sphaerocephalum* from Botswana (Shokoohi, 2021) was also observed in terms of body length (320.8 µm vs. 318.0 µm), vulva position (93.7% vs. 91.3%), stylet length (50.4 µm vs. 48.0 µm), pharynx length (96.6 µm vs. 96.0 µm), VBD (27.6 µm vs. 28.0 µm), R (68.2 vs. 66.0), Rex (20.0 vs. 21.0), and RV (5.1 vs. 4.6). Similitude to *M. sphaerocephalum* from Vietnam (Nguyen *et al*., 2019) was also observed in terms of vulva position (93.7% vs. 94.0-95.0%), stylet length (50.4 µm vs. 51.0-55.0 µm), R (68.2 vs. 64.0 -65.0), Rex (20.0 vs. 21.5-21.6), and RV (5.1 vs. 4.3-4.8), as well as with *M. sphaerocephalum* from the USA (Cordero *et al*., 2012b) in the vulva position (93.7% vs. 92.4%), stylet length (50.4 µm vs. 51.8 µm), R (68.2 vs. 65.7), Rex (20.0 vs. 20.6), and RV (5.1 vs. 5.4).

In both species, *M. onoense* and *M. sphaerocephalum*, differences were observed in some morphological and morphometric parameters such as body length, medium body diameter, number of rings, and tail morphology, compared to other described populations. However, these differences are common in plant-parasitic nematodes (Eskandari *et al*., 2010; Peraza *et al*., 2017) and could be related to their phenotypic plasticity in response to specific environmental conditions in which they are found, such as climate and the plant species on which they feed (Luc, 1970; Van den Berg *et al*., 2014; Peraza *et al*., 2016; Munawar *et al*., 2018; Iqbal *et al*., 2021).

The new cryptic species *M. paraonoense* n. sp. shows morphological and morphometric similarities with *M. onoense* described by Luc (1959) and Loof and De Grisse (1989) in body length (357.8-591.8 µm vs. 370-670 µm), stylet length (44.1-53.9 µm vs. 40-63 µm), R (109-150 vs. 111-138), RV (7-12 vs. 8-14), vulva position (89.9-93.7% vs. 89-94%), and VL/VBD (0.8-1.3 vs. 0.9-1.4). Moreover, this species shows several morphological and morphometric similarities with

M. onoense from this study (Table 1), such as a (8.5-9.5 vs. 9.5) and b ratios (4.5-4.6 vs. 4.1), vulva position (91.5-92.1% vs. 92.4-92.6%), FAD (11.9-13.5 vs. 11.9-12.5), AW (4.0-4.3 vs. 4.2-4.3), KW (9.9-10.5 vs. 10.2-10.6), Pha (95.5-112.2 vs. 112.2-112.9), MBD (50.6-54.5 vs. 48.8), DGO (3.8-4.1 vs. 4.5), Rex (31.5-35.3 vs. 32.9-33.8), RV (9.8-10.2 vs. 8.5-9.3), St%L (9.6-11.6 vs. 12.3-12.7), and VL/VBD (1.0 vs. 1.1). Morphologically, *M. paraonoense* n. sp. was initially identified as *M. onoense*, due to shared morphometric characteristics. However, after sequencing the D2D3, ITS, and COI regions, which are informative regions to differentiate species (Hosseinvand *et al*., 2022), we found low percentages of identity with respect to *M. onoense* sequences from GenBank. Therefore, we consider this nematode as a new species being part of a cryptic complex together with *M. onoense*, as observed in other ring nematodes from the genera *Criconema* and *Criconemoides* (Munawar *et al*., 2020; Clavero *et al*., 2022; Archidona *et al*., 2023). *Mesocriconema paraonoense* n. sp. shows some differences from possible close relatives such as *M. brevistylus* (Singh and Khera, 1976; Loof and De Grisse, 1989) and *M. onostre* (Phukan and Sanwal, 1981; Loof and De Grisse, 1989) by having a shorter stylet (44.1-53.9 µm vs. 56-60 µm and 54-61 µm) and a wider range in the number of body rings (109-150 vs. 140-156 and 133-147), RV (7-12 vs. 9-10 and 7-9), and Rex (30-41 vs. 35-41 and 36-38). However, these three species are within the morphometric ranges observed in *M. onoense*, have well separated submedian lobes, occasional anastomosis, retrograde annulations with smooth edges, and conical tails with morphological diversity in the terminal ring (Luc, 1959; Nguyen *et al*., 2021). This suggests the presence of a cryptic complex or intraspecific diversity in *M. onoense* due to environmental conditions in which they are found (Munawar *et al*., 2019; Clavero *et al*., 2022).

It is important to indicate that *M. onoense* as well as the new species *M. paraonoense* n. sp. coincided with *C. brevistylus* described by Van den Berg *et al*. (2012) in most of the morphometric parameters evaluated. According to the analysis carried out by Nguyen *et al*. (2021) and the molecular data from the present study, we support the hypothesis that the population of *C. brevistylus* from South Africa (Van den Berg *et al*., 2012) could be *M. onoense* (Figs. 5 and 6).

Mesocriconema costarricense n. sp. shares morphological similarities with *M. discus* (Thorne and Malek, 1968; Loof and De Grisse, 1989) and *M. rusticum* (Micoletzky, 1915; Loof and De Grisse, 1989) in terms of vulval position (90.0-93.3% vs. 94-95% and 92-95%), R (95-105 vs. 94-106 and 81-107), and RV (7-10 vs. 7 and 7-10). However, significant differences were observed in body length (300.4-396.1 µm vs. 450-650 µm and 340-520 µm) and stylet length (65.0-76.9 µm vs. 65-72 µm and 50-60 µm). Although all these species have well-developed submedian lobes, in the case of *M. costarricense* n. sp., these lobes are raised and narrowly separated, unlike *M. discus* and *M. rusticum* lobes, which are more open and flattened, resembling a disk in the labial region. Another observable difference that makes it possible to distinguish between these species is the shape of the tail: in *M. costarricense* n. sp. it is conical with a sharp terminal ring, while in *M. discus* and *M. rusticum* it is more rounded, with a slightly flattened, lobed terminal ring. Based on integrative taxonomy carried out in this study, it was possible to determine that *M. costarricense* n. sp. differs morphometrically from other criconematids species described. In addition, based on the molecular data it is also different from other species in the Criconematidae family in GenBank, so it can be classified as a new species.

Analyzed 28S, ITS, and 18S sequences showed a clear relationship between *M. onoense* from Costa Rica and *C. brevistylus* from South Africa (Van den Berg *et al*., 2012) and China (Zhao and Zeng, 2021a) (Figs. 5, 6, and 7). Results of our research agree with Nguyen *et al*. (2021), who indicated that both organisms are the same species. Regarding the COI region, a significant difference was found between *M. onoense* from Costa Rica and *M. onoense* from USA (Powers *et al*., 2014) (Fig. 8). These results suggest the presence of cryptic species with the existence of different genetic lineages within the species, which means, although nematodes share similar morphological characteristics, they belong to genetically different groups. Therefore, morphology alone is not enough to differentiate these genetic variants within species.

Sequences from the 28S gene region from *M. sphaerocephalum* grouped together in a separate clade with sequences from *M. sphaerocephalum* from various countries (Fig. 5). This strongly suggests that they are the same species. Although we only obtained sequences of a single gene, it

allowed for the correct identification of *M. sphaerocephalum* and several studies support this identification (Nguyen *et al*., 2019; Handoo *et al*., 2020; Shokoohi, 2021). In the case of *M. paraonoense* n. sp., the 28S sequences clustered with sequences from *Mesocriconema* sp. from India (Sorokhaibam, 2023) and China (Fig. 5; Zhao and Zeng, 2021b); however, neither study identified the nematode to the species level. Combined, these studies revealed that *M. sphaerocephalum* and *M. paraonoense* n. sp. have a worldwide distribution.

Although 18S sequences were highly similar between *Mesocriconema* species and could not always be used for conclusive correct species identification as mentioned by Hosseinvand *et al*. (2022), our 18S sequence analysis separated them properly (Fig. 7). The two new described species, *M. paraonoense* n. sp. and *M. costarricense* n. sp., were clearly separated from other species by all molecular markers (28S, ITS, COI, and 18S) analyzed in this study (Figs. 5, 6, 7, and 8). The mitochondrial COI region has been shown to be useful in the separation and identification of cryptic species. However, it is important to highlight that low efficiency was observed in the amplification process (PCR) of these gene in some species as mentioned by Rodrigues *et al*. (2023).

Morphometric parameters and molecular markers used in this research allowed accurate and precise identification of four species of nematodes from the Criconematidae family in Costa Rica. Of these species, two new species are described, which expands knowledge of this group of nematodes and provides a valuable contribution to the nematology community worldwide.

Characterization of criconematids based on morphological or molecular methods solely can lead to incorrect identification of these organisms due to the existence of intraspecific similarities and little interspecific variability. Nematodes as well as other organisms have conserved DNA regions with few polymorphisms between species that do not allow for species separation, such as the ITS region. Therefore, the combination of morphological and molecular tools as well as the amplification of different genome regions such as 28S, ITS, 18S, and mitochondrial DNA, provide a broader genome coverage and more conclusive information that allows accurate identification of nematode species, especially cryptic species.

It is important to highlight that in GenBank there is incorrect information, with misattributed or misidentified species names and many without morphometric support, which makes decision making difficult when carrying out nematode identification. In the case of criconematids, they are a complex group in which recent research supports evidence of cryptic diversity present within species and further studies are needed for better understanding of this group.

Costa Rica is a highly biodiverse country, but with little information regarding nematofauna. It is necessary to carry out studies complementary to the identification of species to know how these organisms are associated with natural and agricultural systems. This is the first study of morphological and molecular identification of nematodes belonging to the Criconematidae family in Costa Rica.

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