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Caracterización morfológica y molecular de nematodos anillados (Nematoda: Criconematidae) de Costa Rica: dos nuevas especies descritas

Trabajo Final de Graduación bajo la modalidad de Artículo Científico para optar por el grado de Licenciatura en Ingeniería en Agronomía

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TABLA DE CONTENIDOS

INTRODUCCIÓN

Los nematodos fitoparásitos son organismos microscópicos con forma típica de "gusano" y representan el grupo más abundante de organismos multicelulares en la Tierra (Castro *et al*., 2011). Se caracterizan por la presencia de un estilete en su aparato bucal y destacan por su importancia para la agricultura. Actualmente existen más de 4.100 especies descritas (Poveda *et al*., 2020; Phani *et al*., 2021) y se estima que estos organismos causan pérdidas agrícolas a nivel mundial de entre USD 125 y 173 billones anuales (Watkins *et al*., 2012; Elling, 2013; Ravichandra, 2014; Kim, 2015).

Dentro de los nematodos fitoparásitos se encuentra la familia Criconematidae o "nematodos anillados", los cuales presentan una cutícula con anulaciones transversales característica. Los nematodos fitoparásitos tienen distribución mundial tanto en climas tropicales como templados, donde ectoparasitan muchas especies de plantas (Nguyen *et al*., 2019).

En Costa Rica, se han reportado varios géneros y especies de nematodos anillados en cultivos anuales y perennes, incluidos pastos, arbustos, hortalizas y frutales. En plantaciones de arroz y maíz fueron reportados *Criconemoides* spp. (González, 1978; Guzmán *et al*., 2011), *Criconemella onoensis* (Sancho y Salazar, 1985), *C. ornata* y *C. palustris* (Salazar y López, 1987). Además, Wing Ching *et al*. (2008) mencionaron la presencia de *Criconemella* spp. en diferentes especies de gramíneas. En frutales se han identificado morfológicamente *Criconemella sphaerocephala* (López y Salazar, 1988), *Mesocriconema sphaerocephalum*, *M. anastomoides* (Peraza, 2014), *Crossonema civellae*, *Criconema neopacificum*, *C. graminicola* y *Criconemoides lizarbus* (Peraza y Orozco, 2018).

La identificación de especies de nematodos en nuestro país muestra grandes vacíos, principalmente por la falta de especialistas en nematología. La mayor parte de las investigaciones realizadas en esta área, se basan principalmente en la observación de caracteres morfológicos y morfométricos para el reconocimiento de especies, sin embargo, no fue hasta hace unos años que se inició la identificación mediante técnicas moleculares. La amplia variabilidad intraespecífica y la poca heterogeneidad interespecífica son los principales motivos para utilizar herramientas de identificación molecular (Barsalote *et al*., 2017; Palomares-Rius *et al.*, 2018; Karaca *et al*., 2021). Los métodos de caracterización basados en ADN son ahora más comunes y necesarios para diferenciar especies crípticas, incluidas especies de la familia Criconematidae (Powers *et al*., 2017; Clavero-Camacho *et al*., 2022; Archidona *et al*., 2023).

La identificación correcta y precisa de nematodos fitoparásitos en áreas cultivadas es de suma importancia ya que frecuentemente los síntomas que causan en las plantas se confunden con los causados por otros fitopatógenos o factores abióticos, por lo que el nivel de daño que causan es subestimado (Gandarilla *et al*., 2014). Un diagnóstico adecuado también podría contribuir a la toma de decisiones y generar un impacto positivo en la reducción del uso de agroquímicos y las pérdidas económicas causadas por estos microorganismos. Por lo tanto, el objetivo de esta investigación fue realizar una correcta identificación de especies de nematodos de la familia Criconematidae en Costa Rica, utilizando métodos morfológicos y moleculares para contribuir al conocimiento de estos organismos.

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, and W. Peraza-Padilla. 2023. Morphological and molecular characterization of ring nematodes (Nematoda: Criconematidae) from Costa Rica: two new species described. Nematropica 00:00-00.

Plant-parasitic nematodes are organisms with significant economic impact on agricultural crops. Among these, the Criconematidae family is considered a potential pest due to its strong stylet in their mouthparts and its 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. A morphological study was conducted using a set of measurements, percentages, and ratios to differentiate between 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 discovered 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 family in Costa Rica.

Keywords: D2D3, ITS, 18S, COI. morphology, phylogenetic analysis, plant-parasitic nematodes, sequencing.

RESUMEN

Aráuz-Badilla, J., R. Artavia-Carmona, I. Hilje-Rodríguez y W. Peraza-Padilla. 2023. Caracterización morfológica y molecular de nematodos anillados (Nematoda: Criconematidae) de Costa Rica: dos nuevas especies descritas. Nematropica 00:00-00.

Los nematodos fitoparásitos son organismos que pueden tener un impacto económico significativo en los cultivos agrícolas. Entre estos, la familia Criconematidae se considera 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. 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 del gen 28S, 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 cuatro especies de nematodos anillados incluyendo dos especies nuevas descubiertas las cuales se describen 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: D2D3, ITS, 18S, COI, morfología, análisis filogenético, nematodos fitoparásitos, secuenciación.

INTRODUCTION

Plant-parasitic nematodes are tiny organisms with 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 these organisms cause worldwide agricultural losses between 125 and 173 billion USD annually (Watkins *et al*., 2012; Elling, 2013; Ravichandra, 2014; Kim, 2015).

Within phytoparasitic nematodes is the Criconematidae family or "ring nematodes" which have a cuticle with characteristic transverse wide annulations. Phytoparasitic nematodes have worldwide distribution in tropical and temperate climates, where they ectoparasitize many plant species (Nguyen *et al*., 2019).

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 plantations. Also, Wing Ching *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.

Identification of nematode species in our country shows gaps, 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 recognition. However, it was not until a few years ago that identification with molecular tools began. Wide intraspecific variability and little interspecific heterogeneity 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 of the Criconematidae family (Powers *et al.*, 2017; Clavero-Camacho *et al.*, 2022; Archidona *et al.*, 2023).

The correct and accurate identification of phytoparasitic 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 could also contribute to decision-making and generate a positive impact in reducing the use of agrochemicals, and economic losses caused by these microorganisms. Therefore, the aim of this research was to make a correct identification of nematode species of the Criconematidae family in Costa Rica, through morphological and molecular methods to contribute to their knowledge.

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 from a depth of 5 to 30 cm. Samples were stored in polyethylene plastic bags and transported in thermally insulated containers to the Laboratorio de Nematología from Universidad Nacional 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 "modified rapid Seinhorst" method (Seinhorst, 1959) and preserved in fixed slides following 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 family of the Laboratorio de Nematología, of the Escuela de Ciencias Agrarias from Universidad Nacional.

Morphological and morphometric identification

Female nematodes were measured using a high-resolution microscope connected to a Nikon DS-Fi1 camera to take microphotographs. Measurements, percentages, and ratios commonly used for morphometric identification of ring nematodes were included (Siddiqi, 2000). L (body length), 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 annulus), Rex (number of annulus from to excretory pore), RV (number of annulus from vulva to posterior end), VL (distance from vulva to posterior end), 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 family were used (Geraert, 2010). Microphotographs were edited with Photoshop CS6.

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). 3 µl of proteinase K (20 mg/ml) were added to each tube and then they were 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 15 min cold

incubation at -20 °C for cellular lysis and a 10 min hot incubation 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 2000 rpm for 2 min, and stored at -20 °C for later use in PCR.

For PCR analyses four genome regions were amplified. 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). 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 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), 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 COI region, 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 1% (w/v) TopVision™ agarose gels (100 volts, 1 hour). 4 µl of PCR product were loaded with 2 µl of 6X DNA loading dye stained with 1X GelRedT™. DNA bands were observed under UV light. GeneRuler™ 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 family. Outgroup taxa for each region analyzed was chosen taking as reference 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 was used to generate phylogenetic trees.

RESULTS

Morphological, morphometric, and molecular analyses allowed the identification of four criconematid species (Table 3). Among these, 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 (Figure 1; Table 1)

This nematode species was found in soil samples collected in 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). Females have a robust body (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. Its 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 27and 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.

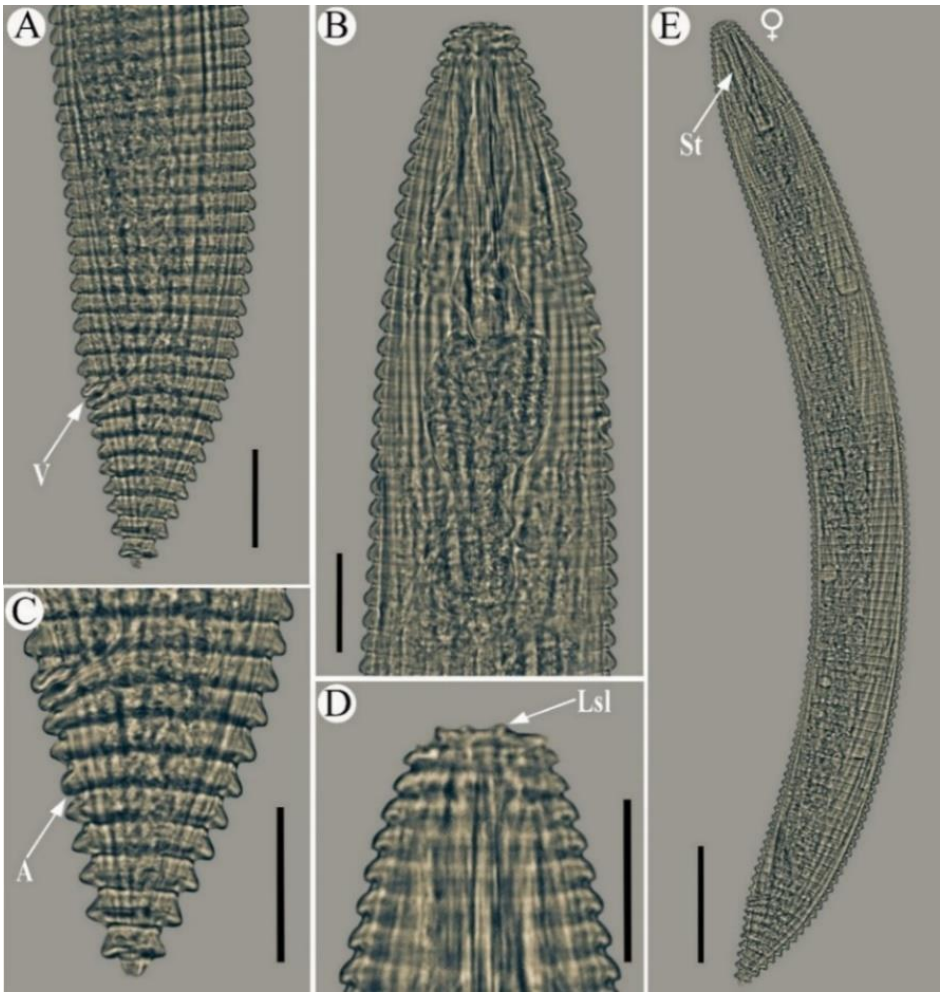

Figure 1. Photomicrographs of *Mesocriconema onoense* females: (A) posterior region showing vulva position, (B) anterior region, (C) tail shape and anus position, (D) cephalic region showing labial submedian lobes, (E) whole female. Abbreviations: V = vulva, A = anus, Lsl = labial submedian lobe, St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

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

This nematode species was found in a cocoa plantation from Puntarenas province (n=32) (type population), southern Costa Rica (coordinates 8°39'55.0"N; 83°01'16.2"W). Additional populations were found in a cocoa plantation from Alajuela province (n=30) and in a sugar cane plantation at 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), that acquires a ventrally curved position when heat-fixed in formaldehyde. They have 109-150 annulations (3.0-5.1 μm wide) along their body, 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) regarding 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 & 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 *Mesocriconema brevistylus* (Singh and Khera, 1976; Loof and De Grisse, 1989) and *Mesocriconema 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).

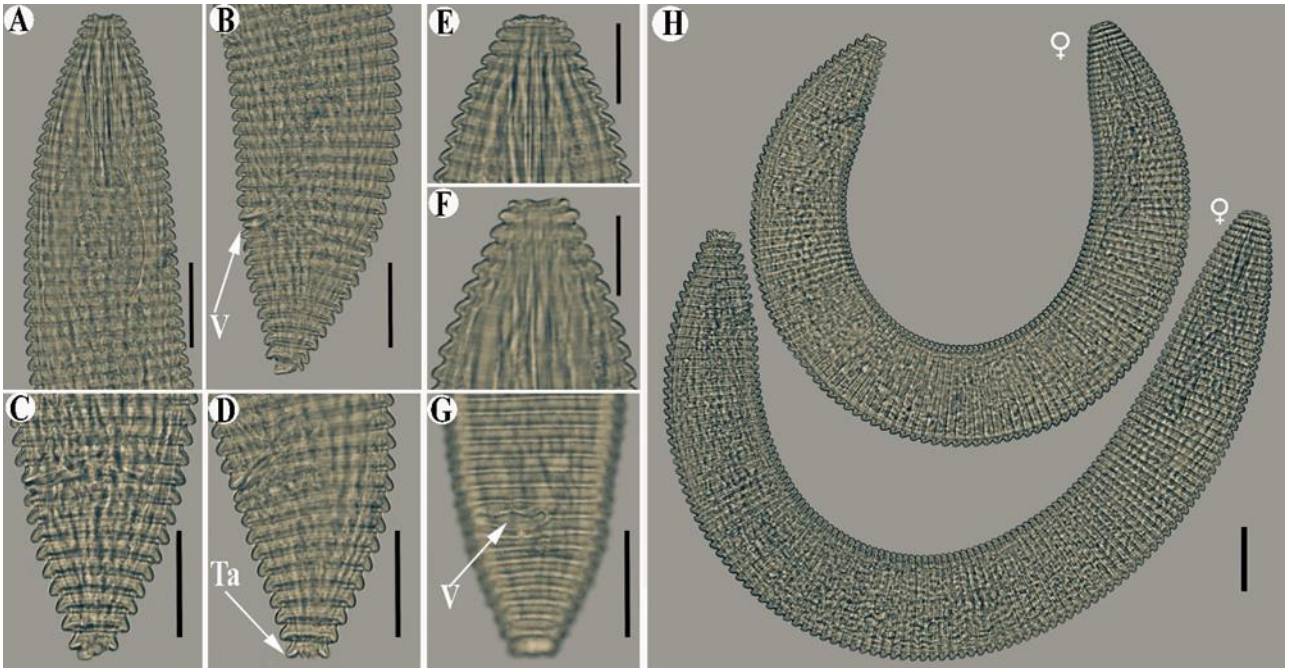

Figure 2. Photomicrographs of *Mesocriconema paraonoense* n. sp. females: (A) anterior region, (B) posterior region showing vulva position, (C, D) tail shape and terminal annulus, (E, F) cephalic region, (G) vulva, (H) whole females. Abbreviations: V = vulva, Ta = terminal annulus. Scales: A, B, C, D, 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 µm and in format: mean ± standard deviation (range).

	Mesocriconema onoense		***Mesocriconema paraonoense* n. sp.**			
Host	Natural area	Natural area	Cocoa		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).

Mesocriconema sphaerocephalum (Taylor, 1936) Loof and De Grisse, 1989 (Figure 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 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.

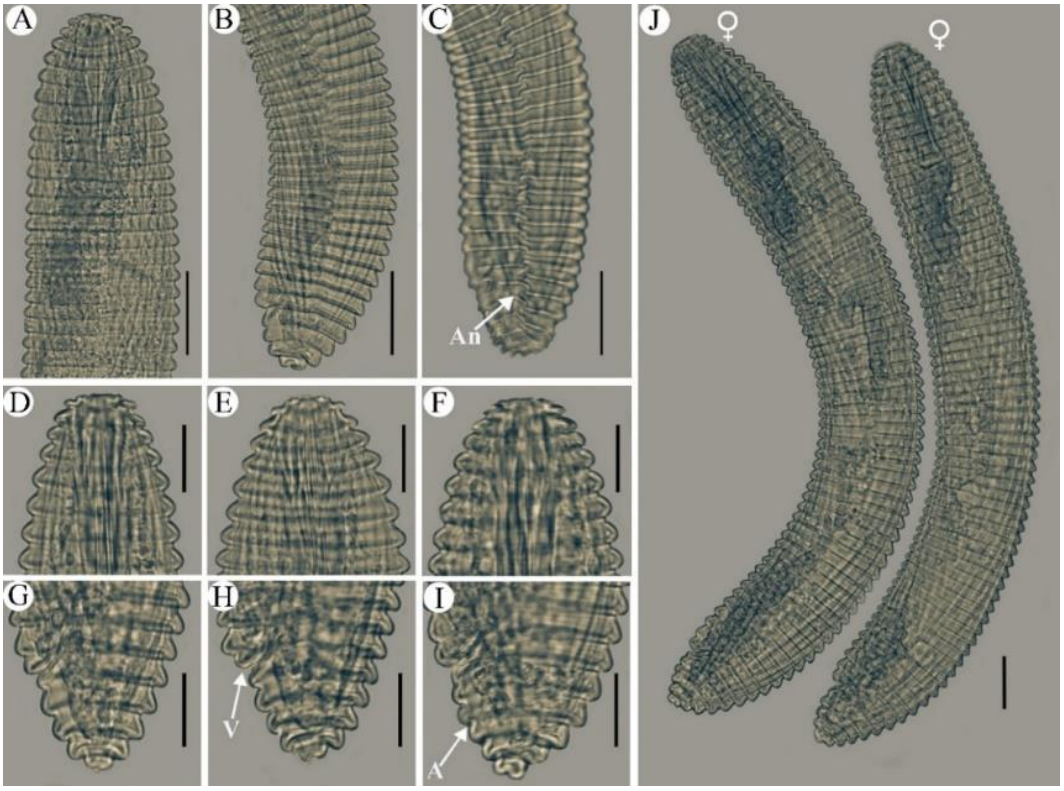

Figure 3. Photomicrographs of *Mesocriconema sphaerocephalum* females: (A) anterior region, (B) posterior region, (C) zigzag pattern anastomosis, (D, E, F) cephalic region, (G) tail shape, (H) vulva position, (I) anus position, (J) whole females. Abbreviations: An = anastomosis, V = vulva, A = anus.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

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

This nematode species was discovered in soil samples from a natural area with herbaceous plants growing on a forest margin at Limón province, Costa Rica Caribbean (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 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 gentilice 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.

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.

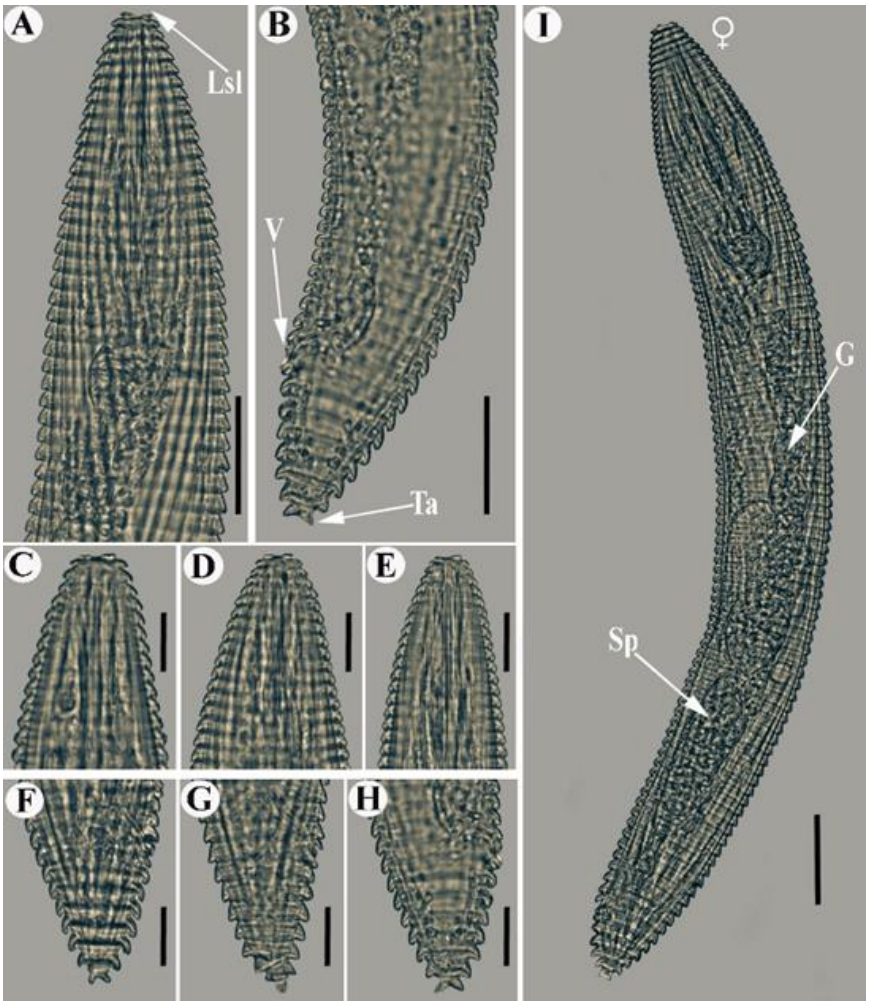

Figure 4. Photomicrographs of *Mesocriconema costarricense* n. sp. females: (A) anterior region showing labial submedian lobes, (B) posterior region showing vulva position and terminal annulus, (C, D, E) cephalic region, (F, G, H) tail shape, (I) whole female showing the gonad and spermatheca. Abbreviation: 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.

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

	Mesocriconema sphaerocephalum	***Mesocriconema costarricense* n. sp.**	
Host	Grass	Natural area	
Location	Aserrí, San José	Limón, 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).

Molecular characterization

Amplification of 28S D2D3, ITS, 18S and COI regions generated fragments of 750, 770, 870 and 740 bp, respectively. Fifteen D2D3, eleven 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). D2D3 sequences of the 28S 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). D2D3 sequences of *M. paraonoense* n. sp. (OR077106 to OR077112) showed low similarity (94.3%) with *M. onoense* from Vietnam (MZ220549) (Nguyen *et al*., 2021) and 100% similarity with unidentified *Mesocriconema* sp. 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. D2D3 sequences of *M. sphaerocephalum* (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). D2D3 sequences of *M. costarricense* n. sp. (OR077119 and OR077120) showed low similarity (95.3-95.5%) with *M. xenoplax* from China (OQ674275) (Zhang *et al*., 2023) and *M. 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). ITS sequences of *M. paraonoense* n. sp. (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). ITS sequences from *M. costarricense* n. sp. (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). 18S sequences of *M. paraonoense* n. sp. (OR077129 and OR077130) showed low similarity (96.2%) with *M. onoense* (MF094909) and *M. xenoplax* (95.7%) from USA (MF095022) (Powers *et al*., 2017). *M. costarricense* n. sp. (OR077126) showed high similarity (99.0%) with *Mesocriconema* sp. (MF094974) from USA (Powers *et al*., 2017) in this genomic region.

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. (OR168958 and OR168959) showed low similarity (90.4%) with *M. rusticum* from USA (KJ787855) (Powers *et al*., 2014).

Table 3. Ring nematodes populations found in this study and its corresponding GenBank® accession number for each sequenced region.

Species	ID *	Location	Host	D2D3	ITS	18S	COI
M. onoense	153C	Río Cuarto, Alajuela	Natural area	OR077113 OR077114	OR077139 OR077140	OR077127	OR167994 OR167995
	173C	Vázquez de Coronado, San José	Natural area	OR077115	-	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	Limón, Limón	Natural area	OR077119 OR077120	OR077141 OR077142	OR077126	OR168958 OR168959

(*) Identification code from Laboratorio de Nematología, Escuela de Ciencias Agrarias, Universidad Nacional.

(-) No sequence obtained.

Phylogenetic trees from 28S D2D3, ITS, 18S and COI regions (Figures 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 discovered species in this study show to be 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 its D2D3 region (Figure 5) were identical to D2D3 sequences from unidentified species of *Mesocriconema* from India and China. *M. costarricense* n. sp. was well separated from other species, however, showed to be close to *M. xenoplax* and *M. nebraskense* in D2D3 and ITS regions (Figures 5 and 6, respectively). *M. onoense*

from this study grouped with *M. onoense* from other countries and with *C. brevistylus* (D2D3, ITS and 18S regions) (Figures 5, 6 and 7). *M. sphaerocephalum* D2D3 sequences (Figure 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).

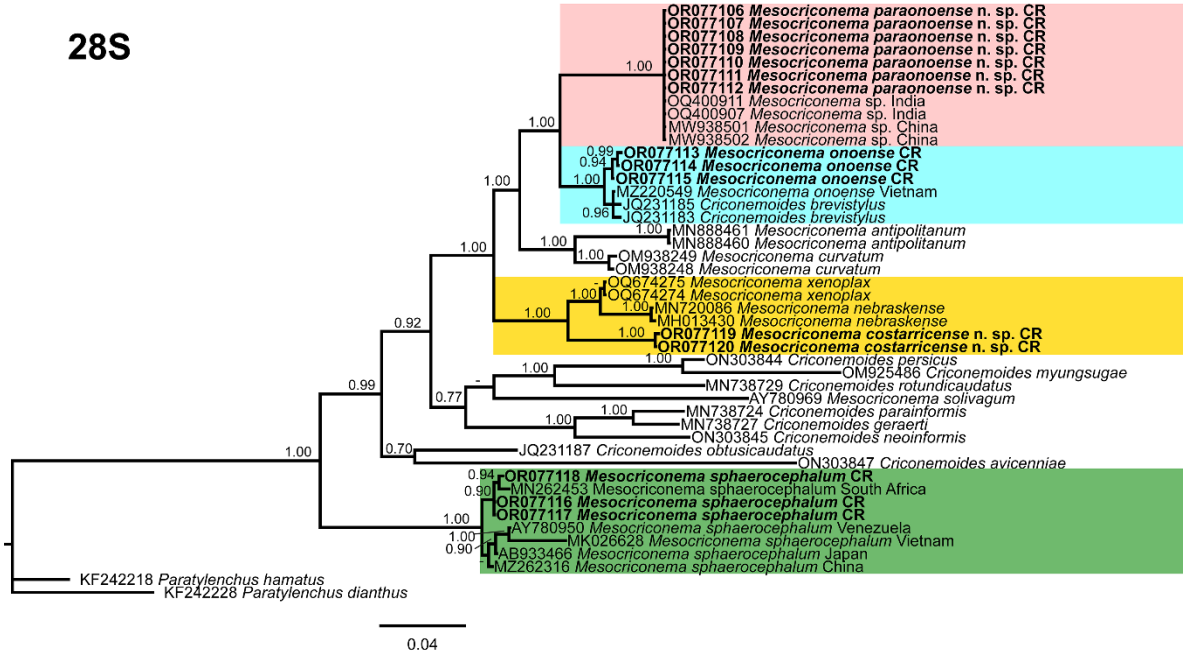

Figure 5. Phylogenetic tree generated from D2D3 sequences of the 28S gene by Bayesian Inference (BI) under TIM3+I+G model. Probabilities for appropriate clades were greater than 0.70. Sequences from this study are marked in bold. Colored boxes indicate related species in the clades.

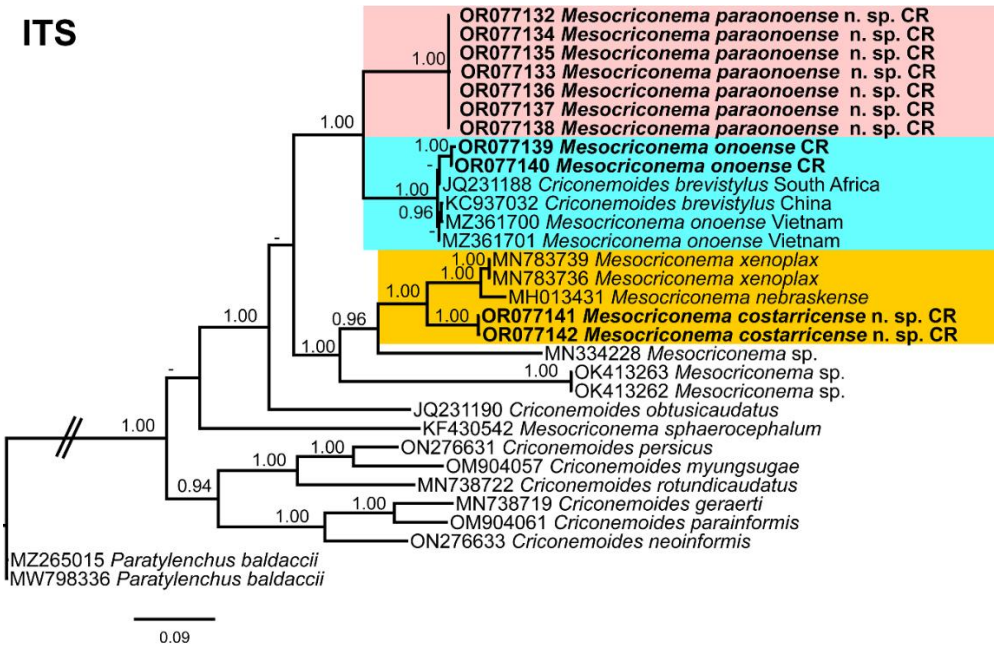

Figure 6. Phylogenetic tree generated from ITS sequences by Bayesian Inference (BI) under TVM+I+G model. The probabilities for appropriate clades were greater than 0.70. Sequences from this study are marked in bold. Colored boxes indicate related species in the clades.

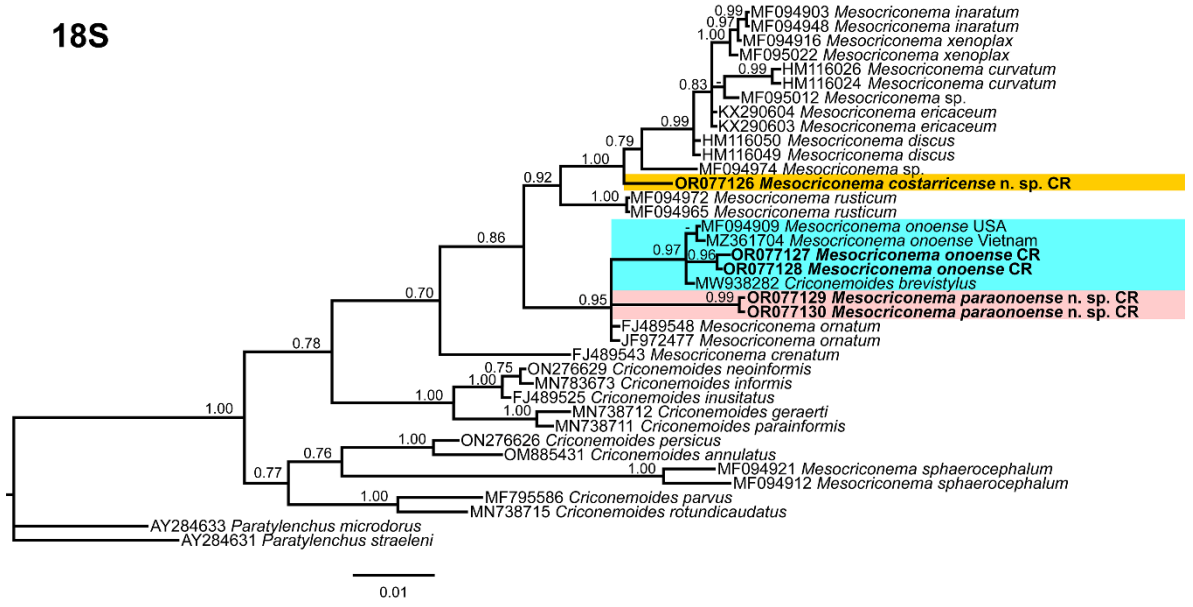

Figure 7. Phylogenetic tree generated from partial 18S gene sequences by Bayesian Inference (BI) under GTR+I+G model. The probabilities for appropriate clades were greater than 0.70. Sequences from this study are marked in bold. Colored boxes indicate related species in the clades.

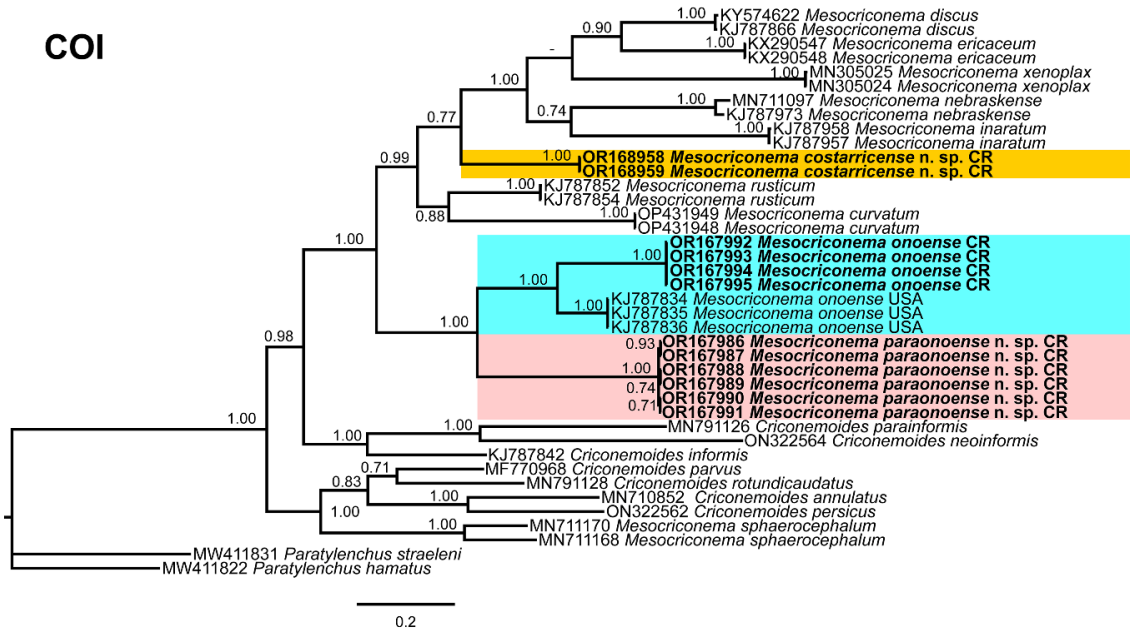

Figure 8. Phylogenetic tree generated from mitochondrial COI sequences by Bayesian Inference (BI) under GTR+I+G model. The probabilities of the appropriate clades are greater than 0.70. Sequences from this study are marked in bold. Colored boxes indicate related in the clades.

DISCUSSION

In Costa Rica, *M. onoense* was previously reported in rice cultivation (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); pineapple in Philippines (Zurbano *et al.*, 2023); grasses, maple (Cordero *et al.*, 2012b) and turf (Powers *et al.*, 2014) in North America; citrus (Crozzoli, 1998), sugar cane (Perichi *et al.*, 2002) and rice in Venezuela (Medina *et al.*, 2009); cotton, corn and soybean in Brazil (Inomoto *et al.*, 2011).

M. 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 in Puerto Rico (Powers *et al.*, 2016a); turf (Munawar *et al.*, 2018), turfgrass, daylily (Cordero *et al.*, 2012b) 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 (Lugo *et al.*, 2010) in Venezuela; 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, according to Peraza (2023) (personal communication), these nematodes can be found in various ecosystems throughout the country, in cultivated areas as well as in natural environments. According to Powers *et al.* (2014) *M. sphaerocephalum* is worldwide distributed and its hosts include a wide variety of crops and native plant species.

Criconematids, being ectoparasites, are characterized by having a well-developed stylet 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, makes 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.

M. paraonoense n. sp. was found in the rhizosphere of sugar cane plantations in the province of Cartago, as well as in cocoa rhizosphere 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 wider distribution

throughout the country (Peraza, 2023; personal communication) since they coincide morphologically with other ring nematodes found. 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.

Worldwide, *Meloidogyne* spp. in cocoa seedlings (Orisajo, 2009; Okeniyi *et al*., 2015.) and *Meloidogyne* spp. and *Pratylenchus* spp. in sugar cane (Alves *et al*., 2023) are reported as the most important genera affecting these crops. However, there is also evidence of the presence of ring nematodes in cocoa plantations (Bustamante and Ordoñez, 2019; Adewale and Dada, 2020; Vera *et al*., 2022) and sugar cane (López *et al*., 2014; Afolami *et al*., 2014), as was also observed in this study.

M. costarricense n. sp. was identified in a natural area of the province of Limón. Similar pristine sites in Costa Rica and other locations around the world harbor nematodes of the family 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 family 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). Our populations coincide morphometrically with the population of *M. onoense* from USA described by 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 are observed with the population of *M. onoense* from Vietnam, described by Nguyen *et al*. (2021) 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 USA (Cordero *et al*., 2012b) in 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 various species of 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 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 (4.5-4.6 vs 4.1) ratios, 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 morphometric characteristics they share. However, after sequencing D2D3, ITS and COI regions, which are quite informative regions to differentiate species (Hosseinvand *et al.*, 2022), we found low percentages of similarity with respect to *M. onoense* sequences. 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 *Criconema* and *Criconemoides* genera (Munawar *et al.*, 2020; Clavero-Camacho *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-Camacho *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 our 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* (Figures 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, it did not show molecular coincidences with species of the Criconematidae family in GenBank, so it can be classified as a new species for science.

Analyzed 28S D2D3, ITS and 18S regions 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) (Figures 5, 6 and 7). Results of our research agree with Nguyen *et al.* (2021) findings, which indicates that both organisms are the same species. Regarding COI region, a significant difference was found between *M. onoense* from Costa Rica and *M. onoense* from USA (Powers *et al.*, 2014) (Figure 8). These results suggest the presence of cryptic species with the existence of different genetic lineages within the species, that 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.

28S sequences from *M. sphaerocephalum* grouped together in a separate clade with sequences from *M. sphaerocephalum* from various countries (Figure 5). This strongly suggests that they are the same species. Although we obtained sequences of a single region of *M. sphaerocephalum* DNA (Table 3), they allowed its correct identification and several studies support it morphologically and molecularly (Nguyen *et al.*, 2019; Handoo *et al.*, 2020; Shokoohi, 2021). In the case of *M. paraonoense* n. sp., 28S sequences clustered with sequences from *Mesocriconema* sp. from India (Sorokhaibam, 2023) and China (Zhao and Zeng, 2021b), (Figure 5), however, both studies did not identify to species level. All these studies mentioned above revealed that *M. sphaerocephalum* and *M. paraonoense* n. sp., have a wide worldwide distribution.

Although 18S is highly similar between *Mesocriconema* species and could not always be conclusive for a correct species identification as mentioned by Hosseinvand *et al*. (2022), our 18S analysis separated them properly (Figure 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 (Figures 5, 6, 7, and 8). The mitochondrial COI region has 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 correspond to new discovered species, which expand knowledge and provide 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 to separate them properly 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 some incorrect information, with misattributed or misidentified species names and many without morphometric support, which makes decision making difficult when carrying out characterization of nematodes. 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 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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