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Oswaldocruzia franciscoensis n. sp. (Nematoda: Molineidae) in *Leptodactylus macrosternum* Miranda-Ribeiro, 1926 (Anura: Leptodactylidae) from Caatinga morphoclimatic domain, Brazil: morphological and molecular characterisation

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Abstract

A new species of *Oswaldocruzia* Travassos, 1917 (Nematoda, Molineidae), parasite of *Leptodactylus macrosternum* Miranda-Ribeiro, 1926 (Anura: Leptodactylidae), from Caatinga morphoclimatic domain, Brazil, is described based on morphological and molecular data. *Oswaldocruzia franciscoensis* n. sp. is characterised by an anterior extremity with a cephalic vesicle divided into two portions, a body covered by cuticular longitudinal ridges, and cervical alae. Males of the new species have caudal bursa of type I with a 2-1-2 pattern, spicules divided into a shoe, bifurcated fork, and blade with two unequal branches, in which the longer branch bifurcates at its distal portion end and the smaller branch with three distal processes, each with distal bifurcations. Females have didelphic and amphidelphic uteri, an ovijector divided into vestibule, anterior and posterior sphincters, and anterior and posterior infundibula. The new species differs from its Neotropical congeners that have caudal bursa of type I, based on the presence of cervical alae and by having a spicular blade distally divided into two unequal branches, with the longer branch bifurcating at its distal portion and smaller branch with three distal processes, each distally bifurcated. The partial 18S rDNA sequence generated for *Oswaldocruzia franciscoensis* n. sp. is the first of a representative belonging to this genus in the Neotropical region.

Introduction

Oswaldocruzia Travassos, 1917 (Nematoda: Molineidae) comprises about 90 nominal species of parasites from amphibians and reptiles distributed worldwide (Guerrero 2013; Svitin 2017; Willkens *et al.* 2021). Currently, 43 species have been reported in the Neotropical region (Willkens *et al.* 2021), in which 11 are widely distributed in Brazil, infecting several species of amphibians and lizards (see Lent and Freitas 1935; Freitas and Lent 1938; Ben Slimane and Durette-Desset 1995; Bursey and Goldberg 2004, 2011; Durette-Desset *et al.* 2006; Ávila and Silva 2010; Campião *et al.* 2014; Larrat *et al.* 2018; Benício *et al.* 2022; Lacerda *et al.* 2023). Although species of *Oswaldocruzia* from Brazil frequently appear in studies of species inventory or parasite ecology, most taxonomic studies regarding morphological descriptions have been published before the last 20 years (Bursey and Goldberg 2004; Durette-Desset *et al.* 2006; Santos *et al.* 2008; Larrat *et al.* 2018). Recent evaluations on these nematodes that include morphological and genetic characterisation are still scarce, especially in Brazil (see Simões *et al.* 2019).

Based on the currently limited knowledge of *Oswaldocruzia* species in Brazil, the present study proposes a new species of this genus, parasitizing *Leptodactylus macrosternum* (*L. macrosternum*) Miranda-Ribeiro, 1926 (Anura: Leptodactylidae), from Caatinga morphoclimatic domain, in the State of Pernambuco, using morphological and genetic characterisation.

Material and methods

Collection and necropsy of hosts

A total of 67 specimens of *L. macrosternum* were collected in a conventional agricultural area (9°20'4.68"S, 40°35'11.25"W), in the municipality of Petrolina, sub-middle region of São

Francisco River, State of Pernambuco, Brazil. Collections were carried out in May 2018, February, September, and October 2019, and September 2021. The sampling area is in the Northeastern semi-arid part of Brazil. It has typical Caatinga vegetation (*sensu stricto*), with a climate characterised by high temperatures and irregular and scarce periods of rain (Prado 2003). Amphibians were collected manually by active search and sent alive to the Laboratório de Morfofisiologia, Centro de Conservação e Manejo de Fauna da Caatinga (CEMAFAUNA-CAATINGA), Universidade Federal do Vale do São Francisco (UNIVASF), municipality of Petrolina, State of Pernambuco. These anurans were identified according to Magalhães *et al.* (2020) and representative specimens (symbiotypes) deposited in the Coleção Herpetológica do Museu de Fauna da Caatinga, UNIVASF, Brazil (MFCH – 5346, 5347, 5349, 5350, 5352, 5354–5357, 5363, 5365, 5381, 5382, 5388, 5402–5404, 5406, 5408, 5411, 5418, 5420, 5423, 5424, 5431, 5432, 5434, 5442).

Hosts were euthanised with an overdose of lidocaine hydrochloride applied topically to their dorsal region, according to the recommendations of the Conselho Nacional de Controle de Experimentação Animal (2018) and necropsied under a stereomicroscope.

Collection, processing and morphological identification of nematodes

A total of 124 nematodes were collected alive. They were placed in Petri dishes containing 0.85% saline and fixed in hot 4% formaldehyde solution, remaining for 15 days at room temperature, and posteriorly transferred to 70° GL ethanol for morphological studies. For molecular studies, some males from each infrapopulation were fixed and preserved in 100% ethanol.

Nematodes were cleared in Amann's Lactophenol for morphological identification, mounted on temporary slides in the same medium, and observed using light microscopy. Drawings were made using a drawing tube attached to a Motic light microscope (Motic, Jiangsu, China). Measurements of parasites are given in micrometres, unless otherwise indicated, and are presented as ranges followed by mean inside parentheses.

Morphological terminology, identification, and description of nematodes follow Ben Slimane *et al.* (1996) and Bursey and Goldberg (2011). The study of the synlophe was according to Durette-Desset (1985). Prevalence, mean intensity, and mean abundance of parasites were calculated according to Bush *et al.* (1997).

Molecular characterisation and phylogenetic analyses

For molecular characterisation, a small tissue sample was excised from the mid body part of a male specimen and subjected to DNA isolation, using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), following manufacturer's instructions. The anterior and posterior parts of the body were processed for morphological identification as previously described. A region of the nuclear 18S rDNA was amplified by polymerase chain reaction (PCR) assay, using the primers Nema18SF (5'-CGCGAATRGCTCATTACAACAGC-3') and Nema18SR (5'-GGGCGGTATCTGATCGCC-3') (Floyd *et al.* 2005). The domains D2–D3 of the 28S rDNA and *cox*1 of the mtDNA were also aimed for amplification, using the genetic markers proposed by De Ley *et al.* (2005) and Folmer *et al.* (1994), respectively, but proper amplicons could not be obtained. PCR reactions were carried out in a final volume of 25 µl consisting of 2.5 µl of 10X PCR buffer minus $MgCl_2$, 1.25 µl of $MgCl_2$ (50 mM), 0.5 µl of dNTP's (10 mM), 0.5 µl of each oligonucleotide primer (10 µM), 0.2 µl of Recombinant Taq DNA polymerase (5 U/µl) (Invitrogen), 1.25 µl of BSA (10 µg/µl), 16.3 µl of H_2O, and 2.0 µl of genomic DNA (about 40 ng). PCR cycling conditions were as follows: denaturation at 94°C for 5 min, followed by 36 cycles of 94°C for 30 s, annealing at 52.7°C for 30 s, and extension at 72°C for 1 min, followed by a post-amplification extension at 72°C for 5 min. Presence of amplicons were verified using agarose gel electrophoresis, positive products were subjected to enzymatic treatment with ExoSAP-IT (Applied Biosystems, Massachusetts, EUA) and sent for sequencing, with the same PCR primers, in ACTGene Molecular Analyses (Rio Grande do Sul, Brazil).

Contiguous sequences were assembled in Geneious Prime (by Dotmatics, Auckland, New Zealand), and the consensus extracted and subjected to BLAST search, in the NCBI database, for confirmation of the genetic proximity between the present sequence and those of *Oswaldocruzia*/Molineidae. This sequence was deposited in the GenBank database (see taxonomic summary).

In order to verify the phylogenetic position of the new species, 18S sequences overlapping the same region obtained in the present study, from all representatives of Molineidae, were taken from the GenBank database. Sequences MG586086 and MG586088, both identified as *Oswaldocruzia filiformis* (Goeze, 1782) parasitizing the smooth newt *Lissotriton vulgaris* (Linnaeus, 1758) (Caudata: Salamandridae) in Germany, had only a very small fragment of the 5' end of the 18S and, consequently, could not be included in the analysis. Since the phylogeny of this family is still unresolved, showing conflicting results (see De Bellocq *et al.* 2001; Simões *et al.* 2019), sequences from representatives of Ancylostomatidae, Angiostrongylidae, Chabertiidae, Diaphanocephalidae, Dictyocaulidae, Strongylidae, Syngamidae, and Trichostrongylidae were also used. *Heterorhabditis bacteriophora* Poinar, 1976 (Nematoda: Heterorhabditidae) was used as the outgroup, based on a previous broad phylogenetic approach including bursate nematodes (Chilton *et al.* 2006). Detailed information on these sequences is presented in Table 1.

Sequences were aligned using the multiple algorithm tool T-Coffee (Notredame *et al.* 2000) and then subjected to Bayesian inference in the software BEAST 2.5 (Bouckaert *et al.* 2019) for phylogenetic reconstruction. The best-fit nucleotide substitution model was chosen based on bModelTest (Bouckaert and Drummond 2017). The molecular clock model was relaxed (log normal), defined using the nested sampling method (Russel *et al.* 2019) and the Yule tree prior, selected according to the posterior densities and the effective sample sizes (ESSs), verified in Tracer (Rambaut *et al.* 2018). The posterior estimates of parameter densities and the ESSs for each parameter of the model, as well as the posterior probability for nodal supports in the majority rule consensus phylogenetic tree, were determined after running the Markov chain Monte Carlo (MCMC), always four chains in two runs, each run with 10×10^6 generations, saving the last 10,001 trees and 25% burn-in. The quality of the analysis (parameter densities, ESSs, and burn-in) and the chain convergence were examined in Tracer (Rambaut *et al.* 2018).

Holotypes, allotypes, paratypes, and hologenophore were deposited in the Coleção Helmintológica do Instituto Oswaldo Cruz (CHIOC), Instituto Oswaldo Cruz (IOC), Fundação Oswaldo Cruz (FIOCRUZ).

Results

Morphological description

Oswaldocruzia franciscoensis n. sp.

(Figures 1 and 2)

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Table 1. Taxa whose 18S rDNA genetic sequences were retrieved from GenBank associated with their accession number (GenBank ID), geographic origin, host, and reference

Parasite taxa	GenBank ID	Geographic origin	Host	Reference
Ancylostoma caninum	MT764922	Brazil	'Dog'	Unpublished
Angiostrongylus costaricensis	EF514913	Lab maintained	*Sigmodon hispidus*	Fontaniella and Wade (2008)
Dictyocaulus viviparus	AY168856	Sweden	*Bos taurus*	Hoglund *et al.* (2003)
Heterorhabditis bacteriophora	AF036593	Lab maintained	Free living	Blaxter *et al.* (1998)
Kalicephalus cristatus	AJ920349	Australia	*Austrelaps superbus*	Chilton *et al.* (2006)
Nematodirella cameli	JX305977	Iran	*Camelus dromedarius*	Unpublished
Nematodirus battus[*1]	AJ920360	Not mentioned	*Ovis aries*	Chilton *et al.* (2006)
Nematodirus battus[2]	U01230	Not mentioned	Not mentioned	Zarlenga *et al.* (1994)
Ransomus rodentorum	OM296295	Not mentioned	Not mentioned	Unpublished
Strongylus equinus	DQ094176	Not mentioned	Not mentioned	Unpublished
Syngamus trachea	AF036606	Not mentioned	Not mentioned	Blaxter *et al.* (1998)
Trilobostrongylus bioccai	ON747286	Canada	*Pekania pennanti*	Carreno and Nadler (2022)
Oswaldocruzia sp.[1]	JX877669	USA	*Bufo americanus*	Unpublished
Oswaldocruzia sp.[2]	LC624873	Japan	*Zhangixalus arboreus*	Kambayashi *et al.* (2022)
Oswaldocruzia sp.[3]	LC624874	Japan	*Buergeria japonica*	Kambayashi *et al.* (2022)

*Superscript numbers make correspondence with taxa in Figure 3.

General: *Oswaldocruzia franciscoensis* n. sp. has small and slender nematodes, and a cuticle with longitudinal ridges along the body. Sexual dimorphism is evident, and females are twice as large as males. Anterior region with cephalic cuticular inflation is divided into two parts, transversally striated, and ending anteriorly to the nerve ring (Figures 1A, 2A). Cervical alae begins slightly anterior to the nerve ring, ending somewhat at the level of the oesophagus intestinal junction (Figures 1A, 2A). Synlophe is composed of uninterrupted longitudinal cuticular crests, without reinforcement (Figures 1B, 1C, 2E, 2F). Mouth is surrounded by three simple, inconspicuous lips: a dorsal lip with two sessile papillae and ventrolateral lips with one sessile papilla and lateral amphid, each (Figure 2D). Oesophageal tooth is absent. Corona radiata is also absent. Oesophagus claviform has a nerve ring just anterior to its midlength. Excretory pore is at the final third oesophagus, anterior to deirids (Figures 1A, 2A).

Male (based on holotype and seven paratypes): Total body length is 4.8–6.85 (5.63) mm, and the body width at the oesophagus intestinal junction is 97–132 (114) mm. Cuticular inflation is 51–84 (67.6) long and 32–42 (38.1) wide. Oesophagus 359–427 (396.5) long. Nerve ring and excretory pore 123–181 (159) and 264–371 (326.3), respectively, from the anterior end. Excretory pore-oesophagus ratio 0.80. Deirids between the excretory pore and the oesophagus intestinal junction (Figure 1A) 280–381 (343) from the anterior end. Synlophe (based on three specimens): dorsal crests starting posteriorly to cephalic inflation, ventral crests starting at level of oesophagus intestinal junction. Dorsal and ventral crests ending close to anterior region of bursa. Number of crests between nerve ring and excretory pore: Ten to eleven dorsal crests, cervical alae, ventral crests absent (Fig. 1B). Thirty two to thirty three crests at mid-body (16-17 dorsal, 16 ventral) (Fig. 1C). Copulatory bursa trilobed, rays are arranged in type 2-1-2 pattern (Figure 1H). Rays 2 and 3 parallel, with a common origin, reaching edge of bursa (Figure 1D, 1F, 1H). Rays 4, 5, and 6 with a common origin (Figure 1D, 1H); ray 4 is not parallel, not reaching the margin of bursa separated from rays 5 and 6; rays 5 and 6 turning caudal, parallel, reaching margin of bursa (Figure 1H). Ray 8, with an independent origin, turns caudal, not reaching margin of bursa (Figure 1F, 1H) (caudal bursa type I). Dorsal ray conical distally divided into rays 9 and 10, reaching the dorsal edge of the bursa; ray 9 lateral and subterminal, ray 10 terminal subdivided into two bilobed branches at each side (Figure 1F, 1H). Genital cone with medial ray 0 on the anterior margin and ray 7 posterior to ray 0 located lateroventrally (Figure 1F). Gubernaculum absent. Spicules equal 149–168 (157.4) long, divided proximally into three main parts: shoe, blade, and fork (Figure 1E). Fork bifurcated at approximately 28% of the spicule; right and left branches not subdivided (Figure 1E). Spicular blade divided into two unequal branches, longer branch bifurcating in distal portion and second branch with three distal processes, each with distal bifurcations (Figure 1G).

Female (based on alloptype and eight paratypes): Total body length is 6.83–11.96 (9.52) mm, and the body width at the oesophagus intestinal junction is 112–172 (137.9). Cuticular inflation is 63–83 (72.9) long and 36–48 (42) wide. Oesophagus is 407–484 (452.9) long. Nerve ring and excretory pore 157–199 (173.3) and 253–392 (332.9), respectively, from the anterior end. Deirids between excretory pore and oesophagus intestinal junction are 274–418 (397). Synlophe (based on three specimens): dorsal crests beginning posteriorly to cephalic inflation, ventral crests beginning at level of oesophagus intestinal junction. Dorsal and ventral crests ending slightly anterior to tail end. Number of crests between nerve ring and excretore pore: Eighteen dorsal crests, cervical alae, ventral crests absent. (Fig 2E). Fifty six to fifty seven crests at mid-body (30-31 dorsal, 26 ventral) (Fig. 2F).

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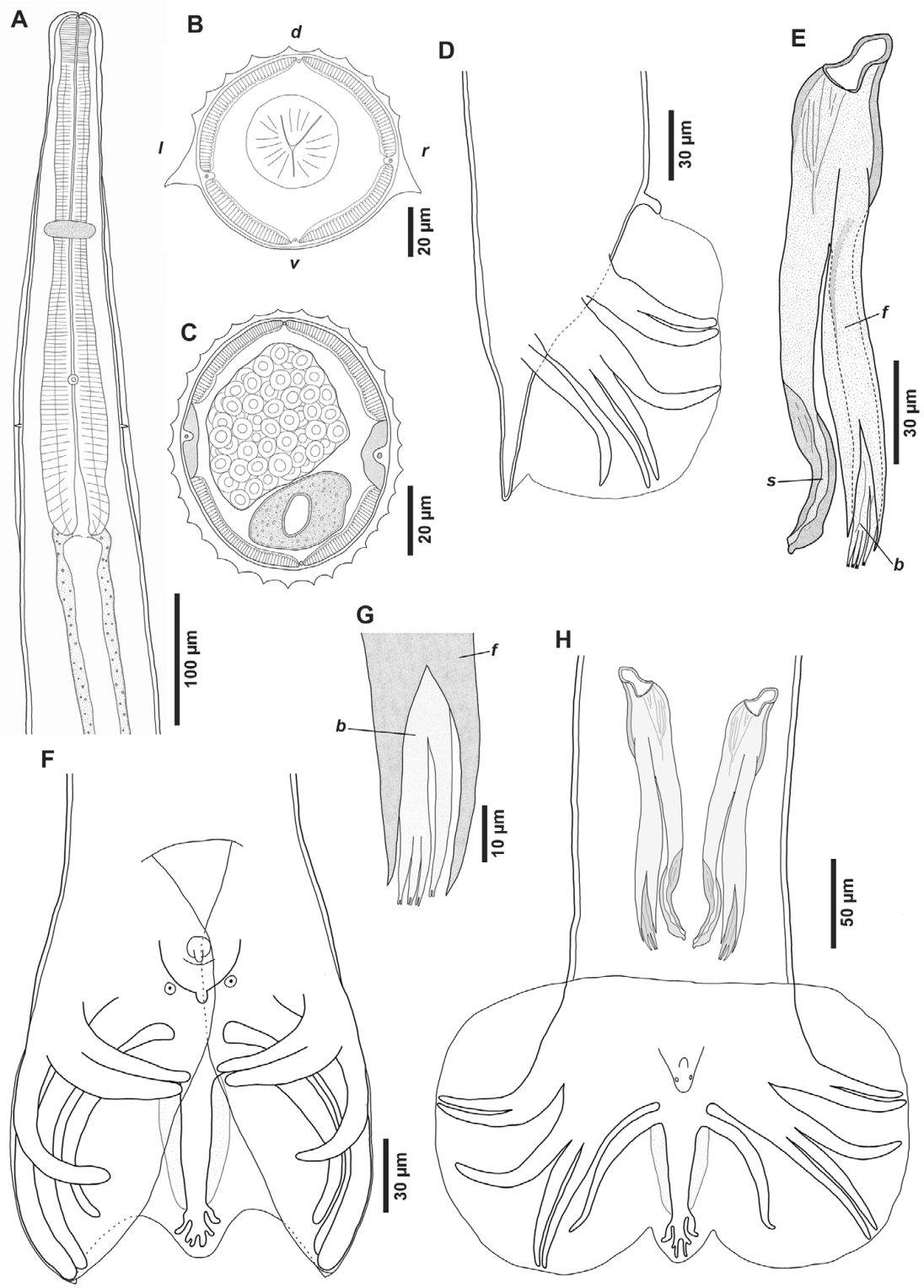

Figure 1. *Oswaldocruzia franciscoensis* n. sp. male. Line drawing. A – The anterior region showing the ventral view. B – The transversal section at the level of the oesophagus region, between the nerve ring and the excretory pore. C – The transversal section at the level of the mid-body. D – The posterior region, caudal bursa, showing the right lateral view. E – The left spicule showing the ventral view. F – The caudal bursa showing the ventral view. G – The distal end of the left spicule blade showing the ventral view. H – The caudal bursa showing the ventral view. (Abbreviations: b – spicule blade; d – dorsal side; f – spicule fork; l – left side; r – right side; s – spicule shoe; v – ventral side).

Vulvar opening at 4.36–7.76 (6.16) mm from the anterior end, vulval lips not prominent as a transverse slit (Figure 2E). Didelphic and amphidelphic uterus (Figure 2B). Vagina short at 87–103 (93.7) long, vestibule is 106–114 (109) long (Figure 2B). Sphincters both 103–112 (108), infundibula both 27–39 (29) long (Figure 2B). Eggs in morula (Figure 2B) 62–111 (78.4) × 38–69 (45.1). Tail conical 123–165 (147.6) long, flexible filament is 11–13 (120) long (Figure 2C).

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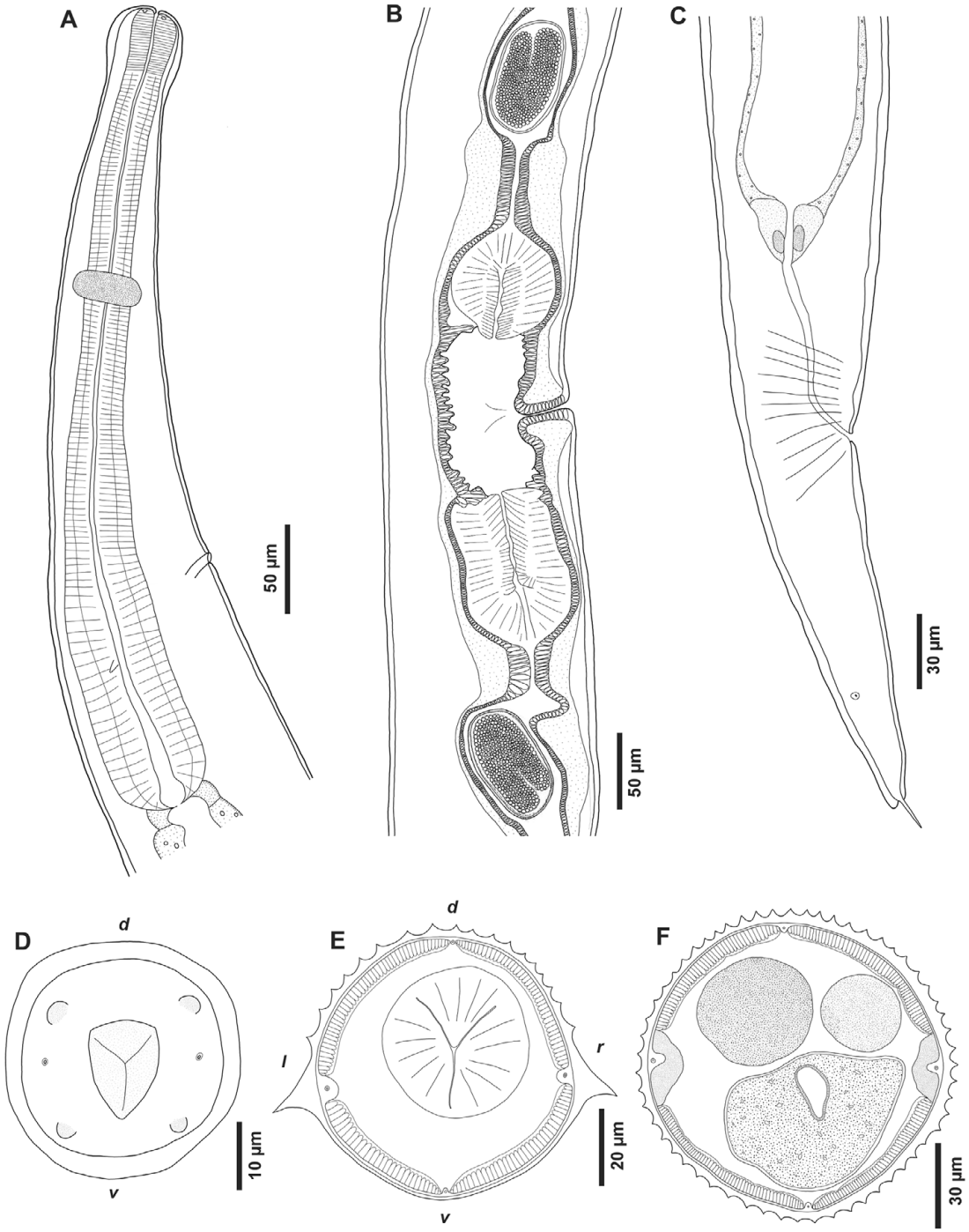

Figure 2. *Oswaldocruzia franciscoensis* n. sp. female. Line drawing. A – The anterior region showing the lateral view. B – The cephalic end showing the apical view. C – The transversal section at the level of the oesophagus region, between the nerve ring and excretory pore. D – The transversal section at the level of the mid-body. E – The female reproductive system region showing the lateral view. F – The posterior region showing the lateral view. (Abbreviations: d – dorsal side; l – left side; r – right side; v – ventral side)

Taxonomic summary

Type host: *Leptodactylus macrosternum* Miranda-Ribeiro (Anura, Leptodactylidae) (Miranda's White-lipped Frog, 'Caçote')

Type locality: conventional agriculture area (9°20'4.68"S, 40° 35'11.25"W), municipality of Petrolina, State of Pernambuco, Brazil

Site of infection: stomach and small intestine

Prevalence: 35.8% (24 infected hosts out of 64 studied)

Mean intensity: 5.9±1.9 parasites per infected host (1–40 specimens)

Mean abundance: 2.1±0.8 parasites per analysed host

GenBank accession: 18S rDNA partial sequence (OR614372)

ZooBank registration: will be provided after the acceptance of the manuscript.

Type specimens: holotype male: CHIOC 39646a; allotype female: CHIOC CHIOC 39646b. Paratypes: CHIOC 39646c (four

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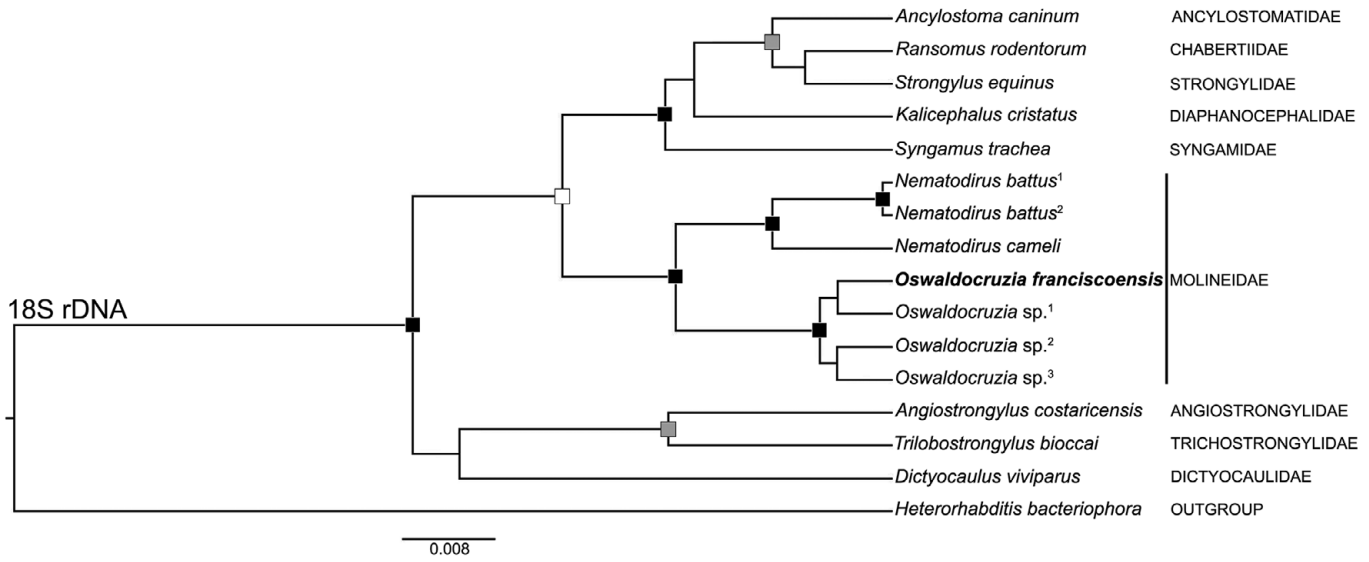

Figure 3. The phylogenetic reconstruction was based on Bayesian inference, from 18S rDNA sequences of representatives from different families of 'bursate nematodes'. The nodal supports were estimated by Bayesian posterior probability (BPP). The dark squares indicate full support (BPP = 1), the grey squares indicate high support (0.96 < BPP < 1), and the white squares indicate moderate support (0.93 < BPP < 0.96). The families of each taxon are indicated in capital letters. The sequence generated in the present study is in bold. Superscript numbers correspond with the information in Table 1.

males and four females). Hologenophore: CHIOC 39646d (anterior and posterior regions of one adult male).

Etymology: The species was named in allusion to the São Francisco River, the primary source of perennial water in the Brazilian Caatinga semi-arid region.

Molecular characterisation

A partial sequence of the 18S rDNA was obtained for *O. franciscoensis* n. sp. (528 bp). This sequence was most closely related to those of the congeners *Oswaldocruzia* sp. (LC624873, LC624874, JX877669), showing 99.24–99.43% of genetic identity, and secondly most closely related to species of *Nematodirus* Ransom, 1907 (AJ920360, JX305977, U01230; genetic identity 96.59–96.78%), a genus also belonging to Molineidae.

In the phylogenetic reconstruction, the new species formed a fully supported monophyletic assemblage with representatives of *Oswaldocruzia* (Figure 3). A fully supported monophyletic lineage was formed by the species of *Nematodirus*, which clustered as a sister group to that of *Oswaldocruzia*, forming an also fully supported monophyletic assemblage and representing the family Molineidae (Figure 3). Representatives of Ancylostomatidae, Chabertiidae, Diaphanocephalidae, Strongylidae, and Syngamidae clustered together with full support, in which this assemblage was sister to representatives of Molineidae, with high support (Figure 3).

Discussion

The new species was assigned to the genus *Oswaldocruzia* because the males have rays of caudal bursa with a 2-1-2 pattern, a gubernaculum is absent, and the spicules are divided into three parts (shoe, blade, and fork); moreover, females are amphidelphic, have post-equatorial vulva, and a tail ending in a small flexible filament (Ben Slimane *et al.* 1996). In addition, both males and females have cephalic vesicles divided into two portions and covered by longitudinal cuticular crests (synlophe) without chitinous support (Ben Slimane *et al.* 1996).

The main morphological features used for differentiating species of *Oswaldocruzia* are the morphology of the caudal bursa and of the spicules in males, the number and arrangement of synlophe crests, as well as the presence or absence of cervical alae in both males and females (see Ben Slimane *et al.* 1996; Bursey and Goldberg 2011; Larrat *et al.* 2018). However, the number and distribution of synlophe crests were not considered in the present differential diagnosis since some authors indicate that such features show intraspecific variations (see Santos *et al.* 2008; Svitin 2017; Kirillova *et al.* 2021; Wilkens *et al.* 2021), which was confirmed after comparing the species as indicated in Table 2. Some authors also use the zoogeographic distribution as a differential character for species of *Oswaldocruzia* (see Ben Slimane *et al.* 1996; Bursey and Goldberg 2011; Larrat et al. 2018).

Currently, 43 species of *Oswaldocruzia* have been reported in the Neotropical region (see Bursey & Goldberg 2011; Guerrero, 2013; Ruiz-Torres *et al.* 2013; Larrat *et al.* 2018), in which eight have type I caudal bursa similar to the new species. Of these, *O. bonsi* Ben Slimane & Durette-Desset, 1993, *O. brasiliensis* Lent & Freitas, 1935, and *O. neghmei* Puga, 1981 differ from the new species because they lack cervical alae, which are present in this latter (see Table 2 for details). The remaining five congeners have cervical alae and will be differentiated from *O. franciscoensis* n. sp. as follows.

Oswaldocruzia franciscoensis n. sp. differs from *O. cartagoensis* Bursey & Goldberg, 2011, *O. lamotheargumedoi* Ruiz-Torres, García-Pietro, Osorio-Sarabia & Violante-Gonzáles, 2013, *O. lopesi* Freitas & Lent, 1938, *O. urubambaensis* Guerrero, 2013, and *O. vitti* Bursey & Goldberg, 2004 mainly based on the morphology of a spicular blade (see Table 2). The new species has a spicular blade divided into two unequal branches, in which the distal end of the longer branch is bifurcated, and the smaller branch has three distal processes, each distally bifurcated (Figure 1E, 1G), contrasting with *O. cartagoensis* that has a spicular blade formed by only one branch, distally divided into eight long narrow pointed

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Table 2. Morphometrics and main morphological characters of Neotropical species of *Oswaldocruzia* Travassos with caudal bursa type I

	O. franciscoensis n. sp.	*O. bonsi* Ben Slimane & Durette-Desset, 1993	*O. brasiliensis* Lent & Freitas, 1935	*Oswaldocruzia cartagoensis* Bursey & Goldberg, 2011	*Oswaldocruzia lamotheargumedoi* Ruiz-Torres, García-Pietro, Osorio-Sarabia & Violante-Gonzáles, 2013	*O. lopesi* Freitas & Lent, 1938	*O. neghmei* Puga, 1981	*O. urubambaensis* Guerrero, 2013	*O. vitti* Bursey & Goldberg, 2004
Type hosts	*Leptodactylus macrosternum* Miranda (Amphibia, Anura)	*Bolitoglossa equatoriana* Brame &Wake (Amphibia, Caudata)	*Palusophis bifossatus* (Raddi) (= *Drymobius bifossatus*) (Squamata, Serpentes)	*Bolitoglossa subpalmata* (Amphibia, Caudata)	*Rhinella marina* (Linnaeus, 1758) (Amphibia, Anura)	*Leptodactylus latrans* (Steffen) (= *Leptodactylus ocellatus* sensu Magalhães et al. 2020) (Amphibia, Anura)	*Hylorina sylvatica* Bell (Amphibia, Anura)	*Rhinella marina* (Linnaeus) (Amphibia, Anura)	*Cercosaura eigenmanni* (Griffin) (Squamata, Sauria) (= *Prionodactylus eigenmanni*)
Type localities	Petrolina, Pernambuco state, Brazil	Napo province, Ecuador	Rio de Janeiro, state of Rio de Janeiro, Brazil	Cartago Province, Costa Rica	Guerrero, México	Rio de Janeiro, state of Rio de Janeiro, Brazil	Puyehue, Osorno province, Chile	San Martin, Cusco, Peru	State of Amazonas, Brazil
Site of infection	Small intestine	Small intestine	Small intestine	Stomach, small and large intestine	Intestine	Small intestine	Small intestine	Small intestine	Small and large intestine
References	Current study	Ben Slimane and Durette-Desset (1993)	Lent and Freitas (1935)	Bursey and Goldberg (2011)	Ruiz-Torres *et al.* (2013)	Freitas and Lent (1938)	Puga (1981)	Guerrero (2013)	Bursey and Goldberg (2004)
Males									
Body length (mm)	4.8–6.85	3.3–5	4.34–4.87	2.9-4	10–11.7	4.9–6.14	5.4–7	6.9–10	2.9–6.4
Cervical alae	present	absent	absent	present	present	present	absent	present	present
Excretory pore-Oesophagus ratio	0.8	0.6	0.72	0.44	0.68	0.85	unknown	unknown	0.69
Synlophe crests at mid-body region	31–34	38–50	unknow	20 crests and 6–8 interlines	54–56	unknow	unknow	44	80
Spicules length	149–168	140–170	112–135	110–122	190–230	126–139	150–160	190–209	120–150

(*Continued*)

Table 2. (*Continued*)

	O. franciscoensis n. sp.	*O. bonsi* Ben Slimane & Durette-Desset, 1993	*O. brasiliensis* Lent & Freitas, 1935	*Oswaldocruzia cartagoensis* Bursey & Goldberg, 2011	*Oswaldocruzia lamotheargumedoi* Ruiz-Torres, García-Pietro, Osorio-Sarabia & Violante-Gonzáles, 2013	*O. lopesi* Freitas & Lent, 1938	*O. neghmei* Puga, 1981	*O. urubambaensis* Guerrero, 2013	*O. vitti* Bursey & Goldberg, 2004
Spicular blade morphology	two unequal branches, the longer branch bifurcating in distal portion and second branch with three distal processes each with distal bifurcations	two long branches, each bifurcating in distal part	one branch, with spatulated distal part	one branch, divided distally into 8 long narrow, pointed processes.	one branch, distally divided into 12 unequal processes	one branch with 3 or 4 distal single blunt distal parts	Spicule divided into two parts, without description of these two parts	six to nine unequal branches, each with single distal parts	three equal branches, with bifurcated distal parts
Females									
Body length (mm)	6.83–11.96	5.4–9	6.97–7.47	3.9–5	12.18–16.8	5.48–8.63	9.08–9.79	12–18.4	7.4–14
Cervical alae	present	absent	absent	present	present	present	absent	present	present
Synlophe crests at mid-body region	55–57	47–59	unknow	30 and 8–10 interlines	74	unknow	unknow	50–60 crests	100
Vagina length	19–25	35	–	27–30	16–18	–	–	62–108	37–46
Eggs length	62–111	85	67	92–104	70–90	80–92	80	79–81	67–90
Eggs width	38–69	45	40	49–67	40–60	50–59	40	36–46	40–52
Tail	123–165	120–180	136–144*	183–220	130–220	120–170	180–210	156–269	100–170
Tail filament	11–13	13	unknown	6–12	12–16	8–13	10	11–19	15

*Authors do not provide data on the length of tail filament.

processes. *Oswaldocruzia lamotheargumedoi* also has one spicular blade formed by only one branch, which is distally divided into 12 unequal processes. *Oswaldocruzia lopesi* has a spicular blade with one branch ending in three or four single distal parts. *Oswaldocruzia urubambaensis* has a spicular blade with six to nine unequal branches, ending in single distal parts. *Oswaldocruzia vitti* has a spicular blade with three equal branches, each with bifurcated distal ends. Therefore, all the previously mentioned species clearly differ from *O. franciscoensis* n. sp.

The present results of genetic characterisation confirmed the allocation of *O. franciscoensis* n. sp. in the genus *Oswaldocruzia*, in which the new species formed a fully supported assemblage with its congeners. Currently, the availability of 18S sequences from representatives of *Oswaldocruzia* is very limited in the GenBank database. These have been originated from hosts of North America and Japan (see Table 1). Therefore, the present 18S sequence of *O. franciscoensis* n. sp. is the first generated for a species of *Oswaldocruzia* in the Neotropical region and may serve for further approaches to the phylogenetic relationships of these nematodes.

Still, on the monophyletic lineage formed by species of *Oswaldocruzia*, it was possible to observe that the representatives from the Americas, including the new species, clustered together, and the same was observed among representatives from Japan. Although these assemblages were weakly supported, the results may be indicative of a biogeographic influence in the genetic relatedness (and probably speciation) of these parasites.

Regarding other genetic markers available for molineid nematodes (including *Oswaldocruzia* spp.) in GenBank, nuclear 28S rDNA and ITS1-5.8S-ITS2 and mitochondrial *cox*1 regions are more numerous. Furthermore, phylogenetic approaches to representatives of Molineidae are limited since they include few species or are focused only on certain genera (De Bellocq *et al.* 2001; Chilton *et al.* 2006; Kirillova *et al.* 2023). In relation to *Oswaldocruzia*, the situation may be considered more critical because only five out of 90 species have been genetically characterised, namely, *O. belenensis* Santos, Giese, Maldonado & Lanfredi, 2008, *O. chabaudi* Ben Slimane & Durette-Desset, 1996, *O. chambrieri* Ben Slimane & Durette-Desset, 1993, and *O. filiformis* and *O. ukrainae* Ivanitzky, 1940, in addition to several sequences labelled as *Oswaldocruzia* sp., in which makers for *cox*1 are by far the most common (see Willkens *et al.* 2016; Kirillova *et al.* 2020, 2023). Therefore, the currently limited genetic evidence does not allow further conclusions about the phylogeny of *Oswaldocruzia*.

Simões *et al.* (2019) suggested the possibility of Molineidae to be artificial, based on phylogenetic reconstructions using 28S and *cox*1 sequences. The authors supported their argument based on the fact that this family of nematodes includes rather complex organisms, with different biological and life history traits, parasitizing hosts from different classes of vertebrates (Simões *et al.* 2019). Nevertheless, the phylogenetic resolution of lower nodes and also of higher taxa (i.e. families) in the results shown by Simões *et al.* (2019) was generally low. The lower degree of conservation observed in some regions of the 28S among the 'bursate nematodes' (see Pereira *et al.* 2019), as well as of mitochondrial genes in comparison with those nuclear, may be overshadowing the real phylogenetic patterns of Molineidae. In this sense, the present 18S-based phylogeny showed good support for lower nodes, indicating that this genetic marker may represent an adequate additional tool for further phylogenetic investigations about Molineidae and other closely related taxa.

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