Research Paper

CAMBRIDGE
UNIVERSITY PRESS

Do host habitat use and cospeciation matter in the evolution of *Oswaldocruzia* (Nematoda, Molineidae) from neotropical amphibians?

Yuri Willkens [iD], Adriano Penha Furtado, Jeannie Nascimento dos Santos [iD] and Francisco Tiago de Vasconcelos Melo [iD]

Laboratório de Biologia Celular e Helmintologia "Profª. Drª Reinalda Marisa Lanfredi" Instituto de Ciências Biológicas - Universidade Federal do Pará, Av. Augusto Corrêa 01 – Guamá CEP: 66075-110 – Belém, Pará, Brasil

Abstract

The genus *Oswaldocruzia* Travassos, 1917 includes approximately 90 species that are parasitic on amphibians and reptiles around the world, of which 43 occur in the neotropical region. However, molecular data supporting the taxonomic status of most species of the genus are scarce, and their phylogenetic relationships remain unknown. Using specimens of four molineid taxa (*Oswaldocruzia belenensis* Santos, Giese, Maldonado Jr. and Lanfredi, 2008; *Oswaldocruzia chabaudi* Ben Slimane & Durette-Desset, 1996, *Oswaldocruzia chambrieri* Ben Slimane & Durette-Desset, 1996 and *Kentropyxia hylae* Feitosa, Furtado, Santos and Melo, 2015) from amphibian hosts collected in different regions of Pará, Brazil, we conducted morphological studies, molecular analyses and phylogenies (maximum likelihood and Bayesian inference) using the cytochrome c oxidase subunity I (*Cox1*) gene. The newly generated sequences were compared with those of ten publicly available *Cox1* sequences of *Oswaldocruzia* from Mexico. Our findings demonstrated significant differences between the sequences of amazonian specimens and sequences from specimens collected in Mexico, and we suggest that host–parasite cospeciation or habitat use might be related to molineid evolution in amphibian hosts. Additionally, this work presents new hosts and new geographical records for species of *Oswaldocruzia* from the neotropics.

Introduction

Oswaldocruzia Travassos, 1917 includes approximately 90 species of parasites of amphibians and reptiles around the world (Guerrero, 2013; Svitin, 2017). There are 43 species formally recognized in the neotropical region, 14 of which have been reported from South American amphibians; only eight of these species have been described as parasites of amphibians and reptiles in Brazil (Bursey & Goldberg, 2011; Guerrero, 2013; Ruiz–Torres *et al.*, 2013; Campião *et al.*, 2014; Willkens *et al.*, 2016).

Ben Slimane *et al.* (1996) established the morphology of the synlophe, the copulatory bursa and the male spicules as the main sources of morphological characters for specific recognition of species within *Oswaldocruzia*. However, intraspecific variability in these characters has been observed for some species of this genus, and morphological similarities with overlapping diagnostic characters between species have also been observed (Bursey & Goldberg, 2005; Santos *et al.*, 2008; Guerrero, 2013). Additionally, there are no recently updated identification keys for the species of *Oswaldocruzia*, and the only identification key for the genus was proposed by Ben Slimane *et al.* (1996), covering 29 of the 43 species currently recognized in the neotropical region.

Eighteen sequences of different genetic regions from *Oswaldocruzia* are publicly available, of which seven are from *Oswaldocruzia filiformis* parasitic in *Lissotriton vulgaris* (Amphibia, Caudata) from Germany (one partial 28S; five with ITS1, 5.8S and ITS2; one with SSU, ITS1, 5.8S and ITS2 from rRNA), nine from an unidentified species parasitic in *Trachycephalus typhonius* (Anura, Hylidae) from Mexico (mitochondrial *Cox1*), one from an unidentified species found in *Anaxyrus americanus* (Anura, Bufonidae) in the United States (partial 18S rRNA) and one sequence from *Oswaldocruzia chambrieri* Ben Slimane & Durette-Desset, 1996 parasitic in *Rhinella gr. margaritifera* (Laurenti, 1768) from Brazil (mitochondrial *Cox1*), the only sequence from the neotropical region with a specific diagnosis by Willkens *et al.* (2016). Consequently, phylogenetic studies supporting the relationships among molineid genera and their species are absent, and no molecular data support the taxonomic status of most of them.

Thus, this study presents a molecular analysis of four molineid species from the eastern Amazon with a phylogenetic approach, including publicly available data and newly generated sequences of the cytochrome oxidase subunit I (*Cox1*) gene from mitochondrial DNA (mtDNA). The main objective was to molecularly characterize three species of *Oswaldocruzia*

and one species of *Kentropyxia* Baker, 1982, from the eastern Amazon, conduct genetic distance-based comparisons and apply a phylogenetic approach to observe the relationships among these taxa and establish molecular bases for species delimitation. Additionally, this study presents new hosts and new geographical records for *Oswaldocruzia belenensis* Santos, Giese, Maldonado jr. and Lanfredi, 2008, *Oswaldocruzia chabaudi* Ben Slimane & Durette-Desset, 1996 and *O. chambrieri* with novel molecular data.

Materials and methods

Host sampling, parasite collection and identification

A total of 250 adult specimens of *Oswaldocruzia* were obtained from the following hosts: *Boana geographica* (Spix, 1824), *Boana wavrini* (Parker, 1936), *Rhinella* gr. *margaritifera* (Laurenti, 1768), *Amazophrynella bokermanni* (Izecksohn, 1994) and *Rhinella marina* (Linnaeus, 1758) in three localities in Pará State, Brazil: Caxiuanã National Forest, Melgaço (Flona Caxiuanã; 1°47′32.3″S, 51° 26′02.5″W), Praia Grande, Salvaterra (0°45′52.1″S, 48°30′43.3″W) and Biological Sciences Institute, UFPA, Belém (ICB-UFPA; 1° 28′23.6″S 48°27.3″W) (fig. 1). All of the collected amphibians were anaesthetized by injection of lidocaine hydrochloride 2%, euthanized and examined for parasites under stereomicroscopes (Leica EZ4). The parasites found were collected, rinsed in saline solution, killed in heated 70% ethanol and stored in 70% ethanol for morphological studies and 96% ethanol for molecular analyses.

For morphological identification, the specimens obtained were clarified in Aman lactophenol, mounted on temporary slides and analysed under an Olympus BX41 light microscope (Olympus, Tokyo, Japan) equipped with a drawing tube and an Olympus BX53 light microscope (Olympus, Tokyo, Japan) with an image capture system at the Laboratory of Cell Biology and Helminthology of Federal University of Pará (LBCH/UFPA). Additionally, to obtain complementary morphological data, we performed scanning electron microscopy (SEM) under VEGA 3 (TESCAN, Brno, Czech Republic) microscopes at the Laboratory of Structural Biology of UFPA and the Laboratory of Histology and Animal Embryology of Federal Rural University of Pará (LHEA/UFRA).

For species diagnosis, we applied the morphological characters established by Ben Slimane *et al.* (1996) and compared our results with the original descriptions. Voucher specimens were deposited in the invertebrate collection of the Emílio Goeldi Museum (MPEG), Belém, Pará, Brazil, and the Helminth Collection of Oswaldo Cruz Institute (CHIOC), Rio de Janeiro, Brazil.

DNA extraction, amplification and sequencing

DNA isolation from the specimens was performed with the QIAGEN QIAamp DNA Mini Kit (Cat No.: 51304) following the manufacturer's recommended protocols. We used polymerase chain reaction (PCR) to amplify the mitochondrial *Cox1* gene using the methodology and the primer cocktail proposed by Prosser *et al.* (2013). The PCR products were observed using agarose gel electrophoresis, and the successfully amplified DNA was purified with the QIAGEN QIAquick PCR Purification Kit (Cat No.: 28104) using the manufacturer's protocols and cycle-sequenced using the Applied Biosystems™ BigDye™ Terminator v3.1 Cycle Sequencing Kit (Cat No.: 4337455). Sequencing was performed using the Applied Biosystems™ 3730 DNA Analyser at the DNA Sequencing Platform of the

Oswaldo Cruz Foundation (RPT01A/PDTIS/FIOCRUZ). The resulting segments were assembled into a contig and edited for errors and ambiguities with the Geneious 9.1.8 suite (https://www.geneious.com).

Molecular and phylogenetic analysis

We built a matrix including our newly generated sequences and ten publicly available *Cox1* sequences from *Oswaldocruzia*: a sequence from *O. chambrieri* parasitic in *Rhinella* gr. *margaritifera* from Caxiuanã National Forest (accession number KU980934), four sequences from *Oswaldocruzia* sp. parasitic of *Trachycephalus venulosus* (Laurenti, 1768) from Mexico (KC130687, KC130698, KC130704 and KC130713) and five sequences from *Oswaldocruzia* sp. parasitic in *Smilisca baudinii* (Duméril & Bibron, 1841) from Mexico (KC130711, KC130712, KC130714, KC130715 and KC130716). Considering the absence of sequences from other molineid taxa parasitic in amphibians from the neotropics, we also obtained a novel sequence from the same sample as the type series of *Kentropyxia hylae* Feitosa *et al.*, 2015, a molineid nematode parasitic in *Osteocephalus taurinus* Steindachner, 1862 from the eastern Amazon for genetic divergence evaluation and we used a sequence of *Amphibiophilus mooiensis* (Nematoda, Amphibiophilidae) Svitin & Du-Preez (2018) from GenBank for tree rooting.

The sequences from our dataset were aligned using the default parameters of the program MUSCLE (Edgar, 2004) under Geneious 9.1.8 (https://www.geneious.com). The alignments were trimmed of poorly aligned regions using the Mesquite 3.51 package (Maddison & Maddison, 2018). Amino acid translation was used to confirm the reading frame positions and find unexpected stop codons. For pairwise nucleotide comparisons, p-distances (the proportion of nucleotide differences by the total number of nucleotides compared between each sequence) were calculated using MEGA X software to estimate the evolutionary distance between sequences (Kumar *et al.*, 2018).

We carried out a phylogenetic reconstruction with maximum likelihood (ML) implemented in PhyML 3.0 (Guindon *et al.*, 2010). The nucleotide evolutionary model was chosen by the Akaike information criterion (AIC) using the JModelTest (Posada, 2008). We determined the node support by applying the approximate likelihood ratio test for branches, the nonparametric branch support based on a Shimodaira–Hasegawa-like (aLRT SH-like) procedure (Anisimova & Gascuel, 2006), and by nonparametric bootstrap percentages (ML-BP) after 1000 pseudoreplications.

We also carried out another phylogenetic analysis using Bayesian inference (BI) in MrBayes version 3.2.6 (Ronquist *et al.*, 2012). To account for different evolutionary processes at each of the three codon positions, BI analyses were performed with distinct models per codon position chosen by the Bayesian information criterion (BIC) (Swofford, 2002). We performed Markov chain Monte Carlo (MCMC) samplings for ten million generations with four simultaneous chains in two runs. Node support was determined by Bayesian posterior probabilities (BPPs) calculated from tree samples every 100 generations after removal of a 'burn-in' fraction of 25%. The robustness of our sampling was assessed using Tracer v1.7.1 software (Rambaut *et al.*, 2018) to calculate the effective sample sizes (ESSs) of the parameters. Values above 100 for effectively independent samples were considered sufficiently sampled. Both the ML and BI trees were visualized and edited using FigTree v. 1.4.4 (Rambaut, 2018).
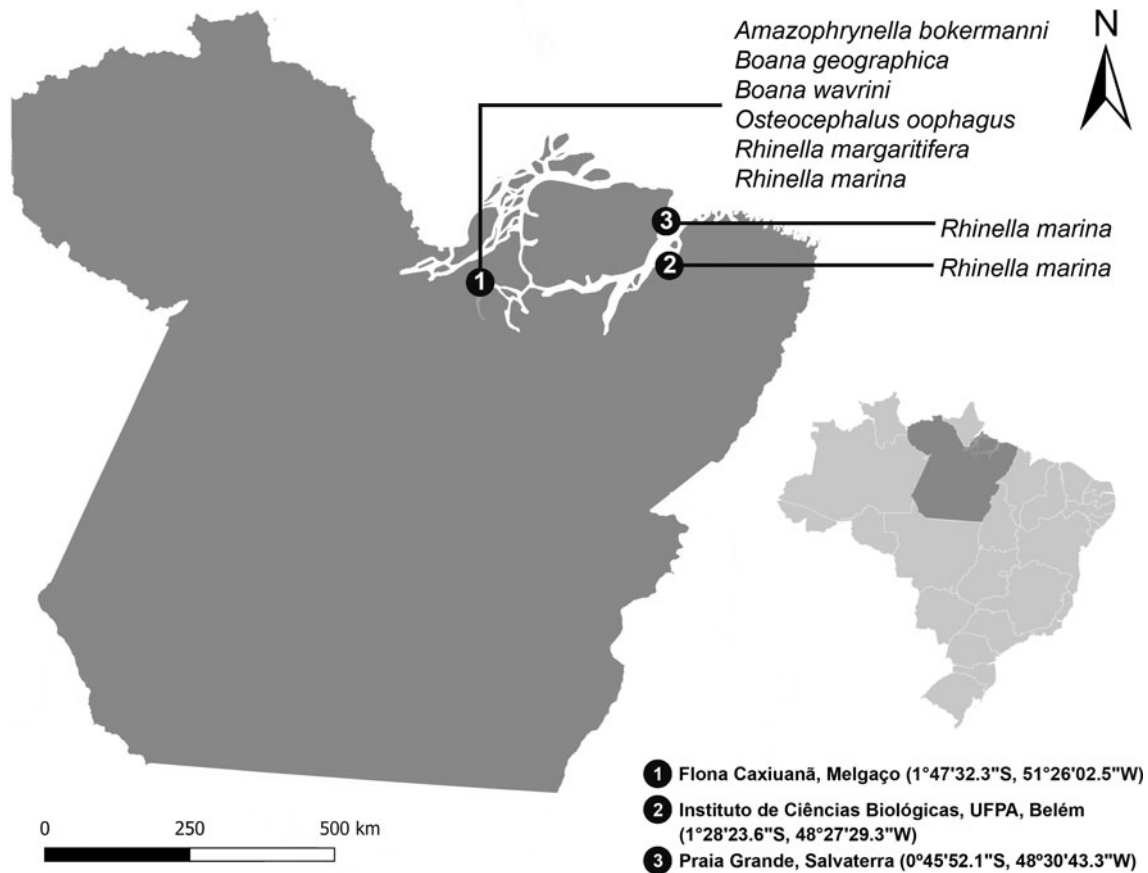
Fig. 1. Map of Pará State, Brazil highlighting the collecting sites of host species harbouring *Oswaldocruzia* used in the present study.

Results

Species identification

We identified three species of *Oswaldocruzia* in the eastern Amazon: *O. belenensis* (voucher specimens: MPEG 000258), *O. chambrieri* (voucher specimens: CHIOC 38717a – 25 males; CHIOC 38717b – 37 females) and *O. chabaudi* (voucher specimens: CHIOC 38718a – seven males; CHIOC 38718b – eight females). Additional morphologic and morphometric data for the species identified in the present work are shown in table 1.

Taxonomic summary

Genus *Oswaldocruzia* Travassos, 1917
Oswaldocruzia belenensis Santos, Giese, Maldonado Jr and Lanfredi, 2008
Hosts: *Rhinella marina* and *Rhinella* gr. *margaritifera*
Site of infection: small intestine
Locality: Caxiuanã National Forest, Pará, Brazil (1°47′32.3″S, 51° 26′02.5″W), Praia Grande-Salvaterra municipality, Pará (0° 45′52.1″S, 48°30′43.3″W), Biological Sciences Institute, UFPA, Belém (1°28′23.6″S 48°27′29.3″W).
Prevalence of infection: Flona Caxiuanã - *R.* gr. *margaritifera* = 0.27 (7 of 26), *R. marina* = 0.9 (9 of 10); Praia Grande – *R. marina* = 0.33 (1 of 3) and ICB-UFPA – *R. marina* = 1.0 (five of five)
GenBank accession numbers: MK492916 and MK492917 (from *R. marina*), MK492915 and MK492918 (from *R.* gr. *margaritifera*)
Voucher specimens: MPEG 000258
Oswaldocruzia chambrieri Ben Slimane & Durette-Desset, 1996
Host: *Amazophrynella bokermanni*
Site of infection: small intestine
Locality: Caxiuanã National Forest, Pará, Brazil (1°47′32.3″S, 51° 26′02.5″W)
Prevalence of infection: Flona Caxiuanã - *A. bokermanni* = 0.89 (eight of nine)
GenBank accession numbers: MK492921 (from *A. bokermanni*)
Voucher specimens: CHIOC 38717a (25 males) and CHIOC 38717b (37 females)
Oswaldocruzia chabaudi Ben Slimane & Durette-Desset, 1996
Hosts: *Boana geographica* and *Boana wavrini*
Site of infection: small intestine
Locality: Caxiuanã National Forest, Pará, Brazil (1°47′32.3″S, 51° 26′02.5″W)
Prevalence of infection: Flona Caxiuanã: *B. geographica* = 0.39 (seven of 18), *B. wavrini* = 1.0 (three of three)
GenBank accession numbers: MK492919 (from *B. wavrini*) and MK492920 (from *B. geographica*)
Voucher specimens: CHIOC 38718a (seven males) and CHIOC 38718b (eight females)

Genetic divergence analysis

We obtained eight sequences of the *Cox1* gene: *O. chabaudi*, one from *B. geographica* (accession number MK492920) and one from *B. wavrini* (MK492919); *O. chambrieri* parasitic in *A. bokermanni* (MK492921); *O. belenensis*, two parasitic in *R. marina* (MK492916 and MK492917) and two from *Rhinella margaritifera* (MK492915 and MK492918) and one sequence from *K. hylae* parasitic on *O. taurinus* (MK492922) (table 2).

Table 1. Morphological and morphometric data of *Oswaldocruzia* spp. used in this study. Measurements are given in ranges followed by mean values in parentheses, and values are given in micrometres unless otherwise stated.

	O. belenensis		*O. chambrieri*		*O. chabaudi*	
	Male	Female	Male	Female	Male	Female
Body length (mm)	6–7 (6)	6.3–11.1 (8.8)	4–6 (5)	7–11 (8)	4–5 (5)	8–11 (10)
Width at midbody	87–119 (102)	103–160 (128)	80–147 (122)	147–187 (169)	91–146 (110)	112–160 (129)
Cephalic vesicle	64–93 (77)	74–107 (82)	61–83 (60)	61–72 (65)	50–57 (54)	42–61 (50)
Oesophagus	389–503 (442)	466–546 (525)	396–445 (430)	440–504 (479)	339–422 (368)	397–479 (426)
Nerve-ring	180–251 (210)	202–288 (224)	140–181 (161)	152–184 (160)	116–199 (156)	183–355 (225)
Excretory pore	246–390 (320)	321–453 (392)	239–312 (287)	256–344 (291)	160–291 (246)	261–355 (313)
Deirids	380 (299–428)	355–500 (419)	261–336 (305)	277–371 (312)	320–380 (345)	334–380 (351)
Excretory pore / oesophagus ratio	0.55–0.82 (0.71)	0.65–0.74 (0.68)	0.57–0.79 (0.67)	0.5–0.76 (0.62)	0.67–0.79 (0.69)	0.7–0.8 (0.8)
Cuticular ridges at midbody	39–45	39–45	41	33	40–46	59–64
Lateral alae	Absent	Absent	Absent	Absent	Absent	Absent
Bursa type	Type II	—	Type III	—	Type III	—
Left spicule	152–181 (162)	—	174–193 (181)	—	151–170 (157)	—
Right spicule	151–178 (161)	—	171–192 (181)	—	153–172 (158)	—
Blade morphology	4 processes, 2 bifurcating	—	5–7 processes	—	5 processes	—
Vulva (mm)	—	4.1–7.8 (6.1)	—	5–8 (5.7)	—	2–3 (3)
Vestibule	—	200–418 (342)	—	327–389 (379)	—	280–411 (322)
Sphincters	—	32–51 (36)	—	21–53 (35)	—	24–36 (28)
Infundibula	—	32–49 (35)	—	34–53 (42)	—	38–82 (54)
Egg width	—	34–45 (37)	—	38–44 (42)	—	29–36 (32)
Egg length	—	64–73 (68)	—	67–74 (71)	—	42–65 (51)
Tail	—	131–182 (175)	—	105–138 (121)	—	75–183 (104)
Caudal Spine	—	7–10 (9)	—	12–24 (15)	—	10–12 (10)

The alignment of the *Cox1* sequences was 655 base pairs (bp) long after trimming and comprised our newly generated sequences and all publicly available *Cox1* sequences from *Oswaldocruzia* spp. available in GenBank. Pairwise nucleotide comparisons among the sequences showed significant divergence levels (*p*-distances) between the novel sequences and most of the previously deposited sequences, presenting the highest values of genetic divergence (11.6–14.2%) compared with the sequences of *Oswaldocruzia* sp. from Mexico. Similarly, the sequence obtained from *K. hylae* showed 12.2–13.5% divergence compared with our novel sequences and 12.2–13.5% divergence compared with the sequences from the Mexican species. The outgroup sequence of *A. mooiensis* was 13.9–14.4% from Mexican species, 13.3–14.4% from Amazon species and 14.7% from *K. hylae*. Lower divergence levels were observed among sequences of the species from the Amazon (0.3–8.2%) and among the sequences obtained from Mexico (0.3–2.4% divergence) (table 2).

Phylogenetic analysis

Alignments of the sequences resulted in a matrix comprising 19 sequences and 655 characters. The best-fit model chosen by AIC was the TPM1uf + I+G model of nucleotide substitution

with optimized frequency parameters, four rate categories, and an estimated gamma shape parameter (1.0560). The ML method resulted in a tree with a score of –l nL = 2186.4931.

The evolutionary models selected through BIC were TrN + I +G in the first codon positions, F81+I in the second codon positions and HKY + G in the third codon positions, all models with unlinking state frequencies and parameters. Markov chains provided highly significant estimated sample sizes (ESSs) for all parameters.

Our phylogenies, based on *Cox1*, inferred using two different methods (ML and BI), resulted in relatively similar topologies with two main clades strongly supported (labelled A, B; figs 2 and 3). Clade A comprised all sequences of *Oswaldocruzia* from Mexico (support values – aLRT SH-like = 99.7%, BPP = 100%), and clade B comprised all species of *Oswaldocruzia* from the Amazon (aLRT SH-like = 96.5%, BPP = 100%), among which our novel sequences were included.

ML and BI topologies diverged regarding the position of *K. hylae*. ML demonstrated *K. hylae* as a separate clade sister to *Oswaldocruzia* spp. from the Amazon (aLRT SH-like = 51.6%), while BI demonstrated it to be a sister to *Oswaldocruzia* from Mexico (BPP = 80%). For clade A, there was no internal resolution, incongruent arrangements of

Table 2. Estimates of genetic divergence (%) of base differences per site between sequences of cytochrome c oxidase I (Cox1) from molineid nematodes parasitic in amphibians from the Brazilian Amazon and Mexico.

| Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. *A. mooiensis* MF460456																			
2. *K. hylae* MK492922	14.7																		
3. *Oswaldocruzia* sp. KC130713	14.1	12.7																	
4. *Oswaldocruzia* sp. KC130711	14.2	12.2	2.3																
5. *Oswaldocruzia* sp. KC130712	14.1	12.8	1.7	1.2															
6. *Oswaldocruzia* sp. KC130715	14.1	13.5	2.3	2.1	1.2														
7. *Oswaldocruzia* sp. KC130687	13.9	13.5	2.1	2.0	1.4	1.1													
8. *Oswaldocruzia* sp. KC130698	13.9	12.7	2.0	1.5	0.9	1.1	0.9												
9. *Oswaldocruzia* sp. KC130714	14.4	13.3	2.4	2.0	1.4	1.1	0.9	1.2											
10. *Oswaldocruzia* sp. KC130704	14.2	13.3	2.3	1.5	1.2	0.9	0.8	1.1	0.5										
11. *Oswaldocruzia* sp. KC130716	14.1	13.3	2.0	1.5	0.9	0.9	0.8	0.8	0.8	0.3									
12. *O. chabaudi* MK492919	14.4	13.5	12.5	12.5	13.0	13.4	13.4	12.8	13.0	13.0	13.0								
13. *O. chabaudi* MK492920	14.2	13.5	12.8	12.8	13.3	13.9	13.9	13.1	13.4	13.4	13.4	0.9							
14. *O. belenensis* MK492916	14.1	13.3	13.9	13.9	13.7	14.2	13.9	13.4	13.9	13.9	13.7	7.9	8.2						
15. *O. chambrieri* KU980934	13.9	12.2	12.7	12.2	12.7	12.8	12.5	12.1	12.8	12.5	12.4	6.7	7.2	3.5					
16. *O. chambrieri* MK492921	14.2	12.5	12.7	12.5	13.0	13.1	12.5	12.4	13.1	12.8	12.7	7.0	7.5	3.8	0.3				
17. *O. belenensis* MK492917	13.7	12.8	11.9	12.5	12.4	12.7	12.4	12.2	12.7	12.7	12.5	7.2	7.6	2.9	3.1	3.4			
18. *O. belenensis* MK492915	13.7	12.2	12.5	12.5	12.4	12.8	12.5	12.1	12.8	12.8	12.7	6.9	7.2	2.8	2.4	2.8	0.8		
19. *O. belenensis* MK492918	13.3	12.3	12.1	12.1	11.9	12.4	12.1	11.6	12.4	12.4	12.2	7.0	7.3	2.8	2.8	3.1	0.8	0.3	
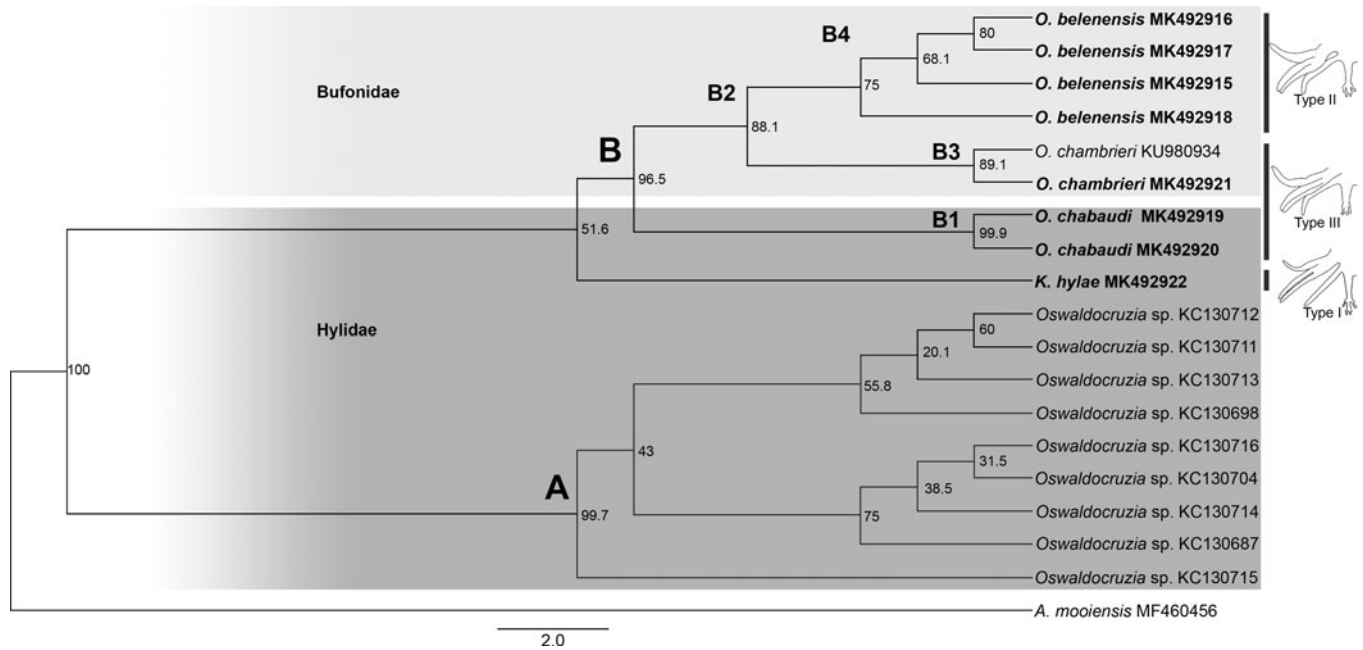
Fig. 2. Phylogenetic relationships of molineid species based on 19 sequences from maximum likelihood (ML) analysis of partial sequences of the mitochondrial cytochrome c oxidase subunit I (*Cox1*) gene. Support values (aLRT SH-like) are indicated at the nodes. The branch-length scale bar indicates the number of substitutions per site.
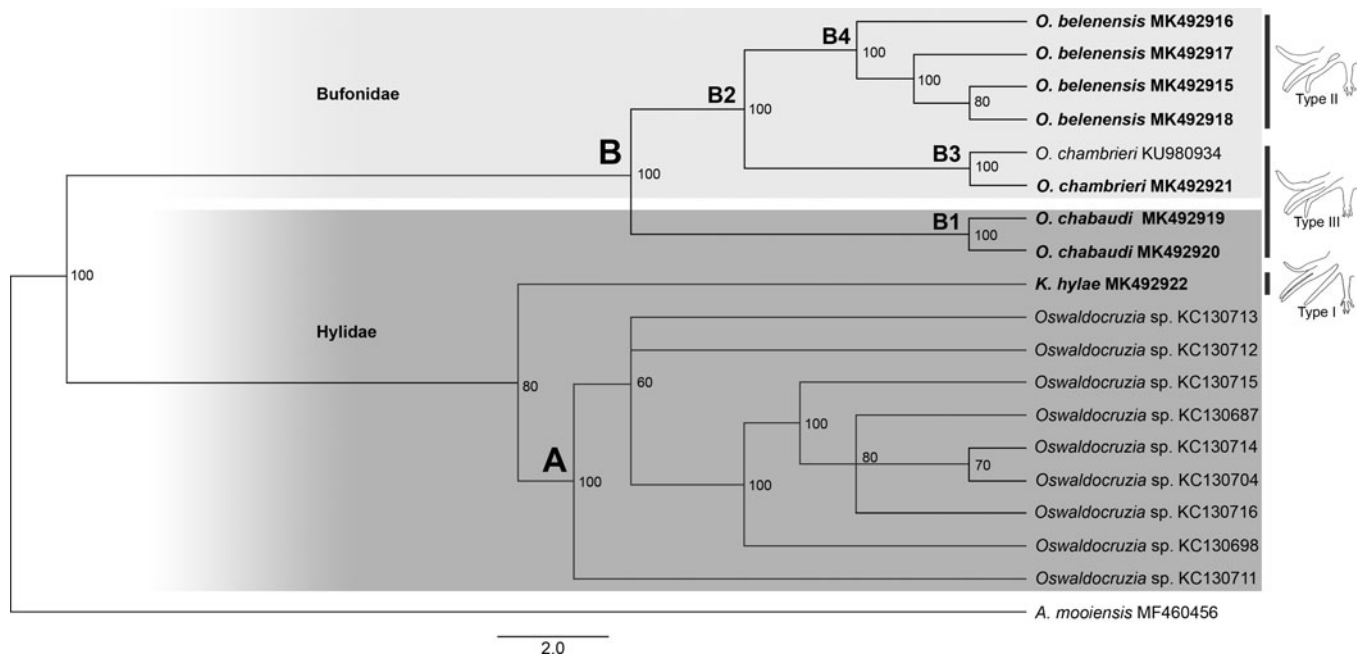

Fig. 3. Phylogenetic relationships of molineid species based on 19 sequences from Bayesian inference (BI) (ten million generations) of partial sequences of the mitochondrial cytochrome c oxidase subunit I (*Cox1*) gene. Support values (BPP) are indicated at the nodes. The branch length scale bar indicates the number of substitutions per site.

nodes and low support values among the taxa. Within clade B, species from the Amazon region, we observed a strong phylogenetic relationship between *O. chabaudi* parasites in *B. geographica* and *B. wavrini*, forming the well-supported clade B1 (aLRT SH-like = 99.9%, BPP = 100%), sister to clade B2 (aLRT SH-like = 88.1%, BPP = 100%), consisting of *O. belenensis* and *O. chambrieri*.

Within clade B2, the sequence from a specimen parasitizing *A. bokermanni* (MK492921) nested along with a sequence of *O. chambrieri* (KU980934), forming the well-supported clade B3 (aLRT SH-like = 89.1%, BPP = 100%), a sister to clade B4 (aLRT SH-like = 75% and BPP = 100%), which is exclusively made up of sequences of *O. belenensis*. For clade B4, we did not observe good internal resolution, and the arrangements were incongruent.

Discussion

Morphology

The genera *Oswaldocruzia* and *Kentropyxia* are morphologically similar, both presenting a club-shaped oesophagus, a cephalic vesicle divided into two portions and longitudinal ridges along the body surface without chitinous support that constitute the synlophe. Males are devoid of gubernaculum, presenting robust spicules surrounded by a hyaline membrane and the caudal bursa with two main lateral lobes supported by rays in a 2–1–2 pattern and a small dorsal lobe. Females have an amphidelphic uterus, a vulva located in the posterior half of the body, and a simple tail ending with a small cuticular spine (Vicente *et al.*, 1991; Anderson *et al.*, 2009). Thus, these similar morphological features may lead to misidentifications of nematodes from both genera.

Ben Slimane *et al.* (1996) presented distinctive morphological characters among those two genera. According to the authors, *Oswaldocruzia* has a simple oral aperture without the corona radiata, and the spicules are divided into three different branches: a simple single pointed branch denominated 'shoe', a bifurcating branch denominated 'fork' and one branch ending with a variable number of distal projections, denominated 'blade' (supplementary figs S1A and S2A). *Kentropyxia* has a complex buccal structure comprised of the vestigial corona radiata with unequal lappets surrounding the cephalic aperture and three-branched spicules with a robust single pointed outer branch ending with small finger-like projections and the two externo-lateral branches ending with numerous distal projections (supplementary figs S1B and S2B). Additionally, regarding the morphology of females, we observed that *Kentropyxia* presents a long and well-developed intermediary portion after the sphincters separating the ovaries from the ovojector and their eggs are not organized within the ovaries, while females of *Oswaldocruzia* have short structures denominated infundibula after the sphincters and their eggs are organized in a row.

Ben Slimane *et al.* (1996) studied several genera of nematodes parasitic in amphibians amongst the Trichostrongylina and divided the species of *Oswaldocruzia* into five biogeographical groups characterized by particularities of the morphology of spicules: (a) 'non-idiomorphic'–Oriento-Ethiopian, Neo-Ethiopian and (b) 'idiomorphic'–Holarctic, Neotropical Caribbean and Neotropical Continental; within these groups, the species are similar and molecular data are absent. The species used in our study included only neotropical continental specimens and sequences from Mexico, for which morphological data are absent. Thus, we could not observe relationships regarding the morphology of spicules of *Oswaldocruzia* and phylogeny.

The species of *Oswaldocruzia* are also divided into three morphological groups based on the types of copulatory bursa, according to the pattern of bursal rays 6 and 8 (types I, II and III) (Ben Slimane *et al.*, 1996). A type I bursa pattern is observed in species of the genus *Kentropyxia* and in most species of *Oswaldocruzia* from the Oriento-Ethiopian, Neo-Ethiopian and Holarctic groups. According to Ben Slimane *et al.* (1996), this morphological trait is considered plesiomorphic in Molineidae. Therefore, the position of *K. hylae* in our phylogeny, the presence of type I bursa and corona radiata in these species reinforce the hypothesis raised by Ben Slimane *et al.* (1996) and Feitosa *et al.* (2015) that *Kentropyxia* spp. emerged first, before the species of *Oswaldocruzia* (figs 2 and 3).

Observations under SEM allowed us to observe ultrastructural characters, such as the cuticular ridges, cephalic structures and the caudal bursa. However, we observed that the morphology of molineid nematodes of amphibians seemed to be highly conserved, and despite the cephalic structures, no distinctive ultrastructural characters were observed between the two genera. We also observed the discontinuous nature of the longitudinal ridges, as pointed out by Santos *et al.* (2008); Feitosa *et al.* (2015) and Willkens *et al.* (2016). The ridges seem to occasionally appear and disappear along the body, resulting in a variable number of ridges for both *Kentropyxia* and *Oswaldocruzia*.

Thus, considering the similar morphology of *Kentropyxia* and *Oswaldocruzia* and among other species from neotropical hosts, we could not assemble the other morphological characters into phylogenies. Increasing the sampling of more molineid taxa will help to improve our comprehension of the morphological characters associated with the phylogenies.

Species divergence and phylogenies

In this study, we analysed four molineid taxa (*O. belenensis*, *O. chabaudi*, *O. chambrieri* and *K. hylae*) using both morphology and molecular data of novel *Cox1* gene sequences from our specimens and sequences previously deposited in GenBank. Genetic distance-based comparisons and phylogenetic analyses under ML and BI, including publicly available *Cox1* sequences, demonstrated significant differences among clades A (including sequences of specimens from Mexico) and B (including sequences of our specimens) and molecular differences among species from arboreal and terrestrial hosts congruent with our morphological observations.

We also observed that *Oswaldocruzia belenensis*, which has a type II bursa, formed a separate clade sister to *O. chabaudi*, which has a type III bursa (a character shared with *O. chambrieri*); this might be evidence that the bursa pattern reflects the evolution of Molineidae, reinforcing the hypothesis of Ben Slimane *et al.* (1996) that the type I bursa is plesiomorphic in comparison to the other types and pointing to the type II bursa as a derivate of the type III bursa. However, these different bursa types also occur in specimens from other biogeographical groups, and further investigations considering not only taxa from different locations but also from biogeographical groups and evolutionary history of their hosts are required.

We could not test the monophyly of the genus because there are no data available from *Oswaldocruzia* spp. from different continents, and our dataset includes genetic data from species from Brazil and Mexico. However, the genetic distance and both phylogenies demonstrated a clear separation of the two main clades, A and B (figs 2 A, B and 3 A, B). For sequences of *Oswaldocruzia* grouped in clade A, the authors did not provide any morphological data regarding their specimens, and we cannot confirm their identification or the biogeographic group in which they are included. Interestingly, the genetic divergence values of *Oswaldocruzia* sequences from Amazon and Mexico (ranging from 11.6 to 14.2%) were similar to those observed among *Kentropyxia* and *Oswaldocruzia* (ranging from 12.23 to 13.5%). Although these sequences were obtained from the region of transition from Neotropical and Nearctic realms (Colima, Mexico), the high divergences found might reflect not only the geographical distance but also suggest that sequences from clade A may represent a lineage that diverged long time ago, or may also represent species from a different biogeographic group of species (Holarctic) or even, a different genus of nematodes, which is reinforced by the position of *K. hylae* within clade A in the BI topology (higher support in comparison to ML).

Within clade B, we observed a strong relationship between *Oswaldocruzia* parasites in *B. geographica* and *B. wavrini,* forming the well-supported clade B1 (figs 1 B1 and 2 B1) (0.9% genetic divergence). This result is congruent with the strong morphological similarity observed between specimens from both host species. *O. chabaudi* was described by Ben Slimane & Durette-Desset, 1996, parasitizing *Boana boans* (Linnaeus, 1758) and *Boana fasciata* (Günther, 1858), and *B. geographica* from Ecuador was also reported (Campião *et al.*, 2014); thus, the occurrence of *O. chabaudi* in different *Boana* spp. suggests that this species is exclusively or mostly associated with arboreal amphibians or the host family, which occupy similar habitats and ecological niches and have overlapping geographical distributions. Additionally, our findings represent the first report of *Oswaldocruzia* for *B. wavrini* and extend the geographical distribution of *O. chabaudi* to the eastern Amazon in Brazil.

The sequence of a specimen of *O. chambrieri* from *A. bokermanni* (MK492921) was most similar to the sequence of a parasite from *R.* gr. *margaritifera* (KU980934) (0.3% genetic divergence), forming well-supported clade B3 (figs 2 B3 and 3 B3). We also observed a strong morphological similarity between specimens from these hosts, especially regarding the spicules, which allowed us to assign them to *O. chambrieri*. This nematode was described by Ben Slimane & Durette-Desset, 1993, parasitizing *R.* gr. *margaritifera* from Ecuador, and was also reported in the same host species in the Brazilian Amazon by Willkens *et al.* (2016). Additionally, the sequence from *O. chambrieri* (KU980934) deposited by Willkens *et al.* (2016) was obtained from the same location as our specimens (Floresta Nacional de Caxiuanã) and was the only available sequence that nested within clade B. The occurrence of this species in different bufonid hosts may reflect an association with terrestrial amphibians, since these bufonids also share similar niches and have overlapping geographical distributions. The report of *O. chambrieri* in *A. bokermanni* represents a new host for this species.

The highly supported clade B4 comprised the sequences of *O. belenensis* parasites in two bufonid hosts: *R. marina* (MK492916 and MK492917) and *R.* gr. *margaritifera* (MK492915 and MK492918) (figs 2 B4 and 3 B4). Both ML and BI phylogenies showed no internal resolution within clade B4 and a genetic divergence ranging from 0.3 to 2.9%, which may reflect intraspecific variation or cryptic diversity. *O. belenensis* was described by Santos *et al.* (2008) from *R. marina* in Belém, Pará, Brazil, the same location for the specimens from the same host in this study. Additionally, *O. belenensis* obtained from *R.* gr. *margaritifera* represent a new host and expand the geographical range known for this species.

Interestingly, the highly supported clade B2 represents a lineage of parasites mostly associated with Bufonid hosts (*O. belenensis* and *O. chambrieri*), completely separated from clade B1, mostly associated with hylids (*B. wavrini* and *B. geographica*) (figs 1 B2 and 2 B2). These findings allowed us to raise two hypotheses: (1) the clades might reflect the cospeciation of *Oswaldocruzia* spp. and their hosts; and (2) it reflects an association of these species with different habitat uses of the hosts. Thus, we will discuss those results and hypotheses below.

Host-parasite cophylogeny hypothesis

We obtained two major clades of *Oswaldocruzia* from the Amazon corresponding to their host families, which may indicate that those species cospeciated with their host families. This raises the hypothesis that events of cospeciation of *Oswaldocruzia* spp.

to their hosts occurred throughout their evolutionary history; however, additional data on *Oswaldocruzia* from different hosts and localities are necessary to support this. Parasites have evolved several specializations to infect, establish and reproduce within their hosts. These specializations may limit the ability of parasites to infect and become established in new hosts (Charleston & Perkins, 2006). This close host–parasite association may involve the joint speciation of ecologically associated species, resulting in congruent phylogenies with similar divergence times (Filipiak *et al.*, 2016). Bufonids and Hylids are included in Hyloidea, a primarily New World clade (which had significant radiations and is distributed globally) sister to the Old World Ranoidea (Cannatella, 2007; Streicher *et al.*, 2018). Different studies have placed anuran families in different positions within Hyloidea (Darst & Cannatella, 2004; Cannatella, 2007; Pyron & Wiens, 2011; Streicher *et al.*, 2018); however, the positions of Bufonidae and Hylidae are congruent, and both families represent long distant lineages.

Ben Slimane *et al.* (1996) considered biogeographical aspects of the host distribution and morphology of spicules to suggest a Gondwanan origin of Trichostrongylina during the Cretaceous and the genus *Kentropyxia* as a South American remnant of a lineage that diverged long time ago, before *Oswaldocruzia* (referred by authors as an 'ancient' species group). Part of this group took refuge in India and probably reached Eurasia by the end of the Cretaceous through southeastern Asia. Similarly, studies of amphibian biogeography and evolution demonstrated the origin of Arboranae, a clade of arboreal frogs, in South America during the latest Cretaceous or early Cenozoic. The three families included in Arboranae (Hylidae, Pelodryadidae, and Phyllomedusidae) differentiated in South America during the early Cenozoic (Duellman *et al.*, 2016). For the terrestrial toads of the Bufonidae family, their origin is estimated to take place in South America after the break-up of Gondwana in the Upper Cretaceous, with further dispersion around the globe and diversification during the early Cenozoic (Graybeal, 1997; Pramuk *et al.*, 2008). Ben Slimane *et al.* (1996) also raised the hypothesis of the origin of *Oswaldocruzia* in southeastern Asia, followed by dispersion events to Africa during the period between the upper Eocene and Miocene. This hypothesis also considers that neotropical species have arisen after the migration of the Nearctic species to the neotropical region during the Pliocene period, and that the Caribbean group arose by the expansion of the former neotropical group into the Caribbean archipelago. The divergence levels among molineid sequences of this study and their phylogenetic positions are congruent with the hypothesis of Ben Slimane *et al.* (1996) that *Kentropyxia* probably emerged first, before *Oswaldocruzia*.

Our results showed that the parasitism in Hylidae (or arboreal hosts) is plesiomorphic (*K. hylae*, clade A, clade B1) in comparison to the parasitism in Bufonidae (terrestrial hosts; clade B2). Therefore, several species of *Oswaldocruzia* have been reported for reptiles and other amphibian families around the world. Additionally, *Kentropyxia sauria* Baker, 1982 was described from the lizard *Kentropyx calcarata* Spix, 1825, and the phylogenetic relationships among these molineids are yet unknown. Thus, the cospeciation hypothesis should be taken into account carefully, first, because the speciation of parasites may occur independently of host speciation, often through host shifts as the parasite comes to occupy a new host environment in isolation from the ancestral lineage (de Vienne *et al.*, 2013) and because data on the phylogenetic relationships among molineid parasites of amphibians and reptiles are still scarce.

Inclusion of the DNA sequences of the remaining genera of Molineidae and *Oswaldocruzia* spp. from different continents and hosts will result in a robust phylogeny, allowing for an understanding of the interrelationships between species of the family, among species of *Oswaldocruzia*, and their distribution and host associations.

Host switching and habitat use hypothesis

We observed that both *O. chambrieri* and *O. chabaudi*, species presenting type III bursa, occurred in different host families. Thus, it seems that our phylogenies do not reflect an association of the bursa type with the host family but only include certain species related to the host family. Additionally, the literature points to no particular correlation of bursa type to the host family, and species with all types of bursa have been reported to several families of amphibian and reptilian hosts (see Bursey & Goldberg, 2011; Willkens *et al.*, 2016). Thus, another possible explanation for our results could be related to the parallelism of the origin of parasitism in Hylidae that might have evolved due to host switching in separated lineages within the genus.

The two different clades of *Oswaldocruzia* observed in our phylogenies could also be explained by the habitat use of the hosts. Araújo *et al.* (2015) suggest that compatibility, the opportunity for infections and adaptations for host–parasite coexistence, are important requirements for the establishment of new host–parasite associations through host switching. Therefore, considering the evolutionary history and the different niches occupied by bufonid (mostly terrestrial) and hylid hosts (mostly arboreal), few physiological compatibilities, contact opportunities or adaptations have recently existed that could lead to host switching/spillover. However, it is possible for semi-arboreal species to have contact with, and harbour nematode species associated with both terrestrial and arboreal hosts. The occurrence of *Oswaldocruzia* spp. infecting hosts from different families with different habitat uses have been reported (see Bursey & Goldberg, 2011; Willkens *et al.*, 2016), but no molecular data are available for those species, and their phylogenetic relationships are still unknown.

Studies on the genus *Rhabdias*, lung parasites of amphibians and reptiles, showed similar associations of some nematode species with one specific amphibian family, with a few exceptions. However, recent studies have shown a greater evolutionary importance of host switching and ecological fitting than an association with host taxa (Tkach *et al.*, 2014; Müller *et al.*, 2018; Willkens *et al.*, 2019; Morais *et al.*, 2020). Considering the sympatric distribution of *Rhabdias* spp. and *Oswaldocruzia* spp. and the frequent cooccurrence of these taxa in the same host species, evidence of host switching might also be found for *Oswaldocruzia* species. Further investigations using morphological and molecular data might clarify whether these clades do in fact represent separated lineages and demonstrate the role of host switching in molineids.

We identified two species of *Oswaldocruzia* in bufonid hosts and only one in hylids. *O. belenensis* occurred in both *R. marina* and *R. margaritifera,* and *O. chambrieri* identified from *A. bokermanni* was previously reported from *R. margaritifera* in Brazil and Ecuador, showing that a higher diversity of molineids was found in terrestrial hosts. Among the neotropical species of *Oswaldocruzia*, 13 are reported from bufonid hosts (terrestrial), 13 from leptodactylid (terrestrial) and only four in Hylidae (arboreal) (see Bursey & Goldberg, 2011; Willkens *et al.*,

2016). Euclydes *et al.* (2021) studied the nematode diversity of parasite communities of Atlantic species of anurans and found a positive correlation of habitat use with the variability in the parasite species composition and their functional diversity. They suggested that the higher functional diversity of parasite communities in terrestrial hosts may be related to the fact that nematodes are the main components of helminth diversity in amphibians and they are transmitted directly through contact with the soil, as pointed out by several studies (Campião *et al.*, 2014; Hamann & González, 2015; da Silva *et al.*, 2017; Gómez *et al.*, 2020). Additionally, although the life cycle of most species of *Oswaldocruzia* remains unstudied, some works have demonstrated that the life cycles of some species are direct and well known (Anderson *et al.*, 2000), and the greater vagility of terrestrial amphibians compared to arboreal species increases the possibility of contact with a higher diversity of parasites, as pointed out by Poulin (2007).

In conclusion, our study is the first attempt to obtain phylogenetic information on molineid nematodes of anurans from the neotropics and it adds new molecular data to four molineid taxa. Here, we present the possible evolutionary importance of host–parasite cospeciation and the importance of habitat use by amphibian hosts as hypotheses to explain the evolution of *Oswaldocruzia* from neotropical amphibians and molineid diversity. Our findings should encourage future studies integrating both morphological and molecular data for future phylogenetic studies within this group. Additionally, we endorse that the diversity of molineid nematodes within the neotropics is likely underestimated, and the possibility of more species, new geographical records, and new questions about their systematics should be investigated.

References

Anderson CR, Chabaud GA and Willmott S (2000) *Nematode parasites of vertebrates their development and transmission.* 2nd edn. 671pp. Wallingford, UK, CAB International.

Anderson CR, Chabaud GA and Willmott S (2009) *Keys to the Nematode Parasites of Vertebrates, Archival Volume*. London, Cab International, 463 p.

Anisimova M and Gascuel O (2006) Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. *Systematic Biology* **55** (4), 539–552. doi:10.1080/10635150600755453

Araújo SBL, Braga MP, Brooks DR, Agosta SJ, Hoberg EP, Von Hartenthal FW and Boeger WA (2015) Understanding host-switching by ecological fitting. *PLoS One* **10**(10), e0139225. doi:10.1371/journal.pone.0139225

Ben Slimane B and Durette-Desset MC (1993) Quatre nouvelles espèces du genre *Oswaldocruzia* travassos, 1917 (Nematoda: Trichostrongyloidea) parasites amphibiens d'Equateur. *Revue Suisse de Zoologie* **100**(1), 113–136.

Ben Slimane B and Durette-Desset MC (1996) New *Oswaldocruzia* (Nematoda, trichostrongylina, molineoidea) parasites of amphibians from French Guyana and Ecuador. *MiscelLània Zoològica* **19**(1), 55–66.

Ben Slimane B, Chabaud AG and Durette-Desset MC (1996) Les nématodes trichostrongylina parasites d'amphibiens et de reptiles: Problèmes taxonomiques, phylétiques et biogéographiques. *Systematic Parasitology* **35**(3), 179–206. doi:10.1007/BF00009639

Bursey CR and Goldberg SR (2005) New species of *Oswaldocruzia* (Nematoda: Molineoidea), new species of *Rhabdias* (Nematoda: Rhabdiasidae), and other helminths in *Rana* cf. *forreri* (Anura: Ranidae) from Costa Rica. *Journal of Parasitology* **91**(3), 600–605. doi:10.1645/GE-3440

Bursey CR and Goldberg SR (2011) New species of *Oswaldocruzia* (Nematoda: Molineidae) and other helminths in *Bolitoglossa subpalmata* (Caudata: Plethodontidae) from Costa Rica. *Journal of Parasitology* **97**(2), 286–292. doi:10.1645/GE-2622.1

Campião KM, Morais DH, Dias OT, Aguiar A, Toledo GM, Tavares LER and da Silva RJ (2014) Checklist of helminth parasites of amphibians from South America. *Zootaxa* **3843**(1), 1–93. doi:10.11646/zootaxa.3843.1.1

Cannatella DC (2007) An integrative phylogeny of Amphibia in Narins PM, Feng AS, Fay RR, Popper AN (Eds) *Hearing and sound communication in amphibians. Springer handbook of auditory research*, **vol 28**. New York, NY, Springer. doi:10.1007/978-0-387-47796-1_2

Charleston MA and Perkins SL (2006) Traversing the tangle: Algorithms and applications for cophylogenetic studies. *Journal of Biomedical Informatics* **39**(10), 62–67. doi:10.1016/j.jbi.2005.08.006

Darst CR and Cannatella DC (2004) Novel relationships among hyloid frogs inferred from 12S and 16S mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution* **31**, 462–475. doi:10.1016/j.ympev.2003.09.003

da Silva HAM, Silva-Soares T and Brito-Gitirana L (2017) Comparative analysis of the integument of different tree frog species from *Ololygon* and *Scinax* genera (Anura: Hylidae). *Zoologia* **34**, 1–17. doi:10.3897/zoologia.34.e20176

de Vienne DM, Refrégier G, Lopez-Villavicencio M, Tellier A, Hood ME and Giraud T (2013) Cospeciation vs host-shift speciation: Methods for testing, evidence from natural associations and relation to coevolution. *New Phytologist* **198**, 347–385. doi:10.1111/nph.12150

Duellman WE, Marion AB and Hedges SB (2016) Phylogenetics, classification, and biogeography of the treefrogs (Amphibia: Anura: Arboranae). *Zootaxa* **4104**(1), 001–109. doi:10.11646/zootaxa.4104.1.1

Edgar RC (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**(5), 1792–1797. doi:10.1093/nar/gkh340

Euclydes L, Dudczak AC and Campião KM (2021) Anuran's habitat use drives the functional diversity of nematode parasite communities. *Parasitology Research*. doi:10.1007/s00436-020-06994-9

Feitosa LAN, Furtado AP, Santos JP and Melo FTV (2015) A new species of kentropyxia Baker, 1982 parasitic in the small intestine of *Osteocephalus taurinus* steindachner (Anura: Hylidae) from the Brazilian eastern Amazon. *Systematic Parasitology* **92**, 251–259. doi:10.1007/s11230-015-9600-1

Filipiak A, Zając K, Kübler D and Kramarz P (2016) Coevolution of host-parasite associations and methods for studying their cophylogeny. *Invertebrate Survival Journal* **13**, 56–65. doi:10.25431/1824-307X/isj.v13i1.56-65

Gómez G, Sánchez L, Ñacari LA and Espínola-Novelo JF (2020) Nematode parasites from Six Species of marsupial gastrotheca (Anura: Hemiphractidae) frogs from the Peruvian Andean highlands. *Pacific Science* **74**(1), 65–73. doi:10.2984/74.1.5

Graybeal A (1997) Phylogenetic relationships of bufonid frogs and tests of alternate macroevolutionary hypotheses characterizing their radiation. *Zoological Journal of the Linnean Society* **119**, 297–338. doi:10.1111/j.1096-3642.1997.tb00139.x

Guerrero R (2013) Two new species of *Oswaldocruzia* (Nematoda: Trichostrongylina: Molineoidea) parasites of the cane toad *Rhinella marina* (Amphibia: Anura) from Peru. *Acta Parasitologica* **58**(1), 30–36. doi:10.2478/s11686-013-0103-4

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W and Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Systematic Biology* **59** (3), 307–321. doi:10.1093/sysbio/syq010

Hamann MI and González CE (2015) Helminth parasites in the toad *Rhinella major* (bufonidae) from Chaco region. *Argentina. Acta Herpetologica* **10**(2), 93–101. doi:10.13128/Acta_Herpetol-16150

Kumar S, Stecher G, Li M, Knyaz C and Tamura K (2018) MEGA x: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* **35**(6), 1547–1549. doi:10.1093/molbev/msy096

Maddison WP and Maddison DR (2018). Mesquite: A modular system for evolutionary analysis. Available at http://mesquiteproject.org

Morais DH, Müller MI, Melo FTV, Aguiar A, Willkens Y, de Sousa Silva C, Giese EG, Ávila RW and da Silva RJ (2020) A new species of *Rhabdias* (Nematoda: Rhabdiasidae), a lung parasite of *Pseudopaludicola pocoto* (Anura: Leptodactylidae) from north-eastern Brazil: Description and phylogenetic analyses. *Journal of Helminthology* **94**. doi:10.1017/S0022149X20000929

Müller MI, Morais DH, Costa-Silva GJ, Aguiar A, Ávila RW and da Silva RJ (2018) Diversity in the genus *Rhabdias* (Nematoda, rhabdiasidae): Evidence for cryptic speciation. *Zoologica Scripta* **47**, 1–13. doi:10.1111/zsc.12304

Posada D (2008) Jmodeltest: Phylogenetic model averaging. *Molecular Biology and Evolution* **25**(7), 1253–1256. doi:10.1093/molbev/msn083

Poulin R (2007) Are there general laws in parasite ecology? *Parasitology* **134** (6), 763–776. doi:10.1017/S0031182006002150

Pramuk JB, Robertson T, Sites JW and Noonan BP (2008) Around the world in 10 million years: Biogeography of the nearly cosmopolitan true toads (Anura: Bufonidae). *Global Ecology and Biogeography* **17**, 72–83. doi:10.1111/j.1466-8238.2007.00348.x

Prosser SWJ, Velarde-Aguilar MG, León-Règagnon V and Hebert PDN (2013) Advancing nematode barcoding: A primer cocktail for the cytochrome c oxidase subunit I gene from vertebrate parasitic nematodes. *Molecular Ecology Resources* **13**, 1108–1115. doi:10.1111/1755-0998.12082

Pyron RA and Wiens JJ (2011) A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. *Molecular Phylogenetics and Evolution* **61**, 543–583.

Rambaut, A. (2018). FigTree v. 1.4.4. Available at http://tree.bio.ed.ac.uk/software/figtree/.

Rambaut A, Drummond AJ, Xie D, Baele G and Suchard MA (2018) Posterior summarization in Bayesian phylogenetics using tracer 1.7. *Systematic Biology* **67**(5), 901–904. doi:10.1093/sysbio/syy032

Ronquist F, Teslenko M, Van der Mark P, et al. (2012) Mrbayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**(3), 539–542. doi:10.1093/sysbio/sys029

Ruiz-Torres N, García-Prieto L, Osorio-Sarabia D and Violante-González J (2013) A new species of nematode (molineidae) from *Rhinella marina* (Amphibia: Bufonidae) in Guerrero, México. *Journal of Parasitology* **99** (3), 509–512. doi:10.1645/GE-3244.1

Santos JN, Giese EG, Maldonado Junior A and Lanfredi RM (2008) A new species of *Oswaldocruzia* (molineidae: Nematoda) in *Chaunus marinus* (amphibian: Bufonidae) (Linnaeus, 1758) from Brazil. *Journal of Parasitology* **94**, 264–268.

Streicher JW, Miller EC, Guerrero PC, Correa C, Ortiz JC, Crawford AJ, Pie MR and Wiens JJ (2018) Evaluating methods for phylogenomic analyses, and a new phylogeny for a major frog clade (hyloidea) based on 2214 loci. *Molecular Phylogenetics and Evolution* **119**, 128–143. doi:10.1016/j.ympev.2017.10.013

Svitin R (2017) Two new species of *Oswaldocruzia* (Nematoda, molineidae) parasitising lizards in Ukraine. *Zootaxa* **4263**(2), 358–368. doi:10.11646/zootaxa.4263.2.9

Swofford DL (2002) Phylogenetic analysis using parsimony (*and other methods). *Journal of Molecular Evolution* **42**, 294–307.

Tkach VV, Kuzmin Y and Snyder SD (2014) Molecular insight into systematics, host associations, life cycles and geographic distribution of the nematode family rhabdiasidae. *International Journal for Parasitology* **44**(5), 273–284. doi:10.1016/j.ijpara.2013.12.005

Vicente JJ, Rodrigues HO, Gomes DC and Pinto RM (1991) Nematodes of Brazil 2nd part: Amphibian Nematodes. *Revista Brasileira de Zoologia* **7**, 549–626.

Willkens Y, Maldonado A, Santos JN, Maschio GF and Melo FTV (2016) Redescription of *Oswaldocruzia chambrieri* (strongylida: Molineidae) from *Rhinella margaritifera* (Anura: Bufonidae) in Caxiuanã National Forest, Brazil. *Acta Parasitologica* **61**(3), 567–575. doi:10.1515/ap-2016-0076

Willkens Y, Rebêlo GL, Santos JN, Furtado AP, Vilela RV, Tkach VV, Kuzmin Y and Melo FTV (2019) *Rhabdias glaurungi* sp. nov. (Nematoda: Rhabdiasidae), parasite of *Scinax* gr. *ruber* (Laurenti, 1768) (Anura: Hylidae), from the Brazilian Amazon. *Journal of Helminthology* **94**, e54, 1–9. doi:10.1017/S0022149X19000476