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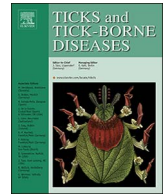
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Description of a new soft tick species (Acari: Argasidae: *Ornithodoros*) associated with stream-breeding frogs (Anura: Cycloramphidae: *Cycloramphus*) in Brazil

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ABSTRACT

In this study, we present a morphological description of immature and adult specimens of *Ornithodoros saraivai* n. sp., a tick associated with the frog *Cycloramphus boraceiensis* (Cycloramphidae) at São Sebastião island, located in the São Paulo state seaboard, Brazil. While larvae of *O. saraivai* are ecologically related to *Ornithodoros faccinii*, another soft tick associated with cycloramphids, the combination of 7 sternal pairs, 16 dorsal pairs, a pyriform dorsal plate and a partially toothed hypostome constitute unique characters of the *O. saraivai* larvae. One undetermined nymphal instar and adults of *O. saraivai* are similar to mature specimens of the *Ornithodoros talaje* species group; however, the *O. saraivai* specimens can be recognized by the presence of a robust bean-shaped spiracle with a large spiracular plate and more than two long seta in palpal article I. Identical partial sequences of the mitochondrial 16S rDNA gene confirmed the identity for all collected stages and for two cohorts of laboratory-reared larvae of *O. saraivai*. A Bayesian and Maximum Parsimony inferred phylogenetic trees support the position of *O. saraivai* in a clade with *O. faccinii*, suggesting the existence of an *Ornithodoros* lineage that evolved in association with amphibians.

1. Introduction

Most species of argasid ticks (Argasidae) are xerophilic hematophagous arthropods, well adapted to parasitize wild vertebrates in dry ecosystems by secreting themselves in burrows, nests and roosting places (Hoogstraal, 1985). This family of ectoparasites is currently composed by 210 species, being the genus *Ornithodoros* Koch the most diverse with 128 representatives (Labruna and Venzal, 2009; Vial and Camicas, 2009; Guglielmone et al., 2010; Nava et al., 2010, 2013; Dantas-Torres et al., 2012; Heath, 2012; Venzal et al., 2012, 2013, 2015; Trape et al., 2013; Barros-Battesti et al., 2015; Muñoz-Leal et al., 2016; Labruna et al., 2016). Among current diversity of the Argasidae family, only few *Ornithodoros* spp. have been documented as parasites of anuran amphibians. Particularly in the Americas, ticks of the

Ornithodoros talaje (Guérin-Méneville, 1849) group were reported parasitizing *Eleutherodactylus cooki* Grant, 1932 (Eleutherodactylidae) in Puerto Rico (Capriles and Gaud, 1977) and *Rhinella marina* (Linnaeus, 1758) (Bufonidae) in Panama (Bermúdez et al., 2013); larvae of an undetermined *Ornithodoros* species parasitizing *Rhinella arenarum* (Hensel, 1867) (Bufonidae) in Argentina (Rivas et al., 2012), and the recently described *Ornithodoros faccinii* Barros-Battesti et al. (2015), feeding upon *Thoropa miliaris* (Spix, 1824) (Cycloramphidae) in Brazil (Barros-Battesti et al., 2015).

Amphibians were common and diverse during the Carboniferous era (Carroll, 2001), a geological period pointed as one of the most probable scenarios for the origin of ticks (Mans et al., 2012). Consequently, this class of vertebrates could have served as primitive hosts for ticks (Oliver, 1989; Dobson and Barker, 1999). In this way and from an

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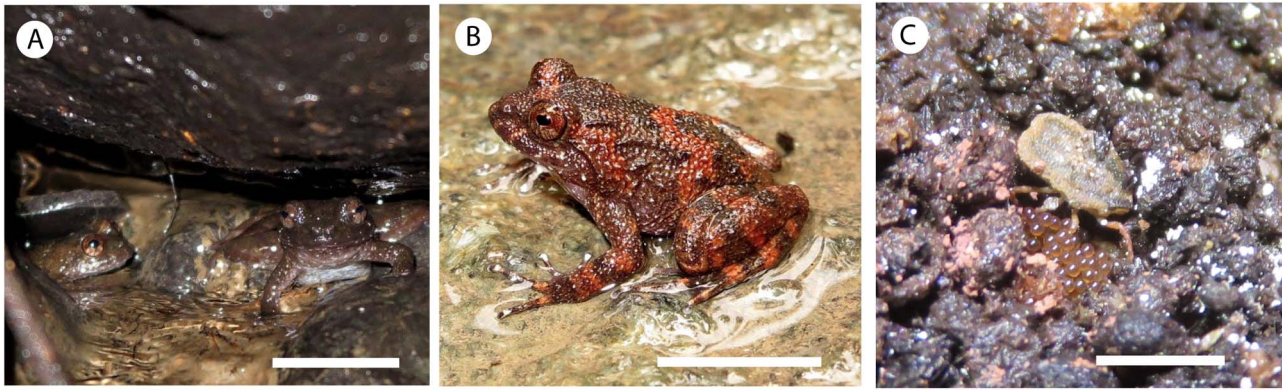


Fig. 1. Type locality of *Ornithodoros saraivai* n. sp.: (A) burrow inhabited by *Cycloramphus boraceiensis* (scale bar equivalent to 2.0 cm), (B) lateral view of a *C. boraceiensis* adult (scale bar equivalent to 2.0 cm), and (C) an *Ornithodoros saraivai* n. sp. female in oviposition (scale bar equivalent to 0.5 cm).

evolutionary point of view, it is of special interest to know the identity of amphibian-associated soft ticks. Based on discrete morphological characters and a phylogenetic approach, in this study we describe a new species of *Ornithodoros*, parasite of a cycloramphid anuran in the Brazilian Atlantic rainforest.

2. Material and methods

2.1. Tick collection

On 20th November 2015, seven larvae of *Ornithodoros* sp. were collected from a frog specimen, *Cycloramphus boraceiensis* Heyer, 1983 (Anura: Cycloramphidae), which was captured on a stream at São Sebastião island (23°51'48"S; 45°25'40"W; elevation: 42 m), municipality of Ilhabela, state of São Paulo, Brazil. Based on this find, in March 2016 we performed a field expedition to the same site, in order to search for ticks in the environment. A total of 30 *Ornithodoros* ticks (5 females, 24 males, 1 nymph) were collected from rock crevices inside a burrow that was inhabited by *C. boraceiensis* (Fig. 1). Two of these females were in the process of oviposition, therefore each one was collected with its eggs in a separate vial. All ticks were taken alive to laboratory, where they were placed in an incubator at 25 °C and 80% relative humidity. Larvae that hatched from the egg batches of the two field-collected females were killed in hot water and preserved in 70% ethanol for morphological analyses.

Amphibian and tick collections for this study have been approved by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), Brazil (permits 17242-3 and 11459).

2.2. Morphological analyses

A total of 20 unfed larvae (10 from each female offspring) was clarified in a 20% KOH solution, and mounted in semi-permanent slides for microscopy using Hoyer's medium. In addition, two larval specimens that were collected from *C. boraceiensis* were also slide-mounted. Slides were photographed with an Olympus DP70 camera implemented in an Olympus BX40 optical microscope and measured with the software Image-Plus Pro v5.1. The totality of adult ticks and the sole nymph were measured in a SteREO Discovery V12 stereomicroscope with the software ZEN 2 pro. All measurements are given in millimetres (mm), with the mean followed by the standard deviation and range in parentheses. Terminology for larval chaetotaxy and measurements followed Venzal et al. (2008, 2013), and we used Cooley and Kohls (1944) terminology for the description of adult and nymphal characters. Subgeneric classification followed Clifford et al. (1964). Females, males, the nymph and unfed larvae were used to obtain micrographs with a JEOL JMS-5900 electron-scanning microscope (JEOL, Tokyo, Japan). External morphology of adults was compared with other

Neotropical ticks deposited in the tick collection "Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva" (CNC) of the Faculty of Veterinary Medicine of the University of São Paulo, Brazil, using the following specimens: *Ornithodoros guaporensis* Nava, Venzal & Labruna 2013 (CNC-2305, CNC-2306, CNC-2307), *Ornithodoros riplatensis* Venzal et al., 2008 (CNC-3254), and *Ornithodoros puertoricensis* Fox 1947 (CNC-3255).

The type-series of the tick species described in this study has been deposited in the following tick collections: United States National Tick Collection, Statesboro, GA, United States (USNMENT); "Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva", University of São Paulo, SP, Brazil (CNC); Acari Collection of the Butantan Institute, University of São Paulo, SP, Brazil (IBSP); Department of Veterinary Parasitology, Faculty of Veterinary, Salto, Uruguay (DPVURU); Instituto Nacional de Tecnología Agropecuaria, Rafaela, Santa Fe, Argentina (INTA).

2.3. Molecular tools

In order to obtain molecular data, one female from which unfed larvae were obtained, one male, one female, two unfed larvae hatched in the laboratory, and one larva collected from *C. boraceiensis* were individually submitted to DNA extraction using the Guanidine Isothiocyanate technique (Sangioni et al., 2005). A conventional PCR was performed using the primers 3'-CCGGTCTCAACTCAGATCAAGT-5' (forward) and 3'-GCTCAATGATTTTTAAATTGCTGT-5' (reverse), which target a ≈460-bp fragment of the tick mitochondrial 16S rRNA gene, as previously described (Mangold et al., 1998). PCR products of the expected size were sequenced in an ABI automated sequencer (Applied Biosystems/Thermo Fisher Scientific, model ABI 3500 Genetic Analyser, Foster City, CA) with the same primers used for the PCR. Obtained sense and antisense sequences were assembled with Geneious R9 (Kearse et al., 2012), and then submitted to BLAST analyses (www.ncbi.nlm.nih.gov/blast) in order to infer closest similarities with other *Ornithodoros* spp. available in GenBank.

2.4. Phylogenetic analysis

Obtained sequences were aligned with other congeneric species available in GenBank using Clustal X (Thompson et al., 1997), and manually adjusted using GeneDoc (Nicholas et al., 1997). With this alignment two phylogenetic analyses were performed. A maximum parsimony tree was constructed in PAUP version 4.0b1 (Swofford, 2002), with 500 bootstrap replicates, random stepwise addition starting trees (with random addition sequences) and TBR branch swapping. A Bayesian analysis was performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001) with four independent Markov chain runs for 1,000,000 metropolis-coupled MCMC generations, sampling a tree

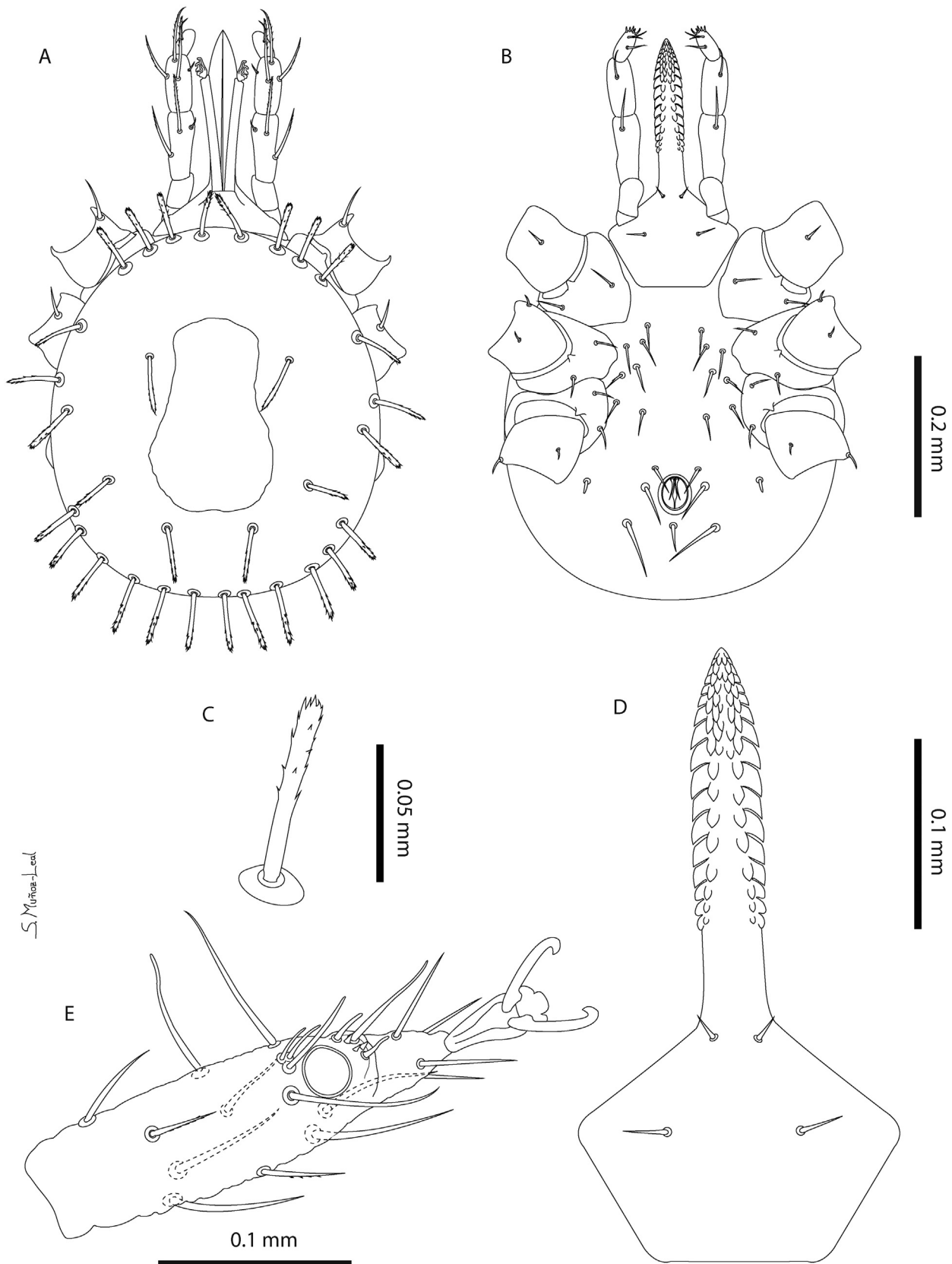


Fig. 2. *Ornithodoros saraivai* n. sp. Drawing of holotype larva: (A) dorsal, (B) ventral, (C) dorsolateral seta, (D) hypostome and basis capituli, (E) tarsus I. Scale bar in millimeters.

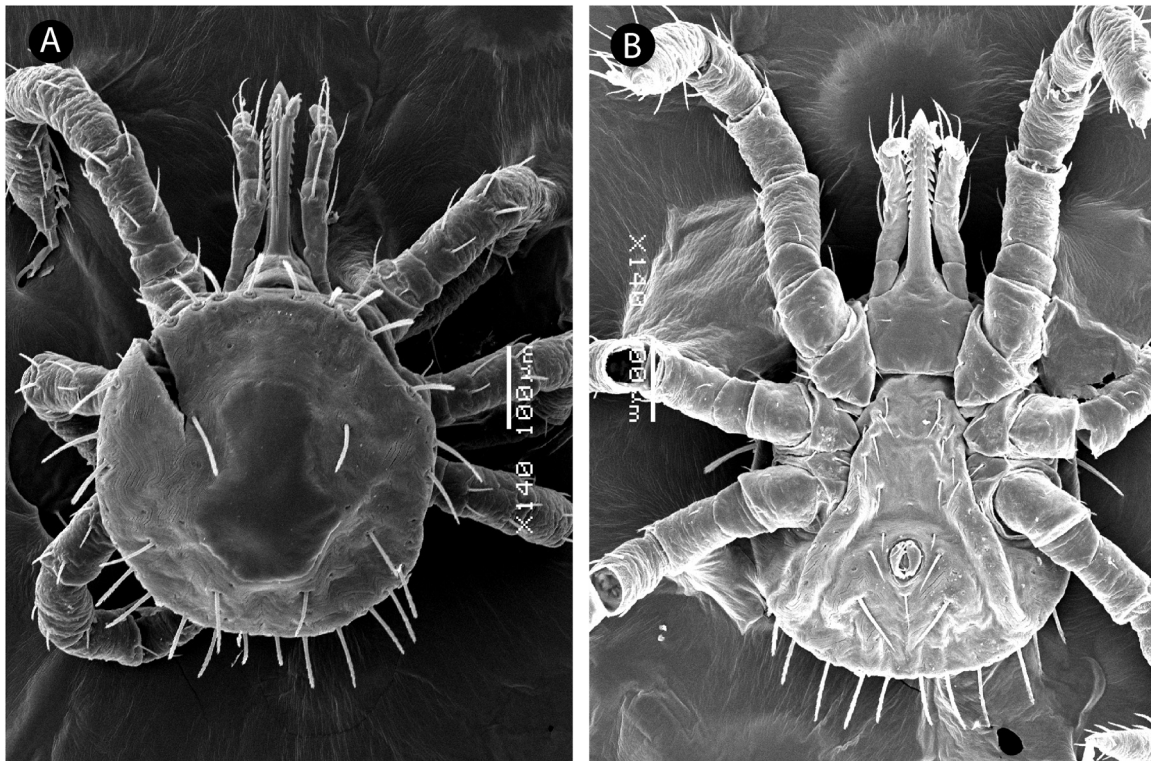


Fig. 3. Scanning electron microscopy of *Ornithodoros saraivai* n. sp. larva: (A) dorsal view, (B) ventral view.

every 100th generation. The first 25% of the trees represented burn-in, and the remaining trees were used to calculate Bayesian posterior probability. Sequences of *Ixodes holocyclus* Neumann 1899 and *Ixodes uriae* White 1852 were used as outgroups (accessions numbers of all sequences are shown in the phylogenetic tree).

3. Results

3.1. Descriptions

Ixodida Leach, 1815
 Argasidae Canestrini, 1890
Ornithodoros Koch, 1844

4. *Ornithodoros saraivai* n. sp. Muñoz-Leal & Labruna

4.1. Larva (Figs. 2 and 3; measurements based on 20 unfed specimens)

Body: idiosoma subcircular. Length including capitulum: 0.771 ± 0.022 (0.735–0.822), and not including capitulum: 0.504 ± 0.016 (0.473–0.526), width: 0.455 ± 0.015 (0.427–0.475).

Dorsum: Dorsal plate pyriform, with irregular margins, wider in the base, length 0.257 ± 0.018 (0.221–0.284), width: 0.170 ± 0.008 (0.151–0.182). Dorsal surface provided with 16 pairs of gross setae, slightly barbed and rather truncated (Fig. 3C), 7 anterolateral, 3 central and 6 posterolateral. Anterolateral setae (Al): Al₁ length 0.073 ± 0.006 (0.060–0.084), Al₂ length 0.068 ± 0.005 (0.052–0.077), Al₃ length 0.072 ± 0.005 (0.063–0.080), Al₄ length 0.072 ± 0.004 (0.062–0.081), Al₅ length 0.075 ± 0.007 (0.063–0.090), Al₆ length 0.079 ± 0.005 (0.069–0.090), Al₇ length 0.075 ± 0.006 (0.064–0.085). Central setae (C): C₁ length 0.085 ± 0.009 (0.059–0.096), C₂ length 0.064 ± 0.006 (0.053–0.074), C₃ length 0.080 ± 0.005 (0.071–0.091). Posterolateral setae (Pl): Pl₁ length 0.067 ± 0.006 (0.055–0.078), Pl₂ length 0.074 ± 0.006 (0.062–0.085), Pl₃ length 0.078 ± 0.006 (0.064–0.090), Pl₄ length 0.082 ± 0.006 (0.073–0.091), Pl₅ length

0.078 ± 0.007 (0.066–0.097), Pl₆ length 0.077 ± 0.005 (0.066–0.089).

Venter: ventral surface provided with 11 pairs of tapering setae plus 1 small pair on anal valves, posteromedian seta present in half of the examined larvae (present in the holotype). Anal valves without exceeding the anal frame. Seven pairs (some larvae with 6) of sternal setae (St): St₁ length 0.040 ± 0.003 (0.032–0.045), St₂ length 0.041 ± 0.004 (0.036–0.051), St₃ length 0.038 ± 0.003 (0.031–0.045), St₄ length 0.040 ± 0.004 (0.032–0.047), St₅ length 0.038 ± 0.004 (0.032–0.049), St₆ length 0.039 ± 0.003 (0.035–0.046), St₇ length 0.039 ± 0.003 (0.033–0.042); three pairs of circumanal setae (Ca): Ca₁ length 0.039 ± 0.006 (0.031–0.055), Ca₂ length 0.057 ± 0.005 (0.049–0.069), Ca₃ length 0.085 ± 0.006 (0.079–0.104); posteromedian setae (Pm) length 0.047 ± 0.007 (0.035–0.060) (absent in some larvae), postcoxal setae (Pc) small, length 0.023 ± 0.003 (0.018–0.027).

Capitulum: basis capituli pentagonal, posterior margin straight (Figs. 2 and 3). Length from posterior margin of basis capituli to posthypostomal setae Ph₁ 0.113 ± 0.008 (0.096–0.128), length from posterior margin of basis capituli to insertion of hypostome 0.122 ± 0.010 (0.101–0.141), length from posterior margin of basis capituli to apex of hypostome 0.328 ± 0.015 (0.295–0.355), width 0.165 ± 0.010 (0.150–0.182). Two pairs of posthypostomal setae; Ph₁ length 0.012 ± 0.002 (0.008–0.015), Ph₂ length 0.018 ± 0.003 (0.014–0.027), distance between Ph₁ setae 0.036 ± 0.003 (0.032–0.040), distance between Ph₂ setae 0.067 ± 0.003 (0.062–0.073). Palpi: total length 0.248 ± 0.010 (0.229–0.267), segmental length/width from I–IV: (I) 0.050 ± 0.003 (0.045–0.055)/ 0.032 ± 0.001 (0.028–0.034), (II) 0.090 ± 0.005 (0.081–0.098)/ 0.033 ± 0.002 (0.029–0.037), (III) 0.085 ± 0.003 (0.077–0.091)/ 0.035 ± 0.002 (0.030–0.038), (IV) 0.047 ± 0.003 (0.042–0.054)/ 0.022 ± 0.001 (0.020–0.023). Setae number on palpal articles I–IV: (I) 0, (II) 4, (III) 5, (IV) 9.

Hypostome: length from Ph₁ to apex 0.216 ± 0.008 (0.200–0.230), length from apex to inferior toothed portion 0.159 ± 0.006 (0.146–0.169), length from insertion of hypostome in basis capituli to

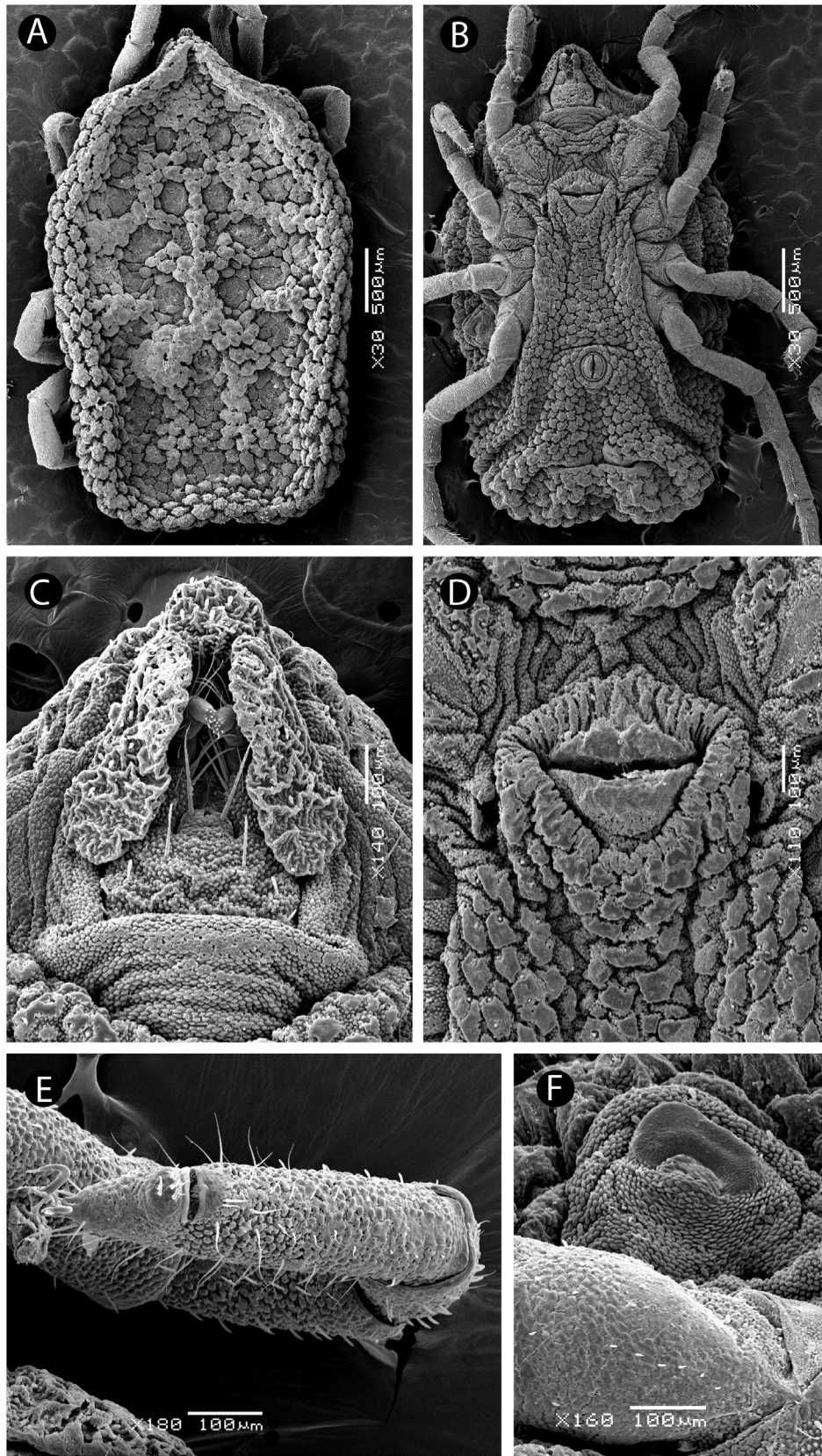


Fig. 4. Scanning electron microscopy of *Ornithodoros saraivai* n. sp. female: (A) dorsal view, (B) ventral view, (C) ventral capitulum, (D), genital opening, (E) tarsus I, (F) spiracle.

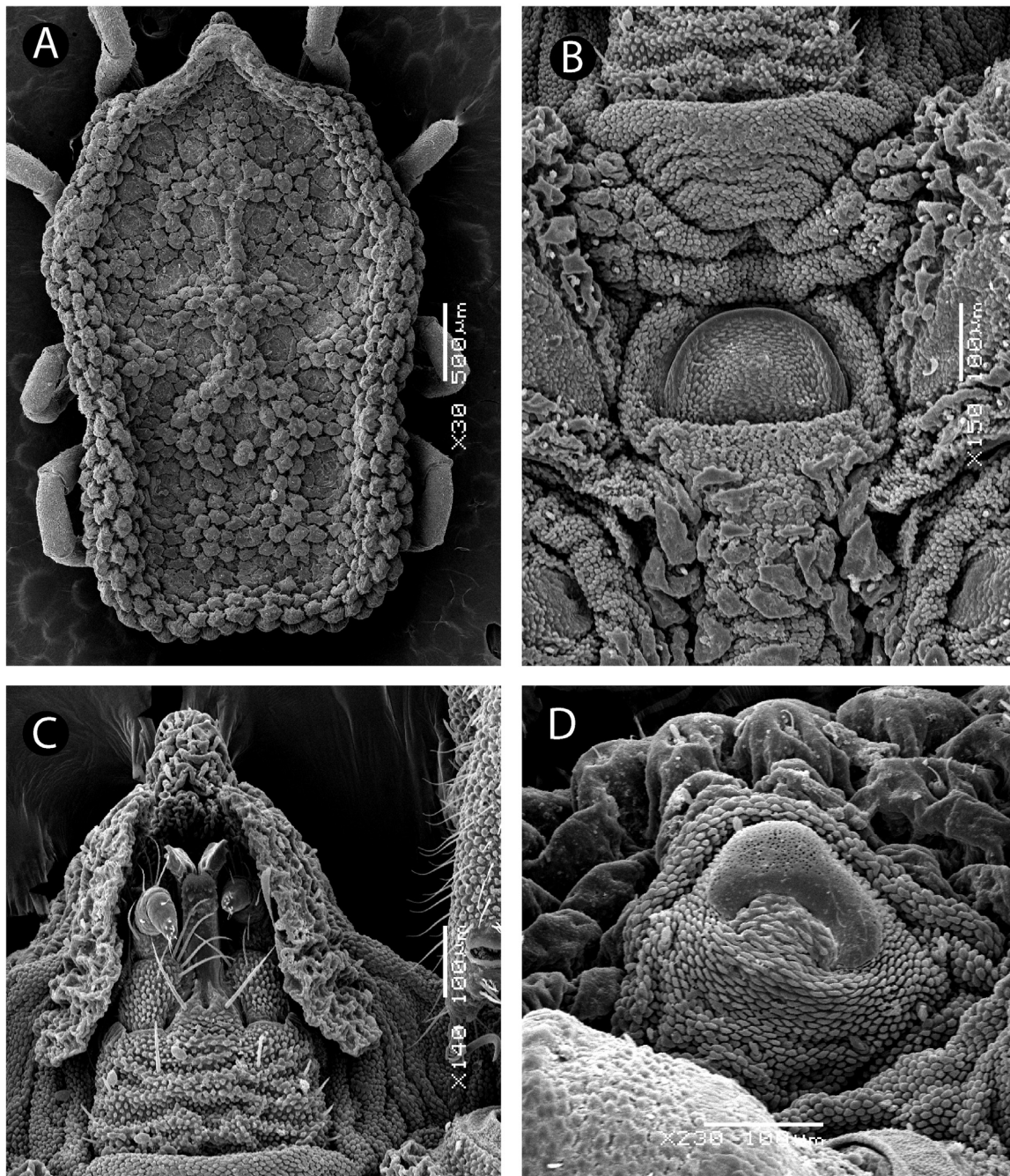


Figure 5. Scanning electron microscopy of *Ornithodoros saraivai* n. sp. male: (A) dorsal view, (B) genital opening, (C) ventral capitulum, (D) spiracle.

apex 0.205 ± 0.007 (0.191–0.216), width in medial basis portion of hypostome 0.037 ± 0.002 (0.033–0.041), width in basis portion of hypostome 0.039 ± 0.002 (0.035–0.043). Denticles present from apex until 2/3 of its total length. Apically pointed. Dental formula 2/2 and 3/3–4/4 in the distal third of the toothed portion, file 1 with 16–17 denticles, file 2 with 15–16 (typically 15), file 3 with 5–7, and file 4 with 1–5.

Legs: covered with wrinkled integument, tarsus I length 0.240 ± 0.011 (0.208–0.259), tarsus I width 0.063 ± 0.003 (0.059–0.069). Setal formula of tarsus I: 1 pair apical (A), 1 distomedian (DM) seta plus two medium sized and three small sensilla, 5 paracapsular setae (PC), 1 posteromedian seta (PM), 1 basal seta (B), 1 pair apicoventral (AV), 1 pair midventral (MV), 1 pair basiventral (BV), and 1 pair posterolateral (PL). Capsule of Haller's organ rounded in shape, without reticulations. Coxa I, II, and III provided with two

ventral setae and one ventral spur projecting towards the trochanter.

4.2. Female (Fig. 4, measurements based on five unengorged specimens)

Body: subrectangular, 4.310 ± 0.231 (4.115–4.674) long, 2.630 ± 0.121 (2.497–2.804) wide. Posterior margin chiefly straight, with a middle concavity in unengorged specimens; lateral margins roughly marked and subparallel, anteriorly converging to an angular apex. Hood slightly exceeding the dorsal apex and visible from above in the majority of the examined specimens. **Dorsum:** integument covered by variable size and shape wrinkled mammillae, with radial ridges on the sides, provided with one to five pores on its dorsal surface, each one with a very small fine tapering or stout truncated seta. Dorsum covered by large mammillae irregular in shape, with truncated conical shape in posterior and lateral margins. Middle of the idiosoma with bilaterally

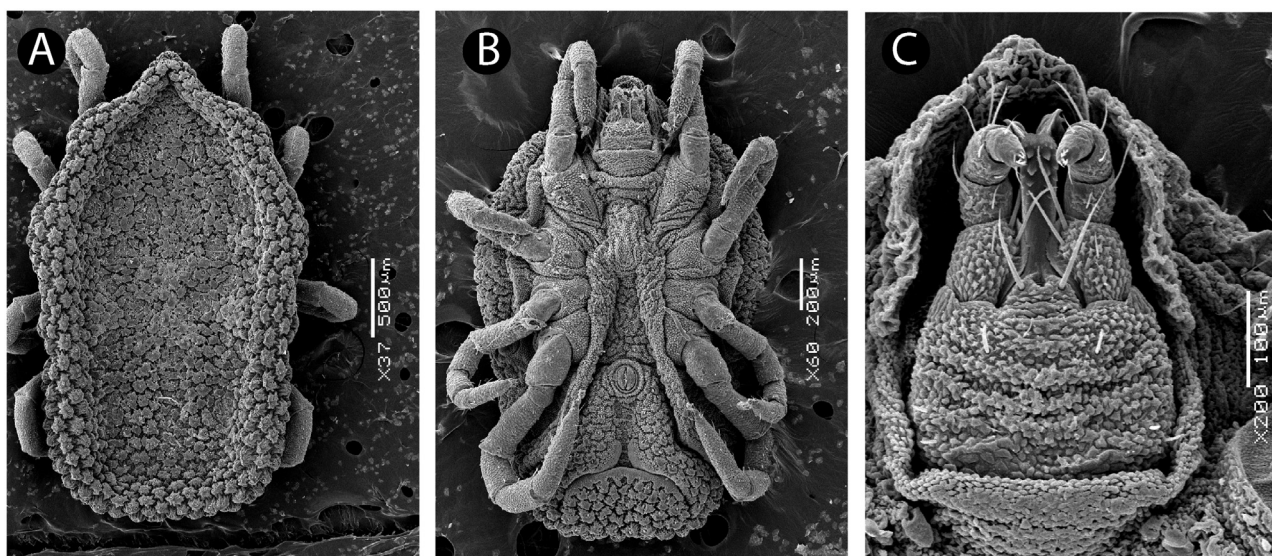


Fig. 6. Scanning electron microscopy of *Ornithodoros saraivai* n. sp. nymph: (A) dorsal view, (B) ventral view, (C) ventral capitulum.

symmetrical depressed regions provided in the middle with barely notable group of disks, below the level of mammillae, and not present at the margins. Lateral suture absent. *Venter*: irregular mammillae covering the ventral surface, some provided with punctuations; presence of very small discs only in preanal and median-postanal grooves. Dorso-ventral groove absent. Preanal groove straight above anus. Coxal fold pronounced. Supracoxal fold small and thin. Genital opening surrounded by a wrinkled integument and located between posterior ends of coxae I; anterior labia with a pore in the middle. Anus surrounded by a ring of irregular mammillae; anal plates with small setae in the internal margin. Intersection of median-postanal and postanal groove with a glabrous smooth fold. Spiracle above coxa IV, provided with a large bean-shaped spiracular plate, with numerous and notorious goblets. Cheeks large and elongate, with irregular transverse wrinkles, covering partially the palps.

Capitulum: situated ventrally to a small hood. Basis capituli rectangular, micromammillated, with one pair of long posthypostomal setae, one pair of postpalpal setae approximately half the size of the posthypostomal setae, and four to five pairs of short lateral setae. Palps with marked micromammillae covering article I, then diminishing their intensity in articles II and III, and absent in article IV. Article I with a ventromedial integumental ridge-like extension along its internal margin, three to four long ventromedial setae projecting towards the hypostome, one small ventral seta inserted between micromammillae, and one small ventrolateral setae. Hypostome with small crenulations apically, dental formula 2/2 in the anterior half, followed by small denticles toward the base, yet not reaching the insertion in the basis capitulum.

Legs: entirely micromammillated; coxae I–II with various mammillae, coxae III–IV with few or rare mammillae over micromammillated surface. Coxae I and II separated by a mammillated integumental portion; coxae II to IV contiguous. Coxal size in decreasing order from I to IV. Tarsi narrow without humps or protuberances, each one provided with bifid claws and a small pulvillus.

Male (Fig. 5, measurements based on 24 unengorged specimens)

Body subrectangular, 3.510 ± 0.139 (3.234–3.798) long, 2.204 ± 0.112 (1.993–2.438) wide. Genital opening with an entirely micromammillated flap in the form of half circle. Other features as in females.

Nymph (Fig. 6, measurements based in one unengorged specimen)

Body: subrectangular, 2.917 long, 1.775 wide. Dorsal and ventral idiosoma similar to adults, except for the presence of a micromammillated integument in the area corresponding to the genital opening of

adults.

Capitulum: basis capituli wrinkled and micromammillated, provided with one pair of posthypostomal setae, one pair of postpalpal setae approximately one third of the size of posthypostomal setae, and two pairs of short lateral setae. Cheeks present. Palps with article I–III micromammillated, and medial margin of article I provided with an integumental ridge-like extension as in adults. Article I provided with two long setae in the anterolateral-medial margin projecting towards the hypostome, and one short ventral seta inserted between micromammillae. Hypostome with small denticles in the corona, 2/2 in the anterior half, and then nude until the insertion in basis capituli.

4.3. Taxonomic summary

Type host: Adult males of *Cycloramphus boraceiensis* Heyer, 1983 (Anura: Cycloramphidae) (Fig. 1B).

Type locality: Ilha de São Sebastião (23°51'48"S; 45°25'40"W), municipality of Ilhabela, state of São Paulo, Brazil.

Type specimens: Holotype: one unfed larva (mounted in a slide), originated from egg laid by a paratype female collected in the type locality, 22 March 2016, deposited in the United States National Tick Collection, Georgia Southern University, Statesboro, USA (USNMENT-00862468), collecting event number RML127891. Paratypes: one unfed larva (mounted in slide), same data as holotype (INTA-2355); two larvae (mounted in separate slides), same data as holotype (IBSP-12576); two larvae (mounted in separate slides), same data as holotype (DPVURU-881); 16 larvae (mounted in separated slides) (CNC-3410) and 24 larvae in ethanol, same as holotype (CNC-3474); seven larvae (one was destroyed for DNA extraction), collected on a *C. boraceiensis*, 20 November 2015, type locality (CNC-3473); 18 males, two females, one nymph (one male and one female were destroyed for DNA extraction; one nymph, two females and two males were used for electron scanning microscopy micrographs) collected in a burrow inhabited by *C. boraceiensis*, 22 and 23 March 2016, type locality (CNC-3411, DPVURU, USNMENT-00862469)

Etymology: this species is named *in memoriam* to Danilo G. Saraiva, who dedicated his professional life to study Brazilian wildlife, with emphasis on ticks and tick-borne diseases.

4.4. Analysis of the mitochondrial 16S rDNA sequences and phylogenetic tree

Adults and larvae submitted to DNA extraction yielded identical 16S

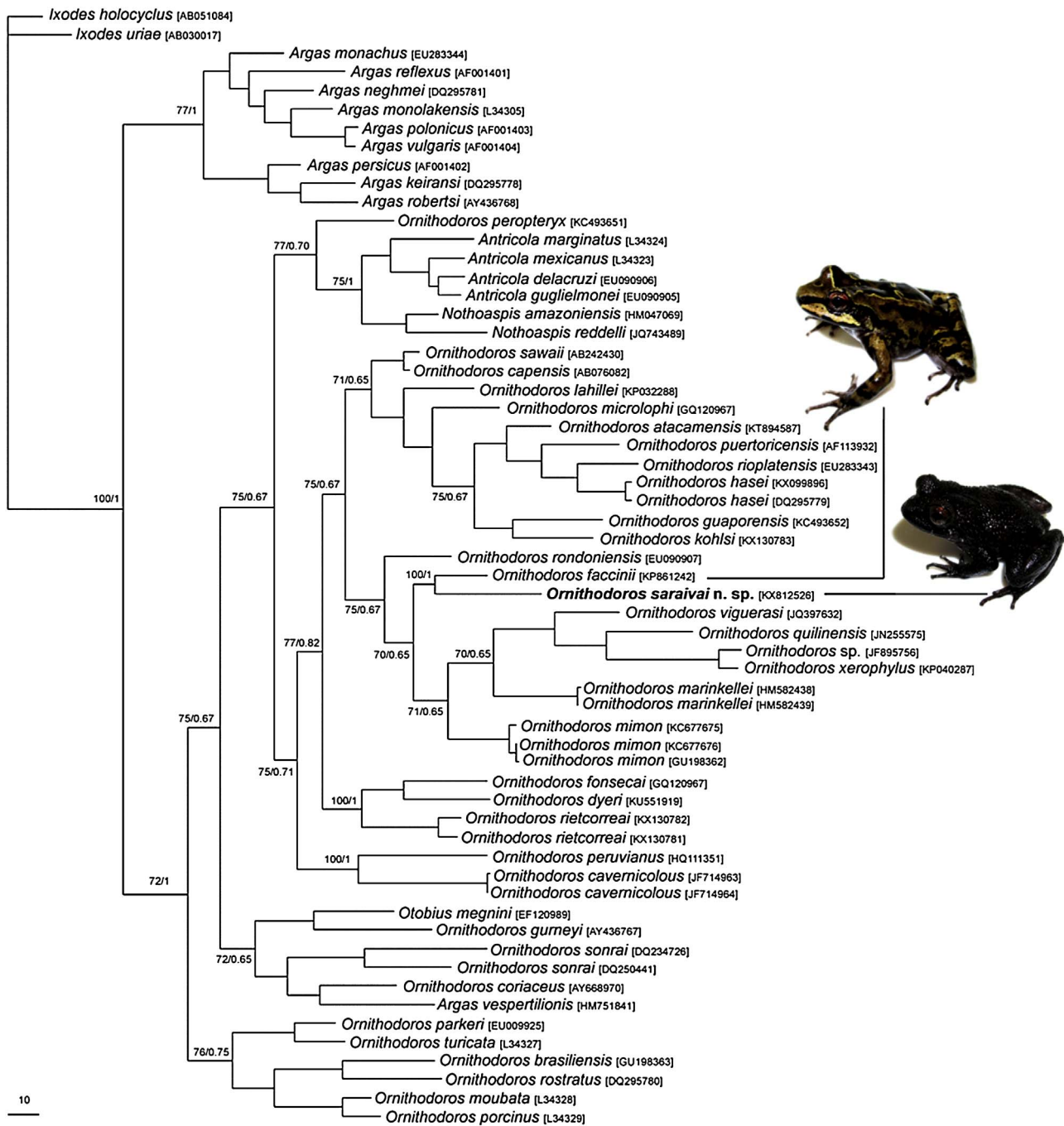


Fig. 7. Phylogenetic tree inferred from tick mitochondrial 16S rDNA partial sequences. The illustrated hosts are of the genus *Thoropa* (above) and *Cycloramphus* (below), both Cycloramphidae anurans.

rDNA sequences, and their consensus was deposited in GenBank database under de accession number KX812526. Pairwise comparisons using BLAST pointed *Ornithodoros sawaii* Kitaoka & Suzuki 1973 and *Ornithodoros capensis* Neumann 1901 as the most similar species with sequences 90–91% similar to *O. saraivai*. Under high bootsraap and posterior probability values, the phylogenetic analysis of the 16S rDNA fragments supports that *O. saraivai* shares a common ancestor with *Ornithodoros faccinii* Barros-Battesti et al. (2015) (Fig. 7). Additionally, both species formed a sister clade related to a major group composed by *Ornithodoros mimon* Kohls, Clifford & Jones 1969, *Ornithodoros marinkellei* Kohls, Clifford & Jones 1969, *Ornithodoros vigerasi* Cooley & Kohls 1941, *Ornithodoros quilinensis* Venzal et al. (2012), *Ornithodoros xerophylus* Venzal et al. (2015), and an undetermined *Ornithodoros* sp. from Bolivia.

5. Discussion

5.1. Species relationships

Larva. Although *O. saraivai* and *O. faccinii* larvae occur in ecologically related niches parasitizing amphibians of the Cycloramphidae family, the two species can be easily separated by larval morphology. Compared with *O. saraivai*, larvae of *O. faccinii* possess 6 rather than 7 pairs of anterolateral setae, 4 rather than 6 pairs of posterolateral setae, 3 instead of 7 sternal pairs, an elliptical instead of a pyriform dorsal plate, and the hypostome with denticles arising from its base (Barros-Battesti et al., 2015). Notably, the posteromedian seta is not a stable character in both species, since it was absent in several specimens used in their respective descriptions. For instance, the presence of 7 sternal

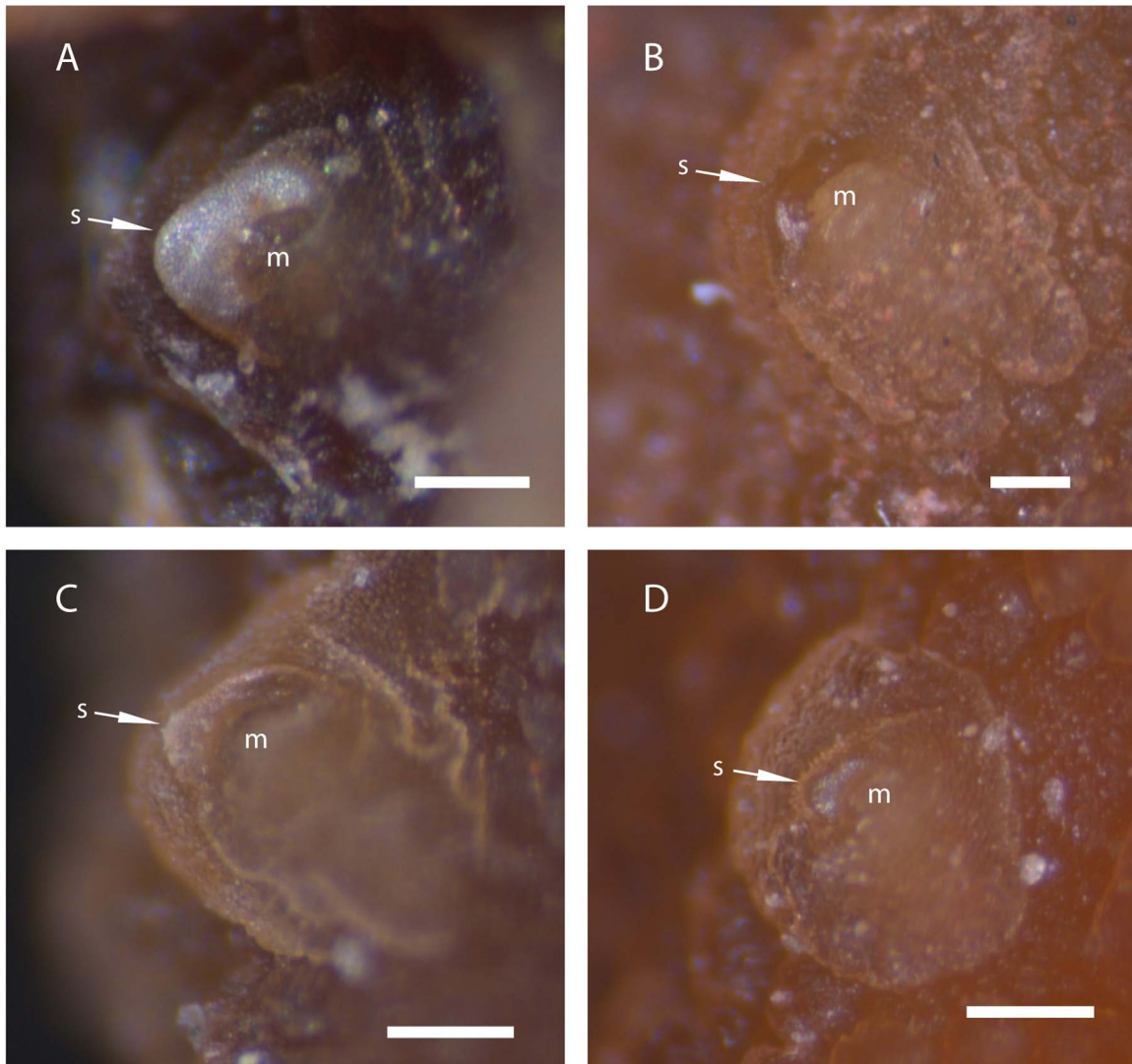


Fig. 8. Right spiracle of *Ornithodoros* spp.: (A) *Ornithodoros saraivai* n. sp., (B) *Ornithodoros rioplatensis*, (C) *Ornithodoros puertoricensis*, and (D) *Ornithodoros guaporensis*. Scale bar of all images is equivalent to 0.1 mm. Abbreviations: s, spiracular plate; m, macula.

setae in *O. saraivai* is a unique feature for a larval stage of *Ornithodoros* genus, only comparable with *O. microlophi*, a reptile-associated tick described from northern shores of Chile, which possesses 6 sternal setae (Venzal et al., 2013). Apart of this difference in sternal setae, *O. microlophi* has a total of 19–21 pairs of dorsal setae, contrasting to 16 pairs in *O. saraivai*.

Larval stages of *O. saraivai* should also be compared with ticks of the *O. talaje* species group that have been described parasitizing amphibians in America, particularly with *O. puertoricensis*, which correspond to the sole species-level diagnosed soft tick of this group associated with this class of vertebrates (Bermúdez et al., 2013). Larvae of *O. saraivai* can be easily differentiated from *O. puertoricensis* and the other four representatives of the *O. talaje* group (Venzal et al., 2008; Nava et al., 2013; Muñoz-Leal et al., 2016) by the presence of 7 sternal setae, and a partially toothed hypostome.

Nymph. Few Neotropical *Ornithodoros* have descriptions of nymphal stages, and when available, the majority of these descriptions refer to an unspecified nymphal instar (Landulfo et al., 2013), a fact that precludes morphological comparisons between species. Following Clifford et al. (1964) definition of the subgenus *Alectorobius* Pocock, 1907, the sole nymph collected in the current study fits morphological features of an *Ornithodoros* (*Alectorobius*) species: developed cheeks, and a configuration of ventral and dorsal mammillae similar to adult

stages. Additionally to these characters, we consider that integumental ridge-like extensions in the medial margin of article I are also indicative of either a late or, at least, not the first *Alectorobius* nymphal instar.

When comparing with other species, the sole nymph of *O. saraivai* resembles known nymphal stages of *Ornithodoros talaje* sensu lato (s. l.). However, it differs by possessing two long medial setae projecting towards the hypostome, and one short ventral seta in article I.

Adult stages. Females and males of *O. saraivai* are morphologically closely related to *O. guaporensis*, *O. puertoricensis*, *O. rioplatensis* and *Ornithodoros talaje* sensu stricto (s. s.), all of them part of the *O. talaje* species group. Differences between these species and *O. saraivai* are difficult to establish except by some notable features. Females and males of *O. saraivai* have a robust spiracle provided with a large spiracular plate, different in shape and size if compared to *O. guaporensis*, *O. puertoricensis*, and *O. rioplatensis* (Fig. 8). Moreover, males of *O. saraivai* have an entirely micromammillated genital flap, a character that separates this species from *O. puertoricensis* males, in which micromammillae cover only the proximal half of the genital flap (Endris et al., 1989), and also from *O. rioplatensis* males, which lack micromammillae on the flap surface. On the other hand, as in *O. saraivai*, genital flap of *O. guaporensis* male is entirely micromammillated. In this case, main difference relies in chaetotaxy of article I, which is composed by at least five setae in *O. saraivai* and only one in *O.*

guaporensis. Although direct comparisons with *O. talaje* s. s. were not possible to perform, external morphology of *O. saraivai* differs by presenting marked and conspicuous dorsal mammillae, which in turn are less pronounced in *O. talaje* s. s., and by an anteriorly pointed idiosoma, which is rather rounded in the latter species (Cooley and Kohls, 1944; Neumann, 1911). Despite of these particular differences, precise discrimination of the Neotropical Ornithodorinae based solely on the morphology of the adult stage remains a difficult task, as previously appointed (Kohls et al., 1965; Jones and Clifford, 1972). In addition, adults of many species lack a proper or detailed description, or in some cases the adult stages remain unknown, as for example, for *O. faccinii*.

The chaetotaxy of palpal article I, and the presence and distribution of micromammillae in the male genital flap seem to constitute valuable postlarval discrete characters for species differentiation. Thus, we propose that these characters should be considered in future descriptions of *Ornithodoros* postlarval stages.

Subgeneric classification. The phenotype of immature and adult stages of *O. saraivai* fit the morphological characters that define the *Alectorobius* subgenus sensu Clifford et al. (1964) with some exceptions. In *O. saraivai*, tarsi I of adults and nymph are provided of pulvilli and in larval stages, ventral setae exceed the number for an *Ornithodoros* (*Alectorobius*), since they present 11 instead of 8–9 pairs. Biological features that also characterize soft ticks of this subgenus, such as several days of larval feeding followed by two successive molts to the second nymphal instar without feeding, were not determined for *O. saraivai*. With this, while partial evidence obtained in the current study supports that *O. saraivai* should be classified as an *Alectorobius* representative, this hypothesis remains to be confirmed by studying biological traits of larval and first nymphal stages.

5.2. Phylogenetic analysis

Ornithodoros saraivai and *O. faccinii* form a monophyletic group together with a larger clade consisting in *O. marinkellei*, *O. mimon*, *O. quilinensis*, *O. vignerasi*, *O. xerophylus*, and an *Ornithodoros* of unknown identity from Bolivia. Although these taxa share a common ancestor, morphological traits of known developmental stages and host preferences differ between them. Compared to *O. saraivai*, morphological traits of larval stages in all the above-mentioned species include three rather than seven sternal pairs, and all of them are associated to volant or non-volant small-mammals. Adult stages of *O. marinkellei* and *O. vignerasi* are readily separated of *O. saraivai* by the presence of idiosomal sclerotized plaques (Labruna et al., 2011; Nava et al., 2012). On the other hand, a larger spiracular plate, a subrectangular rather than oval body outline, and the presence of more than one seta in article I of males, constitute main characters differentiating adults of *O. saraivai* from *O. mimon*. Adult stages of *O. quilinensis* and *O. xerophylus* are still unknown. Only nymphs of *O. mimon* and *O. marinkellei* have been described (Labruna et al., 2011; Landulfo et al., 2013). While nymphal stages of these species share a micro-mammillated idiosoma with the nymph of *O. saraivai*, they differ by having a rounded rather than subrectangular idiosoma.

Although nymphal and adult stages of *O. saraivai* are morphologically similar to ticks of the *O. talaje* group, phylogenetic analysis inferred from a partial fragment of the mitochondrial 16S rRNA gene showed that this new species is not related to *O. talaje* s. l., and rather shares a common ancestor with *O. faccinii*. From an ecological point of view, this phylogenetic relationship is relevant since both *O. saraivai* and *O. faccinii* occur in association with amphibians of the Cycloramphidae family. Theories on the origin of ticks point amphibians of the extinct Labyrinthodontia sub-class as the first hosts of ancestral tick lineages (Oliver, 1989; Dobson and Barker, 1999). From this evolutionary perspective, *O. faccinii* and *O. saraivai* become an interesting group, since all their known stages were found in association with Atlantic rainforest amphibians, contrasting to the xeric habitats

that most *Ornithodoros* species are usually found associated with (Hoogstraal, 1985). However, future phylogenetic reconstructions including a major set of genes must be performed in order to confirm this alleged natural group.

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