

## A new species of *Amblyomma* (Acari: Ixodidae) associated with monkeys and passerines of the Atlantic rainforest biome, Southeastern Brazil

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### ARTICLE INFO

#### Keywords:

*Amblyomma romarioi* n. sp.  
Atlantic rainforest  
*Callicebus nigrifrons*  
Description  
Wild birds

### ABSTRACT

Recent studies have reported several larvae of an unidentified *Amblyomma* species on passerine birds in Atlantic rainforest fragments in southeastern Brazil. These larvae yielded a unique 16S rRNA haplotype designated as *Amblyomma* sp. haplotype Nazaré, which showed nucleotide identity levels of 91% to *Amblyomma parkeri* Fonseca & Aragão, 1952 and 88% to *Amblyomma longirostre* (Koch, 1844). Herein, we describe *Amblyomma* sp. haplotype Nazaré as a new species, *Amblyomma romarioi* n. sp. Martins, Luz & Labruna, through a formal description of the male and female adult stages. *Amblyomma romarioi* is morphologically and genetically most closely related to *A. parkeri*, *A. longirostre* and *Amblyomma geayi* Neumann, 1899. Among males, the rectangular basis capituli and rounded coxa I spurs separates *A. romarioi* from *A. parkeri*, *A. longirostre*, and *A. geayi*, which have basis capituli triangular or slightly hexagonal, and pointed coxa I spurs. Among females, the V-shaped genital aperture and coxa I rounded spurs of *A. romarioi* contrasts to the U-shaped genital aperture and coxa I pointed spurs in *A. parkeri*, *A. longirostre*, and *A. geayi*. Larvae of *A. romarioi* have been collected on 24 species of passerines. The few records of nymphs and adults were on the black-fronted titi monkey *Callicebus nigrifrons* (Spix, 1823). The current distribution of *A. romarioi* is restricted to the Brazilian Atlantic rainforest, southeastern Brazil, in areas with altitude between 363 and 1600 m, within the distribution of *C. nigrifrons*. We discuss ecological features of *Amblyomma romarioi*, comparatively to *A. parkeri*, *A. longirostre* and *A. geayi*. The present study increases the Brazilian tick fauna to 74 species.

### 1. Introduction

Ticks of the genus *Amblyomma* are currently represented by 137 valid species, ≈20% of the Ixodidae family. The great majority of *Amblyomma* species are established in the lands of Gondwanian origin, with the Neotropical region bearing almost half of all species (Nava et al., 2017; Chitimia-Dobler et al., 2017; Apanaskevich and

Apanaskevich, 2018; Barker and Burger, 2018; Guglielmono et al., 2019). During the last two decades, at least 10 *Amblyomma* species have been described or resurrected in South America (Labruna et al., 2005; Barros-Battesti et al., 2007; Nava et al., 2009, 2014a,b; Krawczak et al., 2015).

The tick fauna of Brazil is currently composed by 73 species, 47 Ixodidae and 26 Argasidae (Gianizella et al., 2018; Muñoz-Leal et al.,

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<https://doi.org/10.1016/j.ttbdis.2019.07.003>

Received 2 March 2019; Received in revised form 21 June 2019; Accepted 5 July 2019

Available online 12 July 2019

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2018). The genus *Amblyomma* is the most numerous, with 32 species (Krawczak et al., 2015), comprising 44% of the Brazilian tick fauna. Adult males and females of all these 32 species, as well as the nymphal stage of 31 species have been morphologically described, a situation that has allowed the publication of taxonomic keys for morphological identification of nymphs and adults of the genus *Amblyomma* in Brazil (Onofrio et al., 2006; Martins et al., 2010, 2013; 2015; 2016; Krawczak et al., 2015). In contrast, larvae of most of these species remain undescribed, precluding a precise identification through morphological analysis for most species. For this reason, generation of partial DNA sequences of mitochondrial genes (e.g., 16S rRNA, 12S rRNA) has been widely employed for species level identification of field collected *Amblyomma* larvae (Luz et al., 2017; Zeringóta et al., 2017).

Few years ago, Ogrzewalska et al. (2012) reported several larvae of an unidentified *Amblyomma* species from passerine birds in an Atlantic rainforest fragment in the Municipality of Nazaré Paulista, São Paulo state, southeastern Brazil. These larvae yielded a unique 16S rRNA haplotype that was designated as *Amblyomma* sp. haplotype Nazaré, which showed nucleotide identity levels of 91% to *Amblyomma parkeri* Fonseca & Aragão, 1952 and 88% to *Amblyomma longirostre* (Koch, 1844), two closely related species from which the larval stage is also known to parasitize passerine birds (Ogrzewalska et al., 2012). More recently, two studies reported through molecular analyses (16S rRNA sequences) the presence of *Amblyomma* sp. haplotype Nazaré larvae parasitizing passerine birds in different Atlantic rainforest areas within southeastern Brazil (Luz et al., 2017; Zeringóta et al., 2017). Herein, we describe *Amblyomma* sp. haplotype Nazaré as a new species of the genus *Amblyomma*, through a formal description of the male and female adult stages.

## 2. Material and methods

### 2.1. Tick specimens

In 24 July 2013, three adult males of the genus *Amblyomma* were collected on a black-fronted titi monkey [*Callicebus nigrifrons* (Spix, 1823)] from Nazaré Paulista Municipality, state of São Paulo, southeastern Brazil. This monkey was rescued by the Tietê Ecological Park Wildlife Screening Center of the City of São Paulo. On the 4<sup>th</sup> of June 2018, two *Amblyomma* engorged females were collected on a specimen of *C. nigrifrons* from the Municipality of Atibaia, in the state of São Paulo. In this case, the Wildlife Screening Center of the Department of Parks and Green Areas of the City of São Paulo rescued the monkey. The three males demonstrated the same morphotype, as was the case for the two females, with all specimens shown to be morphologically similar, albeit distinct, to adults of *A. parkeri* and *A. longirostre*. This observation raised the possibility that they could be the adult stage of *Amblyomma* sp. haplotype Nazaré. To evaluate this, DNA was extracted from a distal portion of the idiosoma of one of the males, and from the legs of one of the females. DNA was extracted by the guanidine isothiocyanate-phenol technique (Sangioni et al., 2005) and tested by a PCR protocol that amplifies a  $\approx$  460-bp fragment of the tick mitochondrial 16S rRNA gene (Mangold et al., 1998). PCR products were sequenced and submitted to BLAST analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for comparisons with closest identities. Special attention was paid to the identities with sequences of *Amblyomma* sp. haplotype Nazaré of larval origin, which have been deposited in GenBank (JN800432, KU953954) in previous studies (Ogrzewalska et al., 2012; Zeringóta et al., 2017).

In May 2017, a two-day field trip was undertaken to capture passerine birds by using mist nets in the Itatiaia National Park “Parque Nacional do Itatiaia”, located in the Municipality of Itatiaia, state of Rio de Janeiro, southeastern Brazil, at exactly the same site where Luz et al. (2017) had collected a large number of *Amblyomma* sp. haplotype Nazaré larvae from passerine birds during 2014–2015. Engorged ticks (larvae and nymphs) were collected from birds, and taken to the laboratory, where they were held in an incubator (23 °C, 100% relative

humidity) for molting. All emerged nymphs were allowed to feed inside a cotton sleeve glued to the shaved dorsum of a guinea pig, as described previously (Soares et al., 2012). After detachment, engorged nymphs were held in the same incubator for molting to adults. Emerged unfed adults were killed by immersion in hot water 25 days after molting, preserved in 70% ethanol, and compared morphologically to the adults collected from the afore mentioned monkeys.

In April 2018, a three-day field trip was made to capture passerine birds using mist nets in the State Park “Parque Estadual de Itipococa”, located in Lima Duarte Municipality, state of Minas Gerais, south-eastern Brazil. Engorged ticks (larvae and nymphs) were collected from birds, and taken to the laboratory, where they were reared to the unfed adult stage, as described above. Emerged adults were killed by immersion in hot water, preserved in 70% ethanol, and morphologically compared to the adults that were collected from the afore mentioned monkeys.

On the 19<sup>th</sup> of November 2014, eight engorged nymphs of an unidentified species of *Amblyomma* were collected from the ears of a specimen of *C. nigrifrons* adult female from Barbacena Municipality, state of Minas Gerais; this monkey was rescued by the Wildlife Screening Center of Juiz de Fora Municipality. Six of these nymphs were preserved in 70% ethanol and sent to the laboratory, where they were macerated for DNA extraction and examined by PCR targeting the 16S rRNA gene, as recently reported (Peckle et al., 2019).

### 2.2. Morphological analyses of ticks

Male ticks collected from monkeys, and conspecific adult ticks (males, females) reared from bird-collected larvae were measured using the Image-Pro Plus 5.1 program for analysis of images and morphometry, fitted to an Olympus SZX stereoscope microscope (Olympus Corporation, Tokyo, Japan). In the description that follows, all measurements are provided in mm; a range is given, with a mean  $\pm$  standard deviation in parentheses. Two specimens of each sex (male and female) were prepared for scanning electron microscopy (SEM) following the techniques described by Corwin et al. (1979). Light-microscopy photographs of adult ticks were prepared using a SteREO Discovery V12 stereomicroscope (Carl Zeiss, Munich, Germany) in order to depict the natural scutal ornamentation pattern.

For morphological comparisons, specimens of *A. parkeri*, *A. longirostre*, and *Amblyomma geayi* Neumann, 1899, available at the tick collections “Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva” (CNC) at the Faculty of Veterinary Medicine of the University of São Paulo, São Paulo, SP, Brazil, and “Coleção Acarológica do Instituto Butantan” (IBSP) at the Butantan Institute, São Paulo, SP, Brazil, were examined. In addition, we also examined type specimens of *A. parkeri* (IBSP 609, 4458) and all specimens of *A. parkeri*, *A. longirostre*, and *A. geayi* that were used for the description/redescriptions of these species by Labruna et al. (2009). Finally, all lots of ticks collected on monkeys, deposited in the above tick collections, were revised in order to find the possible new species.

### 2.3. Phylogenetic analyses

The consensus sequence of the new tick species was aligned using Clustal/W v.1.8.1 (Thompson et al., 1994) with 16S rDNA sequences deposited in GenBank as corresponding to 40 different sequences of the genus *Amblyomma*, including the sequences that matched closest BLAST identities to the present new species, namely *A. parkeri*, *A. longirostre*, and *A. geayi*. Alignment was manually adjusted using GeneDoc (Nicholas et al., 1997). The sequence derived from *Amblyomma varanensis* Supino, 1897, was used as the outgroup (accessions numbers of all sequences are provided in the phylogenetic tree). Maximum Parsimony was implemented in PAUP version 4.0b10 (Swofford, 2002) with 500 bootstrap replicates, random stepwise addition starting trees (with random addition sequences) and TBR branch swapping. 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 every 100 generations. The first 25% of the trees represented burn-in, and the remaining trees were used to calculate Bayesian posterior probability.

#### 2.4. Ethical statements

This work was approved by the Ethical Committee in Animal Research of the Faculty of Veterinary Medicine of the University of São Paulo (CEUA n° 7,241,050,318). Field collections of animals and ticks were authorized by the “Instituto Chico Mendes de Conservação da Biodiversidade” (authorization SISBIO nos. 11459-1 and 60873-1), and by the “Instituto Estadual de Florestas” of the state of Minas Gerais (authorization IEF no. 017/2018).

### 3. Results

#### 3.1. Identification of ticks

DNA extracted from one male, one female, and six nymphs collected from *C. nigrifrons* yielded 16S rRNA haplotypes (411 bp) identical to each other, and 100% identical to the sequences of *Amblyomma* sp. haplotype Nazaré of larval origin (JN800432, KU953954) from previous studies (Ogrzewalska et al., 2012; Zeringóta et al., 2017). By BLAST analysis, this sequence was 91–92% identical to *A. parkeri* (JN573300, JN800431), and 88–89% identical to *A. longirostre* (MH513275, KP762568) and *A. geayi* (KM042851, MH513263).

Among the engorged larvae and nymphs collected from passerines in the Parks “Parque Nacional do Itatiaia” and “Parque Estadual de Ibitipoca”, six larvae from the former and one larva from the latter location yielded adults (3 males, 4 females) that were identified morphologically as the same species of ticks collected from the monkeys. The additional engorged larvae and all engorged nymphs of “Parque Nacional do Itatiaia” gave rise to other tick species (*A. parkeri*, *A. longirostre*, *Amblyomma ovale* Koch, 1844), corroborating recent findings of ticks on birds in the same area (Luz et al. (2017)). The additional ticks of “Parque Estadual de Ibitipoca” will be presented in another manuscript.

Detailed analyses of the external morphology of the male and female ticks collected in the present study revealed that they represent *Amblyomma* morphotypes that are morphologically distinct from any other known *Amblyomma* species, justifying their description as a new species.

#### 3.2. Description

Ixodida Leach, 1815

Ixodidae Koch, 1844

*Amblyomma* Koch, 1844

*Amblyomma romarioi* n. sp. Martins, Luz & Labruna (Figs. 1–3)

**Male** (Figs. 1A and 2). Six specimens measured.

**Idiosoma.** Length from apices of scapulae to posterior body margin 3.67–4.40 ( $4.03 \pm 0.26$ ), maximum breadth 2.67–3.18 ( $2.92 \pm 0.18$ ). Outline oval, broadest at the level of spiracular plates, with 11 festoons without ventral plates (Fig. 1A). Genital aperture broadly U-shaped (Fig. 2D). Spiracular plate comma-shaped with elongate macula, numerous minute globlets. Ventral plaques large, the medium and lateral plaques elongate, the intermediate plaque short and rounded. Scutum ornate with pale yellow/orange to green markings on the lateral, median and posterior fields, over a brown background; numerous large and deep punctations uniformly distributed, except for five small

depressions in which punctations are shallow or absent; these depressions consist of a pair at the medium-lateral area, a pair at the postero-lateral area, and a single depression (the deepest one) at the postero-medium area (Figs. 1A and 2A). Cervical grooves short, slightly deep, comma shaped. Eyes flat and elongate. Marginal groove complete, deep, starting at the level of coxa III (Fig. 2A and B). Festoons ornate, with punctations (Fig. 1A and 2B).

**Gnathosoma.** Basis capituli and palpi inornate (Fig. 1A). Palpi short and wide (robust). Length of palpal apices to posterior margin 0.65–0.78 ( $0.71 \pm 0.06$ ), breadth 0.52–0.65 ( $0.56 \pm 0.05$ ), length 0.21–0.30 ( $0.26 \pm 0.04$ ). Basis capituli rectangular, posterior margin concave, giving the impression of having small rounded cornua; numerous small punctations dorsally (Fig. 2E). Palp length 0.43–0.55 ( $0.49 \pm 0.05$ ); length of palpal article I 0.03–0.06 ( $0.05 \pm 0.01$ ); length of palpal article II 0.26–0.30 ( $0.28 \pm 0.01$ ); length of palpal article III 0.07–0.18 ( $0.13 \pm 0.04$ ), suture between II and III distinct. Hypostome (Fig. 2F) moderately elongate, broadly rounded apically (spatulate) with corona of fine denticles. Hypostome slightly shorter than palps, with anterior end at the level of the central portion of palp article IV. Total length of hypostome 0.37–0.39 ( $0.38 \pm 0.01$ ); length of toothed portion 0.18–0.21 ( $0.19 \pm 0.01$ ). Hypostomal dentition 3/3 with 5–6 teeth per row.

**Legs.** Coxa I with 2 unequal rounded spurs, the internal small and the external two times longer than the internal; a single small triangular spur on each of coxae II–IV, slightly longer in the coxa IV. Trochanters without spurs (Fig. 2D). Tarsus I 0.70–0.83 ( $0.76 \pm 0.06$ ) long, 0.24–0.25 ( $0.25 \pm 0.01$ ) broad. Tarsus IV 0.63–0.77 ( $0.71 \pm 0.05$ ) long; 0.18–0.23 ( $0.21 \pm 0.01$ ) broad.

Female (Figs. 1B and 3). Four unfed specimens measured.

**Idiosoma.** Length from apices of scapulae to posterior body margin 4.43–4.74 ( $4.56 \pm 0.16$ ), maximum breadth 3.35–3.47 ( $3.40 \pm 0.06$ ). Outline oval, broadest at the level of spiracular plate, with 11 festoons without tubercles (Figs. 1B and 3A). Genital aperture broadly V-shaped (Fig. 3D). Spiracular plate large, subtriangular with rounded angles, elongate macula surrounded by numerous minute globlets. Scutum length 1.82–1.91 ( $1.88 \pm 0.04$ ), breadth 2.01–2.16 ( $2.09 \pm 0.07$ ). Notably, in molted specimens (unfed), the scutum length is nearly one third of idiosoma length. Scutal ornamentation consisting of pale-yellowish stripes extensively distributed over the light brown background of the scutum (Fig. 1B); a notable dark brown marking on the medial margin of the eyes; numerous large and deep punctations uniformly distributed; cervical grooves deep, converging anteriorly then diverging at the level of eyes as shallow depressions that almost reach the scutal posterior third. Eyes flat, large and elongate, located between the first and second halves of the scutum (Fig. 3B).

**Gnathosoma.** Basis capituli light-coloured, palpi inornate (Fig. 1B), subtriangular, without cornua, posterior margin straight (Fig. 3E); length of palpal apices to posterior margin 0.91–1.05 ( $0.98 \pm 0.07$ ), breadth 0.78–0.81 ( $0.80 \pm 0.01$ ), length 0.33–0.40 ( $0.36 \pm 0.03$ ). Porose areas large, deeply depressed, diameter of one area 0.14–0.23 ( $0.18 \pm 0.04$ ), interporose area 0.09–0.13 ( $0.11 \pm 0.02$ ). Palp length 0.64–0.73 ( $0.69 \pm 0.04$ ); length of palpal article I 0.06–0.07 ( $0.07 \pm 0.01$ ); length of palpal article II 0.40–0.46 ( $0.43 \pm 0.02$ ); length of palpal article III 0.17–0.19 ( $0.18 \pm 0.01$ ), suture between II and III distinct. Hypostome (Fig. 3F) elongate, apically rounded (spatulate) with corona of fine denticles. Total length of hypostome 0.60–0.67 ( $0.64 \pm 0.03$ ); length of toothed portion 0.32–0.42 ( $0.36 \pm 0.05$ ). Hypostomal dentition 3/3 with 6–8 teeth per row.

**Legs.** Coxa I with 2 rounded unequal spurs projecting slightly laterally, the internal small and the external twice longer than the internal; a single small triangular spur on each of coxae II–IV. Trochanters without spurs (Fig. 3C and D). Tarsus I 0.99–1.04 ( $1.02 \pm 0.02$ ) long, 0.21–0.28 ( $0.24 \pm 0.03$ ) broad. Tarsus IV 0.77–0.91 ( $0.83 \pm 0.06$ )

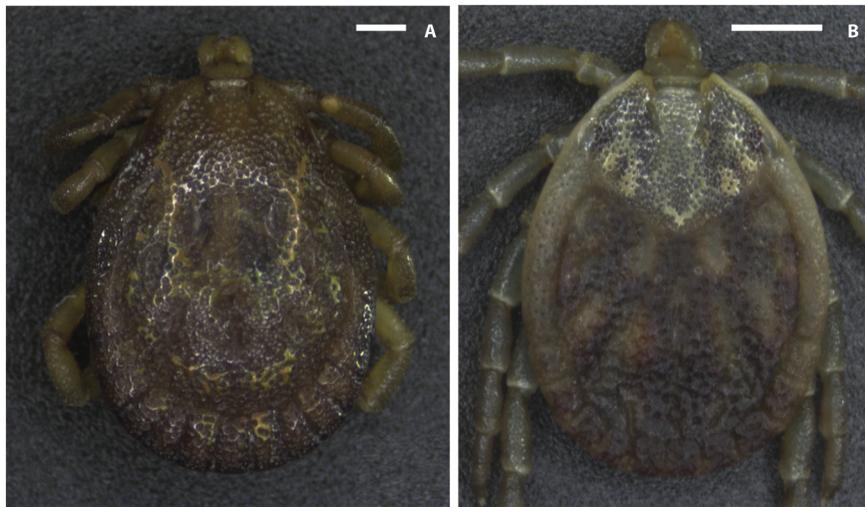


Fig. 1. Dorsal view of the adult stage of *Amblyomma romarioi* by light-microscopy. (A) Male. (B) Female.

long, 0.20–0.23 (0.22 ± 0.01) broad.

### 3.2.1. Types

HOLOTYPE male, ALLOTYPE female, both unfed adults that were collected as engorged larvae ex *Trichothraupis melanops* (Passeriformes: Thraupidae) from the “Parque Nacional do Itatiaia” (22°27′11″S; 44°36′43″W; altitude 800 m), Itatiaia Municipality, state of Rio de Janeiro, Brazil, 26 May 2017; collectors: T.F. Martins, H.R. Luz, S. Muñoz-Leal, I.C.L. Acosta, G.P. Furusawa, J.L.H. Faccini, M.B. Labruna. These engorged larvae were brought live to the laboratory where they molted to nymphs, which were allowed to feed on guinea pigs; the resultant engorged nymphs molted to the above adult ticks. Holotype and Allotype were deposited in the United States National Tick Collection (Statesboro, Georgia) under accession numbers USNMMENT00981860, CEN/RML 129057, and USNMMENT00981861, CEN/RML 129057, respectively.

### 3.2.2. Paratypes

1 male, 1 female, same data as for holotype, deposited in the tick collection “Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva” (São Paulo, SP, Brazil) under accession number CNC-3888; 1 male, 1 female, same data as holotype, except that the engorged larvae were collected on *Turdus flavipes*; deposited in the “Coleção Acarológica do Instituto Butantan” (São Paulo, SP, Brazil) under accession number IBSP-14523; 3 males ex *Callicebus nigrifrons* (Primates: Pitheciidae) from Nazaré Paulista Municipality (23°10′51″S, 46°23′42″W, 845 m), state of São Paulo, Brazil, 24 July 2013, collector L. Milanelo (CNC-3889); 2 engorged females ex *C. nigrifrons* (Primates: Pitheciidae) from Atibaia Municipality (23°07′01″S, 46°33′00″W, 803 m), state of São Paulo, Brazil, 4 June 2018, collector T.C. Sanches (CNC-3890); 1 unfed female that were collected as engorged larva ex *Sittasomus griseicapillus* (Passeriformes: Dendrocolaptidae) from the “Parque Estadual de Ibitipoca” (21°42′47″S; 43°53′50″W; altitude 1350 m), Lima Duarte Municipality, state of Minas Gerais, Brazil, 11 April 2018, collectors D.G. Ramirez, H.R. Luz, I.C.L. Acosta, G.P. Furusawa, W. Flausino. This engorged larva was brought alive to the laboratory where it molted to a nymph, and after feeding on a guinea pig, to the above adult female (CNC-3891).

Half of the body of one paratype male (CNC-3889) and legs of one paratype female (CNC-3890) were processed for DNA extraction. Two paratype males (CNC-3888, 3889) and two paratype females (CNC-3888, IBSP-14523) were used for scanning electronic microscopy.

### 3.2.3. Etymology

The new species is named for Dr. Romário Cerqueira Leite, Professor at the School of Veterinary Medicine of the Federal University of Minas Gerais (UFMG), in recognition of his contribution to the study of Brazilian ticks, with emphasis on integrated control of ticks and tick-borne diseases.

### 3.3. Phylogenetic analyses

Since the male, female, and nymphs of *A. romarioi* yielded 16S rRNA sequences identical to each other, and 100% identical to all sequences of larval origin (formerly *Amblyomma* sp. haplotype Nazaré), we used a single sequence for phylogenetic analysis. In the phylogenetic tree inferred by partial sequences of the mitochondrial 16S rRNA gene (Fig. 4), *A. romarioi* grouped into a monophyletic branch with *A. parkeri* (96% of bootstrap and 1.0 of posteriori probability) within a major clade containing several haplotypes of *A. longirostre* and *A. geayi* (74% of bootstrap and 0.85 of posteriori probability). Congruent topologies were observed for both Maximum parsimony and Bayesian analyses. The partial sequence of the mitochondrial 16S rRNA gene generated in this study for *A. romarioi* has been deposited in GenBank under the accession number [MK440570](#).

### 3.4. Species relationship

*Amblyomma romarioi*, *A. parkeri*, *A. longirostre* and *A. geayi* form a natural group of genetically and morphologically related neotropical ticks that are unique in combining the following morphological characters: idiosoma ovals outlined, coxa I with 2 short spurs being the external significantly longer than the internal, coxae II–IV each with a single triangular short spur, hypostomal dentition 3/3, scutum with distinct ornamentation and obvious punctations, cornua absent or vestigial, trochanters without spurs, festoons without tubercles, and males with 5 ventral plates and marginal groove complete or incomplete. Among males, the rectangular basis capituli and rounded coxa I spurs separates *A. romarioi* from *A. parkeri*, *A. longirostre*, and *A. geayi*, which have basis capituli triangular or slightly hexagonal, and pointed coxa I spurs. Among females, the V-shaped genital aperture and coxa I rounded spurs of *A. romarioi* contrasts to the U-shaped genital aperture and coxa I pointed spurs in *A. parkeri*, *A. longirostre*, and *A. geayi*. In addition, unfed females of *A. romarioi* have the scutum length nearly one third of idiosoma length, in contrast to nearly one half of the

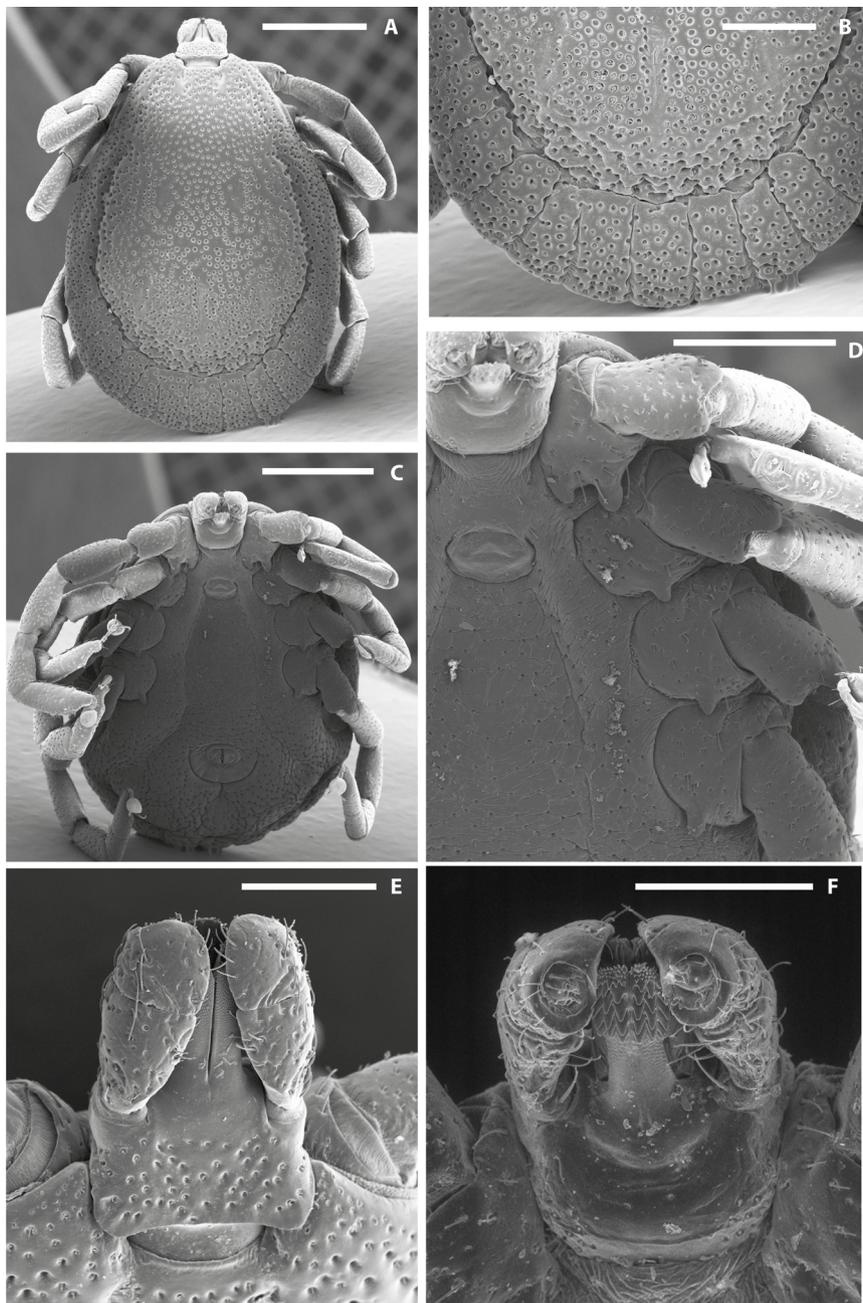


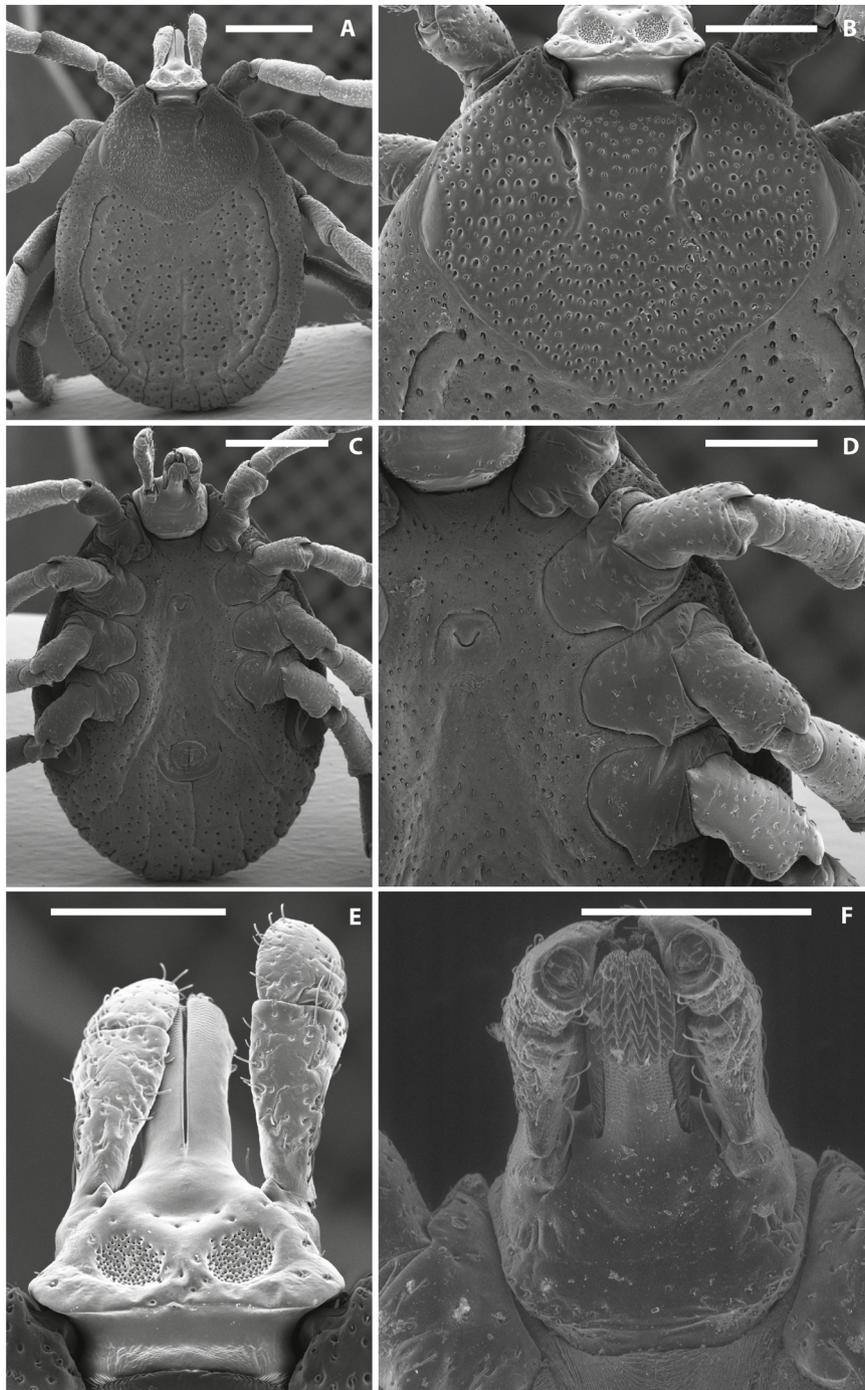
Fig. 2. Scanning electron microscopy of *Amblyomma romarioi* male. (A) Dorsal capitulum (Bar: 200  $\mu$ m). (B) Ventral capitulum (Bar: 200  $\mu$ m). (C) Scutum (Bar: 400  $\mu$ m). (D) Coxae I-IV (Bar: 400  $\mu$ m).

idiosoma length in unfed *A. parkeri*, *A. longirostre*, and *A. geayi*. Detailed redescriptions and illustrations of males and females of *A. parkeri*, *A. longirostre*, and *A. geayi* have been reported by Labruna et al. (2009), who provided main characters for morphological distinction of these three species.

### 3.5. Hosts and distribution

Engorged larvae of *A. romarioi* (which molted to nymphs, and then to adults) were collected from three species of passerines (*T. melanops*, *S. griseicapillus*, and *T. flavipes*). In previous studies (Ogrzewalska et al., 2012; Luz et al., 2017; Zeringóta et al., 2017), larvae of *A. romarioi*

(reported as *Amblyomma* sp. haplotype Nazaré) were collected on multiple species of passerines at different locations, as shown in Table 1. Overall, *A. romarioi* larvae have been collected on 24 species of passerines. These findings indicate that passerines possibly constitute the principal host group for the larval stage of *A. romarioi*. Records of the nymphal and adult stages on the monkey *C. nigrifrons* (Table 1) suggest that these two tick stages are primarily associated with this primate species. This supposition is supported by our findings of fully engorged nymphs and females on *C. nigrifrons*, and by the fact that all larval records of *A. romarioi* were from sites within the known distribution area of this species of monkey (Fig. 5). At present, the distribution of *A. romarioi* is restricted to the Brazilian Atlantic rainforest,



**Fig. 3.** Scanning electron microscopy of *Amblyomma romarioi* female. (A) Dorsal capitulum (Bar: 200 µm). (B) Ventral capitulum (Bar: 200 µm). (C) Dorsal idiosoma (Bar: 400 µm). (D) Coxae I-IV (Bar: 200 µm).

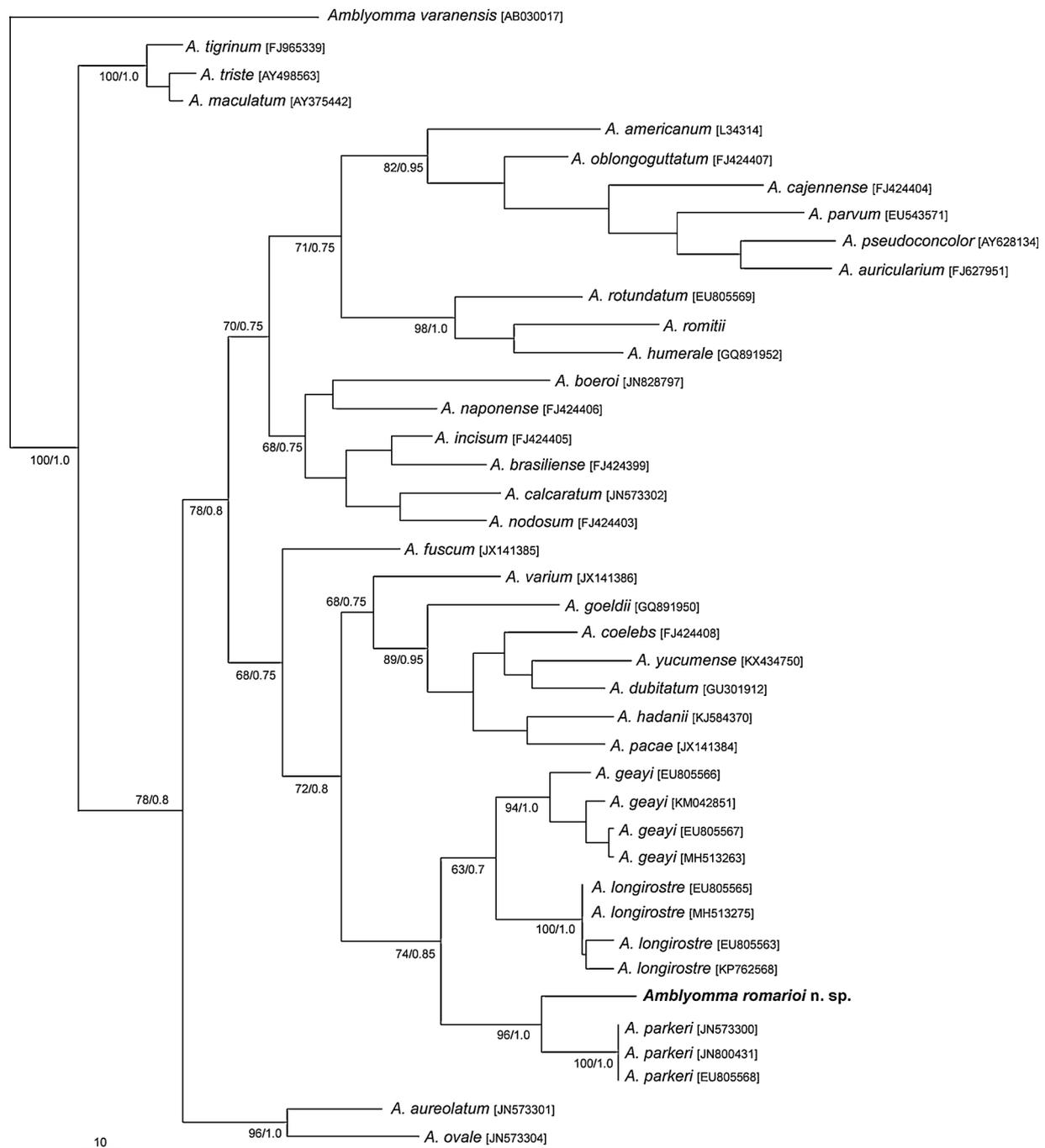
southeastern Brazil, in areas with altitude between 363 and 1600 m.

#### 4. Discussion

The natural group composed by *A. romarioi*, *A. parkeri*, *A. longirostre*, and *A. geayi* share several morphological and genetic characteristics together with many ecological features. In this context, the larval stage of these four species feed chiefly on passerine birds (Labruna et al., 2007; Ogrzewalska et al., 2010, 2012; Luz et al., 2017; Zeringóta et al.,

2017; Table 1), whereas adult ticks are primarily associated with arboreal mammals, namely porcupines (Rodentia: Erethizontidae) for *A. parkeri* and *A. longirostre*, sloths (Pilosa: Bradypodidae, Megalonychidae) for *A. geayi*, and the Black-fronted titi monkey (*C. nigrifrons*) for *A. romarioi* (Labruna et al., 2009; Gianizella et al., 2018; Table 1).

Regarding the nymphal stage, most of the host records of *A. longirostre* have been on passerine birds, (Labruna et al., 2007; Luz et al., 2012, 2017), although there are a few records on porcupines (Luz et al.,



**Fig. 4.** Molecular phylogenetic analysis of *Amblyomma romarioi* n. sp. and closely related Ixodidae. Fragments of  $\approx 400$  nucleotides of the tick mitochondrial 16S rRNA gene were subjected to analysis by maximum-parsimony (MP) and Bayesian (BA) methods. The corresponding sequence of *Amblyomma varanensis* was used as outgroup. Numbers at nodes are support values derived from bootstrap and posterior probability for MP and BA analyses (MP/BA). Bootstrap support values  $\geq 70/\geq 0.7$  for phylogenetic groupings are indicated at the nodes. Numbers in brackets are GenBank accession numbers.

2018). In contrast, most of the host records of the nymphal stage of *A. parkeri* and *A. geayi* have been on arboreal mammals, namely porcupines and monkeys of the genus *Alouatta* for the former (Martins et al., 2013), and sloths for the latter (Gianizella et al., 2018). These findings indicate that passerine birds are major hosts for the larvae of *A. longirostre*, *A. parkeri*, and *A. geayi*, but not for nymphs of the two latter species. The same seems to apply to *A. romarioi*, whose nymphs were never found on passerine birds in areas where its larval stage was found

on these vertebrates (Ogrzewalska et al., 2012; Luz et al., 2017; Zeringóta et al., 2017). Although limited to a single record of 6 nymphs, our observations suggest that *A. romarioi* nymphs would have preference for feeding on *C. nigrifrons*, the same host identified for the adult ticks.

Labruna et al. (2007) observed that most of the host records of larvae and nymphs of *A. longirostre* were from understory passerine birds that nest in trees and rarely visit the ground. Since porcupines

**Table 1**  
List of hosts and geographical records of *Amblyomma romarioi* sp. nov.

Record number	Tick stage <sup>a</sup>	Host species	Municipality (State) <sup>b</sup>	Altitude (m)	References
		PRIMATES			
1	M	<i>Callicebus nigrifrons</i>	Nazaré Paulista (SP)	845	Present study
2	F	<i>C. nigrifrons</i>	Atibaia (SP)	803	Present study
3	N	<i>C. nigrifrons</i>	Barbacena (MG)	1,160	Peckle et al. (2019)
		PASSERIFORMES			
4	L	<i>Attila rufus</i>	Juiz de Fora (MG)	715	Zeringóta et al. (2017)
5	L	<i>A. rufus</i>	Itatiaia (RJ)	800	Luz et al. (2017)
6	L	<i>A. rufus</i>	Teresópolis (RJ)	363	Luz et al. (2017)
7	L	<i>Automolus leucophthalmus</i>	Nazaré Paulista (SP)	845	Ogrzewalska et al. (2012)
8	L	<i>Basileuterus culicivorus</i>	Juiz de Fora (MG)	715	Zeringóta et al. (2017)
9	L	<i>Chiroxiphia caudata</i>	Resende (RJ)	1,052	Luz et al. (2017)
10	L	<i>C. caudata</i>	Itatiaia (RJ)	530	Luz et al. (2017)
11	L	<i>Conopophaga lineata</i>	Juiz de Fora (MG)	715	Zeringóta et al. (2017)
12	L	<i>C. lineata</i>	Itatiaia (RJ)	1600	Luz et al. (2017)
13	L	<i>Cyanoloxia brissonii</i>	Juiz de Fora (MG)	715	Zeringóta et al. (2017)
14	L	<i>Dendrocincla turdina</i>	Itatiaia (RJ)	1600	Luz et al. (2017)
15	L	<i>Habia rubica</i>	Nazaré Paulista (SP)	845	Ogrzewalska et al. (2012)
16	L	<i>Lepidocolaptes falcinellus</i>	Nazaré Paulista (SP)	845	Ogrzewalska et al. (2012)
17	L	<i>Leptopogon amaurocephalus</i>	Juiz de Fora (MG)	715	Zeringóta et al. (2017)
18	L	<i>Platyrinchus mystaceus</i>	Juiz de Fora (MG)	715	Zeringóta et al. (2017)
19	L	<i>P. mystaceus</i>	Teresópolis (RJ)	980	Luz et al. (2017)
20	L	<i>Pyriglena leucoptera</i>	Juiz de Fora (MG)	715	Zeringóta et al. (2017)
21	L	<i>Ramphocelus bresilius</i>	Teresópolis (RJ)	980	Luz et al. (2017)
22	L	<i>Schiffornis virescens</i>	Nazaré Paulista (SP)	845	Ogrzewalska et al. (2012)
23	L	<i>Sclerurus scansor</i>	Nazaré Paulista (SP)	845	Ogrzewalska et al. (2012)
24	L	<i>Sittasomus griseicapillus</i>	Juiz de Fora (MG)	715	Zeringóta et al. (2017)
25	L	<i>S. griseicapillus</i>	Lima Duarte (MG)	1,345	Present study
26	L	<i>Tachyphonus coronatus</i>	Juiz de Fora (MG)	715	Zeringóta et al. (2017)
27	L	<i>T. coronatus</i>	Teresópolis (RJ)	980	Luz et al. (2017)
28	L	<i>Trichothraupis melanops</i>	Juiz de Fora (MG)	715	Zeringóta et al. (2017)
29	L	<i>T. melanops</i>	Resende (RJ)	1,052	Luz et al. (2017)
30	L	<i>T. melanops</i>	Itatiaia (RJ)	1600	Present study
31	L	<i>T. melanops</i>	Teresópolis (RJ)	980	Luz et al. (2017)
32	L	<i>Turdus albicollis</i>	Juiz de Fora (MG)	715	Zeringóta et al. (2017)
33	L	<i>Turdus flavipes</i>	Itatiaia (RJ)	1600	Present study
34	L	<i>Turdus leucomelas</i>	Itatiaia (RJ)	800	Luz et al. (2017)
35	L	<i>Turdus rufiventris</i>	Juiz de Fora (MG)	715	Zeringóta et al. (2017)
36	L	<i>Xenops minutus</i>	Nazaré Paulista (SP)	845	Ogrzewalska et al. (2012)
37	L	<i>Xiphorhynchus fuscus</i>	Juiz de Fora (MG)	715	Zeringóta et al. (2017)
38	L	<i>X. fuscus</i>	Itatiaia (RJ)	1600	Luz et al. (2017)

<sup>a</sup> M: male; F: female; N: nymph; L: larva.

<sup>b</sup> SP: São Paulo; MG: Minas Gerais; RJ: Rio de Janeiro.

(major hosts for the adult stage of *A. longirostre*) are typically arboreal and rarely visit the forest floor (Emmons and Feer, 1997), it was suggested that *A. longirostre* is an arboreal tick species that uses the tree canopy for the free-living developmental stages. Herein, we classified the 24 known host species of the larvae of *A. romarioi* according to the habits of the birds and their nest locations (Table 2). It was observed that, with few exceptions, larvae of *A. romarioi* were collected from passerine birds with arboreal habits and that nest in trees. Considering that *Callicebus* monkeys (so far, the only known host for nymphs and adults of *A. romarioi*) are typically strictly arboreal with preference for the canopy layer (> 15 m high) of the forest (Santana et al., 2008), we propose that in common with *A. longirostre*, *A. romarioi* is an arboreal tick species as well.

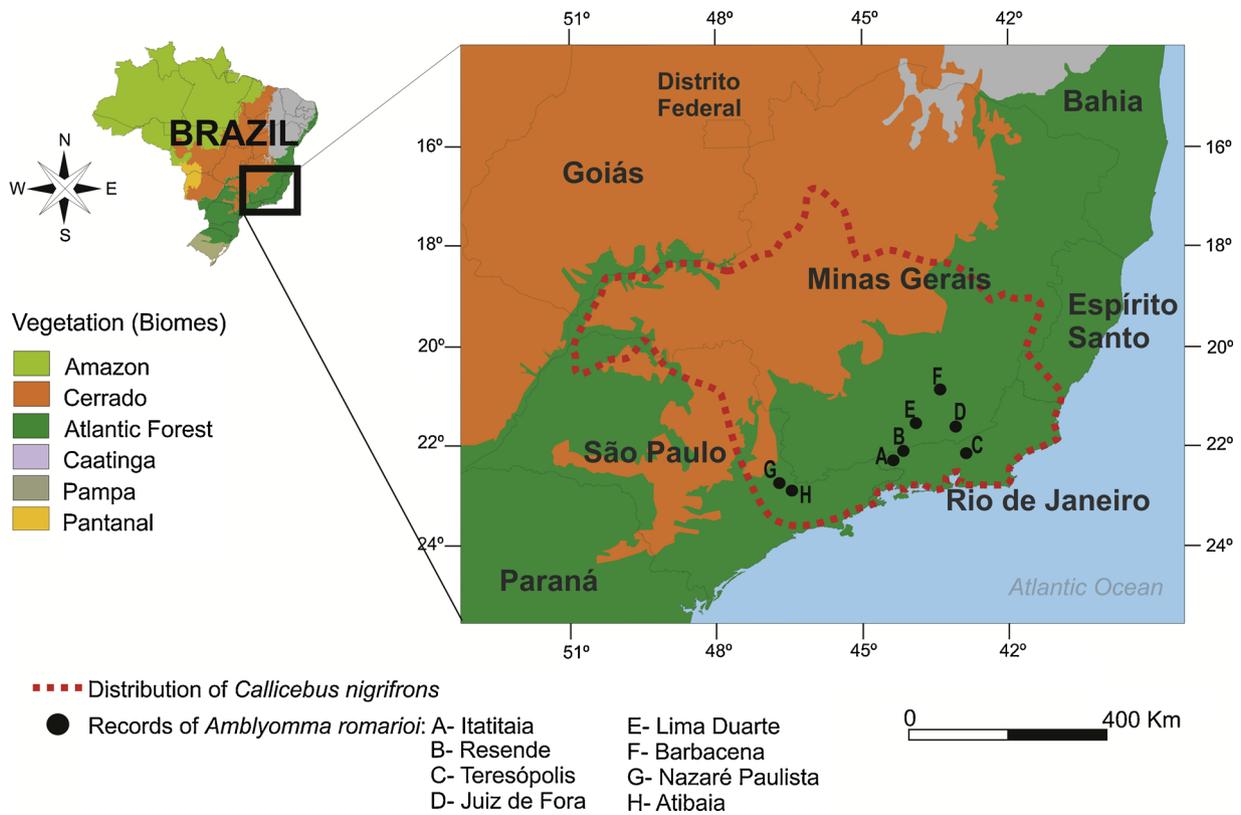
Currently, the distribution of *A. romarioi* is restricted to southeastern Brazil, including the states of Minas Gerais, Rio de Janeiro and São Paulo, in the Atlantic rainforest biome. Previous records of *A. romarioi* (reported as *Amblyomma* sp. haplotype Nazaré) indicate that it occurs sympatrically with *A. parkeri* and *A. longirostre* (Ogrzewalska et al., 2012; Luz et al., 2017; Zeringóta et al., 2017). Finally, it is pertinent to note that *A. romarioi* ticks are known to carry spotted fever group-rickettsial organisms of unknown pathogenicity, namely

"*Candidatus Rickettsia paranaensis*" (formerly *Rickettsia* sp. strain ApPR) (Ogrzewalska et al., 2012; Luz et al., 2017; Zeringóta et al., 2017; Peckle et al., 2019), and *Rickettsia rhipicephali* (Luz et al., 2017; Zeringóta et al., 2017). Indeed, passerine birds show potential for the dissemination of *Rickettsia*-infected *A. romarioi* ticks.

## 5. Dichotomic key for identification of adults of the *Amblyomma longirostre* natural group

### Males

1.	–Basis capituli rectangular, rounded coxa I spurs	<i>A. romarioi</i>
	–Basis capituli triangular or slightly hexagonal, pointed coxa I spurs	2
2.	–Scutum with large punctations restricted to lateral fields, small punctations evenly distributed	<i>A. longirostre</i>
	–Scutum with large punctations evenly distributed	3
3.	–Internal spur on coxa I with prominent rounded medial projection; scutal length > 5 mm	<i>A. geayi</i>
	–Internal spur on coxa I lacks prominent rounded medial projection; scutal length < 5 mm	<i>A. parkeri</i>



**Fig. 5.** Geographical records of *Amblyomma romarioi* in Brazil, in the states of Rio de Janeiro (sites A, B), Minas Gerais (sites C, D), and São Paulo (sites E, F), all within the distribution area of the black-fronted titi monkey *Callicebus nigrifrons*.

**Table 2**

Habits (arboreal, mostly arboreal, terrestrial, or mostly terrestrial) and nest location (tree or ground) of the 24 passerine bird species that were found infested by *Amblyomma romarioi* sp. nov.

Passerine species	Bird habits <sup>a</sup>	Nest location <sup>a</sup>
1 <i>Attila rufus</i>	Arboreal	Tree
2 <i>Automolus leucophthalmus</i>	Arboreal	Ground
3 <i>Basileuterus culicivorus</i>	Arboreal	Tree
4 <i>Chiroxiphia caudata</i>	Arboreal	Tree
5 <i>Conopophaga lineata</i>	Mostly terrestrial	Ground
6 <i>Cyanoloxia brissonii</i>	Arboreal	Tree
7 <i>Dendrocincla fuliginosa</i>	Arboreal	Tree
8 <i>Habia rubica</i>	Arboreal	Tree
9 <i>Lepidocolaptes falcinellus</i>	Arboreal	Tree
10 <i>Leptopogon amaurocephalus</i>	Arboreal	Tree
11 <i>Platyrrinchus mystaceus</i>	Arboreal	Tree
12 <i>Pyrriglena leucoptera</i>	Mostly arboreal	Tree
13 <i>Ramphocelus bresilius</i>	Arboreal	Tree
14 <i>Schiffornis virescens</i>	Arboreal	Tree
15 <i>Sclerurus scansor</i>	Mostly terrestrial	Ground
16 <i>Sittasomus griseicapillus</i>	Arboreal	Tree
17 <i>Tachyphonus coronatus</i>	Arboreal	Tree
18 <i>Trichothraupis melanops</i>	Arboreal	Tree
19 <i>Turdus albicollis</i>	Mostly arboreal	Tree
20 <i>Turdus flavipes</i>	Arboreal	Tree
21 <i>Turdus leucomelas</i>	Mostly arboreal	Tree
22 <i>Turdus rufiventris</i>	Mostly arboreal	Tree
23 <i>Xenops minutus</i>	Arboreal	Tree
24 <i>Xiphorhynchus fuscus</i>	Arboreal	Tree

<sup>a</sup> according to Ridgely and Tudor (1994); Sick (1997), and Del Hoyo et al. (2013).

**Females**

1.	-Eyes located between the 1st and 2nd thirds of the scutum, hypostome apically pointed (lanceolate)	<i>A. longirostre</i>
2.	-Eyes located between the 1st and 2nd halves of the scutum, hypostome apically rounded (spatulate or sublanceolate) - Hypostome apically sub-acute (sublanceolate); scutum with few larger punctations on the lateral field together with median punctations on the rest of the scutum	<i>A. geayi</i>
3.	- Hypostome apically rounded (spatulate); scutal punctations of similar size uniformly distributed - U-shaped genital aperture, pointed coxa I spurs - V-shaped genital aperture, rounded coxa I spurs	<i>A. parkeri</i> <i>A. romarioi</i>

**Acknowledgments**

The authors thank the veterinarians and biologists of the Wildlife Screening Center (CETAS) of the Tietê Ecological Park (PET) and the Department of Parks and Green Areas (DEPAVE-3) of São Paulo City, and Erico Furtado from CETAS Juiz de Fora, for collecting tick species for monkeys. We are very grateful to Gabrielle Ribeiro de Andrade, Maria Cristina Ferreira do Rosário Almeida, and Jairo Alfonso Mendoza Roldan for the assistance with ticks from the Butantan Institute, to Guilherme Pinheiro Furusawa (Universidade Federal Rural do Rio de Janeiro) for assistance during field work, to Lucas Ribeiro dos Santos (Universidade Estadual de Santa Cruz, Ilhéus) and Beatriz Mauricio (Butantan Institute) for assistance with scanning electron microscopy, and Leo Nascimento for logistic support in the Parque Nacional do

Itatiaia. This work was financially supported by the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenadoria de Apoio a Pesquisa e Desenvolvimento (CAPES). This work is dedicated to Dr. Erik Daemon de Souza Pinto, Professor at the School of Veterinary Medicine (UFRRJ) and the Course of Biological Science (UFRJ), who in life collected specimens of this new species.

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