

Original article

New records of soft ticks (Acari: Argasidae) from caves in Brazil, with a morphological study of *Ornithodoros fonsecai* and an analysis of the taxonomic status of *Antricola inexpectata*

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ABSTRACT

In this study, we report soft ticks from bat-inhabiting caves in different areas of Brazil. From 2010 to 2019, we collected 807 tick specimens from nine caves located in four Brazilian states among two biomes. Ticks were morphologically identified as *Antricola guglimonei* (282 specimens), *Ornithodoros cavernicolous* (260 specimens), and *Ornithodoros fonsecai* (265 specimens). Whereas *A. guglimonei* was collected on bat guano in hot caves, *O. cavernicolous* and *O. fonsecai* were collected in cracks and crevices on the walls of cold caves, sometimes in the same chamber. Morphological identifications were corroborated by molecular and phylogenetic analyses inferred from tick mitochondrial 16S rRNA gene partial sequences. The sequences of *A. guglimonei*, *O. cavernicolous* and *O. fonsecai* collected in this study clustered with conspecific GenBank sequences from different localities of Brazil. Remarkably, a clade containing 12 sequences of *O. fonsecai* was clearly bifurcated, denoting a degree of genetic divergence (up to 5 %) of specimens from Cerrado/Atlantic Forest biomes with the specimens from the Caatinga biome. To further evaluate this divergence, we performed morphometric analysis of the larval stage of different *O. fonsecai* populations by principal component analysis, which indicated that the larvae from Caatinga populations were generally smaller than the larvae from other biomes. Some of the present *A. guglimonei* specimens were collected from the type locality of *Antricola inexpectata*. Comparisons of these specimens with the type specimens of *A. inexpectata* and *A. guglimonei* indicated that they could not be separated by their external morphology. Hence, we are relegating *A. inexpectata* to a synonym of *A. guglimonei*. This proposal is corroborated by our phylogenetic analysis.

1. Introduction

Soft ticks (Ixodida: Argasidae) are blood-feeding arthropods that can parasitize all classes of terrestrial vertebrates (Hoogstraal, 1985; Barros-Battesti et al., 2015). The traditional classification of the Argasidae family has included five genera: *Argas*, *Antricola*, *Otobius*, *Nothoaspis* and *Ornithodoros* (Nava et al., 2017). Phylogenetic studies have appointed clear inconsistencies in this classification, highlighted by paraphyly in the genera *Argas* and *Ornithodoros*, and in the proposal of reclassification

of some Neotropical *Ornithodoros* species into the genus *Alectorobius* (Mans et al., 2021). Because this recent proposal is still pending the inclusion of the *Alectorobius* type species [*Ornithodoros talaje* (Guérin-Méneville, 1849)] in further phylogenetic analyses (Kneubehl et al., 2022), herein we adopted the traditional classification in which all *Alectorobius* species are classified in the genus *Ornithodoros* (Nava et al., 2017).

The argasid fauna of Brazil is currently composed by 25 valid species, of which 19 are of the genus *Ornithodoros*. The remaining six species

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belong to the genera *Antricola* (three species), *Nothoaspis* (two species) and *Argas* (one species) (Dantas-Torres et al., 2019; Muñoz-Leal et al., 2019, 2020, 2021a). Indeed, the genus *Ornithodoros* contains the argasid species of greater public health importance in Brazil, where at least nine *Ornithodoros* species are recognized as human-biting ticks (Nogueira et al., 2022). Recently, the spirochete *Borrelia venezuelensis* - a pathogen known to cause relapsing fever in humans - was isolated from *Ornithodoros rudis* Karsch, 1880 in northeastern Brazil (Muñoz-Leal et al., 2018a), where another relapsing fever agent, “*Candidatus* *Borrelia caatinga*”, was isolated from a species phylogenetically closely related to *Ornithodoros tabajara* Muñoz-Leal & Labruna, 2021 (Oliveira et al., 2023). In addition, several novel relapsing fever *Borrelia* agents were molecularly detected in four species of human-biting *Ornithodoros* ticks from different regions of Brazil (Muñoz-Leal et al., 2021b). Finally, distinct rickettsial organisms of unknown pathogenicity, belonging to the *Rickettsia*-spotted fever group, have been reported in *Ornithodoros hasei* (Schulze, 1935), *Ornithodoros faccinni* Barros-Battesti, Landulfo & Luz, 2015, and *Ornithodoros* cf. *mimon* Kohls, Clifford & Jones, 1969 from Brazil (Luz et al., 2019; Peixoto et al., 2021; Dantas-Torres et al., 2022).

Despite of the doubtless relevance of soft ticks for animal and public health in the world (Hoogstraal, 1985; Sonenshine and Roe, 2014), such relevance is still “novelty” in Brazil, given that 13 (52 %) out of the 25 argasid species of the country were described during the last 20 years, and all reports of tick-borne agents infecting Brazilian *Ornithodoros* species were made during the last 6 years. This scenario indicates a vast field to be explored in Brazil. Therefore, the present study aimed to report several additional records of soft ticks in Brazil, performed by morphological and molecular analyses of specimens collected in caves throughout the country.

2. Materials and methods

2.1. Field collection of soft ticks

From 2010 to 2019, soft ticks were collected from the following nine bat-inhabited caves of Brazil: 1- Gruta do Calixto Cave (13°17'35.2"S, 41°03'47.9"W, altitude 474 m), Iramaia municipality, state of Bahia, 01 January 2010. 2- Furna do Fim do Morro do Parafuso Cave (10°38'25.7"S, 37°52'2.9"W, 395 m), Paripiranga municipality, state of Bahia, 13 January 2010. 3- Lapa do Brocotó Cave (17°19'27.9"S, 46°48'19.5"W, 600 m), Paracatu municipality, state of Minas Gerais, 16 October 2010. 4- Meu Rei Cave (08°29'12.0"S, 37°16'48.1"W, 770 m), National Park of Catimbau, Tupanatinga municipality, state of Pernambuco, 09 September 2012 and 27 October 2019. 5- Furna do Gato Cave (08°35'9.9"S, 37°14'26.5"W, 802 m), National Park of Catimbau, Buíque municipality, state of Pernambuco, 09 September 2012 and 26 October 2019. 6- Gruta do Brejinho Cave (07°13'48.8"S, 39°59'48.6"W, 709 m), Araripe municipality, state of Ceará, 13 September 2012. 7- Gruta das Corujas Cave (07°20'07.4"S, 40°09'28.2"W, 701 m), Araripe municipality, state of Ceará, 13 September 2012. 8- Furna do Walimir Cave (08°35'35.0"S, 37°14'07.6"W, 823 m), National Park of Catimbau, Buíque municipality, state of Pernambuco, 26 October 2019. 9- Poço de Areia Cave (08°39'32.8"S, 38°27'58.2"W; 379 m), Floresta municipality, state of Pernambuco, 28 October 2019. All caves belong to the Caatinga biome, except for Lapa do Brocotó Cave, which belongs to the Cerrado biome.

In each prospected cave, ticks were collected from cracks and crevices in the walls close to areas where bat colonies use to live, or on the bat guano on the floor. During our visits to caves in 2012, we also measured the internal temperature of the prospected caves. Collected ticks were held in dry plastic tubes and sent alive to the laboratory, where they were placed in an incubator at 26 °C and 80 % relative humidity. In the laboratory, ticks were visualized and counted under a stereomicroscope (Zeiss Stemi SV 11, Zeiss, Munich, Germany). Ticks were morphologically identified to species according to Dantas-Torres

et al. (2019) and original descriptions (Estrada-Peña et al., 2004; Labruna and Venzal, 2009; Dantas-Torres et al., 2012).

The above field collections of ticks have been authorized by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) Siso permits #34,237-1 and #65,137-1.

2.2. Molecular analyses of ticks

With the purpose to confirm the taxonomic identification of the tick species, one to five specimens from some of the prospected caves were submitted to DNA extraction by the guanidine isothiocyanate-phenol technique (Sangioni et al., 2005). Extracted DNA samples were tested by a conventional polymerase chain reaction (PCR) targeting a \approx 460 bp fragment of the mitochondrial 16S rDNA gene of ticks, as described (Mangold et al., 1998). PCR products of the expected sizes were treated with Illustra ExoProStar 1-Step (GE Healthcare), prepared for sequencing with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), and sequenced in an ABI 3500 instrument (Applied Biosystems). Generated DNA sequences were submitted to BLAST analyses (www.ncbi.nlm.nih.gov/blast) to infer to closest identities to tick DNA sequences available in GenBank.

2.3. Phylogenetic analysis

Partial sequences of the 16S rRNA gene generated in this study were aligned with 59 other sequences of argasid species (mostly from the neotropical region) available in GenBank, including all available 16S rRNA gene sequences of *Ornithodoros fonsecai* (Labruna & Venzal, 2009) and *Antricola* spp. The alignment was constructed with MAFFT (Kato et al., 2002). Best nucleotide substitution models were calculated with MEGA 7 (Kumar et al., 2016). A maximum-likelihood phylogenetic tree was inferred with PhyML (Guindon and Gascuel, 2003) with the Generalized Time-Reversible substitution model with five rates categories of sites. *Ornithodoros rostratus* Aragão, 1911 (DQ295780), and *Ornithodoros brasiliensis* Aragão, 1923 (GU198363) rooted the tree.

2.4. Morphometric analyses of *O. fonsecai* larvae

Because of the relatively high polymorphism found for the 16S rRNA gene sequences between some specimens of *O. fonsecai* from different localities (see Results), we decided to extend our morphological analyses to the larval stage of this species. To do this, unfed larvae were obtained from five populations from five different States and three biomes of Brazil (Fig. 1). In the laboratory, field-collected adult females of *O. fonsecai* from Buíque municipality (Caatinga biome), state of Pernambuco, and from Paripiranga municipality (Caatinga biome), state of Bahia, were fed on guinea pigs as previously described (Oliveira et al., 2023) and held in the incubator for oviposition and egg incubation. Hatched larvae were killed in hot water and immediately preserved in 70 % ethanol. Thereafter, 10 or 20 unfed larvae from each locality were clarified in 20 % KOH solution, mounted in semi-permanent slides for microscopy using Hoyer's Medium, and analyzed using a Nikon Eclipse E200 optical microscope. Measurements are given in millimeters (mm), with the mean followed by the range in parentheses. Terminology for larval chaetotaxy and measurements followed Venzal et al. (2008, 2013).

The same morphometric analysis was performed with unfed larvae from three other localities, as follows: (i) 12 unfed larvae that have been deposited as *O. fonsecai* paratypes in the tick collection “Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva” (CNC) of the University of São Paulo, Brazil, under the accession number CNC1397. These larvae were part of the progeny of paratype females collected in Gruta São Miguel Cave (21°06'29''S, 56°34'49''W), Bonito municipality (Cerrado biome), state of Mato Grosso do Sul, 8 April 2005, as previously reported (Labruna and Venzal, 2009). (ii) 15 unfed larvae obtained from an *O. fonsecai* engorged female from Furna de Araticum Cave, Ubajara

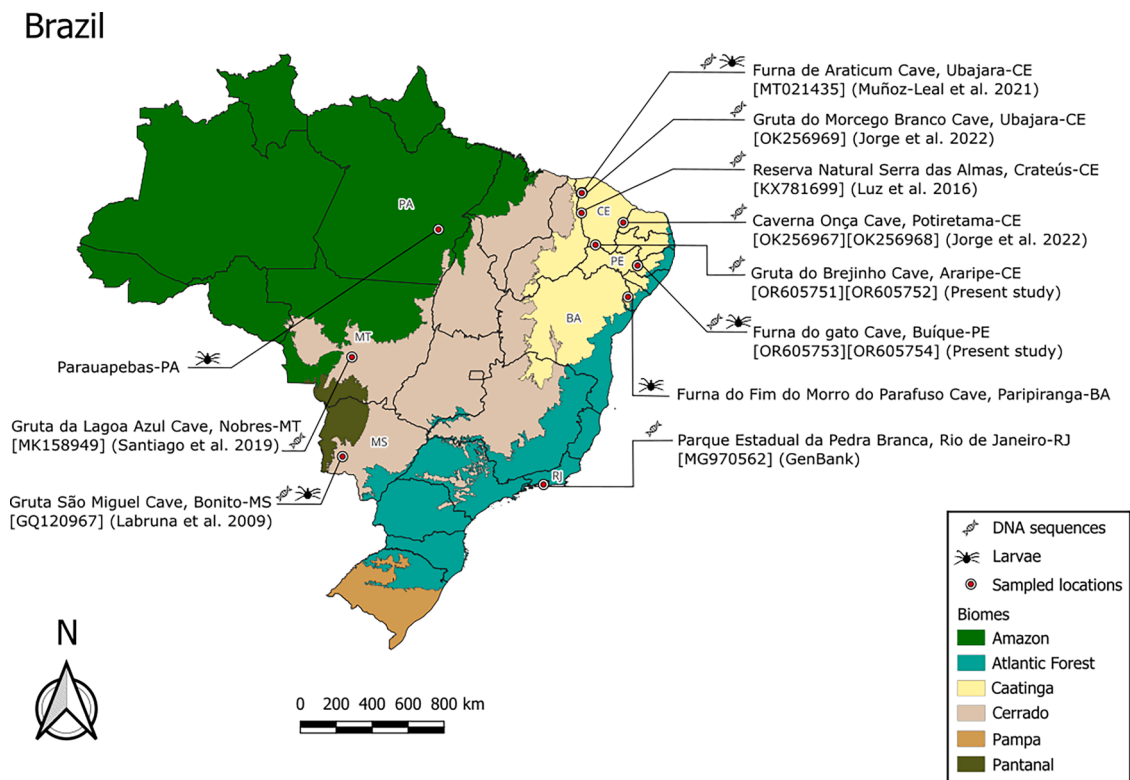


Fig. 1. Map of Brazil indicating the locations where *Ornithodoros fonsecai* ticks were collected, and further evaluated in the laboratory by molecular analysis (partial DNA sequences of the tick mitochondrial 16S rRNA gene; GenBank accession numbers within brackets) and/or by morphological analysis of the larval stage.

municipality (Caatinga biome), Ceará State (03°48'11.5"S, 40°52'40.4"W), which have been obtained in the study of Muñoz-Leal et al. (2021b). (iii) 4 unfed larvae collected from two unnamed caves (1 larva from Cave 1 - 6°20'20.4"S, 49°57'34.3"W; 3 larvae from cave 2 - 6°20'7.2"S, 49°57'33"W) in Parauapebas municipality (Amazon biome), state of Pará, at 20 September 2013; these larvae have been deposited in the Acari Collection of the Butantan Institute (IBSP), São Paulo, Brazil, under the accession numbers IBSP-15,865 and IBSP-15,869.

A principal component analysis (PCA) based on a Pearson's correlation matrix was applied on 62 morphometric variables (Table S1) for unfed larvae of *O. fonsecai* from the above five geographically different populations to elucidate relationships among them. In addition, we included data from the larvae of two geographically (from Piauí and Paraíba states) and genetically (3.3 % difference in the 16S rRNA gene) different populations of *Ornithodoros rietcorreae* Labruna, Nava & Venzal, 2016, retrieved from Labruna et al. (2016). Raw measurements were log ($x + 1$)-transformed to standardize variances and improve normality.

2.5. Morphological analyses of *Antricola* spp

One of the prospected caves of this study was Gruta do Brejinho, the type locality of *Antricola inexpectata* Estrada-Peña, Barros-Battesti & Venzal, 2004. This species was described by Estrada-Peña et al. (2004) based on 7 females collected on 10 July 2002. The holotype female (IBSP-7987) and 5 paratype females (IBSP-7483, -9061) were examined in the present study, together with the holotype female and 2 paratype females (IBSP-7962) of *Antricola guglielmonei* Estrada-Peña, Barros-Battesti & Venzal, 2004. Although the above IBSP accession number for *A. inexpectata* is different from that reported by Estrada-Peña et al. (2004), the above numbers were confirmed by Valeria C. Onofrio (the Curator of IBSP), who also informed that the seventh *A. inexpectata* female specimen of the type series was deposited in the University of Zaragoza, Spain.

The types of *A. inexpectata* and *A. guglielmonei* were morphologically compared to the *Antricola* adult females collected during the present study, and to specimens of *A. guglielmonei* (CNC-4614) collected in the western Brazilian Amazon by Labruna et al. (2008). For the above-mentioned phylogenetic analysis, we included the 16S rRNA haplotype of an *A. guglielmonei* paratype (GenBank #EU090905), previously reported by Labruna et al. (2008). Since there was no available DNA sequence of any *A. inexpectata* specimen, we extracted DNA from the internal contents (the exoskeleton was preserved) of two paratypes of *A. inexpectata* (IBSP-9061), by using the DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA, USA), following the manufacturer's protocol for tissue. Extracted DNA was tested by the PCR assay targeting the tick 16S rDNA gene and the resultant amplicons were sequenced as described above. The generated sequence was included in the present phylogenetic analysis.

3. Results

3.1. Collected ticks

Along the nine bat-inhabited caves from 2010 to 2019, we collected a total of 807 specimens of soft ticks, which were morphologically identified into three species: *A. guglielmonei* (282 specimens), *Ornithodoros cavernicolous* Dantas-Torres, Venzal & Labruna, 2012 (260 specimens), and *O. fonsecai* (265 specimens).

The *A. guglielmonei* specimens were collected on bat guano in three caves (Table 1). The Meu Rei Cave was visited twice, firstly in 2012, and secondly in 2019. In the 2012 visit, ticks were seen abundant on bat guano, whereas uncountable bats were seen on the walls and ceiling over the guano (Fig. 2A-C). The air temperature of the chamber where *Antricola* ticks were collected varied from 30.7 to 31.7 °C, contrasting to the outdoor temperature that was 25 °C. At several instances, we observed *Antricola* ticks moving voraciously over fresh bat droppings, which were visually wetter than the guano as a whole. This affinity of

Table 1

Soft ticks (M: males; F: females; N:nymphs) collected in bat-inhabited caves from different localities of Brazil during 2010–2019.

Tick species	Cave	Municipality (State)	No. of specimens	Year of collection	Specimens processed by molecular analyses (16S rRNA haplotype) ^a
<i>Antricola guglielmonei</i>	Meu Rei	Tupanatinga (Pernambuco)	13 M, 17F, 36N	2012	2 M, 2F (AnG1)
	Meu Rei	Tupanatinga (Pernambuco)	4 M, 1F, 6N	2019	1 N (AnG1)
	Gruta do Brejinho	Araripe (Ceará)	66 M, 47F, 83N	2012	2 M, 2F (AnG1), 1F (AnG2)
<i>Ornithodoros cavernicolous</i>	Gruta das Corujas	Araripe (Ceará)	4 M, 3F, 2N	2012	1 N, 1F (AnG1)
	Furna do Walmir	Buíque (Pernambuco)	6 M, 4F, 3N	2019	
	Furna do Gato	Buíque (Pernambuco)	1N	2019	1 N (OrC1)
<i>Ornithodoros fonsecai</i>	Poço de Areia	Floresta (Pernambuco)	102 M, 69F, 75N	2019	1 M (OrC1), 1 N (OrC2)
	Furna do Gato	Buíque (Pernambuco)	20 M, 25F, 30N	2012	2 N (OrF1)
	Furna do Gato	Buíque (Pernambuco)	44 M, 12F, 69N	2019	1 M (OrF2)
	Furna do Walmir	Buíque (Pernambuco)	1N	2019	
	Furna do Fim M. Parafuso	Paripiranga (Bahia)	3 M, 13F, 14N	2010	
	Gruta do Calixto	Iramaia (Bahia)	6 M, 6F, 7N	2010	
	Gruta do Brejinho	Araripe (Ceará)	5F, 5N	2012	1 N (OrF2), 1 N (OrF3)
	Lapa do Brocotó	Paracatu (Minas Gerais)	1 M, 2F, 2N	2010	

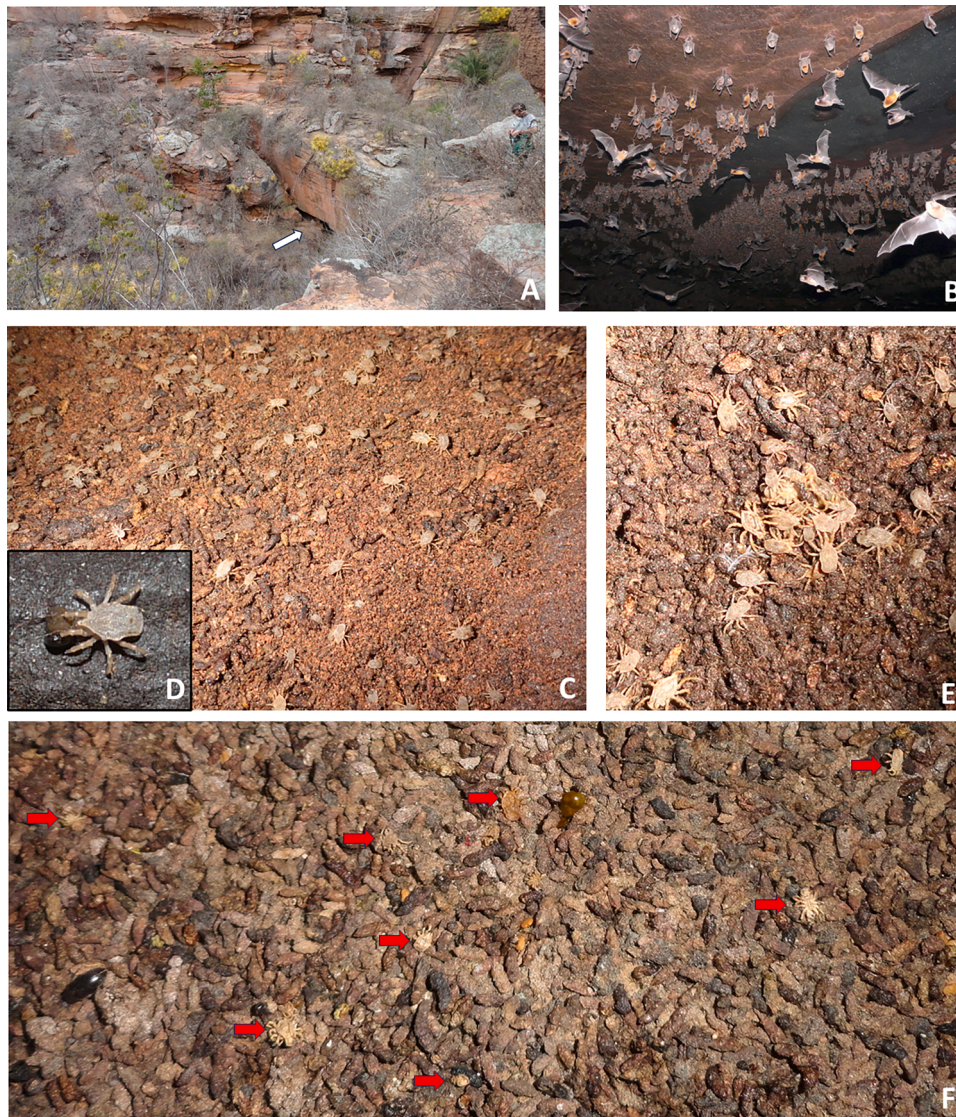
^a Number of specimens that generated 16S rDNA partial sequences, followed by the haplotype code within parentheses.

Fig. 2. Meu Rei cave (A-E) when it was visited in 2012. Overview of the cave entrance (white arrow) (A); numerous bats inside the cave (B); and numerous *Antricola guglielmonei* ticks over the guano (C). An *A. guglielmonei* female nymph with its mouthparts in contact with a fresh bat dropping (D); and a cluster of *A. guglielmonei* ticks as if they were competing for access to the bat fresh droppings (E). The Corujas Cave in 2012, when the guano was dry and all *Antricola* ticks (red arrows) over it were dead (F).

the tick for the fresh droppings was seen both for individual ticks (Fig. 2D) and for clusters of ticks as if they were competing for access to the fresh droppings (Fig. 2E). In the 2019 visit of Meu Rei Cave, only few bats were observed and much fewer *Antricola* ticks were observed over the guano.

During our visit to Gruta do Brejinho Cave, *Antricola* ticks were collected from a single chamber where air temperature was around 31 °C and there were hundreds of bats in the ceiling over the guano. When we visited the Corujas Cave in 2012, the internal air temperature was 27 °C and there were no bats; however, there was an abundant amount of dry guano containing uncountable numbers of dead specimens of *Antricola* over it (Fig. 2F). In fact, among the nine tick specimens collected in this cave, only three were alive; these three specimens were exceptionally collected under a rock where the guano was visually moist, in contrast to the dry appearance of the guano in the rest of the cave.

The specimens of either *O. cavernicolous* or *O. fonsecai* were collected in cracks and crevices on the walls of three or six caves, respectively

(Table 1). Two of these caves (Furna do Walmir, Furna do Gato) had both tick species in the same chamber. Furna do Gato was visited two times, in 2012 and in 2019; *O. cavernicolous* was found only in the second visit. During our 2012 visit, the air temperature in the cave was around 25 °C, similarly to the outside temperature. In the Brejinho Cave, *O. fonsecai* was collected in a chamber where air temperature was 25 °C, contrasting to the 31 °C of the nearby chamber where *Antricola* ticks were collected.

3.2. Molecular analyses of ticks

Partial sequences (413–426 bp) of the mitochondrial 16S rRNA gene were generated for 1–5 specimens from some of the prospected caves, totaling 20 specimens, as depicted in Table 1. Among 12 specimens of *A. guigliemonei* from three caves, 11 specimens had the same haplotype (designated as AnG1) and a single female from Gruta do Brejinho Cave had a distinct haplotype (AnG2), which differed from AnG1 by a single nucleotide polymorphism (SNP). By BLAST analysis, these two

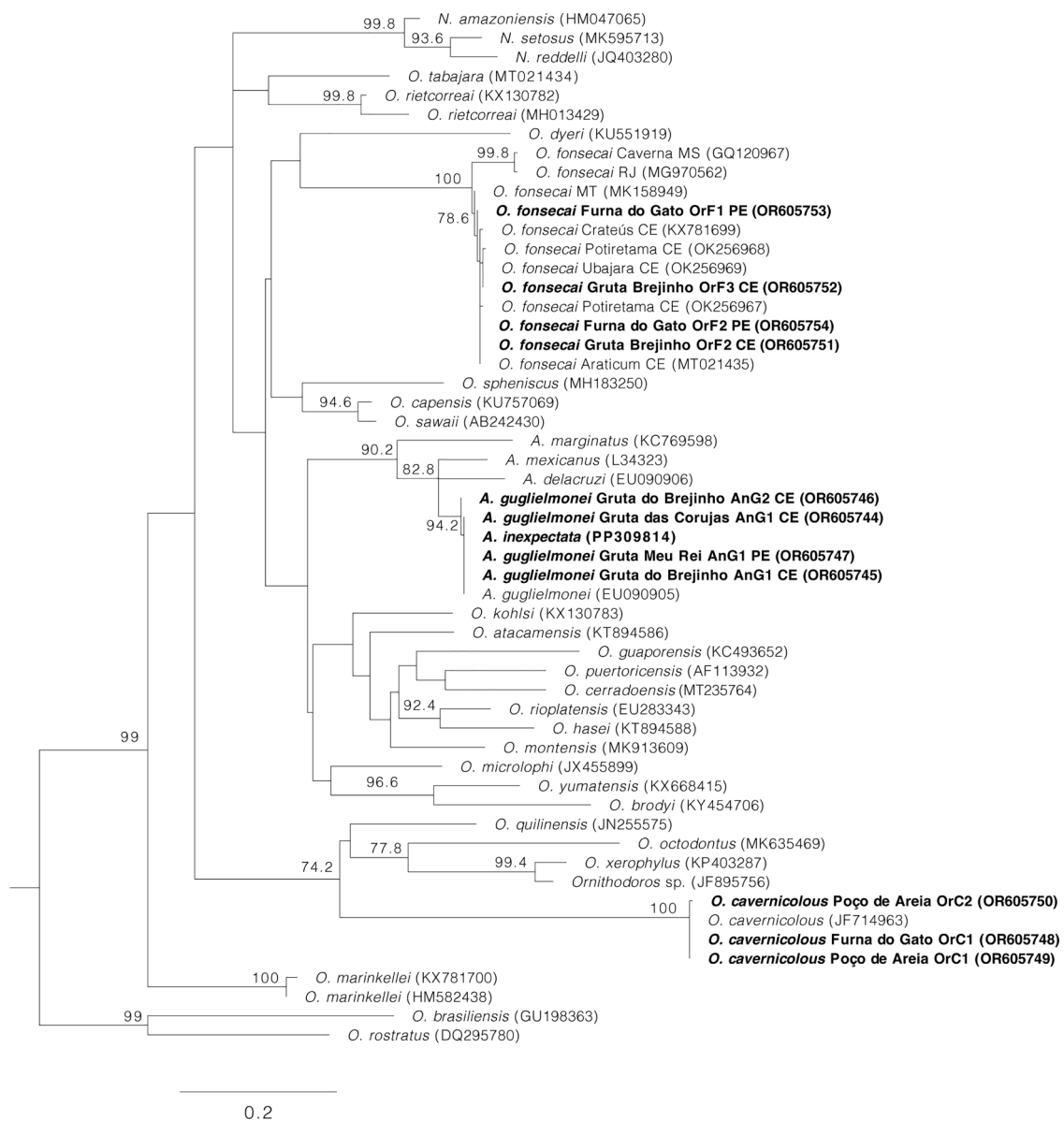


Fig. 3. Maximum likelihood phylogenetic tree inferred for a subset of neotropical Argasidae using an alignment of 439 bp of the mitochondrial 16S rRNA gene. The position of the sequences of *Antricola guigliemonei*, *Ornithodoros cavernicolous* and *Ornithodoros fonsecai* provided by the present study are highlighted in bold. Only support values of relevant clades are showed. Abbreviations: A., *Antricola*; N., *Nothoaspis*; O., *Ornithodoros*.

haplotypes were 99.5 (413/415 bp) to 99.3 % (412/415 bp) identical to the sequence of one *A. guglielmonesi* paratype from the state of Sergipe (EU090905), which was 100 % identical to the sequences of two *A. guglielmonesi* specimens from the state of Rondônia, Brazilian western Amazon (Labruna et al., 2008); these sequences were 95 % identical to *Antricola mexicanus* Hoffmann, 1959 (L34323.1, NC_023340) and *Antricola delacruzi* Estrada-Peña, Barros-Battesti & Venzal, 2004 (EU090906).

From the two paratypes of *A. inexpectata*, viable DNA could be obtained from only one specimen, which generated a 426-bp sequence of the mitochondrial 16S rRNA gene. By BLAST analysis, this sequence was 99.5 % (413/415 bp) identical to *A. guglielmonesi* paratype (EU090905), and 100 % (426/426 bp) identical to AnG1 haplotype of *A. guglielmonesi* generated in the present study.

Three specimens (one male and two nymphs from two caves) were genetically confirmed as *O. cavernicolous* (Table 1); their sequences represented two haplotypes (OrC1 and OrC2), which differed by a SNP and were 99.8–100 % identical to a type sequence of *O. cavernicolous* (JF714963) from Ceará state, Brazil (Dantas-Torres et al., 2012). Five specimens (one male and four nymphs from three caves) were genetically confirmed as *O. fonsecai* (Table 1); they yielded three distinct haplotypes (OrF1, OrF2, OrF3) that differed to each other by one or two SNPs, and were 99.5–100 % identical to several sequences of *O. fonsecai* (MT021435, NC_067910, OK256967, OK256969, KX781699) from other sites in the Caatinga biome (Luz et al., 2016; Muñoz-Leal et al., 2021b; Jorge et al., 2022), and at the same time, 94.6–95.9 % identical to the type sequence of *O. fonsecai* (GQ120967) from the state of Mato Grosso do Sul (Cerrado biome), Brazil (Labruna and Venzal, 2009).

3.3. Phylogenetic analysis

In the phylogenetic analysis inferred from partial sequences of the 16S rRNA gene, the sequences of *A. guglielmonesi*, *O. cavernicolous* and *O. fonsecai* collected in this study clustered with conspecific sequences from different localities of Brazil (Fig. 3). Remarkably, a clade containing 12 sequences of *O. fonsecai* was clearly bifurcated, denoting a degree of genetic divergence of specimens from the states of Mato Grosso do Sul (MS; Cerrado biome, type locality) and Rio de Janeiro (RJ; Atlantic Forest biome) with the specimens from the Caatinga biome [states of Pernambuco (PE) and Ceará (CE)] and Cerrado [state of Mato Grosso (MT)]. On the other hand, the *A. guglielmonesi* sequences grouped without bifurcation in a clade that was sister to the other *Antricola* species (*A. delacruzi* and *A. mexicanus*). Noteworthy, the sequence of the type specimen of *A. inexpectata* was embedded within the clade containing all sequences of *A. guglielmonesi*, including its type sequence EU090905 (Fig. 3).

3.4. Principal components analysis (PCA)

The results of the PCA for the morphological characters showed a clear difference between the group of *O. fonsecai* populations from the states of Pernambuco, Ceará and Bahia and the group of populations from the states of Mato Grosso do Sul and Pará, as well for the additional species included, with only one exception of the outlier specimen *O. fonsecai* PA4 (Fig. 4). The first principal component (explaining 50.92 % of the total variance) was mainly loaded by the variables body length, length of basis capituli, length of capituli, palpal length, hypostome length, and tarsus length, and the second component (explaining 23.58 % of the total variance) was principally loaded by the dorsal

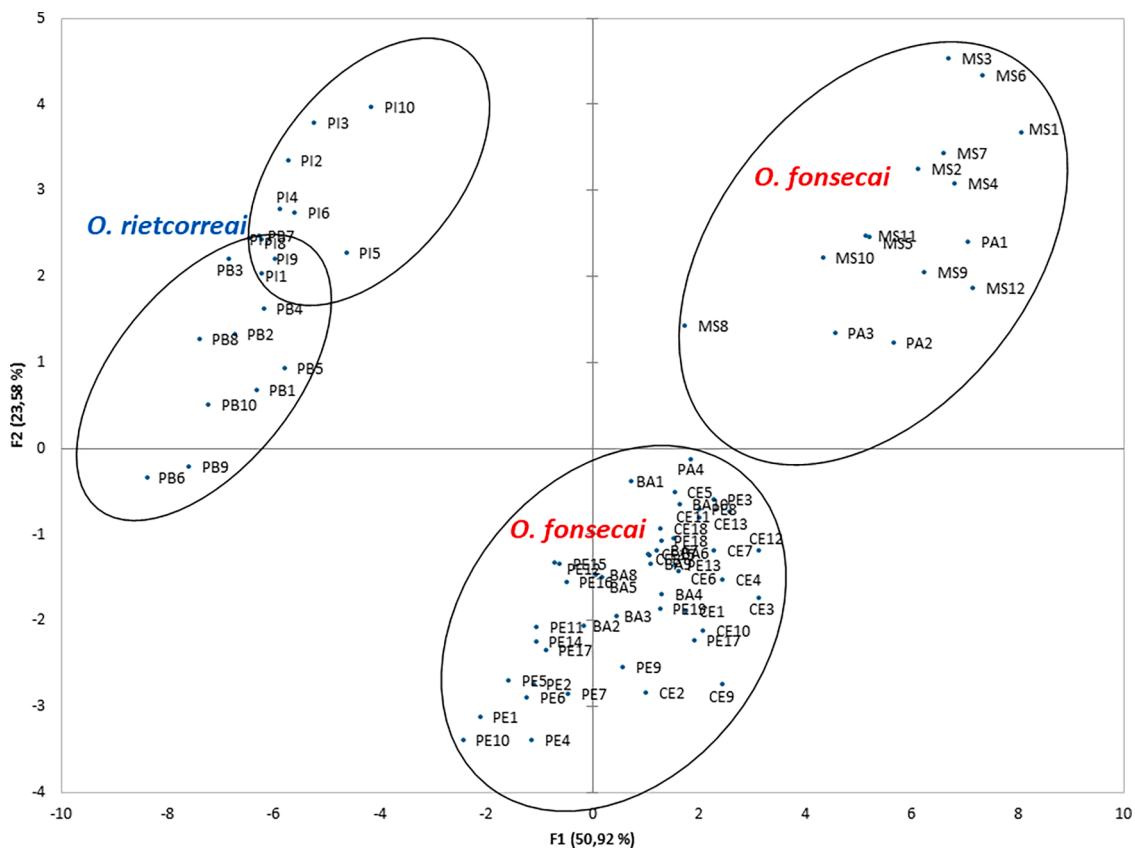


Fig. 4. Principal components analysis (PCA) of the body and setal measurements of the larvae of *Ornithodoros fonsecai* [states of Bahia (BA), Ceará (CE), Mato Grosso do Sul (MS), Pará (PA) and Pernambuco (PE)] and *Ornithodoros rietcorraei* [states of Paiú (PI) and Paraíba (PB)], using the features detailed in Table S1. Morphometric variables for the larvae of *O. rietcorraei* were retrieved from Labruna et al. (2016). Each point represents the position of a measured specimen in the reduced morphometric space.

anterolateral setae and central setae. Overall, these results indicate that the larvae from the states of Pernambuco, Ceará and Bahia (Caatinga biome) were smaller than the larvae from Mato Grosso do Sul (Cerrado) and Pará (Amazon). The separation of these two groups was more distinct than the separation of two populations of *O. rietcorraei*, from the states of Piauí and Paraíba.

3.5. *Antricola inexpectata*

In 2012, 196 *Antricola* specimens (66 males, 47 females, 83 nymphs) were collected in Gruta do Brejinho Cave (Table 1). Although this cave is the type locality of *A. inexpectata* (Estrada-Peña et al., 2004), all specimens from 2012 were morphologically identified as *A. guglielmonei*, corroborated by the molecular analyses of three males and three females.

According to Estrada-Peña et al. (2004), *A. guglielmonei* is morphologically separated from *A. inexpectata* chiefly by the following three characters in adult females: (i) Dorsal tubercles close to each other, without areas of smooth cuticle in *A. guglielmonei*; dorsal tubercles separated by smooth areas of cuticle in *A. inexpectata*. (ii) Zone between coxal insertion and genital shield smooth, with a few posterior striations, never with tubercles in *A. guglielmonei*; zone between coxal insertion and genital shield with small tubercles in *A. inexpectata*. (iii) Setae in several tufts, not well delimited, and never on cuticular thickenings in *A. guglielmonei*; with 3–4 tufts of setae near the spiracular plate, well delimited in the lateral portion of the idiosoma, located over rounded cuticular thickenings in *A. inexpectata*.

Through examinations of female type specimens of *A. guglielmonei* and *A. inexpectata* in the present study, we observed that the three above

characters were not clear to separate the two species. For example, some of the type females *A. guglielmonei* presented dorsal tubercles more separated by smooth areas of cuticle than females of *A. inexpectata* (Fig. 5A, B); the zone between coxal insertion and genital shield could not be discriminated between the two species by light microscopy (Fig. 5C, D), and some of the types of *A. guglielmonei* presented 3–4 tufts of setae near the spiracular plate, well delimited in the lateral portion of the idiosoma, just like observed in *A. inexpectata* (Fig. 5E, F). Regarding the insertion of these setal tufts over rounded cuticular thickenings, we observed that in the holotype of *A. inexpectata*, nearly all setal tufts were over a rounded cuticular thickening (Fig. 6A). In the other type specimens, only some of the setal tufts were clearly observed over a rounded cuticular thickening, for both *A. inexpectata* (Fig. 6B) and *A. guglielmonei* (Fig. 6C, D). Finally, the variability of the above-mentioned characters proved to be intrapopulational, since specimens with one or the other morphotype were observed in each of the *A. guglielmonei* populations examined in the present study. For instance, we have observed that some *A. guglielmonei* specimens from Rondônia (CNC-1041) contained all setal tufts over a rounded cuticular thickening, just like observed in the Holotype of *A. inexpectata* in Fig. 6A. At least six of these *A. guglielmonei* specimens have been assigned to a new accession number in the collection: CNC-4749.

Finally, the 16S rRNA gene partial sequence of a type specimen of *A. inexpectata* (generated in this study) differed by only two nucleotides from the type sequence of *A. guglielmonei* (EU090905), indicating that they were 99.5 % identical to each other (413/415 bp).

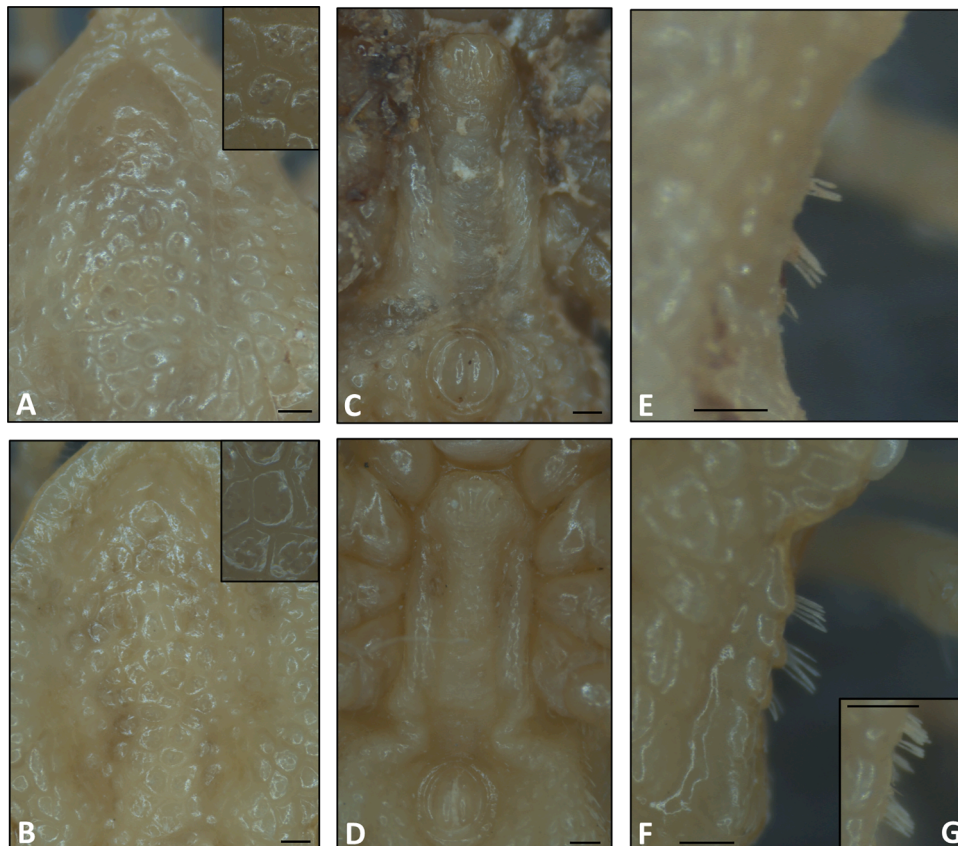


Fig. 5. Light microphotographs of the type specimens of *Antricola inexpectata* (IBSP-7963 and IBSP-7983) and *Antricola guglielmonei* (IBSP-7962). Dorsal tubercles of *A. inexpectata* (A) and *A. guglielmonei* (B); tubercles at higher magnification in the insets. Ventral view of the zone between coxal insertion and genital shield of *A. inexpectata* (C) and *A. guglielmonei* (D). Dorsal view of three tufts of setae posteriorly to spiracular plate in the lateral portion of the idiosoma of *A. inexpectata* (E) and *A. guglielmonei* (F). Another paratype of *A. guglielmonei* showing more than three and not well delimited tufts of setae posteriorly to spiracular plate in the lateral portion of the idiosoma (G). Bars: 0.2 mm.

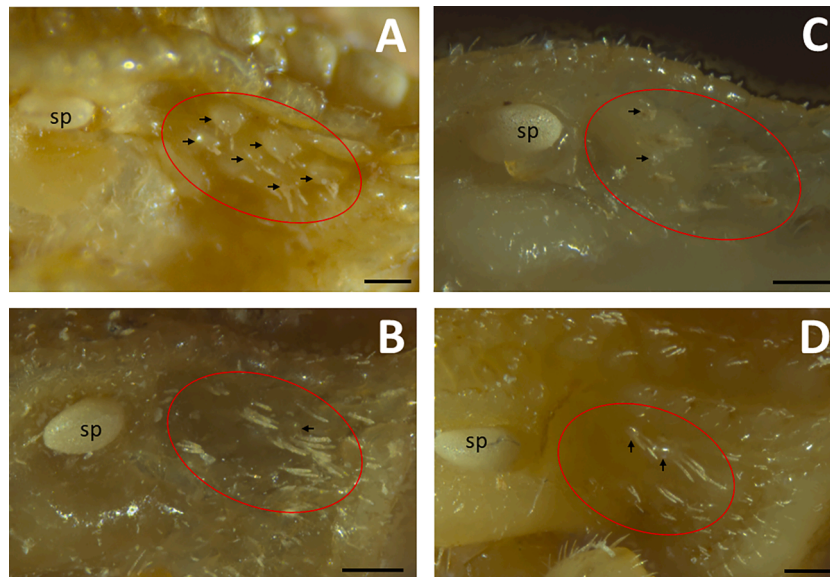


Fig. 6. Light microphotographs of the type specimens of *Antricola inexpectata* (IBSP-7963 and IBSP-7987) and *Antricola guglielmonoi* (IBSP-7962). Lateral view of tufts of setae posteriorly to spiracular plate (sp) in the lateral portion of the idiosoma of *A. inexpectata* (A, B) and *A. guglielmonoi* (C, D). Red circle delimitates the tufts. Each arrow indicates a rounded cuticular thickening on which a setal tuft is inserted. Bars: 0.2 mm.

4. Discussion

The present study reports several records of *A. guglielmonoi*, *O. cavernicolous*, and *O. fonsecai* in caves of Brazil. The two bat-inhabited caves where *A. guglielmonoi* were collected (Meu Rei and Gruta do Brejinho) presented internal temperature of $>30^{\circ}\text{C}$, similarly to other caves where *Antricola* spp. have been collected (De la Cruz, 1992; Labruna et al., 2011). This typical hot atmosphere is generated by the large populations of insectivorous bats (e.g. *Pteronotus* spp.) that colonize internal chambers of the caves (De la Cruz, 1992). Interestingly, there were no bats in the third cave (Gruta das Corujas) during our visit in September 2012, when nearly all *Antricola* ticks were found dead and the cave internal temperature was 27°C . Notably, in 2012 the Caatinga biome was experiencing its worst drought period in 30 years (Novaes et al., 2013). As there was an abundant amount of dry guano in Gruta das Corujas, it is possible that the former resident bat colony abandoned the cave due to the scarcity of food (insects) in the area due to the severe drought period. The high mortality of *Antricola* ticks in this cave was likely associated with the dryness of the guano, since the only three live specimens found during our visit were collected in a small patch of moist guano under a rock.

During our visit to the Meu Rei Cave in 2012, several *Antricola* ticks were observed moving voraciously over fresh droppings that had just been delivered from bats. This clear affinity of ticks for fresh droppings may be related to the extreme drought period that was underway in the Caatinga biome in 2012, which may have decreased bat population and consequently, turned the guano drier than it normally is. Interestingly, a study on the sialotranscriptome of *A. delacruzi* suggested that *Antricola* ticks are guanophagous, i.e., they feed on guano (Ribeiro et al., 2012). The nymphal and adult stages of *Antricola* spp. are non-hematophagous and their mouth parts (scoop like-hypostome) are likely adapted to scavenging rather than blood feeding (De La Cruz, 1992). Hence, our observations on the attractiveness of fresh guano to *A. guglielmonoi* ticks, as illustrated in Fig. 2D-E, support the hypothesis of guanophagy for postlarval stages of *Antricola* ticks. Although we cannot rule out the possibility that the ticks' movement to the fresh feces was primarily to acquire moisture rather than food, this possibility becomes less supported if we consider that the *Antricola*-inhabited hot caves, whose temperatures are $>30^{\circ}\text{C}$, have a highly humid atmosphere (De la Cruz, 1992), which would be in counterpoint to the ticks' voracity for water in

the guano.

Morphological examinations of the type specimens of *A. inexpectata* and *A. guglielmonoi* presented evidence that they represent the same taxon, which was corroborated by analyses of 16S rDNA sequences of specimens from the type localities of *A. inexpectata* and *A. guglielmonoi*. Therefore, based on page priority in the publication of Estada-Peña et al. (2004), we are relegating *A. inexpectata* to a synonym of *A. guglielmonoi*. The Brazilian fauna of *Antricola* ticks is reduced to two valid species, *A. guglielmonoi* and *A. delacruzi*. Although the latter was found with *A. guglielmonoi* in the same caves of the Atlantic Forest (Sergipe state) and Amazon (Rondonia state) biomes (Estrada-Peña et al., 2004; Labruna et al., 2008), it was not found in *A. guglielmonoi*-inhabited caves of the Caatinga (Barbier et al., 2020; present study) and Cerrado biome (Costa et al., 2020).

The other ticks found in the present study, *O. cavernicolous* and *O. fonsecai*, represent the two cave-dwelling tick species with the widest geographical distribution in Brazil, with records in the Amazon, Atlantic Forest, Caatinga, and Cerrado biomes (Munoz-Leal et al., 2018b; Jorge et al., 2022; present study). Our findings of these two tick species cohabiting the same chamber of two caves (Furna do Walmir, Furna do Gato) in the state of Pernambuco agree with another study that reported both species sympatrically in two caves of the state of Ceará, Caatinga biome (Jorge et al., 2022). Within these caves, *O. cavernicolous* and *O. fonsecai* are likely to share the same host species, since bats of the Phyllostomidae family (*Anoura* spp., *Carollia perspicillata*, *Desmodus rotundus*, *Trachops cirrhosus*) have been reported as hosts for both tick species (Labruna and Venzal, 2009; Dantas-Torres et al., 2012; Luz et al., 2016). Although *O. cavernicolous* and *O. fonsecai* are phylogenetically distant (Fig. 3), they are ecologically very similar.

Diversity up to 5 % among 16S rRNA haplotypes of *O. fonsecai* from geographically distinct populations motivated our morphometric analysis of the larval stage by PCA, which indicated that the larvae from the three Caatinga populations were generally smaller than the larvae from Cerrado and Pará populations. This morphological separation was somewhat corroborated by the phylogenetic analysis inferred from 16S rRNA gene partial sequences, which separated the Mato Grosso do Sul population (Cerrado) from all sequences from the Caatinga biome. These two clades of *O. fonsecai* differed by 5 % among the 16S rRNA haplotypes, which seems to be the "grey zone" of polymorphism between 16S rRNA haplotypes of *Ornithodoros* spp. For instance, a 5 % intraspecific

difference was reported between the Brazilian and Colombian/Central American populations of *Ornithodoros marinkellei* Kohls, Clifford & Jones, 1969 (Venzal et al., 2018), whereas a 5 % interspecific difference was reported between *Ornithodoros clarki* Jones & Clifford, 1972 from Central America/Mexico and *Ornithodoros rondoniensis* (Labruna, Terrasini, Camargo, Brandão, Ribeiro & Estrada-Peña, 2008) from Brazil (Labruna et al., 2021).

Despite the morphometric differences pointed out by PCA, we found no remarkable morphological differences between different populations of *O. fonsecai*. Until further studies elucidate these differences by more robust analyses and additional lines of evidence, we consider these populations as conspecific; i.e., they all belong to the species *O. fonsecai*. One of the additional lines of evidence could be cross-mating experiments. For instance, the 16S rRNA haplotypes of the two populations of *O. rietcorreae* included in our PCA analyses (Fig. 4) differed by 3.3 % and they were reproductively compatible (Labruna et al., 2016). On the other hand, *Ornithodoros cerradoensis* Muñoz-Leal, Martins & Labruna, 2020 and *Ornithodoros guaporensis* Nava, Labruna & Venzal, 2013 were reproductively incompatible and their 16S rRNA haplotypes differed by 13.8 % (Muñoz-Leal et al., 2020). It would be interesting to evaluate the reproductive compatibility of two *O. fonsecai* populations showing a 5 % difference in their 16S rRNA haplotypes.

The present findings of *O. fonsecai* larvae with lower size in populations from the Caatinga biome, when compared to larvae from Cerrado and Amazon biomes, suggests environmental influence driving morphological divergence within this tick species, as demonstrated for other arthropod species (Kovacs et al., 2010). Indeed, contrary to shape that tends to be more stable, the size can be influenced by environmental conditions, even more in the case of ticks that spend major part of their life cycle in the off-host environment and require specific conditions of humidity and temperature to survive (Hoogstraal, 1985; Dupraz et al., 2016).

The Caatinga constitutes a unique biome located in the semiarid hinterland of northeastern Brazil, its high temperatures, low air humidity and prolonged dry period when precipitation is scarce, makes this biome much more hostile than the other Brazilian biomes that tend to be more humid (Rios and Thompson, 2013; Silva et al., 2017). Therefore, the reduced size in *O. fonsecai* larvae from Caatinga maybe explained in part by the temperature size rule (TSR), a pattern that consists in an inverse relationship between the temperature at which an ectothermic animal is reared and its body size. This pattern is widely documented among arthropods and although it possesses a generalist nature (with many variables involved), it has been demonstrated that smaller arthropods are more likely to demonstrate a classic TSR (Klok and Harrison, 2013). Although this statement seems to be logic to apply to our *O. fonsecai* data, it remains to be validated for cave-dwelling ticks.

5. Conclusions

This study provides additional geographical records of *A. guigliemonei*, *O. cavernicolous*, and *O. fonsecai* in Brazil. Collection of *Antricola* specimens at the type locality of *A. inexpectata* allowed morphological, molecular and phylogenetic analyses that invalidate the taxon *A. inexpectata*, which is relegated to a synonym of *A. guigliemonei*. A die-off of an *A. guigliemonei* population was reported in a cave, from which the bat colony had abandoned possibly because of a prolonged drought in the region. Morphological and phylogenetic analyses of populations of *O. fonsecai* from different Brazilian biomes indicate that the populations from the Caatinga biome are genetically distinct, and morphologically represented by smaller larvae; this condition could be related to the semiarid weather of the Caatinga biome.

CRedit authorship contribution statement

Glauber M.B. de Oliveira: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software,

Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sebastián Muñoz-Leal:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Santiago Nava:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Maurício C. Horta:** Writing – review & editing, Visualization, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Leopoldo Bernardi:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Data curation. **José Manuel Venzal:** Writing – review & editing, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Marcelo B. Labruna:** .

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2024.102331.

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