GLAUBER MENESES BARBOZA DE OLIVEIRA

Estudo taxonômico e pesquisa de *Borrelia* spp. em carrapatos do gênero *Ornithodoros* no bioma Caatinga

São Paulo

2022

GLAUBER MENESES BARBOZA DE OLIVEIRA

Estudo taxonômico e pesquisa de *Borrelia* spp. em carrapatos do gênero *Ornithodoros* no bioma Caatinga

VERSÃO CORRIGIDA

Tese apresentada ao Programa de Pós-Graduação em Epidemiologia Experimental Aplicada às Zoonoses da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a obtenção do título de Doutor em Ciências.

Departamento:

Medicina Veterinária Preventiva e Saúde Animal

Área de concentração:

Epidemiologia Experimental Aplicada às Zoonoses

Orientador:

Prof. Dr. Marcelo Bahia Labruna

Coorientador:

Prof. Dr. Sebástian Alejandro Muñoz Leal

São Paulo

2022

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO

(Biblioteca Virginie Buff D'Ápice da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo)

4276
FMVZ

Oliveira, Glauber Meneses Barboza de
Estudo taxonômico e pesquisa de Borrelia spp. em carrapatos do gênero Ornithodoros no bioma Caatinga / Glauber Meneses Barboza de Oliveira. – 2022.
60 f. : il.

Tese (Doutorado) – Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Medicina Veterinária Preventiva e Saúde Animal, São Paulo, 2023.

Programa de Pós-Graduação: Epidemiologia Experimental Aplicada às Zoonoses.

Área de concentração: Epidemiologia Experimental Aplicada às Zoonoses.

Orientador: Prof. Dr. Marcelo Bahia Labruna.
Coorientador: Prof. Dr. Sebastián Alejandro Muñoz Leal.

1. Argasidae. 2. Semiárido. 3. Febre Recorrente. I. Título.

Ficha catalográfica elaborada pela bibliotecária Camila Molgara Gamba, CRB 7070-8, da FMVZ/USP.

Faculdade de Medicina Veterinária e Zootecnia Universidade de São Paulo

Comissão de Ética no Uso de Animais

São Paulo, 2nd December 2022

CERTIFIED

We certify that the proposal entitled: "*Taxonomic study and survey of Borrelia spp. in ticks of the genus Ornithodoros in the Caatinga biome.*", protocol number CEUAx 2655061218 (ID 001084), under the responsibility Marcelo Bahia Labruna, agree with Ethical Principles in Animal Research adopted by Ethic Committee in the Use of Animals of School of Veterinary Medicine and Animal Science (University of São Paulo), and was approved in the meeting of day March 07, 2019.

Certificamos que a proposta intitulada: "*Estudo taxon?mico e pesquisa de Borrelia spp. em carrapatos do g?nero Ornithodoros no bioma Caatinga*", protocolado sob o CEUAx nº 2655061218, sob a responsabilidade de Marcelo Bahia Labruna, está de acordo com os princípios éticos de experimentação animal da Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia Universidade de São Paulo, e foi aprovado na reunião de 07 de março de 2019.

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

Profa. Dra. Camilla Mota Mendes
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

FOLHA DE AVALIAÇÃO

Autor: OLIVEIRA, Glauber Meneses Barboza de

Título: **Estudo taxonômico e pesquisa de *Borrelia* spp. em carrapatos do gênero *Ornithodoros* no bioma Caatinga**

Tese apresentada ao Programa de Pós-Graduação em Epidemiologia Experimental Aplicada às Zoonoses da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do título de Doutor em Ciências.

Data: _____/_____/_____

Banca Examinadora

Prof. Dr._____

Instituição:_____ Julgamento:_____

Prof. Dr._____

Instituição:_____ Julgamento:_____

Prof. Dr._____

Instituição:_____ Julgamento:_____

Prof. Dr._____

Instituição:_____ Julgamento:_____

Prof. Dr._____

Instituição:_____ Julgamento:_____

Este trabalho é dedicado aos meus pais, Ana Paula e José Ermano, os quais sempre me incentivaram nos estudos.

AGRADECIMENTOS

Agradeço a Deus primeiramente pelo dom da vida.

Agradeço a minha esposa Laísa pelo carinho e apoio imprescindível.

Agradeço a todos que contribuiram para a realização desse trabalho.

Agradeço a todos funcionários do VPS pelo apoio. Em especial ao Renato e Márcio, por todo apoio laboratorial, Pedrinho por sua dedicação com os animais do infectório, e ao Danival pelo excelente serviço na secretaria.

Agradeço a todos os colegas e amigos. Em especial a Bárbara, Isabela, Carol, Lina, Jaciara, Alejandro, Felipe, Fernando, Silvão, Tiaguinho e Jonas por toda ajuda e amizade.

Agradeço ao professor Maurício e a Eline pelo suporte essencial durante as coletas.

Agradeço ao meu co-orientador Sebastián, por todo apoio e ensinamentos.

Agradeço ao Marcelo, pela oportunidade e orientação.

Agradeço ao CNPq pela bolsa de doutorado concedida através do processo n° 170343/2018-9, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

RESUMO

OLIVEIRA, G.M.B. **Estudo taxonômico e pesquisa de *Borrelia* spp. em carrapatos do gênero *Ornithodoros* no bioma Caatinga**. 2022. 60 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2023.

Os carrapatos argasídeos são ártropodes hematófagos com capacidade de parasitar todas as classes de vertebrados terrestres, sendo incriminados na transmissão de diversos patógenos. No Brasil, até o presente momento são assinaladas 19 espécies de *Ornithodoros*, das quais sete já foram reportadas no bioma Caatinga. O carrapato *Ornithodoros fonsecai* apesar de parasitar preferencialmente os morcegos, tem demonstrado em situações oportunísticas um certo grau de antropofilia. Entre os patógenos transmitidos por carrapatos argasídeos do gênero *Ornithodoros* estão as bactérias do gênero *Borrelia*, principalmente as do grupo da febre recorrente (GFR). Dentre as espécies desse gênero, *Ornithodoros rudis* foi encontrado naturalmente infectado com *Borrelia venezuelensis*, sendo posteriormente realizado o isolamento dessa bactéria. Com base nos recentes avanços na diversidade de argasídeos e ocorrência de borrelias do GFR associadas aos carrapatos *Ornithodoros* no Brasil, o presente estudo teve como objetivo estudar a ocorrência e isolar *Borrelia* spp. de carrapatos *Ornithodoros*, com ênfase no bioma Caatinga, além de caracterizar morfologicamente e molecularmente populações de *O. fonsecai* de diferentes biomas. Em geral, os resultados ampliam a distribuição geográfica de *Ornithodoros rietcorreai* e trazem o primeiro relato de *O.* cf. *tabajara* em Pernambuco, os quais foram alimentados em cobaias no laboratório, na tentativa de isolar microorganismos do gênero *Borrelia*. Embora os animais infestados com *O. rietcorreai* não apresentaram infecção, foram obtidos três isolados de uma nova espécie de *Borrelia* do GFR a partir de *O*. cf. *tabajara*. Em adição, larvas e sequências parciais do gene mitocondrial 16S rRNA de *O. fonsecai* foram amostradas de diferentes estados brasileiros (biomas Amazônia, Caatinga, Cerrado, e Mata Atlântica). Análises morfométricas, filogenéticas e de componentes principais apontam para uma divergência entre populações de *O*. *fonsecai* de diferente biomas, com as larvas do bioma Caatinga apresentando tamanho menor em relação aos demais, e um relativo agrupamento filogenético de populações da Mata Atlântica e Cerrado separadas do bioma Caatinga. Novos estudos são necessários para esclarecer as relações entre as diferentes populações de *O*. *fonsecai*, realizar a descrição formal da nova espécie de *Borrelia* e definir o status taxonômico de *O*. cf. *tabajara*.

Palavras-chave: Argasidae. Semiárido. Febre Recorrente.

ABSTRACT

OLIVEIRA, G.M.B. **Taxonomic study and research on *Borrelia* spp. in ticks of the genus *Ornithodoros* in the Caatinga biome**. 2022. 60 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2023.

Argasid ticks are hematophagous arthropods that can parasitize all classes of terrestrial vertebrates, being incriminated in the transmission of several pathogens. In Brazil, to date, 19 species of *Ornithodoros* are reported, of which seven have been reported in the Caatinga biome. The tick *Ornithodoros fonsecai*, despite preferentially parasitizing bats, has shown a certain degree of anthropophilia in opportunistic situations. Among the pathogens transmitted by argasid ticks of the genus *Ornithodoros* are bacteria of the genus *Borrelia*, especially those of the relapsing fever group (RFG). Among the species of this genus, *Ornithodoros rudis* was found naturally infected with *Borrelia venezuelensis*, and isolation of this bacterium was subsequently performed. Based on recent advances in the diversity of argasids and the occurrence of RFG borrelia associated with *Ornithodoros* ticks in Brazil, the present study aimed to study the occurrence and isolation of *Borrelia* spp. from *Ornithodoros* ticks, with emphasis on the Caatinga biome, and to morphologically and molecularly characterize populations of *O. fonsecai* from different biomes. In general, the results broaden the geographic distribution of *Ornithodoros rietcorreai* and bring the first report of *O.* cf. *tabajara* in Pernambuco, which were fed to guinea pigs in the laboratory in an attempt to isolate microorganisms of the genus *Borrelia*. Although the animals infested with *O. rietcorreai* did not show infection, three isolates of a new *Borrelia* species from the RFG were obtained from *O.* cf. *tabajara*. In addition, larvae and partial sequences of the mitochondrial 16S rRNA gene of *O. fonsecai* were sampled from different Brazilian states (Amazon, Caatinga, Cerrado, and Atlantic Forest biomes). Morphometric, phylogenetic and principal component analyses point to a divergence among populations of *O. fonsecai* from different biomes, with larvae from the Caatinga biome showing smaller size in relation to the others, and a relative phylogenetic clustering of populations from the Atlantic Forest and Cerrado separated from the Caatinga biome. Further studies are needed to clarify the relationships among the different populations of *O. fonsecai*, to perform the formal description of the new species of *Borrelia* and to define the taxonomic status of *O.* cf. *tabajara*.

Keywords: Argasidae. Semiarid. Relapsing fever.

SUMMARY

1. INTRODUCTION

Vector-borne diseases occupy a share with about 22.8% among emerging zoonoses worldwide, of which almost 29% have emerged in the late past decade (Failloux and Moutailler 2015). Ticks hold great importance in the transmission of pathogens, possessing a wide variety in the spectrum of transmitted microorganisms (viruses, bacteria and protozoa) and second only to mosquitoes when it comes to the transmission of diseases to humans (Jongejan & Uilenberg 2004).

Argasids, ticks morphologically characterized by the presence of coxal glands and subterminal mouth apparatus in adults, have great medico-veterinary relevance worldwide, with representatives of the genus *Argas* that can affect avian productions, and *Ornithodoros* species that can transmit pathogens and cause toxicosis when biting humans (Venzal et al. 2006). Similar to the other genera of the argasidae family, *Ornithodoros* species usually inhabit arid or semiarid environments together with their hosts' niches, parasitizing reptiles, birds, and mammals (Estrada-Peña et al. 2018). In Brazil, 19 species of the genus *Ornithodoros* have been reported (Dantas-Torres et al. 2019; Muñoz-Leal et al. 2019, 2020, 2021a), of which seven have already been reported in the Caatinga biome (Luz et al. 2016; Muñoz-Leal et al. 2021a; Jorge et al. 2022).

With regard to disease transmission the argasids are known as main vectors of *Borrelia* species, especially the spirochetes of the relapsing fever group, a disease characterized by recurrence of febrile episodes (Cutler et al. 2015). In Brazil, the isolation of *Borrelia venezuelensis* (Brumpt, 1921) from *Ornithodoros rudis* Karsch, 1880 (Muñoz-Leal et al. 2018) as well as the molecular detection of four supposedly new *Borrelia* species in argasids (Muñoz-Leal et al. 2021b) point to the risk of human exposure to these pathogens. In a research conducted by Oliveira et al. (2018) the parasitism of *Ornithodoros rietcorreai* Labruna, Nava & Venzal, 2016 on humans was reported with the finding of toxicosis. These studies highlight the importance of studies on the acarological fauna and possible related pathogens in the Caatinga biome, which is characterized by the semiarid climate.

Considering this background, this study aimed to extend this knowledge exploring the diversity of argasid ticks in the Caatinga, try to isolate *Borrelia* from these ticks and compare populations of *Ornithodoros fonsecai* Labruna & Venzal, 2009 from different biomes. The results of this study have been adapted for publication into two separated manuscripts.

1.1 REFERENCES

CUTLER, Sally J. Relapsing fever borreliae: a global review. **Clinics in laboratory medicine**, v. 35, n. 4, p. 847-865, 2015.

DANTAS-TORRES, Filipe et al. Ticks (Ixodida: Argasidae, Ixodidae) of Brazil: updated species checklist and taxonomic keys. **Ticks and tick-borne diseases**, v. 10, n. 6, p. 101252, 2019.

ESTRADA-PEÑA, Agustín; MIHALCA, Andrei Daniel; PETNEY, Trevor N. (Ed.). **Ticks of Europe and North Africa: a guide to species identification.** Springer, 2018.

FAILLOUX, Anna-Bella; MOUTAILLER, Sarah. Zoonotic aspects of vector-borne infections. **Rev Sci Technol**, v. 34, n. 1, p. 175-183, 2015.

JONGEJAN, F; UILENBERG, G. The global importance of ticks. Parasitology, v. 129, n. S1, p. S3-S14, 2004.

JORGE, Felipe R. et al. New records of soft ticks (Acari: Argasidae) in the Caatinga biome of Brazil, with a phylogenetic analysis of argasids using the nuclear Histone 3 (H3) gene. **Experimental and Applied Acarology**, v. 86, n. 4, p. 567-581, 2022.

LUZ, Hermes Ribeiro et al. Ticks parasitizing bats (Mammalia: Chiroptera) in the Caatinga biome, Brazil. **Revista Brasileira de Parasitologia Veterinária**, v. 25, p. 484-491, 2016.

MUÑOZ-LEAL, Sebastián et al. Isolation and molecular characterization of a relapsing fever *Borrelia* recovered from *Ornithodoros rudis* in Brazil. **Ticks and tick-borne diseases**, v. 9, n. 4, p. 864-871, 2018.

MUÑOZ-LEAL, Sebastián et al. A third species of *Nothoaspis* Keirans & Clifford, 1975 (Acari: Argasidae): *Nothoaspis setosus* (Kohls, Clifford & Jones, 1969) n. comb. **Systematic Parasitology**, v. 96, n. 7, p. 595-602, 2019.

MUÑOZ-LEAL, Sebastián et al. *Ornithodoros cerradoensis* n. sp.(Acari: Argasidae), a member of the *Ornithodoros talaje* (Guérin-Méneville, 1849) group, parasite of rodents in the Brazilian Savannah. **Ticks and Tick-borne Diseases**, v. 11, n. 5, p. 101497, 2020.

MUÑOZ-LEAL, Sebastián et al. A new species of soft tick from dry tropical forests of Brazilian Caatinga. **Ticks and Tick-borne Diseases**, p. 101748, 2021a.

MUÑOZ-LEAL, Sebastián et al. Relapsing Fever Group Borreliae in Human-Biting Soft Ticks, Brazil. **Emerging infectious diseases**, v. 27, n. 1, p. 321, 2021b.

OLIVEIRA, Stefan Vilges et al. Human parasitism and toxicosis by *Ornithodoros rietcorreai* (Acari: Argasidae) in an urban area of Northeastern Brazil. **Ticks and tick-borne diseases**, v. 9, n. 6, p. 1494-1498, 2018.

VENZAL, J. M. et al. Família Argasidae: características gerais, comentários e chave para gênero e espécies. Barros-Battesti DM, Arzua M, Bechara GH. **Carrapatos de Importância**

Médico-Veterinária da Região Neotropical: Um guia ilustrado para identificação de espécies. São Paulo: Vox/ICTTD-3, p. 223, 2006.

2. NEW RECORDS OF SOFT TICKS IN THE CAATINGA BIOME, WITH A MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION IN *Ornithodoros fonsecai* POPULATIONS

2.1 INTRODUCTION

Soft ticks (Ixodida: Argasidae) are blood-feeding arthropods that can parasitize all classes of terrestrial vertebrates (Hoogstraal 1985). The traditional classification of family Argasidae includes five genera: *Argas*, *Antricola*, *Otobius*, *Nothoaspis* and *Ornithodoros*; however this classification do not represent a consensus among tick taxonomists and the debate remains open until further research provides more answers (Nava et al. 2017; Mans et al. 2019). In Brazil, 19 species of the genus *Ornithodoros* have been reported (Dantas-Torres et al. 2019; Muñoz-Leal et al. 2019a, 2020, 2021a), in addition to the ongoing description of "*Ornithodoros* sp. Ubajara" (Jorge et al. 2022).

The medical importance of *Ornithodoros* ticks has gradually increased in recent years, with the isolation of the tick-borne relapsing fever (TBRF) pathogen *Borrelia venezuelensis* (Brumpt, 1921) from *Ornithodoros rudis* Karsch, 1880 in Maranhão state (Muñoz-Leal et al. 2018a), molecular detection of putatively new species of *Borrelia* in four species of *Ornithodoros* ticks from two brazilian states (Muñoz-Leal et al. 2021b), molecular detection of *Rickettsia* sp. strain Itinguçú in *Ornithodoros faccini* Barros-Battesti, Landulfo & Luz, 2015 (Peixoto et al. 2021) and the molecular detection of a rickettsial organism of the spotted fever group (SFG) in *Ornithodoros* cf. *mimon* (Dantas-Torres et al. 2022).

For medical entomologists, not only the capacity of distinguish species is important, but also the possibility of detect cryptic species and conspecific populations that are geographically or ecologically separated, in order to promote relevant control strategies (Kaba et al. 2012), this importance can be extrapolated for *Ornithodoros* ticks since an strict host specificity with borreliae has been reported for most of the RFG agents (Cutler 2015, Talagrand-Reboul et al. 2018).

Ornithodoros fonsecai Labruna & Venzal, 2009 is a tick that use bats as main hosts, having a geographical distribution in different states of Brazil, including areas of Caatinga and Cerrado biomes, and although the role of *O. fonsecai* as a vector of pathogens is still unknown, this tick is quite anthropophilic and its bite can cause severe inflammatory responses (Labruna and Venzal 2009; Barros-Battesti et al. 2012; Luz et al. 2016).

The state of Pernambuco, Brazil, is almost entirely covered by the Caatinga biome, one of the largest and most diverse seasonally dry tropical forests in Latin America (da Silva et al. 2018). Researchs in the semiarid region of Brazil are important to better determine the geographic distribution and diversity of soft ticks. Thus, the present work aimed to identify soft ticks collected directly from the environment in various regions of Pernambuco State, Brazil and analyze morphologically and molecularly *O. fonsecai* populations from different biomes.

2.2 MATERIAL AND METHODS

2.2.1 Collection of soft ticks

During a field expedition in October 2019, ticks were collected from the environment in nine localities in the state of Pernambuco, northeastern Brazil: five points at National Park of Catimbau, Buíque-PE (i) Furna do Walmir (8°35'35.0''S, 37°14'7.6''W; 823 m); (ii) Brejo de fora (8°35'23.1''S, 37°14'23.6''W; 819 m); (iii) Serra das torres (8°34'0.3''S, 37°14'27.8''W; 777 m); (iv) Furna do gato (08°35'9.9''S, 37°14'26.5''W; 795 m) (v) Caverna meu rei (8°29'12''S, 37°16'48''W; 777 m); two points on private properties in Floresta-PE (vi) Malhada vermelha (8°36'44.2''S, 38°32'29.9''W; 377 m); (vii) Poço de areia (8°39'32.8''S, 38°27'58.2''W; 379 m); one point at (viii) National Forest of Negreiros, Serrita-PE (7°59'22.0''S, 39°24'46.1''W; 475 m); and one in a private property from (ix) Capim district, Petrolina-PE (9°9'40.3''S, 40°26'16.2''W; 454 m).

The eight localities are located within the Caatinga biome, which is characterized by a semiarid climate (temperatures averaging 27°C throughout the year, mean annual rainfall typically <500 mm), and deciduous vegetation composed typically of xeric shrub land and thorn forest that consist primarily of small, thorny trees that shed their leaves seasonally (da Silva et al. 2018). The prospected environments included caves and rock formations with vestiges (e.g., feces) of bats and wild rodents. Ticks were collected with tweezers from under the rocks and stored in punctured plastic vials, which were kept in an improvised environmental chamber (1.5 liter-plastic bottle with a piece of humid cotton on the bottom) until the arrival at the laboratory, where ticks were placed in an incubator at 26°C and 80% relative humidity.

Live adults and nymphs were visualized and counted under a stereomicroscope (Zeiss Stemi SV 11, Zeiss, Münich, Germany). Species were determined according to Dantas-Torres et al. (2019) and original descriptions (Estrada-Peña et al. 2004; Labruna and Venzal 2009; Dantas-Torres et al. 2012; Labruna et al., 2016; Muñoz-Leal et al. 2021a).

2.2.2 Sample sites of *Ornithodoros fonsecai*

For comparative analysis purposes, partial sequences of the 16S rRNA gene (Present study/GenBank) and larvae of *O. fonsecai* were sampled from three localities from Brazil: (i) Gruta São Miguel, Bonito municipality, Mato Grosso do Sul State (21°06'29''S, 56°34'49''W); (ii) Furna de Araticum, Araticum district, Ubajara municipality, Ceará State (03°48'11.5''S, 40°52'40.4''W); (iii) Furna do Gato, Buíque municipality, Pernambuco State (08°35'9.9''S, 37°14'26.5''W). Only sequences were sampled from six localities: (iv) Gruta da Lagoa Azul, Nobres municipality, Mato Grosso State (14°35'18.4''S, 55°58'2.1''W); (v) Parque Estadual da Pedra Branca, Rio de Janeiro municipality, Rio de Janeiro State (22°56'23.1''S, 43°24'12.9''W); (vi) Gruta do Morcego Branco, Ubajara municipality, Ceará State (03°49'53.2''S, 40°54'1.0''W); (vii) Caverna Onça, Potiretama municipality, Ceará State (05°46'12.1''S, 38°10'57.6''W); (viii) Reserva Natural Serra das Almas, Crateús municipality, Ceará State (05°08'30.0''S, 40°54'58.6''W); (ix) Gruta do Brejinho, Araripe municipality, Ceará State (07°13'48.8''S, 39°59'48.6''W). Finally only larvae were sampled from two localities: (x) Furna do Fim do Morro do Parafuso, Paripiranga municipality, Bahia State (10°38'25.7''S, 37°52'2.9''W); (xi) Parauapebas municipality, Pará State (6°20'20.4''S, 49°57'34.3''W) and (6°20'7.2''S, 49°57'33''W) (Fig. 1).

With the exception of samples from Furna do Gato and Gruta do Brejinho localities, which were visited during the general collection of argasids and in a previous expedition respectively, all other samples from other locations were obtained from previously published or otherwise available work (Fig. 1).

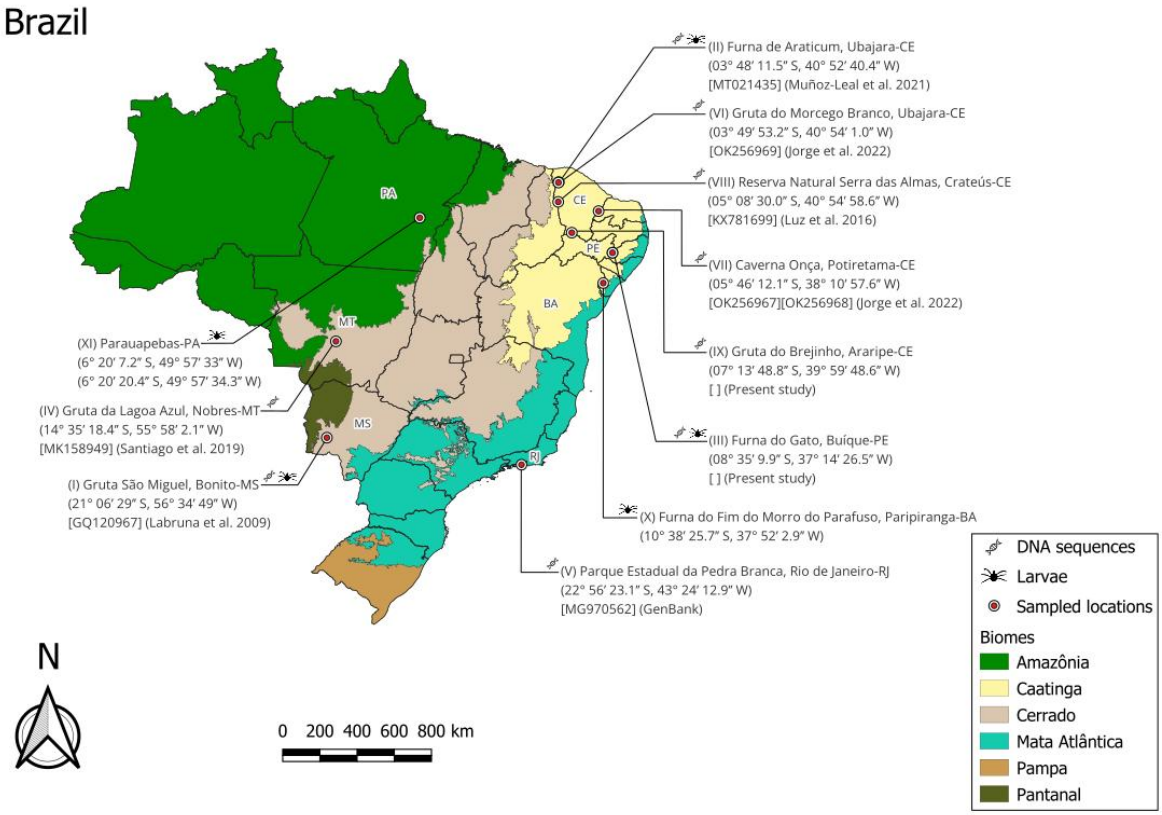

Figure 1. Map of Brazil showing localities where *Ornithodoros fonsecai* were sampled.

2.2.3 Morphological analyses and Principal Component Analysis (PCA)

In order to obtain unfed larvae, females collected in the present and previous studies were taken alive to the laboratory and placed in incubators at 26°C and 80% humidity, and these females subsequently laid eggs. The only exception were the field-collected larvae in the municipality of Parauapebas-PA, which were kindly provided by the Acari Collection of the Butantan Institute (IBSP), access number: IBSP 15865 and IBSP 15869. All larvae were killed and preserved in 70% ethanol, and then 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).

A principal component analysis (PCA) based on a Pearson's correlation matrix was applied on morphometric variables for unengorged larvae from six different populations to elucidate relationships among them. In addition we included data from the larvae of *Ornithodoros rietcorreai* Labruna, Nava & Venzal 2016, retrieved from Labruna et al. (2016). Raw measurements were log (x + 1)-transformed to standardize variances and improve normality.

2.2.4 Molecular and phylogenetic analyses of *O. fonsecai*

One female and a nymph of *O. fonsecai* from Furna do Gato and two nymphs from Brejinho were submitted to DNA extraction by the guanidine isothiocyanate and phenol/chloroform technique (Sangioni et al. 2005), and tested by a PCR assay targeting a ca. 460 bp fragment of the tick 16S rRNA mitochondrial gene, as described by Mangold et al. (1998). PCR amplicons 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 (Altschul et al. 1990).

An alignment of 54 sequences was constructed with MAFFT (Katoh et al. 2002). Best nucleotide substitution models were calculated with MEGA 7 (Kumar et al. 2016). An approximately maximum-likelihood phylogenetic tree was inferred with PhyML (Guindon and Gascuel, 2003) with the Generalized Time-Reversible substitution model, and five rates categories of sites, optimizing the Gamma20 likelihood. Ornithodoros rostratus Aragão, 1911 (DQ295780), and Ornithodoros brasiliensis Aragão, 1923 (GU198363) rooted the tree.

2.2.5 Ethics statement

Field collections of ticks were authorized by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio permit Sisbio 65137-1).

2.3 RESULTS

2.3.1 PCA and analysis of mitochondrial 16S rDNA sequences

The results of the PCA for the morphological characters showed a clear difference between the group of *O. fonsecai* populations from Pernambuco, Ceará and Bahia states and the group of populations from Mato Grosso do Sul and Pará states, as well for the additional specie included, with only one exception of the outlier specimen "OfonsecaiPA-n°6-01" (Table 1, Fig. 2). 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 anterolateral setae and central setae.

DNA from the female from Furna do Gato (PE) and one nymph from Brejinho (CE) yielded identical 16S rDNA sequences, and the others two sequences of nymphs from these localities (one of each locality) were 99.4% (361/363-bp) identical to each other. The Maximum likelihood tree inferred from mithocondrial 16s rDNA sequences of some Neotropical Argasidae are presented in Fig. 2. The maximum-likelihood analysis showed *O. fonsecai* populations forming a monophyletic group. However, *O. fonsecai* from MS and RJ formed a single clade (98.8 bootstrap) grouping within a larger clade (100 bootstrap) formed by *O. fonsecai* from PE, CE and MT.

Table 1. Morphometric variables (mean and range values in millimeters) of *Ornithodoros fonsecai* larvae from Bahia (BA; n=10), Ceará **(**CE; n=15), Mato Grosso do Sul (MS; n=12), Pará (PA; n=4), Pernambuco (PE; n=20) utilized in the principal component analysis (PCA)

	O. fonsecai **BA**	***O. fonsecai*** **CE**	***O. fonsecai*** **MS**	***O. fonsecai*** **PA**	***O. fonsecai*** **PE**
Body length [a]	0.882 (0.866-0.900)	0.931 (0.904-0.956)	0.986 (0.959-1.057)	1.008 (0.953-1.062)	0.874 (0.821-0.909)
Body length [b]	0.502 (0.492-0.529)	0.525 (0.487-0.552)	0.591 (0.551-0.634)	0.619 (0.585-0.660)	0.482 (0.405-0.532)
Body width	0.446 (0.414-0.474)	0.443 (0.417-0.474)	0.529 (0.495-0.565)	0.498 (0.430-0.603)	0.436 (0.386-0.484)
Dorsal plate: length	0.244 (0.228-0.262)	0.239 (0.227-0.253)	0.248 (0.230-0.263)	0.238 (0.236-0.241)	0.243 (0.231-0.261)
Dorsal plate: width	0.172 (0.156-0.185)	0.171 (0.158-0.184)	0.176 (0.167-0.187)	0.156 (0.151-0.166)	0.167 (0.148-0.187)
Dorsal setae pairs: total	14	14	14	14	14
Dorsal setae pairs: dorsolateral	11	11	11	11	11
Dorsal setae pairs: central	3	3	3	3	3
Dorsal anterolateral setae: Al 1	0.136 (0.126-0.149)	0.142 (0.129-0.152)	0.125 (0.115-0.133)	0.137 (0.135-0.141)	0.119 (0.090-0.135)
Dorsal anterolateral setae: Al 2	0.121 (0.105-0.134)	0.130 (0.123-0.137)	0.119 (0.102-0.132)	0.126 (0.122-0.130)	0.113 (0.080-0.126)
Dorsal anterolateral setae: Al 3	0.113 (0.099-0.136)	0.124 (0.115-0.131)	0.106 (0.078-0.118)	0.110 (0.104-0.119)	0.110 (0.087-0.124)
Dorsal anterolateral setae: Al 4	0.109 (0.096-0.126)	0.121 (0.109-0.137)	0.101 (0.086-0.112)	0.111 (0.106-0.118)	0.104 (0.065-0.117)
Dorsal anterolateral setae: Al 5	0.096 (0.090-0.103)	0.110 (0.096-0.121)	0.095 (0.083-0.108)	0.093 (0.090-0.095)	0.096 (0.075-0.108)
Dorsal anterolateral setae: Al 6	0.098 (0.090-0.109)	0.108 (0.102-0.115)	0.097 (0.081-0.108)	0.099 (0.095-0.103)	0.098 (0.086-0.110)
Dorsal anterolateral setae: Al 7	0.099 (0.092-0.110)	0.109 (0.105-0.115)	0.101 (0.088-0.114)	0.100 (0.093-0.108)	0.096 (0.085-0.107)
Dorsal posterolateral setae: Pl 1	0.093 (0.084-0.101)	0.090 (0.079-0.104)	0.080 (0.074-0.091)	0.082 (0.071-0.093)	0.082 (0.068-0.093)
Dorsal posterolateral setae: Pl 2	0.087 (0.079-0.092)	0.087 (0.065-0.102)	0.083 (0.064-0.089)	0.088 (0.080-0.101)	0.082 (0.069-0.093)
Dorsal posterolateral setae: Pl 3	0.085 (0.074-0.093)	0.087 (0.076-0.096)	0.078 (0.047-0.092)	0.083 (0.076-0.087)	0.079 (0.069-0.087)
Dorsal posterolateral setae: Pl 4	0.075 (0.065-0.081)	0.077 (0.067-0.088)	0.079 (0.069-0.090)	0.078 (0.073-0.083)	0.073 (0.058-0.081)
Central setae 1: length	0.107 (0.090-0.117)	0.116 (0.104-0.132)	0.104 (0.080-0.118)	0.106 (0.091-0.119)	0.107 (0.092-0.132)
Central setae 2: length	0.086 (0.073-0.095)	0.093 (0.081-0.102)	0.075 (0.052-0.087)	0.077 (0.071-0.083)	0.081 (0.064-0.095)
Central setae 3: length	0.086 (0.074-0.091)	0.087 (0.079-0.098)	0.070 (0.047-0.081)	0.090 (0.081-0.098)	0.081 (0.062-0.091)
Sternal setae 1: length	0.068 (0.053-0.075)	0.064 (0.054-0.073)	0.072 (0.065-0.077)	0.064 (0.057-0.071)	0.062 (0.052-0.069)
Sternal setae 2: length	0.064 (0.051-0.073)	0.059 (0.053-0.068)	0.072 (0.063-0.079)	0.069 (0.061-0.077)	0.062 (0.055-0.070)
Sternal setae 3: length	0.071 (0.058-0.076)	0.064 (0.059-0.071)	0.072 (0.069-0.076)	0.074 (0.066-0.084)	0.066 (0.052-0.073)
Circumanal setae 1: length	0.055 (0.046-0.067)	0.055 (0.045-0.066)	0.055 (0.045-0.066)	0.053 (0.043-0.059)	0.054 (0.039-0.064)
Circumanal setae 2: length	0.086 (0.074-0.097)	0.087 (0.072-0.094)	0.089 (0.078-0.107)	0.084 (0.067-0.095)	0.087 (0.066-0.095)

Table 1. (Continued).

	O. fonsecai BA	*O. fonsecai* CE	*O. fonsecai* MS	*O. fonsecai* PA	*O. fonsecai* PE
Circumanal setae 3: length	0.109 (0.105-0.117)	0.111 (0.102-0.120)	0.103 (0.084-0.115)	0.105 (0.087-0.112)	0.103 (0.081-0.114)
Posteromedian setae: length	0.078 (0.067-0.087)	0.082 (0.071-0.096)	0.067 (0.048-0.079)	0.085 (0.076-0.092)	0.071 (0.057-0.083)
Postcoxal setae: length	0.036 (0.030-0.045)	0.036 (0.031-0.043)	0.039 (0.028-0.051)	0.035 (0.027-0.043)	0.035 (0.027-0.044)
Anal plate setae: As	0.060 (0.051-0.068)	0.061 (0.053-0.067)	0.052 (0.036-0.062)	0.050 (0.043-0.059)	0.058 (0.051-0.068)
Length of basis capituli [c]	0.173 (0.166-0.184)	0.176 (0.163-0.188)	0.193 (0.169-0.212)	0.186 (0.180-0.199)	0.168 (0.156-0.185)
Length of basis capituli [d]	0.226 (0.212-0.238)	0.216 (0.201-0.233)	0.247 (0.223-0.264)	0.242 (0.233-0.253)	0.221 (0.210-0.236)
Length of capitulum [e]	0.431 (0.419-0.446)	0.441 (0.430-0.457)	0.481 (0.426-0.502)	0.479 (0.470-0.489)	0.419 (0.383-0.449)
Width of basis capituli	0.255 (0.244-0.265)	0.223 (0.209-0.244)	0.258 (0.229-0.270)	0.244 (0.224-0.253)	0.232 (0.200-0.262)
Posthypostomal setae Ph1	0.014 (0.011-0.018)	0.013 (0.011-0.017)	0.016 (0.012-0.021)	0.017 (0.014-0.021)	0.016 (0.012-0.021)
Posthypostomal setae Ph2	0.039 (0.034-0.050)	0.040 (0.032-0.048)	0.037 (0.030-0.046)	0.054 (0.047-0.060)	0.033 (0.022-0.042)
Distance Ph1 - Ph1	0.024 (0.020-0.027)	0.027 (0.021-0.031)	0.027 (0.023-0.031)	0.027 (0.025-0.030)	0.025 (0.019-0.029)
Distance Ph2 - Ph2	0.086 (0.081-0.092)	0.084 (0.073-0.088)	0.089 (0.085-0.096)	0.090 (0.085-0.097)	0.082 (0.072-0.089)
Palpal length	0.302 (0.290-0.311)	0.312 (0.298-0.324)	0.336 (0.297-0.350)	0.330 (0.313-0.341)	0.296 (0.277-0.332)
Length article I	0.072 (0.065-0.080)	0.082 (0.077-0.087)	0.083 (0.075-0.087)	0.091 (0.087-0.092)	0.075 (0.069-0.081)
Length article II	0.101 (0.090-0.108)	0.114 (0.100-0.121)	0.124 (0.115-0.133)	0.110 (0.094-0.121)	0.104 (0.095-0.110)
Length article III	0.095 (0.091-0.099)	0.092 (0.080-0.100)	0.106 (0.099-0.115)	0.105 (0.103-0.108)	0.091 (0.083-0.101)
Length article IV	0.052 (0.049-0.055)	0.052 (0.048-0.058)	0.062 (0.052-0.070)	0.051 (0.041-0.056)	0.052 (0.042-0.064)
Width article I	0.031 (0.031-0.033)	0.036 (0.032-0.038)	0.039 (0.036-0.042)	0.036 (0.034-0.039)	0.034 (0.032-0.038)
Width article II	0.041 (0.037-0.042)	0.043 (0.040-0.045)	0.046 (0.043-0.049)	0.044 (0.040-0.047)	0.041 (0.037-0.046)
Width article III	0.037 (0.035-0.038)	0.038 (0.035-0.041)	0.042 (0.039-0.046)	0.041 (0.036-0.046)	0.035 (0.031-0.039)
Width article IV	0.022 (0.021-0.024)	0.024 (0.023-0.025)	0.031 (0.028-0.032)	0.023 (0.022-0.025)	0.023 (0.021-0.026)
Hypostome: length [f]	0.255 (0.250-0.268)	0.262 (0.251-0.269)	0.278 (0.254-0.292)	0.284 (0.281-0.290)	0.249 (0.221-0.257)
Hypostome: length [g]	0.206 (0.200-0.214)	0.219 (0.209-0.226)	0.224 (0.207-0.233)	0.232 (0.230-0.234)	0.200 (0.178-0.208)
Hypostome: length [h]	0.200 (0.195-0.207)	0.218 (0.209-0.225)	0.220 (0.199-0.227)	0.226 (0.223-0.229)	0.197 (0.177-0.207)
Hypostome: width at midlength	0.054 (0.051-0.059)	0.057 (0.051-0.060)	0.056 (0.051-0.064)	0.057 (0.054-0.059)	0.047 (0.041-0.056)
Hypostome: width at the base	0.067 (0.064-0.073)	0.064 (0.058-0.068)	0.061 (0.054-0.070)	0.063 (0.059-0.067)	0.061 (0.053-0.071)
Apical dental formula	3	3	3	3	3
Median dental formula	3	3	3	3	3
Basal dental formula	2	2	2	2	2
Denticles in hypostomal row 1	18 to 19	20 to 21	19 to 21	18 to 20	17 to 20
Denticles in hypostomal row 2	16 to 17	17 to 20	17 to 19	17 to 18	15 to 18

Table 1. (Continued)

	O. fonsecai **BA**	*O. fonsecai* **CE**	*O. fonsecai* **MS**	*O. fonsecai* **PA**	*O. fonsecai* **PE**
Denticles in hypostomal row 3	9 to 10	10 to 13	9 to 12	10 to 11	10 to 13
Denticles in hypostomal row 4	0 to 1	0 to 3	0 to 1	0 to 1	0 to 2
Tarsus I: length	0.261 (0.250-0.272)	0.276 (0.269-0.282)	0.312 (0.277-0.335)	0.323 (0.314-0.334)	0.262 (0.238-0.280)
Tarsus I: width	0.072 (0.063-0.075)	0.063 (0.059-0.067)	0.084 (0.065-0.099)	0.079 (0.077-0.085)	0.071 (0.059-0.079)

[a] including capitulum.
[b] not including capitulum.
[c] length of basis capituli:measured from the posterior margin of basis capituli to posthypostomal setae 1 (Ph1).
[d] length of basis capituli: measured from the posterior margin of basis capituli to insertion of hypostome.
[e] length of capitulum: measured from the posterior margin of basis capituli to the anterior end of hypostome.
[f] measured to Ph1.
[g] measured to the posterior end of the toothed portion.
[h] measured to the insertion.

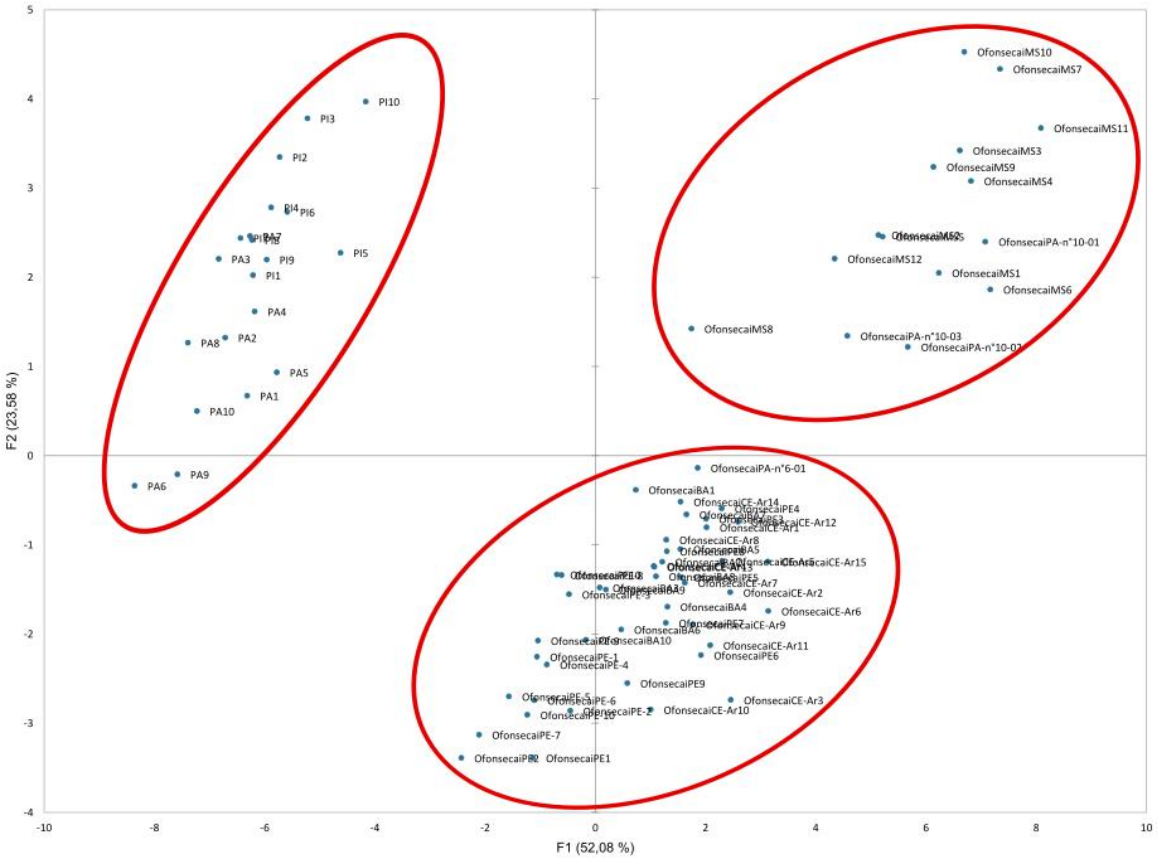

Figure 2. Principal components analysis (PCA) of the body and setal measurements of the larvae of *Ornithodoros fonsecai* (BA, CE, MS, PA and PE States) and *Ornithodoros rietcorreai*, using the features detailed in Table 1. Morphometric variables for the larvae of *O. rietcorreai* were retrieved from Labruna et al. (2016). Each point represents the position of a measured specimen in the reduced morphometric space.

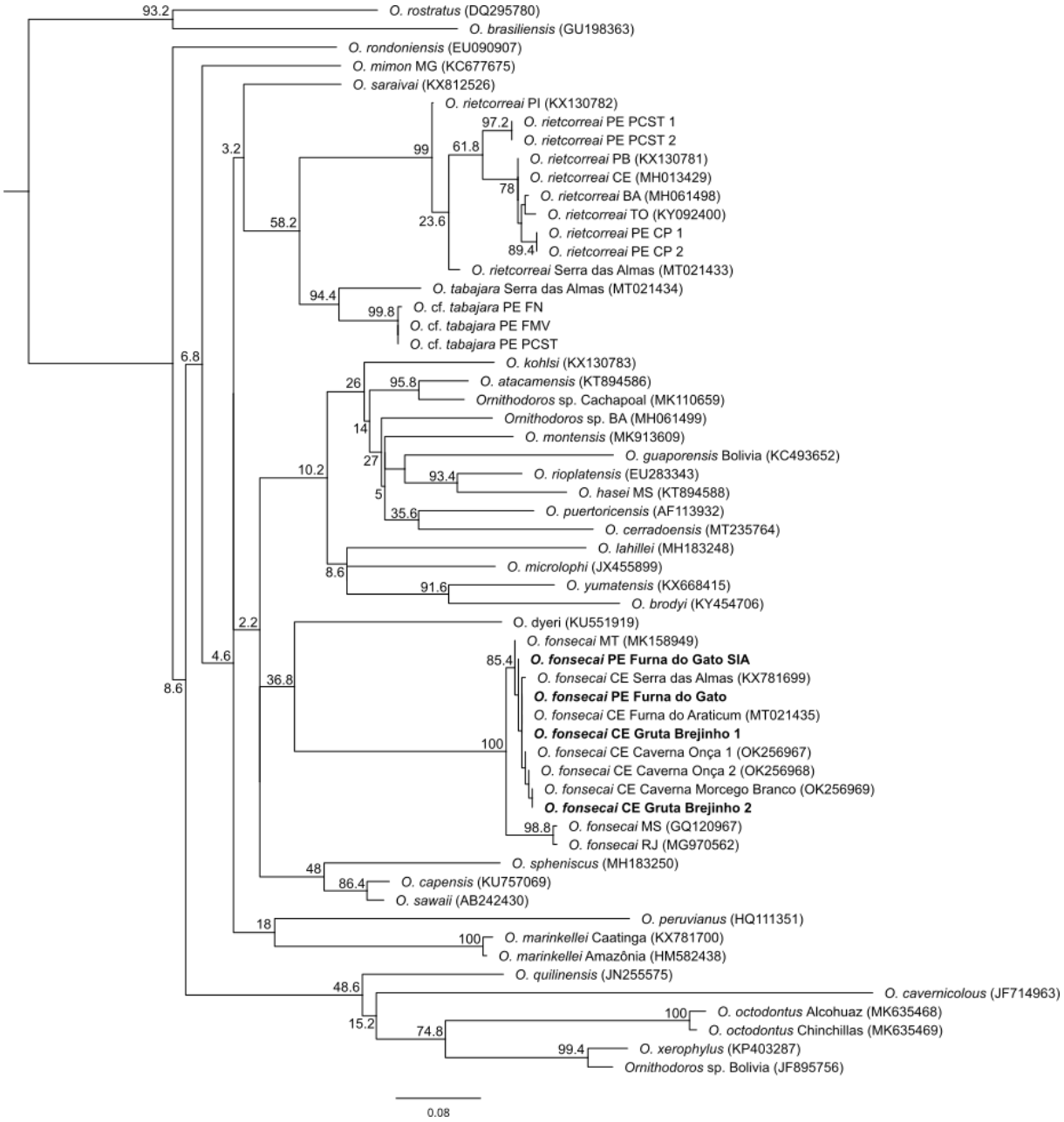

Figure 3. Maximum likelihood phylogenetic tree inferred for a subset of neotropical Ornithodorinae using mitochondrial 16S rDNA sequences. The position of *Ornithodoros fonsecai* provided by the present study are highlighted in bold.

2.3.2 Additional soft ticks collected

In total, 1,904 ticks were collected in this study. One *Antricola* specie and four *Ornithodoros* species were identifed based on external morphology, as follows: *Antricola guglielmonei* Estrada-Peña, Barros-Battesti & Venzal, 2004 (n=11), *Ornithodoros cavernicolous* Dantas-Torres, Venzal & Labruna, 2012 (n=260), *O. fonsecai* (n=126), *O. rietcorreai* Labruna, Nava & Venzal, 2016 (n=932) and *Ornithodoros* cf. *tabajara* (n=575) (Table 2).

Antricola guglielmonei was collected in one cave of Tupanatinga municipality. *Ornithodoros cavernicolous* was collected in two caves and one rock shelter, all inhabited by bats, in two municipalities (Buíque and Floresta). *Ornithodoros fonsecai* was collected in two bat-inhabited caves of Buíque municipality. Specimens of *O. ritecorreai* and *O.* cf. *tabajara* were collected in rock formations with vestiges (e.g., feces) of wild rodents, including visualizations of the rock cavy *Kerodon rupestris* (Wied-Neuwied, 1820) (Rodentia: Caviidae), in four (Buíque, Floresta, Serrita and Petrolina) and three municipalities (Buíque, Floresta, Serrita), respectively.

Table 2. Soft ticks collected in various localities from state of Pernambuco, Caatinga biome, Brazil

Tick species	No. of specimens	Locality	Municipality
Antricola guglielmonei	1F, 4M, 6N	Caverna Meu Rei	Tupanatinga
Ornithodoros cavernicolous	4F, 6M, 3N	Furna do Walmir	Buíque
	1N	Furna do Gato	Buíque
	69F, 102M, 75N	Poço de Areia	Floresta
Ornithodoros fonsecai	12F, 44M, 69N	Furna do Gato	Buíque
	1N	Furna do Walmir	Buíque
Ornithodoros rietcorreai	5F, 3M, 18N	Serra das Torres	Buíque
	21F, 29M, 318N	Malhada Vermelha	Floresta
	18F, 34M, 395N	FLONA Negreiros	Serrita
	4F, 4M, 83N	Capim district	Petrolina
Ornithodoros cf. *tabajara*	34F, 31M, 80N	Serra das Torres	Buíque
	1N	Brejo de Fora	Buíque
	45F, 51M, 194N	Malhada Vermelha	Floresta
	1F	Poço de Areia	Floresta
	20F, 11M, 107N	FLONA Negreiros	Serrita

2.4 DISCUSSION

The present work constitutes the first broader approach on *O. fonsecai* populations using morphological and molecular analyses, including representatives from the Caatinga, Amazon, Cerrado and Atlantic Forest biomes. We also provide new records of soft ticks for the semiarid region of Pernambuco in areas of the Caatinga biome.

Together, the present findings of smaller larvae in populations from Caatinga biome than larvae from Cerrado and Amazon biomes, and the clustering of populations from Cerrado and Atlantic Forest biomes in the phylogenetic analyses suggests an environmental influence driving a 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 the major part of their life-cycle in the off host environment and require specific conditions of humidity and temperature to survive (Hoogstraal et al. 1985; Dupraz et al. 2016). Contrary to expected, the population of MT (also in the Cerrado biome) clustered in the major clade with PE and CE populations (Caatinga biome), a possible explanation would be an variable strength in correlation between morphometric and genetic distances (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; da Silva et al. 2018). Therefore, the reduced size in larvae from Caatinga maybe can be explained in part by the temperature size rule (TSR), a pattern that consist in a 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 posses a generalist nature (with a large number of variables involved), was demonstrated that smaller arthropods are more likely to demonstrate a classic TSR (Klok et al. 2013).

With regards to additional soft ticks collected, we found *Antricola guglielmonei* in the same cave that it was previously reported, however the cave was abandoned by the bats, and just few specimens were collected in the guano, in contrast to the findings of Barbier et al. (2020) that found large amounts of these ticks and just in the ceiling and walls of the cave. Another species found were the two cave-dwelling ticks *O. caverniculous* and *O. fonsecai*, that are primarily associated with phyllostomid bats (Labruna and Venzal 2009; Dantas-

Torres et al. 2012). Our findings of these two species sharing same caves are in accordance to the previously reported by Jorge et al. (2022), including a similar pattern of inverse proportion of specimens collected, so maybe some kind of competition or micro-environmental favoritism can be investigated in further studies.

In our analysis of *O. fonsecai* populations we included samples from Amazon and Atlantic Forest biomes, these results go beyond previous reports, showing that *O. fonsecai* posses a wider distribution within four major Brazilian biomes (Amazon, Atlantic forest, Caatinga, and Cerrado) similar to *O. cavernicolous* (Labruna and Venzal 2009; Dantas-Torres et al. 2012; Luz et al. 2016; Muñoz-Leal et al. 2018b, 2021b; Costa et al. 2020; Jorge et al. 2022). A major source of limitation in *O. fonsecai* analyses is due to lack of morphometrical information about nymphs and adults, host usage, and micro environmental variables.

Another promising finding was the presence of *O. rietcorreai* in their characteristic landscape (rocky cavy habitats) living in sympatry with *O.* cf. *tabajara,* corroborating with previous studies (Labruna et al. 2016; Muñoz-Leal et al. 2021a). The specie *O. rietcorreai* was proved to be mainly associated with wild rodents, with some records in reptiles and a possible usage of bats as hosts (Labruna et al. 2016; Alcantara et al. 2018; Maia et al. 2018; Jorge et al. 2022). In relation to its geographic distribution, *O. rietcorreai* was found in Caatinga and Cerrado biomes (Labruna et al. 2016; Alcantara et al. 2018; Muñoz-Leal et al. 2019b; Muñoz-Leal et al. 2019c; Costa et al. 2020; Muñoz-Leal et al. 2021b), and has called attention to public health with the detection of multiple microorganisms (Muñoz-Leal et al. 2019b; Muñoz-Leal et al. 2021b), associated with some degree of urbanization and anthropophilic behavior (Oliveira et al. 2018; Muñoz-Leal et al. 2019c; Jorge et al. 2022).

The *O.* cf. *tabajara* was found in sympatry in three of the four localities of *O. rietcorreai*, like its closest taxon *O. tabajara* this tick harbors a *Borrelia* of Relapsing Fever Group (RFG), and studies to understand its formal identity are in progress “data not shown”. In the present work, we provide four new records of *O. rietcorreai* and the first report of *O.* cf. *tabajara* in three localities within the Pernambuco state, these localities include conservation units and private properties, therefore possible implications for animal and human health need to be investigated.

In this study we expand the geographical distribution and provide new records of soft ticks in the Caatinga biome. Additionally, we provide evidences of significant morphological divergence in larvae and relative genetic distance between *O. fonsecai* populations from different biomes.

2.5 REFERENCES

ALCANTARA, Edna Paulino et al. Ticks (Acari: Argasidae and Ixodidae) infesting amphibians and reptiles in Northeastern Brazil. **Systematic and applied Acarology**, v. 23, n. 8, p. 1497-1508, 2018.

ALTSCHUL, Stephen F. et al. Basic local alignment search tool. **Journal of molecular biology**, v. 215, n. 3, p. 403-410, 1990.

BARBIER, Eder; BERNARD, Enrico; DANTAS-TORRES, Filipe. Ecology of *Antricola* ticks in a bat cave in north-eastern Brazil. **Experimental and Applied Acarology**, v. 82, n. 2, p. 255-264, 2020.

BARROS-BATTESTI, Darci M. et al. Ornithodoros brasiliensis Aragão (Acari: Argasidae): description of the larva, redescription of male and female, and neotype designation. **Zootaxa**, v. 3178, n. 31, p. 22-32, 2012.

COSTA, Francisco Borges et al. Retrospective and new records of ticks (Acari: Argasidae, Ixodidae) from the state of Maranhão, an Amazon-Cerrado transition area of Brazil. **Veterinary Parasitology: regional studies and reports**, v. 21, p. 100413, 2020.

CUTLER, Sally J. Relapsing fever borreliae: a global review. **Clinics in laboratory medicine**, v. 35, n. 4, p. 847-865, 2015.

DA SILVA, José Maria Cardoso; LEAL, Inara R.; TABARELLI, Marcelo (Ed.). **Caatinga: the largest tropical dry forest region in South America**. Springer, 2018.

DANTAS-TORRES, Filipe et al. Description of a new species of bat-associated argasid tick (Acari: Argasidae) from Brazil. **Journal of Parasitology**, v. 98, n. 1, p. 36-45, 2012.

DANTAS-TORRES, Filipe et al. Ticks (Ixodida: Argasidae, Ixodidae) of Brazil: updated species checklist and taxonomic keys. **Ticks and tick-borne diseases**, v. 10, n. 6, p. 101252, 2019.

DANTAS-TORRES, Filipe et al. *Ornithodoros* cf. *mimon* infected with a spotted fever group Rickettsia in Brazil. **Acta Tropica**, p. 106541, 2022.

DUPRAZ, Marlène et al. Linking morphometric and genetic divergence with host use in the tick complex, *Ornithodoros capensis* sensu lato. **Infection, Genetics and Evolution**, v. 46, p. 12-22, 2016.

ESTRADA-PEÑA, Agustín et al. Three new species of *Antricola* (Acari: Argasidae) from Brazil, with a key to the known species in the genus. **Journal of Parasitology**, v. 90, n. 3, p. 490-498, 2004.

GUINDON, Stéphane; GASCUEL, Olivier. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. **Systematic biology**, v. 52, n. 5, p. 696-704, 2003.

HOOGSTRAAL, Harry. Argasid and nuttalliellid ticks as parasites and vectors. **Advances in parasitology**, v. 24, p. 135-238, 1985.

JORGE, Felipe R. et al. New records of soft ticks (Acari: Argasidae) in the Caatinga biome of Brazil, with a phylogenetic analysis of argasids using the nuclear Histone 3 (H3) gene. **Experimental and Applied Acarology**, v. 86, n. 4, p. 567-581, 2022.

KABA, Dramane et al. Phenetic and genetic structure of tsetse fly populations (*Glossina palpalis palpalis*) in southern Ivory Coast. **Parasites & Vectors**, v. 5, n. 1, p. 1-9, 2012.

KATOH, Kazutaka et al. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. **Nucleic acids research**, v. 30, n. 14, p. 3059-3066, 2002.

KLOK, C. Jaco; HARRISON, Jon F. The temperature size rule in arthropods: independent of macro-environmental variables but size dependent. **Integrative and comparative biology**, v. 53, n. 4, p. 557-570, 2013.

KOVACS, J. L. et al. Environmental and genetic influences on queen and worker body size in the social wasp *Vespula maculifrons*. **Insectes Sociaux**, v. 57, n. 1, p. 53-65, 2010.

KUMAR, Sudhir; STECHER, Glen; TAMURA, Koichiro. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. **Molecular biology and evolution**, v. 33, n. 7, p. 1870-1874, 2016.

LABRUNA, Marcelo B.; VENZAL, Jose M. *Carios fonsecai* sp. nov.(Acari, Argasidae), a bat tick from the central-western region of Brazil. **Acta Parasitologica**, v. 54, n. 4, p. 355-363, 2009.

LABRUNA, Marcelo B. et al. A new argasid tick species (Acari: Argasidae) associated with the rock cavy, *Kerodon rupestris* Wied-Neuwied (Rodentia: Caviidae), in a semiarid region of Brazil. **Parasites & Vectors**, v. 9, n. 1, p. 1-15, 2016.

LUZ, Hermes Ribeiro et al. Ticks parasitizing bats (Mammalia: Chiroptera) in the Caatinga biome, Brazil. **Revista Brasileira de Parasitologia Veterinária**, v. 25, p. 484-491, 2016.

MAIA, Maerle O. et al. Detection of *Rickettsia* spp. in ticks associated to wild mammals in Northeastern Brazil, with notes on an undetermined *Ornithodoros* sp. collected from marsupials. **Experimental and Applied Acarology**, v. 76, n. 4, p. 523-535, 2018.

MANGOLD, A. J.; BARGUES, M. D.; MAS-COMA, S. Mitochondrial 16S rDNA sequences and phylogenetic relationships of species of *Rhipicephalus* and other tick genera among Metastriata (Acari: Ixodidae). **Parasitology research**, v. 84, n. 6, p. 478-484, 1998.

MANS, Ben J. et al. Argasid and ixodid systematics: implications for soft tick evolution and systematics, with a new argasid species list. **Ticks and tick-borne diseases**, v. 10, n. 1, p. 219-240, 2019.

MUÑOZ-LEAL, Sebastián et al. Isolation and molecular characterization of a relapsing fever *Borrelia* recovered from *Ornithodoros rudis* in Brazil. **Ticks and tick-borne diseases**, v. 9, n. 4, p. 864-871, 2018a.

MUÑOZ-LEAL, Sebastián et al. First report of *Ornithodoros peropteryx* in Brazil, and the occurrence of *Ornithodoros cavernicolous* in the western Brazilian Amazon. **Systematic and Applied Acarology**, v. 23, n. 11, p. 2113-2121, 2018b.

MUÑOZ-LEAL, Sebastián et al. A third species of *Nothoaspis* Keirans & Clifford, 1975 (Acari: Argasidae): *Nothoaspis setosus* (Kohls, Clifford & Jones, 1969) n. comb. **Systematic Parasitology**, v. 96, n. 7, p. 595-602, 2019a.

MUÑOZ-LEAL, Sebastián et al. Detected microorganisms and new geographic records of *Ornithodoros rietcorreai* (Acari: Argasidae) from northern Brazil. **Ticks and tick-borne diseases**, v. 10, n. 4, p. 853-861, 2019b.

MUÑOZ-LEAL, Sebastián; COSTA, Francisco B.; FACCINI-MARTÍNEZ, Álvaro A. Mild toxicosis after the bite of *Ornithodoros rietcorreai*: Images of a brief time-line description. **Travel medicine and infectious disease**, p. S1477-8939 (18) 30316-8, 2019c.

MUÑOZ-LEAL, Sebastián et al. *Ornithodoros cerradoensis* n. sp.(Acari: Argasidae), a member of the *Ornithodoros talaje* (Guérin-Méneville, 1849) group, parasite of rodents in the Brazilian Savannah. **Ticks and Tick-borne Diseases**, v. 11, n. 5, p. 101497, 2020.

MUÑOZ-LEAL, Sebastián et al. A new species of soft tick from dry tropical forests of Brazilian Caatinga. **Ticks and Tick-borne Diseases**, p. 101748, 2021a.

MUÑOZ-LEAL, Sebastián et al. Relapsing fever group borreliae in human-biting soft ticks, Brazil. **Emerging infectious diseases**, v. 27, n. 1, p. 321, 2021b.

NAVA, Santiago et al. **Ticks of the Southern Cone of America: diagnosis, distribution, and hosts with taxonomy, ecology and sanitary importance**. Academic Press, 2017.

OLIVEIRA, Stefan Vilges et al. Human parasitism and toxicosis by *Ornithodoros rietcorreai* (Acari: Argasidae) in an urban area of Northeastern Brazil. **Ticks and tick-borne diseases**, v. 9, n. 6, p. 1494-1498, 2018.

PEIXOTO, Maristela Peckle et al. Detection of *Rickettsia* sp. strain Itinguçú in *Ornithodoros faccinii* (Acari: Argasidae) parasitizing the toad *Rhinella ornata* (Anura: Bufonidae) in Brazil. **Ticks and Tick-borne Diseases**, v. 12, n. 3, 2021.

RIOS, Eloci Peres; THOMPSON, Miguel. **Biomas brasileiros**. Editora Melhoramentos, 2013.

SANGIONI, Luis A. et al. Rickettsial infection in animals and Brazilian spotted fever endemicity. **Emerging Infectious Diseases**, v. 11, n. 2, p. 265, 2005.

TALAGRAND-REBOUL, Emilie et al. Relapsing fevers: neglected tick-borne diseases. **Frontiers in cellular and infection microbiology**, v. 8, p. 98, 2018.

VENZAL, José Manuel et al. The *Ornithodoros* (*Alectorobius*) *talaje* species group (Acari: Ixodida: Argasidae): description of *Ornithodoros* (*Alectorobius*) *rioplatensis* n. sp. from southern South America. **Journal of Medical Entomology**, v. 45, n. 5, p. 832-840, 2008.

VENZAL, José M. et al. A new species of *Ornithodoros* (Acari: Argasidae), parasite of *Microlophus* spp.(Reptilia: Tropiduridae) from northern Chile. **Ticks and Tick-borne Diseases**, v. 4, n. 1-2, p. 128-132, 2013.

3. A NOVEL RELAPSING FEVER GROUP *Borrelia* ISOLATED FROM *Ornithodoros* TICKS OF THE BRAZILIAN CAATINGA

3.1 INTRODUCTION

The spirochete genus *Borrelia* constitute bacteria that infect vertebrates, to whom they are transmitted by hematophagous vectors. With the exception of *Borrelia recurrentis,* transmitted by the human clothing louse (*Pediculus humanus*), all *Borrelia* species are primarily transmitted by ticks (Trevisan et al. 2021a,b). Species of the genus *Borrelia* are known to infect a variety of vertebrate hosts (mammals, birds, reptiles) and have been divided into three main groups: (i) the Lyme group (LG), represented by *Borrelia burgdorferi* sensu lato genospecies that are associated with hard ticks (Ixodidae family); (ii) the Reptile-Echidna group (REPG), represented by organisms associated with hard ticks; and (iii) the Relapsing Fever group (RFG), which contains numerous organisms mainly associated with soft ticks (Argasidae family) and a few ones associated with hard ticks, in addition to the louse-borne *B. recurrentis* (Trevisan et al. 2021a,b). Although there has been a recent proposal to split borrelial species into two genera (i.e., *Borrelia* for RFG species, and *Borreliella* for LG species) (Adeolu and Gupta 2014, Barbour and Gupta 2021), this proposal is still controversial; hence, herein we opted to consider all borrelial species as belonging to the genus *Borrelia*, as recently discussed (Margos et al. 2018, 2022).

Most RFG borreliae are primarily associated with soft ticks of the genus *Ornithodoros*, in which the bacteria perpetuate through transstadial and transovarial passages (Cutler 2015). Given the great capacity of *Ornithodoros* ticks to survive for several years without feeding, these arthropods are also pointed out as main reservoirs of RFG borreliae (Cutler 2015). Once transmitted to vertebrates upon tick feeding, RFG borreliae replicate in the blood of competent hosts, which might suffer clinical illness. In humans, this condition is reported as ‘relapsing fever’ (Cutler 2015, Trevisan et al. 2021b). RFG borreliae are maintained in enzootic cycles, mostly between soft ticks (*Ornithodoros* spp.) and rodents (Cutler 2015, Talagrand-Reboul et al. 2018). Once infected, rodents develop an initial peak of spirochetemia that lasts for a few days, and is followed by new spirochete relapses interspaced by a few days, during an infection course of usually one month, when signs of illness might or might not be present (Burgdorfer and Mavros 1970, Boardman et al. 2019).

Over 20 species of RFG borreliae described, the majority is pathogenic to humans and associated with *Ornithodoros* ticks worldwide (Talagrand-Reboul et al. 2018). Although tick-

borne relapsing fever was first reported during the 19th century, it remains neglected as a human disease and little is known on its maintenance in ticks and vertebrates (Cutler 2015, Talagrand-Reboul et al. 2018). In South America, only two *Ornithodoros*-associated *Borrelia* species have been described, *Borrelia brasiliensis* and *Borrelia venezuelensis*; the latter was associated with clinical cases of human relapsing fever in Colombia and Venezuela during the first half of the 20th century (Faccini-Martínez et al. 2022).

In Brazil, the first report of RFG *Borrelia* associated to *Ornithodoros* ticks was performed by Davis (1952), who observed spirochetes in the blood of mice exposed to nymph of *Ornithodoros brasiliensis* Aragão, 1923 from Rio Grande do Sul. Although the spirochetes were named as *B. brasiliensis,* the isolate was lost and never reported again. During this century, Muñoz-Leal et al. (2018) isolated *B. venezuelensis* by feeding *Ornithodoros rudis* from Maranhão state on Vesper mice (*Calomys callosus*). Subsequently, this isolate (designated as *B. venezuelensis* RMA01) was cultured in vitro and its genome sequenced (Kneubehl et al. 2022). *Borrelia venezuelensis* RMA01 constitutes to date the sole isolate of a RFG borreliae transmitted by an *Ornithodoros* tick in South America (Faccini-Martínez et al. 2022). In a recent study, Muñoz-Leal et al. (2021a) reported by molecular methods four novel RFG *Borrelia* genotypes agents in human-biting *Ornithodoros* ticks from Brazil: *Borrelia* sp. Omi2MT and Omi3MT in *Ornihodoros mimon* Kohls, Clifford & Jones, 1969 from Mato Grosso state, *Borrelia* sp. JericoCE in *Ornithodoros hasei* Clifford, Kohls & Sonenshine, 1964, OrietCE in *Ornithodoros rietcorreiai* Labruna, Nava & Venzal, 2016*,* and *Borrelia* sp. TabajaraCE from *Ornithodoros tabajara* Muñoz-Leal & Labruna, 2021; the latter three tick species were collected in Ceará state, within the Caatinga semiarid biome of Brazil. Despite these findings, human infection by RFG borreliae in Brazil remains unreported.

Based on the recent advances on the occurrence of RFG borreliae associated to *Ornithodoros* ticks in Brazil, especially in the Caatinga biome, the present study aimed to isolate borrelial organisms from *Ornithodoros* ticks collected in additional areas of this ecosystem. For this purpose, collected ticks were allowed to feed on guinea pigs, which were monitored for successful borrelial isolation, and subsequent molecular characterization.

3.2 MATERIAL AND METHODS

3.2.1 Study sites and collection of ticks.

During a field expedition in October 2019, ticks were collected from the environment in four localities in the state of Pernambuco, northeastern Brazil: (i) National Park of Catimbau, Serra das Torres, Buíque municipality (08°34’0.3’’S, 37°14’27.8’’W; elevation 777 m); (ii) Malhada Vermelha, Floresta municipality (08°36’44.2’’S, 38°32’29.9’’W; 377 m); (iii) National Forest of Negreiros, Serrita municipality (07°59’22.0’’S, 39°24’46.1’’W; 475 m); (iv) Capim District, Petrolina municipality (09°09’40.3’’S, 40°26’16.2’’W; 454 m) (Fig. 1). The four localities are located within the Caatinga biome, which is characterized by a semiarid climate (temperatures averaging 27°C throughout the year, mean annual rainfall typically <500 mm), and deciduous vegetation composed typically of xeric shrub land and thorn forest that consist primarily of small, thorny trees that shed their leaves seasonally (da Silva et al. 2017). The prospected environments included rock formations with vestiges (e.g., feces) of wild rodents, including visualizations of the rock cavy *Kerodon rupestris* (Rodentia: Caviidae). Ticks were collected with tweezers from under the rocks and stored in punctured plastic vials, which were kept in an improvised environmental chamber (1.5 liter-plastic bottle with a piece of humid cotton on the bottom) until the arrival to the laboratory, where ticks were placed in an incubator at 26°C and 80% relative humidity. Under this condition, field-collected engorged females oviposited fertile eggs that resulted in hatched larvae.

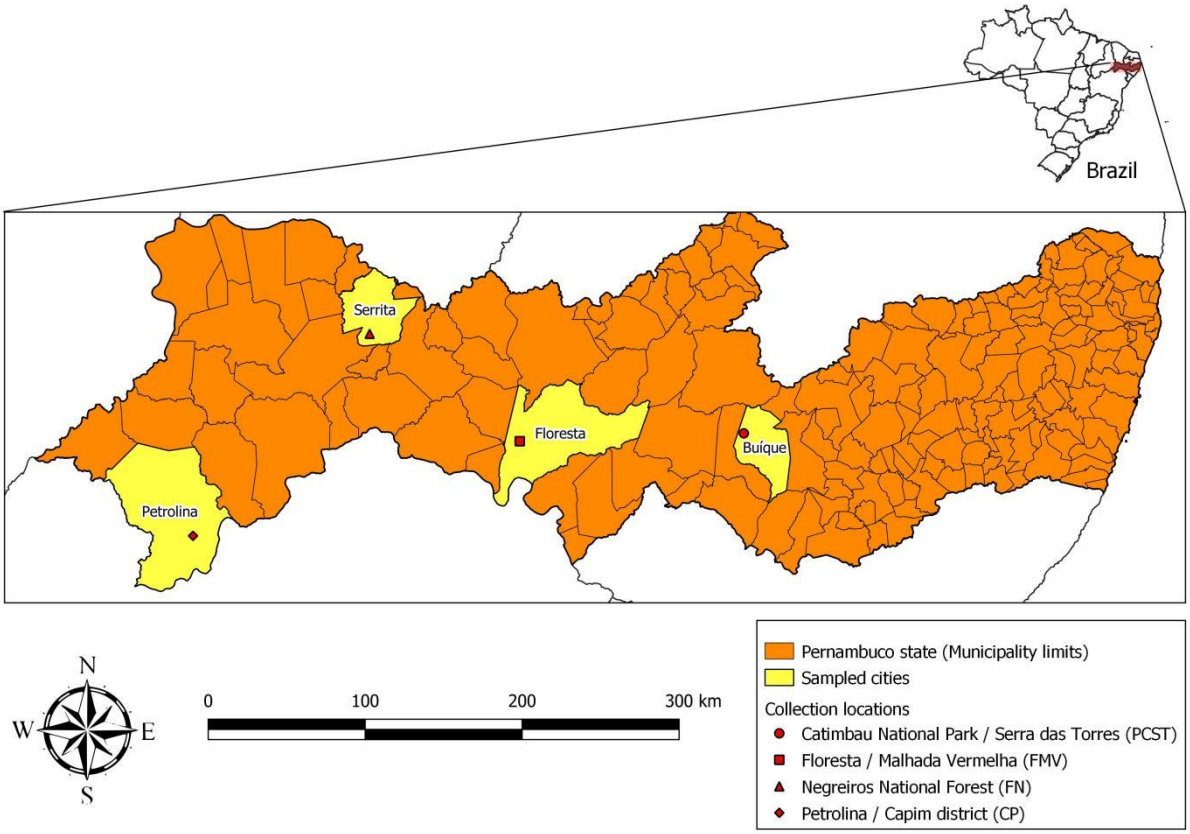

Fig. 1. Locations in the state of Pernambuco, northeastern Brazil, where ticks were collected in the present study.

3.2.2 Taxonomic identification of ticks

Unfed larvae born in the laboratory were killed in hot water, clarified with 25% KOH, and mounted on slides using Hoyer's medium to observe morphological characters by optical microscopy (Olympus BX40 optical microscope, Olympus Optical Co. Ltd., Japan). Living adults and nymphs were visualized and counted under a stereomicroscope (Zeiss Stemi SV 11, Zeiss, Münich, Germany). Species were determined according to Dantas-Torres et al. (2019) and original descriptions (Labruna et al., 2016; Muñoz-Leal et al., 2021b).

Identification of the ticks was complemented by molecular analysis. For this purpose, two or three adult specimens of each species were individually submitted to DNA extraction by the guanidine isothiocyanate and phenol/chloroform technique (Sangioni et al., 2005). A PCR protocol targeting a ≈460 bp fragment of the tick mitochondrial 16S rRNA gene was performed following Mangold et al. (1998). A second PCR protocol targeting a ≈270 bp fragment of the nuclear *Histone 3* (H3) gene was performed as described (Sands et al. 2017). Amplicons of the expected size were prepared for sequencing using Big Dye Terminator

Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), and sequenced in an ABI automated sequencer (Applied Biosystems/Thermo Fisher Scientific, model ABI 3500 Genetic Analyzer, Foster City, CA, USA) with the same primers used for PCR. Obtained sequences were assembled, and primer-trimmed with Geneious R9 (Kearse et al., 2012), and submitted to a BLAST analysis (www.ncbi.nlm.nih.gov/blast) to infer closest identities with congeneric ticks (Altschul et al., 1990).

3.2.3 Isolation of spirochetes

Attempts to isolate viable spirochetes were performed using field-collected ticks, which were separated in groups according to locations and species. Seven guinea pigs were infested, each one with adults and nymphs ticks of one species from a single location. For this purpose, unengorged ticks were released inside a plastic feeding chamber (6 cm diameter) previously glued with a skin compatible-adhesive (Kamar Products, Zionsville, IN, USA) on the shaved dorsum of the guinea pig. Two hours after being released in the feeding chambers, engorged ticks were recovered and placed in the incubator for further studies. A drop of blood (≈2.5μL) was daily obtained from each of the seven guinea pigs by ear vein-puncture, expressed onto glass slides, and observed by dark-field microscopy to detect the presence of motile spirochetes. The mean number of spirochetes per field was calculated by counting the total number of motile spirochetes in 50 microscope fields at 200x magnification, dividing it by 50; results as decimal numbers were always rounded up. Rodents not presenting motile spirochetes during the first 21 days were considered negative and were not bled anymore. If a guinea pig showed motile spirochetes during the first 21 days, daily examinations were extended until 52 days after tick infestations.

Spirochetemic guinea pigs were anesthetized (xylazine 5 mg/kg + ketamine 35 mg/kg) and 2 mL of blood was collected by intracardiac puncture at the 18th day after tick infestation. Part of the blood was submitted to DNA extraction (see below) and the other part was intraperitoneally inoculated into new rodents (three newborn guinea pigs, one mouse and one hamster, each one receiving ≈0.300 mL of blood) to perform the first rodent passage of spirochetes in the laboratory. These inoculated rodents were also evaluated daily through dark-field microscopy of blood samples, as described above. Two newborn guinea pigs, when showing >10 spirochetes/field, were anesthetized (xylazine + ketamine) and euthanized by exsanguination via the intracardiac route. In this case, the collected blood was immediately put in heparin tubes, centrifuged, and the plasma was aliquoted into 2 mL-cryotubes, which

were stored at -80°C, and then at liquid nitrogen for cryopreservation of the isolated spirochetes. Rectal temperature of all animals was measured daily throughout the study with a digital clinical thermometer.

3.2.4 Molecular analyses

DNA extraction of guinea pig blood samples was performed using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA), and tested by the following conventional PCR protocols for amplification of fragments of the borrelial genes 16S ribosomal RNA (*rrs*), flagellin (*flaB*), glycerophosphodiester phosphodiesterase (*glpQ*), and the DNA gyrase subunit B (*gyrB*) (Table 1). In addition, we performed a multilocus sequencing typing (MLST) scheme according to Margos et al. (2008) for amplification of the borrelial genes *clpA*, *clpX*, *pepX*, *pyrG*, *recG*, *rplB, nifS* and *uvrA* (Table S1). DNA of *Borrelia anserina* strain PB (Ataliba et al., 2007) was used as positive control in all reactions. Obtained amplicons were visualized with UV light through 1.5% agarose gels stained with SYBR Safe (Thermo Fisher Scientifific, Waltham, MA, USA). Products containing a single expected size fragment were treated with ExoSAP-IT (USB Corporation, Cleveland, OH, USA) and prepared for sequencing with the BigDye kit (Applied Biosystems, Foster City, CA, USA). An ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) was employed for sequencing using the same primers to perform PCRs. Obtained sequences were assembled, trimmed and translated to amino acid (if applicable) with Geneious R9 (Kearse et al., 2012). Generated DNA sequences were submitted to BLAST analysis (www.ncbi.nlm.nih.gov/blast) to infer closest identities with other spirochetes (Altschul et al., 1990).

Table 1. List of the primer pairs used in the preset study for amplification of five *Borrelia* genes by conventional PCR assays.

Genes and primers	DNA sequences (5'-3') of forward (F) and reverse (R) primers of each pair of primers	Amplicon size (bp)	References
rrs			
FD3	F- AGAGTTTGATCCTGGCTTAG	1540	Ras et al. 1996
T50	R- GTTACGACTTCACCCTCCT		
FD3	F- AGAGTTTGATCCTGGCTTAG	729*	Schwan et al. 2005
16S-1	R- TAGAAGTTCGCCTTCGCCTCTG		
16S-2	F- TACAGGTGCTGCATGGTTGTCG	513*	Schwan et al. 2005
T50	R- GTTACGACTTCACCCTCCT		
Rec-4	F- ATGCTAGAAACTGCATGA	520*	Ras et al. 1996
Rec-9	R- TCGTCTGAGTCCCCATCT		
flaB			
FlaLL	F- ACATATTCAGATGCAGACAGAGGT	665	Stromdahl et al. (2003)
FlaRL	R- GCAATCATAGCCATTGCAGATTGT		
FlaLL	F- ACATATTCAGATGCAGACAGAGGT	485*	Stromdahl et al. (2003)
FlaRS	R- CTTTGATCACTTATCATTCTAATAGC		
FlaLS	F- AACAGCTGAAGAGCTTGGAATG	522*	Stromdahl et al. (2003)
FlaRL	R- GCAATCATAGCCATTGCAGATTGT		
glpQ			
glpQ F+1	F- GGGGTTCTGTTACTGCTAGTGCCATTAC	1386	Schwan et al. 2005
Rev-2	R- CAATACTAAGACCAGTTGCTCCTCCGCC		
glpQ F+1	F- GGGGTTCTGTTACTGCTAGTGCCATTAC	802*	Schwan et al. 2005
glpQ F-1	R- CAATTTTAGATATGTCTTTACCTTGTTGTTTATGCC		
gyrB			
gyrB 5'	F- GGTTTATGAGTTATGTTGCTAGTAATATTCAAGTGC	2026	Schwan et al. 2005
gyrB 3'	R- GGCTCTTGAAACAATAACAGACATCGC		
gyrB 3'	F- GGTTTATGAGTTATGTTGCTAGTAATATTCAAGTGC	542*	Schwan et al. 2005
gyrB 5'+3	R- GCTGATGCTGATGTTGATGG		
rrs-rrlA			
IGS-F	F- GTATGTTTAGTGAGGGGGGTG	1029	Bunikis et al. 2004
IGS-R	R- GGATCATAGCTCAGGTGGTTAG		
IGS-Fn	F- AGGGGGGTGAAGTCGTAACAAG	988*	Bunikis et al. 2004
IGS-Rn	R- GTCTGATAAACCTGAGGTCGGA		

* amplified through a nested or heminested reaction.

Table S1. List of primers used in the multilocus sequencing typing (MLST) scheme according to Margos et al. (2008) for amplification of six genes of *Borrelia* spp. of the relapsing fever group.

Gene	Primers	**DNA sequences (5'-3') of forward (F) and reverse (R) primers of each pair of primers**	**Amplicon size (bp)**	**Reference**
clpA	clpAF1262	F- AAGCTTTTGA**YY**TATTAGATGGTC	1014	http://pubmlst.org/borrelia
	clpAR2276	R- TCATATTT**D**AT**R**GT**D**TCGTC		
	clpAF1264	F- GCTTTTGAYYTATTAGATGGTC	834*	
	clpAR2098	R- CAAAAAAAA**S**ATC**H**A**RRT**TTTCATTTTTTAG		
rplB	rplF3	F- GGAGAAAAATATGGG**K**ATTAAGAC	766	http://pubmlst.org/borrelia
	rplR769	R- G**R**CCCCAAGG**W**GATAC		
	rplF18	F- ATTAAGACTTATA**R**GCCAAAAAC	741*	
	rplF759	R- GATACAGGATG**W**CGACC		
	rplF24	F- GACTTATA**R**GCCAAAAACTTC	741*	
	rplF765	R- CCAAGG**W**GATACAGGATG		
pyrG	pyrF372	F- TGG**R**AA**R**TATTTAGG**K**AGAACTG	1003	http://pubmlst.org/borrelia
	pyrR1375	R- TATTTAGG**K**AGAACTGTACAGC		
	PyrF379	F- TATTTAGG**K**AGAACTGTACAGC	873*	
	PyrR1252	R- TAT**R**CCACAAACATTACG**K**GC		
recG	recF898	F- GC**K**TTTCT**M**TCTAG**Y**ATTCC	881	http://pubmlst.org/borrelia
	recR1779	R- TC**R**GTTAAAGGTTCCTTATAAAG		
	recF908	F- CTAG**Y**ATTCCT**Y**TAATTGA**R**GC	871*	
	recR1779	R- TC**R**GTTAAAGGTTCCTTATAAAG		
clpX	clpXF109	F- GC**Y**ATTTGTTTTGAATGTTCTAAAATATG	859	http://pubmlst.org/borrelia
	clpXR1277	R- TAAAGTTCTTTTGCCCAAGG		
	clpXF268	F- GC**Y**ATTTGTTTTGAATGTTCTAAAATATG	721*	
	clpXR1183	R- CTTTTTTAATTTGCTTA**S**T**W**GAAGG		
pepX	pepXF361	F- AGAGA**Y**TTAAG**Y**TTA**K**CAGG	1168	http://pubmlst.org/borrelia
	pepXR1207	R- C**Y**ATAGTTTCTCTTAAAGA**Y**TGC		
	pepXF364	F- GA**Y**TTAAG**Y**TTA**K**CAGGA**R**TTG	823*	
	pepXR1187	R- TGCATTCCCCACATTGG		
nifS	nifF23	F- TAAAAATAAAGAGTACTAAATAAATG	876	http://pubmlst.org/borrelia
	nifR899	R- CCAAGACCAATAATTCCTGC		
	nifF60	F- AAATAATGGTATAATTCTTAAAATAA	656*	
	nifR716	R- GGAGCAAGCATTTT**Y**TGTC		
uvrA	uvrF1170	F- GAGGCGTTATCTT**W**CAAC	983	http://pubmlst.org/borrelia
	uvrR2153	R- CTAATCTC**D**GTAAAAAATCCAACATAAG		
	uvrF1173	F- GCGTTATCTT**W**CAACTGAATC	974*	
	uvrR2147	R- TC**D**GTAAAAAATCCAACATAAGTTGC		

* amplified through a nested or heminested reaction.

3.2.5 Phylogenetic analyses

Obtained consensus sequences and orthologous sequences retrieved from GenBank were aligned with MAFFT using default parameters (Katoh et al., 2013). Phylogenetics trees were inferred using Maximum likelihood (ML) and Bayesian inference (BI) methods in IQ-TREE v 1.6.12 (Nguyen et al., 2015) and MrBayes v 3.2.6 (Ronquist et al., 2012), respectively. Protein-coding gene present distinct nucleotide exchange rates (heterogeneity) at the first, second, and third codon positions so datasets were partitioned into three codon positions (position-1, position-2, and position-3) (Yang et al., 1996; Ronquist et al., 2012). For ML analyses, the best-fit models for non-coding and protein-coding genes datasets were calculated with the ModelFinder commands "TESTNEWONLY -mrate G" and "TESTNEWONLYMERGE -mrate G", respectively (Kalyaanamoorthy et al., 2017). Trees were run with rapid hill-climbing approach and stochastic disturbance applying 1,000 ultrafast bootstrapsing pseudo-replicates (UFB) to evaluate tree robustness. UFB values < 70%, between 70–94%, and ≥ 95% were considered non-significant, moderate, and high statistical support, respectively (Minh et al., 2013).

BI phylogenies were constructed implementing the MrBayes commands "lset nst=mixed rates=gamma" and "lset= mixed rates=invgamma" for non-coding and protein encoding datasets, respectively (Ronquist et al., 2012; Lanfear et al., 2012; Huelsenbeck, 2004). BI analyses were run with two independent tests of 20 x 106 generations, each with four simultaneous Monte Carlo Markov chains (MCMC), sampling trees every 1,000 generations, removing the first 25% as burn-in. Tracer software was used to confirm the MCMCs correlation as well as reached stationarity and effective sample size (ESS) (Rambaut et al., 2018). All best-fit substitution models and partitions schemes were selected according to the Bayesian Information Criterion (BIC) (Schwarz, 1978). Nodes with Bayesian posterior probabilities (BPP) values ≥ 0.70 were considered of high statistical support (Huelsenbeck and Rannala, 2004).

All trees were visualized and edited using FigTree v 1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape v 1.1 (https://inkscape.org/es/). Congruent topologies between ML and BI analyses were used to produce strict consensus trees in Geneious Prime with the Consensus Tree Builder tool, implementing a support threshold of 100% (www.geneious.com). The consensus phylogram included all monophyletic clades after comparing ML and BI topologies for each dataset.

3.2.6 Ethics statement

Field collections of ticks were authorized by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio permit Sisbio 65137-1). Animal experimentation was approved by the Ethic Committee on Animal Use of the Faculty of Veterinary Medicine of the University of São Paulo (projects number 4425171018 and 2655061218).

3.2.7 Data availability

Partial sequences of ticks and borrelial genes generated in this study are in process of submission and will be available in GenBank.

3.3 RESULTS

3.3.1 Ticks

A total of 1,505 tick specimens were collected in four localities. Morphological analyses resulted in the identification of two *Ornithodoros* species: 932 specimens of *O. rietcorreai* from Buíque, Floresta, Serrita and Petrolina, and 573 specimens of *O. tabajara* from the former three localities (Table 2). Microscopical analyses of laboratory-reared unfed larvae in mounted slides showed morphological characters corresponding to the species of their respective parental specimens.

Table 2. Soft ticks collected in locations of the state of Pernambuco, Caatinga biome, Brazil, during 2019.

Species	Stage	Total	Locality/City
Ornithodoros rietcorreai	(5F, 3M, 18N)	26	PCST/Buíque
	(21F, 29M, 318N)	368	FMV/Floresta
	(18F, 34M, 395N)	447	FN/Serrita
	(4F, 4M, 83N)	91	CP/Petrolina
Ornithodoros cf. *tabajara*	(34F, 31M, 80N)	145	PCST/Buíque
	(45F, 51M, 194N)	290	FMV/Floresta
	(20F, 11M, 107N)	138	FN/Serrita

Partial sequences of the mitochondrial 16S rRNA gene were generated for two *O. rietcorreai* specimens; one from Buíque, which was 99% (422/427 bp) identical to *O. rietcorreai* from Ceará state, Brazil (GenBank MT021433), and the other from Petrolina, which was 99% (425/426 bp) identical to *O. rietcorreai* from Paraíba state, Brazil (GenBank KX130781). The two *O. rietcorreai* haplotypes from this study were 96% identical to each other.

Partial sequences of the mitochondrial 16S rRNA gene were generated for three *O. tabajara* adult specimens, which were 92% (396/429 bp) identical to *O. tabajara* from Ceará

state, Brazil (GenBank MT021434). The sequences of two specimens (one from Buíque and one from Floresta) were 100% identical to each other, whereas the third specimen (from Serrita) generated a second haplotype that differed by a single nucleotide polymorphism (99%; 428/429 bp) from the other two specimens. Although the external morphology of the ticks from Buíque, Floresta and Serrita was compatible with *O. tabajara,* we are provisionally treating them as *Ornithodoros* cf. *tabajara* due to the relatively high polymorphism (8% difference) of their 16S rDNA partial sequences with the type sequence of *O. tabajara* (MT021434) reported by Muñoz-Leal et al. (2021b). Partial sequences (216 bp) of the nuclear H3 gene of the three specimens of *O.* cf. *tabajara* were identical to each other, and by BLAST analysis, they were 99% (174/175 bp) identical to *O. tabajara* from Ceará (OK247605). Ongoing studies are in progress to elucidate the taxonomic status of the *O.* cf. *tabajara* ticks collected in the present study.

3.3.2 Isolation of spirochetes

Four guinea pigs (numbers 1, 3, 5, 7) were infested with 925 *O. rietcorreai* ticks from four localities (25 to 445 ticks per guinea pig), and other three guinea pigs (numbers 2, 4, 6) were infested with 568 *O.* cf. *tabajara* ticks from three localities (136 to 289 ticks per guinea pig) (Table 2). Although we did not count the exact number of ticks that were fully engorged two hours after been released in the feeding chambers, nearly all of them became at least partially engorged. No spirochetes were visualized by dark-field microscopy in the blood of any of the four *O. rietcorreai*-infested guinea pigs (1, 3, 5, 7) during 21 consecutive days after infestation. On the other hand, spirochetes were visualized in the three guinea pigs (2, 4, 6) that were infested with *O.* cf. *tabajara* ticks from three localities (Fig. 2; Supplementary video 1).

During the 53-day course of dark-field microscopy monitoring of guinea pig 2 (infested with *O.* cf. *tabajara* from Buíque), motile spirochetes were visualized in blood at 9 to 11, 14 to 25, 27 to 30, and 39 days after tick infestation; a mean of ≤1 spirochete/microscope field was visualized generally, although the maximum mean count was 5 spirochetes/field at day 17 (Fig. 3). In guinea pig 4 (infested with *O.* cf. *tabajara* from Floresta), motile spirochetes were visualized in blood from 13 to 30 days after tick infestation; while a mean of ≤1 spirochete/field was visualized at most of the times, maximum mean values were 10 spirochetes/field at days 10 and 22 (Fig. 3). In guinea pig 6 (infested with *O.* cf. *tabajara* from Serrita), motile spirochetes were visualized in blood at 11, 14 to 28, and 30

to 31 days after tick infestation; similarly, a mean of ≤1 spirochete/field was visualized at most of the times, yet a maximum mean value of 30 spirochetes/field was observed at day 23 (Fig. 3). The three isolates of spirochetes were recovered from guinea pigs number 2, 4, and 6 infested with *O.* cf. *tabajara* ticks, and were named Buíque-PCST, Floresta-FMV, and Serrita-FN, respectively.

Guinea pigs 2, 4 and 6 were bled at the 18th day after tick infestation (when the mean numbers of spirochetes/field were 4, 5 and 7, respectively), and their blood samples were inoculated into other animals to perform the first rodent passage of the spirochetes. In this case, the blood of guinea pig 2 (isolate Buíque-PCST) was inoculated into a newborn guinea pig and a mouse (Table 2). Blood samples of this newborn guinea pig showed motile spirochetes (mean: ≤1 to 15 spirochetes/field) from the 5th to the 11th day after inoculation (Fig. 4), when it was euthanized by exsanguination and its plasma cryopreserved. Dark-field microscopy of the inoculated mouse revealed spirochetes only at the 8th, 9th and 24th days after inoculation (mean ≤1 spirochete/field), despite of this rodent being daily examined until the 42th day (Fig. 4). The blood of guinea pig 4 (isolate Floresta-FMV) was inoculated into a newborn guinea pig and a hamster (Table 2). Blood samples of this newborn guinea pig showed motile spirochetes (mean: ≤1 to 25 spirochetes/field) from the 4th to the 6th day after inoculation (Fig. 4), when it was euthanized by exsanguination and its plasma cryopreserved. Dark-field microscopy of the inoculated hamster revealed spirochetes only at the 2nd, 3rd and 10th days after inoculation (mean ≤1 or 7 spirochetes/field), despite of this rodent being daily examined until the 39th day (Fig. 4). Finally, the blood of guinea pig 6 (isolate Serrita-FN) was inoculated into a newborn guinea pig, which showed motile spirochetes in blood at 3 to 5, 8 to 17, and 27 days after inoculation, with mean numbers of spirochetes/field varying from ≤1 to 15 (Fig. 4). No rodent developed fever or clinical abnormalities during the present study.

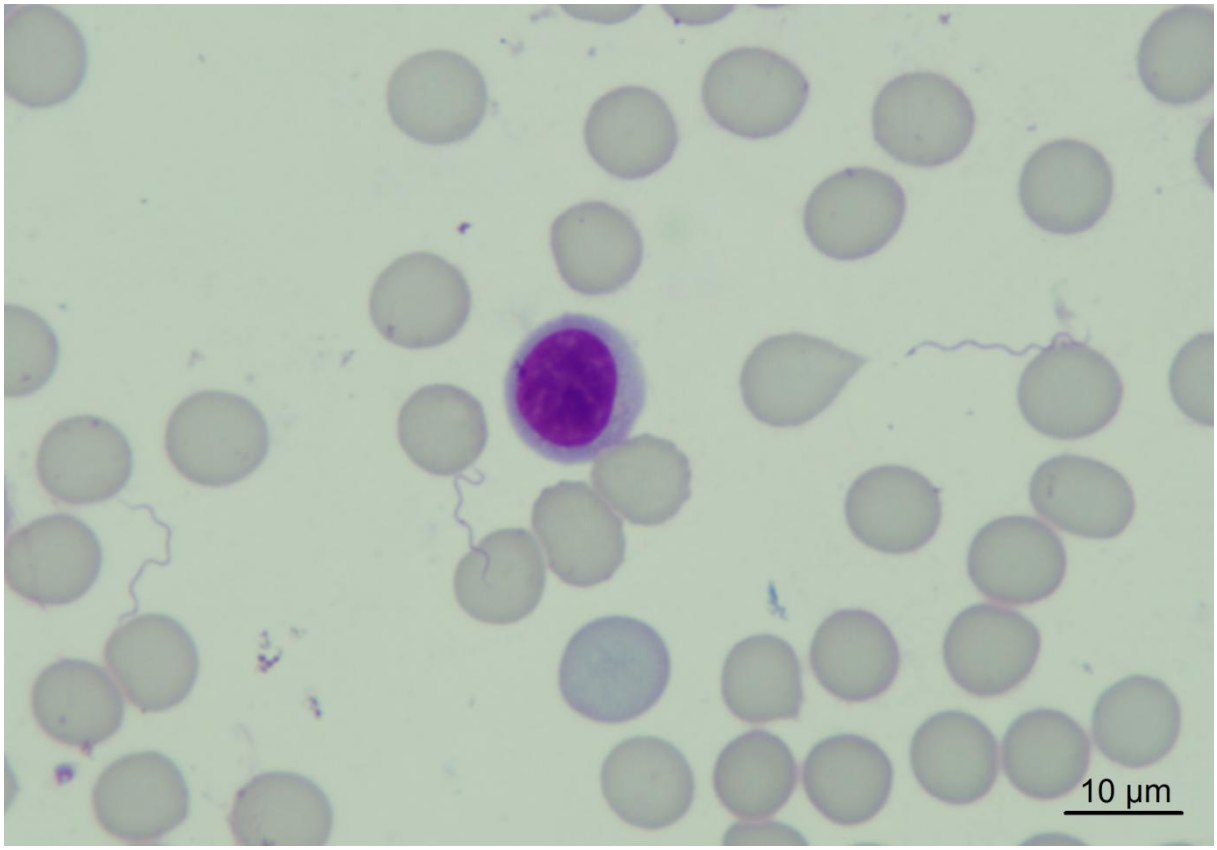

Fig. 2. Giemsa-stained blood smear of guinea pig 4, showing spirochetes (original magnification: 1000x).

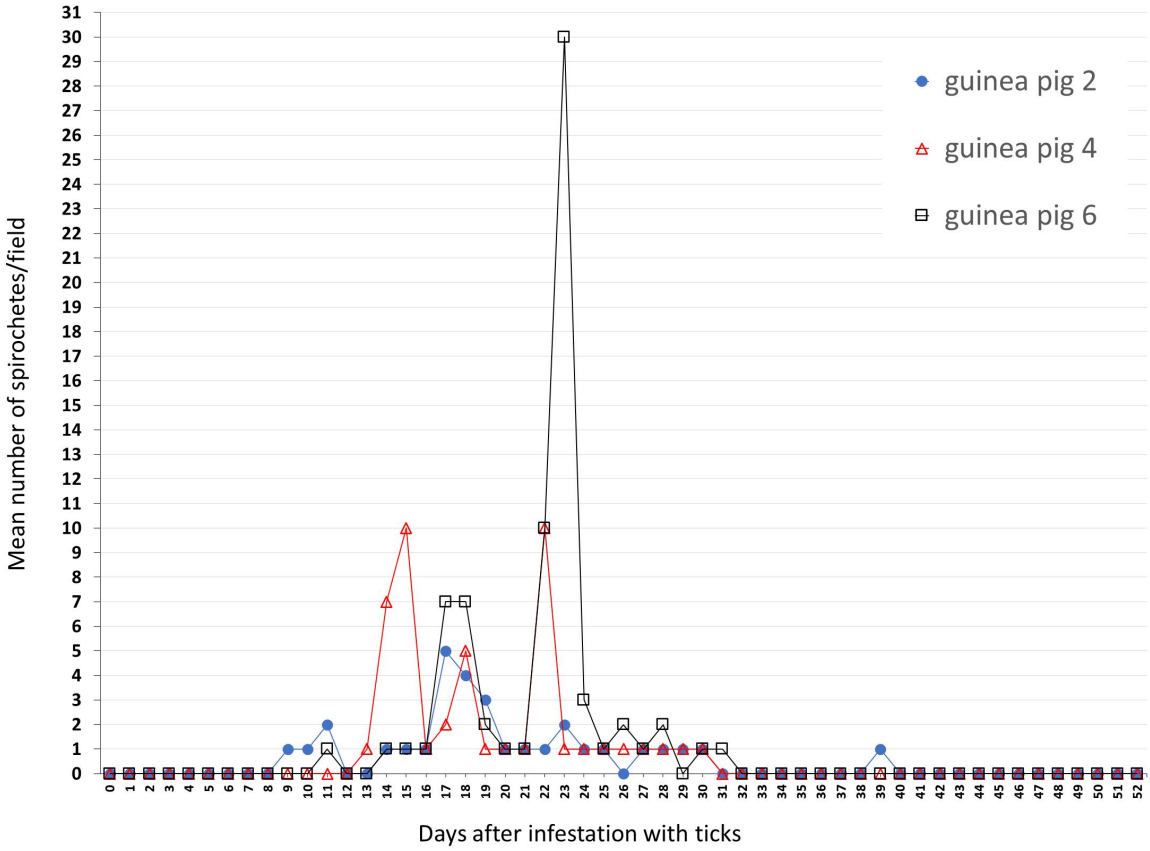

Fig. 3. Results of dark-field examination of blood samples of guinea pigs according to the number of days after infestation with *Ornithodoros* cf. *tabajara* from Buíque (guinea pig 2), Floresta (guinea pig 4) and Serrita (guinea pig 6). Values presented as the mean number of motile spirochetes per microscope field at 200x magnification in each sampled day.

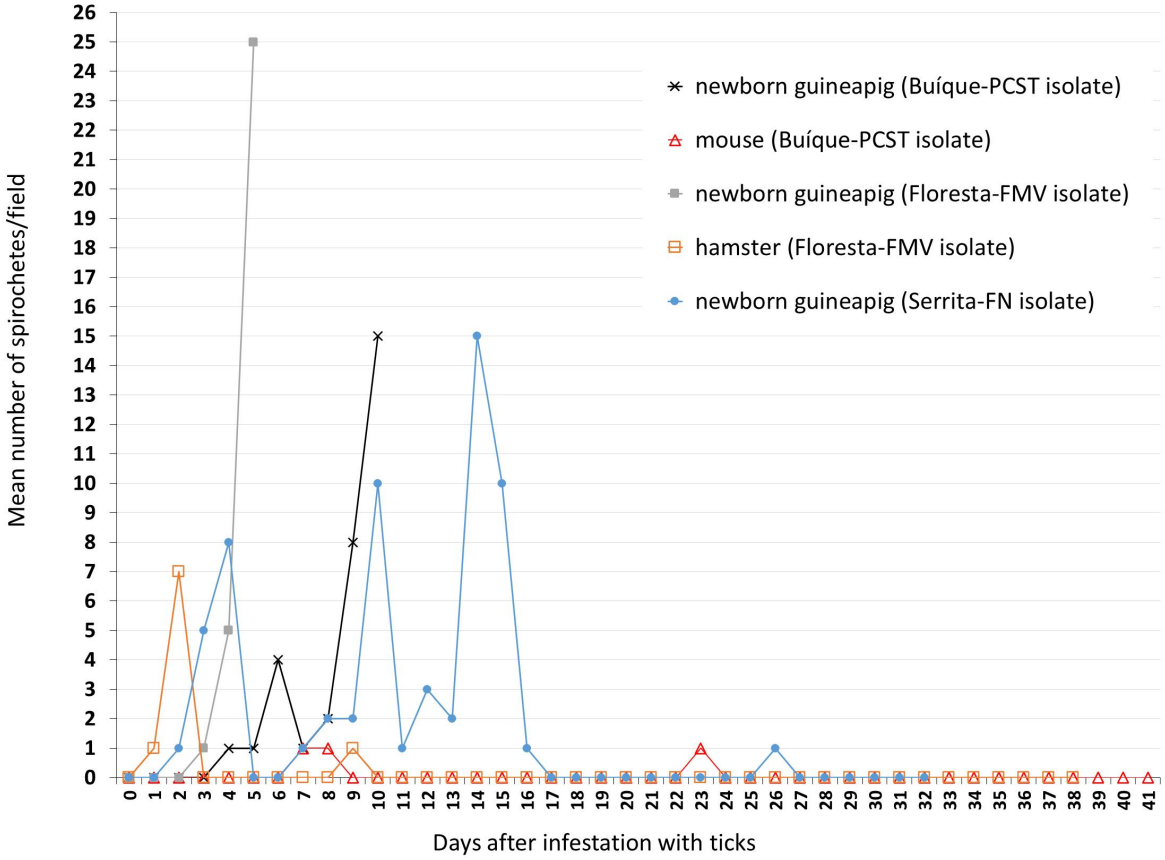

Fig. 4. Results of dark-field examination of blood samples of newborn guinea pigs, hamster and mouse according to the number of days after inoculation with blood samples that were collected from guinea pigs showing spirochetemia due to *Borrelia* sp. isolate Buíque-PCST or Floresta-FMV or Serrita-FN. Values presented as the mean number of motile spirochetes per microscope field at 200x magnification in each sampled day.

3.3.3 Molecular characterization of spirochetes

PCR assays resulted in the successful amplification of fragments of four borrelial genes (*rrs*, *flaB*, *glpQ*, *gyrB*) from blood samples that were collected from spirochetemic guinea pigs nos. 2, 4 and 6. For each borrelial gene, sequences were identical among the three guinea pigs, indicating that isolates Buíque-PCST, Floresta-FMV, and Serrita-FN represented the same *Borrelia* species. Results of BLAST analyses with 100% query cover showed that a 1,410 bp-fragment of the *rrs* gene was >99.2% identical to the sequences of *B. hispanica* (DQ057988, GU350705), *Borrelia duttonii* (CP000976, GU350711), and *Borrelia crocidurae* (CP003426, DQ057990); a 614 bp-fragment of the *flaB* gene was most identical (99.3%) to *Borrelia* sp. clone TabajaraCE from *O. tabajara,* Brazil (MT076263); a 674 bp-fragment of

the *glpQ* gene was most identical (85.1%) to *B. crocidurae* (CP003426); a 507 bp-fragment of the *gyrB* gene was most identical (89.9%) to *B. crocidurae* (CP004267) and *B. duttonii* (CP000976).

Phylogenetic analyses inferred from partial sequences of each of four genes (*rrs*, *flaB*, *glpQ*, *gyrB*) showed that in all cases, the sequences generated for *Borrelia* sp. (isolates Buíque-PCST, Floresta-FMV, and Serrita-FN) grouped within a clade composed by *B. recurrentis* and tick-borne *Borrelia* species of the Old World, such as *B. hispanica*, *B. duttonii*, *B. crocidurae*, and *B. persica* (Fig. 5A-B, 6A-B). For the borrelial genes *rrs, flaB* and *glpQ,* this clade also included genotypes of unnamed *Borrelia* spp. recently reported in soft ticks from Brazil (Muñoz-Leal et al. 2021a), such as *Borrelia* sp. Omi2MT and *Borrelia* sp. Omi3MT from *O. mimon*, *Borrelia* sp. TabajaraCE from *O. tabajara*, and *Borrelia* sp. OrietCE from *O. rietcorreai* (this latter one only for the *rrs* gene). Finally, in the *rrs, flaB* and *glpQ* phylogenetic trees, this large clade was sister to another large clade that contained *Borrelia* species associated to soft ticks from the New World (such as the North American agents *B. turicatae, B. parkeri* and *B. johnsonii*), and two agents from Brazil (*B. venezuelensis* from *O. rudis,* and *Borrelia* sp. JericoCE from *O. hasei*).

PCR amplification and DNA sequences were obtained for six of the eight MLST loci (*clpX*, *pepX*, *pyrG*, *recG*, *rplB,* and *uvrA*) from guinea pig blood samples. Pairwise comparisons proved that the *Borrelia* sequences from guinea pigs nos. 2, 4 and 6 were identical with each other. The phylogenetic analysis of concatenated MLST sequences (Fig. 6C) corroborates the previous trees, indicating that *Borrelia* sp. (isolates Buíque-PCST, Floresta-FMV, and Serrita-FN) belongs to the RFG borreliae, in which it grouped within a clade that included *B. recurrentis* and tick-borne *Borrelia* species of the Old World (*B. hispanica*, *B. duttonii*, *B. crocidurae* and *B. persica*). This clade was sister to a large clade composed mostly by North American agents (*B. turicatae, B. parkeri* and *B. johnsonii*) associated with *Ornithodoros* spp.

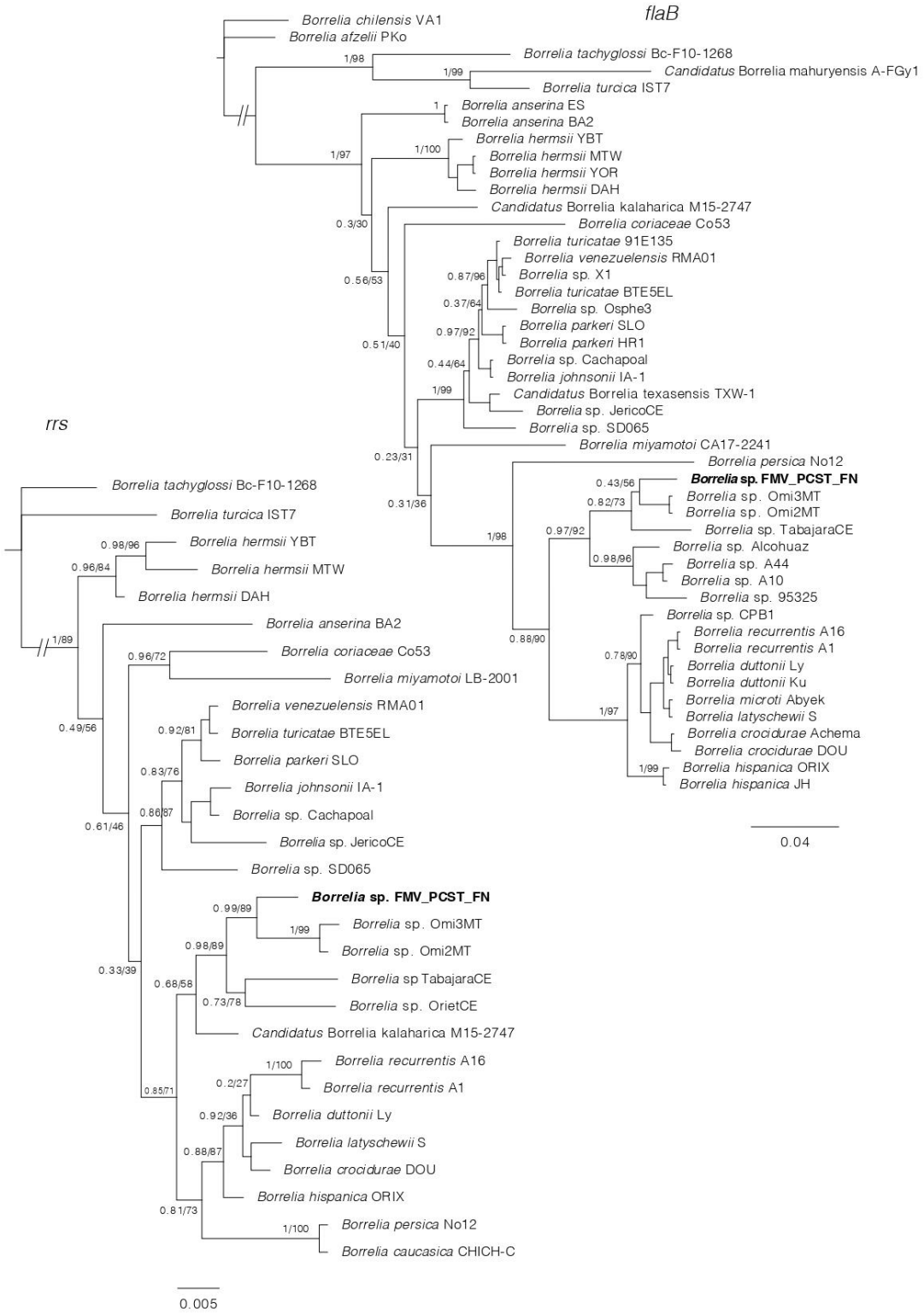

Fig. 5. Phylogenetic analyses of relapsing fever group (RFG) *Borrelia* spp. inferred for *rrs* and *flaB* partial sequences. Bold indicates the *Borrelia* sp. isolated in this study. Trees are drawn to scale. Numbers above or below tree branches represent Bayesian posterior probabilities/ML bootstrap values. Scale bar indicates nucleotide substitutions per site.

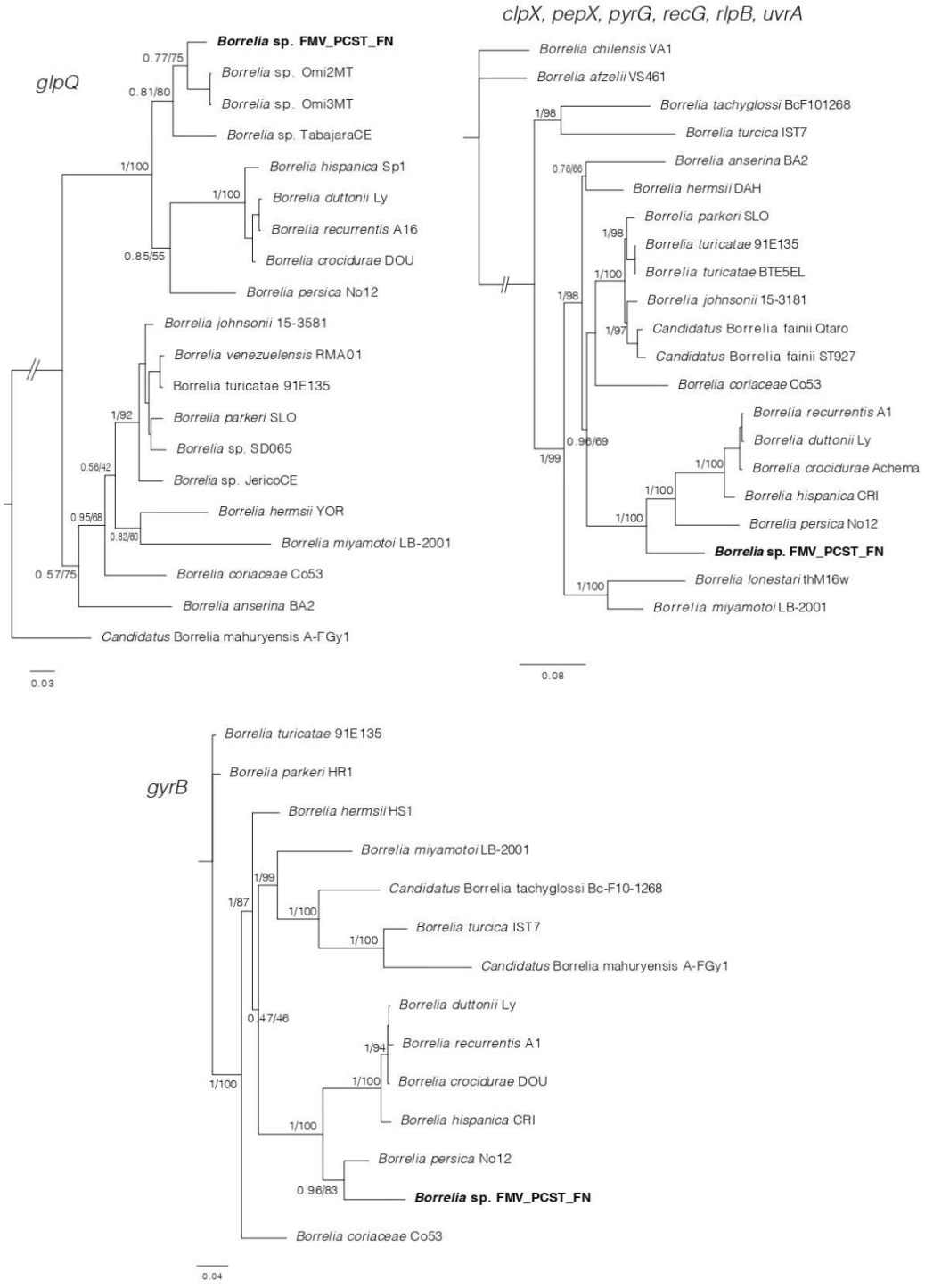

Fig. 6. Phylogenetic analyses of relapsing fever group (RFG) *Borrelia* spp. inferred for *glpQ, gyrB*, and concatenated *clpX, pepX, pyrG, recG, rlpB, uvrA* (MLST). Bold indicates borreliae from this study. Trees are drawn to scale. Numbers above or below tree branches represent Bayesian posterior probabilities/ML bootstrap values. Scale bar indicates nucleotide substitutions per site.

3.4 DISCUSSION

In this study we obtained three primary isolates (Buíque-PCST, Floresta-FMV, and Serrita-FN) of a novel RFG *Borrelia* species through the feeding of *O.* cf. *tabajara* ticks upon guinea pigs, which showed spirochetemia between 9 and 39 days after tick feeding. Guinea pig infection was confirmed by animal passage, based on the inoculation of guinea pig infected blood into newborn guinea pigs, mouse and hamster. Although the three borrelial isolates were from three geographically distinct populations of *O.* cf. *tabajara* ticks, molecular analyses indicated that the three populations were infected by the same borrelial organism, as they showed identical DNA partial sequences of 11 *Borrelia* genes, *rrs*, *flaB*, *glpQ*, *gyrB*, *rrs-rrlA, clpX*, *pepX*, *pyrG*, *recG*, *rplB* and *uvrA.* Phylogenetic analyses based on these partial sequences indicated that isolates Buíque-PCST, Floresta-FMV, and Serrita-FN represent a distinct taxon that is more closely related to Old World *Ornithodoros*-associated *Borrelia* species than to New World borreliae, including *B. venezuelensis,* which was recently isolated from *O. rudis* from Brazil (Muñoz-Leal et al. 2018).

In the phylogenetic analyses inferred from partial sequences of the borrelial genes *rrs*, *flaB* and/or *glpQ*, the isolates Buíque-PCST, Floresta-FMV, and Serrita-FN formed a monophyletic group with borrelial agents recently reported by molecular methods in *Ornithodoros* ticks from Brazil (Muñoz-Leal et al. 2021a). However, the phylogenetic distances between the herein characterized agent and these previous borrelial haplotypes were higher than the distances between several RFG *Borrelia* valid species (Figs. 5A-B, 6A), reinforcing that isolates Buíque-PCST, Floresta-FMV and Serrita-FN represent a new RFG taxon. Interestingly, the insertion of this Brazilian monophyletic group within a large clade composed by *Ornithodoros*-associated RFG borreliae from the Old World (*B. hispanica*, *B. duttonii*, *B. crocidurae*, and *B. persica*) refute the classical paradigm that *Ornithodoros*-associated RFG borreliae are divided into two clades, one composed by Old World species, and another by New World species (Talagrand-Reboul et al. 2018). Undoubtedly, the historical support of this hypothesis was related to the little exploration of the diversity of RFG borreliae in many parts of the world, especially in South America. Indeed, the improvement in phylogenetic tools adopted in recent studies have pointed out this division as rather artificial (Cutler 2015). For instance, at least two novel RFG borrelial agents from Africa (an unnamed *Borrelia* sp. and '*Candidatus* Borrelia fainii') were shown by phylogenetic analyses to belong to the classical New World clade of RFG *Borrelia* spp. (Kisinza et al. 2003, Qiu et al. 2019).

The procedure of feeding field-collected ticks on laboratory animals (i.e., xenodiagnosis) has been used to recover spirochetes before cultivation in axenic media (Cutler et al. 2017). When this procedure is successful, it also demonstrates vector competence. Hence, herein we demonstrated that *O.* cf. *tabajara* is a competent vector of *Borrelia* sp. isolates Buíque-PCST, Floresta-FMV, and Serrita-FN. Previous studies demonstrated that the characterization of RFG borreliae through experimental infection of rodents resulted in variable degrees of clinical signs and borrelial pathogenicity (Talagrand-Reboul et al. 2018). In the present study, none of the infected rodents developed fever or any clinical sign of illness during the evaluated period, including during the highest peaks of spirochetemia. This finding is similar to studies with *B. hermsii,* which induced no signs of illness in chipmunks (*Eutamias amoenus*) and meadow voles (*Microtus pennsylvanicus*) during spirochetemia (Burgdorfer and Mavros 1970), and with *Borrelia crocidurae*, which also did not induce clinical signs in the multimammate rat during spirochetemia (*Mastomys natalensis*) (Boardman et al. 2019). In contrast, *B. hermsii* induced clinical illness coincidently with spirochetemia in pine squirrels (*Tamiasciurus hudsonicus richardsoni*) (Burgdorfer and Mavros 1970). Since there is no correlation between the pathogenicity of a borrelial agent to humans and to laboratory animals, it is not known if *Borrelia* sp. isolates Buíque-PCST, Floresta-FMV, and Serrita-FN are capable of causing relapsing fever in humans.

Even though we used only one laboratory mouse and one hamster for experimental infections, guinea pigs were clearly more susceptible than these two former rodents to the infection by *Borrelia* sp. isolates Buíque-PCST, Floresta-FMV, and Serrita-FN; i.e., during spirochetemia peaks, mean number of spirochetes/microscopy field varied from 10 to 30 in all but one guinea pigs, contrasting to maximal peaks of ≤1 in a mouse and 7 spirochetes/microscopy field in a hamster (Figs. 3, 4). Spirochetemia in guinea pigs were observed up to 26 to 31 days, similarly to previous studies using different rodent species experimentally infected with *B. hermsii* or *B. crocidurae* (Burgdorfer and Mavros 1970, Johnson et al. 2016, Boardman et al. 2019). The spirochetemic period in guinea pigs was characterized by two or three peaks interspersed by few days with no or very low spirochetemia (≤1 spirochete/microscopy field). This pattern has been reported for RFG borreliae and is related to antigenic variation of bacterial major surface immunogenic proteins [variable major proteins (Vmps)] during the infection period (Talagrand-Reboul et al. 2018).

Most of the RFG borreliae are maintained in enzootic cycles between *Ornithodoros* ticks and rodents (Cutler 2015, Talagrand-Reboul et al. 2018). The two tick species of the present study, *O. rietocorreai* and *O.* cf. *tabajara*, were collected from rocky formations inhabited by rodents, including the Caviidae rock cavy. In fact, this rodent species is regarded as one of main hosts for *O. rietcorreai* (Labruna et al. 2016) and possibly also for *O. tabajara* (Muñoz-Leal et al. 2021b). This condition motivated us to test a laboratory Caviidae species (guinea pig, *Cavia porcellus*) for isolation of borreliae, given the phylogenetic close relatedness between rocky cavy and guinea pig (Rowe and Honeycutt 2002). Indeed, our results of spirochetemic guinea pigs claim for additional field studies to explore the occurrence of natural borrelial infection in rocky cavy and other Caviidae species living in rocky formations with the presence of *O.* cf. *tabajara,* and the likely participation of these rodents in the enzootic cycle of *Borrelia* sp. isolates Buíque-PCST, Floresta-FMV, and Serrita-FN.

Although *O.* cf. *tabajara* was collected in sympatry with *O. rietocorreai* in the prospected environments of the present study, borreliae were not observed in the guinea pigs infested with the latter tick species. This condition suggests a specificity affinity of isolates Buíque-PCST, Floresta-FMV, and Serrita-FN to *O.* cf. *tabajara.* In fact, a strict host specificity between borreliae and *Ornithodoros* ticks has been reported for most of the RFG agents (Cutler 2015, Talagrand-Reboul et al. 2018). On the other hand, we cannot exclude the possibility that *O. rietcorreai* specimens were infected by any of the current isolates, since we did not evaluate ticks by direct methods such as PCR targeting borrelial genes. In addition, it is also possible that those *O. rietcorreai* specimens were carrying another borrelial agent not infective for guinea pigs. For instance, Muñoz-Leal et al. (2021a) reported molecular detection of another RFG agent (*Borrelia* sp. OrietCE) in *O. rietcorreai* from Ceará state (Caatinga biome), suggesting that this tick species might also be associated with a specific borrelial agent.

Although the *O.* cf. *tabajara* ticks presented morphological features compatible with the original description of *O. tabajara* from Ceará state, their 16S rDNA sequences were 8% different, indicating that they could represent different species or two different lineages of *O. tabajara.* Interestingly, the original *O. tabajara* ticks from Ceará were found infected by another borrelial agent, *Borrelia* sp. TabajaraCE (Muñoz-Leal et al. 2021a). In our phylogenetic analyses inferred from partial sequences of the *rrs*, *flaB* and *glpQ* genes (Figs. 5A-B, 6A), *Borrelia* sp. TabajaraCE was shown to be distinct but closely related to the *O.* cf. *tabajara* isolates (Buíque-PCST, Floresta-FMV, and Serrita-FN). Indeed, these findings are

coherent with the paradigm of strict host specificity between borreliae and *Ornithodoros* ticks (Cutler 2015, Talagrand-Reboul et al. 2018).

Based on the unique genetic profile of isolates Buíque-PCST, Floresta-FMV, and Serrita-FN, we propose that a formal description and validation of a new taxon needs to be performed in a near future, after its establishment in axenic media and determination of its entire genome.

3.5 REFERENCES

Adeolu M, Gupta RS. A phylogenomic and molecular marker based proposal for the division of the genus *Borrelia* into two genera: the emended genus *Borrelia* containing only the members of the relapsing fever *Borrelia*, and the genus *Borreliella* gen. nov. containing the members of the Lyme disease *Borrelia* (*Borrelia burgdorferi* sensu lato complex). Antonie van Leeuwenhoek. 2014; 105(6):1049–72. https://doi.org/10.1007/s10482-014-0164-x

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of molecular biology*, 215(3), 403-410.

Ataliba, A. C., Resende, J. S., Yoshinari, N., & Labruna, M. B. (2007). Isolation and molecular characterization of a Brazilian strain of *Borrelia anserina*, the agent of fowl spirochaetosis. *Research in veterinary science* 83(2), 145-149.

Barbour, A. G., and R. S. Gupta. 2021. The family *Borreliaceae* (Spirochaetales), a diverse group in two genera of tick-borne spirochetes of mammals, birds, and reptiles. J. Med. Entomol. 58: 1513–1524.

Boardman K, Rosenke K, Safronetz D, Feldmann H, Schwan TG. Host Competency of the Multimammate Rat Mastomys natalensis Demonstrated by Prolonged Spirochetemias with the African Relapsing Fever Spirochete Borrelia crocidurae. Am J Trop Med Hyg. 2019 Dec;101(6):1272-1275. doi: 10.4269/ajtmh.19-0590.

Bunikis, J., Garpmo, U., Tsao, J., Berglund, J., Fish, D., & Barbour, A. G. (2004). Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe. *Microbiology*, 150(6), 1741-1755.

Burgdorfer, W., & Mavros, A. J. (1970). Susceptibility of various species of rodents to the relapsing fever spirochete, *Borrelia hermsii*. *Infection and Immunity*, 2(3), 256-259.

Cutler SJ. Relapsing fever borreliae: a global review. Clin Lab Med. 2015;35:847–65.

Cutler SJ, Rudenko N, Golovchenko M, Cramaro WJ, Kirpach J, Savic S, Christova I, Amaro A. 2017. Diagnosing borreliosis. Vector-Borne Zoonotic Dis. 17:2–11.

da Silva JMC, Leal IR, Tabarelli M. 2017. Caatinga, the largest tropical dry forest region in South America. Springer, Switzerland, 482pp

Dantas-Torres F, Fernandes Martins T, Muñoz-Leal S, Onofrio VC, Barros-Battesti DM. 2019. Ticks (Ixodida: Argasidae, Ixodidae) of Brazil: Updated species checklist and taxonomic keys. Ticks Tick Borne Dis. 10:101252. doi: 10.1016/j.ttbdis.2019.06.012.

Davis GE. Observations on the biology of the argasid tick, *Ornithodoros brasiliensis* Aragão, 1923; with the recovery of a spirochete *Borrelia brasiliensis*, n. sp. J Parasitol. 1952;5:473–6.

Faccini-Martínez, Á. A., C. R. Silva-Ramos, A. M. Santodomingo, A. Ramírez-Hernández, F. B. Costa, M. B. Labruna, and S. Muñoz-Leal. 2022. Historical overview and update on relapsing fever group *Borrelia* in Latin America. Parasit. Vectors 15: 196.

Huelsenbeck JP. Bayesian phylogenetic model selection using reversible jump Markov chain Monte Carlo. Mol Biol Evol. 2004;21:1123–33.

Huelsenbeck JP, Rannala B. Frequentist properties of Bayesian posterior probabilities of phylogenetic trees under simple and complex substitution models. Syst Biol. 2004;53:904–13.

Johnson TL, Fischer RJ, Raffel SJ, Schwan TG. 2016. Host associations and genomic diversity of Borrelia hermsii in an endemic focus of tick-borne relapsing fever in western North America. Parasit Vectors. 9:575. doi: 10.1186/s13071-016-1863-0.

Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat Methods. 2017;14:587–9.

Katoh K, Standley DM. MAFFT Multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013;30:772–80.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., ... & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28(12), 1647-1649.

Kisinza WN, McCall PJ, Mitani H, Talbert A, Fukunaga M. 2003. A new identified tick-borne *Borrelia* species and relapsing fever in Tanzania. The Lancet 362, 1283-1284.

Kneubehl AR, Krishnavajhala A, Leal SM, Replogle AJ, Kingry LC, Bermúdez SE, Labruna MB, Lopez JE. 2022. Comparative genomics of the Western Hemisphere soft tick-borne relapsing fever borreliae highlights extensive plasmid diversity. BMC Genomics. 23, 410. doi: 10.1186/s12864-022-08523-7.

Labruna, M. B., Nava, S., Marcili, A., Barbieri, A. R., Nunes, P. H., Horta, M. C., & Venzal, J. M. (2016). A new argasid tick species (Acari: Argasidae) associated with the rock cavy, *Kerodon rupestris* Wied-Neuwied (Rodentia: Caviidae), in a semiarid region of Brazil. *Parasites & Vectors*, 9(1), 1-15.

Lanfear R, Calcott B, Ho SYW, Guindon S. PartitionFinder: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. Mol Biol Evol. 2012;29:1695–701.

Mangold, A. J., Bargues, M. D., & Mas-Coma, S. (1998). Mitochondrial 16S rDNA sequences and phylogenetic relationships of species of *Rhipicephalus* and other tick genera among Metastriata (Acari: Ixodidae). *Parasitology research*, 84(6), 478-484.

Margos, G., Gatewood, A. G., Aanensen, D. M., Hanincová, K., Terekhova, D., Vollmer, S. A., Cornet M, Piesman J, Donaghy M, Bormane A, Hurn MA, Feil EJ, Fish D, Casjens S, Wormser GP, Schwartz I, Kurtenbach, K. (2008). MLST of housekeeping genes captures geographic population structure and suggests a European origin of *Borrelia burgdorferi*. *Proceedings of the National Academy of Sciences* 105(25), 8730-8735.

Margos, G., A. Gofton, D. Wibberg, A. Dangel, D. Marosevic, S.-M. Loh, C. Oskam, and V. Fingerle. 2018. The genus *Borrelia* re-loaded. PLoS One 13: e0208432.

Margos, G., G. P. Wormser, I. Schwartz, M. Markowicz, A. J. Henningsson, R. Lienhard, B. Stevenson, A. Estrada-Peña, A. Sing, V. Fingerle, and M. Göker. 2022. Evidence of taxonomic bias in public databases: The example of the genus *Borrelia*. Ticks Tick Borne Dis. 13: 101994.

Minh BQ, Nguyen MAT, von Haeseler A. Ultrafast approximation for phylogenetic Bootstrap. Mol Biol Evol. 2013;30:1188–95.

Muñoz-Leal S, Faccini-Martínez ÁA, Costa FB, Marcili A, Mesquita ETKC, Marques EP Jr, Labruna MB. 2018. Isolation and molecular characterization of a relapsing fever *Borrelia* recovered from *Ornithodoros rudis* in Brazil. Ticks Tick Borne Dis. 9:864–71.

Muñoz-Leal, S., A. A. Faccini-Martínez, J. Pérez-Torres, S. M. Chala-Quintero, M. T. Herrera-Sepúlveda, C. L. Cuervo, and M. B. Labruna. 2021a. Novel *Borrelia* genotypes in bats from the Macaregua Cave, Colombia. Zoonoses Public Health 68: 12-18.

Muñoz-Leal, S., Venzal, J. M., Jorge, F. R., Teixeira, B. M., & Labruna, M. B. (2021b). A new species of soft tick from dry tropical forests of Brazilian Caatinga. *Ticks and Tick-borne Diseases*, 12(5), 101748.

Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol. 2015;32:268–74.

Qiu Y, Nakao R, Hang'ombe BM, Sato K, Kajihara M, Kanchela S, Changula K, Eto Y, Ndebe J, Sasaki M, Thu MJ, Takada A, Sawa H, Sugimoto C, Kawabata H. 2019. Human

borreliosis caused by a New World relapsing fever *Borrelia*-like organism in the Old World. Clin Infect Dis. 69:107-112. doi: 10.1093/cid/ciy850.

Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. Susko E, editor. Syst Biol. 2018;67:901–4.

Ras, N.M., Lascola, B., Postic, D., Cutler, S.J., Rodhain, F., Baranton, G., Raoult, D., 1996. Phylogenesis of Relapsing Fever Borrelia spp. Int. J. Syst. Bacteriol. 46, 859–865. doi:10.1099/00207713-46-4-859

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, et al. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol. 2012;61:539–42.

Rowe DL, Honeycutt RL. 2002. Phylogenetic relationships, ecological correlates, and molecular evolution within the Cavioidea (Mammalia, Rodentia). Molecular Biology and Evolution, 19, 263–277. https://doi.org/10.1093/oxfordjournals.molbev.a004080

Sands, A. F., Apanaskevich, D. A., Matthee, S., Horak, I. G., & Matthee, C. A. (2017). The effect of host vicariance and parasite life history on the dispersal of the multi-host ectoparasite, *Hyalomma truncatum*. *Journal of Biogeography* 44(5), 1124-1136.

Sangioni, L. A., Horta, M. C., Vianna, M. C., Gennari, S. M., Soares, R. M., Galvão, M. A., Schumaker TT, Ferreira F, Vidotto O, Labruna, M. B. (2005). Rickettsial infection in animals and Brazilian spotted fever endemicity. *Emerging Infectious Diseases* 11(2), 265.

Schwan, T. G., Raffel, S. J., Schrumpf, M. E., Policastro, P. F., Rawlings, J. A., Lane, R. S., E. B. Breitschwerdt, and S.F. Porcella. 2005. Phylogenetic analysis of the spirochetes *Borrelia parkeri* and *Borrelia turicatae* and the potential for tick-borne relapsing fever in Florida. *Journal of Clinical Microbiology*, 43(8), 3851-3859.

Stromdahl, E. Y., P. C. Williamson, T. M. Kollars, S. R. Evans, R. K. Barry, M. A. Vince, and N. A. Dobbs. 2003. Evidence of *Borrelia lonestari* DNA in *Amblyomma americanum* (Acari: Ixodidae) removed from humans. J. Clin. Microbiol. 41: 5557-5562.

Schwarz G. 1978. Estimating the dimension of a model. Ann Stat. 6:461–4.

Talagrand-Reboul E, Boyer PH, Bergström S, Vial L, Boulanger N. 2018. Relapsing fevers: neglected tick-borne diseases. Front Cell Infect Microbiol. 8, 98.

Trevisan G, Cinco M, Trevisini S, di Meo N, Chersi K, Ruscio M, Forgione P, Bonin S. 2021. Borreliae Part 1: *Borrelia* Lyme group and echidna-reptile group. Biology (Basel). 10, 1036. doi: 10.3390/biology10101036.

Trevisan G, Cinco M, Trevisini S, di Meo N, Ruscio M, Forgione P, Bonin S. 2021. Borreliae Part 2: *Borrelia* relapsing fever group and unclassified *Borrelia*. Biology (Basel). 10, 1117. doi: 10.3390/biology10111117.

Yang Z. Maximum-likelihood models for combined analyses of multiple sequence data. J Mol Evol. 1996;42:587–96.

4. CONCLUSIONS

A significant size divergence in larvae and relative genetic distance was observed among *O. fonsecai* populations from different biomes, and more studies are needed to clarify the possible existence of a complex of species or phenotype plasticity manifestation. New records and geographical distribution are provide for soft ticks in the Caatinga biome, with the addition of a new *Borrelia* sp. of RFG and the first report of *O.* cf. *tabajara,* which will have their taxonomic status clarified from ongoing studies.