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Original article

Detected microorganisms and new geographic records of *Ornithodoros rietcorreai* (Acari: Argasidae) from northern Brazil

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ABSTRACT

Reliable data on distributional ranges of soft ticks (Argasidae) and assessments of putative tick-borne agents enhance the understanding on tick-associated microorganisms. A total of 96 ticks morphologicaly and molecularly identified as *Ornithodoros rietcorreai* were collected in Tocantins State, Brazil, using Noireau traps with living bait as CO2 source. Ninety-six ticks (54 nymphs, 32 males, 10 females) with different engorgement degrees were collected. Fourty-seven (48.9%) of them were individually screened by PCR for detecting bacteria of Anaplasmataceae family and genera *Rickettsia*, and *Borrelia*. The presence of protozoans of the genus *Babesia* was assessed as well. Fourty seven ticks were submitted to analysis. Nine ticks (19.1%) yielded sequences for *gltA* and *htrA* genes most identical with a series of endosymbiont rickettsiae and *Rickettsia bellii*, respectively. Upon two ticks (4.2%) we retrieved DNA of a potential new *Wolbachia* sp., and DNA of a putative novel *Hepatozoon* was characterized from three (6.4%) specimens. No DNA of *Babesia* or *Borrelia* was detected. Remarkably, amplicons of unidentified eukaryotic organisms, most closely related with apicomplexans but also with dinoflagellates (91% of identity after BLAST analyses), were recovered from two ticks (4.2%) using primers designed for *Babesia* 18S rRNA gene. Our records expand the distribution of *O. rietcorreai* into Brazilian Cerrado biome and introduce the occurrence of microorganisms in this tick species.

1. Introduction

Soft ticks (Argasidae) are blood-feeding parasites associated with all classes of terrestrial volant and non-volant vertebrates (Hoogstraal, 1985). In contrast to hard ticks (Ixodidae), phylogeny and taxonomic classification of these ticks remain incompletely solved, resulting in different systematic approaches that lead several species to be assigned into more than one genus (Estrada-Pena et al., 2010; Guglielmone et al., 2010). In the Neotropical Zoogeographical Region 93 species represent this family of ticks, and 26 have been reported in Brazil (Guglielmone et al., 2003; Dantas-Torres et al., 2009; Muñoz-Leal et al., 2018b). Although in last 15 years South American fauna of soft ticks has gained increasing attention collections are still insufficient, a fact that directly underestimates both their geographic distribution and diversity.

Ornithodoros rietcorreai was described upon laboratory-reared larvae and adult specimens collected underneath rocks frequented by rodents (i.e. *Kerodon rupestris*) and bats (Labruna et al., 2016). Recent records point that this tick is also associated with reptiles (Alcantara et al., 2018). While current geographical distribution includes wild arid ecosystems from the Caatinga biome of Paraiba, Piauí and Ceará states (northeastern Brazil) (Labruna et al., 2016; Alcantara et al., 2018), this species might eventually colonize human dwellings and cause toxicosis after its bite (Oliveira et al., 2018; Muñoz-Leal et al., 2019). Despite representing a human parasite, data on microorganisms harbored by *O. rietcorreai* do not exist.

A number of viruses and bacterial agents causing severe disease in humans and animals are currently known to be transmitted by argasid ticks worldwide (Schmidtmann et al., 1976; Hoogstraal, 1985; Labuda and Nuttall, 2004). With the exception of few experimental evidence pointing that soft ticks (i.e. *Ornithodoros parkeri*) can transmit *Rickettsia rickettsii* to laboratory mammals (Davis, 1943), and the recognized role of *Ornithodoros coriaceus* as the vector of the epizootic bovine abortion

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Table 1
List of primers used in the present study.

Organism	Gene		Primers (5'-3')	T (C^{O})	No. of cycles	Product size (bp)	References
Tick mitochondria	16S rRNA	16S + 1	CCGGTCTGAACTCAGATCAAGT	55	35	460	Mangold et al. (1998)
		16S-1	GCTCAATGATTTTTTAAATTGCTGT				
	12S rRNA	T1B	AAA CTAGGATTAGATACCCT	52	35	360	Beati et al. (2012)
		T2A	AATGAGAGCGACGGGCGATGT				
Anaplasmataceae	16S rRNA	EHR16SD	GGTACCYACAGAAGAAGTCC	55	35	345	Inokuma et al. (2000)
		EHR16SR	TGCACTCATCGTTTACAG				
Rickettsia	*gltA*	CSF1	CATCCTATGGCTATTATGCTTGC	55	35	885	Rozental et al. (2017)
		CSR1	TATACTCTCTATG(T/A)AC(A/G)T(A/G)ACC				
	gltA	CSF2[a]	CTTACCGCTATTAGAATGATTGC	63	25	572	Rozental et al. (2017)
		CSR2[a]	GAGCGA(T/G)AGCTTCAAG(T/C)TCTAT				
	htrA	17k-5	GCTTTACAAAATTCTAAAAACCATATA	48	35	549	Labruna et al. (2004)
		17k-3	TGTCTATCAATTCACAACTTGCC				
	ompA	190.70	ATGGCGAATATTTCTCCAAAA	58	35	632	Roux et al. (1996)
		190.701	GTTCCGTTAATGGCAGCATCT				
	ompB	120-M59	CCGCAGGGTTGGTAACTGC	51	35	856	Roux and Raoult (2000)
		120- 807	CCTTTTAGATTACCGCCTAA				
Borrelia	*fla*	BorFlaF1	TACATCAGCTATTAATGCTTCAAGAA	65	25	729	Blanco et al. (2017)
		BorFlaR1	GCAATCATWGCCATTGCRGATTG				
	fla	BorFlaF2[a]	CTGATGATGCTGCTGGWATGG	61	30	410	Blanco et al. (2017)
		BorFlaR2[a]	TCATCTGTCATTRTWGCATCTT				
Babesia	18S rRNA	Bab18F1	GCGGTAATTCCAGCTCCAATAGCGTATAT	63	25	1150	Blanco et al. (2017)
		Bab18R1	TCCGAATAATTCACCGGATCACTCGAT				
	18S rRNA	Bab18F2[a]	AGACGATCAGATACCGTCGTAGTCCTA	66	30	670	Blanco et al. (2017)
		Bab18R2[a]	ATCACTCGATCGGTAGGAGCGACG				

T = anneling temperature.
[a] Primers used in the nested reaction.

agent (Schmidtmann et al., 1976), argasid-borne bacteria are almost exclusively represented by a group of *Borrelia* species that causes relapsing fever in animals and humans (Hoogstraal, 1985). While in Brazil the importance of argasid ticks as vectors of microorganisms is still far from been elucidated, recent research has shed light on the role of *Ornithodoros rudis* as reservoir of human-pathogenic *Borrelia venezuelensis* (Muñoz-Leal et al., 2018a). In order to expand the knowledge on soft ticks and their associated microbial agents, in this study we applied a molecular approach to identify and assess the phylogenetic position of specimens collected in northern Brazil, and to detect the presence of bacteria and protozoans.

2. Materials and methods

2.1. Collection of ticks

In June 2007 and October 2015, prospections for Triatominae insects (Reduviidae: Hemiptera) using Noireau Traps (Noireau et al., 1999) were conducted along a rocky hill located in the Municipality of Paranã, (12°42′40″S, 48°13′13″W; 300 m), Tocantins state, Cerrado biome, northern Brazil. Noireau traps were designed to collect silvatic *Triatoma* spp. in hollow trees and later applied to capture *Rhodnius* spp. in various environments (Noireau et al., 2002). These traps consist into small plastic vials (250 or 500 cm^3) containing living bait as CO2 source. As blood-sucking arthropods are attracted and come closer to the bait, they stick themselves onto a double-sided adhesive tape placed around the external surface of the vial. We used a *Gallus gallus* chick as bait. A total of 675 traps (600 during June 2007 and 75 during October 2015) were mounted. All traps were placed among rocks or crevices, set in the afternoon (15:00–16:00 h) and checked the next morning (9:00–10:00 h) to collect the material. With the exception of two fully engorged females that were kept alive inside individual tubes, collected ticks were placed in 70% ethanol and brought to the laboratory of Hantaviruses and Rickettsioses, Oswaldo Cruz Institute, Fiocruz. Permits for field collections were granted by SISBIO (Sistema de Autorização e Informação em Biodiversidade) license 18014-1.

2.2. Morphological identification of ticks

As in argasid ticks the examination of larval characters is crucial to achieve an accurate specific diagnosis, alive engorged females were individually placed in glass tubes inside an incubator (25 °C, 80% relative humidity) to obtain ovipositions. Both females laid eggs. While part of larvae was preserved in 70% ethanol, six (three per offspring) were clarified in a 25% KOH solution and mounted in Hoyer's medium to make semi-permanent slides for examination under optical microscopy (Olympus BX40 optical microscope). Morphological characters of slide-mounted specimens were compared with descriptions of Neotropical Ornithodorinae (Kohls et al., 1969; Labruna et al., 2016). Micrographs of larvae were obtained with an Olympus DP70 camera using the software Image-Plus Pro v5.1. Collected nymphs and adults were examined using a SteREO Discovery V12 stereomicroscope (Zeiss, Munich, Germany) and compared with descriptions of *Ornithodoros* spp. (Venzal et al., 2008; Nava et al., 2013; Labruna et al., 2016). Additional comparisons were made with nymphs and adults of *O. rietcorreai* deposited in the tick collection "Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva" (CNC) at the University of São Paulo, SP, Brazil (accession numbers CNC-3264, -3265, -3266). Adult *Ornithodoros* were photographed with the software ZEN 2 pro.

2.3. Molecular analyses

To confirm morphological identification of nymphs and adults, and further assess the prescence of bacteria and protozoans on them, molecular tools were implemented as previously described (Ogrzewalska et al., 2012). For this purpose 47 ticks were submitted to DNA extraction using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA). To check successful extractions and further characterize identified ticks, inicial PCRs targeting a portion of the tick mitochondrial 16S rRNA and 12S rRNA genes were performed to all DNA-extracted samples (Mangold et al., 1998; Beati et al., 2012). For six specimens (four nymphs, one male and one female) we sequenced both loci. A battery of conventional and nested PCR assays targeting different genes of *Babesia* (18S rRNA), *Borrelia* (*flaB*), *Rickettsia* (*gltA*, *htrA*, *ompA*, *ompB*) and Anaplasmataceae family (16S rRNA) were implemented for all samples

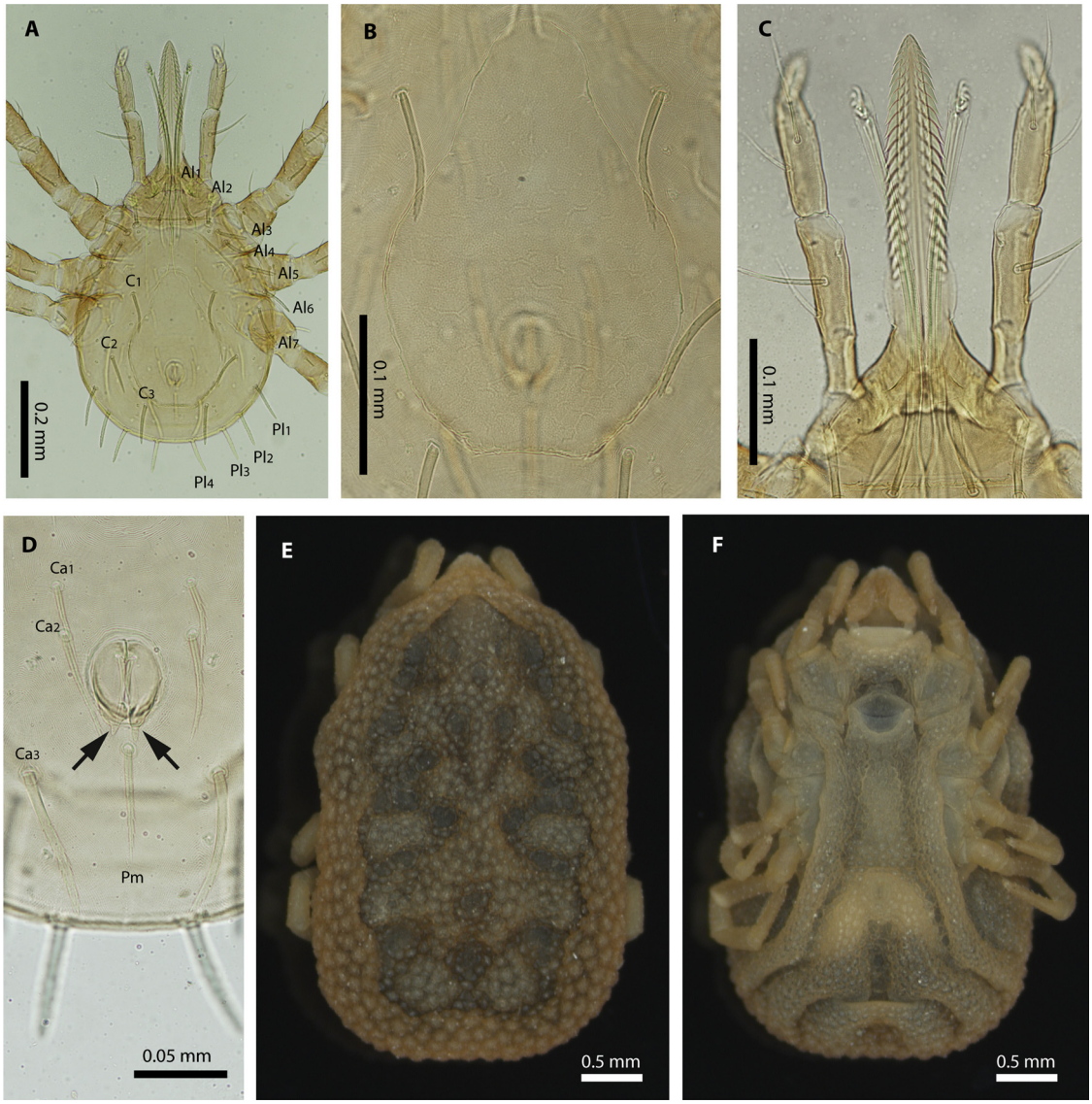

Fig. 1. Micrographs of *Ornithodoros rietcorreai*. Unfed larva: (A) dorsal view, (B) dorsal plate, (C) hypostome, (D) leave-like conspicous projections of anal plates (arrowed). Female: (E) dorsal view, (F) ventral view. Abbreviations of setae: Al, anterolateral; C, central; Pl, posterolateral; Ca, circumanal; Pm, posteromedian.

as well. Primers and PCR conditions are listed in Table 1. All conventional reactions were performed into a final volume of 25 µl per reaction, which contained 12.5 µl of DreamTaq Green PCR Master Mix, 8.0 µl of nuclease-free water (Thermo Fisher Scientific Inc, Waltham, MA, USA), 1 µl of each primer at 10 µM (Invitrogen, Carlsbad, CA, USA), and 2.5 µl of DNA template. For nested PCR, 9.0 µl of nuclease-free water and 1.5 µl of DNA template were used instead. Each PCR assay included negative (nuclease-free water) and appropriate positive controls (DNA of *Babesia vogeli*, *Borrelia anserina*, *Ehrlichia canis*, or *Rickettsia rickettsii*) that were run together with tick samples.

Amplicons were visualized in 1% agarose gels stained with Gel Red Nucleic Acid Gel Stain 10,000× in DMSO (Biotium, Hayward, CA, USA). PCR products of expected size were purified with ExoSAP-IT (Affymetrix, Cleveland, OH, USA), and sequenced in a 96-capillar 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) according to protocols developed by Otto et al. (2008), and using the same primers (forward and reverse) employed for PCRs. Obtained partial sequences were edited and analized in the MEGA 6 (Tamura et al., 2013) and consensuses were submitted to BLAST analyses (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to infer closest identities with sequences of other organisms available in GenBank (Altschul et al., 1990). In the case of two-step PCR, only products of nested rounds were sequenced.

2.4. Phylogenetic analyses

Independent alignments using herein obtained consensus of tick mitochondrial 16S and 12S rDNA sequences and other sequences retrieved from GenBank were constructed with CLUSTAL W (Thompson et al., 1994). Substitution models were calculated with MEGA 5 (Tamura et al., 2011). Maximum Likelihood trees were subsequently inferred using PhyML (Guindon and Gascuel, 2003), with five substitution rate categories and 500 bootstrap replicates. The Nearest Neighbour Interchange (NNI) was used to improve tree topology (Li et al., 1996).

To assess phylogenetic positions of different detected microbial agents, individual alignments were constructed and substitution models calculated with the same softwares as above. Phylogenies were then inferred by Bayesian methods in MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). All trees were constructed with four independent Markov chains using 500,000 metropolis-coupled MCMC generations and sampling a tree every 100th generations. The first 25% of trees

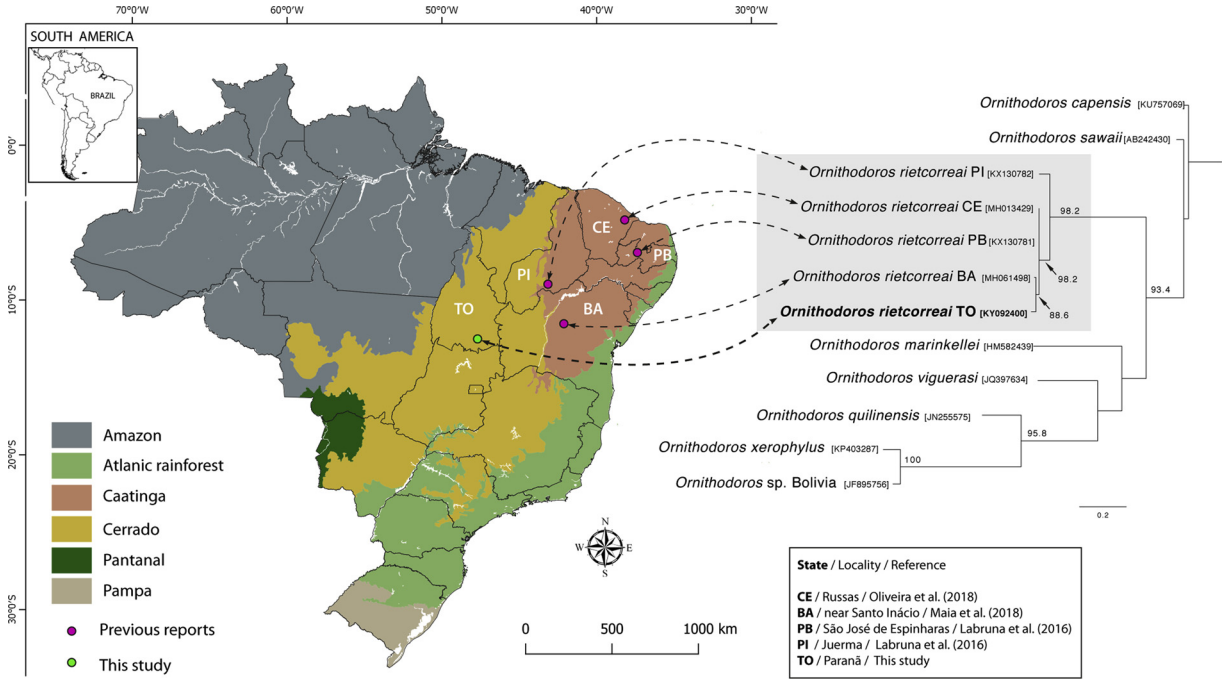

Fig. 2. Mapping and phylogenetic relationships of haplotypes retrieved from different populations of *Ornithodoros rietcorreai* along Brazil. Hasegawa-Kishino-Yano (HKY) substitution model was selected for the phylogenetic analysis. Confidence values for individual branches of the resulting trees were determined by bootstrap analysis with 500 replicates, and it is indicated for every branch. Values < 80.0 were omitted. The tree is drawn to scale and the position of *O. rietcorreai* obtained in this study is highlighted in bold. Brazilian biomes are indicated in different colors as pictured. Abbreviations: BA, Bahia State; CE, Ceará State; PI, Piauí State; PB, Paraíba State; TO, Tocantins State.

Table 2
Number of tested and positive ticks. GenBank accession numbers for sequences generated in the current study are indicated for each microorganism.

Tick stage (n)	No. of positive specimens (%)–Genbank acession number			
	Rickettsia sp.	*Wolbachia* sp.	*Hepatozoon* spp.	Uncultured eukaryotes
Nymphs (23)	5 (21.7)–MF383346[a], MH780988[b]	2 (8.7)–MF383347	1 (4.3)–MF383348	1 (4.3)–MF383351
Females (4)	1 (25)–idem	0 (0.0)	1 (25)–MF383350	0 (0.0)
Males (20)	3 (15)–idem	0 (0.0)	1 (5)–MF383349	1 (5)–MF383352
Total (47)	**9/47 (19.1)**	**2/47 (4.2)**	**3/47 (6.3)**	**2/47 (4.2)**

[a] *gltA* gene.
[b] *htrA* gene.

represented burn-in and remaining trees were used to calculate Bayesian posterior probability values. GenBank accession numbers of sequences are embedded in each phylogentic tree.

3. Results

3.1. Collection, morphological analyses and phylogeny of ticks

Apart from attracting triatomines (data not shown), traps were also efficient to attract soft ticks, allowing us to collect a total of 96 (54 nymphs, 32 males, 10 females) specimens. All collected ticks had an engorgement degree and were identified as *O. rietcorreai* by external morphology and through microscopial observation of laboratory reared larvae (Fig. 1). Slide-mounted and ethanol preserved larvae, adults and nymphs have been deposited in the CNC tick collection under accession numbers CNC-3554, -3555, -3710.

Twenty-three nymphs, 20 males and 4 females were submitted to DNA extraction and all yielded amplicons for mitochondrial 16S and 12S rRNA genes. Sequences of 451 bp obtained for 16S rRNA gene confirmed morphological identifications since a sole haplotype was retrieved from four nymphs, one male and one female. This haplotype resulted most identical with *O. rietcorreai* from Bahía (99.0%; 423/427 bp; e-value = 0.0; MH061498), Ceará (98.5%; 421/427 bp; e-value = 0.0; MH013429), Paraiba (98.5%; 421/427 pb; e-value = 0.0; KX130782) and Piauí (95.5%; 409/428 bp; e-value = 0.0; KX130782) states. Sequencing of mitochondrial 12S rRNA gene resulted into a unique haplotype of 300 bp for all ticks. While no sequence for the 12S rRNA gene was previously available for this species, highest identity values were observed with *Ornithodoros faini* (85.4%; 247/289 bp; e-value = 3e-76; KJ1335889), *Ornithotodors faccinii* (83.3%; 235/282 pb; e-value = 4e-64; KY661387) and *Ornithodoros capensis* (82.3%; 247/300 pb; e-value = 1e-64; AB075953). A phylogenetic reconstruction of soft ticks using mitochondrial 16S rDNA sequences further supports that our specimens correspond to *O. rietcorreai*, since it clusters with all other available sequences for the species into a well sustented monophyletic clade (Fig. 2). Despite low bootstrap support of many branches, a phylogenetic analysis of soft tick mitochondrial 12S rDNA points that *O. rietcorreai* forms a monophyletic group with other Neotropical *Ornithodoros* (Suppl. 1). Mitochondrial 16S and 12S rDNA sequences generated in the current study were deposited in GenBank under the accession numbers KY092400 and KY092401, respectively.

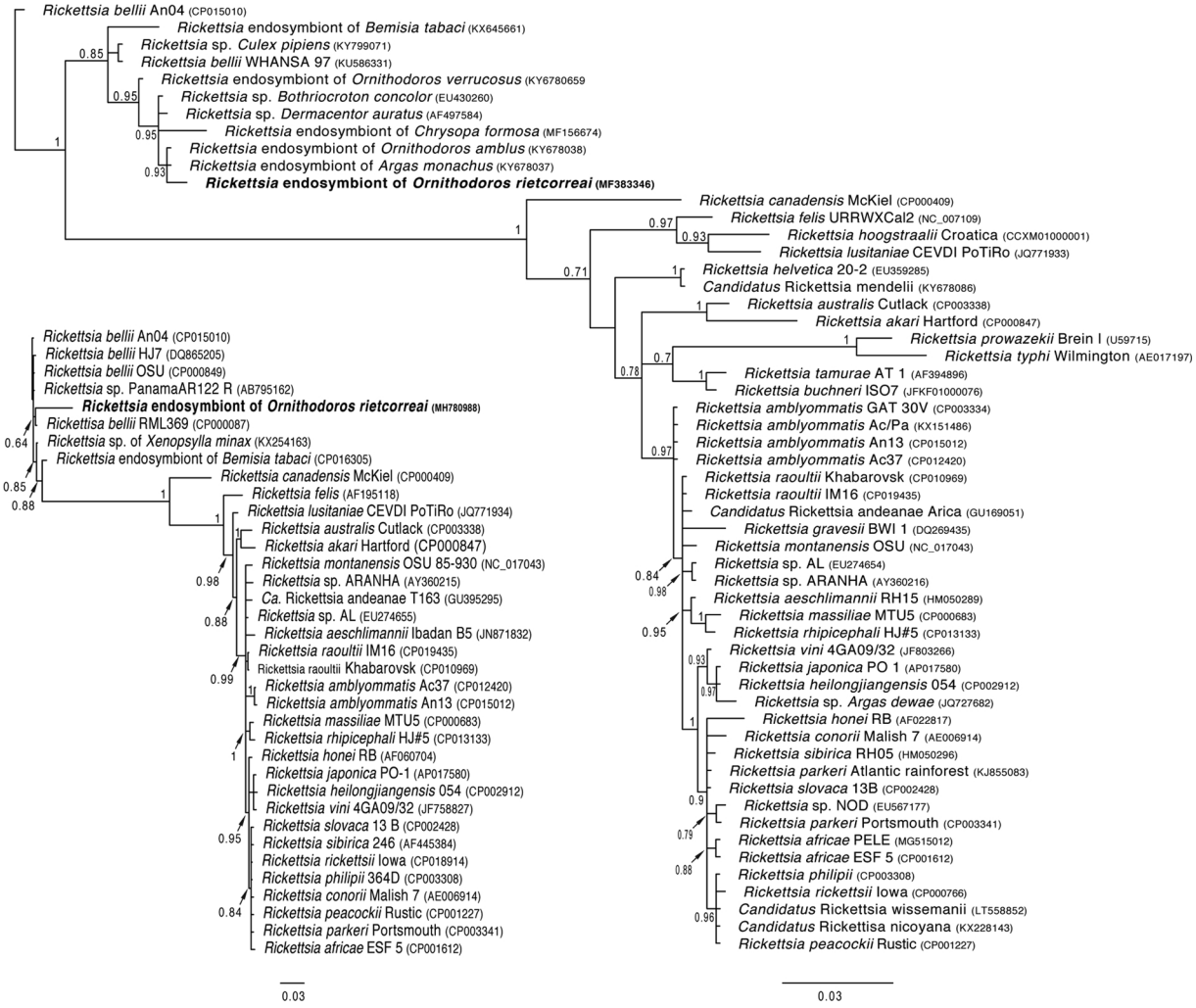

Fig. 3. Phylogenetic trees of *Rickettsia* spp. using the GTR substitution model. Largest tree represents the inference for *gltA* gene and the smaller tree the inference for *htrA* gene. Phylogeny of *gltA* and *htrA* gene included 55 and 35 sequences respectively. *Rickettsia bellii* An04 (CP015010) rooted both trees. Otherwise omitted, numbers above and below the branches represent Bayesian posterior probability ≥0.70. The position of the *Rickettsia* sp. detected in *Ornithodoros rietcorreai* is highlighted in bold.

3.2. Molecular identification and phylogenetic analyses of detected agents

Expected sized amplicons were obtained for Anaplasmataceae, Babesia, and Rickettsia PCRs (Table 2). In particular, negative results were obtained for attempts to amplify Rickettsia ompA and ompB genes. Only gltA and *htrA* genes were successfully amplified and a unique consensus for each locus was retrieved in all positive specimens. BLAST comparisons for obtained 551 bp-*gtlA* haplotype pointed 99.5% (548/551 bp; e-value = 0.0) and 98.7% (544/551 bp; e-value = 0.0) of identity with endosymbiont *Rickettsia* spp. detected in *Ornithodoros amblus* (KY678038) and *Ornithodoros verrucosus* (KY678065), respectively. In turn, a sole 510 bp-haplotype was obtained for *htrA* gene, showing 94.7% (482/509 bp; e-value = 0.0) and 94.5% (482/510 bp; e-value = 0.0) of identity with *Rickettsia bellii* RML369-C (CP000087) and *Rickettsia* sp. ALSK01 (KX254163). Independent phylogenies inferred for both *gltA* and *htrA* genes indicate the *Rickettsia* sp. detected in *O. rietcorreai* as taxon related with the bellii group (Fig. 3).

Obtained 345 bp-sequences of Anaplasmataceae 16S rRNA gene were identical between them and 99.1% (341/344 bp; e-value = 6e-173) identical with an uncultured *Wolbachia* sp. (EU780456) characterized from *Cimex lectularius* (Insecta: Hemiptera). A phylogenetic analysis shows that the *Wolbachia* sp. detected in *O. rietcorreai* indeed clusters with the *Wolbachia* sp. from *C. lectularius*, both within a major clade composed by other species associated with insects (Fig. 4). Wolbachiae detected in other ticks appear forming independent clades (Fig. 4).

Remarkably, *Babesia* PCR assays amplified DNA from other protozoans. Three sequences of 653 bp (two of them identical) matched *Hepatozoon* spp. with 99.2% (647/653 bp; e-value = 0.0) – 99.3% (648/652 bp; e-value = 0.0) with *Hepatozoon* spp. characterized from reptiles (KM234614, KC342523, MG456823). Haplotypes of *Hepatozoon* obtained in this study were denominated as LHR547, LHR579 and LHR560. After a phylogenetic analysis, all tree sequences form a monophyletic clade with *Hepatozoon* sp. B266M12 (MG456823) detected in reptiles. Interestingly, sequences of *Hepatozoon* retrieved from bats and rodents (vertebrates also associated with *O. rietcorreai*) clustered into separated clades, not related with our sequences (Fig. 5).

In addition, *Babesia*-primers amplified 18S rDNA from two uncharacterized eukaryote organisms yielding two haplotypes of 648 bp each. Pairwise comparisons showed these sequences to be 98.7% (zero gaps) identical between them. After BLAST comparisons, both haplotypes showed 90.5% (593/656 bp; e-value = 0.0) and 90.7% (594/655 bp; e-value = 0.0) of identity with *Hepatozoon* spp. (JX531920; JQ762311). Yet moreover, a 90.7% (597/658 bp; e-value = 0.0)–91.7% (594/651 bp; e-value = 0.0) of identity was also observed with *Geneiorhynchus manifestus* (FJ459739), a gregarine (Apicomplexa:

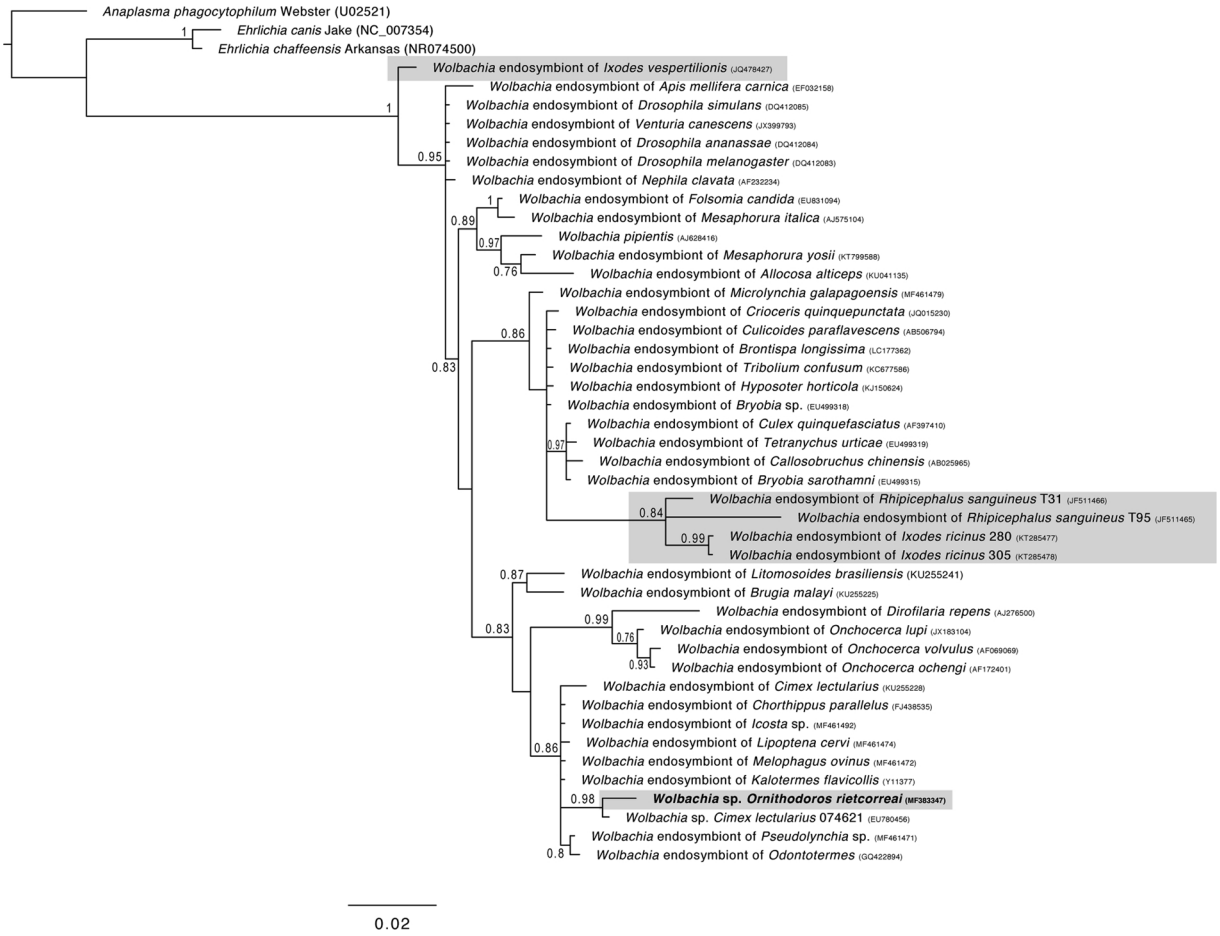

Fig. 4. Phylogentic tree of *Wolbachia* spp. using the K2 + G + I substitution model. The alignement included 46 sequences and *Anaplasma phagocytophilum* Webster (U02521), *Ehrlichia canis* Jake (NC_007354) and *Ehrlichia chaffensis* Arkansas (NR074500) were used as out-groups. Otherwise omitted, numbers above and below the branches represent Bayesian posterior probability ≥ 0.70. Sequences of *Wolbachia* spp. retrieved from ticks are denoted with a grey rectangle. The position of the *Wolbachia* sp. detected in *Ornithodoros rietcorreai* is highlighted in bold.

Eugregarinida) associated with drangonflies (Insecta: Odonata), and with marine dinoflagellates (Dinoflagellata) of genera *Barrufeta* (MH732694), *Gymnodinium* (MH732693; MH732692), and *Polykrikos* (AB466290). These unidentified uncultured eukaryotic organisms were denominated as clones LHR556 and LHR572, and were not included for phylogenetic analyses.

No DNA of *Borrelia* was detected among tested samples.

4. Discussion

The collection of *O. rietcorreai* using Noireau traps is a novelty and a promisory method of collection at least for this species. Takink into account that soft ticks often wait for their hosts in between unaccesible crevices, beneath large rocks or in deep burrows, attractive methods are a useful and effort saving alternative of collection. While *O. rietcorreai* was originally described from three localities in arid ecosystems of the Caatinga biome (Labruna et al., 2016), current results expand its distribution near 640 km towards the southwest into the Cerrado biome of Tocantins State. In the original description of *O. rietcorreai* two populations (one from Piauí State and the other from Paraíba State) were molecularly characterized and a 3.3% of divergence was evidenced between fragments of their mitochondrial 16S rRNA genes. These populations were distanced more than 600 km between them, and bidirectional crosses resulted in viable offsprings (Labruna et al., 2016). Both haplotypes of tick mitochondrial 16S rDNA obtained in this study diverged 1.4%–4.4% with available conspecific sequences in GenBank. While sequences with less than 3.3% of divergence could be considered to be conspecific, there is no evidence on a maximum value useful to trace a specific genetic threshold in *O. rietcorreai*. Thereofore 4.4% might be considered near to an upper limit of divergence. Notwithstanding, a phylogenetic analysis including all mitochondrial 16S rDNA sequences availbale for *O. rietcorreai* suggests that a 4.4% of divergence still reflects conspecificity (Fig. 2). In fact, divergences of 4.3% for 16S mitochondrial rDNA sequences have been evidenced in geographically distanced but conspecific populations of other neotropical *Ornithodoros* (i.e. *Ornithodoros atacamensis*, Muñoz-Leal et al., 2016).

Upon mitochondrial 12S rDNA sequences few conclusions apart from divergences evidenced with other soft tick taxa can be inferred, due to the lack of information concerning this locus among the Argasidae. In fact, a phylogenetic analysis performed for tick mitochondrial 12S rDNA mirrors this scenario since the majority of branches are poorly supported (Suppl. 1).

Bacteria of genus *Rickettsia* are common among the Ixodida (Duron et al., 2017). Although several tick-borne *Rickettsia* spp. are currently classified as human pathogens (Parola et al., 2013), an increasing diversity of agents of this genus has been characterized from different tick taxa and classified as endosymbionts (Weinert et al., 2009; Duron et al., 2017). Herein obtained *gltA* sequence shared an identity higher than 98% with sequences of endosymbiont *Rickettsia* spp. characterized from one *Argas* and two *Ornithodoros* species (Duron et al., 2017). In turn, retrieved *htrA* haplotype showed to be most identical with *R. bellii*, yet high observed divergence (5.3%) for a rather conserved gene (Anderson and Tzianabos, 1989) might denote that our sequences belong to an undescribed rickettsial agent. The fact that *ompA* and *ompB* genes were

Fig. 5. Phylogenetic tree of *Hepatozoon* spp. using the GTR + G substitution model. The alignment included 56 sequences. *Adelina grylli* (DQ096836), *Dactylosoma ranarum* (HQ224958) and *Haemogregarina balli* (HQ224959) rooted the tree. Otherwise omitted, numbers above and below the branches represent Bayesian posterior probability ≥0.70. The position of *Hepatozoon* haplotypes retrieved from *Ornithodoros rietcorreai* are highlighted in bold. As *O. rietcorreai* has been associated with reptiles (Alcantara et al., 2018), rodents and possibly bats (Labruna et al., 2016), we highlighted clades with *Hepatozoon* haplotypes retrieved from blood of these vertebrates to better visualize a possible group for the obtained sequences.

not amplified with primers designed to detect spotted fever group rickettsiae suggests that the *Rickettsia* sp. harbored by *O. rietcorreai* does not belong to this group (Roux et al., 1996; Roux and Raoult, 2000). Indeed, indepenedent phylogentic analyses performed for both genes point that this *Rickettsia* sp. clusters within a group related to *R. bellii*. Contrasted with the prevalence of *Rickettsia* spp. detected in other argasids (Duron et al., 2017), the presence of *Rickettsia* DNA in *O. rietcorreai* was rather low (Table 2). Wheather these low values would represent other than a symbiotic association is far from been elucidated with our data. It is important to note though, that nymphs, males and females yielded identical haplotypes for *gltA* and *htrA* genes respectively, suggesting that this rickettsial agent is at least transtadially perpetuated.

The molecular characterization of a potential *Wolbachia* sp. in two nymphs of *O. rietcorreai* is intriguing and represents the first time that bacteria of this genus have been identified in a soft tick. *Wolbachia* spp. are obligatory intracellular organisms most abundant in insects and nematodes (Zug and Hammerstein, 2012). In contrast, the detection of wolbachiae in ticks has rather low rates of occurrence (Andreotti et al., 2011; Zhang et al., 2011; Subramanian et al., 2012) and in some cases has been linked to natural contamination through endoparasitoid wasps of genus *Ixodiphagus* (Insecta: Hymenoptera) (Plantard et al., 2012). Although with current information inferring the origin of this putative *Wolbachia* sp. harbored by *O. rietcorreai* would be rather speculative, an endoparasitoid way of infection should not be discarded, since only two nymphs were positive. Our phylogenetic analysis positions the *Wolbachia* sp. of *O. rietcorreai* into a major clade with sequences of *C. lectularius* and other insects (Fig. 4). Remarkably, sequences of wolbachiae

retrieved from other tick species form a paraphyletic group, which suggests different origins of infections (Fig. 4). An extended genetic characterization and molecular screenings for *Wolbachia* in further tick species would bring valuable information on the nature of *Wolbachia* spp. associated with ticks.

While most species of *Hepatozoon* have been described to undergo sexual development and sporogony in hard ticks (Smith, 1996), the role of soft ticks as hosts of protozoans of this genus has been scarcely assessed. A sole study documented the observation of *Hepatozoon atticorae* oocysts in haemolymphatic cells of *Ornithodoros peringueyi*, a soft tick associated with cliff swallows in South Africa (Bennett et al., 1992). As in the current study we based our detections only on molecular data it is impossible to ascertain whether positive soft ticks could act as vectors of *Hepatozoon*, since all ticks were engorged and we only detected DNA of this protozoan. However, our phylogentic analysis points that sequences retrieved from *O. rietcorreai* are more related to reptiles, since they clustered with *Hepatozoon* spp. characterized from these vertebrates (Fig. 5). *Ornithodoros rietcorreai* is a parasite of rodents and probably bats (Labruna et al., 2016; Maia et al., 2018). Yet the fact that sequeneces of *Hepatozoon* obtained from rodents and bats grouped apart from our sequences (Fig. 5) suggests again that collected ticks fed upon other vertebrates, probably reptiles. As *O. rietcorreai* was recently reported parasitizing a snake (Alcantara et al., 2018), this is a plausible hypothesis.

The 18S rRNA gene has been widely used to characterize tick-borne apicomplexans and was targeted in this study as well. Interestingly, using primers designed to amplify *Babesia* spp. we amplified not only *Hepatozoon* but also DNA of two unidentified microorganisms exhibiting 90–91% of identity with sequences of apicomplexans and dinoflagellates species available in GenBank. For instance, whether this finding expands the diversity of agents harbored by ticks should be carefully assessed, since it must not be overlooked that tested ticks had an engorgement degree when submitted to PCR assays, so amplified products could also represent DNA of organisms coming from their host's blood.

Finally, the lack of detectable *Borrelia* DNA in *O. rietcorreai* ticks does not imply that this tick species is not involved with spirochetes of this genus under natural conditions since the number of ticks submitted to analyses was rather low. While *O. rietcorreai* is a human parasite, our study did not detected microorganisms that could be linked with zoonotic agents. To perform molecular screenings for tick-borne agents in additional populations of *O. rietcorreai* would be important in order to elucidate whether this tick harbors bacteria or protozoans of public health concern.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2019.04.004.

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