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ARTHROPODS AND MEDICAL ENTOMOLOGY - SHORT COMMUNICATION

Detection of "*Candidatus Rickettsia wissemanii*" in ticks parasitizing bats (Mammalia: Chiroptera) in the northern Brazilian Amazon

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Abstract
A total of 482 bats representing 32 species and two families were captured in the Amazon forests of the Amapá state in northern Brazil. Nineteen *Artibeus planirostris* bats (3.9 %) were infested with 160 ticks, all identified as *Ornithodoros hasei*. Three pools of larvae were screened for rickettsial DNA via polymerase chain reaction (PCR) targeting three rickettsial genes: *gltA*, *ompA* and *htrA*. Only one of them yielded an amplicons of the expected size for all three molecular assays. Comparisons of the obtained sequences including a phylogenetic analysis confirmed the occurrence of "*Candidatus Rickettsia wissemanii*" in Brazil.

Keywords Argasidae · *Ornithodoros hasei* · "*Candidatus Rickettsia wissemanii*" · Amapá · Brazil

Introduction

In Brazil, ten *Rickettsia* spp. have been described in ticks of the Ixodidae family (hard ticks) (Parola et al. 2013; Luz et al. 2018) and one in the Argasidae (soft ticks) (Muñoz-Leal et al. 2019). Soft ticks are endophylic ectoparasites and in South America are common on a variety of bats species (Kohls et al. 1965). In particular, *Ornithodoros hasei* has repeatedly been observed in association with Brazilian bats *Artibeus planirostris* and *Noctylio leporinus* in both the Pantanal and Caatinga biomes (Luz et al. 2016).

Bats exhibit a broad ecological diversity. Despite harbouring more than 160 bat species (López-Baucells et al. 2016), the Amazon biome has been poorly studied in terms of both bat ecology and their associated ectoparasites. Furthermore, recent investigations provided evidence that bat-associated ticks can be infected with members of the spotted fever group rickettsiae, specifically "*Candidatus Rickettsia nicoyana*" (Moreira-Soto et al. 2017) and "*Candidatus Rickettsia wissemanii*" (Tahir et al. 2016).

The current study aimed to improve the knowledge on the relationship between bats, their ticks and rickettsial agents in northern Brazil, specifically, in a portion of the Cerrado biome of Amapá state, a region considered as one of the most threatened Amazon savannah interfaces (Carvalho and Mustin 2017). To this end, we investigated the prevalence and intensity of parasitism by ticks and used molecular methods to detect and characterize the presence of *Rickettsia* DNA.

Material and methods

A total of 21 field trips were conducted between October 2016 and November 2017 to sample bats in 13 sites of a transition Amazon forest/Savannah (Amazonian savannah) area, located in the southeastern region of the Amapá state, northern Brazil. Bats were captured at night using nine mist nets (12 × 3 m;

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14 mm mesh) emplaced along a linear transect of 108 m in each of 13 sampled sites. Each campaign lasted 1–4 days, with an interval of at least 30 days between collection periods. Identification of captured bats followed the recommendations of López-Baucells et al. (2016). Ticks were removed using forceps and placed in individual 1.5 mL screw-capped microcentrifuge tubes containing 96 % ethanol. After completing this procedure, animals were released at the same capture site.

The totality of larvae was observed through a stereomicroscope and two were separated to prepare semi-permanent slides using Hoyer's medium for identification purposes following Kohls et al. (1965). Three pools of larvae, each one composed of ten ticks collected from three different bat specimens, were submitted to DNA extraction using the guanidine isothiocyanate/phenol-chloroform technique (Sangioni et al. 2005).

To obtain genetic data for ticks, a conventional PCR targeting a fragment of approximately 460 base pairs (bp) of the tick mitochondrial 16S rRNA (16S) gene. Samples were subsequently submitted to further PCR assays targeting *Rickettsia* citrate synthase gene (*gltA*) gene. Positive samples were then tested with primers amplifying the 190-kDa outer membrane protein (*ompA*) and the 17-kDa protein (*htrA*) genes. In order to increase the sensitivity of conventional PCR, all *ompA*-negative, or weakly amplified *ompA*-positive samples obtained with the above-mentioned primers, were tested using an *ompA* hemi-nested PCR. Primers and references for thermal cycling conditions used in the current study are listed in Table 1. Conventional PCRs were performed in a 25-μl mix composed by 12.5 μl of DreamTaq Green PCR master mix (2×, Thermo Fischer Scientific Baltics UAB, Vilnius, Lithuania), 1 μl of each primer (10 pmol/μl), 8 μl of ultrapure water and 2.5 μl of template DNA. For hemi-nested rounds, we used 1 μl of the initial amplification and 9.5 μl of ultrapure water. PCR products of the expected sizes were treated with ExoSAP-IT® (USB Corporation, Cleveland, OH, USA), sequenced in an ABI automated sequencer (Applied Biosystems/Thermo Fisher Scientific, model ABI 3500 Genetic Analyser, Foster City, CA). Obtained sequences were trimmed using Geneious R9 (Kearse et al. 2012), and analysed using the BLASTn tool to infer similarities with homologue sequences available in GenBank.

Phylogenetic analysis

A phylogenetic analysis was performed using *gltA* and *ompA Rickettsia* sequences. Sequences of both genes were individually aligned with homologous congeneric sequences available in GenBank, using CLUSTAL W2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/). Ambiguous alignments were manually concatenated and subjected to analysis. A Bayesian phylogenetic tree based on the GTR model with a gamma-distributed rate variation among sites was constructed using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). Four independent Markov chain runs for 500,000 metropolis-coupled MCMC generations and sampling a tree every 100 generations were implemented. The first 25 % of the trees represented burn-in, and the remaining trees were used to calculate the Bayesian posterior probability. Sequences of *gltA* and *ompA* genes from *Rickettsia felis* LSU-lb (JSEL01000034; DQ408668), *Rickettsia australis* Cutlack (CP003338; CP003338) and *Rickettsia akari* Hartford (NC009881; L01461) rooted the tree. GenBank accession numbers for the remaining sequences used in the analysis are indicated within the phylogenetic tree.

Table 1 Primers used to perform PCR in the current study

Gene	Primers	Sequence (5′ – 3′)	Amplicon size	Reference
Tick mitochondria				
16S rRNA	16S+1	CCGGTCTGAACTCAGATCAAGT	PCR 16S+1/16S-1: ~460bp	Mangold et al. (1998)
	16S-1	GCTCAATGATTTTTTAAATTGCTG		
Rickettsia				
gltA	CS78	GCAAGTATCGGTGAGGATGTAAT	PCR: CS78/CS323: 401bp	Labruna et al. (2004)
	CS323	GCTTCCTTAAAATTCAATAAATCAGG AT		
ompA	190.70F	ATGGCGAATATTTCTCCAAAA	PCR 190.70F/190.701R: 617bp	Regnery et al. (1991)
	190.701R	GTTCCGTTAATGGCAGCATCT		
	190.602R	CATTGTTCGTCAGGTTGGCG	hemi-nested PCR 190.70F/190.602R: 512bp	Eremeeva et al. (2006)
htrA	17k-5	GCTTTACAAAATTCTAAAAACCATATA	PCR 17k-5/17k-3: 549bp	Labruna et al. (2004)
	17k-3	TGTCTATCAATTCACAACTTGCC		
	17kD1	GCTCTTGCAACTTCTATGTT	Nested PCR 17kD1/17kD2: 434bp	Webb et al. (1990)
	17kD2	CATTGTTCGTCAGGTTGGCG		

Results and discussion

A total of 482 bats representing 32 species were captured. Only *A. planirostris* were parasitized by 160 larvae of ticks identified morphologically and molecularly as *O. hasei* (Fig. 1). BLASTn pairwise comparisons of obtained sequences showed them to be identical to each other and 99 % identical to 16S rDNA sequences of *O. hasei* deposited in GenBank (KX099896 and KT894588). The consensus sequence of *O. hasei* obtained in this study was deposited in GenBank under the accession number MH600060. *Ornithodoros hasei* has been recorded parasitizing several bats species, including *A. planirostris* in numerous locations throughout the neotropics, especially in the Brazilian Pantanal and Caatinga biomes (Bertola et al. 2005; Luz et al. 2016; Muñoz-Leal et al. 2016).

Only one out of the three pools of tested larvae that were positive for rickettsial DNA. Amplicons of the expected size for the *gltA* PCR were shown to be 100 % identical (350/350bp, 0 gaps, 0.0 *E* value) to the homologous sequence of "*Candidatus Rickettsia wissemanii*" (GenBank LT558852). A conventional reaction to obtain sequences of *ompA* locus applied to the *gltA*-positive sample showed a weak band of the expected size after electrophoresis and UV transillumination, improper for sequencing procedures. Therefore, we used primers to perform an *ompA* hemi-nested reaction (Table 1), and this time obtained a proper band. The sequencing of this product yielded a sequence 100 % identical (491/491bp, 0 gaps, 0.0 *E* value) to the corresponding sequence of "*Ca. R. wissemanii*" (GenBank LT558853). In addition, the sequencing of expected size amplicons of the *htrA* gene-PCR, yielded a 497-bp haplotype 99 % identical (494/497bp, 0 gaps, 0.0 *E* value) with *R. rickettsii* strain Iowa (CP018914). Sequences of *gltA*, *ompA* and *htrA* genes obtained in this study were deposited in the GenBank under accession numbers MH614266, MH614267 and MH614268 respectively.

The concatenated analysis of two rickettsial genes (*gltA*, *ompA*) generated in the current study showed that "*Ca. R. wissemanii*" is phylogenetically close to *R. peacockii* and "*Candidatus R. nicoyana"* (Fig. 2), each of which pertain to the *R. rickettsii* subgroup, as was established previously by Tahir et al. (2016) and Moreira-Soto et al. (2017). Sequences of *htrA* gene of "Ca. *R. wissemanii*" were previously unknown, so comparisons with our detection were impossible to perform. However, BLASTn pairwise comparisons of herein obtained "*Ca. R. wissemanii*" *htrA* sequence point a high similarity with *R. rickettsii*, which is in line with the results of the phylogenetic analysis that we performed. Due to its phylogenetic relatedness with the spotted fever group, the pathogenic role of "*Ca. R. wissemanii*" must not be underestimated.

In Brazil, studies reporting *Rickettsia* spp. transmitted by ticks have focussed mainly on species of the genus *Amblyomma* (Ixodidae) (Parola et al. 2013). At present, *Rickettsia* spp. reported for Brazil consists of at least ten species: "*Candidatus Rickettsia andeanae*", *Rickettsia asemboensis*, *Rickettsia amblyommatis*, *Rickettsia bellii*, "*Candidatus Rickettsia colombianensi*", *Rickettsia felis*, *Rickettsia monteiroi*, *Rickettsia parkeri*, *R. rickettsii* and *Rickettsia rhipicephali* (Parola et al. 2013; Dall'Agnol et al. 2017; Luz et al. 2018). Discounting the recent finding of a *Rickettsia bellii*-like endosymbiont in *Ornithodoros rietcorreai* (Muñoz-Leal et al. 2019), none of these ten species of *Rickettsia* have been detected in association with ticks of the Argasidae family. In this study, we confirmed the presence

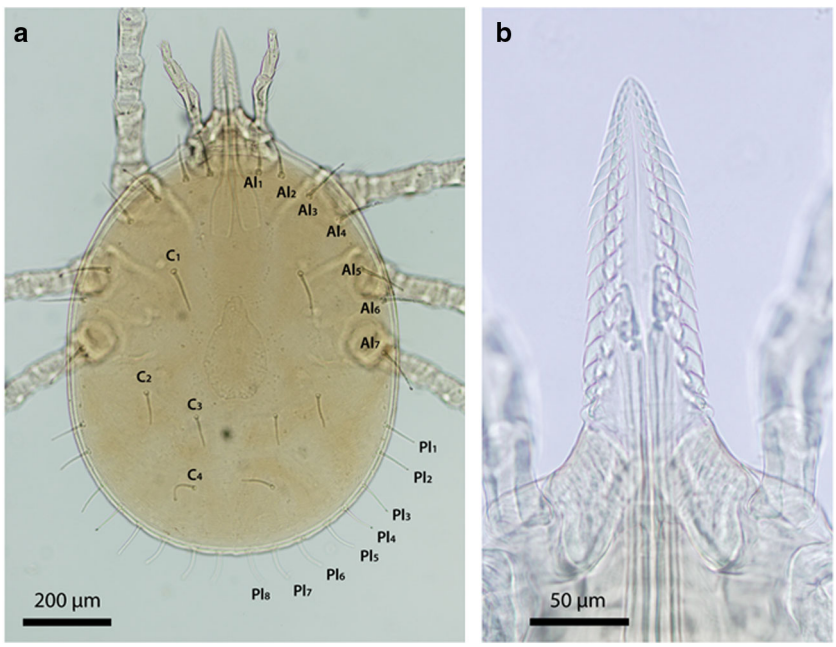

Fig. 1 Micrographs of *Ornithodoros hasei* larva taken with an Olympus DP70 camera implemented in an Olympus BX40 optical microscope, using the software Image-Plus Pro v5.1: dorsal idiosoma (**a**) and hypostome (**b**). Al, anterolateral seta; C, central seta; Pl, posterolateral seta

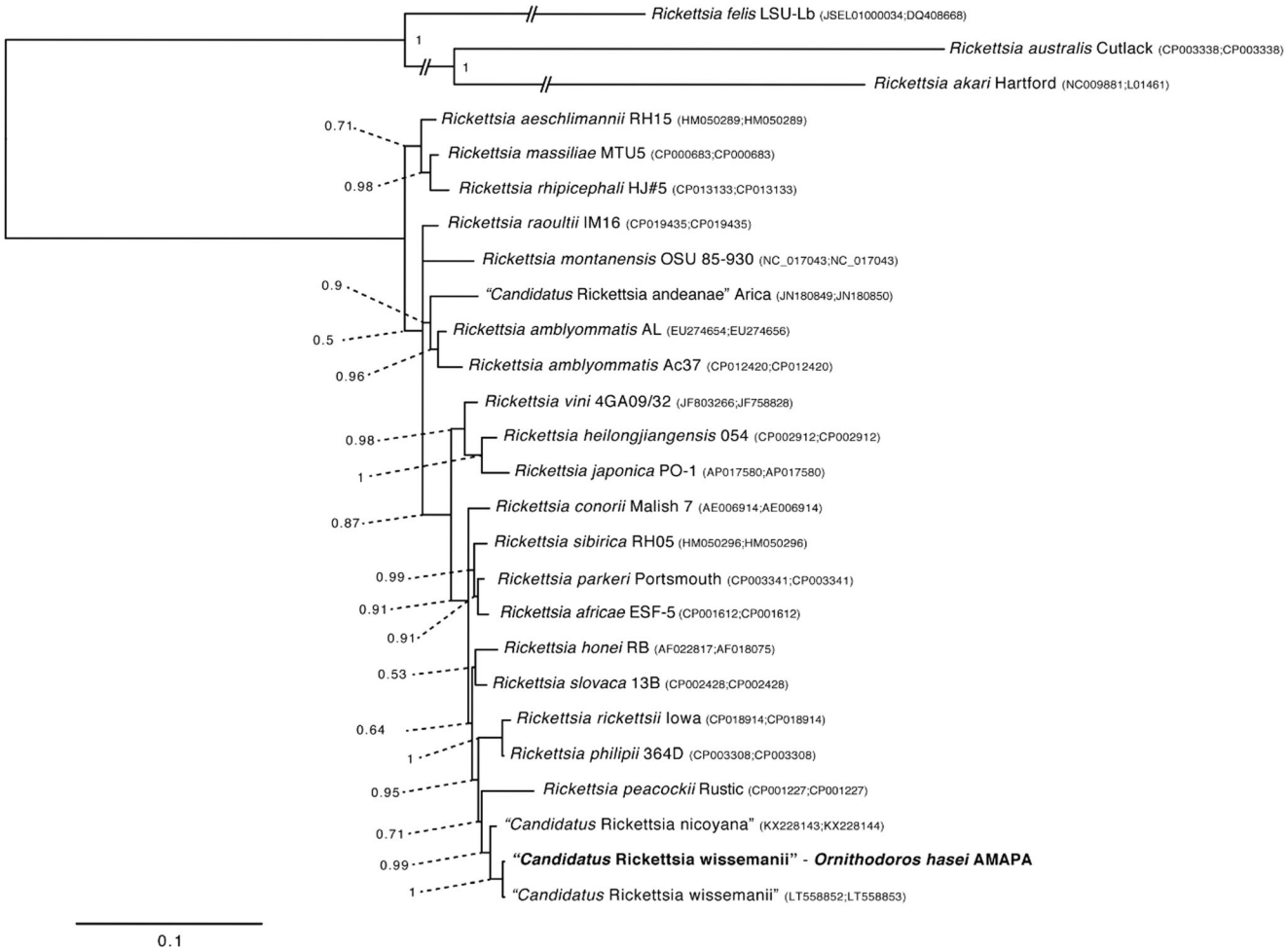

Fig. 2 Molecular phylogenetic analysis of "*Candidatus Rickettsia wissemanii*" from the tick *Ornithodoros hasei* collected on bats, Amapá state, Brazil. Unambiguously aligned nucleotide sites of the rickettsial genes *gltA*, *ompA* and *htrA* were concatenated and subjected to analysis by the Bayesian method. The bootstrap values obtained by 1000 replicates are shown at the nodes

of "*Ca. R. wissemanii*" DNA in *O. hasei*, which constitutes the 12th *Rickettsia* species recorded in Brazil, and the second *Rickettsia* sp. in an *Ornithodoros* tick for the country. Our finding extends the geographical distribution of "*Ca. R. wissemanii*" into the Brazilian Amazon and highlight the need for further studies on bat-tick-*Rickettsia* associations. Finally, we provide the first record of *O. hasei* for the Amapá state.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was evaluated and approved by the Animal Experimentation Ethics Committee of the Federal University of Amapá (process no. 006/201625) and conducted with the permission of Brazilian Institute of the Environment—IBAMA (process no. 55867-1).

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