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Hemogregarine and Rickettsial infection in ticks of toads from northeastern Colombia

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ABSTRACT

The toads *Rhinella* spp. are in constant contact with humans and domestic animals and are commonly parasitized by ticks, which are also potential vectors of pathogenic microorganisms, such as apicomplexans and rickettsia. However, little is known about microorganisms associated with toad ticks. In this work, we molecularly evaluated the presence of *Rickettsia* spp. and hemogregarines in ticks of *Rhinella horribilis* and *R. humboldti* in the Colombian Caribbean, finding two different species of *Rickettsia*: the colombianensi strain and one close to *R. bellii*. In the case of hemogregarines, since only *18S* gene sequences are available, it is difficult to define species and place them correctly in a phylogeny, but most of our samples show a 99% identity with *Hemolivia stellata*, while others identical to each other seem to form another clade within this genre. All collected ticks were identified as *Amblyomma dissimile*, representing the first time that *H. stellata* was recorded in this tick. The prevalence of both microorganisms was very high, which makes it necessary to generate robust phylogenies to clarify their taxonomic diversity and to correctly define their ecological role and pathogenicity, which should be taken into account in amphibian conservation plans and veterinary medicine.

1. Introduction

There are several types of ectoparasites that transmit pathogens to anurans, among which are leeches, mosquitoes and ticks. Within the latter, nine species of the family Ixodidae and one of Argasidae are known; they parasitize toads of the genus *Rhinella* (Burridge, 2011; Bermúdez et al., 2013), and *Amblyomma dissimile* and *Amblyomma rotundatum* are the most common. Both are tri-host ticks that generally use amphibians and reptiles as hosts during their life cycle, although they can accidentally parasitize birds and mammals, including humans (Lampo et al., 1997; Guglielmone et al., 2006); *Amblyomma dissimile* has a greater diversity of hosts, but within anurans, it is only recorded in toads of the Bufonidae family (Guglielmone and Nava, 2010).

The parasitism of these ticks leaves clear cutaneous lesions in the toads, weakening the animal and directly increasing the risk of subsequent infections, whether primary infections, starting from cutaneous lesions, or secondary infections through vector transmission of microorganisms, which is considered a mechanism of population regulation for the hosts (Lampo and Bayliss, 1996; Smith et al., 2008). Although these infections often show no symptoms in wild vertebrates, there is evidence of pathologies, such as anemia, erythrocytic hypertrophy and loss of hemoglobin, along with the possibility of causing phenotypic and phenological changes in the vectors (Desser et al., 1995; Wozniak et al., 1996; Ferguson et al., 2013), especially in infections by hemogregarines (Gupta et al., 2012; Rodrigues-Calil et al., 2017).

This artificial group is composed of six Apicomplexa genera (Barta et al., 2012), of which only *Hepatozoon* and *Hemolivia* parasitize the anurans (Maia et al., 2016). In fact, the latter was described in its natural hosts *Rhinella marina* (s.l.) and the tick *Amblyomma rotundatum*, with the species *Hemolivia stellata* (Petit et al., 1990). Like this one, its congeners use heterothermic vertebrates as intermediate hosts and ticks as definitive hosts (Karadjian et al., 2015) and can also be transmitted through the consumption of other infected vertebrates (Davies and Johnston, 2000).

At the same time, ticks are important vectors of bacteria to multiple vertebrate groups. The genus *Rickettsia* (Rickettsiales: Rickettsiaceae) has been reported more frequently in Latin America, given its importance in public and veterinary health (Witter et al., 2016); despite this incidence, in the country, the knowledge of transmission to amphibians is scarce. Studies focused on vectors have resulted in the discovery of several species of unknown pathogenicity, thanks to the progressive improvement of detection techniques. Such is the case of the *Rickettsia* sp. Colombianensi strain in reptile ticks (Miranda et al., 2012; Santodomingo et al., 2018); this species is phylogenetically related to *R. monacensis*, which was considered nonpathogenic when it was discovered but is currently known to cause disease in humans, as

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Fig. 1. Tree topology of phylogenetic analyses by Maximum Likelihood and Bayesian Inference including concatenated sequences of the genes *gltA*, *ompA* and *16S* obtained in this work and others from GenBank (Table 1). The numbers correspond to the values of Bootstrap/posterior probabilities.

has occurred with other species, such as *R. aeschlimannii*, *R. massiliae* and *R. slovaca* in the old world (Parola et al., 2005; Jado et al., 2007).

Despite the great advances in the study of these bacteria and other microorganisms associated with ticks, very little has been reported about the roles played by amphibians and their ticks in the epidemiology of human and animal diseases. In Colombia, there has been no report of pathogens associated with amphibian ticks, with Bufonidae being one of the most abundant and diverse anuran families in the country, where species such as *Rhinella marina* and *R. horribillis* are present in urban, rural and wild lowland environments, very close to humans and domestic animals (Acevedo-Rincón et al., 2016; Acosta-Galvis, 2017). Therefore, the objective of this work was to evaluate the presence of *Rickettsia* spp., *Hepatozoon* spp. and *Hemolivia* spp. in ticks and some tissues of toads in the department of Magdalena (Colombia).

2. Materials and methods

2.1. Samples studied

The ticks were taken directly from toads from seven localities located in the department of Magdalena in northern Colombia. The toads were captured under the visual encounter search method and through pitfall traps at two locations. Samples taken from each individual were placed in 1.5-ml vials with 96% ethanol and were maintained at −20 °C until their identification. For the taxonomic identification of ticks, the keys from Voltzit (2007), Osorno-Mesa (1940) and Jones et al. (1972) were used for adult specimens, and the keys from Martins et al. (2010) were used for the nymphs. Larvae were identified using the key from Osorno-Mesa (1940) and were corroborated using molecular methods (the *COI* gene). Additionally, six individuals of *Rhinella horribillis* collected at the Universidad del Magdalena were sacrificed, from which ticks, blood, liver, heart and spleen samples were also extracted. Permission for trapping and manipulating the animals as well as collecting the ectoparasites for this study was given by ANLA (Autoridad Nacional de Licencias Ambientales) under the permit no. 1293, and was approved by Universidad del Magdalena Ethical Committee (Acta 001–15).

2.2. DNA extraction

For the adult ticks, the anterior half was used to extract the DNA; for the larvae and nymphs, the complete individual was used. The DNA was extracted individually or in pools, depending on the number of ticks of each stage found in each toad, using the MasterPure ™ DNA Purification Kit (Epicenter, USA). Each pool included up to 5 individuals. DNA extraction of the liver, heart and spleen samples was performed following the instructions of the ISOLATE II Genomic DNA kit, and the MasterPure™ DNA Purification Kit for Blood was used for the blood samples. DNA extraction and its quality were confirmed by means of electrophoresis in agarose gel and with GelRed (Biotium) staining.

2.3. Amplification of COI

Amplification of *COI* was performed through conventional PCR in an Eppendorf Mastercycler® Pro thermocycler, using the universal primers for invertebrates that amplify the gene: LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer et al., 1994). Amplifications were designed with a volume of 25 µL, containing 3 µL of extracted DNA, 0.5 µL of Taq Polymerase (5 U/µL, Bioline), 1 µL of MgCl (50 mM), 2.5 µL of PCR Buffer (10X), 0.5 µL of dNTPs (10 mM) and 1 µL of each primer (10 pmol). The conditions of the amplifications were as

Table 1
Sequences of *Rickettsia* downloaded from GenBank and generated in this study (in bold) that were included in the phylogenetic analyses shown in Fig. 1.

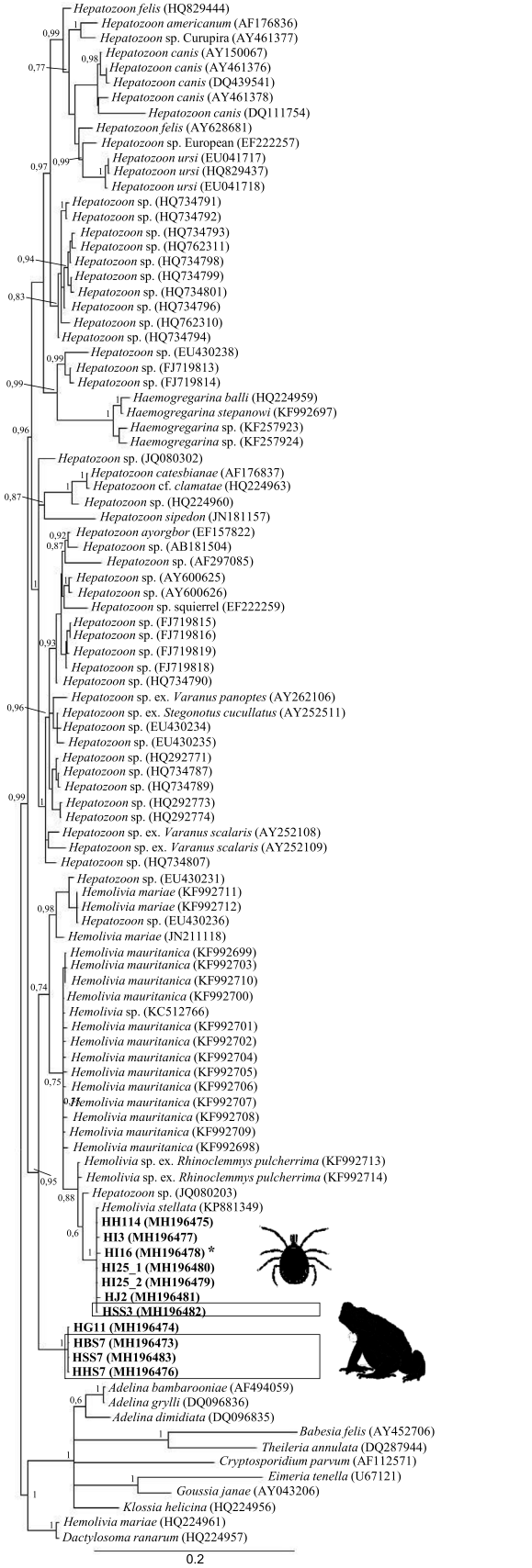
Species	Genes	GenBank accesion numbers
Rickettsia sp. clone RA143	*gltA*	KY996395
Rickettsia sp. Strain Colombianensi	*gltA/ompA/16S*	JF905456/KF691749/KF691750
Rickettsia sp. clone Necocli 190	*gltA*	JX519583
Rickettsia tamurae strain AT-1	*gltA/ompA/16S*	AF394896/DQ103259/NR_042727
Rickettsia monacensis	*gltA/ompA/16S*	DQ100163/LN794217/LN794217
Rickettsia sibirica sibirica	*gltA/ompA/16S*	KM288711/KM288712
Rickettsia mongolotimonae	*gltA/ompA*	DQ097081/DQ097082
Rickettsia slovaca	*gltA/ompA*	U59725/CP002428
Rickettsia conorii	*gltA*	U59728
Rickettsia parkeri	*gltA/ompA*	KJ158742/KJ158741
Rickettsia africae	*gltA/ompA*	U59733/CP001612
Rickettsia japonica	*gltA/ompA/16S*	AY743327/AP011533/AP011533
Rickettsia heilongjiangensis	*gltA/ompA/16S*	AY285776/AH012829/NR_041770
Rickettsia honei	*gltA*	AF018074
Rickettsia raoultii	*gltA/16S*	KU310590/KR608783
Rickettsia aeschlimannii	*gltA/ompA/16S*	AY259084/AY259083/KT318741
Rickettsia massiliae	*gltA/ompA/16S*	HM050293/DQ494551/L36106
Rickettsia rhipicephali	*gltA/ompA*	DQ865206/DQ865208
Rickettsia australis	*gltA/16S*	U59718/U12459
Rickettsia sp. clone Turbo 31	*gltA*	JX519576
Rickettsia sp. clone Necocli 181	*gltA*	JX519577
Rickettsia typhi	*gltA/16S*	U59714/NR_074394
Rickettsia bellii	*gltA/16S*	JQ519684/U11014
Rickettsia rickettsii	*gltA/ompA/16S*	CP006009
Rickettsia bellii	*gltA/16S*	CP015010
Rickettsia felis	*gltA/16S*	CP000053
***Rickettsia* sp. strain Colombianensi (RJ12.2 MIX)**	***gltA/ompA/16S***	MH196484/MH196501/MH196503
***Rickettsia* sp. strain Colombianensi (RH114)**	***gltA***	MH196496
***Rickettsia bellii*-like (RJ41)**	***gltA***	MH196501

follows: an initial denaturation of 95 °C for 1.5 min, followed by 35 cycles of 94 °C for 30 s, annealing at 45 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 7 min.

2.4. Amplification of GltA/16S rRNA/Omp A//from Rickettsia

Amplification was performed by conventional PCR in an Eppendorf Mastercycler® Pro thermal cycler, using the primers with the following sequences: CS-78 (GCAAGTATCGGTGAGGATGTAAT) and CS-323(GCTTCCTTAAAATTCAATAAATCAGGAT) (Labruna et al., 2004). For the PCR, 4 μL of extracted DNA was used, and the final reaction volume was 25 μL, containing 0.5 μL of Taq Polymerase (BIOLASE TM, Bioline), 1 μL of MgCl (50 mM), 2.5 μL of PCR Buffer (10X), 0.5 μL of dNTPs (10 mM) and 1 μL of each primer (10 pmol). The conditions of the amplifications were as follows: an initial denaturation of 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, annealing at 48 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 7 min.

The samples positive for *gltA* from *Rickettsia* were further amplified by conventional PCR with the *16S* rRNA primer genes (Rick-16S− F3 5′-ATCAGTACGGAATAACTTTTA-3′, Rick-16S- R4 5′-TGCCTCTTGCGT TAGCTCAC- 3′) (Anstead and Chilton, 2013) and *ompA* primers (OMPA-F 5′- CAC YAC CTC AAC CGC AGC-3′, OMPA-R 5′- AAA GTT ATA TTTCCT AAA CCY GTA TAA KTA TCR GC -3′) (Phan et al., 2011). The reaction was performed in a total volume of 25 μL with the following conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles



(caption on next page)

Fig. 2. Tree topology of phylogenetic analysis by Bayesian inference, including sequences of the *18S* gene obtained in this work and others from GenBank. The numbers correspond to values of posterior probabilities. Our samples come from blood (HHSS3, HHSS7), spleen (HBS7) and liver (HHS7) from *Rhinella horribilis*. The rest come from *Amblyomma dissimile* larvae (HJ2), nymphs (HI3, HI25_1, HI25_2), a male (HH114) and a female (HG11) collected in *Rhinella horribilis,* and one nymph (HI16) collected in *Rhinella humboldti**.

of denaturation at 95 °C for 30 s, annealing at 58 °C (*16S* rRNA)/52 °C (*ompA*) for 1 min, extension at 72 °C for 2 min and a final extension at 72 °C for 7 min.

2.5. Amplification of 18S gene of Hepatozoon

The amplification was performed by conventional PCR in an Eppendorf Mastercycler® Pro thermocycler, using primers HepF300 (GTTTCTGACCTATCAGCTTTCGACG) and Hep900 (CAAATCTAAGAAT TTCACCTCTGAC) (Ujvari et al., 2004). For the PCR, 4 μL of extracted DNA was used, and the final reaction volume was 25 μL, which contained 0.5 μL of Taq Polymerase (5 U/μL, Bioline), 1 μL of MgCl (50 mM), 2.5 μL of PCR Buffer (10X), 0.5 μL of dNTPs (10 mM) and 1 μL of each primer (10 pmol). The conditions of the amplifications were as follows: an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. The products obtained from the amplification of *COI*, *gltA*, *16S*, *ompA* and *18S* were purified with SureClean Plus (Bioline, USA) following the supplier's instructions. These products were sequenced in both directions.

2.6. Analysis of the sequences

The sequences were checked using the NCBI BLAST tool (www.ncbi.nlm.nih.gov) and were then edited with ProSeq V3 software (Filatov, 2009). Using the program MEGA 7.0 (Kumar et al., 2016) with the ClustalW algorithm (Thompson et al., 1994), the sequences obtained in this study and others available in GenBank were aligned.

2.7. Phylogenetic analyses

For phylogenetic reconstruction, Bayesian inference and maximum likelihood were used in the MrBayes 3.2.2 (Ronquist et al., 2012) and RAxML 8.0.24 (Stamatakis, 2006) programs, respectively. The best nucleotide substitution model for each of the datasets was selected using the Partition Finder program (Lanfear et al., 2012), with the Bayesian Information Criterion (BIC; Schwarz, 1978). The GTR + G model was implemented for the 1st and 2nd codon positions of the *gltA* gene and for 2nd and 3rd codon positions of *OmpA*; the GTR model was used for the 3rd codon position of *gltA*, and the GTR + I + G model was used for the *16S* gene (*Rickettsia* spp.). The *18S* (hemogregarines) was analyzed using the GTR + G model.

Two independent runs of 10, 000, 000 generations were used, sampling the trees every 100 generations, discarding 25% of the trees. The standard deviation of the independent sequences (< 0.01) was used to validate the convergence, grouping the likelihood values over time and using the SumP command in MrBayes. The posterior probability of each clade was measured based on the percentage of trees that recovered that particular clade (Huelsenbeck and Ronquist, 2001). For the maximum likelihood analyses, the fast-scaling algorithm was used with 1000 Bootstrap (BP) pseudoreplicas, taking BP values greater than 70% as a high statistical support (Hillis and Bull, 1993).

3. Results

A total of 208 ticks were collected (16 adults, 90 nymphs and 102 larvae), which were identified as *Amblyomma dissimile* and *Amblyomma* cf. *dissimile*. 4 nymphs and 6 larvae were extracted from 5 R. *humboldti* toads, whereas the rest were collected from 44 R. *horribilis* individuals. Specifically, 1 to 3 ticks were collected per *R. humboldti* toad, whereas we found an average of 4.5 ticks per *R. horribilis* toad. We analyzed 9 adults, 41 pools of nymphs and 32 pools of larvae using molecular methods, including 4 pools of nymphs and 4 pools of larvae from *R. humboldti* and 5 females, 4 males, 37 pools of nymphs and 28 pools of larvae from *R. horribilis*.

Rickettsia DNA was detected in 5/9 (55%) adults, 24/41 (57%) pools of nymphs and 28/32 (88%) pools of larvae, including 2 females, 3 males, 22 pools of nymphs and 24 pools of larvae from *R. horribilis,* and 2 pools of nymphs and 4 pools of larvae from *R. humboldti*. None of the tissues was positive for *Rickettsia*. The percentage of positivity for both *Rickettsia* and for hemogregarines was expressed as a minimum infection rate (MIR), assuming that one tick was positive in each positive pool.

Hemogregarine DNA was detected in 6/9 adults (66%), 33/41 (80%) pools of nymphs and 18/32 (56%) pools of larvae including 3 females, 3 males, 31 pools of nymphs and 16 pools of larvae from *R. horribilis* and 2 pools of nymphs and 2 pools of larvae from *R. humboldti*. Tissues from the six toads were also found positive for hemogregarine DNA; however, we successfully sequenced tissues from only two. The first was a blood sample (cod HSS3) of an individual who also had a positive female tick (cod NH114). The second individual was found positive for liver, blood and spleen tissues (cod HHS7, HSS7, HBS7 respectively). Among the infected ticks, 5 adults, 13 pools of nymphs and 18 pools of larvae from *R. horribilis* and 2 pools of nymphs and 4 pools of larvae from *R. humboldti* were coinfected with both *Rickettsia* and hemogregarine DNA.

All *COI* sequences showed 99% identity with *A. dissimile*. The sequences of the genes *gltA* and *ompA* showed identities of 100% with *Rickettsia* sp. strain Colombianensi (e.g., JF905456.1/MF034497.1) and 99% with *R. tamurae* sequences (e.g., KT753265.1) and *R. monacensis* (e.g., KU586332.1). *GltA* sequence corresponding to the J41 sample (pool of larvae from *R. horribilis*) had 99% identity with *R. bellii* (e.g., NR_074484.2). The phylogenetic analyses also showed the proximity between our sequences with the group conformed by *R. monacensis*, *Rickettsia* strain Colombianensi and *R. tamurae*, and the sample J41 with *R. belli* (Fig. 1, Table 1).

The sequences of the fragments amplified with the *Hepatozoon* primers showed identities of 99% with *Hemolivia stellata* sequences (e.g., KP881349.1) and 98% with *Hemolivia* sp. (e.g., KX347435.1). Bayesian Inference and Maximum Likelihood analyses grouped 6/7 of the samples obtained from ticks with *H. stellata* and one taken from sample blood. By Bayesian Inference, the remaining sample taken from ticks and the others obtained from the tissues appeared within the clade of *Hemolivia* as sisters to all of the others (Fig. 2); by Maximum Likelihood, they appeared to be sisters to all of the others except to the group of *Hemolivia marie*. All our sequences were deposited in GenBank under accession nos. **MH196473-MH196505.**

4. Discussion

This is the first report of parasitism by *Amblyomma dissimile* in *Rhinella humboldti*; a previous report described *A. dissimile* using *R. horribillis* and *R. marina* as hosts (Osorno-Mesa, 1940; Guglielmone and Nava, 2010). Likewise, this report represents the first time that *Hemolivia stellata* has been detected in *Amblyomma dissimile* and the third time that *Rickettsia* spp. has been reported (Miranda et al., 2012; Santodomingo et al., 2018), with two possible different species detected: *Rickettsia* sp. strain Colombianensi and another close to *Rickettsia bellii*. The latter was detected in only one pool of larvae, in contrast, *R. bellii* had been found in *A. rotundatum* taken from *Rhinella* spp. from Brazil and Panama, with high infestation rates (Labruna et al., 2004; Andoh et al., 2015; Horta et al., 2015; Silva et al., 2016).

Despite the high prevalence of *Rickettsia* spp. in the tick samples,

none of the toad tissue samples analyzed here were positive for these bacteria, as shown by Horta et al. (2015), which, although the finding must be corroborated with a larger number of samples, may be due to *Rickettsia* spp. being transmitted transovarially and interstadially maintained in the vector (Horta et al., 2006; Zemtsova et al., 2010; Sakai et al., 2014), which may mean that ticks had been previously infected and that horizontal transmission does not occur with these hosts, given that *Rickettsia* spp. most commonly infect homeothermic vertebrates (Parola et al., 2013).

However, De Sousa et al. (2012) found *Rickettsia monacencis* and *Rickettsia helvetica* in muscle tissue of the lizard *Teira dugesii* (Lacertidae) and its *Ixodes ricinus* ticks. Therefore, it cannot be ruled out that heterothermic hosts such as amphibians and reptiles are sentinels or reservoirs of *Rickettsia* spp., since there are some studies that demonstrate the presence of Rickettsia in amphibians, though they are not very precise in their identification (Desser and Barta, 1984, 1989; Desser, 1987; Bataille et al., 2018). It is possible that the molecular detection of *Rickettsia* spp. is not sensitive enough, though, as proposed by Levin et al. (2016), this may vary depending on the stage in which the infection is found.

In contrast, infection by hemogregarines was found in all types of tissue analyzed, and there was also a high prevalence in ticks. Our phylogenetic analyses showed that the samples analyzed corresponded to the genus *Hemolivia*, with the majority being grouped with *H. stellata* and the remaining ones forming an independent clade within this genus, using the phylogenetic framework suggested by Kvičerová et al. (2014) and including the only sequence of *H. stellata* available in GenBank (KP881349). This species of hemogregarine has only been described in *Amblyomma rotundatum* as the definitive host, *R. marina* as an intermediate host and, more recently, in *Ameiva ameiva* (Petit et al., 1990; Lainson et al., 2007).

Although not previously reported in *Amblyomma dissimile*, *Amblyomma rotundatum* and *Amblyomma dissimile* share much of their geographic distribution and hosts, including the toads *Rhinella* spp. (Guglielmone and Nava, 2010; Nava et al., 2017); therefore, it is possible that *H. stellata* is also associated with these ticks. However, many authors agree that the identification and phylogenetic positions of these hemogregarines, especially with respect to the genus *Hepatozoon*, are very imprecise given the lack of biogeographic, morphological, and especially molecular information (Barta et al., 2012; Kvičerová et al., 2014; Karadjian et al., 2015; Maia et al., 2016; O'Donoghue, 2017). Sequences other than those for the *18S* gene are necessary, given that 18S sequences are insufficient to resolve the relationship between these groups; in our study, the differences were evidenced by obtaining different topologies with the Maximum Likelihood and Bayesian Inference methods.

In this study, tissue histopathological examinations were not performed, but the tissues studied did not present detectable macroscopic lesions. Shutler et al. (2009) suggest that infection by *Hepatozoon* sp. in *Lithobates clamitans* (Ranidae) is benign or undetectable to the immune system. In contrast, Sailasuta et al. (2011) show an infection by two morphotypes of *Hepatozoon* sp. caused pathological lesions with subacute to chronic inflammation in the liver of *Hoplobatrachus rugulosus* (Dicroglossidae). These reports illustrate the need to develop reliable phylogenies given their importance in areas such as conservation biology, especially in a group such as amphibians, which have suffered considerable population declines for several decades (Stuart et al., 2004). In addition, it has been shown that *R. marina,* in areas such as Australia where it is considered invasive, has propitiated the expansion of its natural pathogens, which entails a risk for native amphibians that were previously not exposed (Selechnik et al., 2017).

5. Conclusions

In this study, it is shown that the prevalence of *Rickettsia* spp. associated with toads is quite high; however, *Rickettsia* spp. were either not present or not detectable in some tissues of these hosts. Therefore, future studies should focus on elucidating whether or not the toads may be infected with these bacteria to understand the ecological relationship that exists between these bacteria and the toads. In addition, it is imperative that sequences from regions other than the *18S* gene be obtained for the phylogenetic identification and location of hemogregarines and to clarify the true vectors and intermediate hosts for these species, their transmission dynamics and their life cycles, to clarify the true diversity in these taxa and their role in the population dynamics of their hosts, which are fundamental for the conservation of a group that is particularly sensitive to climate change, habitat fragmentation and habitat loss.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2018.06.003.

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