IJP: Parasites and Wildlife 7 (2018) 75–84

Contents lists available at ScienceDirect

IJP: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Molecular detection of hemogregarines and haemosporidians in Brazilian free-living testudines

Juliana Paula de Oliveira[a], Marcos Rogério André[a], José Roberto Ferreira Alves Júnior[b], Ana Paula Gomes Lustosa[c], Karin Werther[a,*]

[a] São Paulo State University (Unesp), School of Agricultural and Veterinarian Studies, Jaboticabal Campus, Via de Acesso Professor Paulo Donato Castellane s/n, 14.884-900, Jaboticabal, SP, Brazil
[b] Instituto Federal Goiano, Campus Urutaí, Rodovia Geraldo Silva Nascimento Km 2.5, 75.790-000, Urutaí, GO, Brazil
[c] Chico Mendes Institute for Biodiversity Conservation (ICMBio), National Center for Research and Conservation of Reptiles and Amphibians (RAN), Rua 229, nº 95, Setor Leste Universitário, 74.605-090, Goiânia, GO, Brazil

ARTICLE INFO

Keywords:
Haemogregarina
Turtle
PCR
Phylogeny
Phrynops geoffroanus
Podocnemis expansa

ABSTRACT

Morphological and molecular techniques were used to investigate the presence of hemogregarines and haemosporidians in biological samples of free-living Geoffroy's side-necked turtles (*Phrynops geoffroanus*) and Giant Amazon turtles (*Podocnemis expansa*) from Brazil. No evolutionary form of haemosporidians or hemogregarines were observed in the blood smears of 83 *P. geoffroanus* samples, and there were no meronts in the histological sections of 31 necropsied *P. geoffroanus* samples. All DNA samples extracted from *P. geoffroanus* tissues and blood aliquots were negative in haemosporidian PCR assays (based on the mitochondrial cytochrome *b* gene) and hemogregarine PCR assays (based on the 18S rRNA gene). In the analysis of blood smears of all seven *Podocnemis expansa* evaluated, gametocytes of hemogregarines were observed. The seven *P. expansa* were negative in the haemosporidian PCR assays. Moreover, hemogregarine DNA was detected in blood samples from all of the sampled *P. expansa*. The phylogenetic maximum likelihood inference and probabilistic Bayesian inference revealed five closely related genotypes that formed a monophyletic group. There was also a sister group to the lineage that consisted of *Haemogregarina* spp. of freshwater turtles from Canada, Italy, Mozambique, Kenya, Gabon, Vietnam, and China. The findings suggest that free-living *P. expansa* were parasitized by a new genotype or even a possible new species of the genus *Haemogregarina*. Haemosporidians and hemogregarines are not frequently found in *P. geoffroanus* in the studied region under the local conditions of that period.

1. Introduction

Geoffroy's side-necked turtle (Chelidae: *Phrynops geoffroanus*) and the Giant Amazon turtle (Podocnemididae: *Podocnemis expansa*) are freshwater turtle species that have a widespread geographical distribution throughout South America (Molina, 2001; Rueda-Almonacid et al., 2007; Van Dijk et al., 2014). It is known that testudines, in addition to other species of reptiles, are hosts of intracellular hemoparasites of the Phylum Apicomplexa (Jacobson, 2007), which have a typical heteroxene evolutionary cycle of coccidia (Telford2009; Nardini et al., 2013). Among these protozoa, hemogregarines (Suborder Adeleorina: Families Haemogregarinidae, Karyolysidae and Hepatozoidae) and haemosporidians (Order Haemosporida: Family Haemoproteidae), typically parasite red blood cells and occasionally the leukocytes of reptiles (Telford, 2009). The hemogregarines have already been described as parasitizing tortoises [*Hemolivia* spp. (Karyolysidae) (Široký et al., 2004, 2007; Harris et al., 2013; Kvičerová et al., 2014)] and freshwater turtles [*Hemolivia* spp. (Karyolysidae) (Kvičerová et al., 2014), *Hepatozoon* sp. (Hepatozoidae) (Soares et al., 2017) and *Haemogregarina* spp. (Haemogregarinidae) (Jakes et al., 2001; Telford Jr. et al., 2009; Davis and Sterrett, 2011; Pineda-Catalan et al., 2013; Rossow et al., 2013; Dvořáková et al., 2014, 2015; Picelli et al., 2015; Arizza et al., 2016; Rakhshandehroo et al., 2016)]. With regard to haemosporidians, while *Haemoproteus* spp. and *Haemocystidium* spp. (Haemoproteidae) have been detected in freshwater turtles (Jacobson, 2007; Telford2009; Pineda-Catalan et al., 2013), *Haemoproteus* spp. have been also described in tortoises (Telford2009; Cook et al., 2010; Örkun and Güven, 2013; Javanbakht et al., 2015; Martinele et al., 2016).

Hemogregarines can be morphologically classified based on the

* Corresponding author.
E-mail addresses: julepaulavet@posgrad.fcav.unesp.br (J.P.d. Oliveira), marcos_andre@fcav.unesp.br (M.R. André), jose.junior@ifgoiano.edu.br (J.R.F. Alves Júnior), ana-paula.lustosa@icmbio.gov.br (A.P.G. Lustosa), werther@fcav.unesp.br (K. Werther).

https://doi.org/10.1016/j.ijppaw.2018.01.008
Received 18 October 2017; Received in revised form 11 December 2017; Accepted 24 January 2018

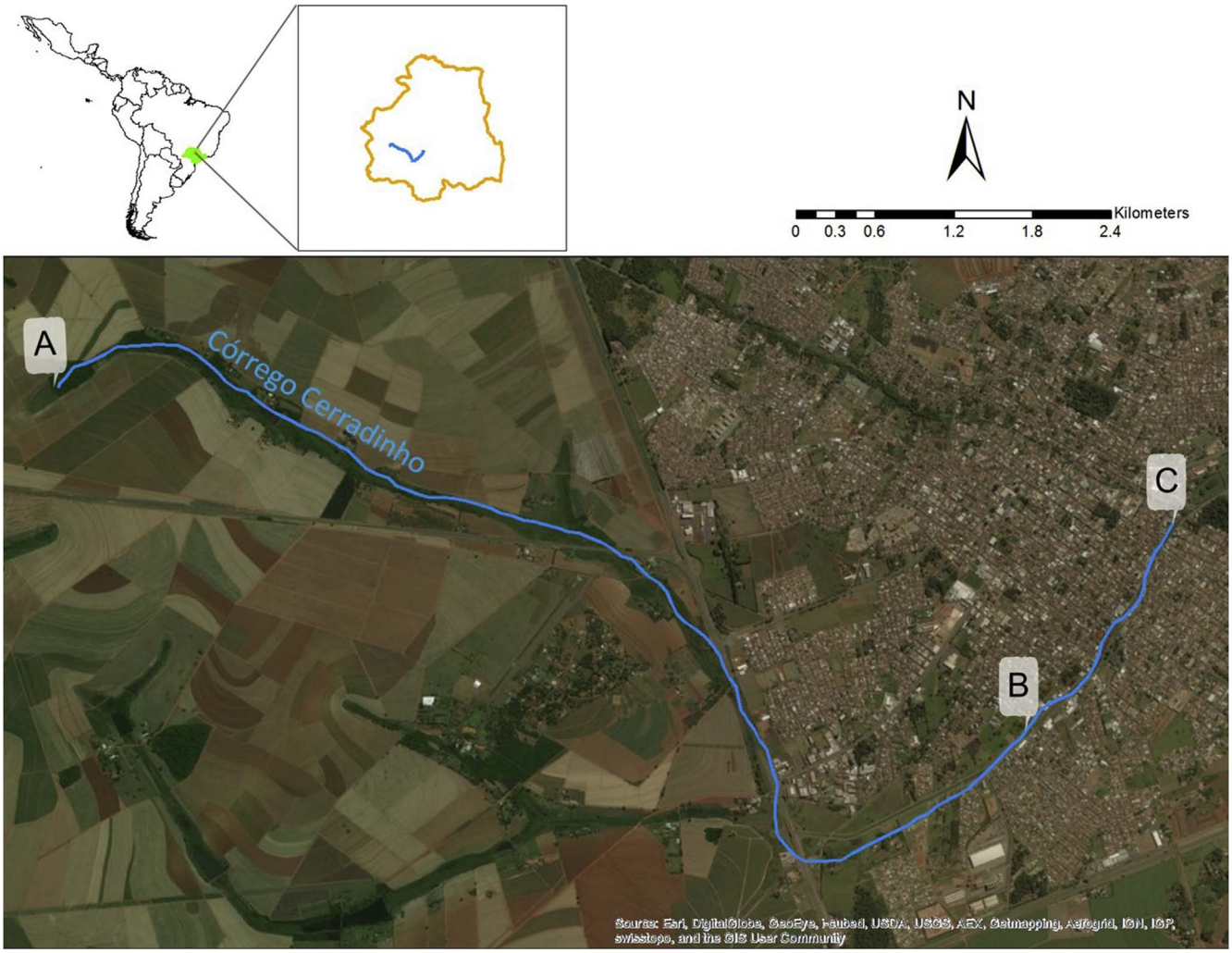

Fig. 1. Capture region of Geoffroy's side-necked turtle (*Phrynops geoffroanus*). The location of the state of São Paulo, Brazil is shown in green in the top left of the map. The municipality of Jaboticabal is shown in the top right in orange, and the Córrego Cerradinho is shown in blue. A satellite image of Jaboticabal, São Paulo, with the Córrego Cerradinho (A–C) course highlighted in blue. The section considered in this study (B–C) represents 1.9 kilometers of the stream. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

developmental details of sporogonic phases of the parasite in the vector, that provide the main characters for classification, the morphology of gametocytes in the red blood cells and an evaluation of the stages of development (Široký et al., 2004; Jacobson, 2007). Although useful, this methodology is not sufficient for a taxonomic diagnosis (Pineda-Catalan et al., 2013; Haklová-Kočíková et al., 2014) in addition the classical systematics has been problematic because of the variability to which morphological details are subjected (Barta et al., 2012). Therefore, the use of molecular methods (Ujvari et al., 2004; Pineda-Catalan et al., 2013) from blood or tissue samples from biopsy or necropsy (Johnson et al., 2007), with appropriate molecular phylogeny study, became an essential adjunct to existing morphological and biological characters for use in the inference of evolutionary history relationships among haemoprotozoan parasites (Barta, 2001; O'Donoghue, 2017).

Molecular investigations of hemogregarines in testudines are mainly based on the 18S ribosomal RNA gene (Wozniak et al., 1994). Phylogenetic inferences of hemogregarines have already been made based on biological samples of testudines and vectors (Harris et al., 2013; Dvořáková et al., 2014, 2015; Kvičerová et al., 2014; Arizza et al., 2016; Rakhshandehroo et al., 2016). For instance, *Hemolivia* spp. was characterized molecularly from blood samples of freshwater turtles in Nicaragua, tortoises in Greece and countries in the Middle East (Turkey, Syria, and Iraq) (Kvičerová et al., 2014), and ticks collected in tortoises in Algeria (Harris et al., 2013). *Haemogregarina* spp. were characterized molecularly from blood samples from freshwater turtles in Canada (Barta et al., 2012), the Middle East (Turkey, Syria, and Iran) (Dvořáková et al., 2014; Rakhshandehroo et al., 2016), North Africa (Morocco, Algeria) (Dvořáková et al., 2014), Southeast Asia (Vietnam, Indonesia, Thailand, and China) (Dvořáková et al., 2015), and Europe (Bulgaria and Italy) (Dvořáková et al., 2014; Arizza et al., 2016). In South America, DNA from hemogregarines have been detected by PCR in blood samples from captive freshwater turtles [Geoffroy's side-necked turtle (Chelidae: *Phrynops geoffroanus*) and the Giant Amazon turtle (Podocnemididae: *Podocnemis expansa*)] in Brazil (Pessoa et al., 2016) and molecularly characterized from blood samples of wild Scorpion Mud turtles (*Kinosternon scorpioides*) in the Brazilian Amazon (Soares et al., 2017). Based only on the morphology of gametocytes in blood smears and results of molecular tests (Pessoa et al., 2016), without phylogenetic inferences, the hemogregarines could be confounded with *Hepatozoon* sp., this misidentification may also happen when the phylogenetic analyses are performed without *Haemogregarina* spp. sequences as ingroup (Soares et al., 2017).

The molecular assays for detecting DNA from haemosporidians in testudines are predominantly based on the mitochondrial gene *citocromo b*. Javanbakht et al. (2015) have characterized morphologically and phylogenetically *Haemoproteus anatolicum* and *H. caucasica* from blood samples of free-living tortoises from Afghanistan, Georgia, Iran, and Turkey. Örkun and Güven (2013) characterized morphologically

and molecularly *H. anatolicum* in blood samples from a specimen of *Testudo graeca* in Anatolia, Turkey. Phylogenetic inferences were performed on *Haemocystidium* (*Simondia*) *pacayae* and *H.* (*S.*) *peltocephali* from blood, liver, and muscle tissue samples of free-living freshwater turtle species belonging to the genus *Podocnemis* in Peru [Yellow-spotted Amazon river turtle (Podocnemididae: *Podocnemis unifilis*) and *P. expansa*] (Pineda-Catalan et al., 2013). Martinele et al. (2016) detected *Haemoproteus* sp. in captive tortoises in Brazil [Red-footed tortoise (*Chelonoidis carbonaria*) and Yellow-footed tortoise (*C. denticulata*)].

The present search aimed to investigate the presence of hemogregarines and haemosporidians in biological samples of free-living *P. geoffroanus* and *P. expansa* from two distinct environments using morphological and molecular techniques.

2. Material and methods

2.1. Animals, period, study area, and ethics statement

Between February and May 2012, *Phrynops geoffroanus* (Geoffroy's side-necked turtle) specimens were captured using net poles, along 1.9 km of an urban stream section of Córrego Cerradinho (total length: 11 km) in the city of Jaboticabal (21°15‘22“S and 48°18’58“W), São Paulo state, southeastern Brazil (Fig. 1). The city is located in a transition area between the Cerrado and Atlantic Forest biomes. Upstream, the stream is bordered by rural properties, and there is practically no riparian forest. In the urban stretch, the stream is partially contained on its sides by stone walls.

Podocnemis expansa (Giant Amazon turtles) were captured in October 2014 in the Environmental Protection Area (APA) Meandros do Araguaia, Araguaia River, Goiás State, Brazil (13°17′49.0″S 50°36′16.5″W to 13°30′00.0″S 50°43′29.51″W) (Fig. 2). This area of the Cerrado biome is located in the northwest of the state of Goiás, near the District of Luiz Alves, Municipality of São Miguel do Araguaia, Goiás State, Brazil and includes the Araguaia River with its lakes and abundant riparian vegetation.

Animal handling consisted of collecting biological material and then releasing the animals in the same place. This search was performed in accordance with the regulations of the Ethics Committee for Animal Use of São Paulo State University (Unesp), School of Agricultural and Veterinarian Sciences, Jaboticabal (protocol no. 2935/17) and the Brazilian Government Institute for Wildlife and Natural Resources Care (ICMBIO) (license no. 28440-1 and 13447-6).

During the capture of *P. geoffroanus*, in April 2012, a high testudine mortality rate was observed. *P. geoffroanus* individuals were collected for other reasons and organ samples (liver, spleen, lungs, and heart) were made available to this study. The cause of mortality was identified as carbamate compounds intoxication (data not shown). No insects were noted during the collecting of the death animals and also two weeks later. Before the mortality of the reptiles, a high quantity of insects was observed near the stream.

2.2. Inspection of ectoparasites and collection of biological samples

P. geoffroanus ($n = 83$) and *P. expansa* ($n = 7$) *specimens* were manually restrained, given a physical examination, and searched for ectoparasites.

P. geoffroanus blood samples were collected from the cervical vertebral venous plexus (Avila Junior et al., 2007). For *P. expansa*, blood access was through the dorsal coccigeal vein (Nardini et al., 2013). Blood aliquots were collected using EDTA (ethylenediaminetetraacetic acid) to perform molecular analyses, and the aliquots were stored in microtubes at −80 °C. Additionally, blood samples without anticoagulant were collected for preparation of smears to research hemoparasites (Campbell, 2006; Almosny and Monteiro, 2007).

Of the 83 *P. geoffroanus* blood samples collected in EDTA to search for hemoparasites, 31 of the corresponding animals died as a result of environmental contamination. All were necropsied (Matushima, 2007; Terrell and Stacy, 2007). During necropsy, liver, spleen, lungs, and heart fragments were collected, stored individually in 2-mL microtubes and kept at −20 °C for subsequent DNA extraction and investigation of hemoparasite genomic material. In parallel, organ samples were fixed for 24 h in 10% buffered formalin solution for subsequent histopathological processing.

2.3. Sample processing

2.3.1. Evaluation of parasitemia and hemoparasite morphology

The blood smears without anticoagulant and stained with Rosenfeld method dye (May Grunwald Giemsa method) (Campbell, 2006) were evaluated under light microscopy (Olympus BX-43; Olympus Corp., Tokyo, Japan) coupled to a camera (Olympus DP73). The search for evolutionary forms of haemosporidians and hemogregarines were performed via inspection of 2×10^3 erythrocytes under 400× magnification (Picelli et al., 2015). The evaluation was conducted using a total of 5×10^3 erythrocytes (Harris et al., 2011; Rakhshandehroo et al., 2016), and no cases of hemoparasites were observed. When no hemoparasites was detected using this method, the blood smear was considered to be negative.

A morphological evaluation of hemoparasites was performed following the characteristics described by Telford et al. (2009). Twenty fields were evaluated under an immersion objective. The image register and morphometry analysis [mean size (μm), length *versus* width, and standard deviation (SD)] of parasitized cells and gametocytes, as well as the description of the changes caused by the parasites in the infected erythrocytes, were analyzed in a computer system of image analysis and measured using Olympus cellSens™ Microscope Imaging Standard software. In order to compare normal and parasitized erythrocyte measurements (length, width, and area of cells and nuclei) and parasitized erythrocytes with centralized and displaced nuclei measurements (length, width, and core area of cells, nuclei, and gametocytes), the variables were subjected to analysis of variance and differences were noted when the F test averages were compared using the Tukey test ($P < 0.05$). The analyses were performed using SAS statistical software (version 9.1; SAS Institute, Cary, NC, USA).

2.3.2. Histopathological examinations

From the fragments of fixed organs of *P. geoffroanus* histological slides were prepared and stained with hematoxylin and eosin (Jeckel Neto, 2007). The histological slides were examined under a light microscope (Olympus BX-43) coupled to a camera (Olympus DP73) using 100× and 400× magnification. The histopathological analysis took into account the location, distribution, and intensity of changes in the liver, spleen, lungs, and heart. Concurrently, structures suggestive of merozoites and meronts were investigated in tissues and structures suggestive of gametocytes in erythrocytes (Telford Jr. et al., 2001; Telford2009). The photographic record in an immersion objective was performed using Olympus cellSens™ Microscope Imaging Standard software.

2.3.3. Detection of hemoparasite genomic material

DNA was extracted from 10 μL of each EDTA whole blood and frozen tissue (10 mg of spleen and 25 mg of the other organs) sample using the QIAamp DNeasy Blood & Tissue Kit (protocols 1a and 1c, cat. no. 69504; QIAGEN®, Valencia, CA, USA), according to the manufacturer's instructions. DNA concentration and quality was measured using the 260/280 nm absorbance ratio (Nanodrop®, Thermo Fisher Scientific, Waltham, MA, USA). In order to verify the existence of amplifiable DNA in the samples, internal control PCR assays targeting the intron from the Chelidae-RNA fingerprint 35 (R35) protein gene (Fujita et al., 2004) were performed. R35-positive samples were submitted to PCR assays to amplify haemosporidian cytochrome *b* and

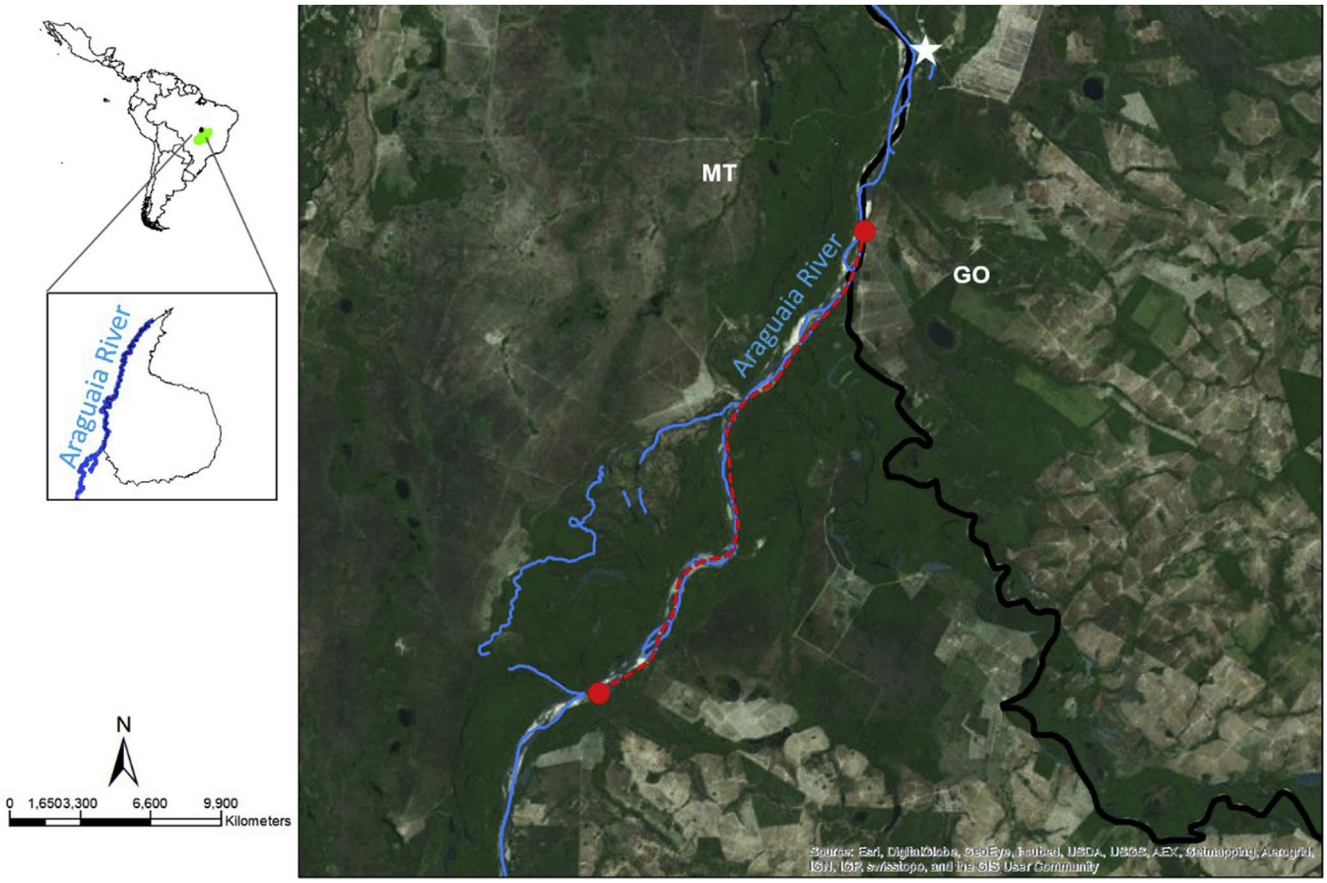

Fig. 2. Cartographic representation of the site of capture of the Giant Amazon turtle (*Podocnemis expansa*). In the top left, the location of the state of Goiás in Brazil is shown along with the outline of the municipality of São Miguel do Araguaia and the Araguaia River. Main Picture: Satellite image of the area between Mato Grosso (MT) and Goiás (GO). The red extent of the Araguaia River was covered, and the white star corresponds to the location of the Chico Mendes Institute for Biodiversity Conservation (ICMBio) in the District of Luiz Alves, Municipality of São Miguel do Araguaia, Goiás State, Brazil. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

hemogregarines 18S rRNA gene fragments.

For the molecular detection of DNA fragments of haemosporidians, we used nested PCR protocols with slight modifications (Hellgren et al., 2004; Vardo et al., 2005; Javanbakht et al., 2015) and primer oligonucleotides based on the 5′ terminal portion of the mitochondrial cytochrome *b* gene (Creasey et al., 1993; Escalante et al., 1998; Bensch et al., 2000; Perkins and Schall, 2002; Hellgren et al., 2004) (Table 1).

The amplification assays were performed in a reaction volume of 25 µL containing a mixture of 5 µL DNA template for the initial cPCR and 1 µL of the amplification product in the nested PCR. 1 × PCR buffer (Life Technologies®, Carlsbad, CA, USA), 0.2 mM of each deoxynucleotide triphosphate (dNTPs) (Life Technologies®), 1.5 mM $MgCl_2$, 0.5 µM of each primer (Síntese Biotecnologia®, Belo Horizonte, MG, BR), 0.75U Taq DNA Polymerase (Life Technologies®) and ultra-pure sterile water (Life Technologies®) were used for "Reaction A" and "Nested Reaction A". For the amplification of "Reaction B" and "Nested Reaction B", "Reaction C", and "Nested Reaction C", we used 1 × PCR buffer (Life Technologies®), 0.2 mM of each dNTPs (Life Technologies®), 2 mM $MgCl_2$, 0.4 µM of each primer (Síntese Biotecnologia®), 1U Taq DNA Polymerase (Life Technologies®) and ultra-pure sterile water (Life Technologies®). The amplified fragments had approximately 480, 1,200, and 673 bp of *Haemoproteus* spp. and *Haemocystidium* spp., based on the Hellgren et al. (2004), Vardo et al. (2005), and Javanbakht et al. (2015) cycling conditions protocols, respectively. *Haemoproteus* DNA

Table 1
Oligonucleotides sequences used in the amplification reactions of haemosporidians DNA fragments based on the mitochondrial cytochrome *b* gene, references and the size of flanked fragments.

cPCR	Primers		Reference	Fragment
Reaction A	HaemNFI	5′-CAT ATA TTA AGA GAA ITA TGG AG-3′	Hellgren et al. (2004)	
	HaemNR3	5′-ATA GAA AGA TAA GAA ATA CCA TTC-3′		
Nested Reaction A	HAEMF	5′-ATG GTC CTT TCG ATA TAT GCA TG-3′	Bensch et al. (2000)	~480 bp
	HAEMR2	5′-GCA TTA TCT GGA TGT GAT AAT GGT-3′		
Reaction B	DW2	5′-TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG-3′	Perkins and Schall (2002)	
	DW4	5′-TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG-3′		
Nested Reaction B	DW1	5′-TCA ACA ATG ACT TTA TTT GG-3′	Perkins and Schall (2002)	~1.2 kb
	DW6	5′-GGG AGC TGT AAT CAT AAT GTG-3′		
Reaction C	DW2	5′-TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG-3′	Perkins and Schall (2002)	
	DW4	5′-TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG-3′		
Nested Reaction C	DW1	5′-TCA ACA ATG ACT TTA TTT GG-3′	Perkins and Schall (2002)	~673 bp
	DW3	5′-TGC TGT ATC ATA CCC TAA AG-3′		

positive control was obtained from a naturally infected wild goose (Werther et al., 2017). Ultra-pure sterile water (Life Technologies®) was used as negative control.

In order to generate a large concatenated sequence of hemogregarines 18S rRNA gene (1100 bp), each sample of extracted DNA was submitted to two cPCR assays aiming at amplifying 581 bp and 900 pb fragments using two distinct pairs of primers: first pair HepF300 (5' - GTT TCT GAC CTA TCA GCT TTC GAC G - 3′) and HepR900 (5' - CAA ATC TAA GAA TTT CAC CTC TGA C - 3′) (Ujvari et al., 2004), and second pair HEMO1 (5' - TAT TGG TTT TAA GAA CTA ATT TTA TGA TTG - 3′) and HEMO2 (5' - CTT CTC CTT CCT TTA AGT GAT AAG GTT CAC - 3′) (Perkins and Keller, 2001), respectively. For the HepF300/HepR900 primer pair, the amplification conditions were performed with 25-μL PCR reactions containing 5 μL DNA template, 1× PCR buffer (Life Technologies®), 0.2 mM of each dNTPs (Life Technologies®), 1.5 mM $MgCl_2$, 0.6 μM of each primer (Síntese Biotecnologia®), 1U Taq DNA Polymerase (Life Technologies®), and ultra-pure sterile water (Life Technologies®). The cycling conditions were conducted following the cycling conditions protocol of O'Dwyer et al. (2013). For the HEMO1/HEMO2 primer pair reactions, mix amplification contained 5 μL DNA template, 1× PCR buffer (Life Technologies®), 0.2 mM of each dNTPs (Life Technologies®), 2.5 mM $MgCl_2$, 0.3 μM of each primer (Síntese Biotecnologia®), 1.5U Taq DNA Polymerase (Life Technologies®), and ultra-pure sterile water (Life Technologies®). for a 25-μL reaction. PCR amplifications were performed following the cycling conditions protocol of Harris et al. (2011). *Hepatozoon* DNA obtained from naturally infected wild carnivores (André et al., 2010) and negative (ultra-pure sterile water) controls were used as positive and negative controls, respectively.

PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide (Life Technologies®). In order to prevent PCR contamination, DNA extraction, reaction setup, PCR amplification, and electrophoresis were performed in separate rooms. The gels were imaged under ultraviolet light using Image Lab Software version 4.1 (*ChemiDoc™ XRS + System with Image Lab™ Software*, BioRad®, Richmond, CA, USA). The reaction products were purified using the Silica Bead DNA gel extraction kit (Thermo Fisher Scientific®). The sequencing of amplified fragments were carried out using the BigDye® Terminator v3.1Cycle Sequencing Kit (Thermo Fisher Scientific®) and ABI PRISM 3700 DNA Analyzer (Applied Biosystems®, Foster City, CA, USA) (Sanger et al., 1977).

The sequences obtained from the positive samples were first submitted to a screening test using Phred-Phrap software version 23 (Ewing and Green, 1998; Ewing et al., 1998) to evaluate the electropherogram quality and to obtain consensus sequences from the alignment of the sense and antisense sequences. The BLAST program (Altschul et al., 1990) was used to analyze the sequences of nucleotides (BLASTn) with the aim of browsing and comparing with sequences from an international database (GenBank) (Benson et al., 2006). Samples exhibiting positive results for both PCR protocols had their sequences concatenated (HepF300/HepR900 and HEMO1/HEMO2) using Fragment Merger software version 1 (Bell and Kramvis, 2013). The sequences were aligned with sequences published in GenBank using MAFFT software, version 7 (Katoh and Standley, 2013, 2016; Yamada et al., 2016). *Eimeria sevilletensis* (Genbank access no. AF311644), *Isospora suis* (U97523), *Sarcocystis* sp. (U97524), and *Theileria mutans* (FJ213586) were used as outgroups. Aligned sequences were edited using BioEdit Sequence Alignment Editor version 7.0.5.3 (Hall, 1999).

The best model of evolution was selected using IQ-TREE software (Nguyen et al., 2015) by W-IQ-TREE (Trifinopoulos et al., 2016), under the Akaike Information Criterion (AIC) (Posada and Buckley, 2004). The Maximum-likelihood (ML) analysis was inferred with the IQ-TREE (Nguyen et al., 2015) using W-IQ-TREE (Trifinopoulos et al., 2016), which includes an estimation of bootstrap node support, and 1000 bootstrapping replicates. Phylogenetic inference was based on Bayesian (BI) and ML methods. The Bayesian inference (BI) analysis was performed with MrBayes 3.2.2 *on XSEDE* (Ronquist and Huelsenbeck, 2003) using CIPRES Science Gateway (Miller et al., 2010). Markov Chain Monte Carlo (MCMC) simulations were run for 10^6 generations with a sampling frequency of every 100 generations and a burn-in of 25%. The trees were examined in Treegraph 2.0.56–381 beta (Stöver and Müller, 2010).

Additionally, an analysis of nucleotide polymorphisms of the 18S rRNA sequences obtained in the present study was performed. The sequences were aligned using MAFFT software, version 7 (Katoh and Standley, 2013, 2016; Yamada et al., 2016). The number of haplotypes, haplotype diversity (Hd), nucleotide diversity (Pi), and DNA divergence between samples were estimated to explore the levels of genetic differentiation among the population. These data were determined using the program DnaSP 5, version 5.10.01 (Librado and Rozas, 2009).

3. Results

No ectoparasites were found in any of the freshwater turtle species at the time of sampling. From the blood smears of 83 *P. geoffroanus* samples, no evolutionary stages of haemosporidians or hemogregarines were observed. We furthermore did not observe meronts in the histological sections of liver, spleen, lungs, or heart of 31 necropsied *P. geoffroanus* animals. In the analysis of blood smears of *P. expansa*, gametocytes of hemogregarines were observed in the seven *P. expansa* (100%) samples evaluated (Fig. 3). The mean level of parasitemia among the sampled *P. expansa* specimens was 1.62% (range: 0.98–3.01%). The intracellular gametocytes presented dimensions of 9.03 ± 2.59 μm × 4.52 ± 1.10 μm and an area of 34.93 ± 12.05 μm^2. Among the parasitized erythrocytes, rare cells hosted two (7.77%) or three (0.43%) gametocytes (0.43%), and the dimensions of the gametocytes of the "multiparasited-erythrocytes" were 8.69 ± 1.52 μm × 4.28 ± 0.84 μm and an area of 33.93 ± 10.43 μm^2. The morphometric characteristics of the parasitized and non-parasitized cells are listed in Table 2. There were no significant differences between the morphometric data of the nuclei of non-parasitized and parasitized erythrocytes ($P > 0.05$). The one exception was in terms of erythrocyte length, which exhibited statistical differences between parasitized and non-parasitized cells ($P < 0.05$). These were slight and subtle distortions of host cells caused by parasitism that could hardly be observed during the data collection.

Among the parasitized erythrocytes, 26.8% presented nucleus displacement. A comparative evaluation was performed between the morphometric parameters of the erythrocytes parasitized with and

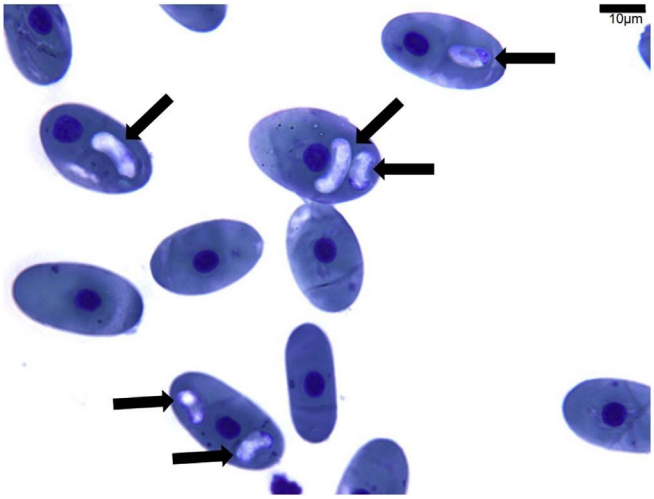

Fig. 3. Photomicrograph of blood smear of a Giant Amazon turtle (*Podocnemis expansa*), stained by the Rosenfeld method dye (May Grunwald Giemsa method), observed in an immersion objective. The presence of intracellular hemogregarines gametocytes in erythrocytes is indicated by arrows.

Table 2
Comparative morphometric analysis (mean, standard deviation and dimension value range) of non-parasitized and *Haemogregarina* sp.-parasitized erythrocytes found in free-living Giant Amazon turtle (*Podocnemis expansa*) blood.

	Cells			Nuclei		
	Length (μm)	Width (μm)	Area (μm^2)	Length (μm)	Width (μm)	Area (μm^2)
N	22.26 ± 0.89(0.19) [b]	15.33 ± 0.96(0.20) [a]	272.89 ± 20.58(4.39) [a]	5.48 ± 0.44(0.09) [a]	4.90 ± 0.36(0.08) [a]	23.35 ± 2.04(0.43) [a]
PA	24.11 ± 1.81(0.39) [a]	15.01 ± 0.94(0.20) [a]	290.33 ± 37.27(8.13) [a]	5.44 ± 0.95(0.21) [a]	4.84 ± 0.84(0.18) [a]	21.54 ± 5.30(1.17) [a]

All of the data are mean ± standard deviation (standard error); the same letters in columns indicate no statistically significant differences (P ≥ 0.05); different letters in columns indicate statistically significant differences (P ≤ 0.05); N = non-parasitized erythrocytes; PA = parasitized erythrocytes.

Table 3
Morphometric analysis of hemogregarine gametocytes from parasitized erythrocytes with and without nucleus displacement observed in free-living Giant Amazon turtle (*Podocnemis expansa*) blood smears.

Morphometric parameters			Nuclei of erythrocytes	
			Centralized	Displaced
Erythrocytes	*Cell*	Length (μm)	24.89 ± 2.93(0.35) [a]	23.77 ± 3.00(0.36) [b]
		Width (μm)	14.94 ± 1.83(0.22) [a]	15.04 ± 1.34(0.16) [a]
		Area (μm^2)	298.13 ± 58.77(7.13) [a]	287.07 ± 49.21(5.97) [a]
	Nuclei	Length (μm)	5.34 ± 0.77(0.09) [b]	5.72 ± 1.08(0.13) [a]
		Width (μm)	4.79 ± 0.80(0.10) [a]	4.99 ± 0.54(0.06) [a]
		Area (μm^2)	21.09 ± 6.52(0.79) [a]	23.04 ± 6.12(0.74) [b]
Hemogregarine gametocytes		Length (μm)	8.44 ± 1.83(0.22) [b]	10.26 ± 1.93(0.23) [a]
		Width (μm)	4.42 ± 0.86(0.10) [b]	4.84 ± 0.73(0.09) [a]
		Area (μm2)	31.15 ± 8.22(1.00) [b]	44.83 ± 12.61(1.53) [a]

All of the data are mean ± standard deviation (standard error); the same letters in columns indicate no statistically significant differences (P ≥ 0.05); different letters in columns indicate statistically significant differences (P ≤ 0.05).

without nucleus displacement (Table 3). It is possible to affirm that the erythrocytes with nucleus displacement presented smaller cellular dimensions, larger nuclei, and that the parasites that were found inside these cells were also larger. Statistically significant differences were observed in all gametocyte parameters and the length of the cells and the nuclei (P < 0.05).

All 83 DNA blood samples (exhibiting an average concentration of 32.4 [SD = 38.7] ηG/μL) from *P. geoffroanus* amplified the predicted product for the R35 nuclear intron. Of the 31 *P. geoffroanus* tissue organs (average DNA concentration of 44.8 (SD = 122.5) ηG/μL), R35 nuclear intron amplification was observed in 45.16% (14/31) of the livers, 32.26% (10/31) of the spleens, 41.93% (13/31) of the lungs, and 29.03% (9/31) of the cardiac tissue. Tissues (62.9% [78/124]) that showed negative results for the R35 nuclear intron were excluded from the analysis. All seven DNA *P. expansa* blood samples (average concentration of 28.1 [SD = 8.4] ηG/μL) amplified the predicted product for R35 nuclear intron.

All of the DNA samples extracted from the *P. geoffroanus* tissues and blood aliquots were negative in the haemosporidians PCR assays (based on the mitochondrial cytochrome *b* gene) and hemogregarines (based on the 18S rRNA gene). The seven *P. expansa* samples were negative in the haemosporidians PCR assays. Moreover, hemogregarine DNA was detected in blood samples from all of the sampled *P. expansa* for both of the PCR protocols employed. Consensus sequences obtained from the HepF300/HepR900 and HEMO1/HEMO2 primer oligonucleotides were combined, thereby yielding approximately 1100-bp sequences from five animals. Sequences from two *P. expansa* had only one of the two consensus sequences, precluding the combination of HepF300/HepR900 and HEMO1/HEMO2; sequences from these both animals were then removed from the phylogenetic analysis. All of the combined sequences that exhibited appropriate qualities and identity with hemogregarines were deposited in the Genbank international database under the following accession numbers: MG031173 - MG031177 and MG031181 - MG031185.

In the BLASTn analysis, the 18S rRNA sequences of hemogregarines detected in *P. expansa* exhibited 98% identity with *Haemogregarina pellegrini* (KM887508 and KM887509) of *Malayemys subtrijuga* (snail-eating turtle) from Vietnam, and *Platysternon megacephalum* (Big-headed turtle) from China, respectively, and *Haemogregarina sacaliae* [KM887507] of *Sacalia quadriocellata* (Four-eyed turtle) from Vietnam.

The phylogenetic ML inference revealed five closely related genotypes (shown in bold in Fig. 4), which formed a monophyletic group. Sister taxa to this lineage were also observed, with 85% clade support (bootstrap), made up of *Haemogregarina* spp. of freshwater turtles, including *H. stepanowi*, *H. balli*, *H. sacaliae*, and *H. pellegrini*. Distinct from the afore mentioned clades, there are formed of *Hepatozoon* spp. sequences detected in mammals and lizards and *Hemolivia* spp. sequences found in tortoises, lizards, and freshwater turtles. In probabilistic BI, the hemogregarine sequences detected in *P. expansa* (shown in bold in Fig. 5) were grouped into a monophyletic group. However, four sequences presented polytomy. A topology similar to that found in the ML inference was observed, and we noted a sister group to the lineage consisting of *Haemogregarina* spp. of freshwater turtles, which presented a Bayesian posterior probability of 74%.

Nucleotide polymorphisms were also analyzed among all of the sequences obtained in the present study. The alignments were analyzed separately because the HepF300/HepR900 (Ujvari et al., 2004) and HEMO1/HEMO2 (Perkins and Keller, 2001) PCR protocols amplify different regions of the 18SrRNA gene, resulting in a total of six samples for each protocol. Both DNA fragments obtained from two different regions from the 18SrRNA gene were shown to be quite conserved. The analysis of nucleotide polymorphisms of the 18S rRNA sequences obtained from both protocols revealed a small number of haplotypes (4 [HepF300/HepR900 protocol] and 3 [HEMO1/HEMO2 protocol]) among the *P. expansa* sampled. The alignment of sequences obtained from the HepF300/HepR900 protocol yielded an alignment size of 620 nucleotide sites, with 10 variable sites, showing four haplotypes (Hd: 0.800; SD = 0.172). Three sequences shared the same haplotype (T1Hep, T5Hep, and T3Hep) with a Pi of 0.00853 (SD = 0.00253) and an average number of nucleotide differences of 3.53333. The alignment of 18S rRNA *Haemogregarina* sequences obtained using the HEMO1/HEMO2 protocol yielded 839 nucleotide sites, with three variable sites,

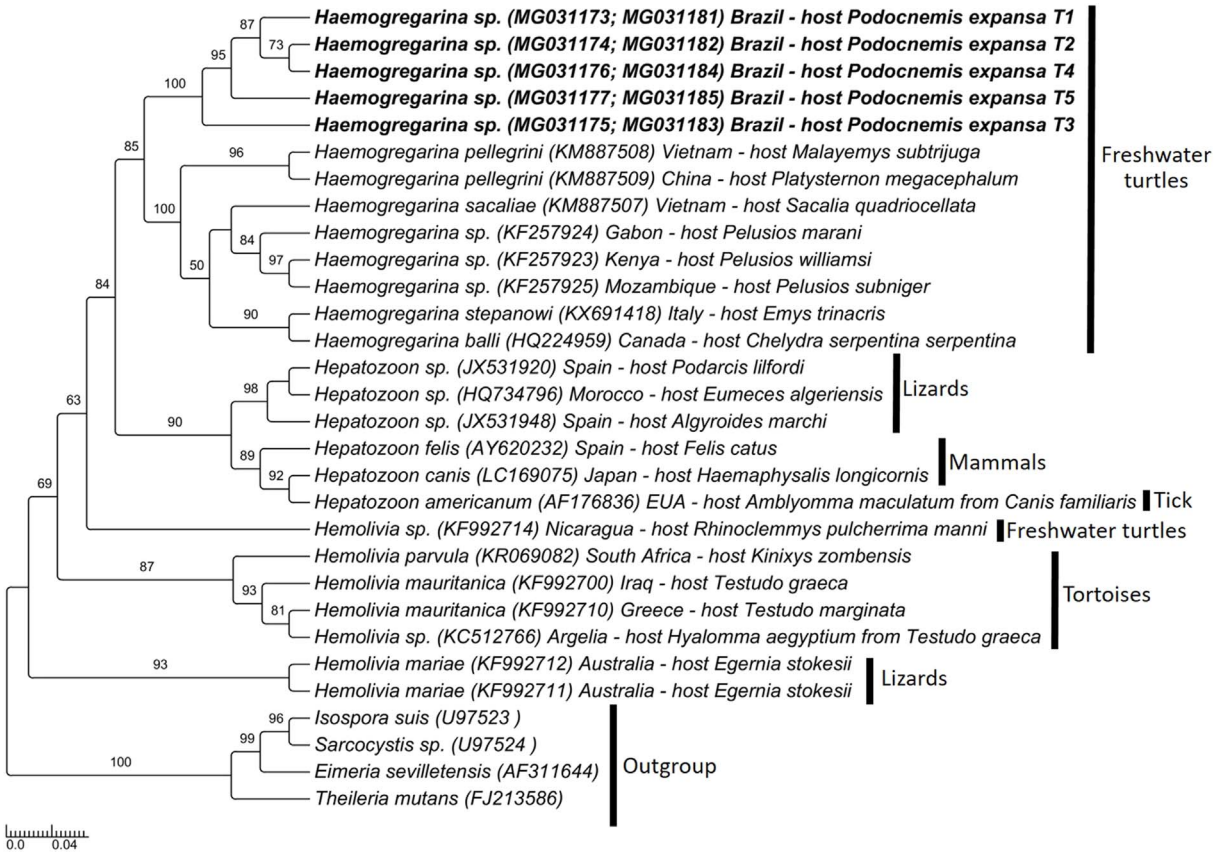

Fig. 4. Phylogenetic tree based on an alignment of a 1100-bp fragment of hemogregarines 18S rRNA sequences using the Maximum Likelihood (ML) method and a TVM + G + I evolutionary model. Bootstrap values for ML (≥50) accessed with 1000 replicates are above the relevant nodes. The sequences indicated in bold represent those from this study, and original sample codes were referred to as T1–T5. *Isospora suis*, *Sarcocystis* sp., *Eimeria sevilletensis*, and *Theileria mutans* were used as an outgroup.

showing three haplotypes (Hd: 0.600; SD = 0.215). Three sequences shared the same haplotype (T1HEMO, T2HEMO, T4HEMO and T5HEMO), Pi of 0.00993 (SD = 0.00350), and average number of nucleotide differences of 1.40000.

4. Discussion

Hemogregarines were detected both by blood smears and by molecular methods in free-living *P. expansa* individuals. Although it was possible to verify the presence of hemoparasites, the ectoparasites, which would act as possible vectors, were not found at the moment of capture. Small leeches have been already observed in *P. expansa* captured between December and April, which corresponds to the period of elevation of the Araguaia River bed. The presence of hemoparasites and the absence of leeches in *P. expansa* has already been observed by Picelli et al. (2015). These authors suggested that the presence of ectoparasites was related to, among other factors, climatic factors, seasonality, and river bed level. In this study, the capture of *P. expansa* coincided with the end of the dry season and the beginning of the rainy season, which would explain the absence of leeches in the sampled animals. Leeches are incriminated vectors of hemogregarines among aquatic reptiles (Brites and Rantin, 2004; Jacobson, 2007; Dvořáková et al., 2014, 2015; Arizza et al., 2016).

Although in this study we did not find ectoparasites in *P. expansa*—which is most likely due to the season of the year in which the animals were captured—it is suggested that the tissue schizogony provided merozoites that became gametocytes observed in the blood smears (Široký et al., 2004). Široký et al. (2004) suggested that the consecutive release of dormant merozoites in the organs and their transformation into gametocytes could explain the long-term parasitemia due to the continuous and long cyclical merogony of hemogregarines in the parenchymatous organs of *T. marginata*. Such a phenomenon could allow hemoparasites to overcome the seasonal nature of testudines' and vector' activity (Široký et al., 2004).

Although morphological alterations in parasitized erythrocytes by hemogregarines have been reported in freshwater turtles and tortoises (Jakes et al., 2001; Široký et al., 2007; Rossow et al., 2013; Davis and Sterrett, 2011; Pessoa et al., 2016), no alterations were observed in this study in terms of the dimensions of the parasitized *versus* non-parasitized erythrocytes, except of parasitized erythrocyte length ($P < 0.05$). This fact is most likely due to a morphological peculiarity of *P. expansa* erythrocytes. The dimensions (length and width) of *P. expansa* erythrocytes are larger and their nuclei smaller when compared with erythrocytes of other terrestrial and aquatic testudines that have already had such documented measurements (Uğurtaş et al., 2003). Therefore, *P. expansa* erythrocytes would present greater intracytoplasmic space for the gametocyte to be lodged without significantly affecting the cell morphology.

In this study, haemosporidians were not noted in biological samples of *P. expansa* using either morphological or molecular methods. While *Haemocystidium* spp. have been reported in freshwater turtles from Peru (Pineda-Catalan et al., 2013), *Haemoproteus* spp. have been described in free-living freshwater turtles from Australia (Jakes et al., 2001) and free-living tortoises from Anatolia, Iran, Georgia, Afghanistan (Javanbakht et al., 2015), the Republic of South Africa (Cook et al., 2010), and Turkey (Örkun and Güven, 2013; Javanbakht et al., 2015).

In addition, the presence of leeches, ectoparasite arthropods, and hemoparasites (hemogregarines and haemosporidians) were not observed in free-living *P. geoffroanus* from an anthropic area. The absence of hemoparasites may be related not only to the absence of ectoparasites but also to the absence of schizogonic tissue forms in the histological sections of these animals, which would justify the lack of

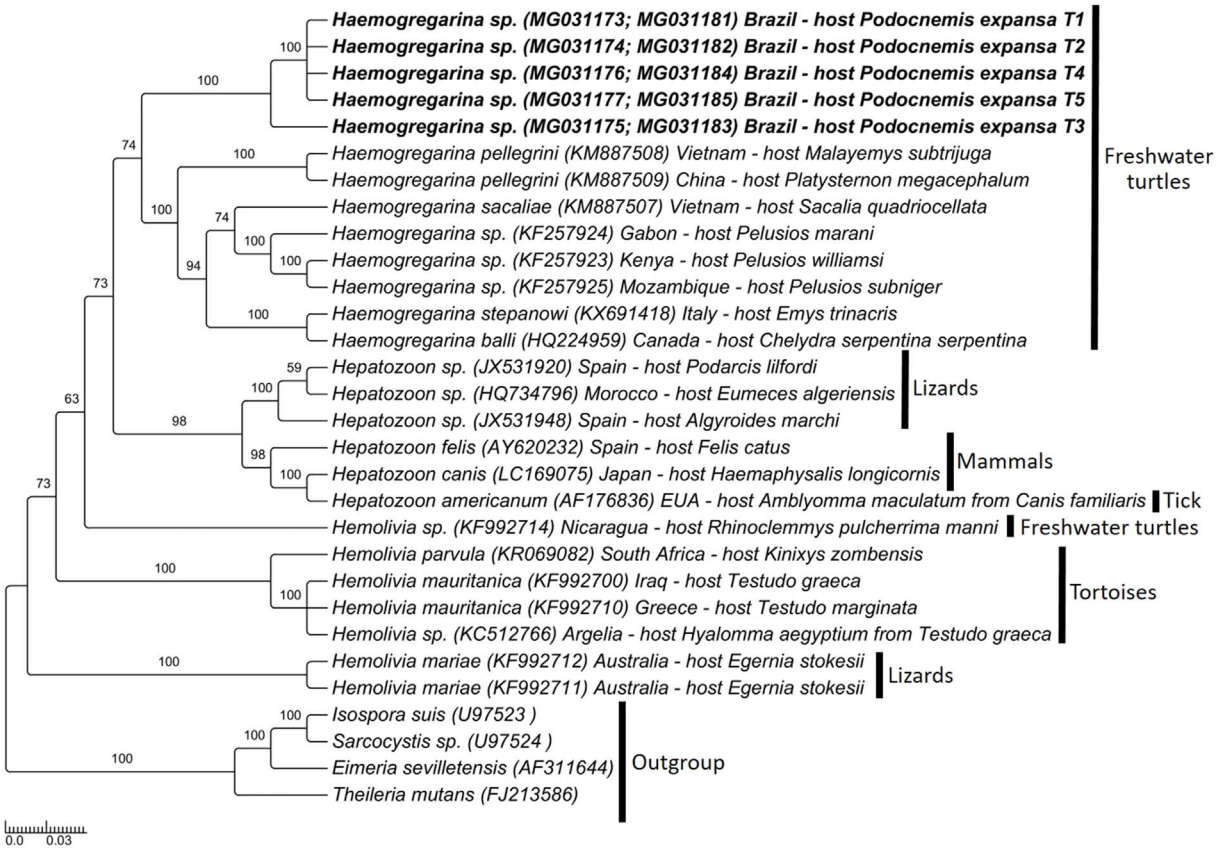

Fig. 5. Phylogenetic tree based on an alignment of a 1100-bp fragment of hemogregarines 18S rRNA sequences using Bayesian inference (BI) and a TVM + G + I evolutionary model. Bayesian posterior probabilities values were above the relevant nodes accessed with 10^6 replicates. The sequences indicated in bold represent those from this study, and original sample codes were referred to as T1–T5. *Isospora suis*, *Sarcocystis* sp., *Eimeria sevilletensis*, and *Theileria mutans* were used as an outgroup.

merozoite release and consequent absence of gametocytes in erythrocytes. Contamination of Córrego Cerradinho stream with carbamates, which results in high animal mortality, may have occurred due to pesticide spraying equipment being washed directly in the stream. Córrego Cerradinho is impacted by anthropic effects, both due to the rural influence of agricultural practice and animal husbandry, as well as the urban influence of domestic sewage, rubble, surface runoff, chemical products, and domestic animals (Borges et al., 2003). Additionally, only rare and isolated strips of riparian forest occur along its 11-kilometer route. It is suggested that environmental degradation may have had an impact on the biodiversity of vertebrates, vectors, and parasites, which influenced the parasitic fauna of these testudines. Research of parasites in freshwater turtles in anthropic environments is necessary to further our knowledge of these animals and their habitat (Brites, 2002). Venancio et al. (2013) suggested that as an environmental sentinel, *P. geoffroanus* may suffer from contamination of domestic and industrial wastewater effluents and it may impact this animal's health and physiological condition. In turn, leeches may serve as indicators of environmental quality because they act as potential biomonitors of organic contaminants in aquatic environments (Metcalfe et al., 1988).

In terms of the molecular detection of hemogregarines in *P. expansa*, a more frequent occurrence of amplification for pairs of the oligonucleotides HepF300/HepR900 than for pairs of HEMO1/HEMO2 was noted. Previous studies have also reported such a difference in the amplification efficiency of these two pairs of primers (Harris et al., 2011, 2013; Maia et al., 2011; O'Dwyer et al., 2013; Harris et al., 2015). When it was not possible to obtain the combination of sequences of both pairs (HepF300/HepR900 + HEMO1/HEMO2), we opted to exclude the sample from the phylogenetic analyses because the short extension of the consensus sequences obtained by only one of the pairs of oligonucleotides could compromise the phylogenetic inferences (Harris et al., 2011). Amplicons of hemogregarine 18S rRNA genes from free-living *P. expansa* generated sequences that enabled phylogenetic positioning close to parasites of the genus *Haemogregarina* spp. of captive and free-living freshwater turtles from Canada, Italy, Vietnam, China, Gabon, Kenya, and Mozambique (Barta et al., 2012; Dvořáková et al., 2014, 2015; Arizza et al., 2016). It was observed that for both phylogenetic analyses the sequences obtained in this study were positioned in a distinct clade of the single sequence of *Hemolivia* sp. obtained from freshwater turtles from Central America, *Rhinoclemmys pulcherrima manni* (Painted Wood turtle) [KF992714] (Kvičerová et al., 2014). These authors reported that although *R. pulcherrima manni* came from Nicaragua, at the time the samples were obtained, the testudine was in captivity as a pet in the Czech Republic. In the analysis of haplotypes, we observed that four distinct haplotypes were obtained from the six sequences of the *Haemogregarina* 18S rRNA gene obtained using the HepF300/HepR900 primers pair. On the other hand, three different haplotypes were obtained from the six sequences generated with the HEMO1/HEMO2 primers pair. Such a finding emphasizes the need for more research aimed at analyzing the genetic diversity of hemoparasites in populations of free-living reptiles (Readel et al., 2008). This situation motivates the molecular investigation of hemogregarines of free-living aquatic testudines from geographically nearby South American countries to collaborate in a more robust way the existence of a new species of the genus *Haemogregarina*. For this purpose, increased surveillance of wildlife populations and an ecological approach to studying hosts, wild and domestic, and environmental ecosystems are much needed (Thompson, 2013).

In conclusion, under the conditions in which this research was carried out, it is proposed that free-living *Podocnemis expansa* (Giant Amazon turtles) were parasitized by a new genotype or even a possible

new species of the genus *Haemogregarina*. Haemosporidians and hemogregarines are not frequent in *Phrynops geoffroanus* (Geoffroy's side-necked turtle) in the region of this stream under local conditions. Quality and environmental preservation can directly influence animal, parasite, and vector diversity.

Conflicts of interest

None.

Acknowledgments

The authors are very thankful to the São Paulo Research Foundation (FAPESP) for the financial support to K. Werther (process numbers #2015/22851-8) and for the Coordination for the Improvement of Higher Education Personnel (CAPES) Doctorate Scholarship to J. P. Oliveira. The authors thank Prof. Dr. Rosangela Zacarias Machado for technical and laboratory support; Dr. Keyla Carstens Marques de Sousa, M.V. Mayara de Cássia Luzzi, Msc. Priscila Ikeda, Msc. Luiz Ricardo Gonçalves, Msc. Jyan Lucas Benevenute, Msc. Mariele de Santi (São Paulo State University -Unesp, School of Agricultural and Veterinarian Sciences, Jaboticabal) for scientific collaboration; Prof. Dr. Alanna do Socorro Lima da Silva (Universidade Federal do Oeste do Pará - UFOPA, Santarém-PA, Brazil), Prof. Dr. Aline Eyko Kawanami (Centro Universitário Barão de Mauá, Ribeirão Preto-SP, Brazil) and MsC. Denise Granato Chung (São Paulo State University - Unesp, School of Agricultural and Veterinarian Sciences, Jaboticabal) for Geoffroy's side-necked turtle (*Phrynops geoffroanus*) capture support; Dra. Alessandra Bertassoni da Silva (São Paulo State University - Unesp, Institute of Biosciences, Humanities and Exact Sciences, São José do Rio Preto) for building maps; and Msc. Ana Carolina Ortegal Almeida (São Paulo State University - Unesp, School of Agricultural and Veterinarian Sciences, Jaboticabal) for the histological evaluation support. The authors also thank the National Center for Research and Conservation of Reptiles and Amphibians (RAN) of the Chico Mendes Institute for Biodiversity Conservation (ICMBio) (13°13′02.1″S 50°34′37.8″W) in District of Luiz Alves, Municipality of São Miguel do Araguaia, Goiás State, Brazil, for the infrastructure support of the Giant Amazon turtle (*Podocnemis expansa*) capture. We also appreciate the picture from Wellington Adriano Moreira Peres of *P. expansa* on the Araguaia River sandbank (Goiás, Brazil), in the graphical abstract.

References

Almosny, N.R.P., Monteiro, A.O., 2007. Patologia Clínica. In: Cubas, Z.S., Silva, J.C.R., Catão-Dias, J.L. (Eds.), Tratado de animais selvagens: medicina veterinária, pp. 939–966 Roca, São Paulo.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

André, M.R., Adania, C.H., Teixeira, R.H.F., Vargas, G.H., Falcade, M., Sousa, L., Salles, A.R., Allegretti, S.M., Felippe, P.A.N., Machado, R.Z., 2010. Molecular detection of *Hepatozoon* spp. in Brazilian and exotic wild carnivores. Vet. Parasitol. 173 (1–2), 134–138. https://doi.org/10.1016/j.vetpar.2010.06.014.

Arizza, V., Sacco, F., Russo, D., Scardino, R., Arculeo, M., Vamberger, M., Marrone, F., 2016. The good, the bad and the ugly: *Emys trinacris, Placobdella costata* and *Haemogregarina stepanowi* in Sicily (Testudines, Annelida and Apicomplexa). Folia Parasitol. 63 029. https://doi.org/10.14411/fp.2016.029.

Avila Junior, R.H., Santos, A.L.Q., Pachaly, J.R., 2007. Contenções Farmacológicas E Anestesia. In: Vilani, R.G.D.˙O.C. (Ed.), Grupo Fowler avanços na medicina de animais selvagens: medicina de répteis. Grupo Fowler - Associação Paranaense de Medicina de Animais Selvagens, Curitiba, pp. 174–177.

Barta, J.R., 2001. Molecular approaches for inferring evolutionary relationships among protistan parasites. Vet. Parasitol. 101 (3–4), 175–186. https://doi.org/10.1016/S0304-4017(01)00564-7.

Barta, J.R., Ogedengbe, J.D., Martin, D.S., Smith, T.G., 2012. Phylogenetic position of the adeleorinid coccidia (Myzozoa, Apicomplexa, Coccidia, Eucoccidiorida, Adeleorina) inferred using 18S rDNA sequences. J. Eukaryot. Microbiol. 59 (2), 171–180. https://doi.org/10.1111/j.1550-7408.2011.00607.x.

Bell, T.G., Kramvis, A., 2013. Fragment merger: an online tool to merge overlapping long sequence fragments. Viruses 5 (3), 824–833. https://doi.org/10.3390/v5030824.

Bensch, S., Stjernman, M., Hasselquist, D., Östman, Ö., Hansson, B., Westerdahl, H., Pinheiro, R.T., 2000. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proc. Biol. Sci. 267 (1452), 1583–1589. https://doi.org/10.1098/rspb.2000.1181.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Rapp, B.A., Wheeler, D.I., 2006. GenBank. Nucleic Acids Res. 34 (1), D16–D20. https://doi.org/10.1093/nar/gkj157.

Borges, M.J., Galbiatti, J.A., Ferraudo, A.S., 2003. Monitoramento de qualidade hídrica e eficiência de interceptores de esgoto em cursos d'água urbanos da bacia hidrográfica do Córrego Jaboticabal. Rev. Bras. Recur. Hídricos 8 (2), 161–171. https://doi.org/10.21168/rbrh.v8n2.p161-171.

Brites, V.L.C., 2002. Hematologia, bioquímica do sangue, parasitologia, microbiologia, algas epizoárias e histopatologia de *Phrynops geoffroanus* (Schweigger, 1812) (Testudinata, Chelidae) expostos a diferentes influências antrópicas no rio Uberabinha, Minas Gerais. 196 f. Dissertation (Doutorado em Ecologia e Recursos Naturais) – Centro de Ciências Biológicas e da Saúde. Universidade Federal de São Carlos, São Carlos.

Brites, V.L.C., Rantin, F.T., 2004. The influence of agricultural and urban contamination on leech infestation of freshwater turtles, *Phrynops geoffroanus*, taken from two areas of the Uberabinha River. Environ. Monit. Assess. 96 (1–3), 273–281. https://doi.org/10.1023/B: EMAS.0000031733.98410.3c.

Campbell, T.W., 2006. Hematologia de répteis. In: Thrall, M.A., Baker, D.C., Campbell, T.W., DeNicola, D., Fettman, M.J., Lassen, E.D., Rebar, A., Weiser, G. (Eds.), Hematologia e Bioquímica Clínica Veterinária. Roca, São Paulo, pp. 248–263.

Cook, C.A., Smit, N.J., Davies, A.J., 2010. Hemoproteids (Apicomplexa: Haemoproteidae) from South African tortoises (Cryptodira: Testudinidae). J. Parasitol. 96 (6), 1168–1172. https://doi.org/10.1645/GE-2527.1.

Creasey, A.M., Ranford-Cartwright, L.C., Moore, D.J., Williamson, D.H., Wilson, R.J.M., Walliker, D., Carter, R., 1993. Uniparental inheritance of the *mitochondrial* gene cytochrome b in *Plasmodium falciparum*. Curr. Genet. 23 (4), 360–364. https://doi.org/10.1007/BF00310900.

Davis, A.K., Sterrett, S.C., 2011. Prevalence of haemogregarine parasites in three freshwater turtle species in a population in Northeast Georgia, USA. Int. J. Zool. Res. 7 (2), 156–163. https://doi.org/10.3923/ijzr.2011.156.163.

Dvořáková, N., Kvičerová, J., Papoušek, I., Javanbakht, H., Tiar, G., Kami, H., Široký, P., 2014. Haemogregarines from western Palaearctic freshwater turtles (genera *Emys, Mauremys*) are conspecific with *Haemogregarina stepanowi* Danilewsky, 1885. Parasitology 141 (4), 522–530. https://doi.org/10.1017/S0031182013001820.

Dvořáková, N., Kvičerová, J., Hostovský, M., Široký, P., 2015. Haemogregarines of freshwater turtles from Southeast Asia with a description of *Haemogregarina sacaliae* sp. n. and a redescription of *Haemogregarina pellegrini* Laveran and Pettit, 1910. Parasitology 142 (6), 816–826. https://doi.org/10.1017/S0031182014001930.

Escalante, A.A., Freeland, D.E., Collins, W.E., Lal, A.A., 1998. The evolution of primate malaria parasites based on the gene encoding cytochrome *b* from the linear mitochondrial genome. Proc. Natl. Acad. Sci. U. S. A. 95 (14), 8124–8129. http://www.pnas.org/content/95/14/8124.full.

Ewing, B., Green, P., 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res. 8 (3), 186–194. https://doi.org/10.1101/gr.8.3.186.

Ewing, B., Hillier, L., Wendl, M.C., Green, P., 1998. Base-calling of automated sequencer traces using Phred. I. Accuracy assessment. Genome Res. 8 (3), 175–185. https://doi.org/10.1101/gr.8.3.175.

Fujita, M.K., Engstrom, T.N., Starkey, D.E., Shaffer, H.B., 2004. Turtle phylogeny: insights from a novel nuclear intron. Mol. Phylogenet. Evol. 31, 1031–1040. https://doi.org/10.1016/j.ympev.2003.09.016.

Haklová-Kočíková, B., Hižňanová, A., Majláth, I., Račka, K., Harris, D.J., Földvári, G., Tryjanowski, P., Kokošová, N., Malčeková, B., Majláthová, V., 2014. Morphological and molecular characterization of Karyolysus – a neglected but common parasite infecting some European lizards. Parasites Vectors 7, 555. https://doi.org/10.1186/s13071-014-0555-x.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. (Oxf) 41, 95–98. http://brownlab.mbio.ncsu.edu/JWB/papers/1999Hall1.pdf.

Harris, D.J., Borges-Nojosa, D.M., Maia, J.P.M.C., 2015. Prevalence and diversity of *Hepatozoon* in native and Exotic Geckos from Brazil. J. Parasitol. 101 (1), 80–85. https://doi.org/10.1645/14-522.1.

Harris, D.J., Graciá, E., Jorge, F., Maia, J.P.M.C., Perera, A., Carretero, M.A., Giménez, A., 2013. Molecular detection of *Hemolivia* (Apicomplexa: Haemogregarinidae) from ticks of North African *Testudo graeca* (Testudines: Testudinidae) and an estimation of their phylogenetic relationships using 18S rRNA sequences. Comp. Parasitol. 80 (2), 292–296. https://doi.org/10.1654/4594.1.

Harris, D.J., Maia, J.P.M.C., Perera, A., 2011. Molecular characterization of *Hepatozoon* species in reptiles from the Seychelles. J. Parasitol. 97 (1), 106–110. https://doi.org/10.1645/GE-2470.1.

Hellgren, O., Waldenström, J., Bensch, S., 2004. A new PCR assay for simultaneous studies of *Leucocytozoon, Plasmodium*, and *Haemoproteus* from avian blood. J. Parasitol. Lawrence 90 (4), 797–802. https://doi.org/10.1645/GE-184R1.

Jacobson, E.R., 2007. Parasites and parasitic diseases of reptiles. In: Jacobson, E.R. (Ed.), Infectious Diseases and Pathology of Reptiles: Color Atlas and Text. CRC Press, Taylor & Francis Group, Boca Raton, pp. 579–580.

Jakes, K.A., O'donoghue, P., Munro, M., Adlard, R., 2001. Hemoprotozoa of freshwater turtles in Queensland. J. Wildl. Dis. 37 (1), 12–19. https://doi.org/10.7589/0090-3558-37.1.12.

Javanbakht, H., Kvičerová, J., Dvořáková, N., Mikulíček, P., Sharifi, M., Kautman, M., Maršíková, A., Široký, P., 2015. Phylogeny, diversity, distribution, and host specificity of Haemoproteus spp. (Apicomplexa: Haemosporida: Haemoproteidae) of palaearctic tortoises. J. Eukaryot. Microbiol. 62 (5), 670–678. https://doi.org/10.1111/jeu.12227.

Jeckel Neto, E.A., 2007. Técnicas de rotina em histologia. In: Carli, A.A. (Ed.), Parasitologia clínica: seleção de métodos e técnicas de laboratório para o diagnóstico das parasitoses humanas, Second ed. Atheneu, São Paulo, pp. 591–608.
Johnson, A.J., Origgi, F.C., Wellehan Jr., J.F.X., 2007. Molecular diagnostics. In: Jacobson, E.R. (Ed.), Infectious Diseases and Pathology of Reptiles: Color Atlas and Text. CRC Press, Taylor & Francis Group, Boca Raton, pp. 351–380.
Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30 (4), 772–780. https://doi.org/10.1093/molbev/mst010.
Katoh, K., Standley, D.M., 2016. A simple method to control over-alignment in the MAFFT multiple sequence alignment program. Bioinformatics 32 (13), 1933–1942. https://doi.org/10.1093/bioinformatics/btw108.
Kvičerová, J., Hypša, V., Dvořáková, N., Mikulíček, P., Jandzik, D., George Gardner, M., Javanbakht, H., Tiar, G., Široký, P., 2014. *Hemolivia* and *Hepatozoon*: haemogregarines with tangled evolutionary relationships. Protist 165 (5), 688–700. https://doi.org/10.1016/j.protis.2014.06.001.
Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25 (11), 1451–1452. https://doi.org/10.1093/bioinformatics/btp187.
Maia, J.M.C., Harris, D.J., Perera, A., 2011. Molecular survey of *Hepatozoon* species in lizards from North Africa. J. Parasitol. 97 (3), 513–517. https://doi.org/10.1645/GE-2666.1.
Martinele, I., Tostes, R., Castro, R., D'agosto, M., 2016. Prevalence of *Haemoproteus* spp. (Apicomplexa: Haemoproteidae) in tortoises in Brazil and its molecular phylogeny. Parasitol. Res. 115 (1), 249–254. https://doi.org/10.1007/s00436-015-4741-3.
Matushima, E.R., 2007. Técnicas Necroscópicas. In: Cubas, Z.S., Silva, J.C.R., Catão-Dias, J.L. (Eds.), Tratado de animais Selvagens: medicina veterinária. Roca, São Paulo, pp. 980–990.
Metcalfe, J.L., Fox, M.E., Carey, J.H., 1988. Freshwater leeches (Hirudinea) as a screening tool for detecting organic contaminants in the environment. Environ. Monit. Assess. 11 (2), 147–169. https://doi.org/10.1007/BF00401727.
Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Gateway Computing Environments Workshop (GCE), pp. 1–8. New Orleans. https://doi.org/10.1109/GCE.2010.5676129.
Molina, F.B., 2001. Class Reptilia, Order Chelonia (Testudinata) (Chelonians): turtles, tortoises biology, management, and free-living populations. In: Fowler, M.E., Cubas, Z.S. (Eds.), Biology, Medicine, and Surgery of South American Wild Animals. Iowa State University Press, Ames, pp. 15–20.
Nardini, G., Leopardi, S., Bielli, M., 2013. Clinical hematology in reptilian species. Vet. Clin. North. Exot. Anim. Pract. 16 (1), 1–30. https://doi.org/10.1016/j.cvex.2012.09.001.
Nguyen, L.T., Schmidt, H.A., Von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 32 (1), 268–274. https://doi.org/10.1093/molbev/msu300.
O'Donoghue, P., 2017. Haemoprotozoa: making biological sense of molecular. Int. J. Parasitol. Parasites Wildl. 6 (3), 241–256. https://doi.org/10.1016/j.ijppaw.2017.08.007.
O'Dwyer, L.H., Moço, T.C., Paduan, K.S., Spenassatto, C., Silva, R.J., Ribolla, P.E.M., 2013. Description of three new species of *Hepatozoon* (Apicomplexa, Hepatozoidae) from Rattlesnakes (*Crotalus durissus terrificus*) based on molecular, morphometric and morphologic characters. Exp. Parasitol. 135 (2), 200–207. https://doi.org/10.1016/j.exppara.2013.06.019.
Örkun, O., Güven, E., 2013. A new species of *Haemoproteus* from a tortoise (*Testudo graeca*) in Turkey, with remarks on molecular phylogenetic and morphological analysis. J. Parasitol. 99 (1), 112–117. https://doi.org/10.1645/GE-3100.1.
Perkins, S.L., Schall, J.J., 2002. A molecular phylogeny of malarial parasites recovered from cytochrome *b* gene sequences. J. Parasitol. 88 (5), 972–978. https://doi.org/10.1645/0022-3395(2002)088[0972:AMPOMP]2.0.CO;2.
Perkins, S.L., Keller, A.K., 2001. Phylogeny of nuclear small subunit rRNA genes of hemogregarines amplified with specific primers. J. Parasitol. 87 (4), 870–876. https://doi.org/10.1645/0022-3395(2001)087[0870:PONSSR]2.0.CO;2.
Pessoa, L.M.B., Scalon, M.C., Carneiro, F.T., Lima, M.G.M., Silva, T.F., Monteiro, R.V., Paludo, G.R., 2016. Ocorrência de hemogregarinas em duas espécies de cágados brasileiros. Acta Sci. Vet. 44 (0), 1381. http://revistas.bvs-vet.org.br/actascivet/article/view/31451.
Picelli, A.M., Carvalho, A.V., Viana, L.A., Malvasio, A., 2015. Prevalence and parasitemia of *Haemogregarina* sp. in *Podocnemis expansa* (Testudines: Podocnemididae) from the Brazilian Amazon. Rev. Bras. Parasitol. Vet. 24 (2), 191–197. https://doi.org/10.1590/S1984-29612015033.
Pineda-Catalan, O., Perkins, S.L., Peirce, M.A., Engstrand, R., Garcia-Davila, C., Pinedo-Vasquez, M., Aguirre, A.A., 2013. Revision of hemoproteid genera and description and redescription of two species of chelonian hemoproteid parasites. J. Parasitol. 99 (6), 1089–1098. https://doi.org/10.1645/13-296.1.
Posada, D., Buckley, T.R., 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and bayesian approaches over likelihood ratio tests. Syst. Biol. 53 (5), 793–808. https://doi.org/10.1080/10635150490522304.
Rakhshandehroo, E., Sharifiyazdi, H., Ahmadi, A., 2016. Morphological and molecular characterisation of *Haemogregarina* sp. (Apicomplexa: Adeleina: Haemogregarinidae) from the blood of the Caspian freshwater turtle *Mauremys caspica* (Gmelin) (Geoemydidae) in Iran. Syst. Parasitol. 93 (5), 517–524. https://doi.org/10.1007/s11230-016-9643-y.
Readel, A.M., Phillips, C.A., Wetzel, M.J., 2008. Leech parasitism in a turtle assemblage: effects of host and environmental characteristics. Copeia 2008 (1), 227–233. https://doi.org/10.1643/CH-06-212.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19 (12), 1572–1574. https://doi.org/10.1093/bioinformatics/btg180.
Rossow, J.A., Hernandez, S.M., Sumner, S.M., Altman, B.R., Crider, C.G., Gammage, M.B., Segal, K.M., Yabsley, M.J., 2013. Haemogregarine infections of three species of aquatic freshwater turtles from two sites in Costa Rica. Int. J. Parasitol. Parasites Wildl. 2, 131–135. https://doi.org/10.1016/j.ijppaw.2013.02.003.
Rueda-Almonacid, J.V., Carr, J.L., Mittermeier, R.A., Rodríguez-Mahecha, J.V., Mast, R.B., Vogt, R.C., Rhodin, A.G.J., Ossa-Velásquez, J., Rueda, J.N., Mittermeier, C.G., 2007. Las tortugas y los cocodrilianos de los países andinos del trópico. Conservación Internacional. Serie de guías tropicales de campo, No. 6. Editorial Panamericana, Bogotá pp. 194–198 and 206-211.
Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. 74 (12), 5463–5467. https://doi.org/10.1073/pnas.74.12.5463.
Široký, P., Kamler, M., Frye, F.L., Fictum, P., Mondrý, D., 2007. Endogenous development of *Hemolivia mauritanica* (Apicomplexa: Adeleina: Haemogregarinidae) in the marginated tortoise *Testudo marginata* (Reptilia: Testudinidae): evidence from experimental infection. Folia Parasitol. (Praha) 54 (1), 13–18. https://doi.org/10.14411/fp.2007.002.
Široký, P., Kamler, M., Modrý, D., 2004. Long-term occurrence of *Hemolivia* CF. *mauritanica* (Apicomplexa: Adeleina: Haemogregarinidae) in the captive *Testudo marginata* (Reptilia: Testudinidae): evidence for cyclic merogony? J. Parasitol. 90 (6), 1391–1393. https://doi.org/10.1645/GE-3306.
Soares, H.S., Marcili, A., Barbieri, A.R.M., Minervino, A.H.H., Moreira, T.R., Gennari, S.M., Labruna, M.B., 2017. Novel piroplasmid and *Hepatozoon* organisms infecting the wildlife of two regions of the Brazilian Amazon. Int. J. Parasitol. Parasites Wildl. 6 (2), 115–121. https://doi.org/10.1016/j.ijppaw.2017.05.002.
Stöver, B.C., Müller, K.F., 2010. TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. BMC Bioinf. 11 (7), 1–9. https://doi.org/10.1186/1471-2105-11-7.
Telford Jr., S.R., 2009. Hemoparasites of the Reptilia: Color Atlas and Text. CRC Press, Taylor & Francis Group, Boca Raton, pp. 01–260.
Telford Jr., S.R., Norton, T.M., Moler, P.E., Jensen, J.B., 2009. A new *Haemogregarina* species of the alligator snapping turtle, *Macrochelys temminckii* (Testudines: Chelydridae), in Georgia and Florida that produces macromeronts in circulating erythrocytes. J. Parasitol. 95 (1), 208–214. https://doi.org/10.1645/GE-1696.1.
Telford Jr., S.R., Wozniak, E.J., Butler, J.F., 2001. Haemogregarine specificity in two communities of Florida snakes, with descriptions of six new species of *Hepatozoon* (Apicomplexa: Hepatozoidae) and a possible species of *Haemogregarina* (Apicomplexa: Haemogregarinidae). J. Parasitol. 87 (4), 890–905. https://doi.org/10.1645/0022-3395(2001)087[0890:HSITCO]2.0.CO;2.
Terrell, S.P., Stacy, B.A., 2007. Reptile necropsy techniques. In: Jacobson, E.R. (Ed.), Infectious Diseases and Pathology of Reptiles: Color Atlas and Text. CRC Press, Taylor & Francis Group, Boca Raton, pp. 219–256.
Thompson, R.C.A., 2013. Parasite zoonoses and wildlife: one Health, spillover and human activity. Int. J. Parasitol. 43 (12–13), 1079–1088. https://doi.org/10.1016/j.ijpara.2013.06.007.
Trifinopoulos, J., Nguyen, L.T., Von Haeseler, A., Minh, B.Q., 2016. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res. 44 (W1), W232–W235. https://doi.org/10.1093/nar/gkw256.
Uğurtaş, I.H., Sevinç, M., Yildirimhan, H.S., 2003. Erythrocyte size and morphology of some tortoises and turtles from Turkey. Zool. Stud. 42 (1), 173–178. http://zoolstud.sinica.edu.tw/Journals/42.1/173.pdf.
Ujvari, B., Madsen, T., Olsson, M., 2004. High prevalence of *Hepatozoon* spp. (Apicomplexa, Hepatozoidae) infection in water pythons (*Liasis fuscus*) from tropical Australia. J. Parasitol. 90 (3), 670–672. https://doi.org/10.1645/GE-204R.
Van Dijk, P.P., Iverson, J.B., Rhodin, A.G.J., Shaffer, H.B., Bour, R., 2014. Turtles of the world, 7th edition: annotated checklist of taxonomy, synonymy, distribution with maps, and conservation status. In: Rhodin, A.G.J., Pritchard, P.C.H., Van Dijk, P.P., Saumure, R.A., Buhlmann, K.A., Iverson, J.B., Mittermeier, R.A. (Eds.), Conservation Biology of Freshwater Turtle and Tortoises: a Compilation Project of the IUCN/SSC Tortoise and Freshwater Turtle Specialist Group, Chelonian Res Monogr, 5(7):329–479. https://doi.org/10.3854/crm.5.000.checklist.v7.2014.
Vardo, A.M., Wargo, A.R., Schall, J.J., 2005. PCR detection of lizard malaria parasites: prevalence of *Plasmodium* infections with low-level parasitemia differs by site and season. J. Parasitol. 91 (6), 1509–1511. https://doi.org/10.1645/GE-589R.1.
Venancio, L.P.R., Silva, M.I.A., Da Silva, T.L., Moschetta, V.A.G., De Campos Zuccari, D.A.P., Almeida, E.A., Bonini-Domingos, C.R., 2013. Pollution-induced metabolic responses in hypoxia-tolerant freshwater turtles. Ecotoxicol. Environ. Saf. 97, 1–9. http://doi.org/10.1016/j.ecoenv.2013.06.035.
Werther, K., Luzzi, M.C., Gonçalves, L.R., Oliveira, J.P., Alves-Júnior, J.R.F., Machado, R.Z., André, M.R., 2017. Molecular and serological detection of arthropod-borne agents in wild Orinoco geese (*Neochen jubata*) in Brazil. Comp. Immunol. Microbiol. Infect. Dis. 55, 30–41. https://doi.org/10.1016/j.cimid.2017.09.003.
Wozniak, E.J., Telford Jr., S.R., Mclaughlin, G.L., 1994. Employment of the Polymerase chain reaction in the molecular differentiation of reptilian hemogregarines and its application to preventative zoological medicine. J. Zoo Wildl. Med. 25 (4), 538–547. http://www.jstor.org/stable/20095414.
Yamada, K.D., Tomii, K., Katoh, K., 2016. Application of the MAFFT sequence alignment program to large data—reexamination of the usefulness of chained guide trees. Bioinformatics 32 (21), 3246–3251. https://doi.org/10.1093/bioinformatics/btw412.