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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

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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).

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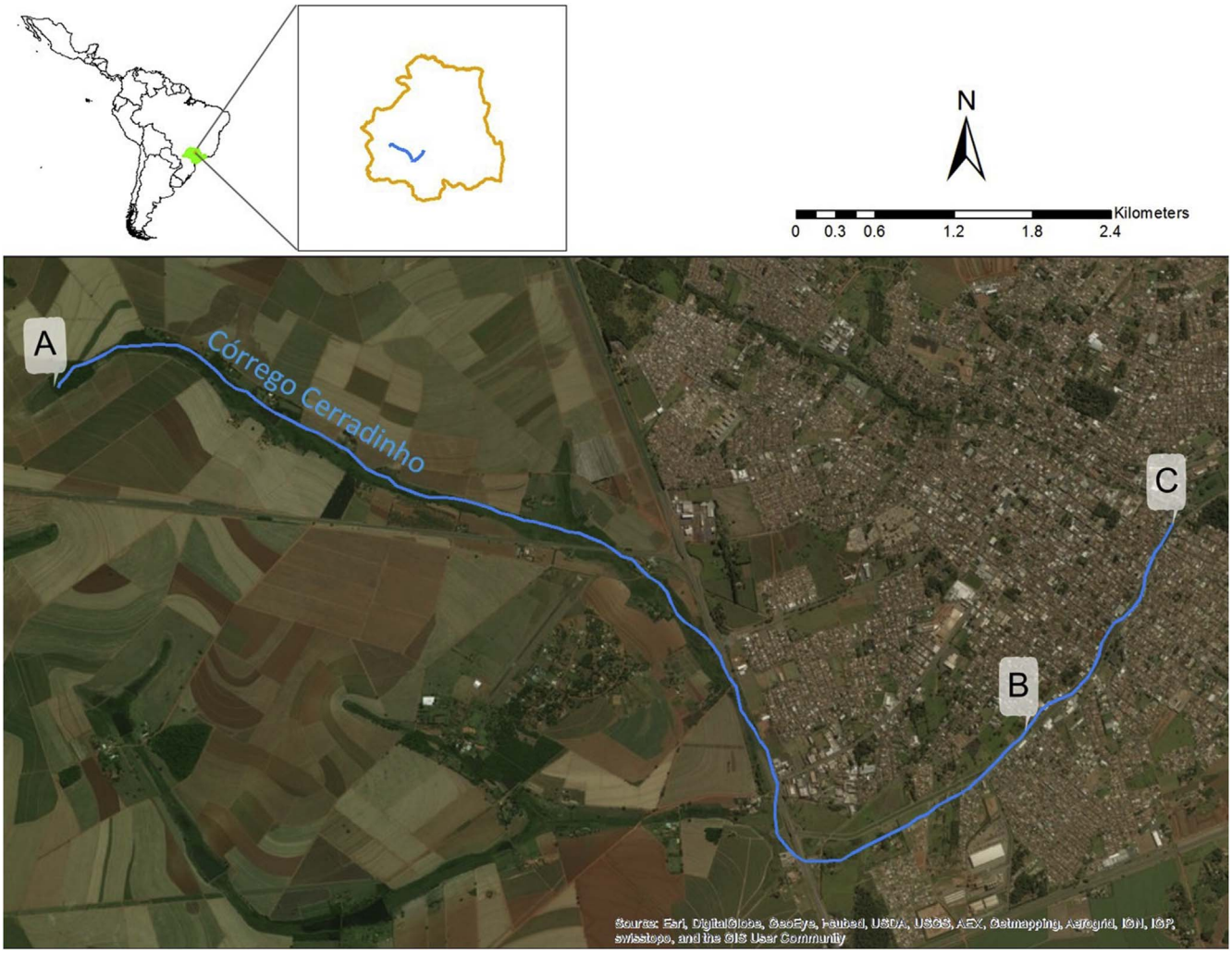

Fig. 1. Capture region of Geoffroy's side-necked turtle (*Phrynops geoffroanus*). The location of the state of São Paulo, Brazil is shown in green in the top left of the map. The municipality of Jaboticabal is shown in the top right in orange, and the Córrego Cerradinho is shown in blue. A satellite 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