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Sinopse:
Esta tese investigou a prevalência e a diversidade de hemoparasitos em lagartos da Amazônia Central. Além disso, traz informações sobre distribuição, taxonomia e relações ecológico-evolutivas desses parasitos e seus hospedeiros.

Palavras-chave: Biodiversidade, herpetofauna, morfologia, parasitos de sangue, relações filogenéticas, taxonomia

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“I’ve always said to young Brazilian students what a wonderful place they’re in. If you turn over a stone you’ll find four new species underneath it. The Amazon region is a veritable mine of parasitological information, yet very, very few people were engaged in parasitological studies in this region.”

Ralph Lainson

(1927-2015)

Resumo

Os parasitos são reconhecidos pela sua grande capacidade de influenciar a evolução e ecologia de seus hospedeiros, tanto ao nível de indivíduo quanto de comunidade. Entretanto, no Brasil, um dos países com a maior biodiversidade do planeta, estudos sobre parasitismo em populações silvestres ainda são relativamente escassos. Na região amazônica, levantamentos anteriores constataram uma rica fauna de parasitos de sangue em lagartos e deram indícios sobre um elevado potencial para descoberta de novas espécies. Nesse contexto, a presente tese investigou a ocorrência de hemoparasitos em lagartos da Amazônia Central, explorando ao longo de três capítulos aspectos relacionados à diversidade, taxonomia e suas relações ecológico-evolutivas. Para tanto, foram obtidas amostras sanguíneas de diversas espécies de lagartos capturadas em áreas de floresta de terra-firme, localizadas próximas aos munícipios de Manaus, Presidente Figueiredo e Rio Preto da Eva, no Estado do Amazonas, Brasil. No **primeiro capítulo** foram reunidos os resultados sobre a prevalência e a riqueza de hemoparasitos encontrados nessas localidades, apresentando também uma lista atualizada dos estudos realizados no Brasil sobre hemoparasitos em lagartos. O **segundo capítulo** traz a redescrição taxonômica, usando dados morfológicos e moleculares, de uma espécie de hemogregarina, *Hepatozoon ameivae*, detectada em lagartos *Ameiva ameiva*. Para o **terceiro capítulo** foi levantada a hipótese de que a ecologia do lagarto *Uranoscodon superciliosus* está moldando a diversidade dos tripanossomas que os parasitam, o que pode ser evidenciado pelas relações filogenéticas de dois novos genótipos de tripanossomas isolados nessa espécie de hospedeiro. Por fim, os resultados obtidos nessa tese ampliaram o conhecimento sobre a diversidade e distribuição dos hemoparasitos no Brasil, além de terem gerado informações inéditas sobre o sistema parasito-hospedeiro formado pelos lagartos e seus hemoparasitos na região amazônica.

Palavras-chave: Biodiversidade; Filogenia; Floresta Amazônica; Morfologia; Parasitos de Sangue; Squamata.

Abstract

Parasites are recognized for their great ability to influence the evolution and ecology of their hosts, at the individual and community levels. However, in Brazil, one of the countries with the greatest biodiversity on the planet, studies on parasitism in wild populations are still relatively scarce. In the Brazilian Amazonia, previous surveys found a rich fauna of blood parasites in lizards and gave indications of a high potential for the discovery of new species. In this context, the present thesis investigated the occurrence of hemoparasites in lizards from Central Amazonia, exploring over three chapters aspects related to the diversity, taxonomy and their ecological-evolutionary relationships. For this purpose, blood samples were obtained from different lizard species captured in areas of upland forests (*terra-firme*), located near the municipalities of Manaus, Presidente Figueiredo and Rio Preto da Eva, in the State of Amazonas, Brazil. In the **first chapter**, results on the prevalence and richness of hemoparasites found in these locations were gathered, also presenting an updated list of studies carried out in Brazil involving hemoparasites in lizards. The **second chapter** presents the taxonomic redescription, using morphological and molecular data, of a hemogregarine species, *Hepatozoon ameivae*, detected in *Ameiva ameiva* lizards. In the **third chapter**, we raised the hypothesis that the ecology of the lizard *Uranoscodon superciliosus* is shaping the diversity of the trypanosomes that parasitize it, which can be evidenced by the phylogenetic relationships of the two new trypanosome genotypes isolated from this host species. Finally, the results obtained in this thesis expanded the knowledge about the diversity and distribution of hemoparasites in Brazil, in addition to novel information about the host-parasite system formed by lizards and their hemoparasites in the Amazonian region.

Keywords: Biodiversity; Phylogeny; Amazon rainforest; Morphology; Blood Parasites; Squamata.

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Introdução Geral

Com origens e histórias evolutivas independentes, o estilo de vida parasitário é complexo e, possivelmente, o mais bem-sucedido entre os seres vivos, estando presente em praticamente todos os grandes grupos taxonômicos (De Meeûs e Renaud 2002, O'Donoghue 2017). Há estimativas que sugerem que mais da metade das espécies viventes podem ser consideradas, em sentido amplo, como parasitos (Dobson et al. 2008, Poulin 2014, Morand 2015). Tradicionalmente, os parasitos (gr. *parasitos* - quem come à mesa do outro) são definidos como organismos que passam a maior parte ou toda a vida associados a outros organismos (hospedeiros) alimentando-se deles e, como consequência, causando algum tipo de prejuízo a esses indivíduos (Price 1977, Poulin e Morand 2004). Contudo, dada a particularidade de cada parasito e das características que envolvem a relação com seu hospedeiro, é possível aprofundar esse significado: além de obter nutrientes, muitas espécies dependem de seus hospedeiros para seu desenvolvimento, reprodução e dispersão, estratégias parasitárias que podem ou não levar seus hospedeiros à morte (Erbet e Herre 1996, Poulin e Morand 2004).

Ecologicamente, parasitos são considerados como verdadeiros “engenheiros” da natureza, pois, além de intervirem sobre a coexistência ou a exclusão das espécies, podem atuar em diferentes níveis e processos dentro das comunidades, influenciando variáveis que estruturam os ecossistemas (Poulin 1999, Thomas et al. 2000, Hatcher et al. 2012, Buck 2019). São organismos-chave para a composição de algumas teias alimentares, aumentando a complexidade e afetando os fluxos de energia e a ciclagem de nutrientes (Lafferty et al. 2008, Hatcher et al. 2012, Anaya-Rojas et al. 2019). Em seus hospedeiros são capazes de influenciar vários processos ecológicos e evolutivos (e.g. seleção sexual, migração, competição e predação, especiação e extinção) que, por sua vez, moldam a dinâmica da população hospedeira e levam a efeitos diretos ou indiretos sobre outras espécies que interagem com seus hospedeiros (e.g.

predadores e competidores) (Schall 1992, Tompkins e Begon 1999, Hatcher et al. 2012, Buck 2019). Dessa forma, os parasitos são importantes preditores da biodiversidade e da saúde dos ecossistemas (Hudson et al. 2006, Dobson et al. 2008, Thompson et al. 2018).

Por outro lado, os parasitos geralmente são reconhecidos pelos diversos efeitos negativos que produzem sobre seus hospedeiros, principalmente quando associados às populações humanas (e.g. malária, Doença de Chagas, filariose, entre outras) com severos impactos na saúde pública e economia mundial (Perkins 2014, Telleria e Tibayrenc 2017, WHO 2019). Como resultado, grande parte das pesquisas estão concentradas em poucos grupos de hospedeiros animais, sobretudo parasitos de mamíferos com interesse econômico ou envolvendo animais silvestres associados a doenças zoonóticas (Valkiunas 2005, Spodareva et al. 2018). Entretanto, negligenciar espécimes pode ter implicações sobre o entendimento da virulência e evolução dos patógenos de importância médica (Rambaut et al. 2001, Galen et al. 2018). A acurácia das inferências sobre processos evolutivos depende de uma amostragem ampla dos taxa (Heath et al. 2008), e especialmente da reconstrução das transições entre grupos de hospedeiros que levaram à origem da doença (Liu et al. 2010).

Embora sejam hospedeiros subestimados, os répteis (Chordata, Reptilia) se destacam pela grande variedade de parasitos sanguíneos (hemoparasitos) que albergam, com uma riqueza de espécies registradas superior àquelas conhecidas para aves e mamíferos (Davies e Johnston 2000, Telford 2009). Isso provavelmente se deve à antiga idade filética dos répteis [final do período Carbonífero (~315 M.a)] e também à elevada diversidade ecológica e taxonômica desses hospedeiros (Poinar e Poinar 2004, Vitt e Caldwell 2013). Nesse aspecto, os lagartos (Lepidosauria, Squamata) podem ser considerados como hospedeiros potencialmente diversos em espécies de hemoparasitos, uma vez que, além de possuírem 60% das 11 mil espécies descritas da classe Reptilia Laurenti, 1768, apresentam hábitos de vida bastante diversificados

e são encontrados em uma ampla variedade de ambientes (Vitt et al 2008, Faria et al. 2019, Peixoto et al. 2020, Uetz et al. 2020).

Nos répteis, os hemoparasitos encontrados com maior frequência pertencem ao filo Apicomplexa Levine, 1970 e à família Trypanosomatidae Doflein, 1901 (Telford 2009, O'Donoghue 2017). Esses dois taxa contabilizam juntos nesses hospedeiros aproximadamente 570 espécies, divididas em 18 gêneros e 10 famílias, das quais mais da metade (ca. 320) foram identificadas em lagartos (Tabela 1). Há também outros organismos menos frequentes, como os estágios larvais (microfilárias) do filo Nematoda Diesing, 1861 e, também, inclusões virais e bacterianas (Telford 2009, Halla et al. 2014). Além disso, entre os hemoparasitos, à exceção de alguns vírus e bactérias, há uma convergência adaptativa ao uso de invertebrados hematófagos como principais vetores para transmissão entre seus hospedeiros vertebrados (O'Donoghue 2017; Tabela 1).

O filo Apicomplexa (Chromista, Alveolata) detém mais de 6 mil espécies descritas (Votýpka et al. 2017). Todas são endossimbiontes obrigatórias e apresentam um conjunto de estruturas na extremidade anterior, denominado complexo apical, que possibilita a invasão e sobrevivência dentro da célula hospedeira (Levine et al. 1980, Morrissette e Sibley 2002, Baum et al. 2008, Tardieux e Baum 2016). O desenvolvimento dos Apicomplexa é único entre os eucariotos por apresentar uma ontogenia reprodutiva cíclica, que contém duas fases assexuadas, merogonia e esporogonia, e uma sexuada, chamada de gametogonia (Striepen et al. 2007, Baum et al. 2008, Votýpka et al. 2017). Os membros desse filo com estágios de desenvolvimento intracelular nas células sanguíneas dos répteis são (Tabela 1): as hemogregarinas (Coccidia, Adeleorina), os hemococcídios (Coccidia, Eimeriorina), os hemosporídeos (Hematozoa, Haemosporida) e os piroplasmas (Hematozoa, Piroplasmida). Destes, os mais encontrados em lagartos são as hemogregarinas do gênero *Hepatozoon* Miller, 1908 e os hemosporídeos do

gênero *Plasmodium* Marchiafava & Celli 1885 (Smith 1996, Telford 2009, Lainson 2012, Perkins et al. 2014).

Tabela 1. Diversidade de hemoparasitos do Filo Apicomplexa e de tripanosomatídeos descritos em répteis ao redor do mundo e seus vetores (Telford 1995, 2009, Smith 1996, Lainson 2012, Megía-Palma et al. 2017, O'Donoghue 2017, Úngari et al. 2018, Fermino et al. 2019, Tomé et al. 2019).

Parasito (No. spp.)	Hospedeiro	Vetor
Kinetoplastea		
Trypanosomatidae		
Trypanosoma (85)	crocodilianos, quelônios, lagartos e serpentes	sanguessugas e artrópodes
Leishmania (Sauroleishmania) (18)	lagartos e serpentes	flebotomíneos
Coccidia		
Hepatozoidae		
Hepatozoon (220)	crocodilianos, jabutis, tuataras, anfisbênias, lagartos e serpentes	diversos artrópodes
Haemogregarinidae		
Haemogregarina (46)	quelônios aquáticos	sanguessugas
Dactylosomatidae		
Dactylosoma *(1)	lagartos	?
Karyolysidae		
Hemolivia (4)	quelônios e lagartos	ácaros
Karyolysus (13)	lagartos	ácaros
Lankesterellidae		
Lankesterella (4)	lagartos	?
Lainsonia (2)	lagartos	mosquitos
Schellackia (10)	lagartos	mosquitos e ácaros
Hematozoa		

Cont. Tabela 1

Parasito (No. spp.)	Hospedeiro	Vetor
Plasmodiidae		
Plasmodium (107)	lagartos e serpentes	mosquitos e flebotomíneos
Saurocytozoon (2)	lagartos	mosquitos
Haemoproteidae		
Haemocystidium (28)	quelônios, lagartos e serpentes	tabanídeos
Garniidae		
Garnia (11)	lagartos	?
Progarnia (1)	crocodilianos	?
Fallisia (11)	lagartos	?
Haemohormidiidae		
*Sauroplasma**(3)	quelônios, lagartos e serpentes	?
*Haemohormidium**(1)	quelônios	?

*Espécies desses gêneros nesses hospedeiros foram consideradas de natureza incerta (Barta 1991, Telford 2009).

No caso dos tripanosomatídeos (Kinetoplastea, Trypanosomatidae), esses são parasitos unicelulares caracterizados principalmente pela presença de um flagelo único e de uma organela chamada cinetoplasto, que é formada por uma grande rede de DNA circular (kDNA) e está localizada na base do flagelo (Stevens et al. 2001, Simpson et al. 2006). A reprodução é assexuada por fissão binária com alternância das formas celulares (pleomorfismo) durante o ciclo de vida, as quais podem variar desde estágios de desenvolvimento intracelulares nos tecidos do hospedeiro (amastigotas), até formas extracelulares flageladas com membranas ondulantes (tripomastigotas) observadas no plasma sanguíneo dos vertebrados (O'Donoghue 2017). Os gêneros *Trypanosoma* Gruby, 1843 e *Leishmania* Ross, 1903 são os únicos flagelados heteroxenos dessa família com representantes infectando répteis (Tabela 1) e outros vertebrados (Telleria e Tibayrenc 2017). Dentre esses dois, *Trypanosoma* se sobressai pela grande

diversidade genética e morfológica de suas espécies em lagartos e serpentes (Viola et al. 2008, 2009, Telford 2009, Fermino et al. 2019).

Com os avanços das ações antrópicas sobre os ambientes naturais e em face a uma eminente crise climática, há uma crescente preocupação sobre os possíveis impactos da perda em massa das espécies (Ceballos et al. 2017). Mas, apesar de serem componentes essenciais da biodiversidade, os parasitos são inconspícuos para a maioria dos esforços conservacionistas (Thompson et al. 2018, Milotic et al. 2020). No entanto, são extremante vulneráveis à extinção e frequentemente correm maiores riscos de desaparecem do que seus hospedeiros (Dunn et al. 2009, Thompson et al. 2018, Milotic et al. 2020). Isso se deve ao fato de estes poderem ser extintos em decorrência da extinção dos seus hospedeiros (co-extinção) ou através do declínio da população hospedeira (Milotic et al. 2020). Além disso, assim como na recente pandemia causada pelo vírus Sars-CoV-2 (novo coronavírus), alguns patógenos podem se beneficiar com as atividades humanas e seus efeitos, uma vez que proporcionam a esses organismos oportunidades de propagação e o estabelecimento em novos hospedeiros (Jones et al. 2008, Cizauskas et al. 2017, Zohdy et al. 2019). Dessa forma, alterações na dinâmica da fauna parasitária produzem consequências profundas sobre a saúde de populações humanas e silvestres (Dobson et al. 2008, Corlett et al. 2020, FAO 2020).

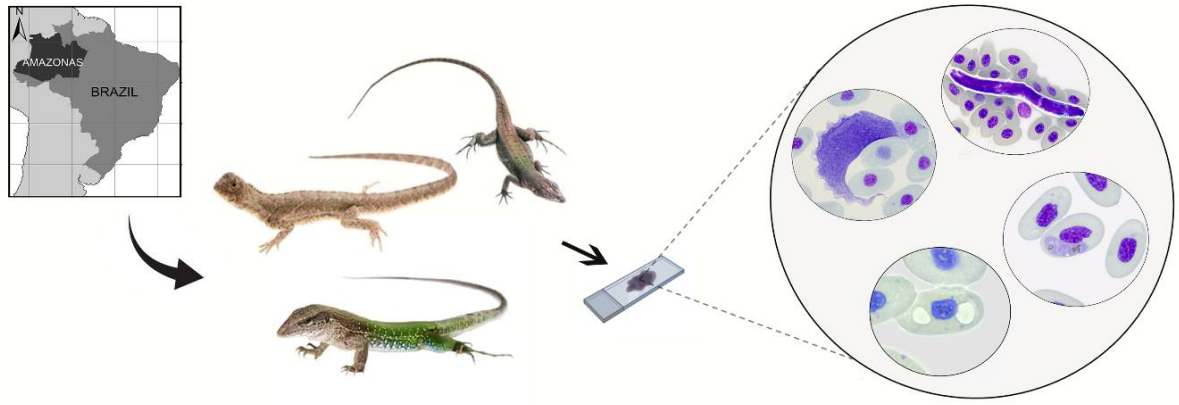
Nesse aspecto, a Floresta Amazônica, a qual concentra grande parte da biodiversidade do planeta, tem sido irreversivelmente destruída e modificada no Brasil sob o amparo de uma legislação ambiental e governos que incentivam a exploração de recursos e o desmatamento (Novaes e Souza 2013, Magnusson et al. 2018). Apenas no ano de 2019 houve um aumento de quase 34% (10.129 km²) em relação à taxa de desmatamento de 2018 na Amazônia brasileira (INPE 2020). Uma condição bastante dramática, tendo em vista que nesse bioma há constantemente descrições de espécies novas de hospedeiros reptilianos, como os lagartos, cujos hemoparasitos ainda são pobremente conhecidos (Costa e Bérnils 2018, Ribeiro-Júnior et

al. 2020). De fato, há registros de hemoparasitos em somente 10% (n = 16/152) das espécies de lagartos que ocorrem na Amazônia brasileira (Lainson 2012, Costa e Bérnils 2018).

Apesar dessa quantidade relativamente baixa de animais amostrados, os estudos pioneiros conduzidos pelo Dr. Ralph Lainson, principalmente entre os anos de 1966 e 1992, mostraram a existência de uma rica fauna de hemoparasitos nesses hospedeiros, sugerindo assim que a região amazônica no Brasil detém um potencial elevado para novas espécies destes parasitos (Lainson 2012). Entre os hemoparasitos registrados nesses levantamentos, foram 27 espécies do filo Apicomplexa e duas espécies de tripanosomas (para mais detalhes ver Tabela 1 do Capítulo 1). Porém, a maioria dessas foram detectadas em lagartos oriundos da Amazônia Oriental e, em sua maioria, descritas apenas com base na taxonomia tradicional, sem o uso de ferramentas moleculares. Isso pode levar a um desconhecimento dos padrões de distribuição e identidade das espécies de parasitos que ocorrem no Brasil, além de refletir sobre uma baixa compreensão da interação desses organismos com seus hospedeiros lagartos (Heath et al. 2008, Morand 2018).

Nesse contexto, a presente tese investigou a diversidade de hemoparasitos e caracterizou, através de dados morfológicos e moleculares, a composição desta comunidade em hospedeiros lagartos da Amazônia Central. Os resultados alcançados encontram-se organizados em três capítulos:

- **Capítulo 1** trata da prevalência e diversidade dos hemoparasitos encontrados nos largartos da região da Amazônia Central;
- **Capítulo 2** apresenta a redescrição da hemogregarina *Hepatozoon ameivae* (Carini e Rudolph, 1912) do lagarto *Ameiva ameiva*;
- **Capítulo 3** aborda as relações filogenéticas dos tripanosomas que parasitam *Uranoscodon superciliosus* (Linnaeus, 1758).



CAPÍTULO 1

Amanda M. Picelli, Adriane C. Ramires, Gabriel S. Masseli, Felipe A. C. Pessoa, Lucio A. Viana e Igor L. Kaefer. **Under the light: high prevalence of haemoparasites in lizards (Reptilia: Squamata) from Central Amazonia revealed by microscopy**. Manuscrito publicado no periódico Anais da Academia Brasileira de Ciências em 20 de julho de 2020. **DOI 10.1590/0001-3765202020200428**.

Under the light: high prevalence of haemoparasites in lizards (Reptilia: Squamata) from Central Amazonia revealed by microscopy

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Abstract: Blood samples from 330 lizards of 19 species were collected to investigate the occurrence of haemoparasites. Samplings were performed in areas of upland (*terra-firme*) forest adjacent to Manaus municipality, Amazonas, Brazil. Blood parasites were detected in 220 (66%) lizards of 12 species and comprised four major groups: Apicomplexa (including haemogregarines, piroplasms, and haemosporidians), trypanosomatids, microfilarid nematodes and viral or bacterial organisms. Order Haemosporida had the highest prevalence, with 118 (35%) animals from 11 species. For lizard species, *Uranoscodon superciliosus* was the most parasitised host, with 103 (87%; n = 118) positive individuals. This species also presented the highest parasite diversity, with the occurrence of six taxa. Despite the difficulties attributed by many authors regarding the use of morphological characters for taxonomic resolution of haemoparasites, our low-cost approach using light microscopy recorded a high prevalence and diversity of blood parasite taxa in a relatively small number of host species. This report is the first survey of haemoparasites in lizards in the study region. It revealed a high diversity of lizard haemoparasites and highlights the need to understand their impacts on hosts.

INTRODUCTION

The protozoologist Dr. Ralph Lainson (1992) two decades ago in his work on neglected parasites in the Amazonia basin quoted a phrase from P.C.C. Garnham, his former advisor: "There is a serious danger that malarial parasites become extinct." Since that time, very few efforts have been made to contain the threats to the diversity of these parasites and other organisms (Ferrante & Fearnside 2019). In fact, these threats have been aggravated by increased habitat destruction in recent years, particularly in tropical regions (INPE 2019). Extinction, alteration in the abundance or introduction of parasites can have profound impacts on the health of a large number of free-living species (Dobson et al. 2008), because parasites are ecologically involved in important mechanisms that regulate wildlife populations and structure communities (Tompkins & Begon 1999, Thomas et al. 2000). Moreover, they may influence their host biological processes, such as sexual selection (Ehman & Scott 2002, Megía-Palma et al. 2018), predation and competition dynamics (Schall 1992, Garcia-Longoria et al. 2015), as well as speciation and extinction processes (Anderson & May 1978, Poulin 1999, Prenter et al. 2004).

Reptiles are hosts for a wide variety of parasites, especially for diverse groups that parasitise blood cells (Davies & Johnston 2000, Telford 2009). These blood parasites may be intra- or extracellular organisms that range from protozoan kinetoplastids (Killick-Kendrick et al. 1986, Telford 1995) and apicomplexan parasites (Levine 1988, O'Donoghue 2017), to microfilarid nematodes (Thoisy et al. 2000, Halla et al. 2014) as well as viral and bacterial inclusions (Telford 2009). Except for the last two pathogens, whose transmission is not yet clear, the other three parasitic taxa share a common feature by using a range of haematophagous invertebrates as the main vectors for transmission between vertebrate hosts (Smallridge & Paperna 1997, Viana et al. 2012, Van As et al. 2015, Fermino et al. 2019). Furthermore, the haemoprotozoans of Phylum Apicomplexa Levine, 1970 are apparently the most studied of all

and also represent the taxon with the largest number of species parasitising reptiles (Levine 1988). Only in lizards (Squamata: Sauria), approximately 14 genera were recorded (O'Donoghue 2017); haemogregarines and haemosporidians are the most frequently identified groups (Smith 1996, Perkins 2014).

Although Brazil is a megadiverse country and has the third richest reptilian fauna in the world (Costa & Bérnils 2018), approximately 795 species, knowledge about haemoparasite diversity in these hosts consists of mainly a few concentrated studies in the eastern Amazon region (Lainson 1992, 2012). These studies recorded a rich haematozoan fauna in lizards and also suggest that the Amazon biome has a great potential for the discovery of new haemoparasitic species in these vertebrates, as 29 (80%) of the 36 known protozoan species in the country occur in this region (Table I). However, these records are limited to a total of 20 lizard species (Table I), which represent 7% (n = 276) of the described Brazilian lizard fauna and 10% (n = 16/152) for the Amazon region (Costa & Bérnils 2018). This small number is probably due to the difficulties in collecting these hosts and also the lack of specialists interested in working with haemoparasites from herpetofauna.

Light microscopy is an important tool for diagnosing infections that has crossed centuries and generations of scientists, still being the fastest and most accessible technique for searching parasites (Halla et al. 2014). This is especially true for studies adopting horizontal approaches that aim to estimate parasitism in poorly known groups. In this sense, we sought to investigate using light microscopy the presence and diversity of haemoparasites in lizards from Central Amazonia.

MATERIALS AND METHODS

STUDY AREA

The study was conducted in four upland (*terra-firme*) forest sites in Brazilian Central Amazonia, all located in the State of Amazonas, Brazil (Figure 1). The first study area was the Federal University of Amazonas forest fragment campus (UFAM; 3º4'34"S, 59º57'30"W), located in the eastern part of the city of Manaus. The three remaining study areas were located, respectively, 38 km (UFAM Experimental Farm; 2°38'57.6"S, 60°3'11"W), 80 km (Biological Dynamics of Forest Fragments Project [BDFFP]; 2°25'S, 59°50'W), and 160 km (Agrovila Rio Pardo; 1°48'S, 60°19' W) north of Manaus. These sampling regions present a mean annual temperature of approximately 26°C with relative air humidity over 80% (Araujo et al. 2002). The yearly precipitation is over 2,000 mm and mostly concentrated in a rainy season that usually occurs from December to May (Marques-Filho et al. 1981). The vegetation of the sampling sites is mainly composed of a mosaic of upland Amazonian rainforest, which varies from primary and secondary forests to open areas. The average elevation is 40–160 m above sea level (Laurance et al. 2011). Some of these landscapes are relatively undisturbed (Deichmann et al. 2010, Rojas-Ahumada et al. 2012), but most exhibit anthropogenic alterations (Rocha et al. 2004, Ramos et al. 2014).

LIZARD AND BLOOD SAMPLING

A total of 330 lizards from 19 species distributed in 17 genera and 10 families were sampled between 2016 and 2019 (Table II). Animals were captured using several methods, such as active search (Doan 2003) and traps, i.e., pitfalls with drift-fences (Jenkins et al. 2003), funnels made out of PVC pipes (Abrahão et al. 2019) and live-traps (Vieira et al. 2015). Lizards were identified through specialised literature (Ávila-Pires 1995, Vitt et al. 2008), and taxonomic

nomenclature was adopted following Costa & Bérnils (2018). The blood samples were obtained by tail or cardiac puncture using a sterile insulin syringe (Samour et al. 1984). A portion of collected blood was used to make smears, which were fixed with absolute methanol and stained with 10% Giemsa. The other portion was applied to a filter paper for molecular analyses. Lizards were released within 24 h of capture, but in the case of cardiac puncture, the blood was collected after euthanasia (via injection of 2% lidocaine). Specimens were preserved in 10% formalin and deposited in the Zoological Collections of the National Institute of Amazonian Research (INPA) and UFAM in Manaus, Brazil.

Lizard sampling and access to the genetic data were authorised by the Brazilian Ministry of the Environment (SISBIO n° 53851-4 and SISGEN AA6199D, respectively). All procedures were approved by the ethics committee on animal use from Universidade Federal do Amazonas (protocol number 012/2016).

MICROSCOPIC ANALYSES

Blood smears were examined for up to 20 min under a Leica DM4B microscope (Leica Microsystems, Heerbrugg, Switzerland) at × 400 and × 1000 total magnification. The slides with parasites were carefully examined and images were captured with an attached Leica DMC4500 digital camera and processed with LAS V4.8 (Leica Microsystems Suiza Limited 2015). Morphometric measurements were taken with this same system. However, they will not be presented here in this work, as they are part of ongoing taxonomic studies. Haematozoan parasites were taxonomically identified by comparing their morphologies to the descriptions from the guides of Telford (2009) and Lainson (2012), besides original description articles. Additionally, to confirm the identification of some haemosporidian species, we compared our material with that of the collection of Dr. Ralph Lainson, deposited at the Evandro Chagas Institute (IEC) in Belém, Brazil.

RESULTS

Haemoparasite infections were detected in 220 (66%) out of 330 lizards of 12 species distributed among seven families (Table II). Mixed infections occurred in 91 positive specimens. For sampling sites, BDFFP had 78% (n = 156/200) of the infected lizards, UFAM Experimental Farm had 68% (n = 13/19), Agrovila Rio Pardo had 47% (n = 50/105) and UFAM urban forest fragment had 16% (n = 1/6). Parasites were grouped into four major groups (Figure 2), with the following prevalence: (i) intracellular apicomplexan parasites at 173 (52%) individuals; (ii) trypanosomatids at 84 (25%); (iii) microfilarial worms at 38 (11%); (iv) unidentified viral or bacterial inclusions at 30 (9%).

Among the positive lizards, Tropiduridae and Teiidae were the families that showed the highest prevalence, with 86% (n = 112/130) and 66% (n = 90/135) positive animals, respectively. With regards to lizard species, *Uranoscodon superciliosus* Linnaeus, 1758 stood out for presenting a high prevalence, with 87% (n = 103/118) of infected individuals, and also because it was the species with the greatest diversity of parasites, with the occurrence of six different taxa: Haemohormidiidae, Plasmodiidae, Garniidae, Trypanosomatidae, microfilarial worms and unidentified inclusions.

Parasites of phylum Apicomplexa (Table III) were found in all infected lizard species; 14 species from five families were identified. Two morphotypes of the genus *Hepatozoon* (Hepatozoidae) were observed in 40 *Ameiva ameiva* Linnaeus, 1758 (55%; n = 72) and one was identified as *Hepatozoon ameivae* Carini & Rudolph, 1912 (Figure 2a). *H. ameivae* was recorded overlapping the nucleus of the parasitised cells, whereas the other morphotype caused lateral displacement of the nucleus to one end of the red blood cell (Figure 2b). Both parasites were restricted to erythrocytes. *Sauroplasma*-like (Haemohormidiidae) infections (Figure 2c) appeared in 14% (n = 49/330) of individuals from six lizard species (Table III). Notably, *U.*

superciliosus had the highest number of parasite occurrences, with 32 (27%; n = 118) positive specimens.

Haemosporidian parasites presented the highest prevalence, with 35% (n = 118/330) animals infected and, except for *Alopoglossus angulatus* Linnaeus, 1758, all positive host species were parasitised by malaria. Based on blood stage morphology, 13 species from two families, Plasmodiidae and Garniidae, were identified (Table III; Figure 2d-j). It is important to note that despite some authors (e.g., Levine 1988, Telford 2009), here we recognise the family Garniidae as well as the genera *Garnia* and *Fallisia* as valid taxa diagnosed by absence of pigment and ultrastructural characteristics (Lainson et al. 1971, Boulard et al. 1987).

Plasmodium spp. (Figure 2d-g) were detected in 64 (19%; n = 330) lizards from nine species, with the highest number of positive specimens seen in *A. ameiva* (36%; n = 72). At least 11 morphotypes were visualised, and five *Plasmodium* species could be recognised (Table III). Gametocytes of *Saurocytozoon* cf. *tupinambi* Lainson & Shaw, 1969b were observed in leucocytes (Figure 2h) from five (20%; n = 25) *Tubinambis teguixin* Linnaeus, 1758. Non-pigmented malaria parasites from the genera *Fallisia* (Figure 2i) and *Garnia* (Figure 2j) were found in four (1%; n = 330) and 46 (14%; n = 330) lizards, respectively (Table III). Two *Fallisia* species were detected in *Plica umbra* Linnaeus, 1758, *Fallisia* cf. *simplex* Lainson et al., 1975 and *Fallisia* cf. *audaciosa* Lainson et al., 1975. In *Neusticurus bicarinatus* Linnaeus, 1758, we found *Fallisia* cf. *effusa* Lainson et al., 1974a. Parasites of the genus *Garnia* were mainly recorded in *U. superciliosus* (22%; n = 118). We also detected four unidentified morphotypes and four species of this genus (Table III).

Extracellular parasites of the family Trypanosomatidae (Table II) were found in 83 *U. superciliosus* (70%; n = 118) and one *P. umbra* (8%; n = 12): each tropidurid species had one *Trypanosoma* morphotype. The trypanosome of *U. superciliosus* had an elongated body and diffuse nucleus (Figure 2k), while the observed *P. umbra* had a rounded shape and compact

nucleus (Figure 2l). Microfilarial worms (Nematoda) occurred in five lizard species (Table II), with higher prevalence in *A. ameiva* with 37% (n = 27/72) positive specimens. These blood parasites exhibited highly variable sizes and shapes (Figure 2m-n) and were very similar to the genus *Piratuba*. However, accurate diagnoses of filarial worms is mainly based on morphological features of adult worms. Thus, identification of this group in the present study remains indeterminate.

The last of the four major groups, inclusions of uncertain nature (Figure 2o-p), were detected in erythrocytes of five lizard species and showed little morphological variation. They consisted of a large spherical shape with a rarely darker stained margin. These vacuoles resemble rickettsial parasites recorded for other reptilian hosts, although without ultrastructural study it was not possible to confirm this identification.

DISCUSSION

We observed a high prevalence of blood parasites among lizards from Central Amazonia: More than half of the sampled individuals and species were infected. We also demonstrated that lizards are the hosts for a wide variety of haemoparasites. Indeed, we observed great parasite richness in a small number of host species and in a limited sampling area. This finding reinforces that the neotropical region holds a rich haemoparasite fauna, as shown by studies conducted in other localities across the Amazon Basin (Renjifo et al. 1952, Telford 1970, 1973, 1980, Ayala et al. 1973, Lainson 1992, Thoisy et al. 2000, Matta et al. 2018). Furthermore, it is important to note that we sampled lizard species with diversified microhabitat use, ranging from terrestrial (e.g., *A. ameiva*), semi-aquatic (e.g., *Neusticurus bicarinatus)*, scansorial (e.g., *P. umbra*) to arboreal (e.g., *U. superciliosus*) (Vitt et al. 2008). This environmental diversity may imply determinant characteristics for the composition of the haemoparasite assemblages found

in these lizards because different species of vectors, including mosquitoes, sandflies and ticks, are likely distributed along the gradient occupied by these hosts.

Parasite and host checklists are crucial in expanding our knowledge on species distribution. Nonetheless, surveys and descriptive studies of haemoparasite species on lizards conducted in the Amazonian biome have decreased considerably in recent years. In Brazil, it has been 10 years since a haematozoan species from a lizard has been described (Lainson et al. 2010). Most access to this haemoparasitic diversity in the Neotropics departs from the classical approach by using light microscopy to investigate prevalence and parasite identity. The exclusive use of morphological attributes for the diagnosis of species has been strongly criticised as unreliable because molecular tools have advanced in solving taxonomic problems (Pineda-Catalan et al. 2013, Perkins 2014). In fact, molecular biology constitutes a modern and acurate tool in parasitology, but its use still faces financial and technical limitations —i.e. difficulties in developing protocols and molecular markers—, especially in megadiverse and developing countries such as Brazil (Perkins et al. 2011, Morand 2018). Nevertheless, in comprehensive multi-species approaches like ours, whose main objective is not to solve systematic and phylogenetic questions, observations of blood smears under a microscope still prove to be a feasible method to access the prevalence and parasite diversity hidden in these hosts, despite some taxonomic limitations.

Among the 12 infected lizard species, haemoparasites were recorded for the first time in three of them: *Arthrosaura reticulata* O'Shaughnessy, 1881, *Norops planiceps* Troschel, 1848 and *A. angulatus.* However, we did not find blood parasites in nine lizard taxa (Table II), even though parasites have already recorded in some of these hosts in other localities (Table I). This discrepancy may simply reflect unequal sampling efforts. The methods we used for capture were diversified and effective for certain hosts groups, such as Teiidae and Tropiduridae, but are limited for many lizard species, mainly those that access subterranean microhabitats (Faria

et al. 2019). Indeed, Teiidae, Tropiduridae and a lizard species, *U. superciliosus,* were the hosts with highest parasite prevalence. However, with the myriad known problems in obtaining samples (Perkins et al. 2011)—financial, technical and logistical difficulties in accessing remote areas—and the need to move forward on other parasitology research fronts, such as vectors and life cycle, landscape and epizootiology studies, those abundant taxa may be an interesting choice to be included in ecological parasitic systems as model organisms. Additionally, for many reasons lizards are considered model organisms (Huey et al. 1983, Camargo et al. 2010), as they respond very well when testing ecological and evolutionary hypotheses (Schall 1996).

Most of the parasites found in this study belong to phylum Apicomplexa. Indeed, all host species had some representative of this group. One of them was the genus *Hepatozoon*, relatively common parasite in reptiles and, despite the great diversity of lizards sampled in this study, was found exclusively infecting *A. ameiva*. *Hepatozoon ameivae* was described by Carini & Rudolph 1912 in *A. ameiva* in the State of Minas Gerais and later recorded in the municipality of São João da Barra, State of Rio de Janeiro, both in southeastern Brazil (Carini & Rudolph 1912, Sabagh et al. 2015). Lainson et al. (2003) also probably recorded *H. ameivae* in lizards from the municipality of Capanema, State of Pará, northern Brazil. This parasite has an outstanding feature: its gametocytes are found in the erythrocyte nucleus, a relatively uncommon developmental pattern in the Apicomplexa that can lead to severe distortion and even lysis of the infected cell nucleus (Telford 2009). It is important to note that *H. ameivae* found here was morphologically and molecularly characterized, and the analysis of its phylogenetic position clearly showed that this parasite belongs to the genus *Hepatozoon* (Picelli et al., unpublished data).

Our results showed a relatively low prevalence for *Sauroplasma*-like and we thought that positive lizard species were not previously recorded for piroplasms (Table III). *Sauroplasma* infections are common in lizards, even though there are only three species described for these

hosts: *Sauroplasma thomasi* du Toit, 1938, *Sauroplasma zonurum* Pienaar, 1962, and *Sauroplasma boreale* Svahn, 1976 (Telford 2009, Halla et al. 2014). In Brazil, these parasites were recently recorded in the freshwater turtle *Podocnemis expansa* (Picelli et al. 2016). Morphologically, they are small (2.5-4 μm) vacuole-shaped intraerythrocytic parasites with chromatin granules associated (Halla et al. 2014, Picelli et al. 2016). These morphological features mislead many authors to identify *Sauroplasma*-like inclusions as *Chelonoplasma*, *Nuttalia* or *Pirhemocyton* (Bardi et al 2019). They can also be overlooked as artefacts or bacterial and viral infections (Telford 2009). Parasitologists always pay attention to this conflicting taxonomic situation, but no molecular data is yet known for this genus.

Haemosporidian were the most predominant and richest taxon detected on lizards, mainly from Plasmodiidae parasites. It is well known that malaria parasites are widely distributed geographically, ubiquitous for most lizard families and are morphologically diverse, with over 100 species reported to infect reptiles (Schall 1996, Telford 2009). In the Eastern Brazilian Amazonia, 21 species of lizard malaria are known, and 13 (61%) of them were found in our research. For some of these (*Garnia* cf. *uranoscodoni* Lainson et al. 1975, *Garnia* cf. *multiformes* Lainson et al. 1975, *Garnia* cf. *utingensis* Lainson et al. 1971, *Fallisia* cf. *audaciosa* and *F.* cf. *effusa*), this finding is the first occurrence record away from their type localities. Recently, Matta et al. (2018) reported the presence of *Plasmodium kentropyxi* Lainson et al., 2001 and *Plasmodium carmelinoi* Lainson et al., 2010 in Teiidae lizards, at a low prevalence, in the Colombia Orinoco-Amazon basin. The difference between our findings is that here *P.* cf. *kentropyxi* was found at a relatively high prevalence only in its type host, *Kentropyx calcarata* Spix, 1825. Another interesting species seen in our study is *Plasmodium* cf. *tropiduri* Aragão & Neiva, 1909. It was the only haemosporidian species found in two different host species: *K. calcarata* and *Copeoglossum nigropunctatum* Spix, 1825. This haemoparasite was one of the world's first reptilian malaria parasites, described by Aragão &

Neiva (1909) in the lizard *Tropidurus torquatus* Wied-Neuwied, 1820. Since then, it was observed across many lizard families and can be considered one of the most widespread saurian malaria species in South America (Telford 2009). In fact, most of the haemosporidians present here were previously reported in other Amazonian locations (Telford 2009, Matta et al. 2018), evidence that malaria species rediscovered here may be widely distributed throughout the biome. Phylogenetic and phylogeographic studies that involve samples from different Amazonian localities may provide insights regarding the diversification and evolution of this group.

Parasites of the genus *Trypanosoma* were restricted to two lizard species and at a low prevalence when compared to protozoans of the phylum Apicomplexa, which parasitised more than half of the captured lizards. However, these flagellates were found in several *U. superciliosus.* Both *P. umbra* and *U. superciliosus* already had trypanosomatids recorded by Walliker (1965) and Lainson et al. (1975), respectively. The first author provided a poor morphological description of *Trypanosoma superciliosae* Walliker, 1965 without reporting their prevalence in *U. superciliosus* from the municipality of Codajás, Amazonas State. Interestingly, Lainson et al. (1975) mentioned that they searched, in Pará state, for this parasite in a large number of *U. superciliosus* individuals but were unsuccessful. Nevertheless, the same authors described *Trypanosoma plicae* Lainson et al., 1975 in *P. umbra.* Besides these species, there is only one other species described for this genus on Brazilian lizards: *Trypanosoma rudolphi,* recorded just once in *C. nigropunctatum* (Carini & Rudolph 1912). This low species richness is probably due to the lack of studies conducted on these parasites in Brazilian lizards. Indeed, trypanosome species have been reported worldwide in lizards more than in any other reptilian group (Fermino et al. 2019). Although trypanosomes have a unique stage of their life cycle by circulating in the blood of reptiles, trypomastigote forms exhibit high polymorphism and

plasticity (Spodareva et al. 2018). Therefore, it is not possible to confirm that we found the same species described for those hosts, even with some morphological similarities.

Our data revealed a low prevalence of microfilaria, which are larval stages from nematodes of the superfamily Filaroidea. These vector-borne parasite larvae are commonly found in the peripheral blood of vertebrates and here, except for *U. superciliosu*s, all lizard species that we found positive for these parasites already had records for adult worms from many Onchocercidae species in other locations (Ávila & Silva 2010, Macedo et al. 2017). For *U. superciliosu*s, the occurrence of microfilariae has been vaguely reported in eastern Amazonia and these studies did not provide morphological characterisation of these nematodes (Lainson et al. 1975). In reptiles, Oswaldofilariinae, a onchocercid subfamily, stands out as the main filarid group that parasitise these hosts. Some genera that infected lizards include *Oswaldofilaria*, *Piratuboides* and *Piratuba* (Pereira et al. 2010). Adult worms from this taxon are recognised by the long distance between the head and vulva, and a series of other characters are used for species identification (Pereira et al. 2010). Given that there is scarce information on their larval morphology and we did not collect data related to the adult phase of these helminths, we are unable to advance the identification of this group in this study.

One of the most intriguing findings of our work was the intraerythrocytic inclusions of an uncertain nature. These vacuole-like inclusions appeared at a low prevalence and resembled some bacterial infections, caused by *Rickettsia*, and also to the viruses of the Lizard Erythrocytic Virus (LEV) group, such as *Pirhemocyton* (Telford & Jacobson 1993, Telford 2009). In fact, pirhemocytonosis are commonly found in lizards, mainly green iguanas (*Iguana iguana*), as white square vacuole-like cytoplasmic inclusions (Harr et al. 2001, Halla et al. 2014). Viral or bacterial infections have been reported in many amphibians and reptiles across the world and some of them can cause diseases in these hosts (Davies & Johnston 2000, Ariel 2011). However, these organisms are poorly studied and their diagnosis can be complex because

it involves several approaches, including electron microscopy, serological surveys and molecular tools (Ariel 2011). Unfortunately, our knowledge about these inclusions and its occurrence throughout the Amazonian biome is very limited, and therefore we were unable to deepen in their identification.

Parasites commonly co-occur in the same host (Vaumourin et al. 2015, Galen et al. 2019), and we detected a high prevalence of this interaction. Indeed, we observed the co-occurrence of very distinct groups of haemoparasites in terms of life cycles, evolutionary history and in the exploitation of their hosts. The presence of an infracommunity in a host may be the result of a random occurrence of these parasites or a consequence modulated by the existence of a previous infection (Vaumourin et al. 2015, Hernandes-Córdoba & Braga 2019). Meanwhile, there are several challenges to understanding these interactions. Most previous studies ignored them, and only recently has the importance of such multiparasitism been recognised (Vaumourin et al. 2015). For lizards, parasitic ecological systems are frequently based on the one-on-one interactions and focus mainly on ecology of coccidian or malarial parasitism (Schall 1996, Amo et al. 2005, Hernandes-Córdoba & Braga 2019, Megía-Palma et al. 2020). From our perspective, there is still a long and curious path to explore until we can better understand haemoparasites and their lizard hosts.

This study is the first multi-species haemoparasite survey performed on lizard assemblages in Central Amazonia. We also present the most complete and updated list of haematozoan species described for these hosts in this region. Furthermore, our low-cost investigation using light microscopy demonstrates that Central Amazonia has a high prevalence and significant diversity with potential for new records of haemoparasites, especially malaria species. These findings might support future taxonomic characterisation of the parasites reported here, as well as further studies in parasite ecology and evolution. At last, our work emphasizes the importance

of screening parasites in wildlife animals to allow a better understanding of the biodiversity of this biome.

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AUTHOR CONTRIBUTIONS

AMP, LAV, FAC and ILK conceived and designed the study. AMP, ACR and GSM performed the fieldwork. AMP and ACR processed the data and performed the microscopic analysis. AMP interpreted the results and worked on the manuscript. LAV, FAC and ILK

contributed to critical reading of the manuscript and supervised the findings of this work. All authors took part on the preparation, revised and approved the final version of the manuscript.

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TABLE I

Checklist of haematozoan parasite species occurring in Brazilian lizards.

Parasite species	Host species	Prevalence	Locality	Author/Year
Coccidia				
Hepatozoidae				
Hepatozoon ameivae	*Ameiva ameiva*	Not inf.	Minas Gerais	Carini & Rudolph, 1912*
		Not inf.	Capanema/PA	Lainson et al., 2003
		1 of 10	São João da Barra/RJ	Sabagh et al., 2015
Hepatozoon cnemidophori	*Cnemidophorus* sp.	1 of 11	Porto Nacional/TO	Carini, 1941a*
Hepatozoon missoni	*Tupinambis teguixin*	Not inf.	São Paulo/SP	Carini, 1909*
Hepatozoon sinimbui	*Iguana iguana*	1 of 1	Porto Nacional/TO	Carini, 1942*
Hepatozoon tupinambis	*Tupinambis teguixin*	2 of 3	Ilha do Governador/RJ	Laveran & Salimbeni, 1909*
		Not inf.	São Paulo/SP	Carini, 1909
		3 of 10	Ilha Solteira/SP	Pessôa et al., 1974
		3 of 7	Fortaleza/CE	Pessôa et al., 1974
Karyolysidae				
Hemolivia stellata	*Ameiva ameiva*	3 of 20	Capanema/PA	Lainson et al. 2007
Lankesterellidae				
Lainsonia iguanae	*Iguana iguana*	3	Exu/PE	Landau, 1973*
		1	Belém/PA	Landau, 1973*
Lainsonia legeri	*Tupinambis teguixin*	Not inf.	Belém/PA	Landau et al., 1974*
Schellackia landaue	*Polychrus marmoratus*	17 of 148	Capanema/PA	Lainson et al., 1976*

Table I (continued)

Parasite species	Host species	Prevalence	Locality	Author/Year
Haemosporida				
Garniidae				
Fallisia audaciosa	*Plica umbra*	4 of 235	Belém/PA	Lainson et al., 1975*
Fallisia effusa	*Neusticurus bicarinatus*	26 of 32	Capanema/PA	Lainson et al., 1974a*
Fallisia modesta	*Tropidurus oreadicus*	1 of 1	Belém/PA	Lainson et al., 1974a*
Fallisia simplex	*Plica umbra*	20 of 235	Belém/PA	Lainson et al., 1975*
Garnia gonatodi	*Gonatodes humeralis*	26 of 52	Belém/PA	Lainson et al., 1971
Garnia karyolytica	*Thecadactylus rapicauda*	1 of 4	Novo Repartimento/PA	Lainson & Naiff, 1999*
Garnia morula	*Copeoglossum nigrupunctatum*	18 of 63	Belém/PA	Lainson et al., 1974b
Garnia multiformis	*Plica umbra*	41 of 235	Belém/PA	Lainson et al., 1975*
Garnia telfordi	*Ameiva ameiva*	1 of 1	Nova Xavantina/MT	Lainson et al., 1971*
Garnia uranoscodoni	*Uranoscodon superciliosus*	46 of 167	Belém/PA	Lainson et al., 1975*
Garnia utingensis	*Dactyloa punctata*	2 of 6	Belém/PA	Lainson et al., 1971*
Haemoproteidae				
*Haemocystidium catenatus***	*Enyalius catenatus*	1 of 1	Juquitiba/SP	Pessoa & Cavalheiro, 1970*
Plasmodiidae				
Plasmodium carmelinoi	*Ameiva ameiva*	12 of 206	Pará	Lainson et al., 2010*
Plasmodium cnemidophori	*Cnemidophorus* sp.	2 of 4	Porto Nacional/TO	Carini, 1941b*
	Ameiva ameiva	29 of 66	Belém/PA	Lainson & Shaw, 1969a
Plasmodium dipoglossi	*Diploglossus fasciatus*	2 of 2	Xerém/RJ	Aragão & Neiva, 1909*

Table I (continued)

Parasite species	Host species	Prevalence	Locality	Author/Year
Plasmodium dipoglossi	*Copeoglossum nigrupunctatum*	6 of 20	Belém/PA	Lainson & Shaw, 1969a
Plasmodium kentropyxi	*Kentropyx calcarata*	31 of 39	Outeiro/PA	Lainson et al., 2001*
		36 of 55	Capanema/PA	Lainson et al., 2001*
		35 of 57	Belém/PA	Lainson et al., 2001*
Plasmodium minasense	*Copeoglossum nigrupunctatum*	Not inf.	Minas Gerais	Carini & Rudolph, 1912*
	Polychrus acutirostris	1 of 1	Mateus Leme/MG	Cordeiro, 1977
	Iguana iguana	Not inf.	Salvador/BA	Telford, 1979
	Tupinambis teguixin	5 of 6	Belém/PA	Landau et al., 1973
Plasmodium neusticuri	*Neusticurus bicarinatus*	134 of 465	Belém/PA	Lainson & Paperna, 1996*
Plasmodium rhadinurum	*Iguana iguana*	Not inf.	Porto Nacional/TO	Carini, 1945
		3 of 3	Codajás/AM	Walliker, 1966
Plasmodium tropiduri	*Tropidurus torquatus*	1 of 1	Bicudos/MG	Aragão & Neiva, 1909*
		4 of 39	Jacobina/BA	Pessôa & Lopes, 1963
		10 of 51	Davinolândia/SP	Silva & Rodrigues, 1974
		2 of 31	Pinhal/SP	Silva & Rodrigues, 1974
		6 of 49	Águas da Prata/SP	Silva & Rodrigues, 1974
		1 of 1	Porto Nacional/TO	Carini, 1941c
		10 of 89	Belo Horizonte/MG	Hernandes-Córdoba & Braga, 2019
		50 of 87	Ouro Preto/MG	Hernandes-Córdoba & Braga, 2019

Table I (continued)

Parasite species	Host species	Prevalence	Locality	Author/Year
Plasmodium tropiduri	*Tropidurus itambere*	3 of 12	Ibitipoca/MG	Nunes et al., 2010
	Copeoglossum nigrupunctatum	8 of 20	Belém/PA	Lainson & Shaw, 1969a
	Kentropyx calcarata	20 of 151	Pará	Lainson et al., 2001
Plasmodium vacuolatum	*Plica umbra*	31 of 235	Belém/PA	Lainson et al., 1975*
Plasmodium vautieri	*Urostrophus vautieri*	1 of 1	São Paulo/SP	Pessôa & de Biasi, 1973*
Saurocytozoon mabui	*Copeoglossum nigrupunctatum*	2 of 31	Ananindeua/PA	Lainson et al., 1974b*
Saurocytozoon tupinambi	*Tupinambis teguixin*	5 of 6	Belém/PA	Lainson & Shaw, 1969b*
	Crocodilurus amazonicus	1 of 1	Bacarena/PA	Lainson et al., 1974b
Kinetoplastea				
Trypanosomatidae				
Trypanosoma plicae	*Plica umbra*	27 of 235	Belém/PA	Lainson et al., 1975*
Trypanosoma superciliosae	*Uranoscodon superciliosus*	Not inf.	Codajás/AM	Walliker, 1965*
Trypanosoma rudolphi	*Copeoglossum nigrupunctatum*	Not inf.	Minas Gerais	Carini & Rudolph, 1912*

***Species description studies.**
****Originally described as *Haemoproteus catenatus*.**

TABLE II

Prevalence of haemoparasites in lizards from Central Amazonia.

Species	No. examined	No. infected	No. (%) lizards infected			
			Apicomplexa parasites	Trypanosomes	Microfilarial worms	Viral or bacterial inclusions
Alopoglossidae						
Alopoglossus angulatus	1	1	1 (100)	—	—	—
Dactyloidae						
Dactyloa punctata	3	2	2 (66)	—	—	—
Norops fuscoauratus	1	—	—	—	—	—
Norops ortonii	1	—	—	—	—	—
Norops planiceps	9	2	2 (22)	—	1 (11)	—
Gekkonidae						
Hemidactylus mabouia	1	—	—	—	—	—
Gymnophthalmidae						
Arthrosaura reticulata	11	2	1 (9)	—	—	2 (18)
Cercosaura argulus	1	—	—	—	—	—
Loxopholis sp.	1	—	—	—	—	—
Loxopholis percarinatum	6	—	—	—	—	—
Neusticurus bicarinatus	3	2	2 (66)	—	—	—

Table II (continued)

Species	No. examined	No. infected	No. (%) lizards infected			
			Apicomplexa parasites	Trypanosomes	Microfilarial worms	Viral or bacterial inclusions
Iguanidae						
Iguana iguana	1	—	—	—	—	—
Mabuyidae						
Copeoglossum nigropunctatum	8	6	6 (75)	—	2 (25)	—
Phyllodactylidae						
Thecadactylus rapicauda	10	—	—	—	—	—
Sphaerodactylidae						
Gonatodes humeralis	8	3	3 (37)	—	—	—
Teiidae						
Ameiva ameiva	72	60	58 (80)	—	27 (37)	3 (4)
Cnemidophorus sp.	2	—	—	—	—	—
Kentropyx calcarata	36	24	23 (63)	—	2 (5)	—
Tupinambis teguixin	25	6	6 (24)	—	—	3 (12)
Tropiduridae						
Plica umbra	12	9	7 (58)	1 (8)	—	1 (8)

Table II (continued)

Species	No. examined	No. infected	No. (%) lizards infected			
			Apicomplexa parasites	**Trypanosomes**	**Microfilarial worms**	**Viral or bacterial inclusions**
Uranoscodon superciliosus	118	103	62 (52)	83(70)	6 (5)	21 (18)
Total	330	220	173 (52)	84 (25)	38 (11)	30 (9)

TABLE III

Infections of Apicomplexa parasites in 12 lizard species sampled in this study.

Host species (N)	Parasites	*n* infected (% infected)
Alopoglossidae		
Alopoglossus angulatus (1)	*Sauroplasma*-like	1 (100)
Dactyloidae		
Dactyloa punctata (3)	*Garnia* cf. *utingensis*	1 (33)
	Plasmodium sp.	1 (33)
Norops planiceps (9)	*Plasmodium* sp.	2 (22)
	Sauroplasma-like	1 (11)
Gymnophthalmidae		
Arthrosaura reticulata (11)	*Plasmodium* sp.	1 (9)
Neusticurus bicarinatus (3)	*Fallisia* cf. *effusa*	2 (66)
Mabuyidae		
Copeoglossum nigropunctatum (8)	*Plasmodium* sp.	2 (25)
	Plasmodium cf. *tropiduri*	3 (37)
	Sauroplasma-like	1 (12)
Sphaerodactylidae		
Gonatodes humeralis (8)	*Garnia* sp.	3 (37)
Teiidae		
Ameiva ameiva (72)	*Hepatozoon ameivae*	40 (55)
	Hepatozoon sp.	11 (15)
	Garnia cf. *telfordi*	10 (13)
	Garnia sp.	4 (5)
	Plasmodium cf. *carmelinoi*	4 (5)
	Plasmodium cf. *cnemidophori*	1 (1)
	Plasmodium sp.	21 (29)
	Sauroplasma-like	13 (18)
Kentropyx calcarata (36)	*Plasmodium* cf. *kentropyxi*	12 (33)
	Plasmodium cf. *tropiduri*	2 (5)
	Plasmodium sp.	6 (16)

Table III (continued)

Host species (N)	**Parasites**	***n* infected (% infected)**
	Sauroplasma sp.	13 (36)
Tupinambis teguixin (25)	*Plasmodium* sp.	1 (4)
	Saurocytozoon cf. *tupinambi*	5 (20)
Tropiduridae		
Plica umbra (12)	*Fallisia* cf. *audaciosa*	1 (8)
	Fallisia cf. *simplex*	1 (8)
	Garnia cf. *multiformes*	2 (16)
	Plasmodium cf. *vacuolatum*	4 (33)
Uranoscodon superciliosus (118)	*Garnia* cf. *uranoscodoni*	16 (13)
	Garnia sp.	10 (8)
	Plasmodium sp.	2 (1)
	Sauroplasma-like	32 (27)

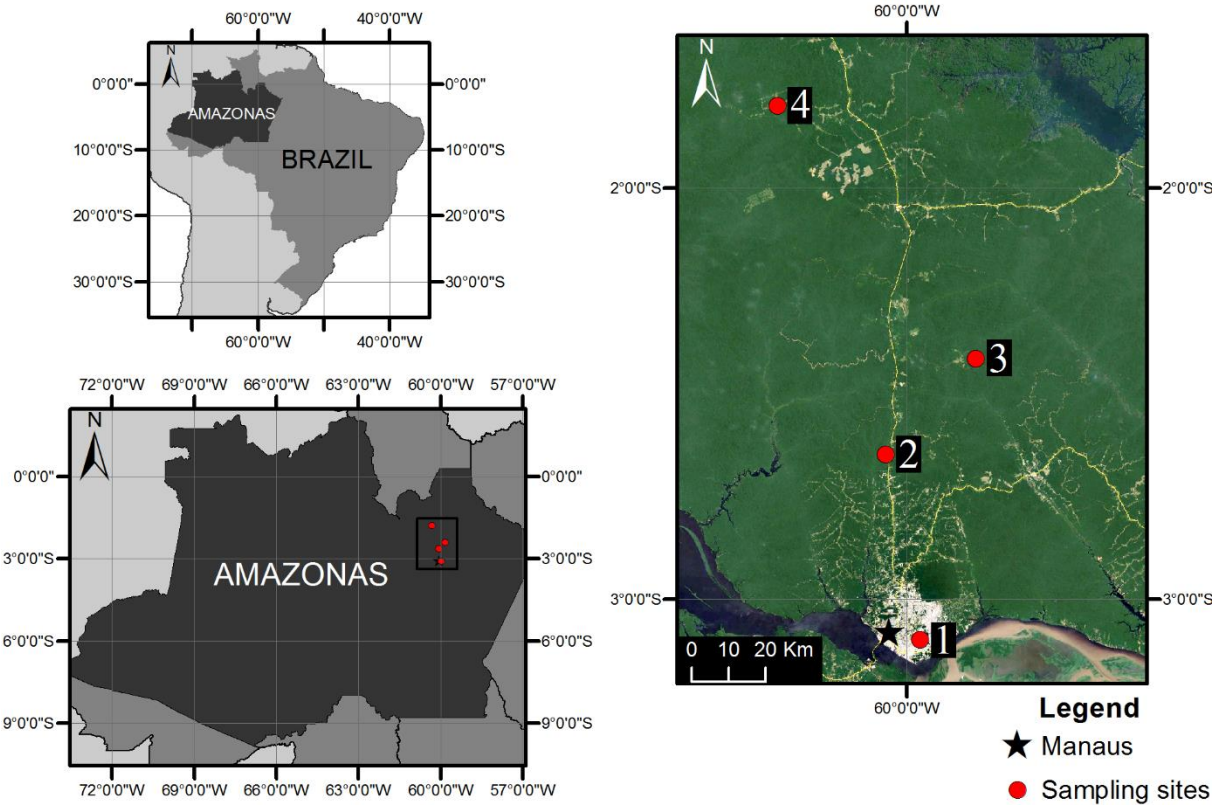


Figure 1. Sampling areas in Central Amazonia: (1) Campus of the Federal University of Amazonas (UFAM); (2) UFAM Experimental Farm; (3) Biological Dynamics of Forest Fragments Project (BDFFP) Reserve; (4) Agrovila Rio Pardo.

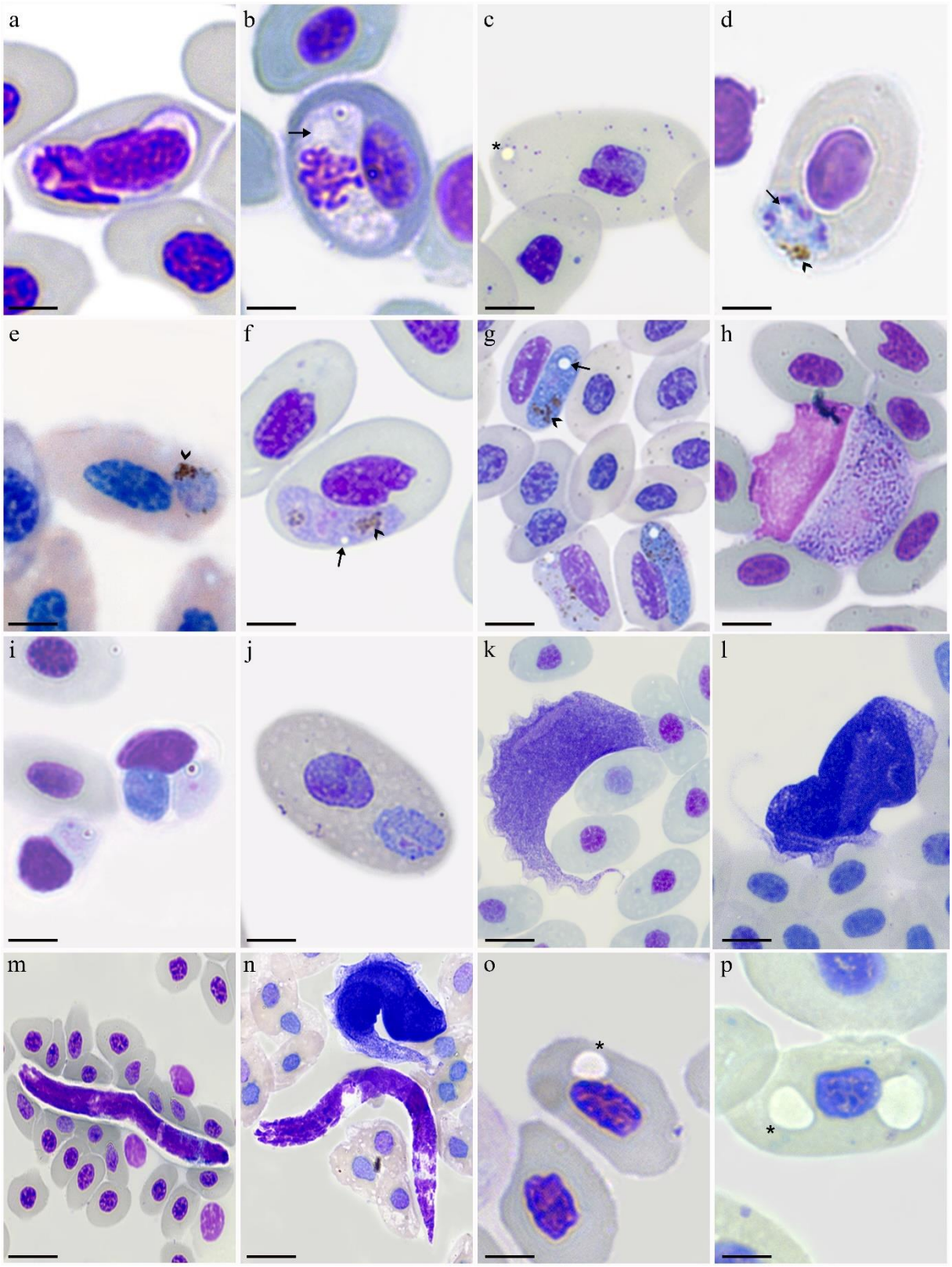
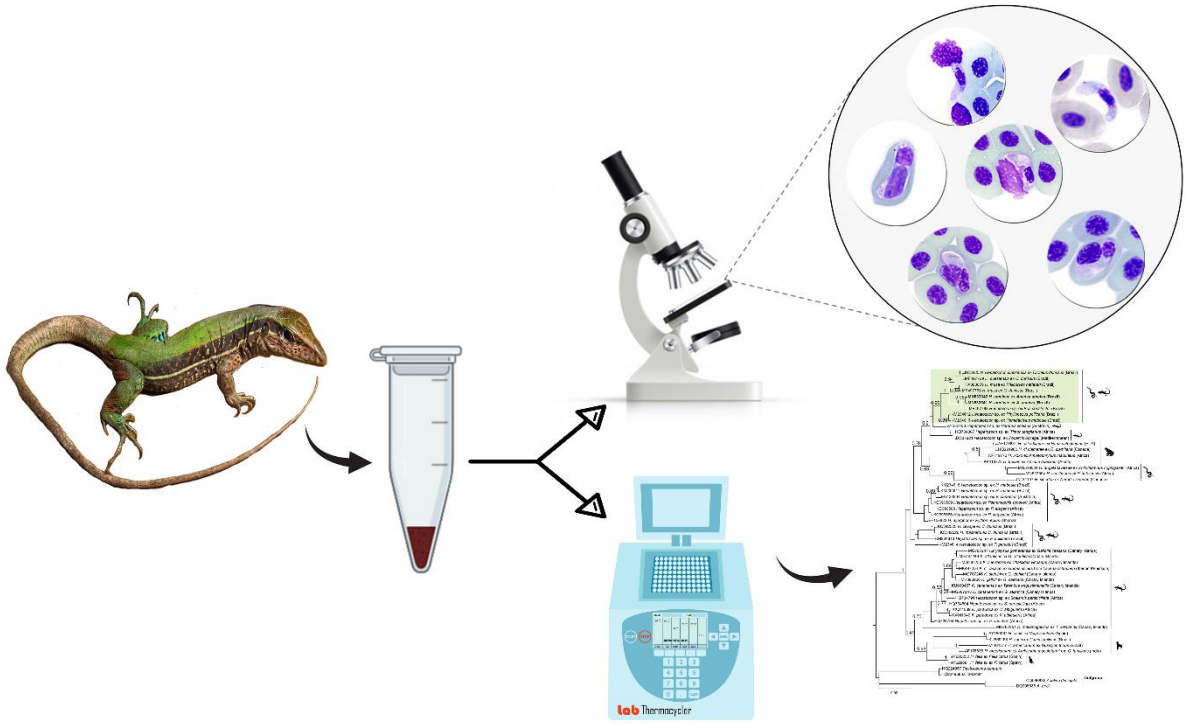


Figure 2. Parasites and inclusions found in lizards from Central Amazonia. Gametocytes of (a) *Hepatozoon ameivae* and (b) *Hepatozoon* sp. in *Ameiva ameiva.* (c) *Sauroplasma*-like infection in *Uranoscodon superciliosus*. (d) Trophozoite with nuclear division of *Plasmodium carmelinoi*

from *A. ameiva.* (e) Trophozoite and mature (f) gametocyte of *Plasmodium* sp. in *Norops planiceps.* (g) Macrogametocytes and microgametocyte of *Plasmodium kentropyxi* in *Kentropyx calcarata*. (h) Gametocyte of *Sarocytozoon tupinambi* in a lymphocyte from *Tupinambis teguixin*. (i) *Fallisia simplex* in *Plica umbra,* showing single and double gametocyte infections in the thrombocytes. (j) Gametocyte of *Garnia uranoscodoni* from *U. superciliosus*. *Trypanosoma* spp. infections in (k) *U. superciliosus* and (l) *P. umbra.* (m) Microfilaria in *A. ameiva* and in (n) mixed infection in *U. superciliosus*. Vacuole-like inclusions in erythrocytes from (o) *U. superciliosus* and (p) *A. ameiva*. Arrow heads indicate pigment granules; black arrows indicate parasite vacuoles and asterisks indicate inclusions. Micrographs are from Giemsa-stained thin blood films. Scale bar is 10 μm.



CAPÍTULO 2

Amanda M. Picelli, Maria R. L. da Silva, Adriane C. Ramires, Túllio R. R. da Silva, Felipe A. C. Pessoa, Lucio A. Viana e Igor L. Kaefer. **Redescription of *Hepatozoon ameivae* (Carini and Rudolph, 1912) from the lizard *Ameiva ameiva* (Linnaeus, 1758)** Manuscrito publicado no periódico Parasitology Research em 12 de junho de 2020. **DOI 10.1007/s00436-020-06760-x**

Redescription of *Hepatozoon ameivae* (Carini and Rudolph, 1912) from the lizard *Ameiva ameiva* (Linnaeus, 1758)

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Abstract

Blood samples from 72 *Ameiva ameiva* lizards from Central Amazonian upland forests were collected and thin smears of 40 (55.5%) animals were positive for gamonts of *Hepatozoon* with a mean level of intensity of infection of 14 parasites/2,000 blood erythrocytes (0.73%). The gametocytes were found attached with host cells nuclei and their dimensions were 14.28 ± 1.05 µm in length and 4.50 ± 0.80 µm in width. Phylogenetic analyses of the 18S rRNA gene showed that the new sequences obtained from *A. ameiva* constitute a monophyletic sister clade to the *Hepatozoon* spp. from Brazilian snakes. Based on morphological features and new molecular data we redescribe this haemogregarine as *Hepatozoon ameivae.* This study also provides the first molecular characterization of a *Hepatozoon* species from a Brazilian lizard.

Keywords: Adeleorid taxonomy · *Ameiva ameiva* · *Hepatozoon ameivae* · Molecular description · Reptiles · 18S ribosomal RNA gene

Introduction

Haemogregarines of the genus *Hepatozoon* (Miller, 1908) are a group of heteroxenous apicomplexan parasites (Apicomplexa, Adeleorina) recognized by the remarkable morphology of their gametocytes, present in the blood cells of a wide variety of vertebrate hosts (Smith 1996). Hepatozoids have been reported worldwide in reptiles (Telford 2009) and are uniquely diverse among squamates, especially in snakes that hold most of the known species (Úngari et al. 2018). In addition, these parasites can use a wide range of invertebrate definitive hosts (i.e. flies, mosquitoes, phlebotomines, ticks and leeches) which implies in a complex variety of life cycles (Telford 2009). Although there are still few elucidated life cycles involving reptilian hosts, *Hepatozoon* parasites are generally transmitted among reptiles through ingestion of the

vector or by predation of the paratenic vertebrate hosts, such as anurans, lizards and rodents (Smith 1996; Paperna and Lainson 2004; Viana et al. 2012).

Phylogenetic estimates using 18S rRNA gene sequences indicated that *Hepatozoon* is a paraphyletic taxon (Maia et al. 2012; Kvičerová et al. 2014; Cook et al. 2016), however there is a consensus that this species group should still be considered as belonging this genus (Maia et al. 2016). In Brazil, there are records of ca. 50 species described as *Hepatozoon* infecting reptiles (Úngari et al. 2018). Nevertheless, there are molecular data attributed to only 10% of them and these are derived from alligators: *Hepatozoon caimani* (Carini, 1909) Pessoa, de Biase, de Souza 1972 (Soares et al. 2017); and snakes: *Hepatozoon cevapii* O'Dwyer et al. 2013, *Hepatozoon massardi* O'Dwyer et al. 2013 and *Hepatozoon cuestensis* O'Dwyer et al. 2013; *Hepatozoon musa* Borges-Nojosa et al. 2017 (O'Dwyer et al. 2013; Borges-Nojosa et al. 2017). For species that parasitize lizards the situation is even worse. There are no molecular data for any of the six species described in lizards: *Hepatozoon tupinambis* Laveran and Salimbeni 1909, *Hepatozoon missoni* Carini, 1909, *Hepatozoon ameivae* Carini and Rudolphi 1912, *Hepatozoon goianense* Carini, 1941, *Hepatozoon cnemidophori* Carini, 1941 and *Hepatozoon sinimbui* Carini, 1942. Their insufficient morphological descriptions were based almost exclusively on the characteristics of the intraerythrocytic gamonts. Furthermore, except for *H. sinimbui* that was reported in *Iguana iguana* Linnaeus, 1758 (Carini 1942), these *Hepatozoon* species were described from lizards of the Teiidae family, with *Ameiva ameiva* (Linnaeus, 1758) appointed as host for at least one of them, *H. ameivae*.

The neotropical species *A. ameiva* is a heliothermic terrestrial lizard widely distributed in South America (Vitt et al. 2008). It has an active foraging habit and is considered an opportunistic predator consuming a wide variety of food categories (e.g. anurans, arthropods and even plants) (Vitt and Colli 1994). It lives associated with open areas and can be found from clearings and edges of primary forests to anthropized environments (Sartorius et al. 1999).

In Amazonia this lizard is abundant and has been object of previous studies with apicomplexan parasites (Lainson 2012). However, most of them focused mainly on haemosporidians and intestinal coccidians, providing scarce information about hepatozoids in these lizard hosts.

Here we aimed to investigate the presence of *Hepatozoon* parasites in *A. ameiva* from Central Amazon region. In addition, we redescribe *H. ameivae* based on morphological and molecular characteristics.

Material and methods

Lizard capture and blood sampling

Specimens of *A. ameiva* (n = 72) were captured using pitfalls with drift-fences (Jenkins et al. 2003), live-traps baited with eggs (Vieira et al. 2015) and by noose with fishing line (García-Muñoz and Sillero 2010), at upland (*terra-firme*) forest sites nearby the municipalities of Manaus (2°20'S, 60°6'W), Presidente Figueiredo (1°48'S, 60°19'W) and Rio Preto da Eva (2°25'S, 59°50'W), all located in the State of Amazonas, Brazil. Using a sterile insulin syringe approximately 0.5 to 1 mL of blood was collected by cardiac or tail venipuncture (Samour et al. 1984). Afterwards, a portion of collected blood was used to make thin smears, which were air-dried, fixed with absolute methanol and stained with 10% Giemsa. The other portion was applied to a filter paper or stored in 96% ethanol for molecular analyses. Lizards were released within 24 h to their collection sites, but in the case of cardiac puncture individuals were euthanized with 2% lidocaine, preserved in 10% formalin and deposited in the Zoological Collections of the National Institute of Amazonian Research (INPA) and Amazonas Federal University (UFAM) in Manaus, Brazil.

Microscopic analyses

Blood smears were screened for *Hepatozoon* gamonts, using a Leica DM4B microscope (Leica Microsystems, Heerbrugg, Switzerland) at × 400 and × 1000 total magnification for up to 20 min (Bromwich and Schall 1986). The slides with parasites were carefully examined and images were captured with an attached Leica DMC4500 digital camera and processed with LAS V4.8 (Leica Microsystems Suiza Limited 2015). Measurements of the length, width and area of the gamonts and host cells (infected and uninfected) were performed under this system. Morphometric data are given in micrometres (μm) and for each metric the averages, ranges and standard deviations were also analysed. The intensity of infection was estimated by the number of forms observed in 2,000 erythrocytes, in 20 replicates of 100 erythrocytes per field examined (Godfrey et al. 1987).

DNA extraction, amplification and sequencing

Blood samples that were positive for *Hepatozoon* infection by microscopic examination had total DNA extracted by using DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA), following the manufacturer's instructions. Detection of *Hepatozoon* DNA was performed by nested PCR using the primers 4558 (5′-GCT AAT ACA TGA GCA AAA TCT CAA-3′) and 2733 (5′-CGG AAT TAA CCA GAC AAA T-3′), followed by HepF300 (5′-GTT TCT GAC CTA TCA GCT TTC GAC G-3′) and Hep900 (5′-CAA ATC TAA GAA TTT CAC CTC TGA C-3′), which targeted a fragment of 600 base pairs (bp) of the 18S rRNA gene, for sequencing and phylogenetic analysis (Silva et al. 2018). The polymerase chain reaction (PCR) conditions for the primary PCR (primers 4558/2733) consisted of a pre-PCR step at 94 °C for 3 min, followed by 45 cycles of 94 °C for 1 min, 55 °C for 2 min, an extension at 72 °C for 1 min and 10 s, and a final extension at 72 °C for 10 min. The secondary PCR (primers HepF300/Hep900) consisted of a pre-PCR step at 94 °C for 3 min, followed by 45 cycles of 94 °C for 45 s, 56 °C for 1 min,

an extension at 72 °C for 40 s, and a final extension at 72 °C for 10 min. In each PCR assay, a negative (distilled water) and a positive control were used. Amplified products were visualized by electrophoresis in 2% agarose and visualized by GelRed™ (Biotium, Hayward, USA) staining and UV transillumination. The amplicons were purified using Wizard® SV Gel and PCR Clean-Up System in agreement with the manufacturer's instructions. PCR products were sequenced using the BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

DNA sequences from four positive samples were edited using the BioEdit software v7.2.5 (Hall 1999) and compared for similarity with sequences available in GenBank® using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST). Two sequences obtained in this study were aligned with 52 sequences retrieved from GenBank®, using the MUSCLE algorithm and the GENEIOUS v.7.1.3 software (Kearse et al. 2012). Phylogenetic hypothesis was estimated using Bayesian Inference (BI) implemented in MrBayes Ver. 3.2.1 (Huelsenbeck and Ronquist 2001) and best evolutionary model was identified using the jModelTest v.2.1.10 program (Darriba et al. 2012). The chosen parameter of the substitution model, based on the Akaike information criterion (AIC), was GTR+I+G. Markov chain Monte Carlo simulations were run for 10^7 generations in two parallel runs, saving one tree each 1,000 generations with burn-in of 25%. Strict consensus tree was generated and visualized using FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). *Dactylosoma ranarum* Lankester, 1892 (HQ224957; HQ224958), *Adelina dimidiata* Schneider, 1885 (DQ096836) and *Adelina grylli* Butaeva, 1996 (DQ096835) were used as outgroups. To estimate the percentage of nucleotide divergence of *H. ameivae* isolates from this study and *Hepatozoon* spp. sequences from reptiles available on

GenBank®, an alignment of 461 bp was used. Pairwise *p* distances were calculated using MEGA6 software (Tamura et al. 2013).

Results

Hepatozoon were detected in the blood of 40 out of 72 (55.5%) *A. ameiva* specimens examined by light microscopy. For the sampled areas, the prevalence was as follows: Manaus had 81% (n = 9/11) of the lizards infected; Rio Preto da Eva had 67% (n = 21/31); and Presidente Figueiredo had 33% (n = 10/30). The average intensity of infection was 14 parasites/2,000 blood erythrocytes (0.73%; ± 14.5), ranging from 1 to 55 parasites. Gametocytes were elongated, large and wide, with an eccentric nucleus and rounded extremities (Fig. 1; Table 1). Parasites were also found frequently overlapping the nuclei of the parasitised erythrocytes (Fig. 1). These morphological attributes matched with the characteristics described for *H. ameivae* at the same host species (Carini and Rudolph 1912).

The four isolated *Hepatozoon* sequences of 18S rRNA obtained from the blood of *A. ameiva* were identical among them and a BLAST search showed they were distinct from the *Hepatozoon* spp. sequences available in GenBank®. The percentage of nucleotide divergence (*p* distance) between *Hepatozoon* sequences from this study and *Hepatozoon* spp. from other lizard and snake species ranged from 0.7% to 3% (Online Resource 1). Based on the morphological characteristics, similarity search and genetic divergence, we identified the isolated sequences as belonging to *H. ameivae.* The sequences obtained in this study were deposited in GenBank® under the following accession numbers: MN833639 - MN833642.

The phylogenetic tree (Online Resource 2) derived from a Bayesian Inference analysis of the 445-bp fragment of the *Hepatozoon* 18S rRNA gene, demonstrated that *H. ameivae* clustered in a major monophyletic clade with other *Hepatozoon* spp. sequences from lizards, snakes and

frogs from different geographical locations. These new sequences were placed in a subclade composed exclusively of haemogregarines from Brazilian squamates hosts and grouped into a well-supported smaller clade with *Hepatozoon* sp., recently isolated from *Boa constrictor* Linnaeus, 1758 (Úngari et al. 2018). This indicated that this subgroup is a sister taxa to the lineage comprised by *H. cuestensis* and *H. musa* (O'Dwyer et al. 2013; Borges-Nojosa et al. 2017; Úngari et al. 2018). Furthermore, *H. ameivae* was positioned distinct of *Hepatozoon* spp. sequences from carnivores and *Karyolysus* spp. sequences from lizards.

Redescription of *Hepatozoon ameivae* (Carini and Rudolph, 1912)

This parasite was first described infecting the lizard *A. ameiva* in southeast Brazil; here it was found in the peripheral blood of the same host species from Central Amazonia, Brazil. Gametocytes were found inside of mature erythrocytes (Fig. 1a-c) and in leukocytes (Fig. 1d-e) at higher levels of infection intensity (up to 1.5%); and were also detected free in blood at low frequency (Fig. 1f). In the original description, parasites were seen infecting only erythrocytes and the free forms were detected in the organ smears.

Gamonts were elongated, large and wide, with uniform cytoplasm and dimensions 16–11 × 6–3 μm (Fig. 1; Table 1). Both the extremities were rounded and slightly arched, but one of them was wider than the other. Parasite nucleus was densely stained with strongly condensed chromatin strands, rounded or irregular in shape with dimensions 10–3 × 5–2 μm (Table 1), and was located at the narrow end of the parasite. Gamonts were associated with the erythrocytes' nuclei being positioned over (Fig. 1a) or laterally (Fig. 1c) to it. Infected erythrocytes exhibited few visible cytopathological effects when compared to the size of uninfected erythrocytes (Table 1), but were slightly elongated or more irregularly shaped than these (Fig. 1a-c). The nuclei of infected erythrocytes were somewhat hypertrophied and those that were fully enveloped by the parasite became longer and thinner (Fig. 1a); or may also have been displaced

laterally as a result of the position assumed by the parasite in the cell (Fig. 1b-c). Overall, these characteristics were similar to the original species description and yet no encapsulated form as described by Carini and Rudolph (1912) was seen in our study.

Taxonomic status

Host type: *Ameiva ameiva* (Linnaeus, 1758 - Teiidae) (Fig. 1; Table 1).

Other Hosts: Unknown.

Vector: Unknown.

Type Locality: State of Minas Gerais, southeast Brazil (Carini and Rudolph 1912).

Other Localities: In northern Brazil: Capanema, Pará State (Lainson et al. 2003b), Presidente Figueiredo (1°48'S, 60°19'W), Rio Preto da Eva (2°25'S, 59°50'W) and Manaus (2°20'S, 60°6'W) municipalities, Amazonas State (present study); São João da Barra, Rio de Janeiro State, southeast Brazil (Sabagh et al. 2015).

Prevalence (present study): Presidente Figueiredo: 10 out of 30 (33%); Rio Preto da Eva: 21 out of 31 (67%); and Manaus: 9 out of 11 (81%).

Infection site: Mature erythrocytes and leukocytes at low frequency.

Intensity of infection: Average intensity of 14 parasites/2,000 blood erythrocytes (0.73%; ± 14.5), ranging from 1 to 55 parasites.

DNA sequences: The 18S ribosomal gene sequences were deposited in GenBank® (accession numbers: MN833639 - MN833642).

Material: Four blood slides from *Ameiva ameiva* were deposited at the National Institute of Amazonian Research (INPA), Manaus, AM, Brazil (nº INPA 18a, INPA 18b, INPA 18c and INPA 18d).

Remarks: Gamonts of *H. ameivae* reported in different locations presented morphological variation in their dimensions: Minas Gerais with 13–11 × 4–3 μm (Rudolphi and Carini 1912); Eastern Amazonia 14–11 × 3 μm (Lainson et al. 2003); and Central Amazonia (this study) with 16–11 × 6–3 μm. As detailed below, *H. ameivae* is compared with four species described for teiid lizards (gamont dimensions). *Hepatozoon tupinambis* (16 × 6 μm) infected erythrocytes are always distorted and greatly hypertrophied to about three times and have a thin, dehemoglobinized cytoplasm (Laveran and Salimbeni 1909; Telford 2009). In contrast, *H. missoni* (9 × 3 μm) and *H. goianense* (6 × 4 μm) develop smaller gametocytes (Carini 1909; Carini 1941a) and the last species was recorded only in leukocytes (Carini 1941a). *Hepatozoon cnemidophori* (17 × 4 μm) (Carini 1941b) are very similar to *H. ameivae* gamonts, but this exhibit uniform cytoplasm and nuclei in a polar position with condensed chromatin. Regarding the phylogenetic relationships found here, we considered relevant to compare it with the close species (gamont dimensions). Despite the resemblance to size and curved shape of *H. musa* (17.2 × 4.0 μm), *H. cuestensis* (15.6 × 3.0 μm), and *Hepatozoon* sp. from *B. constrictor* (13.3 × 4.6 μm) to *H. ameivae*, the main diagnostic feature is its unique interaction with the host cell nucleus, being positioned over or completely attached to it.

Discussion

To our knowledge, this is the first molecular and morphological data attributed to a species of *Hepatozoon* in Brazilian lizards, and based on this we redescribed *H. ameivae*. Indeed, there is still paucity of sequences available for this taxon, despite the blood parasite richness found in neotropical lizard species (Picelli et al. *in press*). In South America, knowledge on herpetofauna's *Hepatozoon* began to be accessed not long ago, with the first sequences of these parasites in snakes (O'Dwyer et al. 2013). Harris et al. (2015) were the first to investigate at the molecular level haemoparasites from Phylum Apicomplexa in saurian hosts from Brazil, and

showed a great diversity of *Hepatozoon* haplotypes in a few native and exotic gecko species. Due to the type of material analyzed (tissue fragments), there were no morphological characteristics associated with these sequences and therefore no species were designated. This approach has been frequently employed in haemogregarine studies (Harris et al. 2015, 2018; Tomé et al. 2016), however, an exclusively molecular view does not provide all the information necessary for species-level identification and to understand ecological and life cycle aspects of the organism.

Here, we found *H. ameivae* with a relatively high prevalence and a moderate level of infection in *A. ameiva* from Central Amazonian upland forests. This parasite was first observed in 1912 by Carini and Rudolph in this lizard species from an imprecise location in Minas Gerais State, southeastern Brazil. Since then it has been found in coastal sand dune (‘*restinga*’) and forest environments, always infecting the same vertebrate host (Lainson et al. 2003; Sabagh et al. 2015). Our record increases the known geographic distribution range for *H. ameivae*, which appears to be as widespread as its lizard host. In fact, this teiid species is a ubiquitous lizard with very generalist habits (Vitt and Colli 1994). These characteristics may increase the probability of infection and facilitate the spread of parasites, mainly those with low host specificity such as hepatozoids (Smith 1996; Tomé et al. 2012). In fact, *A. ameiva* was assigned as a haemogregarine carrier in Caribbean Island of Providencia (Ayala 1975), Eastern Colombia (Ayala et al. 1973) and Panama (Telford 1977), but none of these records properly identified the parasites.

Our morphometric analyses showed that *H. ameivae* gametocytes from this study region are relatively larger than those observed in other locations where the parasite was registered (see Remarks). Nevertheless, its diagnostic feature, the overlap of the nucleus, was found in all reports (Carini and Rudolph 1912; Lainson et al. 2003; Sabagh et al. 2015). Perhaps, this size variation may be effect of the different environmental pressures faced by the hosts from each

landscape (Bower et al. 2019) or simply a difference between how the measurements were made by the different authors (Perkins et al. 2011). To resolve this issue, it would be necessary to access the material deposited by those authors or collect new data from those locations to test such host-induced variation hypothesis.

Another interesting finding in our study was that *H. ameivae* gamonts were parasitizing leukocytes from animals with high levels of infection. The infection of leukocytes by *Hepatozoon* spp. in lizards is rare, but common in birds (Valkiūnas et al. 2016) and mammals (Silva et al. 2018). Some authors argue that parasites in reptiles have been phagocytized by leukocytes of older and larger hosts (Laird 1950; Godfrey et al. 2011). However, as we observed in this study, this type of parasitism in reptiles was associated with high levels of infection and therefore the presence of parasites in white blood cells would be a consequence of this (Telford 2009).

Overall, the phylogenetic relationships based on analysis of 18S rRNA gene reported here were similar to those observed in previous works (Harris et al. 2015, 2018; Borges-Nojosa et al. 2017; Úngari et al. 2018), maintaining a main clade composed by *Hepatozoon* from reptiles and amphibians. In addition, it also reinforced the hypothesis of a possible biographical pattern proposed by Harris et al. (2015), with lineages from squamate hosts from South America within a *Hepatozoon* subclade. Nevertheless, the sequences obtained in this study were clustered in a lineage considered hitherto exclusive of hepatozoids from snakes (Úngari et al. 2018). Truly, *H. ameivae* was most closely related to *Hepatozoon* sp. (MF497768) from *B. constrictor* from southern Brazil (Úngari et al. 2018), differing by only three nucleotides (0.7%). This is a very intriguing relationship which may be reflecting prey-predator transmission (Tomé et al. 2012; Cook et al. 2018) or low vertebrate host specificity (Barta et al. 2012). It is possible that *A. ameiva* may be serving for *H. ameivae* as both intermediate (presence of gamonts in blood cells) and paratenic hosts. Carini and Rudolph (1912) reported cystic forms in *A. ameiva* liver and it

is well known that this lizard is part of the diet of several ophidian species, including *B. constrictor* (Sanches et al. 2018). However, the fact that the lizard has cysts in its tissues does not necessarily make it a paratenic host (Telford 2009). Further cross-infection experiments, such as those performed by Paperna and Lainson (2004) and Lainson et al. (2007), will be needed to elucidate the possibility of *H. ameivae* occurrence in *B. constrictor*. On the other hand, as has been shown in other studies (Barta et al. 2012; Cook et al. 2015; Borges-Nojosa et al. 2017), 18S rRNA gene is a conserved marker and genetic distances close to 1% could be considerable for species distinction. Moreover, *H. ameivae* gamonts did not share morphological and morphometric characteristics with those from *B. constrictor* (Úngari et al. 2018). Therefore, for the reasons given here we do not consider *H. ameivae* to be the same species found in *B. constrictor*.

In sum, our study redescribes *H. ameivae* through an integrative taxonomic approach, using morphological, morphometric and molecular data of this species. We also provided the first genetic sequence to this haemoparasite and expanded its known geographic distribution. Furthermore, these novel sequences contribute with new information on phylogenetic relationship among *Hepatozoon* spp. from Brazilian squamate hosts.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval All procedures performed in this study involving animals were approved by the ethics committee on animal use from Universidade Federal do Amazonas (protocol number 012/2016), and lizard sampling and access to the genetic data were authorised by the Brazilian Ministry of the Environment (SISBIO number 53851 and SISGEN AA6199D, respectively).

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Table 1. Morphometric characteristics of the *Hepatozoon ameivae* found in the lizard *Ameiva ameiva* from Central Amazonia, Brazil. Measurements are presented as mean ± standard deviation (SD) followed by the range (maximum and minimum values).

Characteristic	*n*	Length (μm)	Width (μm)	Area (μm^2)
Parasite	30	14.28 ± 1.05 (16.33–11.11)	4.50 ± 0.80 (6.17–3.16)	51.09 ± 9.85 (67.88–29.65)
Parasite nucleus	30	4.90 ± 1.27 (10.33–3.31)	3.19 ± 0.69 (5.07–2.14)	12.48 ± 3.28 (20.69–7.89)
Infected erythrocyte	30	16.48 ± 1.38 (19.18–12.90)	8.47 ± 1.17 (11.25–6.27)	111.20 ± 15.36 (145.87–79.29)
Infected erythrocyte nucleus	30	7.87 ± 0.81 (9.38–6.12)	3.92 ± 0.62 (5.61–3.03)	26.25 ± 4.42 (37.71–18.34)
Uninfected erythrocyte	30	14.31 ± 0.93 (17.66–13.15)	8.87 ± 0.58 (10.24–7.86)	108.05 ± 10.04 (122.79–91.14)
Uninfected erythrocyte nucleus	30	6.40 ± 0.75 (8.05–5.02)	3.88 ± 0.50 (4.90–2.74)	20.73 ± 3.6 (28.52–15.63)

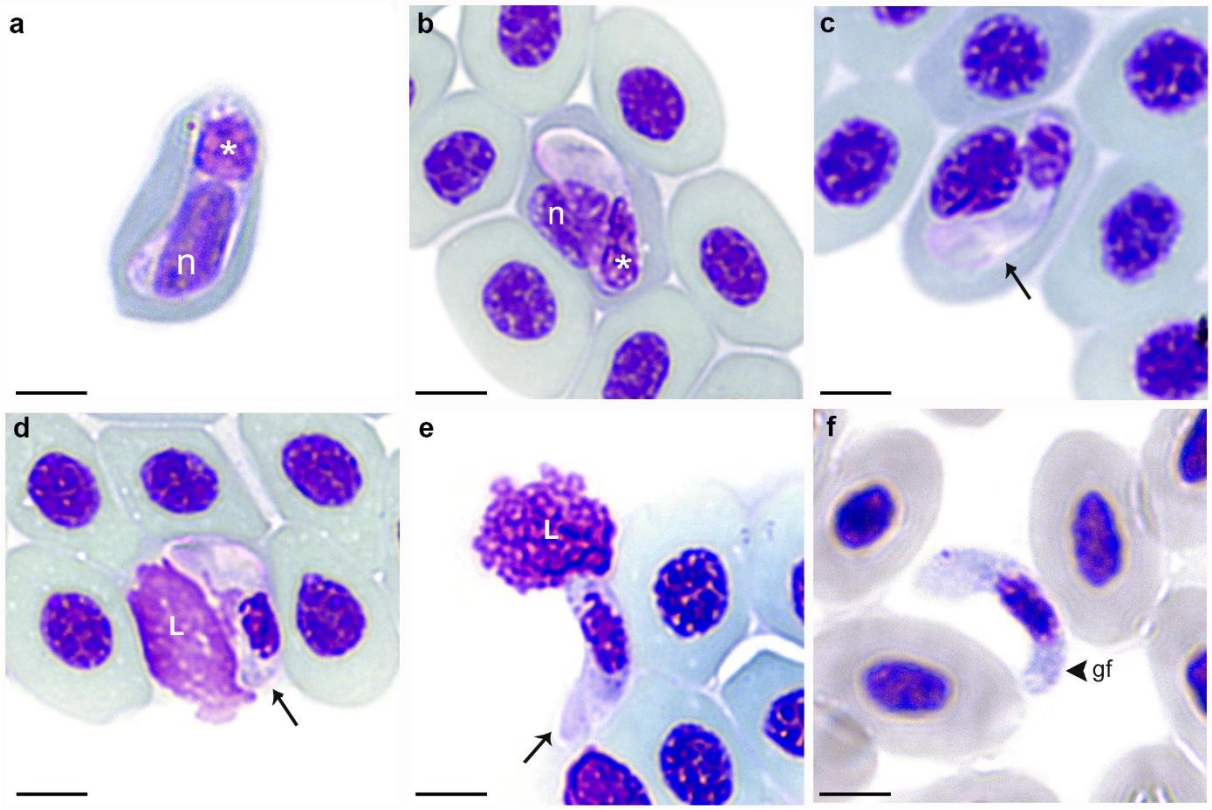


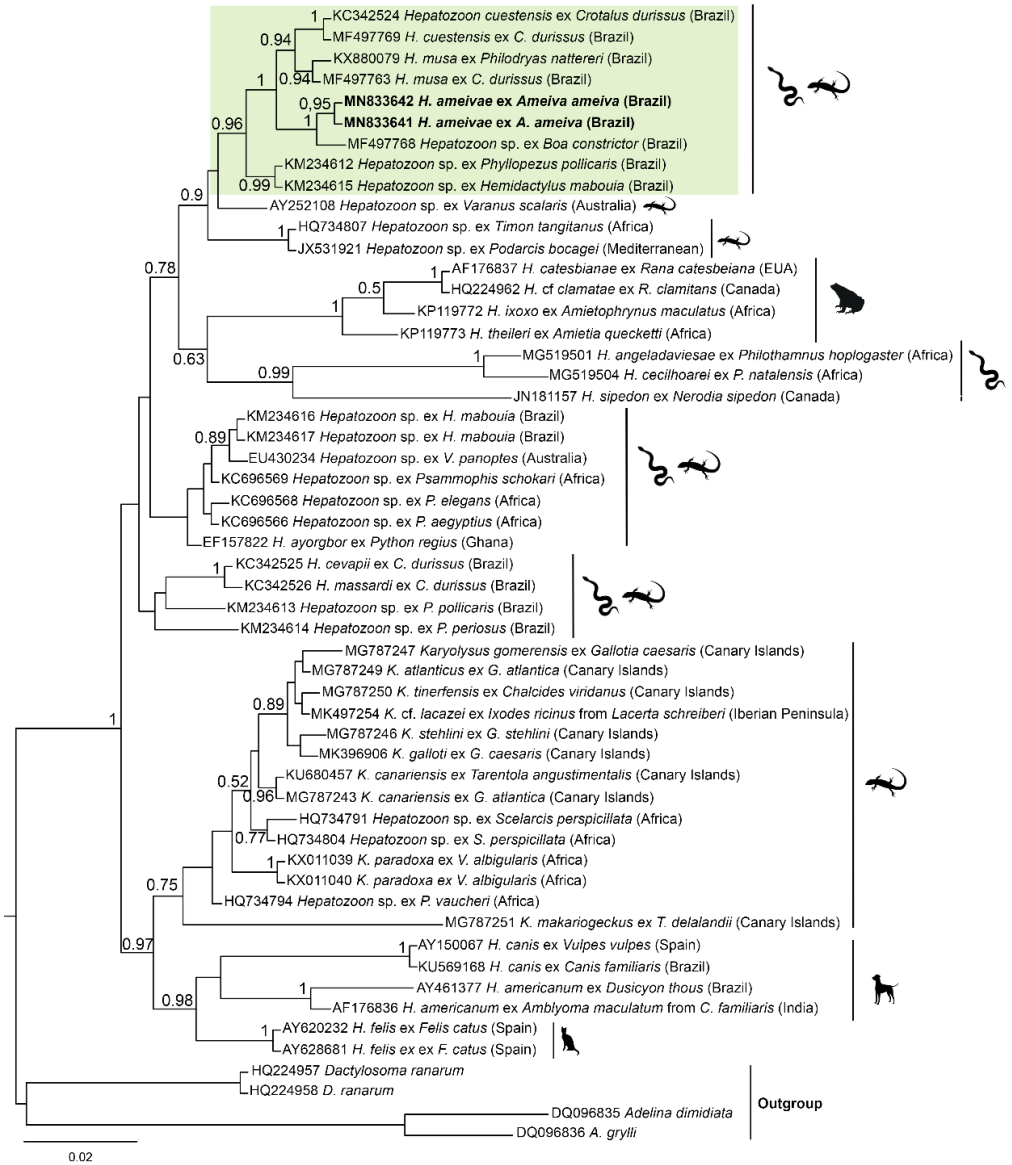
Fig. 1 Gametocytes of *Hepatozoon ameivae* in the blood of *Ameiva ameiva* from Central Amazonia, Brazil. **a**, **b** and **c** — Intraerythrocytic gamonts; **d** and **e** — Parasites invading leukocytes (L); **f**— Gamont free in blood (gf). Arrows indicate parasites; asterisks indicate gamont nuclei; and (n) indicates host cell nucleus. Micrographs are from Giemsa-stained thin blood films. Scale bar is 20 µm.

Online Resource 1 Distance matrix among partial 18S rRNA sequences of *Hepatozoon ameivae* obtained in this study and isolates of *Hepatozoon* spp. from the GenBank® database (461 base pairs). The upper matrix shows the uncorrected pairwise distance (*p* distance) among the sequences, while the lower matrix shows the number of nucleotide differences.

Sequences	1	2	3	4	5	6	7	8	9	10	11	12
1. *Hepatozoon ameivae* (MN833641)*		0.000	0.007	0.017	0.017	0.030	0.030	0.022	0.015	0.015	0.015	0.013
2. *Hepatozoon ameivae* (MN833642)*	0		0.007	0.017	0.017	0.030	0.030	0.022	0.015	0.015	0.015	0.013
3. *Hepatozoon* sp. (MF497768)	3	3		0.020	0.020	0.033	0.033	0.028	0.017	0.017	0.017	0.015
4. *Hepatozoon* sp. (KM234612)	8	8	9		0.000	0.026	0.026	0.017	0.015	0.015	0.015	0.013
5. *Hepatozoon* sp. (KM234615)	8	8	9	0		0.026	0.026	0.017	0.015	0.015	0.015	0.013
6. *Hepatozoon* sp. (HQ734807)	14	14	15	12	12		0.000	0.026	0.033	0.033	0.028	0.026
7. *Hepatozoon* sp. (JX531921)	14	14	15	12	12	0		0.026	0.033	0.033	0.028	0.026
8. *Hepatozoon* sp. (AY252108)	10	10	13	8	8	12	12		0.020	0.020	0.024	0.022
9. *Hepatozoon cuestensis* (KC342524)	7	7	8	7	7	15	15	9		0.000	0.009	0.007
10. *Hepatozoon cuestensis* (MF497769)	7	7	8	7	7	15	15	9	0		0.009	0.007
11. *Hepatozoon musa* (KX880079)	7	7	8	7	7	13	13	11	4	4		0.002
12. *Hepatozoon musa* (MF497763)	6	6	7	6	6	12	12	10	3	3	1	

Redescription of *Hepatozoon ameivae* (Carini and Rudolph, 1912) from the lizard *Ameiva ameiva* (Linnaeus, 1758) - Parasitology Research – Picelli et al - Programa de Pós-Graduação em Zoologia, Instituto de Ciências Biológicas, Universidade Federal do Amazonas, Manaus 69067-005, AM, Brazil. E-mail: amanda.mpicelli@gmail.com

Online Resource 2 Bayesian phylogenetic hypothesis based on an alignment of 445 bp fragment of *Hepatozoon* spp. 18S rRNA from *Ameiva ameiva* isolated in this study and sequences deposited in GenBank®. Bootstrap values (≥50) are given above the nodes. The branch length scale represents 0.02 substitutions per site. *Dactylosoma ranarum*, *Adelina dimidiata* and *Adelina grylli* were used as outgroup. The sequences indicated in bold represent those from this study.



CAPÍTULO 3

Amanda M. Picelli, Bruno R. Fermino, Adriane C. Ramires, Felipe A. C. Pessoa, Lucio A. Viana, Igor L. Kaefer e Marta M. G. Teixeira **Trypanosome phylogenetic relationships from the Amazonian Diving Lizard indicate host ecology as a driver of parasite diversification.** Manuscrito em preparação para revista Parasitology Research.

Trypanosome phylogenetic relationships from the Amazonian Diving Lizard indicate host ecology as a driver of parasite diversification

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Abstract

Lizard trypanosomes are notable for their species richness and for presenting lineages associated with both the Aquatic and Terrestrial clades. However, they remain one of the least understood groups within the evolutionary history of the genus *Trypanosoma*. In this study, we explored the diversity of trypanosomes in the Amazonian Diving Lizard *Uranoscodon superciliosus* through phylogenetic and morphological approaches. Blood samples from 116 lizards were collected and smears of 83 (71%) individuals were positive for *Trypanosoma.* Trypomastigotes forms observed showed striking morphological (i.e. rounded, elliptical, leaf-shaped and fusiform bodies) and morphometric (dimensions: 32-124 × 13.3-32.9 µm) variation. Phylogenies based on Small Subunit rDNA (*SSU* rDNA) and glycosomal Glyceraldehyde 3-Phosphate Dehydrogenase (*gGAPDH*) genes sequences revealed two novel trypanosome genotypes among eleven isolates obtained here, with more than one haplotype each that were grouped into two different subclades. The new genotypes clustered into a monophyletic group within the Aquatic clade, and formed a sister-taxon of the trypanosomes from neotropical frogs. To our knowledge, this study provides the first molecular data for lizard trypanosomes from the Amazonian biome and also suggests that hosts using similar microhabitats could harbor more closely-related trypanosomes.

Keywords: *gGAPDH* gene · Morphology · Phylogeny · *Uranoscodon superciliosus* · *SSU* rDNA gene · *Trypanosoma*

Introduction

Organisms with parasitic lifestyle are most often analyzed from the perspective of their deleterious effects and how they affect the ecological and evolutionary processes of their hosts (Tompkins and Begon 1999, Botero et al. 2013, Kubacka et al. 2019). However, as the parasite-host interaction is a two-way road, the evolutionary history of the parasites depends, among

other factors, on the ecological features of their hosts, such as foraging habits, population density, geographical range and habitat use (Arriero and Møller 2008, Bordes et al. 2009, Leung and Koprivnikar 2016). The role and degree of importance of these traits on parasite diversification varies among systems and is also conditioned by the transmission mode of the parasites (Poulin and Morand 2004). Properties associated with host microhabitat can act as selective filters for parasites (Combes and Théron 2000, Pérez et al. 2019), and in this respect flagellate parasites of the genus *Trypanosoma* Gruby, 1843 (Kinetoplastea, Trypanosomatidae) stand out for presenting a marked dichotomy in its phylogeny: the division between the aquatic and terrestrial habitats used by their vertebrate hosts and vectors (Stevens et al. 2001, Hamilton et al. 2004).

Those two major evolutionary clusters are known as Aquatic and Terrestrial clades and are strongly supported by phylogenetic reconstructions based on *SSU* rDNA (small subunit of ribosomal DNA) and *gGAPDH* (glycosomal glyceraldehyde 3-phosphate dehydrogenase) gene sequences (Hamilton et al. 2004, Simpson et al. 2004). In the Aquatic clade are included lineages predominantly from aquatic or semi-aquatic hosts, such as leeches, fishes, platypus, anurans, chelonians, caimans and some lizards (Haag et al. 1998, Dvořáková et al. 2015, Fermino et al. 2015, Lemos et al. 2015, Spodareva et al. 2018). Differently, the Terrestrial clade contains mostly trypanosomes from mammals, birds and also reptilian hosts (Votýpka et al. 2012, Fermino et al. 2019, Pérez et al. 2019, Rodrigues et al. 2019). These phylogenetic relationships do show that trypanosomes have a very interesting evolutionary history with high capacity for switching and adaptation to new hosts (Hamilton et al. 2007, Telleria and Tibayrenc 2017, Pérez et al. 2019). Such traits can be evidenced by the current high species richness (ca. 500 species) that infect all class of vertebrate hosts around the world (Spodareva et al. 2018, Ortiz and Solari 2019), associated with a wide variety of blood-sucking invertebrates (i.e., insects, ticks and leeches) that act as vectors (Viola et al. 2008a, Fermino et al. 2015). However,

despite the recent contributions from evolutionary studies with the genus, there is still a gap in sampling non-mammalian hosts, which renders poorly resolved relationships among minor trypanosome clades (Telleria and Tibayrenc 2017).

This sample bias is very pronounced in Neotropical lizards, especially for those that occur in the Amazonian biome (Picelli et al. 2020). Despite the great diversity of lizards in the Neotropics (Ribeiro-Júnior and Amaral 2016a), trypanosome species that were recognized in these hosts represent only a small part of the described species for this genus. There are at least 11 trypanosome species and a single molecular sequence available, without a nominal species, attributed to neotropical lizards (Telford 2009, Cabalero et al. 2015). Moreover, these flagellates as well as most trypanosomatid species were named using classical methods based on trypomastigote morphology present in the bloodstream of their hosts, and also by association with host species and geographic region (Telford 2009). This probably mislead species identifications, causing under or overestimation of trypanosome diversity because trypanosomes, besides being pleomorphic organisms, exhibit a high degree of polymorphism and low specificity with their hosts (Ferreira et al. 2007, Spodareva et al. 2018).

From Amazonian lizards belonging to the family Tropiduridae Bell, 1843, there are three species formally described (Telford 2009): *Trypanosoma superciliosae* Walliker, 1965 in *Uranoscodon superciliosus* (Linnaeus, 1758), *Trypanosoma plicae* Lainson, Shaw and Landau 1975 in *Plica umbra ochrocollaris* (Linnaeus, 1758), and *Trypanosoma plicaplicae* Telford 1996 in *Plica plica* (Linnaeus, 1758). All of them lack molecular data and only trypomastigotes were characterized. Among these we can highlight *T. superciliosae* for being the largest trypanosome species ever described infecting lizards, with the trypomastigote forms measuring between 87-124 µm in length (Walliker 1965). Apparently, it was found occurring under a heavy infection in *U. superciliosus* from Central Amazonia (Walliker 1965). This host is an arboreal species widely distributed throughout the Amazon Basin and lives in close association

with water bodies, mainly small streams of primary rainforests (Faria et al. 2019, Picelli et al. 2019). It has an interesting escape behavior, jumping into the water and diving at the any sign of a predator – because of this it is called Diving Lizard (Howland et al. 1990). Although it is considered abundant in the microhabitat that it occurs, there have been no further studies with *U. superciliosus* and their trypanosomes, except for a recent survey of hemoparasites that we performed on saurian hosts from Brazilian Amazonia (Picelli et al. 2020). In that study we recorded trypomastigotes in blood smears of this lizard species, but without analyzing them in a phylogenetic context.

Herein, we aimed to investigate the diversity and phylogenetic relationships of trypanosomatids that parasitize the Diving Lizard *U. superciliosus* from the Central Amazonian region. Based on the association between the evolutionary history of the parasites and the ecological features of their hosts, we hypothesized that the trypanosomes found in this species would belong to the Aquatic Clade.

Material and methods

Collection sites, lizard and blood sampling

From 2016 to 2019, we conducted the study in six areas of primary upland (‘*terra-firme*’) forest distributed across the municipalities of Manaus and Rio Preto da Eva, State of Amazonas, Brazil (Fig. 1). Five of these localities belong to Area of Relevant Ecological Interest of the Biological Dynamics of Forest Fragments Project (AREI-BDFFP; 2°25'S, 59°50'W), and the other site is the Experimental Farm of the Federal University of Amazonas (FEX-UFAM; 2°38'57.6"S, 60°3'11"W) (Laurance et al. 2017, Faria et al. 2019). Lizards were captured through nocturnal active search while resting on branches or tree trunks along the banks of small streams. Approximately 0.5 to 1 mL of blood was collected from these animals by tail

venipuncture using a sterile insulin syringe (Samour et al. 1984). A portion of these blood samples was used to make thin smears, which were air-dried, fixed with absolute methanol and stained with 10% Giemsa (Picelli et al. 2020). The other part was applied to a filter paper or stored in 96% ethanol for molecular analyses. Most lizards were returned to the collection site at 24 hours after capture, while others were euthanized (via injection of 2% lidocaine), preserved in 10% formalin, and deposited as voucher specimens in the Zoological Collection of the Federal University of Amazonas (UFAM).

Microscopic analyses

The search for trypanosomes in blood smears was performed under a Leica DM4B microscope (Leica Microsystems, Heerbrugg, Switzerland) at × 200, × 400 and × 1000 total magnification. Positive slides were examined in their entirety for the capture of images by using Leica DMC4500 digital camera and processed with LAS V4.8 (Leica Microsystems Suiza Limited 2015). Morphometric analysis of sanguineous trypomastigote forms found were taken with ImageJ software (Schneider et al. 2012). Measurements of the body length, body width, free flagellum and distance form kinetoplast to posterior end of the body are given in micrometers (μm).

DNA extraction, PCR amplification and sequencing

Total genomic DNA from positive blood samples, determined by microscopic examination, were extracted using the ammonium acetate method, following a previously described protocol (Fermino et al. 2019). Detection of trypanosomes DNA was performed by nested-PCRs (polymerase chain reactions) for amplification of non-coding V7V8 *SSU* rDNA and *gGAPDH* gene fragments. The PCR amplification of *SSU* rDNA sequences [~900 base pairs (bp)] was carried out using the primers 609 F (5'-CAC CCG CGG TAA TTC CAG C-3')

and 706 R (5'-CTG AGA CTG TAA CCT CAA-3') (Borghesan et al. 2013). For nested-PCR amplification of *gGAPDH* (~608 bp) sequences a set of primers was employed, the first round of primers were GAPDH SF (5'-GTG GCG GTKGTYGAC ATG AAC A-3') and GAPDH SR (5'-TTG GAG TCR TAG ATR GAG CT-3'), followed by GAP 3F (5'-GTG AAG GCG CAG CGC AAC-3') and GAP 5R (5'-CCG AGG ATG YCC TTC ATG-3') (Fermino et al. 2015). For both molecular markers, PCR reaction mixtures and conditions were performed as described previously (Borghesan et al. 2013, Fermino et al. 2019). Sequences from 5–10 clones of each amplified sample were determined, screened for chimera by the RDP4 package, and those that were representatives of the whole genetic diversity will be deposited in GenBank®.

Phylogenetic analyses of *SSU* rDNA and g*GAPDH* sequences

The newly generated sequences were aligned with sequences retrieved from GenBank® using Clustal W and MUSCLE programs (Thompson et al. 1994). Three alignments were created: (i) consisting of the V7V8 region of the *SSU* rDNA gene; (ii) including g*GAPDH* sequences; and (iii) concatenated V7V8 *SSU* rDNA and g*GAPDH* genes sequences. We employed sequences from trypanosomes found in *U. superciliosus* and specimens representing the main clades, plus using non-trypanosome trypanosomatids as an outgroup. For the reconstruction of phylogenetic relationships, Maximum Likelihood (ML) and Bayesian inference (BI) analyses were employed as described previously (Teixeira et al. 2011, Fermino et al. 2013, 2015, 2019). The ML analysis was performed using RAxML v8.2 (Stamatakis et al. 2014). Tree searches employed GTRGAMMAI model and proportion of invariable sites, model parameters were estimated in RAxML over the duration of tree search, and bootstrap supports were estimated with 500 replicates. The BI analysis was conducted using MrBayes (Ronquist et al. 2012) with GTRGAMMAI; the first 25% of the tree was discarded as 'burn in'. The specimens included in the phylogenetic trees analyses and their respective GenBank

accession numbers are shown in the phylogenetic trees. Divergence between g*GAPDH* sequences were calculated using the *p*-distance model (Nei and Kumar 2000) and were conducted in MEGA7 (Kumar et al. 2016).

Results

Prevalence and morphology of blood trypanosomes

We detected trypanosomes in blood smears of 83 (71%) out of 116 *U. superciliosus*. The prevalence for the two collection sites, FEX-UFAM and AREI-BDFFP (Fig. 1), were 72% (n = 8/11) and 71% (n = 75/105), respectively.

Trypomastigote forms observed through light microscopy presented morphological variation, including rounded, elliptical, leaf-shaped and fusiform parasites (Fig. 2). It is important to note that these morphologies occurred together in some blood smears (37%; n = 44/116). Below we highlight the main morphological (Fig. 2) and morphometric (Table 1) characteristics by comparing shape, size, kinetoplast position and features of the nucleus and undulating membrane of these flagellates.

Rounded and elliptical trypanosomes (Fig. 2a-d; Table 1) present broad bodies (mean dimensions 45.2 ± 9 × 22.5 ± 4.7 μm) and short free flagellum (12.2 ± 6.9 μm). The sausage-shaped nucleus is in a marginal position and situated close to the small kinetoplast. Spherical trypomastigotes (Fig. 2a) has conspicuous undulating membranes and the presence of many cytoplasmic vacuoles. Flagellates with ellipsoid bodies (Fig. 2b-d) present many-folded undulating membranes and inside to it the flagellum with a prominent line.

Leaf-shaped flagellates (Fig. 2e-h; Table 1) possess large and wide cells (mean dimensions 78.2 ± 12.7 × 21.6 ± 4.9 μm), with rounded posterior ends. The nucleus is long, thin, diffuse and located at posterior end of the body with the kinetoplast appended to it (Fig.

2f). Free flagellum is long (21 ± 10.7 μm) and undulating membrane is well-developed and many-folded. Cytoplasm is dark stained and, in some cases, with granules or vacuoles (Fig. 2g).

Fusiform trypomastigotes (Fig. 2i-l; Table 1) show large and elongated bodies tapered at both ends (mean dimensions 98.9 ± 10.8 × 17.8 ± 3.4 μm). The nucleus has an irregular appearance or, in some cases, sausage-shaped, and it is disposed laterally in the middle part of the body. Kinetoplast is small dark stained and placed posterior to the nucleus. The undulating membrane was well-developed and the free flagellum was short (11.7 ± 5.6 μm). The dark stained cytoplasm sometimes made the nucleus and kinetoplast hard to discern, and in several parasites contained granules or vacuoles (Fig. 2i-j). Both extremities are generally lighter stained than the rest of body and the posterior end exhibited cytoplasmic projections of filamentous aspects. These morphologies were the most predominant compared to the previous ones, occurring at greater numbers in the blood samples.

Barcoding and phylogenetic analyses

The comparison of the alignment of V7V8 *SSU* rDNA barcode sequences and their position in the derived phylogenetic tree (ML; Fig. 3a), revealed the presence of two different genotypes of the genus *Trypanosoma* (Genotype 01 and Genotype 02) among 11 isolates obtained from the blood of *U. superciliosus.* The novel isolates clustered in the main monophyletic Aquatic clade with other trypanosomes lineages from herpetofauna, fishes, platypus and leeches. These genotypes grouped into a well-supported monophyletic clade within a major assemblage of *Trypanosoma* spp. from anurans.

Inferred phylogenetic relationships (ML and BI analyses) using g*GAPDH* sequences (Fig. 3b) and concatenated *SSU* rDNA and g*GAPDH* sequences (Fig. 4) supported the position of the newly generated sequences in the Aquatic clade. They also showed a new monophyletic cluster composed by the two new genotypes plus the lineage obtained from the South American lizard

Notomabuya frenata (COPE, 1862) (TCC878; Caballero et al. 2015). This lizard clade was closely related to the trypanosome lineages from neotropical anurans comprised by An01 and An02 clades (Attias et al. 2016). Interestingly, although there were slight differences on the position of the subclades of this group, the topology recovered in our g*GAPDH* and concatenated analysis indicated that the Anuran clade comprised a polyphyletic group with reasonable support nodes values (Figs. 3 and 4), forming four monophyletic clades: (i) An04; (ii) *Trypanosoma loricatum* (Mayer, 1843) (MH428670; Spodareva et al. 2018); (iii) An03/An05; and (iv) An01/An02. Furthermore, the sequences from our study were positioned distinct of trypanosome isolates from other reptilian hosts placed into Terrestrial clade. The divergence of g*GAPDH* sequences between trypanosomes isolated in this study and *Trypanosoma* spp. from other lizards and snakes ranged from ~4.3% (*N. frenata* [TCC878]) to ~24% (Lizard-Snake/Rodent-Marsupial clade). The distance separating Genotype 01 and Genotype 02 was ~3.3%. Intraspecific divergence of g*GAPDH* sequences was 1.5% in Genotype 01 and 0.25% in Genotype 02.

Based on the phylogenetic positioning and molecular data we considered the two trypanosome genotypes isolated from *U. superciliosus* as two species, with at least one of them being new (see the Discussion Section below). Unfortunately, as we could not to associate the trypomastigote forms with the obtained sequences, we are unable to taxonomically describe them at this moment. Lizards with Genotype 01 and Genotype 02 shared trypanosomes with the same morphological features in their blood smears. In addition, we noted that in g*GAPDH* analyzes some specimens (L285 and L331) had trypanosome sequences from both genotypes, indicating mixed infections.

Discussion

In this study, we provide the first molecular data of trypanosomes from an Amazonian lizard species and we were able to detected two novels genotypes. Lizard trypanosomes, despite their relative high species richness (ca. 50 species), still constitute one of the least researched groups within the genus *Trypanosoma*, with few molecular information assessed thus far (Telford 2009, Hamilton et al. 2004). Precisely, there are only three sequences associated with described species (Lukeš et al. 1997, Haag et al. 1998): *Trypanosoma varani* Wenyon, 1908, *Trypanosoma therezieni* Brygoo, 1963 and *Trypanosoma scelopori* Ayala, 1970. In addition, beyond the new isolates from our research, the current knowledge about the distribution and evolution of reptilian trypanosomes in the Neotropical region lies mostly on species that parasitize caimans (Viola et al. 2008b, Fermino et al. 2013, 2015, 2019), with few lineages derived from squamates (Viola et al. 2009, Caballero et al. 2015). This scarcity of phylogenetic data can lead to a superficial understanding of virulence, host switches and diversification among trypanosomatid species (Heath et al. 2008). Consequently, on a large-scale view, such gap may have implications for the development of more efficient treatments to combat diseases caused by pathogenic trypanosome species (Telleria and Tibayrenc 2017).

Another interesting finding concerns to the high prevalence of trypanosomes observed through light microscopy: most of the sampled *U. superciliosus* were positive and many of them had large numbers of parasites. This is an intriguing result, because usually trypanosomes were found at low prevalence on lizard hosts (Telford 2009), probably due to low parasitemia in peripheral blood which can lead to a false negative. Furthermore, as previously discussed (see Picelli et al. 2020), there are two other studies involving trypanosomes research in this host species at the Brazilian Amazon (Walliker 1965, Lainson et al. 1975). However, there are differences between these two surveys and ours. Lainson et al. (1975) examined blood slides of a reasonably larger number of *U. superciliosus* (n = 167) in the Eastern Amazonia than we did here, and yet no parasitized individuals were found, which is quite curious given the authors'

extensive research on hemoparasites carry out in that region (Lainson 1992, Shaw 2019). In the case of the Walliker's study (1965) in the municipality of Codajás (Amazonas, Brazil) despite the author have found trypanosomes, we cannot compare the prevalence data, as there was no mention on the number of lizards examined, but it was reported that parasites were abundant in the blood smears and also in liver tissue sections. For now, we cannot explain why this peculiar prevalence of trypanosomes is occurring on *U. superciliosus* in this study region, since we have not tested any hypothesis related to this here. However, it is possible to speculate that intrinsic characteristics of the sampling sites may be determining the presence of parasites and as well as vectors. In fact, in many circumstances landscape features were considered as a proxy for hemoparasites prevalence and parasitemia in wild fauna (Ferreira et al. 2017, Carbayo et al. 2019, Kubacka et al. 2019, Pérez et al. 2019, Werner et al. 2020) but, to date, no ecological study has used landscape metrics to predict interactions between trypanosomes and lizards.

With regard to trypomastigote forms, we found a remarkable morphological diversity in *U. superciliosus*, which can be considered as evidence for the occurrence of co-infections as much as phenotypic plasticity. Here, the molecular data confirmed the presence of mixed infections in these hosts, which we believe to be the first record in lizards. But, as trypanosomes species commonly exhibit polymorphic trypomastigotes (Ferreira et al. 2007, Spodareva et al. 2018), we consider that both situations are actually happening in our study. Although we are aware of the problems attributed to trypomastigote morphologies (Gu et al. 2007, Thompson et al. 2013, Hayes et al. 2014), we think it is taxonomically relevant to compare them with trypanosome species formerly described from tropidurid lizards (*T. plicaplicae*, *T. plicae* and *T. superciliosae*). In *T. plicaplicae*, trypomastigotes have undeveloped undulating membrane, lightly stained cytoplasm and compacted oval nucleus (Telford et al. 1996), characteristics markedly different from those observed here. We also noted that the leaf-shaped parasites (Fig. 1e-h) resembled in some aspects with *T. plicae*, such as shape, presence of cytoplasmic granules

and diffuse elongated nucleus, however the dimensions of the trypomastigotes of *T. plicae* (49–64 ×10–21 μm [Lainson et al. 1975]) differed from those of our study (47–102.6 × 13.3–32.9 μm). With respect to *T. superciliosae*, its morphological description matches the fusiform flagellates (Fig. 1i-l) that we observed here: both trypomastigotes had large bodies lengths (this study: 74.7–124 μm; and description made by Walliker [1965]: 87–124 μm), filamentous cytoplasmic extensions and irregular shaped nuclei (Walliker 1965). Those features could be considered, according to Walliker (1965), as the main keys for the diagnosis of *T. superciliosae* and, based on these attributes, we think that we might rediscovered this taxon. However, as there is no previous molecular data to *T. superciliosae* and due to the variety of morphologies observed, we are not sure of which the two new isolated genotypes would belong to this species and whether the fusiform trypomastigotes can be considered as the only representatives of *T. superciliosae*. To solve this puzzle, it is necessary to isolate those parasites in culture media, which we unsuccessfully tried to do so far, and obtain morphological data from other life cycle stages as well as reliable molecular information from pure isolates.

Overall, our phylogenetic analyzes emphasized the polyphyletic origin of lizard trypanosomes, demonstrating that there are at least three different evolutionary adaptations for these hosts: (i) a terrestrial lineage that together with trypanosomes from snakes and mammals form the Lizard-Snake/Rodent-Marsupial clade (Viola et al. 2008a, Ortiz et al. 2018); and two aquatic lineages that constitute two independent subclades related to anuran trypanosomes – a (ii) subclade consisting of *T. therezieni* plus anuran sequences (clade An04) from North America, Europe and Africa (Haag et al. 1998, Spodareva et al. 2018); and the (iii) subclade comprising trypanosomes from lizards *U. superciliosus* (this study) and *N. frenata* (TCC878) and anurans (clade An01/An02) from South America (Attias et al. 2016, Cabalero et al. 2015). An interesting point about this last aquatic subclade is that all sequences come from several Brazilian biomes, including other samples from the Amazonian region (Caballero et al. 2015,

Ferreira et al. 2008). This could be a possible biogeographic pattern; however, a larger sampling of lizard trypanosomes is needed to test such hypothesis. Moreover, phylogenetic relationships among lizard trypanosomes are still poorly understood, but some studies have proposed that similar ecological features of host species and/or the use of the same vector group may shape their evolutionary history (Stevens et al. 2001, Viola et al. 2008a).

Host ecology has a considerable importance in shaping evolution among parasites (Poulin and Morand 2004) and for lizard trypanosomes it is possible to recognize that there are certain similarities on natural history of hosts positioned within the same clade as well as differences between those who are in distant clades. For example, lizards [*Varanus exanthematicus* (Bosc, 1792), *Tarentola annularis* (Geoffroy Saint-Hilaire, 1827) and *Sceloporus jarrovii* Cope, 1875] and snakes [*Crotalus durissus* Linnaeus, 1758 and *Pseudoboa nigra* (Duméril et al. 1854)] harboring parasites from the Terrestrial clade are hosts species commonly found on the ground in open areas of savannas or in desertic environments (Gadsden et al. 2007, Orofino et al. 2010). Differently, *U. superciliosus*, *N. frenata* and *Calumma brevicorne* (Günther, 1879), host species from Aquatic clade, are arboreal and semi-arboreal lizards that inhabit riparian areas of dense or sparse humid forests (Raxworthy and Nussbaum, 2006, Ribeiro-Júnior and Amaral 2016b, Faria et al. 2019). However, trypanosomes of lizard in the Aquatic clade did not form, like squamates in Terrestrial clade, a monophyletic group and the use of analogous microhabitats by those hosts may be just the result of an adaptive convergence between them. Furthermore, the sequences positioning within one of those major clades does not necessarily reflect the environment used by the reptilian host, as in the case of the South American caimans, which exhibit mostly aquatic habit use and the lineages that infect them are allocated in both Terrestrial and Aquatic clades (Fermino et al. 2015, Fermino et al. 2019).

Subclades formed by lizard isolates in both Terrestrial and Aquatic clades do not constitute exclusive groups of hosts from the order Squamata. Actually, lizard trypanosomes were more

closely related to other classes of vertebrates than with other reptilian flagellates (Haag et al. 1998, Dario et al. 2017, Ortiz et al. 2018). Previous studies demonstrated that in Terrestrial clade, the cluster composed by trypanosomes from lizards, snakes, bats, rodents and marsupials most likely presented sandflies as vectors (Ayala 1970, Minter-Goedbloed et al. 1993, Hamilton et al. 2007, Viola et al. 2008a, Ortiz et al. 2018). For the aquatic lineage comprised by *T. therezieni* the vector incrimination remains unclear, but it is expected to be by an insect given the microhabitat of its chameleon host (Hamilton et al. 2007). Further, anuran trypanosomes from clade An04 that are related to *T. therezieni* do not share a same group of vectors: some species are transmitted by leeches and others by mosquitoes (Desser et al. 1973, Martin and Desser 1991), which hampers any inference for this lizard/anuran clade. With respect to trypanosomes from *U. superciliosus* and *N. frenata* ithey are likely transmitted by sandflies, because their sister-group clade An01/An02 are anuran trypanosomes transmitted by these hematophagous insects (Ferreira et al. 2008). Therefore, clades with lizard trypanosomes may be showing also a common vector association, which may elucidate the proximity between trypanosomes from phylogenetically distant vertebrate hosts. In this sense, it is possible that hosts, such as lizards and frogs, with similar lifestyles living within the same environmental gradient, could harbor more closely-related trypanosomes because at some point in their evolutionary stories they may have shared vectors. To test this hypothesis, it would be necessary to return to our study areas and collect samples from arboreal frogs and also from potential vectors, to see where they fit phylogenetically.

Taken together, our analyzes also supported that trypanosomes from hosts such as *U. superciliosus*, whose life history depends mostly on aquatic environments, are positioned within the Aquatic clade. The relationship of the two major trypanosome clades with the environments, from which they carry their names, was observed in the first molecular phylogenies that revealed a small monophyletic group composed by isolates of leech-transmitted fish

trypanosomes that were named as Aquatic clade (Stevens and Gibson 1999, Stevens et al. 2001, Hamilton et al. 2004). Despite the continuous debate on the drivers of trypanosome diversity (Pérez et al. 2019), recent phylogenetic reconstructions reinforce the strong connection of these parasites with the microhabitat of their hosts (Fermino et al. 2013, Lemos et al. 2015, Ortiz et al. 2018, Pérez et al. 2019). Furthermore, the presence of some "intrusive" host species, whose predominant habitat does not correspond exactly with the trypanosome clade that it is inserted, it is absolutely plausible considering the vagility of the hosts and the fact that in many natural systems, like the continuous environmental gradient of tropical forests, there are no barriers that constraint aquatic and terrestrial environments (Faria et al. 2019, Pérez et al. 2019). Interestingly, this absence of boundaries is quite strong among the lizards in the Central Amazonia, as demonstrated by Faria et al. (2019), where few species are restricted to riparian zones and most of them can be found occurring along the entire forest gradient.

In conclusion, this study revealed the presence of an intriguing morphological and molecular diversity of trypanosomes from the diving lizard *U. superciliosus* in Central Amazonia. Thus, we demonstrate that this lizard species harbors more than one genotype of trypanosome at the same geographic area, as well as within same host individuals. Finally, our study also contributed with new data on the phylogenetic relationship of *Trypanosoma* in lizards and other vertebrates and their association to host's habitat use, emphasizing how the knowledge about the diversity of Amazonian biodiversity remains neglected for certain group of organisms such as trypanosomes.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval All procedures on lizards were performed according to the recommendations and approval of the ethics committee on animal use from UFAM (protocol number 012/2016), and were authorized by the Brazilian Ministry of the Environment (SISBIO number 53851 and SISGEN AA6199D).

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Table 1. Morphometric characteristics of the trypomastigotes found in the lizard *Uranoscodon superciliosus* from Central Amazonia. Brazil. Measurements are presented as mean ± standard deviation (SD) followed by the range (maximum and minimum values).

Morphology	*n*	Body length [a] (μm)	Body width (μm)	Free flagellum (μm)	Kinetoplast to posterior end of the body (μm)
Rounded or elliptical	15	45.2 ± 9 (32.1–65.7)	22.5 ± 4.7 (13.4–31.8)	12.2 ± 6.9 (2.5–25.8)	16.2 ± 4.5 (5.9–23.6)
Leaf-shaped	30	78.2 ± 12.7 (47–102.6)	21.6 ± 4.9 (13.3–32.9)	21 ± 10.7 (5.1–45.9)	13.1 ± 8.1 (3.5–38.8)
Fusiform	30	98.9 ± 10.8 (74.7–124)	17.8 ± 3.4 (11–24)	11.7 ± 5.6 (4.7–26.6)	42.2 ± 7.4 (29.9–63)

[a] Measure taken without the free flagellum.

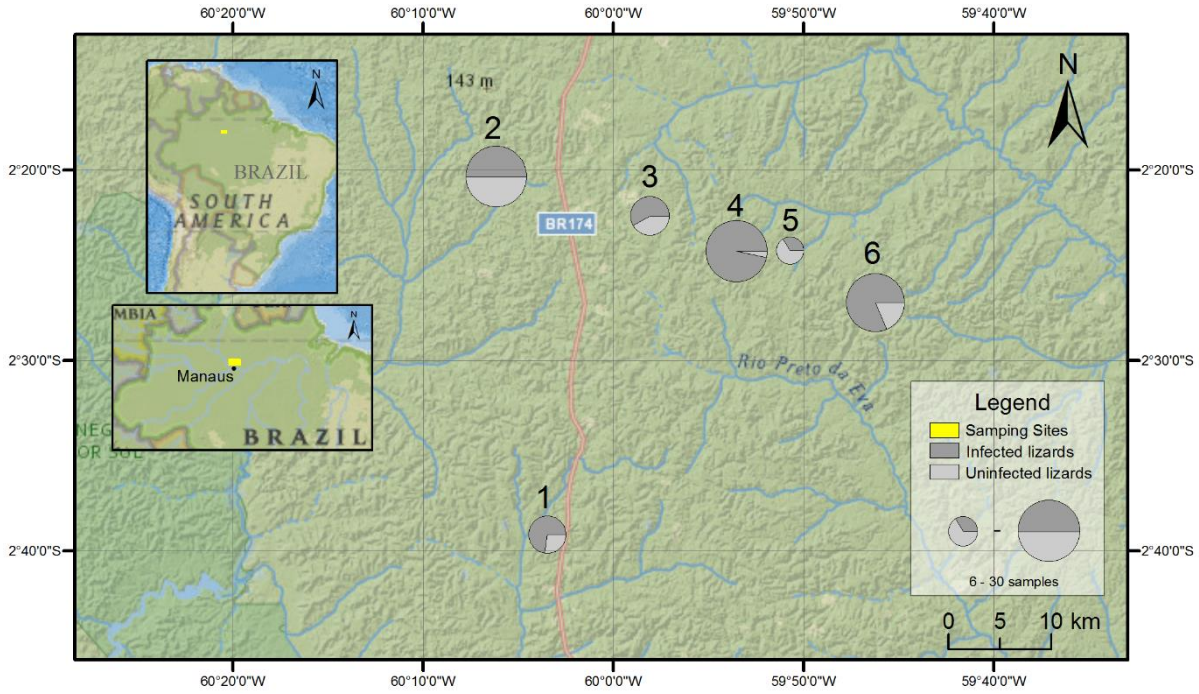


Fig. 1 Geographical origin of isolates of *Uranoscodon surpercilosus* trypanosomes in Central Amazonia, Brazil. (1) Experimental Farm of the Federal University of Amazonas – FEX-UFAM; and (2-6) Area of Relevant Ecological Interest of the Biological Dynamics of Forest Fragments Project – AREI-BDFFP. Tones of gray indicate the trypanosome prevalence, and the sizes of the circles indicate the number of individuals analyzed per site.

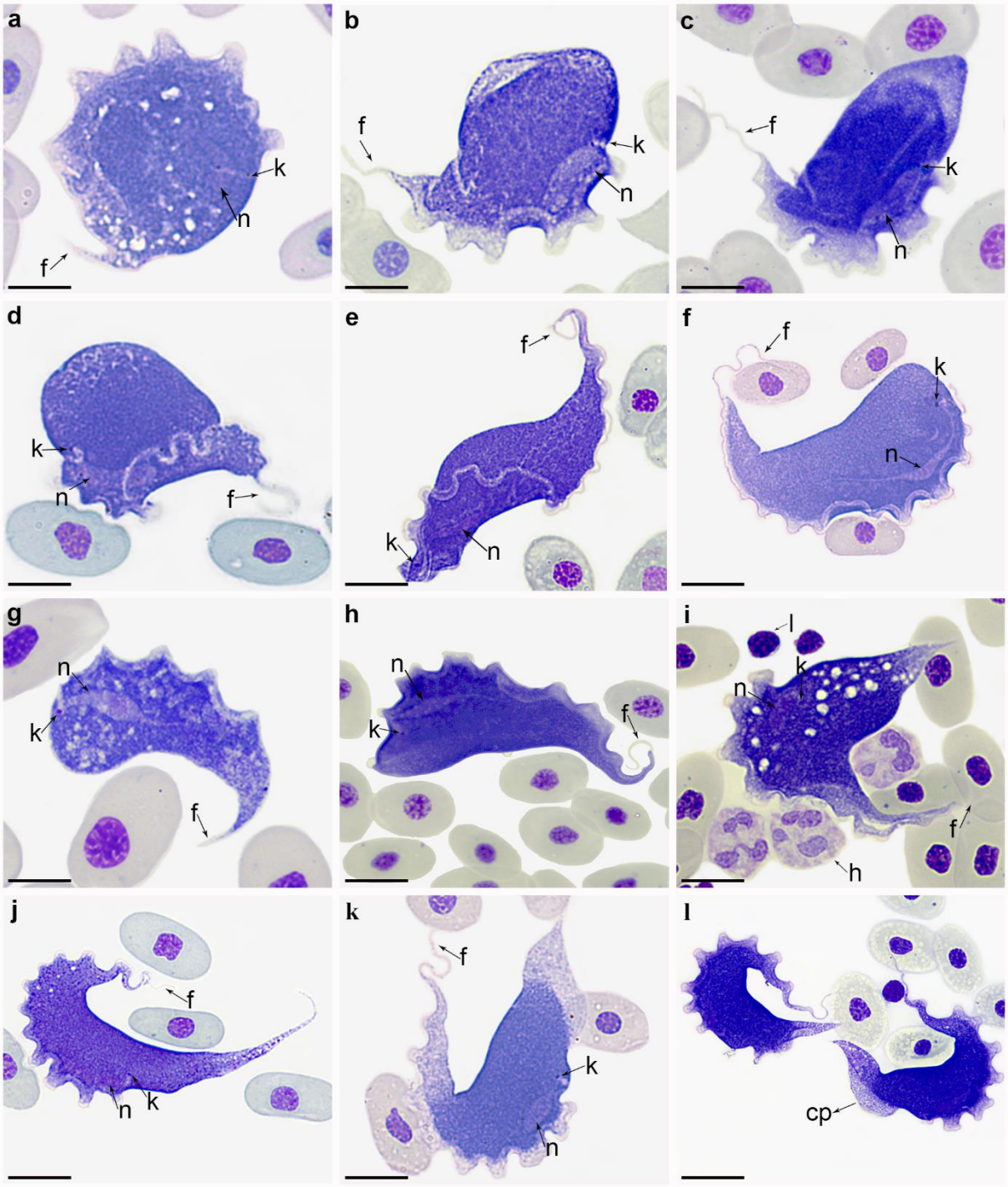


Fig. 2 Trypomastigote forms found in the peripheral blood of *Uranoscodon superciliosus* from Central Amazonia, Brazil. (**a – d**) Rounded or elliptical morphologies; (**e – h**) leaf-shaped trypanosomes; (**i – l**) elongated forms with cytoplasmic projections. Abbreviations: n, nucleus; k, kinetoplast; f, flagellum; cp, cytoplasmic projection. Micrographs are from Giemsa-stained thin blood films. Scale bar is 10 μm.

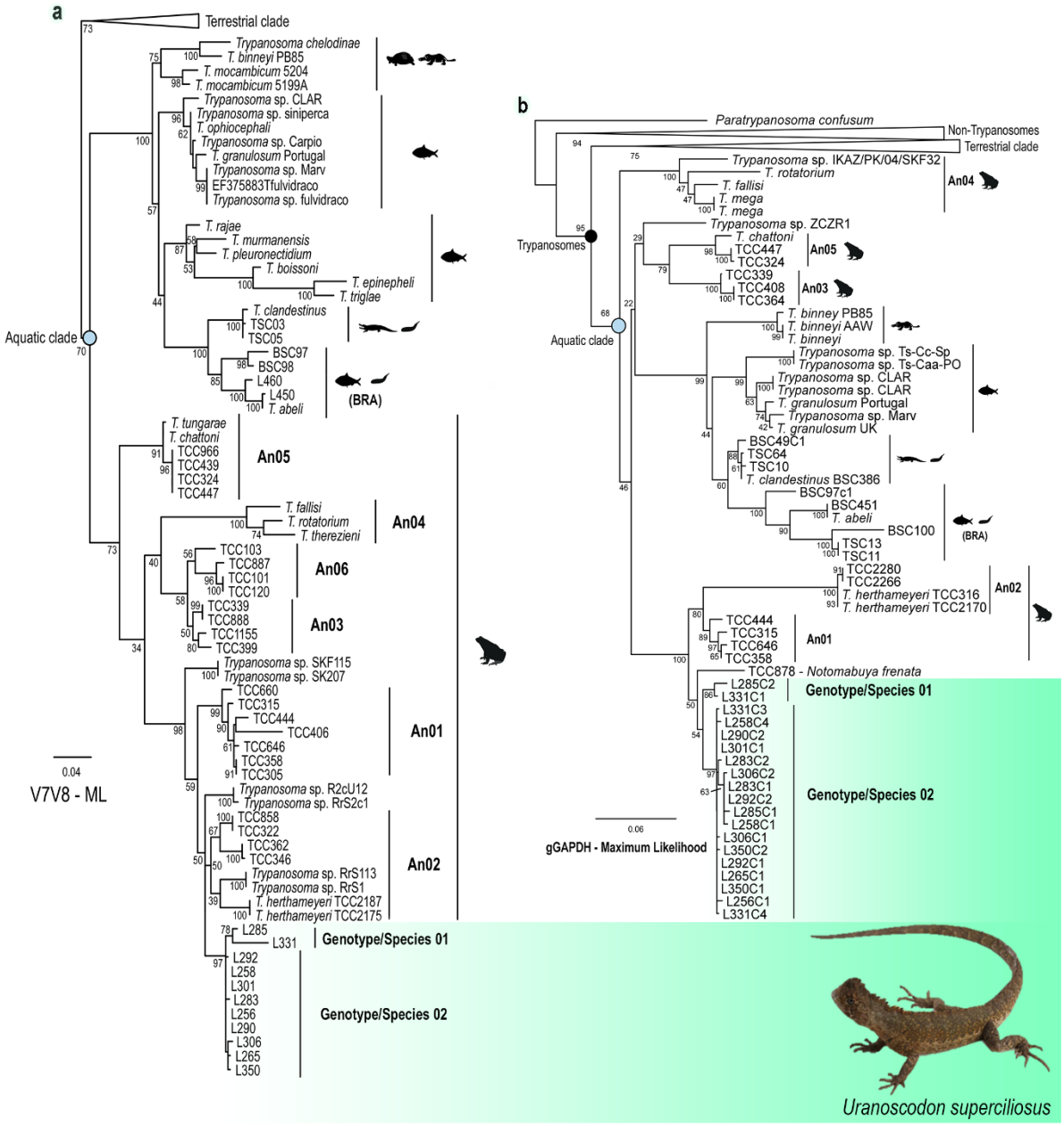


Fig. 3 Phylogenetic positioning of trypanosomes of *Uranoscodon superciliosus* from Central Amazonia. Phylogenetic trees (ML) inferred from V7V8 *SSU* rDNA (**a**) and by *gGAPDH* (characters 812, Ln = −8826.807868) (**b**) gene sequences. Maximum Likelihood inference (characters 606, Ln = −5963.950862) supported the Genotype 01 and Genotype 02 in the Aquatic clade. Trypanosomes of the terrestrial lineages and trypanosomatids of other genera were used as outgroups. Bootstrap values are given under the nodes.

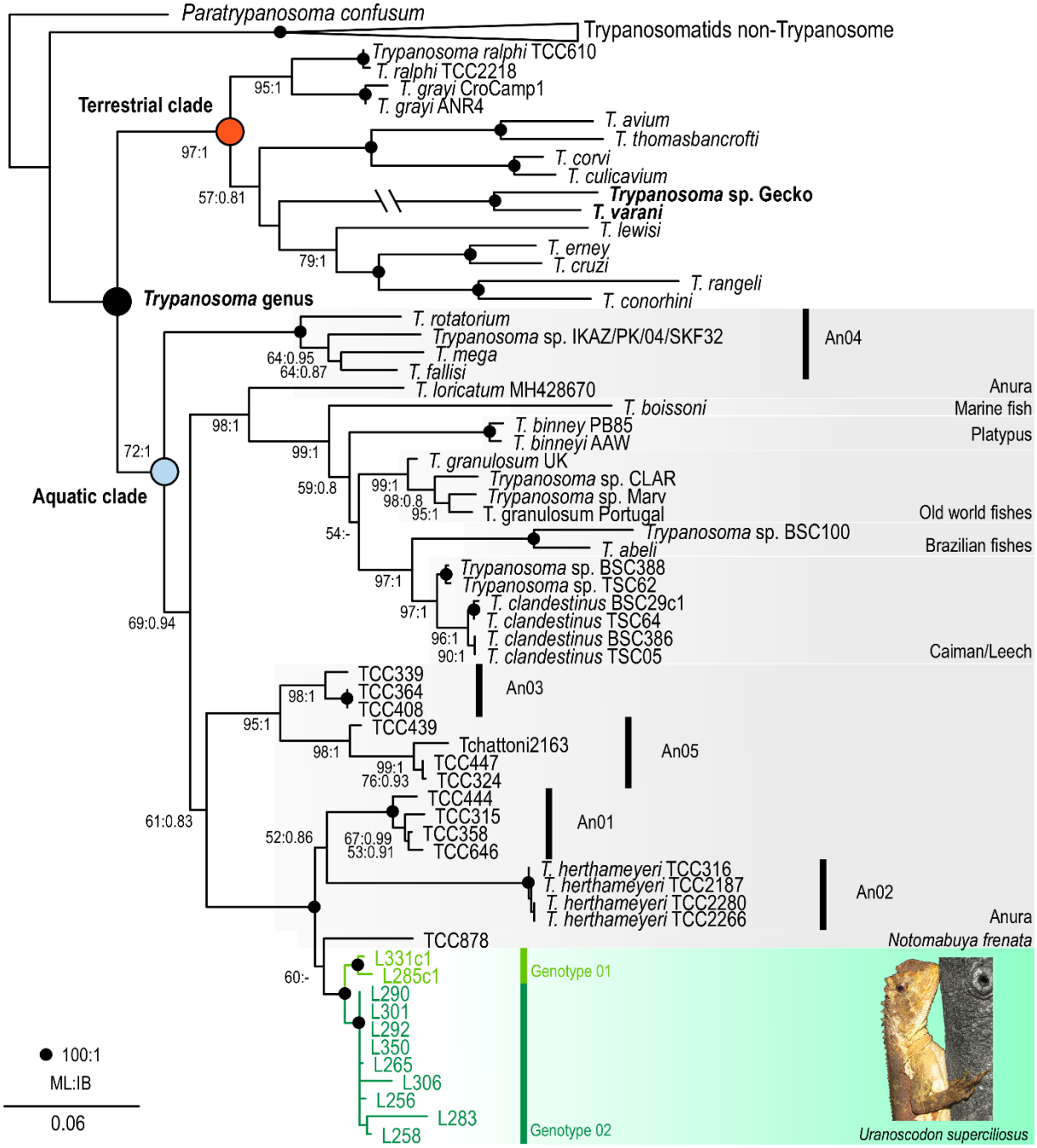
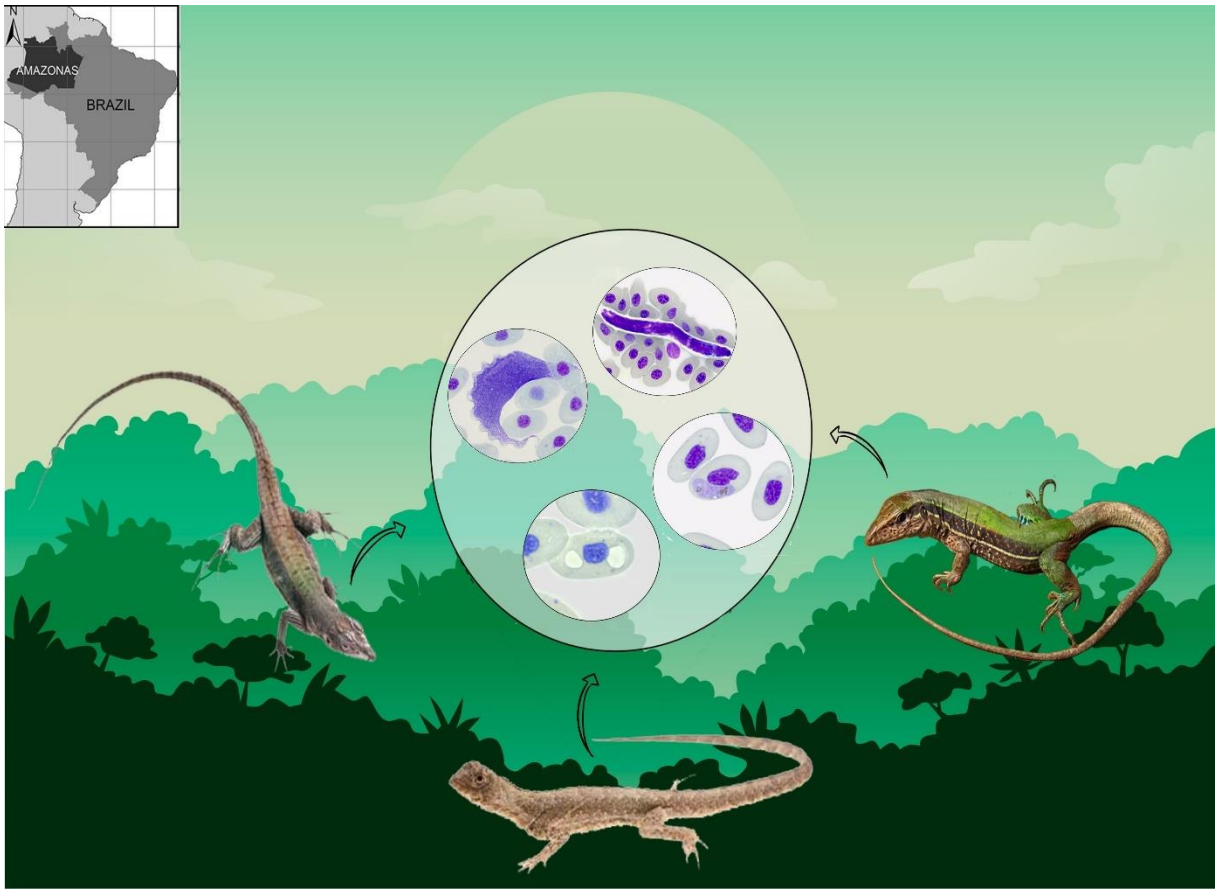


Fig. 4 Phylogenetic analysis (ML) based on concatenated *SSU* rDNA and *gGAPDH* gene sequences of *Trypanosoma* spp. from *Uranoscodon superciliosus* isolated in this study. The analyses were inferred by Maximum Likelihood (ML, 1,318 characters, Ln = −17092.752369) and Bayesian Inference (BI). The analyses include species representative of all major clades within the genus *Trypanosoma*, and trypanosomatids of other genera as outgroups. Numbers at nodes (ML/BI) are bootstrap supports (> 50%) and Bayesian posterior probabilities (> 0.8) derived from 500 replicates.



CONSIDERAÇÕES FINAIS

De um modo geral, os resultados obtidos nesta tese contribuíram para o conhecimento sobre os hemoparasitos que infectam lagartos, ampliando as informações sobre a diversidade e distribuição desses organismos e seus hospedeiros, um sistema de estudo até então negligenciado por grande parte dos zoólogos e parasitólogos no Brasil.

No **Capítulo 1**, realizamos o primeiro levantamento de hemoparasitos em assembleia de lagartos na Amazônia Central, demostrando que essa região não abriga apenas uma fauna diversificada desses hospedeiros, mas também uma elevada riqueza de hemoparasitos com potencial para novas espécies. Tal diversidade pôde ser acessada mesmo sem o uso das modernas ferramentas moleculares, dada a alta prevalência nesses hospedeiros. Neste capítulo também produzimos uma lista com os estudos realizados até o momento no Brasil sobre esses parasitos e hospedeiros, mostrando que há uma grande lacuna nessa área de estudo no país, uma vez que a maior dos biomas brasileiros e de suas espécies de lagartos ainda não tiveram seus hemoparasitos investigados.

Com relação ao **Capítulo 2**, fornecemos pela primeira vez dados moleculares e morfológicos de uma hemogragarina de lagarto no Brasil, *Hepatozoon ameivae*, o que levou à redescrição dessa espécie. Além disso, inferimos a relação filogenética desse parasito com outras hemogregarinas da herpetofauna, mostrando uma proximidade com espécies que infectam serpentes e a existência de um provável padrão biogeográfico para esses parasitos.

O **Capítulo 3** revelou que *Uranoscodon supercilisous* é hospedeiro de pelo menos dois novos genótipos de tripanosomas, sendo que um deles provavelmente pertence a uma espécie já descrita nesse lagarto, *Trypanosoma superciliosae*. Esses tripanosomas, além de serem geneticamente diversos, exibiram grande variabilidade em suas formas e tamanhos. Além disso, os dados sobre a relação filogenética desses parasitos demonstraram uma forte associação com o uso do habitat do hospedeiro, reforçando nossa hipótese ecológica-evolutiva.

Por fim, os resultados encontrados aqui poderão embasar futuros estudos taxonômicos, ecológicos e evolutivos com esses hemoparasitos e seus lagartos hospedeiros. Esperamos que essa tese incentive a outros pesquisadores a se engajarem nesse fascinante tema.

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Poder Executivo
Ministério da Educação
Universidade Federal do Amazonas
Comissão de Ética no Uso de Animais

UFAM

CERTIFICADO

Certificamos que o Projeto de Protocolo n° **012/2016– CEUA / UFAM**, intitulado como "Diversidade de hemoparasitos (Protozoa: Apicomplexa) em lagartos amazônicos", sob a orientação do **Dr**. Igor Luis Kaefer e na responsabilidade de Amada Maria Picelli, aluna do Programa de Pós- Graduação em Zoologia- UFAM, está de acordo com a Legislação Federal pertinente ao uso científico de animais e foi **APROVADO** pelo COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL (CEEA-UFAM).

Manaus, 10 de abril de 2017.

Profa. Dra. Cinthya Iamile Frithz Brandão de Oliveira
Presidente do CEUA-UFAM

Portaria 1595/2014 - GR

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº AA6199D

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**AA6199D**
Usuário:	**Fiocruz**
CPF/CNPJ:	**33.781.055/0001-35**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie
Ameiva ameiva
Kentropyx calcarata
Tupinambis teguixin
Cnemidophorus sp
Uranoscodon superciliosus
Plica umbra
Arthrosaura reticulata
Leposoma percarinatum
Neusticurus bicarinatus
Cercosaura argulus
Anolis punctatus
Anolis fuscoauratus
Anolis planiceps

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 53851-4	Data da Emissão: 11/12/2017 15:17	Data para Revalidação*: 10/01/2019

* De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: AMANDA MARIA PICELLI	CPF: 373.027.428-71
Título do Projeto: Diversidade de hemoparasitos (Protozoa: Apicomplexa) em anfíbios e lagartos amazônicos	
Nome da Instituição : FUNDAÇÃO UNIVERSIDADE DO AMAZONAS - FUA	CNPJ: 04.378.626/0001-97

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coleta e identificação de potenciais vetores	09/2016	09/2017
2	Captura de lagartos e amostragem sanguínea	09/2016	09/2018
3	Levantamento bibliográfico	09/2016	12/2019
4	Monitoramento da infecção	09/2016	09/2017
5	Identificação dos hemoparasitos	09/2016	09/2018
6	Análise dos dados	01/2017	08/2019
7	Preparação e submissão de artigos	07/2017	08/2019
8	Caracterização do desenvolvimento endógeno	09/2017	03/2018
9	Caracterização molecular do parasitos	03/2018	06/2019
10	Redação da tese	05/2019	11/2019
11	Defesa	01/2020	01/2020

Observações e ressalvas

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8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Outras ressalvas

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