



UNIVERSIDADE FEDERAL DO PARÁ
CAMPUS UNIVERSITÁRIO DE ALTAMIRA
FACULDADE DE CIÊNCIAS BIOLÓGICAS

GLEICIERLE RODRIGUES PAIVA

ABORDAGEM MOLECULAR NA IDENTIFICAÇÃO DE ESPÉCIES
CANDIDATAS DE *Hepatozoon* sp.(APICOMPLEXA: HEPATOZOIDAE)

ALTAMIRA-PA

2018

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Trabalho de Conclusão de Curso
apresentado à Faculdade de Ciências
Biológicas da Universidade Federal do
Pará, Campus de Altamira, como
requisito parcial para obtenção do grau de
Licenciado em Ciências Biológicas.

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Trabalho de Conclusão de Curso
submetido à aprovação como requisito
parcial do grau de Licenciado em
Ciências Biológicas, pela banca
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Altamira – PA, ____Dezembro de 2018

Dedico este trabalho à minha mãe que nunca
desiste de mim.

AGRADECIMENTOS

Ao professor Emil Hernández pelo suporte e incentivo durante o processo de definição e orientação, que através das críticas, cobranças e conselhos me mostrou que se dedicar é o melhor caminho e que sempre podemos fazer melhor.

Ao Elciomar Araújo por em todo esse período, acreditar que seria possível, pela amizade, paciência e risos no laboratório durante as etapas que percorremos para que esse trabalho pudesse ser concluído.

A Jamille Corrêa por todo apoio, paciência e atenção com que me “aturou” e se dispôs a ajudar e colaborar mesmo distante geograficamente.

A minha mãe, agradeço imensamente por nunca desistir de mim e mesmo sem poder ajudar financeiramente, sempre se mostrou forte para poder ser o meu alicerce.

A minha irmã que nos dois últimos anos da graduação veio morar comigo e me ajudou de forma significativa a segurar a barra por aqui.

A toda família Costa Carneiro, que me acolherem e me deram não somente um abrigo, mas um lar muito acolhedor, destaco em especial Cristiane Costa pela confiança e pelo incentivo na vida profissional.

Ao Aderson Alcântara um grande amigo que sempre deu todo apoio e incentivo. Obrigada pela participação que têm na minha vida.

A Gilcilene Cornélio pelo companheirismo e cumplicidade.

Ao meu grupo particular; por me suportarem na crises de estresse, pelos abraços e por toda diversão nos rolês, especialmente à Gabriela Wemilly, Maria Karoline, Tamires Pereira, Adriele Praia, Andressa Nogueira e Raryana Leite por todo companheirismo, apoio, paciência, cumplicidade e carinho, mesmo nos momentos em que eu fui “grossa”. Não posso deixar de acrescentar a dona Francisca Alves.

Aos colegas e amigos do Laboratório de zoologia, da Faculdade de Ciências Biológicas da UFPA de Altamira e aos professores do curso pelos ensinamentos.

Aos colegas do trabalho que foram parceiros e sempre se preocuparam verdadeiramente comigo.

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O manuscrito será submetido ao periódico *Systematic Parasitology*.

Abordagem Molecular na Identificação de espécies candidatas de *Hepatozoon* sp.

(Apicomplexa: Hepatozoidae)

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Resumo

PAIVA, G.R. **Abordagem Molecular na Identificação de espécies candidatas de *Hepatozoon* sp. (Apicomplexa: Hepatozoidae)**. Trabalho de Conclusão de Curso, Universidade Federal do Pará, Altamira, 2018, 11 p.

Hepatozoon spp. são protozoários intracelulares mais frequentes em serpentes. O objetivo deste estudo foi a utilização da abordagem molecular pelo método Automatic Barcode Gap Discovery, para identificar espécies candidatas do gênero *Hepatozoon*, parasitando as serpentes: *Clelia clelia* e *Drymarchon corais*. Das serpentes foram extraídas amostras sanguíneas e delas o DNA, do qual foi amplificado um fragmento do gene nuclear 18S por meio da Reação de Cadeira em Polimerase. A análise filogenética, indicou que o *Hepatozoon* sp. de das duas espécies de serpentes formam grupos distintos das outras espécies do gênero. O alto valor da probabilidade posterior (99%), indica que *Hepatozoon* sp. de *C. clelia* e *D. corais* representam linhagens irmãs, embora mais dados sejam necessários para inferir as relações filogenéticas entre elas. O resultado do ABGD identificou a existência de 33 grupos (prior maximal distance $P= 0.001$) para a partição inicial, classificando *Hepatozoon* sp. de *C. clelia* e de *D. corais* como sendo dois taxa distintos, podendo ser consideradas como espécies candidatas não confirmadas. Poucos estudos tratam do relacionamento entre as espécies de *Hepatozoon*, sendo assim os dados morfológicos tradicionalmente usados na taxonomia desses grupos possibilita significativamente a comparação com outras espécies de *Hepatozoon*, contribuindo para a descrição da diversidade de espécies do gênero.

Abstract

PAIVA, G.R. **Molecular Approach in Identification of candidate species of *Hepatozoon* sp. (Apicomplexa: Hepatozoidae)**. Trabalho de Conclusão de Curso, Universidade Federal do Pará, Altamira, 2018, 11 p.

Hepatozoon spp. are more frequent intracellular protozoa in snakes. The objective of this study was the use of the molecular approach by the Automatic Barcode Gap Discovery method, to identify candidate species of the genus *Hepatozoon*, parasitizing the snakes: *Clelia clelia* and *Drymarchoncorais*. From the snakes were extracted blood samples and from them the DNA, from which a fragment of the 18S nuclear gene was amplified by means of the Polymerase Chain Reaction. Phylogenetic analysis indicated that *Hepatozoon* sp. of *C. clelia* and *D. corals* form distinct groups of the other species of the genus. The high posterior probability value (99%) indicates that *Hepatozoon* sp. of *C. clelia* and *D. corals* represent sister lines, although more data are needed to infer the phylogenetic relationships between them. The ABGD result identified the existence of 33 groups (prior maximal distance $P = 0.001$) for the initial partition, classifying *Hepatozoon* sp. *C. clelia* and *D. corais* as two distinct taxa, and may be considered as unconfirmed candidate species. Few studies deal with the relationship between *Hepatozoon* species, thus the morphological data traditionally used in the taxonomy of these groups makes it possible to compare them with other species of *Hepatozoon*, contributing to the description of the species diversity of the genus.

1. INTRODUÇÃO

O filo Apicomplexa Levine 1970, apresenta a maior diversidade de espécies de protozoários, com cerca de 6.000 espécies (Levine, 1988), sendo que 99% dessas espécies ainda são poucos conhecidas (Morrison 2009). Os organismos do grupo representam um dos principais parasitos registrados em animais domésticos e silvestres (Levine, 1988). Seus representantes apresentam um conjunto de estruturas conhecidas como “complexo apical”, facilitando a entrada desses parasitas nas células de seus hospedeiros, constituído por anéis polares, conóide, roptrias e microtúbulos subpeliculares, (Levine et al. 1980).

O gênero *Hepatozoon* Miller 1908, é composto por parasitas intracelulares com ciclo heteroxênico, pertencentes ao filo Apicomplexa; subordem Adeleina Léger 1911, família Hepatozoidae Wenyon 1926, (Levine et al. 1980). São hospedeiros de vertebrados e invertebrados (hematófagos), tendo como vetores mosquitos, moscas da areia, carrapatos e alguns ácaros (Desser, 1995). Em serpentes, infecções com *Hepatozoon* spp. são geralmente adquiridas por meio de transferência horizontal por ingestão de invertebrados infectados ou presas intermediárias (Smith 1996; Tome 2012). Até 1996, foram descritas 121 espécies de *Hepatozoon* parasitando serpentes, enquanto que 46 espécies como parasitas de mamíferos e 42 de anfíbios (Smith 1996). A identificação das espécies de *Hepatozoon* são comumente baseadas apenas em características morfológicas, morfométricas dos gamontes e oocistos no sangue de seus hospedeiros (Moço 2008).

A existência de discordâncias entre a taxonomia clássica de morfologia e as filogenias moleculares dentro de parasitas (Smith, 1996) levantam questionamentos sobre a real diversidade de espécies de protozoários neotropicais. O marcador 18S rRNA vem sendo utilizado na literatura para a descrição de novas espécies de *Hepatozoon*, representando uma ferramenta a ser integrada a taxonomia clássica (Sloboda et al. 2007). O método Automatic Barcode Gap Discovery (ABGD) aparece como uma abordagem para a delimitação de espécie candidatas, utilizando a largura do Barcode Gap entre as sequências (Puillandre et al. 2012). Esse método é muito utilizado em trabalhos com anuros (Ferrão et al. 2016, Correa et al. 2017, da Silva et al. 2017), sendo que a aplicação em estudos com parasitos é escasso (Locke et al. 2015a, b, Pérez-Ponce De León et al. 2016).

Assim, o objetivo deste trabalho foi delimitar espécies candidatas de *Hepatozoon* spp. em *Clelia clelia* (Daudin 1803) e *Drymarchon corais* Boie 1827, utilizando o marcador nuclear 18S rDNA e a abordagem molecular pelo método ABGD.

2. METODOLOGIA

2.1 ÁREA DE ESTUDO

Um exemplar de *Clelia clelia* foi coletado no Projeto de Desenvolvimento Sustentável (PDS) Virola Jatobá (-3.17407 S; -51.298 W) no município de Anapu, Pará – Brasil. O PDS possui uma área territorial de 38.423,97 ha, no qual as famílias assentadas nesta região sobrevivem do uso alternativo da agricultura (Silva et al., 2015), apresentando uma formação de floresta ombrófila densa com árvores de médio e grande porte (Correa et al. 2018).

Já, o exemplar de *Drymarchon corais* foi coletado na zona periurbana do município de Altamira, Pará – Brasil, em Maio de 2016.

2.2 EXTRAÇÃO E AMPLIFICAÇÃO DO DNA

O sangue dos hospedeiros foi coletado com auxílio de seringa (10 ml). Para visualização foi realizado esfregão sanguíneo e em seguida os hospedeiros foram sacrificadas com anestésico injetável de uso veterinário, fixadas em formalina 10%, armazenados em álcool 70% e depositadas na coleção do laboratório de Zoologia Adriano Giorgi da Faculdade de Ciências Biológicas, Campus de Altamira-PA.

A extração do DNA foi realizada através do protocolo fenol Clorofórmio (Sambrook et al., 1989) e ressuspendido com 30 µl de água ultrapura. A qualidade do DNA foi verificada em gel de agarose a 1% em cuba de eletroforese. Reação em Cadeia de Polimerase (PCR) foi realizada para amplificar um fragmento de 500 pb do gene nuclear 18S com os primers HEP f300/ HEP f 900 (Ujvari et al. 2004).

A reação para amplificação do fragmento do gene nuclear 18S seguiu os seguintes volumes: (15 µL volume total):

6.7 µL de H ₂ O	1.5 µL de MgCl ₂ (25 mM)
1.25 µL de dNTPs (10 mM)	1.25 µL de Tampão 10x (75 mM Tris HCl, 50 mM KCl, 20 mM (NH ₄) ₂ SO ₄)
1.5 µL do primer HEP f 300	1.5 µL do primer HEPf 900
1 µL de DNA (30–50 ng/µL)	0.3 µL de Taq (1U)

A reação para amplificação do fragmento do gene nuclear 18S seguiu as seguintes condições:

Temperatura inicial de 92°C (60 sec.);	Seguidos por 35 ciclos de 92°C (60 sec.), 50°C (50 sec.) e 72°C (1.5 min.);
Extensão final à 72 °C por 7 min.	

As reações de sequenciamento foram realizadas utilizando-se o kit BigDye Terminator (Life Technology) seguindo o protocolo do fabricante. O primer forward 18S, foi utilizado na reação de

sequenciamento. Os produtos das reações de sequenciamento foram precipitados com etanol de acordo com as recomendações do fabricante (Applied Biosystems), ressuspensos em 10 µL de formamida deionizada e posteriormente injetados no sequenciador automático ABI 3500xl (Applied Biosystems).

2.3 ALINHAMENTO DAS SEQUÊNCIAS E ANÁLISES FILOGENÉTICAS

As sequências foram alinhadas no software BioEdit (Hall, 1999) utilizando o algoritmo Clustal W (Thompson et al. 1994) e editadas manualmente.

A árvore filogenética Bayesiana foi inferida através do software MrBayes v.3.2.6 (Altekar et al. 2004), com os valores padrões para duas corridas e quatro cadeias, correndo 10⁶ gerações, com uma árvore amostrada a cada 1000 gerações. O valor do “burn in” foi selecionado por visualização do log likelihoods associado com a distribuição posterior da árvore no software Tracer v 1.5 (Drummond et al. 2012). Acessamos a convergência examinando a média do desvio padrão da frequência split entre as corridas (< 0.01). Toda árvore gerada antes do nivelamento da curva do log likelihood foram descartadas, correspondendo a 20% das amostras. O número de amostras independentes foram consideradas suficientes, quando a estacionaridade foi alcançada e o *Effective Sample Sizes* (ESS) foram maiores que 200. Foi utilizado como grupo externo *Babesiosoma stableri* Schmittner & McGhee 1961 e *Dactylosoma ranarum* Labbe 1894. As distâncias genéticas par a par não corrigida entre as espécies foram obtidas pelo programa Mega 6.0 (Tamura et al. 2011).

Para a delimitação de espécies, utilizamos o método Automatic Barcode Gap Discovery (ABGD) que detecta automaticamente as diferenças significativas entre a divergência intra e interespecífica (Barcoding gap) e define o limite dessas distâncias com base no padrão par-a-par da distância genética (Puillandre et al. 2012) e outros dados considerados como evidência de apoio. As análises no ABDG foram realizadas no site (<http://www.abi.snv.jussieu.fr/public/abgd/>) utilizando o modelo de distâncias simples (Kimura 2.0). O limiar para a diversidade intraespecífica foi definido num mínimo de 0.001 e máximo de 0.1. O valor padrão do barcoding gap foi de $X = 1.0$.

3. RESULTADOS

Foi amplificado dois fragmentos de 500 pb do gene nuclear 18S de *Hepatozoon* sp. parasitando as serpentes *Clelia clelia* e *Drymachon corais*. A análise bayesiana revelou que os

Hepatozoon deste estudo formam grupos distintos das outras espécies do gênero (Figura 2), sendo mais próximas de HQ734807 *Hepatozoon* sp. e *H. seychellensis* Harris, Damas-Moreira, Maia e Perera, 2014. O alto valor da probabilidade posterior (99%), indica que *Hepatozoon* sp. de *C. clelia* e *D. corais* representam linhagens irmãs. A distância genética entre as duas linhagens de *Hepatozoon* deste estudo foi de 1,5%, enquanto que o valor para as outras espécies de *Hepatozoon* variou de 2,1 – 6.6% (Apêndice 1).

O resultado do ABGD identificou a existência de 33 grupos (prior maximal distance P= 0.001) para a Initial Partition, classificando *Hepatozoon* sp. de *C. clelia* e *Hepatozoon* sp. de *D. corais* como sendo dois taxa distintos, podendo ser consideradas como espécies candidatas não confirmadas. A análise ainda identificou diversas sequências do Genbank classificadas como *Hepatozoon* sp., como espécies candidatas não confirmadas (Apêndice 2).

4. DISCUSSÃO

Até 1996, 121 espécies de *Hepatozoon* eram conhecidas parasitando serpentes em todo o mundo (Smith 1996), desde então 18 novas espécies foram descritas (Telford et al. 2004, 2005, Sloboda et al. 2007, O’Dwyer et al. 2013, Borges-Nojosa et al. 2017, Netherlands et al. 2018). A utilização de dados moleculares na taxonomia de *Hepatozoon* vem contribuindo com a descrição de novas espécies (O’Dwyer et al. 2013, Borges-Nojosa et al. 2017, Netherlands et al. 2018). Nossos resultados corroboram com essa crescente abordagem no estudo da diversidade de espécies de *Hepatozoon*, chamando a atenção para a taxonomia mais integrativa (dados morfológicos, morfométricos e moleculares) para a correta identificação das espécies de parasitos.

O método de delimitação de espécies ABGD em estudos com parasitos, tem encontrado resultados discordantes. Por ser uma abordagem nova em grupos taxonômicos com histórico de descrições baseadas na taxonomia tradicional (Smith 1996) é normal a divergência dos resultados. De acordo com (Pérez-Ponce De León et al. 2016), a delimitação de limites de espécies não pode ser baseada unicamente por esse método, pois pode ocorrer superestimação de espécies em trematóides, sugerindo a análise concatenada para a construção de uma species tree. Resultado similar foi encontrado em *Leucocytozoon* (parasita da malária nas aves), onde o método de “barcode gap” não foi hábil para delimitar espécies, sugerindo o uso de abordagens multi-locus para acessar linhagens evolutivas independentes (Galen et al. 2018). Enquanto que na delimitação de espécies de carrapatos, um dos principais vetores do

Hepatozoon, essa abordagem teve sucesso na delimitação de espécies candidatas.

Hepatozoon spp. de *Clelia clelia* e *Drymachon corais* formam grupos distintos das outras espécies do gênero, sugerindo que são espécies distintas podendo ser utilizada como uma abordagem exploratória quando possuindo apenas dados moleculares. Sugere-se a realização de outros estudos com as espécies candidatas encontradas neste, para descrever estas espécies, associados a outros marcadores moleculares, por exemplo, o DNA mitocondrial Citocromo Oxidase Subunidade I – COI, considerado o DNA barcoding para as espécies do mundo (Hebert et al. 2003). E a Dados morfológicos tradicionalmente usados na taxonomia desses grupos, pois possibilita a comparação com outras espécies de *Hepatozoon*.

Agradecimentos:

Agradecemos ao SISBIO por fornecer o número de autorização 32401 para capturar, coletar e transportar o material biológico.

Conflito de interesses: Os autores declaram que não há conflito de interesses.

Aprovação ética: Foram seguidas todas as diretrizes institucionais, nacionais e internacionais aplicáveis para o cuidado e uso de animais.

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39	6.6%	6.4%	5.8%	6.2%	6.4%	6.2%	5.8%	5.5%	6.4%	6.0%	6.4%	6.6%	5.8%	5.8%	6.8%	4.9%	5.1%	4.9%	5.8%	5.5%	5.5%	4.7%	6.2%	5.5%	7.0%	7.0%	5.1%	5.3%	5.3%	4.7%	4.7%	5.8%	5.3%	7.5%	7.5%	4.7%	7.2%	0.6%		
40	6.6%	6.4%	5.3%	5.8%	6.0%	5.8%	5.3%	5.1%	6.0%	6.2%	6.4%	6.2%	5.3%	5.3%	6.4%	4.7%	5.1%	4.7%	5.3%	5.5%	5.5%	4.5%	6.2%	5.3%	6.2%	5.8%	4.9%	5.1%	5.3%	4.3%	4.3%	5.1%	5.3%	7.5%	7.5%	4.7%	7.7%	6.0%	6.2%	
41	7.2%	7.0%	6.4%	6.8%	7.2%	7.0%	6.4%	6.4%	7.0%	6.6%	7.0%	7.2%	6.4%	6.4%	7.5%	5.5%	5.8%	5.8%	6.8%	6.8%	6.2%	6.2%	7.5%	7.0%	7.5%	7.5%	6.6%	6.8%	6.6%	6.8%	6.8%	7.2%	7.5%	8.5%	8.5%	6.6%	5.5%	8.5%	8.3%	8.7%

- 1 = Hepatozoon sp. (*Clelia_clelia*) - 1
- 2 = Hepatozoon sp. (*Drymarchon_corais*) - 2
- 3 = KF939622Hepatozoon_sp - 3
- 4 = HQ292771Hepatozoon_sp - 4
- 5 = AB181504Hepatozoon_sp - 5
- 6 = HQ734787Hepatozoon_sp - 6
- 7 = HQ734790Hepatozoon_sp - 7
- 8 = EF157822Hepatozoon_ayorgbor - 8
- 9 = HQ734789Hepatozoon_sp - 9
- 10 = HQ734807Hepatozoon_sp - 10
- 11 = KF246565Hepatozoon_seychellensis - 11
- 12 = HQ292774Hepatozoon_sp - 12
- 13 = HQ734806Hepatozoon_sp - 13
- 14 = HQ734809Hepatozoon_sp - 14
- 15 = HQ292773Hepatozoon_sp - 15
- 16 = KF992710Hemolivia_mauritanica - 16
- 17 = FJ719813Hepatozoon_sp - 17
- 18 = KP881349Hemolivia_stellata - 18
- 19 = FJ719817Hepatozoon_sp - 19
- 20 = KR069084Hepatozoon_fitssimonsi - 20
- 21 = FJ719814Hepatozoon_sp 21
- 22 = HQ734794Hepatozoon_sp - 22
- 23 = EF222259Hepatozoon_sp - 23
- 24 = FJ719816Hepatozoon_sp - 24
- 25 = AY600625Hepatozoon_sp - 25
- 26 = AY600626Hepatozoon_sp - 26
- 27 = HQ734805Hepatozoon_sp - 27
- 28 = HQ734804Hepatozoon_sp - 28
- 29 = KJ461940Karyolysus_sp - 29
- 30 = AY628681Hepatozoon_felis - 30
- 31 = AY620232Hepatozoon_felis - 31
- 32 = HQ734791Hepatozoon_sp - 32
- 33 = HQ734803Hepatozoon_sp - 33
- 34 = AF130361Hepatozoon_catesbiana - 34
- 35 = HQ224962Hepatozoon_cf - 35
- 36 = EF222257Hepatozoon_sp - 36
- 37 = HQ224959H_bali - 37
- 38 = AY731061Hepatozoon_canis - 38
- 39 = AY461375Hepatozoon_canis - 39
- 40 = AY461377Hepatozoon_sp - 40
- 41 = HQ224958Dactylosoma_ranarum - 41

2. APÊNDICE II

RESULTADO AUTOMATIC BARCODE GAP DISCOVERY

Resultado do ABGD para as sequências do gene nuclear 18S deste estudo e Genbank.
Initial Partition with prior maximal distance $P=1.00e-03$ Distance K80 Kimura MinSlope=1.000000

- Group [1] n: 1; id:** *Hepatozoon* sp. (*Clelia clelia*)
- Group [2] n: 1; id:** *Hepatozoon* sp. (*Drymarchon corais*)
- Group [3] n: 1; id:** AB181504*Hepatozoon*_sp
- Group [4] n: 2; id:** AF130361*Hepatozoon_catesbiana* HQ224962*Hepatozoon_cf*
- Group [5] n: 1; id:** AY461375*Hepatozoon_canis*
- Group [6] n: 1; id:** AY461377*Hepatozoon*_sp
- Group [7] n: 1; id:** AY600625*Hepatozoon*_sp
- Group [8] n: 1; id:** AY600626*Hepatozoon*_sp
- Group [9] n: 2; id:** AY620232*Hepatozoon_felis* AY628681*Hepatozoon felis*
- Group [10] n: 1; id:** AY731061*Hepatozoon_canis*
- Group [11] n: 1; id:** EF157822*Hepatozoon_ayorgbor*
- Group [12] n: 1; id:** EF222257*Hepatozoon*_sp
- Group [13] n: 1; id:** EF222259*Hepatozoon*_sp
- Group [14] n: 1; id:** FJ719813*Hepatozoon*_sp
- Group [15] n: 1; id:** FJ719814*Hepatozoon*_sp
- Group [16] n: 1; id:** FJ719816*Hepatozoon*_sp
- Group [17] n: 1; id:** FJ719817*Hepatozoon*_sp
- Group [18] n: 1; id:** HQ224959*H_bali*
- Group [19] n: 1; id:** HQ292771*Hepatozoon*_sp
- Group [20] n: 1; id:** HQ292773*Hepatozoon*_sp
- Group [21] n: 1; id:** HQ292774*Hepatozoon*_sp
- Group [22] n: 1; id:** HQ734787*Hepatozoon*_sp
- Group [23] n: 1; id:** HQ734789*Hepatozoon*_sp
- Group [24] n: 3; id:** HQ734790*Hepatozoon*_sp HQ734806*Hepatozoon*_sp
HQ734809*Hepatozoon*_sp
- Group [25] n: 1; id:** HQ734791*Hepatozoon*_sp
- Group [26] n: 1; id:** HQ734794*Hepatozoon*_sp
- Group [27] n: 1; id:** HQ734803*Hepatozoon*_sp
- Group [28] n: 1; id:** HQ734804*Hepatozoon*_sp
- Group [29] n: 1; id:** HQ734805*Hepatozoon*_sp
- Group [30] n: 1; id:** HQ734807*Hepatozoon*_sp
- Group [31] n: 1; id:** KF246565*Hepatozoon_seychellensis*
- Group [32] n: 1; id:** KF939622*Hepatozoon*_sp
- Group [33] n: 1; id:** KR069084*Hepatozoon_fitzsimonsi*

ANEXO I

FIGURAS DO ARTIGO

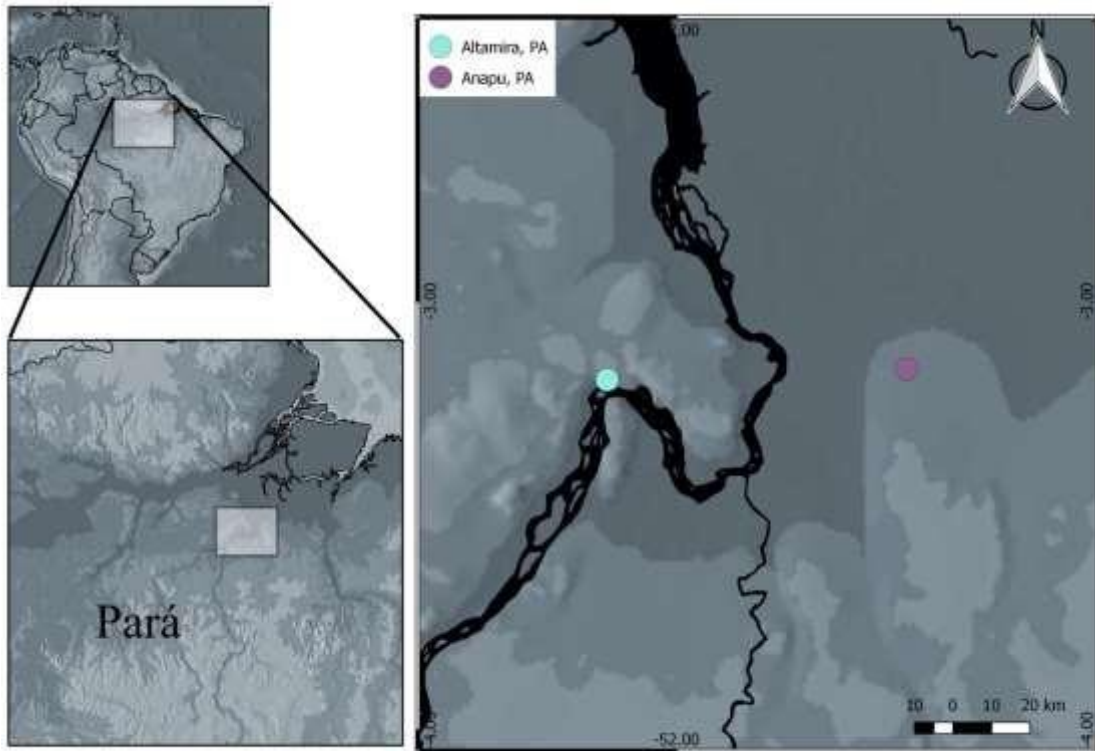


Figura 1. Localidades nas quais foram coletadas as serpentes de estudo, uma na margem direita e outra na margem esquerda do Rio Xingu.

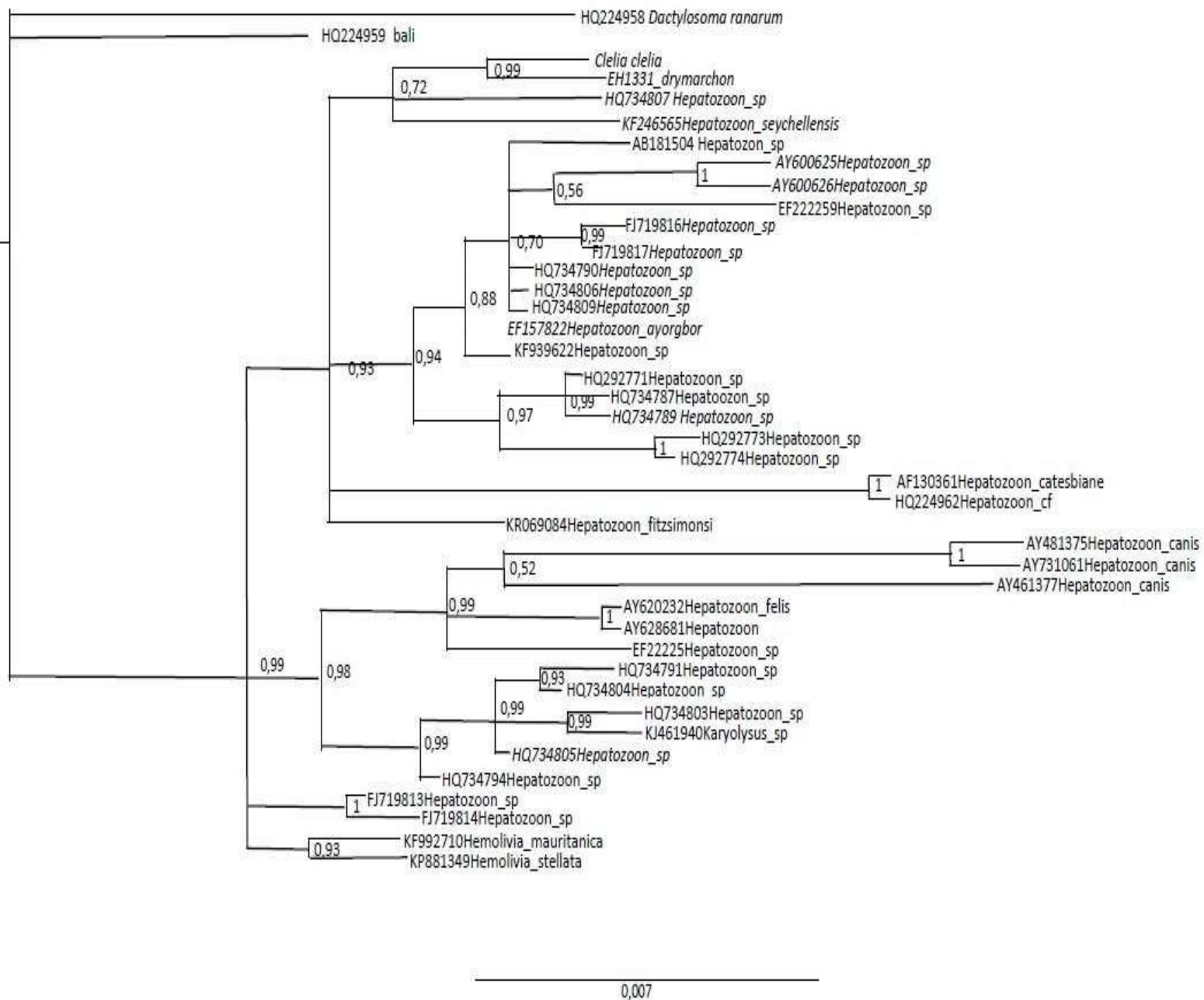


Figura 2: Árvore Bayesiana dos *Hepatozoon* spp. Os valores nos ramos representam a Probabilidade Posterior do suporte dos ramos.

ANEXO II

NORMAS DE PUBLICAÇÃO DA REVISTA

Systematic Parasitology

An International Journal EditorinChief: Aneta Kostadinova

ISSN: 01655752 (print version)

ISSN: 15735192 (electronic version)

Journal no. 11230

Systematic Parasitology publishes papers on the systematics, taxonomy and nomenclature of the following groups: Nematoda (including plant-parasitic), Monogenea, Digenea, Cestoda, Acanthocephala, Aspidogastrea, Cestodaria, Arthropoda (parasitic copepods, hymenopterans, mites, ticks, etc.), Protozoa (parasitic groups), and parasitic genera in other groups, such as Mollusca, Turbellaria, etc. *Systematic Parasitology* publishes fully illustrated research papers, brief communications, and fully illustrated major revisions. In order to maintain high standards, all contributors describing new taxa are asked to state clearly where the holotype is deposited and to make paratypes available for examination by the referees. It is recognized that, in some cases, this may cause problems for the authors, but it is hoped that by adhering to this rule authors may be protected against rapid synonymy of their taxa, and the types will be preserved for posterity.

5-Year Impact Factor: 1.087

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- Conceived of or designed study
- Performed research
- Analyzed data
- Contributed new methods or models
- Wrote the paper

GENERAL

The following types of communication will be considered for publication: - papers of about 6,000 words (fully illustrated) - brief communications or research notes (about 2,000 words), not normally illustrated - major revisions (about 24,000 words), fully illustrated. Any communication which contains descriptions of new taxa (genera or species) should be accompanied by specimens (preferably paratypes) for scrutiny by the referees and by a statement where the holotypes are deposited. Papers and major revisions should include, at LaTeX macro package (zip, 182 kB) the beginning, a summary (approximately 250 words for papers and 500 words for major revisions).

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The title page should include: The name(s) of the author(s) A concise and informative title The affiliation(s) and address(es) of the author(s) The email address, telephone and fax numbers of the corresponding author

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

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Text Formatting

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- Use italics for emphasis.
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- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.

- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).
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Please use no more than three levels of displayed headings.

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Abbreviations should be defined at first mention and used consistently thereafter.

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Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, 25 and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

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The authorities and date of all parasite taxa should be given when the names (full binomen) are first cited in the abstract and the text. Subsequently, the generic component is abbreviated except at the beginning of a sentence. The authorities, but not the date, should be given for the hosts of described parasites. Publications cited only as authorities for taxa should not be included in the References. The International Code of Zoological Nomenclature will be strictly adhered to. Important: Museum accession numbers for all deposited type and voucher specimens are required at submission. The museum should be a recognised national or international museum which is protected by

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REFERENCES

Citation

Cite references in the text by name and year in parentheses. Some examples:

Negotiation research spans many disciplines (Thompson 1990).

This result was later contradicted by Becker and Seligman (1996).

This effect has been widely studied (Abbott 1991; Barakat et al. 1995; Kelso and Smith 1998; Medvec et al. 1999).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

Reference list entries should be alphabetized by the last names of the first author of each work.

➤ Journal article

Harris, M., Karper, E., Stacks, G., Hoffman, D., DeNiro, R., Cruz, P., et al. (2001). Writing labs and the Hollywood connection. *Journal of Film Writing*, 44(3), 213–245.

➤ Article by DOI

Slifka, M. K., & Whitton, J. L. (2000) Clinical implications of dysregulated cytokine production. *Journal of Molecular Medicine*, doi:10.1007/s001090000086

➤ Book

Calfee, R. C., & Valencia, R. R. (1991). *APA guide to preparing manuscripts for journal publication*. Washington, DC: American Psychological Association.

➤ Book chapter

O'Neil, J. M., & Egan, J. (1992). Men's and women's gender role journeys:

Metaphor for healing, transition, and transformation. In B. R. Wainrib (Ed.), *Gender issues across the life cycle* (pp. 107–123). New York: Springer.

➤ Online document

AbouAllaban, Y., Dell, M. L., Greenberg, W., Lomax, J., Peteet, J., Torres, M., & Cowell, V. (2006). Religious/spiritual commitments and psychiatric practice. Resource document. American Psychiatric Association.

http://www.psych.org/edu/other_res/lib_archives/archives/200604.pdf.

Accessed 25 June 2007.

Journal names and book titles should be italicized.

For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

EndNote style (zip, 3 kB)

TABLES

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lowercase letters (or asterisks for significance values and other statistical data) and included beneath the table body.

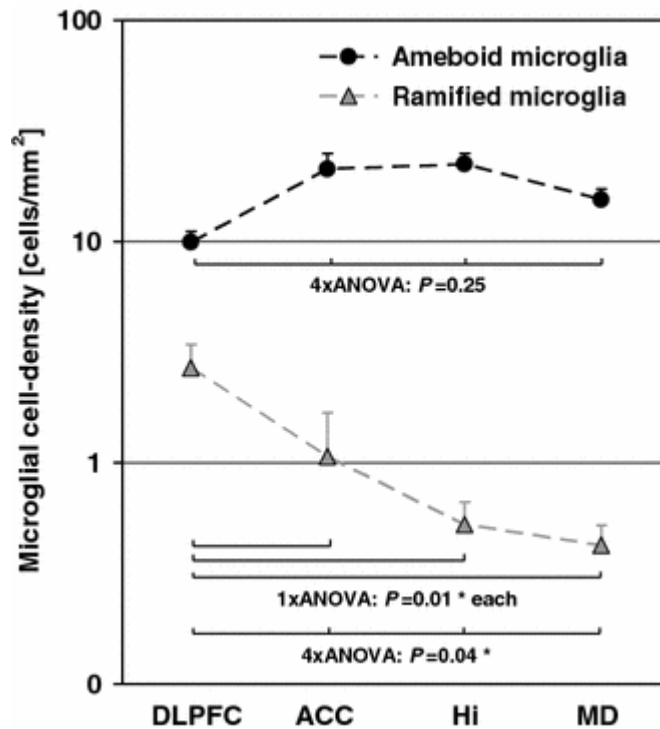
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- Supply all figures electronically.
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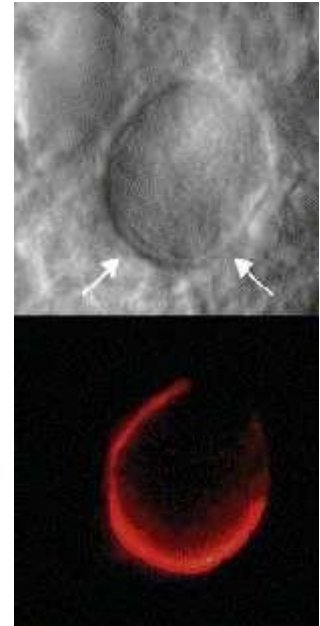
- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
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Halftone Art

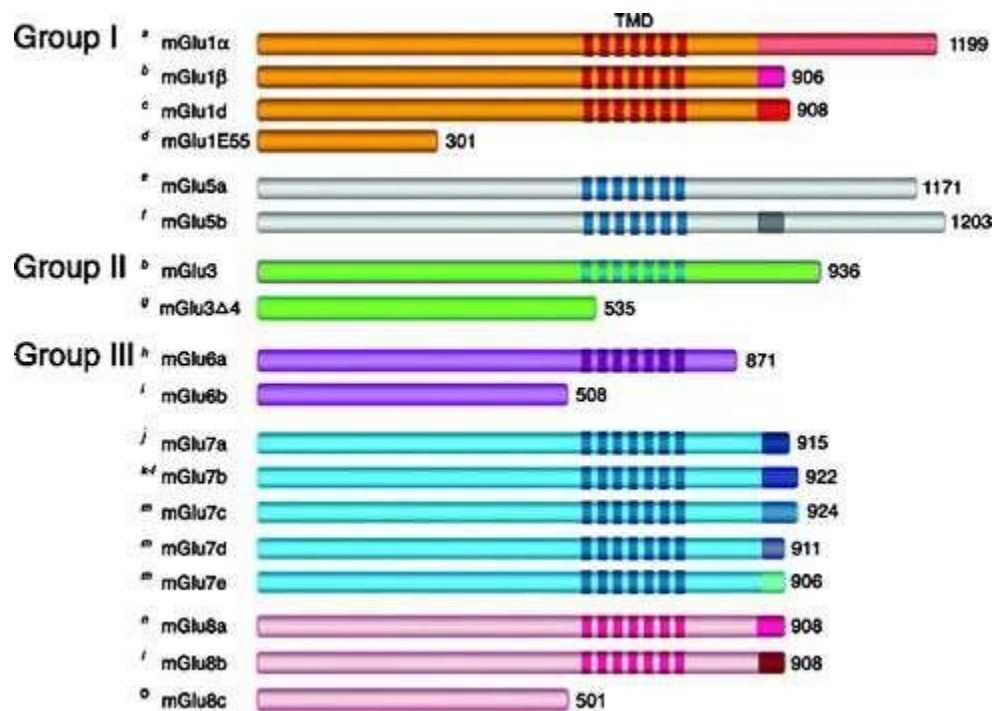
Definition: Photographs, drawings, or paintings with fine shading, etc.

If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.

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If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures, "A1, A2, A3, etc." Figures in online appendices (Electronic Supplementary Material) should, however, be numbered separately.

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- Figure captions begin with the term Fig. in bold type, followed by the figure

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- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
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Figures should be submitted separately from the text, if possible.

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