UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO” (UNESP)
FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS (FCAV)
Campus Jaboticabal
Programa de Pós-Graduação em Medicina Veterinária

DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE HEMOPARASITOS EM ANTAS (*Tapirus terrestris*) DE VIDA LIVRE NO BRASIL

Anna Claudia Baumel Mongruel

Médica Veterinária

2024

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Orientador: Prof. Dr. Marcos Rogério André

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para obtenção do título de Doutora em Medicina Veterinária, área: Patologia Animal.

2024

M743d

Mongruel, Anna Claudia Baumel
Detecção e caracterização molecular de hemoparasitos em antas (Tapirus terrestris) de vida livre no Brasil / Anna Claudia Baumel Mongruel. -- Jaboticabal, 2024
314 f. : il., tabs., fotos, mapas

Tese (doutorado) - Universidade Estadual Paulista (UNESP), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal
Orientador: Marcos Rogério André
Coorientadora: Rosangela Zacarias Machado

1. Doenças de animais selvagens. 2. Diagnóstico. 3. Biologia molecular. 4. Filogenia. 5. Parasitologia. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Universidade Estadual Paulista (UNESP), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal. Dados fornecidos pelo autor(a).

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Impacto potencial dessa pesquisa

A anta (*Tapirus terrestris*) é o maior mamífero terrestre nativo do Brasil. Essa espécie é amplamente distribuída na América do Sul, ocorrendo em pelo menos quatro biomas brasileiros (Amazônia, Cerrado, Mata Atlântica e Pantanal). Possui um papel ecológico importante na biodiversidade da flora dos habitats onde vive devido à capacidade de dispersar sementes de plantas e frutos. A anta é considerada um animal vulnerável à extinção em todo o país e os principais desafios relacionados à sua conservação estão ligados às consequências das ações humanas, como a caça ilegal, incêndios florestais, perda de habitat e atropelamentos em rodovias e estradas. Considerando a necessidade de entender quais outras ameaças esses animais podem enfrentar, o estudo dos hemoparasitos e agentes transmitidos por vetores que podem infectar as antas se faz importante, visto que ainda há pouca informação sobre essa temática. No presente trabalho, foi dectado DNA de bactérias das famílias Anaplasmataceae, Mycoplasmataceae, Bartonellaceae e Borreliaceae em amostras de sangue de antas de vida-livre do Pantanal e Cerrado. Além disso, detectou-se também DNA de protozoários da família Theileriidae. Alguns dos agentes encontrados foram relatados nessa espécie animal pela primeira vez, além da descrição de novas e *Candidatus* espécies e também de genótipos inéditos.

Potential impact from this research

The lowland tapir (*Tapirus terrestris*) is the largest land mammal from Brazil. This species is widely distributed in South America, occurring in at least four Brazilian biomes (Amazon, Cerrado, Atlantic Forest and Pantanal). It has an important ecological role in the flora biodiversity in the habitats where it lives due to its ability to disperse plant and fruits seeds. The lowland tapir is considered an animal vulnerable to extinction throughout the country and the main challenges related to its conservation are associated to the consequences of human actions, such as illegal hunting, forest fires, loss of habitat and being run over on highways and roads. Considering the need to unraveling other threats these animals may face, the study of blood and vector-borne agents that can infect tapirs is important, as there is still little information on this topic. In the present research, DNA from bacteria from the Anaplasmataceae, Mycoplasmataceae, Bartonellaceae and Borreliaceae families was detected in blood samples from free-living tapirs from the Pantanal and Cerrado. Furthermore, DNA from protozoa of the Theileriidae family was also detected. Some of the agents found were reported in this animal species for the first time, in addition to the description of novel Candidatus species and genotypes.

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Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE HEMOPARASITOS EM ANTAS (*Tapirus terrestris*) DE VIDA LIVRE NO BRASIL

AUTORA: ANNA CLAUDIA BAUMEL MONGRUEL
ORIENTADOR: MARCOS ROGÉRIO ANDRÉ
COORIENTADORA: ROSANGELA ZACARIAS MACHADO

Aprovada como parte das exigências para obtenção do Título de Doutora em Medicina Veterinária, área: Patologia Animal pela Comissão Examinadora:

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DADOS CURRICULARES DA AUTORA

Anna Claudia Baumel Mongruel – Filha de Isabela Baumel Mongruel e Roberto Alfredo Pietrobelli Mongruel, nasceu em 23 de outubro de 1993, na cidade de Ponta Grossa, no Estado do Paraná. Em 2011, ingressou no curso de Medicina Veterinária da Universidade Estadual do Centro-Oeste (UNICENTRO), em Guarapuava (PR), graduando-se em fevereiro de 2016. Durante a graduação, realizou duas iniciações científicas, voltadas paras as temáticas de diagnóstico coproparasitológico e diagnóstico molecular de hemoparasitoses em animais selvagens, sob orientação dos Profa. Dra. Meire Christina Seki e do Prof. Dr. Adriano de Oliveira Torres Carrasco. Em 2016, ingressou na Residência Multiprofissional e em Área da Saúde pela Universidade Federal do Paraná (UFPR), campus Curitiba, na área de Patologia Clínica Veterinária – atuação em Biologia Molecular, sob orientação do Prof. Dr. Rafael Felipe da Costa Vieira. Durante a residência, refinou conhecimentos práticos e teóricos acerca do diagnóstico molecular de hemoparasitoses, coleta e identificação de carrapatos e organização e gerenciamento de ambiente laboratorial. Também durante a residência, visitou o Department of Comparative Pathobiology, da Purdue University (West Lafayette, Indiana, EUA), sob supervisão da Dra. Andrea Santos. Em 2018 ingressou no mestrado pelo Programa de Pós-Graduação em Ciências Veterinárias, em área de Saúde Única, da UFPR, novamente sob orientação do Prof. Dr. Rafael Vieira, com bolsa do CNPq, e defendendo sua dissertação em maio de 2019. Em 2021 completou a Pós-graduação (*lato sensu*) em Microbiologia Clínica pela Universidade Positivo, campus Curitiba, refinando e adquirindo conhecimentos técnicos e práticos acerca do diagnóstico microbiológico. Em 2020 ingressou no Programa de Pós-Graduação em Medicina Veterinária, em área de Patologia Veterinária, sob orientação do Prof. Dr. Marcos Rogério André e coorientação da Prof. Dra. Rosangela Zacarias Machado, na UNESP campus Jaboticabal, inicialmente com bolsa CAPES e, posteriormente, sendo contemplada com bolsa FAPESP (#2019/26403-0). Entre fevereiro a agosto de 2023, realizou período de pesquisa no exterior, com bolsa BEPE FAPESP (#2022/13016-1), no Department of Ecology and Evolutionary Biology da Tulane University em New Orleans (Louisiana, EUA), sob supervisão do Dr. Keith Clay. Durante esse período, teve contato com técnicas de bioinformática voltadas para análises de microbiomas.

EPÍGRAFE

"There can be 100 people in one room, and 99
don’t believe in you, but all it takes is one person
to believe in you and that can change everything."

– Lady Gaga

DEDICATÓRIA

Dedico esse trabalho à Anna Claudia que
estava no primeiro ano de Medicina Veterinária.

AGRADECIMENTOS

Seriam as nossas escolhas uma simples soma de circunstâncias ou será que essa coisa de destino realmente existe? Em árabe, existe a palavra *maktub* que significa "estava escrito" ou "tinha que acontecer". Sendo bastante sincera, em diversos momentos durante o doutorado, eu não sabia o que eu estava fazendo. Eu não sabia se aquilo era pra mim, se eu era boa o suficiente ou se todo o tempo que eu estava dedicando a isso valeria a pena. Afinal, o doutorado toma muito tempo e todo mundo fala que o tempo é valioso. Por muitas vezes, eu desejei que o doutorado passasse rápido. Mas não tinha como: é um processo longo. E a prova disso é que muita coisa aconteceu enquanto eu estava nele. Quando eu fui aprovada para ingressar no programa, no final de 2019, ninguém sabia direito o que era passar por uma pandemia. Hoje, cinco anos depois, a gente lembra de 2020 e 2021 e quase não acredita em tudo o que aconteceu. Quando eu comecei o doutorado, eu tinha meus quatro avós. Hoje eu tenho um avô. Quando eu comecei o doutorado, eu não tinha nenhum sobrinho. Hoje eu tenho duas. Quando eu comecei o doutorado, eu estava namorando. Hoje eu estou noiva. Assim como várias outras coisas que aconteceram de forma simultânea na minha vida durante esses anos, o doutorado também aconteceu. E o principal: eu sentindo se aquilo era pra mim ou não, eu sabendo o que eu estava fazendo ou não, eu pensando em desistir ou não, eu tendo dias bons ou não... o doutorado aconteceu. E, por isso, eu penso que, talvez, era pra acontecer mesmo. Afinal, quais outras forças, se não uma tal força universal que se encarrega de desenhar o destino das pessoas, faria isso dar certo? Pois eu citaria sim algumas outras e é à elas que eu quero agradecer:

Obrigada aos meus pais, Roberto e Isabela, por acreditarem em mim. Ninguém acredita em mim tanto quanto vocês e isso me faz imaginar, um pouquinho, como é grande o amor que um pai e uma mãe sentem pelos seus filhos.

Obrigada ao meu orientador Prof. Dr. Marcos Rogério André, por ter confiado em mim e aceitado me orientar. Por ter me conduzido até aqui com paciência, educação e atenção. A eficiência, seriedade e paixão com as quais o senhor conduz o seu trabalho como professor e pesquisador é um exemplo para mim.

Obrigada às professoras Dra. Rosangela Zacarias Machado e Dra. Darci Moraes Barros-Battesti pelo pioneirismo e esforços dedicados à parasitologia veterinária que permitem que alunos como eu usufruam do conhecimento, infraestrutura e legado consequentes do trabalho das senhoras.

Obrigada ao meu noivo Guilherme Sagrillo, por ter sido, e sempre ser, tão paciente, cuidadoso e carinhoso. Durante os anos do doutorado, tivemos muitas despedidas dolorosas em rodoviárias e aeroportos. E, mesmo com o incômodo do relacionamento a distância, você sempre me apoiou, incentivou e dizia que tudo ia ficar bem (e ainda me pediu em casamento no meio disso tudo). Amo muito você.

Obrigada aos meus irmãos Felipe, Daniel, Cynthia e Marcello. Eu adoro contar pra todo mundo que eu tenho quatro irmãos. Vocês são parte de quem eu sou e eu amo vocês.

Obrigada também às minhas amigas da vida Katherinne, Salua e Nathália, por estarem sempre por perto há tantos anos (mesmo a quilômetros de distância).

Obrigada à todos os meus colegas pós-graduandos (atuais ou que já defenderam) e estagiários do Vector-Borne Bioagents Laboratory; em especial: Ana Calchi, Ana Carolina Santiago, Amir Alabi, Bianca Cardenal, Clara Dias, Lorena Freitas e Ricardo Bassini-Silva, pelas várias vezes em que vocês me emprestaram material, me passaram um protocolo ou revelaram um gel pra mim. Além de ajudarem a tornar essa caminhada mais leve com risadas, desabafos, almoços, cafézinhos e fofoquinhas.

Many thanks to Dr. Keith Clay and all members from Clay Lab (Paul, Jake and Peyton) at Tulane University. My time in New Orleans was one of the most special and unforgettable chapters of my life! And you guys are part of it, always being very kind and helpful to me. Thank you, thank you, thank you!!

Obrigada à Dra. Patrícia Medici e todo o time de pesquisadores e profissionais da Iniciativa Brasileira para a Conservação da Anta Brasileira (INCAB/IPÊ), pelo trabalho sério realizado em prol do estudo da anta brasileira e pelo fornecimento das amostras para que esse projeto pudesse acontecer. Queria poder também agradecer às antas cujas as amostras de sangue vieram parar na minha mão e que, graças à esses animais, pude aprender tanta coisa nova.

Obrigada à CAPES, pelo provimento de bolsa no início do doutorado. O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

Obrigada à Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) por acreditar no meu trabalho, concedendo bolsa de pesquisa (2019/26403-0) e bolsa de estágio (2022/13016-1) no exterior.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pelos Auxílios à Pesquisa concedidos ao Prof. Marcos R. André para a realização do presente trabalho (Processos números 2020/12037-0 e 2022/08543-2).

Obrigada aos meus bichinhos de estimação: Antônia, Banzé e Peteco (esse último adotado na UNESP). Se hoje eu sou médica veterinária, é porque sempre gostei muito de bicho. E ter os meus bichinhos na minha vida é um reforço diário desse sentimento.

Por fim, mas não menos importante, meu agradecimento especial à essa força que pincelou meu caminho e me trouxe até aqui hoje, independente das minhas inseguranças e incertezas, fazendo com que tudo que tivesse que acontecer para o meu crescimento e evolução, acontecesse. Algumas pessoas chamam ela de Deus.

Sumário

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

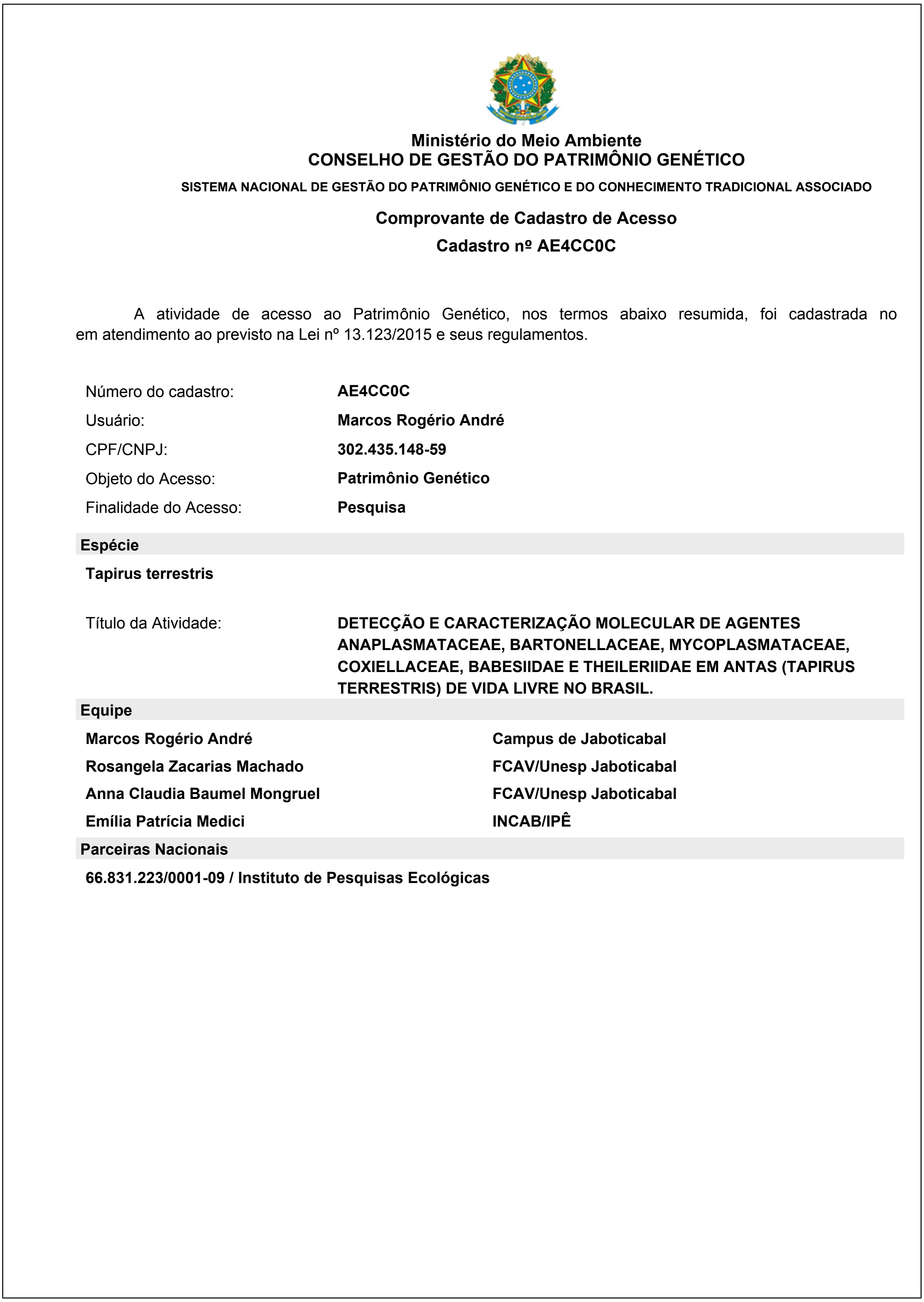
Certificamos que o projeto de pesquisa intitulado **"Detecção e caracterização molecular de agentes Anaplasmataceae, Bartonellaceae, Mycoplasmataceae, Coxiellaceae, Babesiidae e Theileriidae em antas (*Tapirus terrestris*) de vida livre no Brasil"** protocolo nº 4558/20, sob a responsabilidade do Prof. Dr. Marcos Rogério André, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 15 de outubro de 2020.

Vigência do Projeto	01/11/2020 a 29/02/2024
Espécie / Linhagem	*Tapirus terrestris*
Nº de animais	102
Peso / Idade	Variável
Sexo	Variável
Origem	Animais capturados pela equipe da Iniciativa Nacional para a Conservação da Anta Brasileira (INCAB), projeto do Instituto de Pesquisas Ecológicas (IPÊ), para a instalação de colares satelitais de monitoramento e coleta de amostras biológicas para estudos de saúde e genética e animais que foram à óbito por atropelamento em rodovias

Jaboticabal, 15 de outubro de 2020.

Fabiana Pilarski
Profª Drª Fabiana Pilarski
Coordenadora – CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n CEP 14884-900 - Jaboticabal - SP - Brasil
Tel. 16 3209-7100 - www.fcav.unesp.br



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 AE4CC0C

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

Número do cadastro:	**AE4CC0C**
Usuário:	**Marcos Rogério André**
CPF/CNPJ:	**302.435.148-59**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Tapirus terrestris

Título da Atividade:	**DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE AGENTES ANAPLASMATACEAE, BARTONELLACEAE, MYCOPLASMATACEAE, COXIELLACEAE, BABESIIDAE E THEILERIIDAE EM ANTAS (TAPIRUS TERRESTRIS) DE VIDA LIVRE NO BRASIL.**

Equipe

Marcos Rogério André	**Campus de Jaboticabal**
Rosangela Zacarias Machado	**FCAV/Unesp Jaboticabal**
Anna Claudia Baumel Mongruel	**FCAV/Unesp Jaboticabal**
Emília Patrícia Medici	**INCAB/IPÊ**

Parceiras Nacionais

66.831.223/0001-09 / Instituto de Pesquisas Ecológicas

Resultados Obtidos

Divulgação de resultados em meios científicos ou de comunicação

Identificação do meio onde foi divulgado:	**https://doi.org/10.3390/microorganisms1003061**

Data do Cadastro:	**08/07/2022 10:58:57**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **10:59** de **08/07/2022**.

SISTEMA NACIONAL DE GESTÃO
DO PATRIMÔNIO GENÉTICO
E DO CONHECIMENTO TRADICIONAL
ASSOCIADO - **SISGEN**

DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE HEMOPARASITOS EM ANTAS (*Tapirus terrestris*) DE VIDA LIVRE NO BRASIL

RESUMO – A anta brasileira (*Tapirus terrestris*) é o maior mamífero terrestre do Brasil. Atualmente, essa espécie é considerada vulnerável à extinção em todo o país. O presente trabalho teve como objetivos detectar e caracterizar agentes das famílias bacterianas Mycoplasmataceae, Bartonellaceae, Anaplasmataceae, Coxiellaceae e Borreliaceae; e também das famílias de protozoários Babesiidae, Theileriidae e Hepatozoidae em amostras de sangue de antas de vida-livre do Cerrado e Pantanal, por meio de técnicas moleculares. Entre 2013 a 2018, amostras de sangue de *T. terrestris* de vida-livre foram coletadas de 94 indivíduos vivos e 8 atropelados, totalizando 125 amostras (alguns animais vivos foram amostrados mais de uma vez em momentos diferentes), no Estado do Mato Grosso do Sul. A coleta de sangue foi realizada pelos profissionais da Iniciativa Nacional para a Conservação da Anta Brasileira (INCAB-IPÊ). Desse total, 78 (78/125; 62.4%) amostras foram coletadas de 61 antas (61/94; 64,89%) no Pantanal e 39 amostras (39/125; 31,2%) foram coletadas de 33 antas (33/94; 35,11%) provenientes do Cerrado. Além disso, todas as antas atropeladas (8/125; 6,4%) foram amostradas no Cerrado, totalizando 41 indivíduos deste bioma. As amostras foram submetidas à protocolos de PCR (convencional) e qPCR (PCR quantitativa). Ainda, 86 amostras foram submetidas à amplificação de um fragmento de aproximadamente 400 pb da região V3-V4 do gene bacteriano 16S rRNA, por meio de sequenciamento de nova geração, para caracterização do microbioma. Como resultado, 33,61% demonstraram resultados positivos para a pesquisa de *Mycoplasma* spp. (PCR para os genes 16S rRNA, 23S rRNA, *RNAse* P e *dnaK*), 52,2% para piroplasmídeos (PCR para os gene 18S rRNA, *hsp70* e *cox*-1), 6,55% para *Bartonella* sp. (qPCR para o gene *nuoG* e PCR para o gene *ribC*), 18% para *Anaplasma* spp. (PCR para o gene 16S rRNA e espaçador intragênico ITS 23S-5S), 25,4% para *Neorickettsia* spp. (PCR para o gene 16S rRNA), e 2% para *Borrelia* spp. (qPCR para o gene 16S rRNA e PCR para o gene *flaB*). Nenhuma amostra foi positiva para os protocolos testados para *Ehrlichia* spp. (PCR para o gene *dsb*), *Coxiella burnetii* (qPCR para o elemento gênico IS1111) e *Hepatozoon* spp (PCR para o gene 18S rRNA). As análises filogenéticas das sequências obtidas demonstraram a presença de duas novas espécies *Candidatus* nesses animais: *'Candidatus* Mycoplasma haematoterrestris' e *'Candidatus* Mycoplasma hematotapirus'; uma nova espécie de *Theileria,* nominada *Theileria terrestres* nov. sp.; ocorrência de um genótipo similar à *Bartonella henselae*; ocorrência de um novo genótipo de *Anaplasma* sp., ocorrência de *Neorickettsia* sp.; e *Borrelia theileri*, patógeno anteriormente descrito somente em animais domésticos no Brasil. Na análise do microbioma do sangue das antas, os filos Pseudomonadota, Bacillota, Mycoplasmatota, Bacterioidota e Actinomycetota mostraram-se dominantes. A análise da diversidade *alpha* de amostras de animais vivos demonstrou que todos os grupos avaliados compartilham as mesmas comunidades bacterianas principais. O presente trabalho descreveu a ocorrência de diferentes hemoparasitos (*Mycoplasma* spp., *Theileria terrestris*, *Bartonella* sp., *Anaplasma* sp., *Neorickettsia* sp. e *Borrelia theileri*) em *T. terrestris* de vida livre no Brasil, além da primeira caracterização do microbioma do sangue dessa espécie.

Palavras-chave: Animais selvagens, diagnóstico, filogenia, hemoparasitos.

MOLECULAR DETECTION AND CHARACTERIZATION OF HEMOPARASITES IN WILD LOWLAND TAPIRS (*TAPIRUS TERRESTRIS*) FROM BRAZIL

ABSTRACT - The lowland tapir (*Tapirus terrestris*) is the largest land mammal in Brazil and is considered vulnerable to extinction throughout the country. The present research aimed to detect and characterize agents from the bacterial families Mycoplasmataceae, Bartonellaceae, Anaplasmataceae, Coxiellaceae, and Borreliaceae; and also the protozoan families Babesiidae, Theileriidae, and Hepatozoidae in blood samples of wild tapirs from the Cerrado and Pantanal biomes, using molecular techniques. Between 2013 and 2018, blood samples were collected from 94 living and 8 road-killed wild tapirs, totaling 125 samples (some living animals were sampled more than once at different times), in Mato Grosso do Sul State. Blood collection was carried out by professionals from the National Initiative for the Conservation of the Brazilian Tapir (INCAB-IPÊ). Out of this, 78 (78/125; 62.4%) samples were collected from 61 tapirs (61/94; 64.89%) in the Pantanal and 39 samples (39/125; 31.2%) were collected from 33 tapirs (33/94; 35.11%) from the Cerrado. Furthermore, all road-killed tapirs (8/125; 6.4%) were sampled in the Cerrado, totaling 41 individuals from this biome. Samples were subjected to PCR (conventional) and qPCR (quantitative PCR) protocols targeting the studied agents. Furthermore, 86 samples were subjected to amplification of a fragment of approximately 400 bp from the V3-V4 region of the bacterial 16S rRNA gene, through next generation sequencing, for blood microbiome characterization. From the tested samples, 33.61% obtained positive results for *Mycoplasma* spp. (PCR for the 16S rRNA, 23S rRNA, *RNAse* P and *dnaK* genes), 52.2% for piroplasmids (PCR for the 18S rRNA, *hsp70* and *cox*-1 genes), 6.55% for *Bartonella* sp. (qPCR for the *nuoG* gene and PCR for the *ribC* gene), 18% for *Anaplasma* spp. (PCR for the 16S rRNA gene and ITS 23S-5S intragenic space), 25.4% for *Neorickettsia* spp. (PCR for the 16S rRNA gene), and 2% for *Borrelia* spp. (qPCR for the 16S rRNA gene and PCR for the *flaB* gene). None of the samples was positive for the protocols tested for *Ehrlichia* spp. (PCR for the *dsb* gene), *Coxiella burnetii* (qPCR for the IS1111 gene elemento) and *Hepatozoon* spp (PCR for the 18S rRNA gene). Phylogenetic analyses of obtained sequences demonstrated the presence of two *Candidatus* species: '*Candidatus* Mycoplasma haematoterrestris' and '*Candidatus* Mycoplasma hematotapirus'; detection of a novel species of *Theileria*, named *Theileria terrestris* nov. sp.; detection of a *Bartonella* sp. genotype similar to *Bartonella henselae*; a novel genotype of *Anaplasma* sp.; detection of *Neorickettsia* sp.; occurrence of *Borrelia theileri*, a pathogen previously described only in domestic animals in Brazil, was also detected. Regarding the analysis of the tapir blood microbiome, the phyla Pseudomonadota, Bacillota, Mycoplasmatota, Bacterioidota and Actinomycetota demonstrated to be dominant. When analyzing the diversity of living animals' samples, tapir populations from all analyzed groups demonstrate that they share the dominant bacterial community members. The present research described the occurrence of different hemoparasites (*Mycoplasma* spp., *Theileria terrestris*, *Bartonella* sp., *Anaplasma* sp., *Nerickettsia* sp. and *Borrelia theileri*) in free-living *T. terrestris* in Brazil, in addition to the first characterization of the blood microbiome from this species.

Keywords: Diagnosis, hemoparasites, phylogeny, wildlife.

CAPÍTULO I – CONSIDERAÇÕES GERAIS

1. Introdução

Considerando as perspectivas futuras para o diagnóstico de doenças infecciosas em animais selvagens, tecnologias inovadoras podem melhorar a detecção de patógenos, bem como contribuir com o conhecimento acerca de tais agentes em diferentes espécies (Michel et al., 2021). As zoonoses são doenças infecciosas compartilhadas entre os seres humanos e outras espécies animais e são causadas por cerca de 60% dos patógenos que infectam seres humanos (Aggarwal e Ramachandran, 2020). Áreas onde ocorre o contato próximo entre seres humanos, animais domésticos e animais selvagens são aquelas com a maiores chances de ocorrer a transmissão de agentes zoonóticos (Kalema-Zikusoka, 2005).

A ocorrência de patógenos transmitidos por vetores abrange desde áreas com climas tropicais e subtropicais até locais de clima temperado (Martens et al., 1995). O aumento das populações de artrópodes vetores, que ocorre principalmente em decorrência de alterações ambientais, influencia não apenas o aparecimento de novas doenças, mas também o ressurgimento de doenças já conhecidas (Gratz, 1999). Desde o início do século 20, as doenças transmitidas por carrapatos estão em ascensão devido ao aumento dos impactos ao meio ambiente. Embora os carrapatos sejam considerados os artrópodes mais relacionados à transmissão de patógenos, após os mosquitos, os estudos acerca da transmissão de agentes infecciosos por carrapatos são considerados negligenciados quando comparados àqueles transmitidos por artrópodes voadores (Boulanger et al., 2019).

O Pantanal é considerado a maior área alagada contínua de água doce do planeta, abrangendo cerca de 179.300 km^2 de planícies aluviais de baixa altitude. As estações de chuva e seca são bem definidas, com as chuvas concentrando-se durante o verão e favorecendo a ocorrência de cheias sazonais (Tomas et al., 2019). O Cerrado é o segundo maior bioma do Brasil, abrangendo a parte predominante das savanas neotropicais (Delitti et al., 2006). Diferentes tipos de vegetação podem ser encontradas no Cerrado. As paisagens são caracterizadas por florestas de savana que variam desde pastagens quase sem árvores, dominadas por gramíneas e arbustos, até florestas de árvores semidecíduas (Furley et al., 1999). A área onde ocorre o Cerrado se estende por 2 milhões de km^2 da região central do planalto brasileiro e representa 21% da área terrestre do Brasil, o que o torna a região mais extensa de savana da América do Sul (Delitti et al., 2006). Considerando seu vasto vasto, as condições climáticas do Cerrado variam de acordo com a região na qual o bioma ocorre. Na região que abrange o Estado do Mato Grosso do Sul, o Cerrado é descrito apresentando estação chuvosa mais intensa entre os meses de Dezembro à Fevereiro, com pequenos períodos de estiagem no mês de Janeiro. As médias de temperatura anual para essa região podem variar entre 21^{o}C a 25^{o}C, sendo esta uma região que apresenta médias de temperaturas mais baixas quando comparadas à outras regiões onde esse bioma pode ser encontrado (Silva et al., 2008).

A principal causa de desmatamento nos países da América do Sul é a expansão de áreas de atividades ligadas à produção agropecuária (Arraes et al., 2012), assim como ocorre no Brasil (Domingues et al., 2014). A fragmentação de habitats naturais está relacionada a uma perda consistente da abundância de espécies selvagens (Haddad et al., 2015) e ao aumento no risco de extinção para mamíferos (Crooks et al., 2017). Nos últimos anos, tanto o Pantanal quanto o Cerrado têm enfrentado altas taxas de destruição no Brasil. Estima-se que os incêndios florestais destruíram aproximadamente 23% do Pantanal em 2020 (LASA/UFRJ, 2020), e em 2021, 7,9% do a vegetação natural foi reportada como suprimida no Cerrado (PRODES/INPE, 2021). Incêndios florestais podem causar ferimentos diretos e morte de animais, mas também danos indiretos causados pela perda de habitat e esgotamento de recursos (Berlinck et al., 2021).

O interesse na vida selvagem tem crescido por diferentes razões: desde a necessidade de traçar planos para a conservação de espécies ameaçadas até mesmo o interesse econômico nesses animais, visto que, em países que apresentam uma alta taxa de biodiversidade, a fauna é considerada um importante atrativo e contribuinte da indústria do turismo (Michel et al., 2021). De fato, em 2022, a cadeia produtiva do turismo no Estado do Mato Grosso do Sul, na qual uma expressiva porção é relacionada ao ecoturismo, movimentou cerca de R$21,6 bilhões na região (Fecomércio MS, 2023).

A espécie *Tapirus terrestris*, popularmente conhecida como anta brasileira, faz parte da ordem Perissodactyla e família Tapiridae (Medici et al., 2012) e é considerada o maior mamífero terrestre silvestre do Brasil (Rodrigues, 2007). A anta é considerada uma espécie mantenedora da biodiversidade da flora, visto a sua capacidade de dispersar sementes nos territórios onde habita (Barcelos et al., 2013). Dentre os países da América do Sul, o Brasil é o país com maiores perdas de habitat para a espécie (Cordeiro et al., 2016). Embora ainda possa ser encontrada em quatro biomas brasileiros (Mata Atlântica, Pantanal, Amazônia e Cerrado), a anta está classificada como vulnerável no país pela avaliação do risco de extinção da espécie (Medici et al., 2012).

Alterações de habitat são a principal causa do surgimento de doenças infecciosas em antas (Mangini et al., 2012). Os incentivos às pesquisas relacionadas à saúde e aos agentes infecciosos que podem afetar a espécie são listados como metas nos esforços que visam a conservação da anta brasileira (Medici et al., 2007). Entretanto, embora exista uma diversidade de estudos envolvendo hemoparasitos de animais silvestres, poucos são os relatos que descrevem a detecção de patógenos em antas.

2. Objetivos

2.1. Objetivo Geral

O presente estudo teve como objetivo investigar a ocorrência molecular e diversidade genética de agentes das famílias Mycoplasmataceae, Babesiidae, Theileriidae, Bartonellaceae, Anaplasmataceae, Coxiellaceae, Hepatozoidae e Borreliaceae em amostras de sangue de antas (*Tapirus terrestris*) de vida livre nos biomas Cerrado e Pantanal, no estado do Mato Grosso do Sul. Adicionalmente, o estudo objetivou caracterizar o microbioma do sangue dessa espécie animal.

2.2. Objetivos específicos

- Investigar, por meio de métodos moleculares, a ocorrência de agentes das famílias Mycoplasmataceae, Babesiidae, Theileriidae, Bartonellaceae, Anaplasmataceae, Coxiellaceae, Hepatozoidae e Borreliaceae em amostras de sangue de antas de vida livre nos biomas Cerrado e Pantanal, no estado do Mato Grosso do Sul;

- Traçar inferências filogenéticas com genótipos dos agentes sob estudo comparativamente àqueles previamente detectados em animais domésticos, selvagens e seres humanos no Brasil e no mundo;
- Investigar a diversidade de genótipos dos agentes sob estudo circulantes em antas de vida livre nos biomas Cerrado e Pantanal, no estado do Mato Grosso do Sul;
- Determinar a composição bacteriana presente no sangue de antas por meio da amplificação de regiões hipervariáveis do gene 16S rRNA e determinar as variáveis que possam influenciar de maneira estatisticamente significativa as comunidades *alpha* e *beta*.

CAPÍTULO II – REVISÃO DE LITERATURA

1. O gênero *Tapirus* e a anta brasileira

A Superordem Ungulata divide os mamíferos que andam apoiando-se sobre a ponta dos dedos em dois grupos: Perissodactyla (espécies que possuem dedos em número ímpar) e Arctiodactyla (espécies que possuem dedos em número par). Além do andar ungulígrado, os ungulados são caracterizados por serem animais terrestres e adaptados a coletar alimentos exclusivamente usando a boca, visto que a estrutura dos pés evoluiu primariamente para a locomoção (Berkoviz e Shellis, 2018).

A Ordem Perissodactyla, por sua vez, compreende herbívoros monogástricos de duas subordens: Hippomorpha, que possui como representante único a família Equidae; e Ceratomorpha, que alberga as famílias Rhinoceratidae e Tapiridae. Comparativamente à Artiodactyla, Perissodactyla possui um número muito menor de espécies representantes atualmente. As famílias Equidae e Tapiridae são representadas por seus gêneros únicos *Equus* e *Tapirus*, respectivamente. Já a família Rhinoceratidae possui quatro gêneros, sendo eles: *Ceratotherium*, *Dicerorhinus*, *Diceros* e *Rhinoceros* (Berkoviz e Shellis, 2018).

Teoriza-se que os ancestrais do gênero *Tapirus* surgiram inicialmente na Europa, durante o período Oligoceno e, posteriormente, distribuiram-se pela Ásia e Américas (García et al., 2012). Atualmente, quatro espécies fazem parte desse gênero. Dentre elas, três fazem parte da fauna da América do Sul e Central (*Tapirus pinchaque* [Roulin, 1829], *Tapirus terrestris* [Linnaeus, 1758] e *Tapirus bairdii* [Gill, 1865]), enquanto uma é encontrada na região sudeste da Ásia (*Tapirus indicus* [Desmarest, 1819]). Acredita-se que o distanciamento evolutivo entre a espécie asiática e as neotropicais ocorreu há cerca de 20 a 30 milhões de anos atrás (Ashley et al., 1996). Embora uma quinta espécie de anta tenha sido proposta recentemente, nominada como *Tapirus kabomani* (Cozzoul et al., 2013), estudos posteriores apontaram a ausência de evidências filogenéticas que diferenciem *T. kabomani* de *T. terrestris* (Ruiz-Garcia et al. 2015). Adicionalmente, diferenças morfológicas entre essas duas espécies parecem não ser totalmente esclarecidas (Dumbá et al. 2018). Portanto, a existência, de fato, de uma quinta espécie permanece em discussão.

Em relação à distribuição das espécies neotropicais, duas linhagens genéticas são propostas baseadas em estudos filogenéticos do gene mitocondrial citocromo c oxidase e do gene 12S rRNA (Ahsley et al., 1996; Norman e Ahsley, 2000). A linhagem neotropical 1 engloba as espécies *T. pinchaque* e *T. terrestris*. A primeira ocorre na região noroeste da América do Sul, englobando os territórios da Colômbia, Equador e Peru, limitando-se a regiões montanhosas. Já a espécie *T. terrestris* possui território mais amplo, ocorrendo na maior parte da América do Sul, desde as regiões de chaco na Bolívia e Paraguai e norte Argentino até a região norte da Colômbia; além de Venezuela, Guianas e regiões de Mata Atlântica no Brasil (Padilla e Dowler, 1994; García et al., 2012) (**Figura 1**). A linhagem neotropical 2 é representada pela espécie *T. bairdii*, cujo território concentra-se na América Central, desde a região sul do México até a Colômbia, na América do Sul (García et al., 2012).

O representante do gênero *Tapirus* no Brasil é a espécie *T. terrestris*, também conhecida como anta brasileira. Pesando até 300 quilogramas e medindo entre 1,7 a 2,0 metros, a anta brasileira é o maior mamífero terrestre autóctone do Brasil (Rodrigues, 2007). Assim como seus ancestrais, são animais forrageiros que se alimentam principalmente de folhas e plantas (Henry et al., 2000; García et al., 2012). Ainda, alimentam-se de uma quantidade expressiva de frutas, atendendo às demandas energéticas do seu grande tamanho corporal (Bodmer, 1990). Com isso, torna-se uma espécie mantenedora da biodiversidade de flora dos locais onde habita, devido à capacidade de espalhar sementes viáveis em suas fezes (Rodrigues et al., 1993; Barcelos et al., 2003). Devido a essa característica, as antas são conhecidas como "jardineiras da floresta".

Dentre todas as espécies de antas, a anta brasileira é que a possui a maior e mais diversa extensão territorial (Padilla e Dowler, 1994; Cordeiro et al., 2016). Entretanto, a espécie enfrenta declínios populacionais e também em extensão territorial (Taber et al., 2008). De fato, *T. terrestris* está incluído na Lista Vermelha da União Internacional para Conservação da Natureza e dos Recursos Naturais (IUCN Red List) como "vulnerável" devido à redução contínua das populações estimada em aproximadamente 30% nos últimos 33 anos (Varela et al., 2019).

Embora ainda seja encontrada em quatro biomas brasileiros (Amazônia, Cerrado, Mata Atlântica e Pantanal), a espécie é considerada vulnerável à extinção no país (Medici et al., 2012). Ainda, os biomas brasileiros apresentam as maiores taxas de perda de habitat para a anta brasileira na América do Sul, principalmente nas áreas de Cerrado e Mata Atlântica, além de ser considerada tecnicamente extinta na Caatinga e nos Pampas (Cordeiro et al., 2016). As principais causas do declínio da espécie são aquelas ligadas, de forma direta ou indireta, a ações antrópicas, como a caça, a destruição do habitat, a competição de território com as produções agropecuárias, incêndios florestais e atropelamentos em rodovias (Medici et al., 2012; Varela et al., 2019).

As antas vivem em territórios com tamanhos médios de 8.3 km^2 (Medici et al., 2022). É um animal cujo comportamento é solitário e tímido, buscando alimentos durante a noite e descansando ou se escondendo durante o dia (Tapir Specialist Group, 2024). A sobreposição de territórios entre diferentes indivíduos da espécie é descrita e existem evidências de que a tolerância e convívio entre tais animais não está necessariamente associado à uma relação de parentesco entre eles (Pinho et al., 2014).

Figura 1. Distribuição da espécie *T. terrestris* na América do Sul (fonte: Carlos Pedraza, Tapir Specialist Group, 2008; Disponível online em: https://tapirs.org/tapirs/lowland-tapir/ > acessado em 28 de Fevereiro de 2024).

2. Agentes bacterianos estudados neste trabalho

2.1. Hemoplasmas

O gênero *Mycoplasma* faz parte da Classe Mollicutes, que alberga bactérias com ausência de parede celular (Razin et al., 1998; Tratchenberg, 2005). Os gêneros *Haemobartonella* e *Eperythrozoon* foram reclassificados no gênero *Mycoplasma* após estudos baseados no gene 16S rRNA, que demonstraram uma maior proximidade genética de tais agentes com o gênero em questão (Rikihisa et al., 1997; Neimark et al., 2001). As espécies do gênero *Mycoplasma* capazes de infectar a superfície dos eritrócitos de hospedeiros vertebrados são comumente chamadas de hemoplasmas. Tais bactérias possuem como característica um formato pleomórfico e um genoma circular de tamanho bastante pequeno, codificando apenas genes essenciais para a sobrevivência das mesmas (Messick et al., 2004).

No Brasil, a ocorrência das principais espécies de hemoplasmas que acometem animais domésticos é descrita. As espécies *Mycoplasma haemofelis*, '*Candidatus* Mycoplasma haemominutum' e '*Candidatus* Mycoplasma turicensis', que infectam felinos domésticos, são comumente descritas no país (Braga et al., 2012; Firmino et al., 2016; Munhoz et al., 2018), assim como relatos de infecção por *Mycoplasma haemocanis* e '*Candidatus* Mycoplasma haematoparvum' em cães domésticos também são frequentes (Valle et al., 2014; Soares et al., 2016; Miranda et al., 2021).

Em relação a animais de produção, relatos da infecção por *Mycoplasma ovis* podem ser encontrados em ovinos (Souza et al., 2019a; Mongruel et al., 2020), caprinos (Machado et al., 2017), equinos (Kakimori et al., 2023), assim como relatos de *Mycoplasma suis* (Guimarães et al., 2007b; Toledo et al., 2016; Gatto et al., 2019) e *Mycoplasma parvum* (Gatto et al., 2019) em suínos. As espécies '*Candidatus* Mycoplasma haemobos' e *Mycoplasma wenyonii* são relatadas em bovinos (Mello et al., 2019).

As consequências da infecção por hemoplasmas ainda são desconhecidas para muitos hospedeiros (Maggi et al., 2013). Manifestações clínicas são mais conhecidas em gatos, principalmente infectados por *M. haemofelis*. Nesses hospedeiros, é relatada a ocorrência de febre, letargia, palidez de mucosas, esplenomegalia e anorexia. Em cães, infecção por *M. haemocanis* está associada à crise hemolítica em animais esplenectomizados (Kemming et al., 2004), co-infectados por outros agentes ou em situação de imunossupressão (Santos et al., 2018). Já em animais de produção, a infecção por hemoplasmas geralmente possui caráter crônico, com consequências na produtividade e índices de reprodução dos animais (Guimarães et al., 2018).

Animais selvagens, tanto de cativeiro quanto de vida-livre, também são hospedeiros de hemoplasmas no Brasil. Felídeos (Guimarães et al., 2007a; André et al., 2011a; Ribeiro et al., 2017; Sousa et al., 2017a; Furtado et al., 2018; Carneiro et al., 2020) e canídeos selvagens (André et al., 2011a; Sousa et al., 2017a; Carneiro et al., 2020) foram diagnosticados com as mesmas espécies e/ou genótipos muito semelhantes a aqueles detectados em gatos e cães domésticos. Primatas não-humanos, tanto de cativeiro (Ramalho et al., 2017; Melo et al., 2019; Mongruel et al., 2022) quanto de vida-livre (Bonato et al., 2015), são descritos como hospedeiros de genótipos conhecidos ou novos de hemoplasmas. Adicionalmente, diferentes grupos taxonômicos de mamíferos de vida-livre têm sido relatados como hospedeiros para esses agentes no Brasil, como cervídeos (Grazziotin et al., 2011; André et al., 2020a), capivaras (Vieira et al., 2009; Gonçalves et al., 2019; Vieira et al., 2021), roedores (Gonçalves et al., 2015), morcegos (Ikeda et al., 2017; Santos et al., 2020; Mello et al., 2024), pinípides (Sousa et al., 2017a, Battisti et al., 2024), cetáceos (Duarte-Benvenuto et al., 2023), procionídeos (Sousa et al., 2017a; Perles et al., 2023a; Fagundes-Moreira et al., 2023), marsupiais (Gonçalves et al., 2019; Pontarolo et al., 2021; Oliveira et al., 2023a; Braga et al., 2023), ouriços (Valente et al., 2021) de vida-livre e representantes da Superordem Xenarthra (Oliveira et al., 2022).

A rota de transmissão dos hemoplasmas ainda é discutida. Em gatos, a transmissão de *M. haemofelis* e '*Ca*. M. haemominutum' por pulgas da espécie *Ctenocephalides felis* foi descrita como uma consequência do repasto sanguíneo destes ectoparasitos em apenas um entre nove animais testados (Woods et al., 2005). A transmissão por meio da ingestão de pulgas e ovos de pulgas infectados por gatos, envolvendo as mesmas espécies de hemoplasmas, não possui evidência da sua viabilidade (Woods et al., 2006). Já em relação aos cães, o carrapato *R. sanguineus* sensu lato é descrito como vetor de *M. haemocanis* para esses hospedeiros, além da transmissão por transfusão sanguínea (Saneviratna et al., 1973; Lester et al., 1995).

Diferentes espécies e genótipos de hemoplasmas já foram detectados em possíveis vetores, tais como carrapatos da espécie *Amblyomma dubitatum* (Vieira et al., 2021) e piolhos *Polyplax spinulosa* (Gonçalves et al., 2019). Adicionalmente, a competência vetorial de *Rhipicephalus microplus* na transmissão de '*Ca*. M. haematobos' já foi relatada em camundongos (Shi et al., 2019). Entretanto, o papel dos artrópodes ainda não é totalmente desvendado para todas as espécies de hemoplasmas visto que hospedeiros sem histórico de infestação por ectoparasitos apresentaram-se positivos para hemoplasmas (Mongruel et al., 2022; Huggins et al., 2023). Ainda, ectoparasitos coletados de hospedeiros positivos para hemoplasmas nem sempre apresentam resultados positivos para estes agentes (Sousa et al., 2017). A transmissão vertical por via uterina já foi descrita em animais domésticos (Girotto-Soares et al., 2016) e selvagens (Millán et al., 2024), o que pode significar uma rota importante na disseminação do agente. De fato, acredita-se que os hemoplasmas utilizem vias de transmissão diversas, envolvendo artrópodes vetores, transmissões diretas via interações sociais/agressivas e transmissão vertical (Millán et al., 2021).

Adicionalmente, existe um relato da co-infecção por *M. haemofelis*, *Bartonella henselae* e vírus HIV em uma pessoa no Brasil (Dos Santos et al., 2008). Em outras regiões do mundo, espécies de hemoplasmas originalmente de animais, como *M. suis* (Yuan et al., 2009), *M. ovi*s (Sykes et al., 2010) e *'Ca*. M. haematoparvum' (Steer et al., 2011) também já foram relatadas infectando seres humanos, demonstrando o potencial zoonótico de tais agentes. Portanto, embora a gama de espécies hospedeiras possa ser considerada ampla no país, não haviam relatos da infecção por hemoplasmas em antas brasileiras até o presente trabalho.

2.2. *Bartonella* sp.

O gênero *Bartonella* compreende bactérias Gram-negativas, intracelulares facultativas e pertencentes à classe Alphaproteobacteria. Tais agentes infectam eritrócitos e células endoteliais de diferentes espécies animais, incluindo seres humanos (Morse et al., 2012; Harms e Dehio, 2015), e são transmitidos primariamente por vetores artrópodes (Breitschwerdt, 2014). Em seres humanos, as principais espécies patogênicas são *Bartonella bacilliformis*, *B. henselae* e *Bartonella quintana* (Dehhaghi et al., 2019).

Bartonella bacilliformis é o agente etiológico da Doença de Carrion e Veruga Peruana e ocorre nas regiões andinas da América do Sul, principalmente no Peru, onde seu vetor, o mosquito *Lutzomyia verrucarum*, pode ser encontrado. A manifestação clínica ocorre de duas formas: a forma potencilamente fatal, caso não instaurada antibioticoterapia, caracterizada por uma anemia severa (febre Oroya) e a forma não-fatal, caracterizada por erupções cutâneas nodulares (verruga peruana) (Alexander, 1995; Ihler, 1996).

Bartonella henselae possui gatos domésticos como seus principais hospedeiros (Lamas et al., 2008; Breitschwerdt et al., 2020) e é o agente causador da Doença da Arranhadura do Gato (DAG), a qual é comumente caracterizada por linfadenopatia regional e febre (Florin et al., 2008). Em seres humanos, a transmissão ocorre por meio da inoculação de bactérias viáveis provenientes de arranhaduras ou mordeduras de gatos domésticos. Entre os gatos, a transmissão ocorre principalmente por meio de fezes de pulgas da espécie *C. felis* (Guptill, 2010).

Bartonella quintana, agente etiológico da Febre das Trincheiras, é transmitida pelo piolho *Pediculus humanus corporis*, encontrado em roupas e comumente associado a situações de higiene pessoal precária, baixas temperaturas climáticas e pobreza. As principais alterações clínicas causadas por *B. quintana* cursam com febre e endocardite (Focault et al., 2006).

O diagnóstico das infecções por *Bartonella* spp. é desafiador (Breitschwerdt et al., 2010), haja vista que estes agentes apresentam níveis baixos de bacteremia. Ensaios de qPCR demonstraram uma maior sensibilidade na detecção de *Bartonella* sp., tanto em animais domésticos quanto selvagens, quando as amostras de sangue de tais hospedeiros foram submetidas à cultura líquida de pré-enriquecimento utilizando meio BAPGM (*Bartonella Alpha Proteobacteria Growth Medium*) durante cerca de sete dias (Furquim et al., 2021; Amaral et al., 2022a; Amaral et al., 2022b; Dias et al., 2023), previamente à extração de DNA. Além disso, estudos que conseguiram cultivar *Bartonella* sp. diretamente de amostras de sangue de animais positivos descreveram a utilização de sangue fresco e/ou conservado a pelo menos -

70ºC ou em nitrogênio líquido (Tea et al., 2004; Amaral et al., 2022a; Amaral et al., 2022b), de modo a manter a viabilidade das bactérias até o momento do cultivo.

Presume-se que a complexidade envolvida na transmissão de *Bartonella* sp. em condições naturais seja maior do que a previamente considerada, haja vista a descrição cada vez maior de novas espécies e subespécies, assim como hospedeiros animais e vetores envolvidos (Breitschwerdt, 2014). Material genético de *Bartonella* sp. já foi detectado em pulgas coletadas de animais domésticos e selvagens no Brasil (Sousa et al., 2018b; Raimundo et al., 2022). Adicionalmente, as espécies *B. henselae*, *Bartonella clarridgeae* e *Bartonella koehlerae* já foram descritas em gatos domésticos de diferentes regiões no país (Miceli et al., 2013; André et al., 2014; 2015; Drummond et al., 2018; Silva et al., 2019; Raimundo et al., 2019).

Em relação a animais selvagens, genótipos similares à *B. henselae* e *B. koehlerae* foram descritas em felídeos selvagens mantidos em zoológicos (Filoni et al., 2012). Além disso, *Bartonella* spp. já foi detectada molecularmente em roedores selvagens (Gonçalves et al., 2016; Sousa et al., 2018b; Gonçalves-Oliveira et al., 2020), moscas Hippoboscidae parasitas de cervídeos (Souza et al., 2017) e morcegos (Amaral et al., 2018), e diretamente de morcegos (Ikeda et al., 2017; Ferreira et al., 2018; André et al., 2019; Gonçalves-Oliveira et al., 2020; Pacheco et al., 2024). Ainda, novas espécies de *Bartonella* foram recentemente descritas em roedores e marsupiais do Pantanal sul-matogrossense denominadas, respectivamente, *Bartonella harrusi* (Amaral et al., 2022b) e *Bartonella machadoae* (Amaral et al., 2022a). Uma possível nova subespécie de *Bartonella washoensis* (*Bartonella washoensis* subesp. *brasiliensis*) foi descrita em tatus e tamanduás (Calchi et al., 2020b).

Embora material genético de *B. bacilliformis* tenha sido detectado em carrapatos das espécies *Amblyomma ovale* e *Amblyomma scalpturatum* coletados em *T. terrestris* no Peru (Del Valle-Mendoza et al., 2018), a detecção direta de *Bartonella* spp. nesses animais, até o presente trabalho, não havia sido ainda relatada.

2.3. Família Anaplasmataceae

Em 2001 a ordem Rickettsiales foi reclassificada e, com isso, o gênero *Anaplasma* foi expandido. Anteriormente, tal gênero compreendia apenas patógenos específicos de ruminantes (*Anaplasma marginale*, *Anaplasma centrale* e *Anaplasma bovis*). Após a reclassificação, as espécies *Anaplasma phagocytophilum* (uma unificação das espécies anteriormente denominadas *Ehrlichia equi*, *Ehrlichia phagocytophila* e o agente da erliquiose granulocítica humana), *Anaplasma bovis* (anteriormente *Ehrlichia bovis*) e *Anaplasma platys* (anteriormente *Ehrlichia platys*) passaram a fazer parte do gênero.

Embora tais organismos sejam relacionados geneticamente, alguns aspectos referentes à sua biologia são distintos, tais como a especificidade de hospedeiros, o tipo de célula-alvo infectado e as espécies de carrapatos vetores (Dumler et al., 2001; Kocan et al., 2005). Enquanto as espécies de *Anaplasma* infectam eritrócritos, plaquetas e leucócitos, as espécies de *Ehrlichia* infectam leucócitos e células endoteliais (Cohn, 2003; Rymaszewska e Grenda, 2008; Marcelino et al., 2021). A transmissão biológica de tais patógenos ocorre por meio de diferentes espécies de carrapatos Ixodidae (Cohn, 2003).

Atualmente, o gênero *Anaplasma* possui oito espécies reconhecidas, sendo elas: *A. bovis*, *A. centrale*, *A. marginale*, *A. ovis*, *A. phagocytophilum*, *A. platys* (Rymaszewska, Grenda, 2008), *A. odocoilei* (Tate et al., 2013) e *A. capra* (Yang et al., 2018), embora diferentes genótipos e espécies *Candidatus* sejam também descritas (Caudill, Brayton, 2022). Dentre as espécies citadas, *A. phagocytophilum*, *A. platys*, *A. ovis* e *A. capra* são descritas como espécies capazes de infectar seres humanos (Dumler et al., 2005; Chochlakis et al., 2010; Arraga-Alvarado et al., 2014; Li et al., 2015). No Brasil, a ocorrência de *A. marginale* é considerada endêmica em bovinos, causando perdas econômicas na bovinocultura, tendo como vetor biológico o carrapato *R. microplus* (Kessler, 2001; Almeida et al., 2006) e dípteros hematófagos (tabanídeos e *Stomoxys calcitrans*) como vetores mecânicos. Recentemente, esta espécie foi detectada molecularmente em caprinos da região nordeste do Brasil (Da Silva et al., 2018; Barbosa et al., 2021; Silva et al., 2024). Em relação aos animais domésticos, *A. platys* ocorre em cães do país e sua transmissão está associada principalmente a carrapatos *R. sanguineus* sensu stricto (Snellgrove et al., 2020) e *R. sanguineus* sensu lato (Cordeiro et al., 2020). A detecção dessa espécie em felinos (Lima et al., 2010) e bovinos de corte (André et al., 2020b) também já foi relatada no Brasil.

Com a reorganização da ordem Rickettsiales, as espécies conhecidas como *Ehrlichia phagocytophila*, *Ehrlichia equi* e o agente causador da eliquiose granulocítica humana (*Ehrlichia* 'HGE agent') foram agrupados e redefinidos como representantes do gênero *Anaplasma*, sendo então nominada a espécie *Anaplasma phagocytophilum* (Dumler et al., 2001). Tal espécie é capaz de infectar os granulócitos de diferentes hospedeiros animais como ruminantes, equinos, cães, animais selvagens e seres humanos (Rymaszewska e Grenda, 2008). A transmissão está relacionada principalmente aos carrapatos do gênero *Ixodes* spp., em especial às espécies *Ixodes scapularis, Ixodes pacificus* e *Ixodes persulcatus* (Khatat et al., 2021). Atualmente, sabe-se que diferentes variantes de *A. phagocytophylum* ocorrem em diferentes regiões, culminando em discrepâncias em relação à manifestações clínicas, distribuição e hospedeiros susceptíveis. Por exemplo, o genótipo de *A. phagocytophilum* capaz de causar doença em pequenos ruminantes na Europa é considerado distinto daquele que causa doença em seres humanos nos Estados Unidos (Woldehiwet, 2010). Embora a ocorrência das principais espécies de carrapatos relacionadas à transmissão deste agente não seja descrita no Brasil, alguns estudos apontam para a circulação de genótipos semelhantes à *A. phagocytophilum* no país. A detecção molecular de fragmentos genéticos semelhantes e filogeneticamente próximos a *A. phagocytophilum* já foi relatada em cães domésticos (Santos et al., 2013; Silveira et al., 2015; Silveira et al., 2017a), equinos (Santos et al., 2019), aves selvagens (Machado et al., 2012; Mongruel et al., 2017a) e em carrapatos *Amblyomma sculptum* e *Rhipicephalus sanguineus* coletados em cães (Santos et al., 2013).

Em relação ao gênero *Ehrlichia*, seis espécies são descritas: *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia muris*, *Ehrlichia ruminantium* e *Ehrlichia minasensis* (Dumler et al., 2001; Cabezas-Cruz et al., 2016), sendo as espécies *E. chaffeensis*, *E. ewingii*, *E. canis* e *E. ruminantium* relacionadas à infecção em seres humanos (Dumler e Walker, 2001; Allsopp et al., 2005; Thomas et al., 2009; Bouza-Mora et al., 2017). No Brasil, *E. canis* é a espécie mais difundida, infectando células mononucleares de cães e sendo transmitida por *R. linnaei* (anteriormente denominada como carrapatos da linhagem tropical de *R. sanguineus*) (Moraes-Filho et al., 2015). Ainda, *E. canis* é relata em gatos de diversas regiões do Brasil (Braga et al., 2014; André et al., 2015, 2017; Guimarães et al., 2019). *Ehrlichia minasensis*, inicialmente descrita na haemolinfa de *R. microplus*, é filogeneticamente relacionada à *E. canis* (Cabezas-Cruz et al., 2016; Aguiar et al., 2019) e já foi descrita infectando e causando sinais clínicos em bovinos do estado do Mato Grosso (Aguiar et al., 2014) e em bovinos de corte assintomáticos no estado do Mato Grosso do Sul (André et al., 2020b). A transmissão transestadial de *E. minasensis* em carrapatos da espécie *R. microplus* foi relatada (Carvalho et al., 2016). Ainda, um genótipo filogeneticamente relacionado a *E. ruminantium* foi detectado em um equino na região sul do Brasil (Vieira et al., 2018).

As espécies do gênero *Neorickettsia* também podem causar enfermidades em animais e humanos. Neste sentido, *Neorickettsia risticii* e *Neorickettsia findlayensis* são agentes causadores da Febre do Cavalo de Potomac (FCP) (Pusterla et al., 2003; Teymournejad et al., 2020), enquanto *Neorickettsia sennetsu* é o agente etiológico da neorickettsiose Sennetsu, que afeta seres humanos (Gibson et al., 2006). *Neorickettsia helminthoeca* é relacionada à intoxicação por consumo de salmão em cães (Headley et al., 2011; Furtado et al., 2022). Diferentemente dos gêneros *Ehrlichia* e *Anaplasma*, as bactérias do gênero *Neorickettsia* são transmitidas por interações entre hospedeiros vetebrados e trematódeos (Dumler, 2001). *Neorickettsia risticii* é capaz de infectar primariamente os monócitos, causando a FCP, que cursa com anorexia, diarreia e cólica em equinos (Palmer, 1993). A deteção molecular de *N. risticii* já foi relatada em cavalos sintomáticos no sul (Coimbra et al., 2006; Marutani et al., 2019) e sudeste (Paulino et al., 2020) do Brasil. Supõe-se que a transmissão de *N. risticii* esteja relacionada à ingestão de pastagens contendo metacercárias infectadas por equinos susceptíveis (Dumler et al., 2001). No sul do país, a transmissão foi relacionada à cercaria *Parapleurolophocercous cercariae* infectando caramujos do gênero *Heleobia* (Coimbra et al., 2005).

Nos últimos anos, com o avanço das técnicas de biologia molecular, um notável número de genótipos, novos ou filogeneticamente relacionados à espécies já conhecidas, de *Anaplasma* e *Ehrlichia* têm sido detectados em diferentes grupos taxonômicos de hospedeiros selvagens no Brasil, como aves (Machado et al., 2012; Werther et al., 2017; Mongruel et al., 2017a; Sacchi et al., 2021), cervídeos (Sacchi et al., 2012; Mongruel et al., 2017b; Soares et al., 2017b), canídeos selvagens (André et al., 2012; Sousa et al., 2017b; André, 2018), felídeos selvagens (André et al., 2012; Sousa et al., 2017b; Mazzotti et al., 2018), quatis (Sousa et al., 2017b), roedores (Benevenute et al., 2017; Vieira et al., 2022), javalis (Santana et al., 2022) e representantes da Superordem Xenarthra (Calchi et al., 2020a). Recentemente, uma possível nova espécie de *Ehrlichia,* denominada '*Candidatus* Ehrlichia dumleri' foi descrita em quatis (*Nasua nasua*) no centro-oeste do Brasil (Perles et al., 2022b). Adicionalmente, '*Candidatus* Anaplasma amazonensis'e *'Candidatus* Anaplasma brasiliensis' foram descritos em mamíferos da Superordem Xenarthra no Brasil (Calchi et al., 2020a).

Recentemente, genótipos 16S rRNA filogeneticamente relacionados a *Neorickettsia risticii* foram detectados em morcegos (Ikeda et al., 2021; Mello et al., 2023b) e quatis (Perles et al., 2023c). Entretanto, até o presente trabalho, não haviam descrições da infecção por agentes Anaplasmataceae em antas.

2.4. *Coxiella burnetii*

Coxiella burnetii é uma bactéria intracelular Gram-negativa, pertencente à ordem Legionellales e que infecta monócitos e macrófagos. Essa espécie é o agente etiológico da enfermidade denominada "febre Q" em seres humanos, uma zoonose reportada mundialmente (Honarmand, 2012; Eldin et al., 2017). A transmissão deste agente ocorre, de forma mais comum, por via aerógena. Entretanto exposição a produtos de aborto e ingestão de leite não-pasteurizado também são citados como meios de transmissão. Trata-se de uma zoonose de caráter ocupacional, para a qual médicos veterinários e funcionários de abatedouros e fazendas estão sob maiores riscos para infeção (Tissot-Dupont e Raoult, 2008; Lemos et al., 2011; Honarmand, 2012). Além do contato direto, carrapatos já foram apontados como possíveis participantes na transmissão vetorial de *C. burnetii* (Eldin et al., 2017).

A infecção por *C. burnetii* em seres humanos é geralmente assintomática. Entetanto, no caso de pessoas imunossuprimidas ou que apresentam alguma comorbidade, a doença pode trazer complicações como pneumonia e hepatite. Ainda, está associada a casos de abortamento em gestantes (Maurin, Raoult, 1999). Embora a ocorrência de *C. burnetii* seja relatada mundialmente (Tissot-Dupont e Raoult, 2008), relatos no Brasil ainda são escassos. Por meio de métodos moleculares e sorológicos, o agente já foi relatado em seres humanos (Lemos et al., 2011; Rozental et al., 2018), bovinos (Zanatto et al., 2019a) e caprinos (Oliveira et al., 2018). De fato, uma variada gama de espécies animais domésticos são descritos como hospedeiras e potenciais fontes de infecção para seres humanos, sendo os ruminantes os animais mais associados à dispersão do agente (Celina, Cerny, 2022).

Acredita-se que os animais selvagens não possuam um impacto tão relevante para a transmissão zoonótica do agente. Entretanto, possuem papel importante na manutenção do patógeno no meio ambiente, especialmente com o envolvimento da transmissão por carrapatos (Kazar, 2005; Celina, Cerny, 2022). No Brasil, *C. burnetii* foi molecularmente relatada apenas em roedores (Rozental et al., 2017) e morcegos (Ferreira et al., 2018). Entretanto, evidências sorológicas da exposição ao agente já foram relatadas em cervídeos (Zanatto et al., 2019b), roedores (*Wiedomys pyrrhorhinos*) e gambás (*Didelphis albiventris*) (Oliveira et al., 2020).

Considerando que as antas são encontradas frequentemente infestadas por diferentes espécies de carrapatos em diferentes biomas no Brasil (Labruna et al., 2021), a vigilância epidemiológica acerca da infecção por *C. burnetii* nesses animais mostra-se importante. Entretanto, embora um estudo tenha citado um resumo de congresso que descreve a detecção molecular de *C. burnetii* em *T. terrestris* na Guiana Francesa (Epelboin et al., 2023), o referido trabalho não foi publicado e não pôde ser encontrado na literatura. Portanto, até o presente momento, não existem relatos disponíveis sobre a infecção por *C. burnetii* em antas.

2.5. *Borrelia* spp.

As bactérias do gênero *Borrelia* são espiroquetas transmitidas por vetores capazes de causar doenças em humanos e animais, podendo infectar as células endoteliais de seus hospedeiros (Trevisan et al., 2021a; Trevisan et al., 2021b). Desde a sua primeira descrição, no início do século 20, diferentes espécies e cepas, que podem causar enfermidades com aspectos clínicos e epidemiológicos distintos, já foram relatadas. Atualmente, as espécies desse gênero podem ser divididas em três principais grupos: (1) Grupo Lyme: representadas pela espécie *Borrelia burgdorferi* sensu lato e transmitidas por carrapatos Ixodidae e o piolho *Pediculus humanus humanus*; (2) Grupo da Febre Recorrente (FR): representado por diferentes espécies que são transmitidas principalmente por carrapatos Argasidae, com algumas poucas espécies sendo transmitidas por carrapatos Ixodidae; (3): Grupo Réptil-Equidna: grupo recentemente descrito capaz de infectar répteis, anfíbios e equidnas (Tachyglossidae), filogeneticamente distante dos demais, e transmitido por carrapatos Ixodidae (Margos et al., 2018; Trevisan et al., 2021a; Trevisan et al., 2021b).

Uma das espécies patogênicas mais conhecidas do gênero *Borrelia* é *B. burgdorferi*, o agente etiológico da Doença de Lyme (DL). A DL é uma zoonose que ocorre principalmente na Europa e Estados Unidos, sendo transmitida por carrapatos do gênero *Ixodes*. Em seres humanos, alterações decorrentes da resposta imune do hospedeiro frente à infecção, como artrites, glomerulonefrites e encefalites, são relatadas. Com o avanço das ferramentas moleculares, percebeu-se a existência de variantes genéticas da espécie. Portanto, atualmente, o grupo *B. burgdoferi* sensu lato é composto por mais de vinte genoespécies (Pinheiro, 2018; Wolcott et al., 2021). A ocorrência da DL é considerada incerta no Brasil (Faccini-Martínez et al., 2021).

A Febre Recorrente transmitida por piolhos é causada pela espécie *Borrelia recurrentis* e tem como vetor o piolho da espécie *P. humanus humanus*, que transmite a espiroqueta não por picadas, mas sim quando é esmagado e seus fluidos penetram a pele do hospedeiro susceptível durante episódios de prurido. A infecção pode desencadear episódios febris (cerca de três a cinco), dor de cabeça, icterícia, confusão mental e hemorragias. mental e hemorragias. O agente não é relatado em outros mamíferos que não o ser humano (Cutler, 2015; Warrel, 2019).

Os carrapatos Argasidae do gênero *Ornithodoros* são os vetores mais comuns da Febre Recorrente. As espécies de *Borrelia* desse grupo possuem como reservatório pequenos mamíferos, sendo que seres humanos são acidentalmente infectados quando estes se expõem à ambientes onde o vetor é encontrado, como cavernas e ninhos de aves (Telegrand-Reboul, et al., 2018; Faccini-Martínez et al., 2021). A infecção em seres humanos pode cursar com mais de 10 episódios de febre, caso não seja iniciado o tratamento, além de sintomas e sinais inespecíficos, como dor de cabeça e esplenomegalia (Cutler, 2015). Algumas espécies de *Borrelia* do grupo da FR podem ser transmitidas por carrapatos duros (Ixodidae), como é o caso da espécie *Borrelia theileri,* cujo vetor principal são carrapatos do gênero

Rhipicephalus. Esse patógeno foi descrito inicialmente em 1904 como causador de febre, apatia e anorexia em bovinos (Theiler, 1904).

Recentemente, genótipos de *Borrelia* sp. do grupo da FR têm sido descritos em carrapatos e hospedeiros vertebrados no Brasil. Neste sentido, *Borrelia venezuelensis* foi descrita em carrapatos da espécie *Ornithodoros rudis* coletados no Cerrado brasileiro, após isolamento *in vitro* e caracterização molecular (Muñoz-Leal et al., 2018). Genótipos distintos de *Borrelia* sp. foram detectados em órgãos de morcegos hematófagos na região Nordeste (Jorge et al., 2023). *Borrelia theileri* foi detectada em carrapatos da espécie *R. microplus* da região Sudeste e Centro-Oeste (Yparraguirre et al., 2007; Cordeiro et al.., 2018) e em bovinos das regiões Sudeste, Centro-Oeste e Norte do país (Paula et al., 2022; Figueiroa et al., 2023).

Embora estudos sorológicos tenham apontado evidências de exposição à *B. burgdorferi* por *T. terrestris* mantidos em cativeiro na Alemanha (Stoebel et al., 2003), a ocorrência de reação cruzada entre anticorpos produzidos por *B. theileri* e antígenos brutos de *B. burgdorferi* é descrita (Rogers et al., 1999) e deve ser levada em consideração na interpretação de testes sorológicos. Em 2015, tentativas de detectar *B. burgdorferi* em carrapatos do gênero *Amblyomma* spp. coletados de *T. pinchaque* no Equador resultaram em resultados negativos (Pesquera et al., 2015). Até o presente trabalho, não haviam relatos da detecção de *Borrelia* sp. em amostras biológicas de *T. terrestris*.

3. Protozoários estudados neste trabalho

3.1. Piroplasmídeos

A origem da palavra "Piroplasmida" vem do grego (pera = pêra) e descreve o formato do estágio intraeritrociário dos agentes apicomplexos que compõe a ordem Piroplasmida. Comumente designados piroplasmídeos, tais agentes são representados pelas espécies pertencentes aos gêneros *Babesia*, *Theileria*, *Cytauxzoon* e *Rangelia*. Assim como outros representantes do Filo Apicomplexa, os piroplasmídeos são parasitos intracelulares obrigatórios e necessitam de dois hospedeiros para completar seu ciclo: um vertebrado (aves e mamíferos) e outro invertebrado (carrapatos Ixodidae) (Schnittger et al., 2022).

Jalovecka e colaboradores (2019) definiram a existência de dez clados diferentes nos quais estão distribuídas as espécies da ordem Piroplasmida, baseando-se em fragmentos longos do gene 18S rRNA (>1.600 pb). De acordo com essa proposta, a divisão taxonômica da Ordem Piroplasmida, seguindo a topologia filogenética obtida, é: Clado I – grupo *Babesia microti-like* (*Babesia* sensu lato) contendo as espécies *B. microti*, *Babesia vulpes*, *Babesia rodhaini*, *Babesia felis*; Clado II – grupo Monotremata (*Theileria* sensu lato) contendo a espécie *Theileria ornythorynchi*; Clado III – grupo Western (*Babesia* sensu lato) contendo as espécies *Babesia duncani*, *Babesia lengau* e *Babesia conradae*; Clado IV – grupo Marsupialia (*Theileria* sensu lato) contendo espécies de *Theileria* spp. detectadas em marsupiais na Austrália; Clado V – grupo Peircei (*Babesia* sensu lato) contendo as espécies *Babesia peircei*, *Babesia poelea* e *Babesia ugwidiensis*; Clado VI – grupo Rhinocerotidae (*Theileria* sensu lato) contendo a espécie *Theileria bicornis*; Clado VII – grupo *Cytauxzoon* contendo as espécies *Cytauxzoon felis* e *Cytauxzoon manul* e, mais recentemente, *Cytauxzoon otrantorum, Cytauxzoon europaeus e Cytauxzoon banethi* (Panait et al., 2021); Clado VIII – grupo Equus (*Theileria* sensu lato) contendo as espécies *Theileria equi* e *Theileria haneyi*; Clado IX – grupo *Theileria* sensu stricto contendo as espécies *Theileria annulata*, *Theileria parva*, *Theileria lestoquardi* e *Theileria orientalis*; Clado X – grupo *Babesia* sensu stricto contendo as espécies *Babesia bovis*, *Babesia bigemina*, *Babesia caballi, Babesia vogeli, Babesia rossi Babesia canis* e *Babesia ovis* (Jalovecka et al., 2019).

Nos últimos anos, o número de espécies hospedeiras de piroplasmídeos têm se expandido, o que, consequentemente, expandiu também o número de clados presentes nas análises filogenéticas da ordem Piroplasmida. Os clados Phyllostomidae *group*, composto por piroplasmídeos detectados em morcegos não-hematófagos da família Phyllostomidae (Ikeda et al., 2021); South America Marsupialia *group*, composto por *Babesia* spp. detectada em *Didelphis aurita* (Oliveira et al., 2023b), Capybara *group*, composto por *Babesia goianiaensis* descrita em capivaras (*Hydrochoerus hydrocharis*) (Krawczak et al., 2023) e *Tapirus terrestris* *group* (representado por *Theileria terrestris* e descrito no presente trabalho) foram descritos em estudos realizados no Brasil

Os piroplasmídeos possuem ciclos de vida que envolvem reprodução sexuada e assexuada em carrapatos ixodídeos vetores (hospedeiros definitivos) e assexuada em hospedeiros vertebrados (hospedeiros intermediários). Todos os membros da ordem Piroplasmida realizam reprodução assexuada dentro de células sanguíneas dos hospedeiros vertebrados (Jalovecka et al., 2018). A infecção do hospedeiro vertebrado começa com a invasão do mesmo por esporozoítos transmitidos pela picada do carrapato infectado. Para os parasitos da família Theileriidae (*Theileria* e *Cytauxzoon*), ocorre a invasão e o consequente processo de esquizogonia em leucócitos ou macrófagos. Para as espécies do gênero *Babesia* acredita-se que todo o ciclo dentro do hospedeiro vertebrado ocorra exclusivamente em eritrócitos, entratanto, a ocorrência de esquizogonia não é confirmada para esse gênero (Jalovecka et al., 2019). Interessantemente, *Rangelia vitalii*, um membro do grupo de *Babesia* sensu stricto, apresenta esquizogonia em células endoteliais de diversos órgãos antes de invadirem hemácias (Carini e Maciel, 1914).

A esquizogonia é considerada uma fase do ciclo na qual os parasitos multiplicam-se consideravelmente no interior de leucócitos/macrófagos/células endoteliais e dão origem aos esquizontes (também chamados de corpúsculos de Koch) (Jalovecka et al., 2018). Além disso, em algumas espécies de *Theileria* sensu stricto, o processo de invasão dos leucócitos pelo parasito é complexo e a modulação da reposta imune leva à uma transformação neoplásica da célula, devido à inibição dos mecanismos de apoptose. As espécies de *Theileria* que apresentam tal mecanismo são conhecidas como "transformadoras" e dentro do grupo das espécies transformadoras estão aquelas que infectam principalmente ruminantes: *T. annulata*, *T. parva*, *T. lestoquardi* e *Theileria taurotragi* (Bishop et al., 2004; von Schubert et al., 2010; Sivakumar et al., 2014; Mans et al., 2015).

A merogonia, fase seguinte do ciclo, ocorre exclusivamente dentro dos eritrócitos (Jalovecka et al., 2019), com exceção de *R. vitalii*, para a qual também ocorre em leucócitos (Silva et al., 2011; França et al., 2014). Após adentrar a célula, e permanecer livre no meio citoplasmático, os esporozoítos (no caso das espécies de *Babesia*) ou merozoítos (no caso de Theileriidae) se transformam em trofozoítos.

Estes, por sua vez, multiplicam-se por fissão binária gerando o merozoíto (Jalovecka et al., 2019). Esse é o momento do ciclo no qual é possível visualizar formas intraeritrocitárias nas hemácias dos hospedeiros, por meio de esfregaços sanguíneos corados (**Figura 2**).

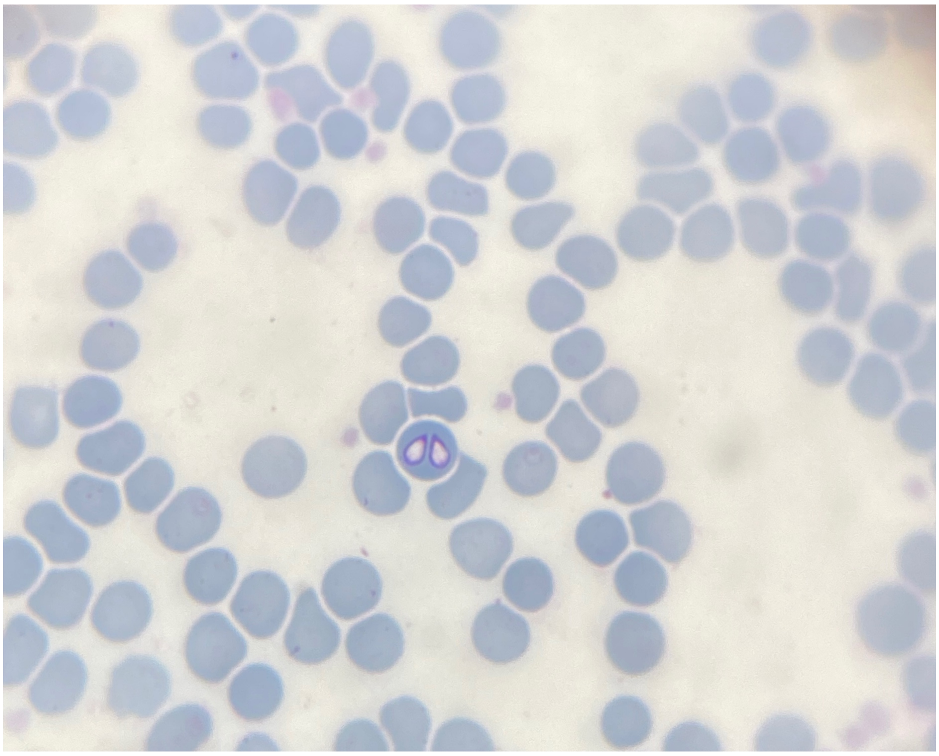


Figura 2. Merozoíto de *Rangelia vitalii* em hemácia de cão. Visualização em microscópio de luz, em aumento de 1000x. Lâmina corada com coloração de Giemsa (Fonte: arquivo pessoal da autora).

A multiplicação do parasito ocorre até que a hemácia se rompa e merozoítos sejam liberados na corrente sanguínea novamente para infectar novos eritrócitos (Takahira, 2018; Jalovecka et al., 2018). Tal multiplicação é assíncrona e diferentes estágios divisionais podem ser encontrados ao mesmo tempo em um mesmo hospedeiro (Chauvin et al., 2009). Durante a invasão, alguns merozoítos irão se transformar em gametócitos e circular na corrente sanguínea do hospedeiro até serem ingeridos durante o repasto sanguíneo de um carrapato competente, dando início à próxima fase do ciclo: a gamogonia (Jalovecka et al., 2018).

Os gametócitos ingeridos pelo carrapato irão transformar-se em gametas maduros no lúmen intestinal destes artrópodes, para dar início à reprodução sexuada do parasito. Novamente, nessa fase do ciclo ocorre uma diferença entre Babesiidae e Theileriidae: os gametas formados pelas espécies de *Babesia* são considerados morfologicamente indistinguíveis, denominados corpos raiados ("*ray bodies*"), enquanto os gametas formatos por *Theileria* e *Cytauxzoon* distinguem-se morfologicamente em microgametas e macrogametas. Após a fusão e fertilização dos gametas, forma-se um zigoto que irá penetrar a matriz peritrófica das células epiteliais do carrapato. Nesse local, os zigotos entram em divisão meiótica que irá resultar na formação de cinetos (Jalovecka et al., 2018).

Nessa fase, existe mais uma diferença entre Babesiidae e Theileriidae: os cinetos primários dos gêneros *Theileria* e *Cytauxzoon* invadem diretamente a glândula salivar do carrapato; já os cinetos primários de *Babesia* spp. continuam a se multiplicar assexualmente em vários tecidos, gerando assim os chamados cinetos secundários que irão, por sua vez, invadir a glândula salivar. Neste ponto, existe também uma diferença entre as espécies de *Babesia*: os cinetos primários dos parasitos do grupo *Babesia* sensu stricto invadem também os tecidos ovarianos dos carrapatos, o que irá permitir sua transmissão transovariana. Já os cinetos primários das espécies do grupo da *B. microti* (sensu lato) não invadem o ovário e, consequentemente, não há transmissão transovariana. Logo, dentre os piroplasmídeos, a transmissão transovariana ocorre somente para as espécies do grupo *Babesia* sensu stricto (Jalovecka et al., 2018; Jalovecka et al., 2019).

Na glândula salivar dos carrapatos, ocorre a fase de esporogonia. Após a invasão da glândula, os cinetos formam o esporonte que irá, posteriormente, diferenciar-se em esporoblasto. De forma geral, o esporoblasto permanece dormente durante a ecdise do carrapato e sua maturação inicia-se após o carrapato fixar-se na pele do hospedeiro vertebrado, resultando em esporozoítos que serão liberados na corrente sanguínea do hospedeiro por meio da saliva do carrapato (Jalovecka et al., 2018). A **Figura 3** resume as principais diferenças entre os ciclos das espécies do gênero Babesiidae e daquelas da família Theileriidae.

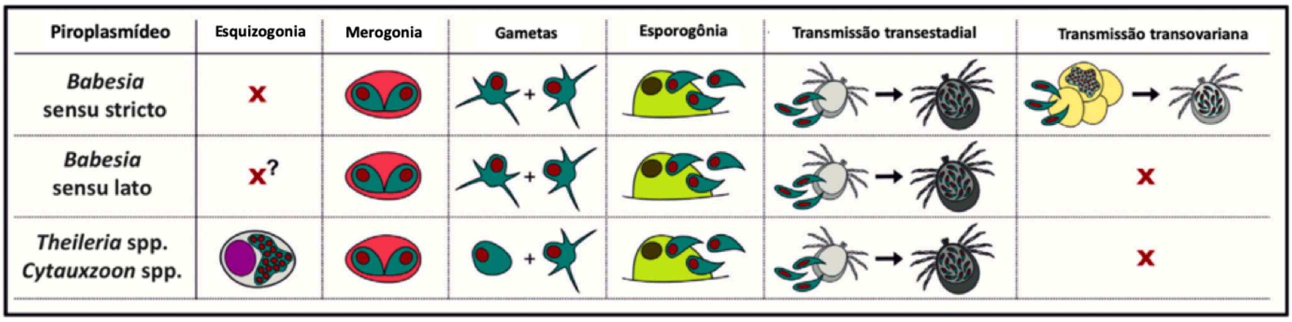


Figura 3. Esquema adaptado de Jalovecka e colaboradores (2019) resumindo as principais diferenças entre os ciclos das espécies do grupo *Babesia* sensu stricto (incluindo *R. vitalii*), *Babesia* sensu lato e daquelas da família Theileriidae (*Theileria* spp. e *Cytauxzoon* spp.). Enquanto as espécies dos gêneros *Theileria* e *Cytauxzoon* realizam a fase esquizôgonica em leucóticos e macrófagos, respectivamente, após a invasão dos esporozoítos na corrente sanguínea, a presença de tal fase não é totalmente compreendida para espécies do grupo de gênero *Babesia* sensu stricto (com exceção de *R. vitalii*, na qual tal fase foi observada em células endoteliais) e sensu lato. Outra diferença marcante entre *Theileria*/*Cytauxzoon* e *Babesia* são os gametas morfologicamente indistinguíveis formados por este último. Em relação ao tipo de transmissão, todas es espécies realizam perpetuação transestadial, enquanto somente as espécies do grupo *Babesia* sensu stricto realizam, também, transmissão transovariana (Fonte: Jalovecka et al., 2019 modificado).

Considerando a vasta distribuição de carrapatos vetores em todo o mundo, a babesiose é considerada uma das doenças infecciosas mais comuns que acometem as células sanguíneas dos animais (Hunfeld et al., 2008; Schnittger et al., 2022). De fato, no Brasil, a babesiose canina é uma doença endêmica e têm como principal agente etiológico *Babesia vogeli*, sendo transmitida pelos carrapatos *R. sanguineus* sensu stricto e *Rhipicephalus linnaei*. Embora a espécie *Babesia gibsoni* também tenha sido detectada no país, sua ocorrência é considerada de menor importância quando comparada a *B. vogeli* (Takahira, 2018). Ainda, *B. vogeli* já foi detectada molecularmente em felinos domésticos no país (André et al., 2014; 2015; Malheiros et al., 2016). No contexto dos animais de produção, as espécies *B. bovis* e *B. bigemina* possuem importância ecônomica por serem os agentes causadores da tristeza parasitáriaia bovina (TPB) - juntamente com *Anaplasma marginale* - e transmitidas por carrapatos *R. microplus* (Mendes et al., 2019). A babesiose bovina, que pode cursar em conjunto à anaplasmose (caracterizando a TPB) ou de maneira isolada, é apontada como uma das principais causas de mortalidade e morbilidade nos rebanhos do país (Alves, 2012).

O gênero *Theileria* representa piroplasmas de importância principalmente para animais de produção e que podem ser transmitidos por carrapatos dos gêneros *Amblyomma, Rhipicephalus, Hyalomma* e *Haemaphysalis* (Mans et al., 2015). No Brasil, diferentes genótipos são descritos em animais domésticos e selvagens. A ocorrência de *T. equi*, espécie capaz de infectar equinos, é considerada endêmica no país (Campos et al., 2019), podendo causar febre, inapetência, emagrecimento e anemia nesses animais (Schnittger et al., 2022). Ainda, genótipos de *Theileria* sp. filogeneticamente associados a aqueles encontrados em equídeos e ruminantes e animais selvagens já foram detectados em gatos domésticos (André et al., 2014; André et al., 2015). Genótipos de *Theileria* spp. relacionados às espécies que infectam ruminantes foram detectados em um rebanho de búfalos (*Bubalus bubalis*) no Pará (Silveira et al., 2016). Já a espécie *Theileria cervi* é relatada em cervídeos (*Subulo [Mazama] gouazoubira* e *Blastocerus dichotomus*) no país (Silveira et al., 2011; Silveira et al., 2014; Fava et al., 2022).

A espécie *Cytauxzoon felis* é um piroplasmídeo que infecta os eritrócitos e macrófagos teciduais de gatos domésticos (Cohn e Birkenheuer, 2015), causando febre, inapetência, letargia, dor generalizada e comumente levando esses animais a óbito (Wang et al., 2017). Nos Estados Unidos, linces-pardos (*Lynx rufus*) são indicados como reservatórios do agente visto que normalmente não desenvolvem formas graves da doença devido à esquizogonia curta e auto-limitante (Wang et al., 2017). No Brasil, existem relatos da detecção de *Cytauxzoon* sp. em felídeos selvagens de cativeiro (Peixoto et al., 2007; André et al., 2009) e também de vida livre (Soares et al., 2017a; Sousa et al., 2018a), além de gatos domésticos (Maia et al., 2013; André et al., 2015; André et al., 2017). Entretanto, relatos de infecções letais causadas por *Cytauxzoon* sp. são incomuns no país, o que sugere que genótipos diferentes daqueles encontrados nos Estados Unidos circulam no Brasil (André et al., 2017). Até o momento, casos fatais de cytauxzoonose no Brasil foram relatados somente em leões (*Panthera leo*) mantidos em cativeiro no estado do Rio de Janeiro

(Peixoto et al., 2007) e onça-pintada (*Panthera onca*), também mantida em cativeiro, no estado do Mato Grosso do Sul (Guizelini et al., 2021). Adicionalmente, hipotetiza-se que a onça-pintada seja a espécie nativa que atua como reservatório de *Cytauxzoon* sp. no Brasil (Furtado et al., 2017; Fagundes-Moreira et al., 2022), sendo os carrapatos da espécie *A. sculptum* apontados como os possíveis vetores do agente (Fagundes-Moreira et al., 2022).

A ordem Piroplasmida também contempla o protozoário da espécie *R. vitalii*. Embora esta seja filogeneticamente posicionada no grupo de *Babesia* sensu stricto (Soares et al., 2011), *R. vitalii* é capaz de infectar outras células além de eritrócitos, incluindo leucócitos e células endoteliais (Loretti e Barros, 2005; França et al., 2010). Esse patógeno infecta cães domésticos e canídeos selvagens; estes últimos são apontados como possíveis reservatórios do agente nas regiões sul e sudeste do Brasil (de Lorenzo et al., 2021). A ocorrência deste protozoário já foi confirmada na Argentina (Eiras et al., 2014), Uruguai (Soares et al., 2015) e Paraguai (Inácio et al., 2019). A distribuição geográfica do agente é limitada à distribuição dos carrapatos vetores da espécie *Amblyomma aureolatum* (Soares et al., 2018). A rangeliose é considerada uma doença hemolítica aguda, podendo causar anemia, icterícia, anorexia e hemorragia em cães (Fighera et al., 2010).

Possivelmente, um número maior de parasitos da ordem Piroplasmida existe quando comparados ao número de hospedeiros vertebrados que são capazes de albergar tais patógenos (Schnittger et al., 2022). Recentemente, por meio de técnicas moleculares, novos genótipos de piroplasmídeos têm sido descritos em animais selvagens no Brasil, como cervídeos (Silveira et al., 2011; Silveira et al., 2014), aves (Werther et al., 2017), gambás (Soares et al., 2017a; Gonçalves et al., 2021; Oliveira et al., 2023b; Braga et al., 2023), morcegos não-hematófagos (Ikeda et al., 2021) e hematófagos (Mello et al., 2023a), canídeos selvagens (Sousa et al., 2018a; Souza et al., 2019b), felídeos selvagens (André et al., 2011b; Sousa et al., 2018a), búfalos (Silveira et al., 2016), roedores (Sousa et al., 2018a), capivaras (Krawczak et al., 2023) e representantes da Superordem Xenarthra (Soares et al., 2017a; Calchi et al., 2023).

Em relação às antas, a espécie *Theileria equi* foi relatada em um animal, atendida após atropelamento, no Estado do Mato Grosso do Sul. O animal apresentava hipertermia, anemia e leucocitose. No relato em questão, uma sequência parcial do gene 18S rRNA, com aproximadamente 414 pb, foi amplificada e sequenciada. Na análise via *software* BLASTn para comparação de similaridade com outras sequências depositadas na base de dados GenBank, 98% de identidade com *T. equi* foi observada (Silveira et al., 2017b). Alguns anos depois, DNA de *T. equi* foi detectado em 11/19 antas mantidas em cativeiro na região amazônica, também por meio da amplificação de fragmentos curtos (392–475 bp) do gene 18S rRNA, dessa vez seguido por análises filogenéticas de Máxima Verossimilhança e Inferência Bayesiana (Gonçalves et al., 2020). DNA de *Theileria* sp. também foi detectado em amostras de sangue e baço coletadas de cadáveres de antas atropeladas na região do Pantanal, no Estado do Mato Grosso State. Tais sequências, com aproximadamente 740 pb, mostraram-se filogeneticamente próximas à *Theileria* sp. detectadas em gatos no Brasil (Silva et al., 2021). Considerando a natureza conservada do gene 18S rRNA, a determinação de espécies baseando-se exclusivamente em fragmentos curtos deste gene pode ser considerada inacurada, visto a incapacidade de se obter análises robustas (Wu et al., 2015; Jalovecka et al., 2019). Até o presente trabalho, sequências longas do gene 18S rRNA de piroplasmídeos não haviam sido amplificadas em amostras de *T. terrestris*.

3.2. *Hepatozoon* spp.

O gênero *Hepatozoon* compreende hemoparasitos apicomplexos que possuem como hospedeiros intermediários animais vertebrados (mamíferos, anfíbios, répteis e aves) e invertebrados (artrópodes) como hospedeiros definitivos. Tal gênero é representado por mais de 300 espécies diferentes (Smith, 1996; Aguiar, 2018). A infecção dos hospedeiros vertebrados ocorre quando estes ingerem artrópodes contendo oocistos esporulados do parasito (Smith, 1996). Em cães, os agentes etiológicos da hepatozoonose são bastante conhecidos. *Hepatozoon canis* é transmitido por carrapatos das espécies *R. sanguineus*, *A. ovale, Haemaphysalis flava* e *Haemaphysalis longicornis* (Murata et al., 1995; Baneth e Shkap, 2007; Rubini et al., 2009) e é responsável por causar uma doença mais branda nos cães, sendo considerado um parasito oportunista que geralmente causa doença em animais imunossuprimidos, ocorrendo na África, Américas, Ásia e Europa. Por outro lado, *Hepatozoon americanum* (transmitido por *Amblyomma maculatum*) é responsável por causar uma doença grave e quase sempre fatal, tendo sua distribuição geográfica limitada à América do Norte (Baneth et al., 2013; Aguiar, 2018).

No Brasil, a espécie infecta cães é *H. canis* e seu vetor definitivo carrapatos *R. sanguineus* (Aguiar, 2018). Genótipos filogeneticamente associados a *H. canis* e *H. americanum* foram descritos em felinos domésticos (Rubini et al., 2006; Bortoli et al., 2012; André et al., 2015; Braga et al., 2016). Genótipo filogeneticamente associado a *H. americanum* também foi detectado em cães na região norte do Brasil (Gomes et al., 2016). Em animais selvagens, *Hepatozoon* sp. já foi descrito em canídeos (André et al., 2010; Perles et al., 2019a), felídeos (André et al., 2010), roedores (Perles et al., 2019b; Weck et al., 2020), morcegos (Perles et al., 2020), quatis (Sousa et al., 2017c; Perles et al., 2022a), gambás (André et al., 2022; Braga et al., 2023), anfíbios (Ferreira et al., 2020) e répteis (Bouer et al., 2017; Clemente et al., 2023) no país.

A hepatozoonose é comumente diagnosticada como um achado acidental em laboratório, quando o parasito, em sua forma de gamonte, é encontrado em neutrófilos ou monócitos de mamíferos durante análise de esfregaços sanguíneos corados (**Figura 4**). Entretanto, a identificação etiológica definitiva depende de técnicas moleculares, como PCR e sequenciamento de fragmentos genéticos conservados como o gene 18S rRNA (Aguiar, 2018). Em 2021, amostras de sangue e baço de antas do Estado do Mato Grosso, coletadas *post-mortem*, foram testadas quanto à presença de material genético de *Hepatozoon* sp. Entretanto, nenhum animal foi positivo (Silva et al., 2021). Fava et al. (2022) relataram a detecção molecular de um genótipo 18S rRNA filogeneticamente próximo à *H. canis* em uma anta atendida em um centro de reabilitação de animais selvagens no Brasil.

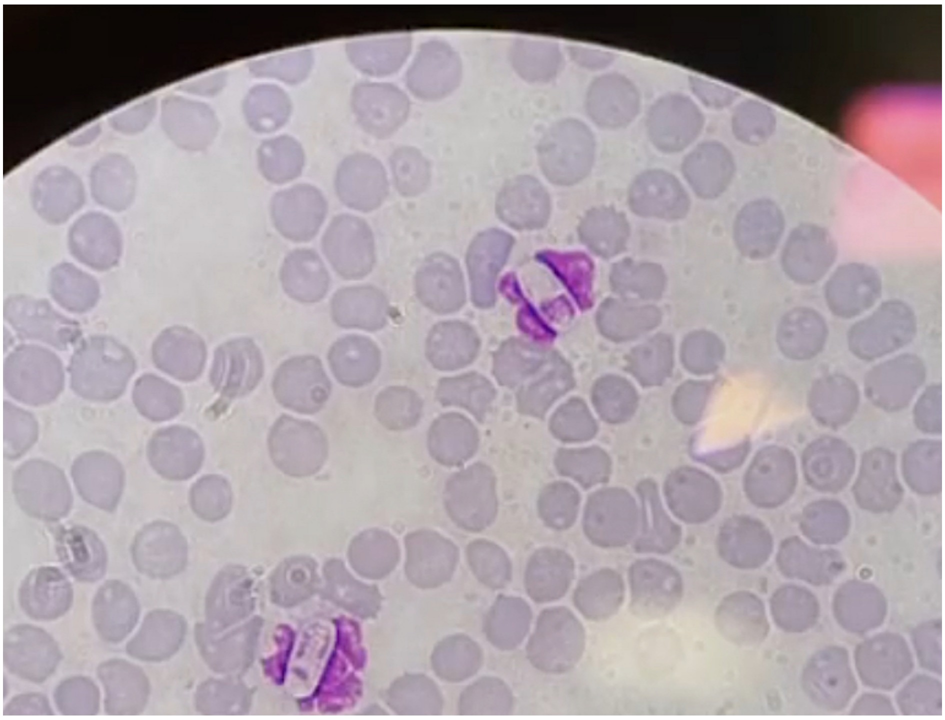


Figura 4. Gamontes de *H. canis* em neutrófilos de cão. Visualização em microscópio de luz, em aumento de 1000x. Lâmina corada com coloração rápida (Panótico) (Fonte: arquivo pessoal da autora).

3. Análise do microbioma

O microbioma é definido como uma comunidade microbiana característica que ocupa um habitat bem definido e com propriedades físico-químicas características, podendo incluir hospedeiros eucarióticos como um macro ecossistema no qual o microbioma está integrado. O microbioma não limita-se apenas aos microrganismos envolvidos, mas abrange também seu nichos ecológicos específicos (Berg et al., 2020). De fato, a relação entre uma determinada comunidade microbiana e o seu hospedeiro é descrita como uma das comunidades biológicas mais complexas e íntimas (Christian et al., 2015).

O aumento do número de pesquisas relacionadas a esse tema está ligado principalmente à crescente acessibilidade às técnicas de sequenciamento de nova geração (SNG) e ferramentas de bioinformática (Cullen et al., 2020). A utilização de SNG é particularmente útil uma vez que não é necessário ter suposições prévias sobre a comunidade bacteriana presente nas amostras estudadas, incluindo espécies não-cultiváveis (Galan et al., 2016). Tanto o sequenciamento de genoma completo (SGC) e o sequenciamento de regiões informativas do gene 16S rRNA podem ser utilizados para identificação microbiana em estudos de microbioma. De fato, os genes do RNA ribossômico (rRNA) são considerados alvos universais e conservados que provaram ser úteis para descrição e identificação de bactérias (Petrosino et al., 2019).

Além da detecção de uma ampla gama de patógenos, estudos focados na análise do microbioma utilizando regiões hipervariáveis do gene 16S rRNA são utilizados para investigar diferentes interações entre animais domésticos hospedeiros e seus microorganismos associados, como a eficiência alimentar em touros (McGovern et al., 2018), efeitos da raça e mudanças de dieta na composição da microbiota intestinal em ovelhas (Chang et al., 2020; Wei et al., 2021), e a associação entre comportamento agressivo em cães e a microbiota intestinal (Kirchoff et al., 2019).

No que diz respeito a animais selvagens, os estudos do microbioma estão fortemente ligados aos esforços focados na conservação de espécies. O estudo da microbiota oral e/ou intestinal de espécies criticamente ameaçadas de extinção, como anfíbios (Mann et al., 2021), rinocerontes de vida livre e cativeiro (Gibson et al., 2019) e aves (Ran et al., 2021; West et al., 2022) têm sido utilizados para elucidar como as mudanças dietéticas e ambientais podem afetar a composição do microbioma e, consequentemente, a saúde do hospedeiro. Além disso, a vigilância de agentes patogênicos em animais selvagens é importante para prevenir ou identificar o (re)surgimento de doenças infecciosas que possam afetar seres humanos e animais domésticos (Galan et al., 2016). De fato, estudos utilizando fragmentos hipervariáveis do gene 16S rRNA relataram a troca de componentes da microbiota da pele entre cães, lobos e seus cuidadores humanos (Wetzels et al., 2021).

As análises do microbioma também podem ser realizadas utilizando amostras de sangue. Em seres humanos, estudos sobre o microbioma sanguíneo mostraram resultados interessantes, como a existência de microrganismos “saudáveis” em um ambiente que, até então, acreditava-se ser completamente estéril (Castillo et al., 2019). Além disso, o estudo do microbioma do sangue também é útil para compreender condições patológicas, como a translocação de microrganismos de sua localização usual para a corrente sanguínea, condição conhecida como "atopobiose" (Potgieter et al., 2015).

Em medicina veterinária, muitos estudos acerca da avaliação do microbioma intestinal são realizados. No entanto, evidências da existência de populações microbianas no sangue de animais saudáveis e doentes foram recentemente relatadas. Em cães, a presença de populações bacterianas em amostras de sangue de animais aparentemente saudáveis tem sido descrita, em conjunto com evidências de sua origem como uma mudança na estrutura populacional de microrganismos intestinais (Scarsella et al., 2020). Em suínos, a análise do microbioma sanguíneo revelou composições diferentes de populações bacterianas na corrente sanguínea antes e após a indução de peritonite nesses animais (Hyun et al., 2021).

Poucos estudos realizaram a análise do microbioma sanguíneo de animais selvagens. Em roedores de um mesmo ecossistema, porém de espécies diferentes, a análise do microbioma revelou que a diversidade de componentes do microbioma é influenciada positivamente pela diversidade de espécies hospedeiras (Gavish et al., 2014). Outro estudo utilizando amostras de sangue de duas espécies de peixes (*Hipoglossus hipoglossus* e *Reinhardtius hipoglossus*) detectou precocemente evidências de disbiose, indicando o surgimento de doenças nos animais (Fronton et al., 2023). Em camelos dromedários (*Camelus dromedarius*), a presença de *Anaplasma* spp. foi detectada em amostras de sangue por meio da amplificação do gene 16S rRNA. Ainda, a abordagem SNG foi capaz de detectar amostras positivas identificadas como negativas para *Anaplasma* spp. com base na PCR convencional, mostrando maior uma maior sensibilidade do SNG (Mohamed et al., 2021).

Comunidades bacterianas também podem ser avaliadas em vetores artrópodes encontrados infestando animais selvagens (Clay et al., 2008; Steiner et al., 2008; André et al., 2023). O uso de ensaios baseados na amplificação do gene 16S rRNA pode ser útil para a detecção de espécies bacterianas e potenciais novas espécies transmitidas por vetores parasitando animais selvagens, de modo a realizar a vigilância de doenças emergentes (Egan et al., 2020).

No Brasil, descrição do microbioma intestinal de animais selvagens já foi relatada em diferentes espécies de aves marinhas (Ewbank et al., 2021), saguis (*Callithrix* spp.) (Malukiewicz et al., 2022) e capivaras (*Hydrochoerus hydrochaeris*)

(Cabral et al., 2022). Recentemente, André e colaboradores (2023) descreveram o microbioma de amostras de suabe retal e oral de morcegos não-hematófagos, e também de ectoparasitos encontrados infestando esses animais (moscas Streblidae e Macronyssidae e ácaros Spinturnicidae), no Estado do Mato Grosso do Sul. Entretanto, descrições do microbioma do sangue em animais selvagens são limitados e escassos na literatura.

Considerando a importância da anta brasileira e a condição de vulnerabilidade da espécie no país, associadas a relatos recentes descrevendo a ocorrência de diferentes patógenos nesses animais, as análises do microbioma lançariam luz sobre as diferentes comunidades bacterianas que podem ser encontradas em amostras de sangue desses animais. Embora análises do microbioma intestinal de *T. bairdii* (Yanez-Montalvo et al., 2021) e *T. indicus* (Arumugam et al., 2023) já tenham sido descritas, assim como o microbioma de carrapatos *Amblyomma sculpturatum* e *Amblyomma ovale* coletados de *T. terrestris* no Perú (Rojas-Jaimes et al., 2022), até o presente trabalho não haviam descrições do microbioma do sangue de antas.

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CAPÍTULO III* – Expanding the Universe of Hemoplasmas: Multi-Locus Sequencing Reveals Putative Novel Hemoplasmas in Lowland Tapirs (*Tapirus terrestris*), the Largest Land Mammals in Brazil*

*Esse capítulo corresponde à publicação científica publicada no periódico Microorganisms 2022, 10, 614. doi: https://doi.org/10.3390/microorganisms10030614

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Abstract: The lowland tapir (*Tapirus terrestris*) is the largest land mammal in Brazil and classified as a vulnerable species, according to the assessment of the risk of extinction. The present study aimed at investigating the occurrence and genetic diversity of hemoplasmas in free-ranging T. terrestris from the Brazilian Pantanal and Cerrado biomes. Blood samples were collected from 94 living and eight road-killed tapirs, totalizing 125 samples Conventional PCR targeting four different genes (16S rRNA, 23S rRNA, RNAse P, and dnaK) were performed, and the obtained sequences were submitted for phylogenetic, genotype diversity, and distance analyses. The association between hemoplasma positivity and possible risk variables (age, gender, and origin) was assessed. Out of 122 analyzed samples, 41 (41/122; 33.61% CI: 25.84–42.38%) were positive in the 16S rRNA-based PCR assay for hemoplasmas. Positivity for hemoplasmas did not differ between tapirs' gender and age. Tapirs from Pantanal were 5.64 times more likely to present positive results for hemoplasmas when compared to tapirs sampled in Cerrado. BLASTn, phylogenetic, genotype diversity, and distance analyses performed herein showed that the sampled lowland tapirs might be infected by two genetically distinct hemoplasmas, namely '*Candidatus* Mycoplasma haematoterrestris' and '*Candidatus* Mycoplasma haematotapirus'. While the former was positioned into "*Mycoplasma haemofelis* group" and closely related to '*Candidatus* Mycoplasma haematoparvum, the latter was positioned into "*Mycoplasma suis* group" and closely related to '*Candidatus* Mycoplasma haematobos'. The impact of both putative novel species on tapir health status should be investigated.

Keywords: hemotropic *Mycoplasma*; lowland tapirs; Pantanal; Cerrado

1. **Introduction**

The lowland tapir (*Tapirus terrestris*) is the largest land mammal in Brazil and the species is widely distributed throughout the country. Although the lowland tapir can still be found in four Brazilian biomes (Atlantic Forest, Pantanal wetlands, Amazon, and Cerrado), this species is classified as vulnerable according to extinction risk assessments [1,2].

The emergence of infectious diseases in tapir populations may occur as a conse- quence of habitat loss and increased contact between animals. In this instance, preventive medicine should be applied for both in situ and ex situ conservation attempts [3]. In the last decades, studies focusing on direct detection of different pathogens, such as *Trypanosoma terrestris* [4,5], *Theileria* sp. [6,7], and *Mycobacterium* sp. [8] and also serological evidence for bacterial, viral [9,10], and rickettsial [11] exposure have been reported in tapirs from Brazil.

The *Mycoplasma* genus (Mollicutes: Mycoplasmataceae) comprises bacteria lacking a cell wall and presenting a small genome (under 1 Mbp) [12–14].

Belonging to the *Mycoplasma* genus, hemoplasmas (hemotropic mycoplasmas) encompass species that attach to erythrocytes' cell membranes of different mammalian hosts [15]. Even though the conse- quences of hemoplasma infection are still unrevealed for many vertebrate species [16–18] it has been associated with anemia in cats infected by *Mycoplasma haemofelis* [19,20] and sheep infected by *Mycoplasma ovis* [21].

Cultivation in vitro has not been achieved for hemoplasmas up to now. For this reason, the diagnosis mainly relies on the molecular detection of selected gene fragments. Recently, newly described genotypes and *Candidatus* species have been reported in wild animals from Brazil based on PCR amplification of selected molecular markers, namely 16S rRNA and 23S rRNA genes. Potentially novel species have been identified in capybaras (*Hydrochoerus hydrochaeris*) [22] hairy dwarf porcupines (*Sphiggurus villosus*) [23], opossums (*Didelphis albiventris*) [24], and coatis (*Nasua nasua*) [25] from Brazil. Moreover, putative new genotypes have also been reported in free-ranging animals from different regions of the country, such as non-human primates from the Brazilian Amazon [26] rodents from several Brazilian biomes [27] and bats [28,29].

To the best of the authors' knowledge, this is the first report of hemoplasma infection in lowland tapirs. The present study aimed to investigate the occurrence and genetic diversity of hemotropic *Mycoplasma* in free-ranging *T. terrestris* from the Pantanal and Cerrado biomes in Brazil.

2. Materials and Methods

2.1. Study Areas

The Cerrado biome is the second largest of the Brazilian biomes, after the Amazon. This biome spreads across 2 million km^2 of the central Brazilian plateau and accounts for 21% of the country's land area, representing the most extensive savannah region in South America and the one harboring the highest biodiversity worldwide. It consists largely of a mosaic of different ecosystems and vegetation types, including tree and scrub savannah, grassland with scattered trees, and occasional patches of a dry, closed-canopy forest called 'Cerradão'. Gallery forests are found throughout the region along rivers and streams [30].

The Pantanal is the largest continuous freshwater wetland on the planet, encompassing 179,300 km^2 of low elevation floodplains of the upper Paraguay River, in the center of South America (Brazil, 78%; Bolivia, 18%; Paraguay, 4%). Dry and wet seasons are well defined and rainfall concentrates between November to March, during the summer, favoring the occurrence of a seasonal flood pulse. The landscape is known to include open woodlands, forests, floodable, and nonfloodable grasslands, and also temporary or permanent aquatic habitats [31]. The Pantanal wetland is one of the most important strongholds for tapirs in South America as it holds a large, continuous, healthy tapir population [32].

Most of the Pantanal wetland is held in private lands (93% of the land on the Brazilian side) [31]. Baía das Pedras Ranch is a private property of 145 km^2 in the Nhecolândia subregion of the Southern Pantanal, Municipality of Aquidauana, Mato Grosso do Sul State. The main activity is extensive cattle raising over native grasses, the traditional method. This ranch includes a mosaic of seasonally inundated grasslands, lakes, gallery forests, scrub, and deciduous forests that supports an abundance of wildlife which is also exploited through ecotourism by the owners.

Currently, Brazil is facing a historic scenario of natural areas destruction. In 2020, 23% of Pantanal were estimated to be burned out by wildfires [33]. In 2021, the Cerrado presented an increase of 7.9% in the suppression of natural vegetation when compared to the previous year, totaling ~8531.44 km^2 [34]. It is estimated that wildfires may have affected at least 65 million native vertebrates and 4 billion invertebrates, including endangered species such as the jaguar (*Panthera onca*), giant anteater (*Myrmecophaga tridactyla*), marsh deer (*Blastocerus dichotomus*), crowned solitary eagle (*Buteogallus coronatus*), and hyacinth macaw (*Anodorhynchus hyacinthinus*). The impacts caused by wildfires are related as direct injuries and death, or indirect injuries caused by habitat loss and resource depletion [35].

2.2. Sampling

This study was approved by the Ethics Committee for Animal Experimentation of FCAV/UNESP (Faculty of Agricultural and Veterinary Sciences of the São Paulo State University) under protocol number 4558/20. The “Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO)” provided the required annual permits for the capture and immobilization of tapirs and collection of biological samples (SISBIO# 14,603). All protocols for the capture, anesthesia, handling, and sampling of tapirs have been reviewed and approved by the Veterinary Advisors of the Association of Zoos and Aquariums (AZA)—Tapir Taxon Advisory Group (TAG), and the Veterinary Committee of the IUCN SSC Tapir Specialist Group (TSG).

From 2013 to 2018, blood samples from free-ranging *T. terrestris* were collected from 94 living and 8 road-killed individuals (collected from corpses with average time of 24h after death) totalizing 125 samples. From this total, 78 (78/125; 62.40% CI: 53.66–70.40%) samples were collected from 61 tapirs (61/94; 64.89%; CI: 54.83–73.78%) in the Pantanal wetland and 39 samples (39/125; 31.20% CI: 23.74–39.78%) were collected from 33 tapirs (33/94; 35.11% 26.22–45.17%) from the Cerrado. Both study areas are located in Mato Grosso do Sul State, central-western Brazil. Sampling was per- formed during tapir anesthesia for the installation of GPS collars by professionals from the “Iniciativa Nacional para a Conservação da Anta Brasileira (INCAB-IPÊ)” (Lowland Tapir Conservation Initiative (LTCI-IPÊ)). Some living animals (*n* = 20) were sampled more than once. Moreover, eight samples (8/125; 6.40% CI: 3.28–12.12%) belonging to eight road-killed animals on highways BR-267 and MS-040 in the Cerrado biome were collected during necropsy procedures.

The sampled living animals included females (46/94; 48.94% CI: 39.07–58.88%), males (48/94; 51.06% CI: 41.12–60.93%), adults (>48 months old) (50/94; 53.19% CI: 43.18–62.95%), and sub-adults (<48 months old) (44/94; 46.81% CI: 37.05–56.82%). The road killed animals included females (3/8; 37.50% CI: 13.68–69.43%), males (5/8; 62.50% CI: 30.57–86.32%), adults (>48 months old) (5/8; 62.50% CI: 30.57–86.32%), and sub-adults (<48 months old) (3/8; 37.50% CI: 13.68–69.43%). In total, 125 biological samples were obtained from 102 in-dividual tapirs (living or road killed) identified as females (49/102; 48.04% CI: 38.59–57.63), males (53/102; 51.96% CI: 42.37–61.41), adults (55/102; 53.92% CI: 44.28–63.28%), and sub-adults (47/102; 46.08% CI: 36.72–55.72%).

2.3. DNA Extraction and PCR Protocols for Mammals' Endogenous Genes

DNA extraction was performed using a commercial kit (InstaGene™ Matrix, Biorad®, Hercules, CA, USA) and following the manufacturers' instructions. In order to ensure suc-cessful DNA extraction, a conventional PCR for the mammal-endogenous gene glyceraldehyde-3-phosphate dehydrogenase (gapdh) targeting a 450 bp fragment [36] was performed in all samples. Samples that were not successfully amplified by the gapdh- based PCR protocol were submitted to a second protocol targeting a 227 bp fragment of the irpb (interphotoreceptor retinoid-binding protein) gene [37]. Samples that did not yield amplicons in either of the used PCR protocols were excluded from the subsequent analysis.

2.4. Conventional Polymerase Chain Reaction (cPCR) Assays for Hemoplasmas Based on the 16SrRNA, 23S rRNA, RNAse P and dnaK Gene Fragments

Samples were screened for the presence of hemoplasmas DNA using a semi-nested PCR protocol targeting a fragment of approximately 1107 bp of the 16S rRNA gene [38,39]. Positive samples were submitted to PCR protocols targeting the 23S rRNA (approximately 800 bp) [18], *RNAse P* (approximately 165 bp) [16], and *dnaK* (approximately 544 bp) genes [40] for additional molecular characterization. Thermal conditions and PCR reagent concentrations were slightly modified from the originally published protocols for *RNAse P* and *dnaK* genes (**Table 1**). DNA obtained from a naturally infected sheep with *Mycoplasma ovis* [18] and ultrapure RNAse and DNAse-free water (Promega, Madison, WI, USA) were used as positive and negative controls, respectively, in all PCR assays for hemoplasmas.

Table 1. Target genes, primers, thermal conditions, and reagent protocol used in the PCR assays for hemoplasmas based on the 16S rRNA, 23S rRNA, *RNAse P*, and *dnaK* genes.

Target gene	Primer sequences	Thermal conditions	Reagents volumes and concentration	Fragment size	Primers reference
16S rRNA	1st round: 5’-AGAGTTTGATCCTGGCTCAG -3’ 5’-ACCGCAGCTGCTGGCACATA -3’ 2nd round: 5’-ATATTCCTACGGGAAGCAGC -3’ 5’-ACCGCAGCTGCTGGCACATA -3’	95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec, extension at 72°C for 1 min, and final extension at 72°C for 10 min for both rounds.	1st reaction: 2,5 µl from 10X Buffer, 0,75 µl from 50mM MgCl^{2}, 2 µl from 10mM dNTP mix, 1 µl from each primer at 10mM, 0,25 µl from 5U/ µl Taq polymerase, 12,5 µl from ultrapurified water and 5 µl from template DNA. 2nd reaction: Ultrapurified water (16,5 µl) and template DNA (1 µl) quantities changes.	~1,107 bp	Harasawa et al., 2014; Di Cataldo et al., 2020
23S rRNA	5′-TGAGGGAAAGAGCCCAGAC-3’ 5′-GGACAGAATTTACCTGACAAGG -3′	94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, extension at 72°C for 1 min, and final extension at 72°C for 10 min.	2,5 µl from 10X Buffer, 0,75 µl from 50mM MgCl^{2}, 2 µl from 10mM dNTP mix, 1 µl from each primer at 10mM, 0,25 µl from 5U/µl Taq polymerase, 12,5 µl from ultrapurified water and 5 µl from template DNA.	~800 bp	Mongruel et al., 2020
RNAseP	5’-GATKGTGYGAGYATATAA AAAATAAARCTCRAC -3’ 5’-GMGGRGTTTACCGCGTTTCAC -3’	95° C for 2 min, followed by 50 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 1 min.	2,5 µl from 10X Buffer, 1,0 µl from 50mM MgCl^{2}, 2 µl from 10mM dNTP mix, 1 µl from each primer at 10mM, 0,25 µl from 5U/ µl Taq polymerase, 12,25 µl from ultrapurified water and 5 µl from template DNA.	~164 bp	Maggi et al., 2013
dnaK	5’-GGGTGGAGATGATTGAGA CCA -3’ 5’-GGGTGGAGATGATTGAGACCA -3’	95° C for 5 min, followed by 45 cycles of denaturation at 95°C for 20 sec, annealing at 55.5°C for 30 sec, extension at 72°C for 45 sec and final extension at 72°C for 7 min.	2,25 µl from 10X Buffer, 1,0 µl from 50mM MgCl^{2}, 2 µl from 10mM dNTP mix, 1 µl from each primer at 10mM, 0,15 µl from 5U/µl Taq polymerase, 12,6 µl from ultrapurified water and 5 µl from template DNA.	~544 bp	Descloux et al., 2020

The products obtained in PCR assays were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide (Life Technologies™, Carlsbad, CA, USA) at 100 V/150 mA for 50 min. The gels were imaged under ultraviolet light (ChemiDoc MP Imaging System, Bio Rad™, Hercules, CA, USA) using the Image Lab Software v4.1 (Biorad, Hercules, CA, USA).

2.5. Sequencing

Amplified products were purified using a commercial kit (Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, WI, USA) and sequenced using the BigDye™ Termi- nator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific™, Waltham, MA, USA) and ABI PRISM 310DNA Analyzer (Applied Biosystems™, Foster City, CA, USA) [41].

2.6. Sequence Analysis and Phylogeny

The obtained sequences were first submitted to a screening test using Bioedit v7.0.5.3 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html, accessed on 8 December 2021) [42] to evaluate the electropherogram quality and generate the consensus sequences. The BLASTn program (National Center for Biotechnology Information, Bethesda, MD, USA) [43] was used to analyze the nucleotide sequences (BLASTn), aiming to browse and compare with se- quences from an international database (GenBank) [44]. The consensus sequences obtained in the current study and those retrieved from GenBank were aligned using the ClustalW software [45] via Bioedit v7.0.5.3 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html, ac- cessed on 8 December 2021) [42] and also improved by an MAFFT alignment performed using GUIDANCE2 online server (http://ww.guidance.tau.ac.il, accessed on 8 December 2021) [46]. Phylogenetic inferences were based on Bayesian analysis via CIPRES online server (https://www.phylo.org/index.php/, accessed on 8 December 2021). The best-fit model was determined using jModeltest v2.1.6 via CIPRES online server (https://www.phylo.org/index.php/, accessed on 8 December 2021) [47]. The phylogenetic analyses were performed using the obtained sequences from the 16S rRNA, 23S rRNA, *RNAse P*, and *dnaK*-based PCR protocols. All the sequences obtained from the present work were submitted to the GenBank database.

2.7. Genetic Diversity Assessment

The genetic diversity assessment was performed using only the obtained 16S rRNA sequences. In order to calculate nucleotide diversity (π), polymorphism level (haplotype diversity—[dh]), number of haplotypes (h), and the average number of nucleotide differ- ences (K) among the sequences obtained, the DnaSP program v5 (http://www.ub.edu/ dnasp/, accessed on 21 December 2021) [48] was used. The Genotype network was con- structed in PopART (http://popart.otago.ac.nz, accessed on 11 December 2021), using the TCS inference method [49]. GPS coordinates collected for each sampling location were used to develop a map representing the genotype distribution using PopART software. Additionally, a distance-based analysis was performed using SplitsTree v4.14.6 (University of Tubingen, Tubingen, Germany) [50] to investigate the genetic relationship among hemo- plasma genotypes detected in the present study and those previously deposited in GenBank. The pairwise distance matrix from the alignment of the 16S rRNA sequences detected in tapirs in the current study was calculated using the *p*-distance model and included Tran- sitions + Transversions substitutions with uniform rates. Analysis was performed using MEGAX software (https://www.megasoftware.net, accessed on 17 December 2021) [51,52]. Data were transferred to a Microsoft Excel 2016 sheet to construct the heat map according to the rates obtained on the pairwise distance matrix.

2.8. Statistical Analysis

The chi-square test was used to determine associations between variables (gender, location, and age) and the outcomes (positive or negative PCR results for hemoplasmas). Odds ratio (OR), 95% confidence interval, and *p*-values were calculated for each variable. Results considered significantly different when $p < 0.05$. Data were compiled and ana- lyzed in Epi Info™ software (v7.1.5, Centers for Disease Control and Prevention, Atlanta, GA, USA).

3. Results

3.1. PCR for Mammals' Endogenous Genes

Out of 125 blood samples tested for the presence of the gapdh gene fragment, 9 (9/125; 07.20% CI: 03.83–13.12%) did not amplify fragments of the expected size and were submitted to the irpb-based PCR protocol. Out of 9 analyzed samples, 3 (3/125; 02.40%; CI: 00.82–06.82%) did not successfully amplify fragments of the expected size and were excluded from the following analyses. All excluded samples were obtained from road-killed tapirs.

3.2. PCR Assays for Hemoplasmas

From 122 analyzed samples, 41 (41/122; 33.61% CI: 25.84–42.38%) presented bands of expected size on electrophoresis after being processed by the partial 16S rRNA-based PCR protocol. The positive samples were obtained from 34 living animals and two road-killed animals. Out of the 34 positive living animals, 20 were identified as males (20/34; 58.82% CI: 42.22–73.63%) and 14 were identified as females (14/34; 41.18% CI: 26.37–57.78%). Moreover, 30 animals were from the Pantanal (30/34; 88.24% CI: 73.38–95.33%) and 4 from the Cerrado (04/34; 11.76% CI: 04.67–26.62%). Fifteen (15/43; 44.12% CI: 28.88–60.55%) were adults and 19 (19/34; 55.88% CI: 39.45–71.12%) sub-adults. Some animals (*n* = 3) presented positive samples in more than one sampling, totalizing seven positive samples (mean value: 2.3 positive samples/tapir) from the same animals. The two samples from road-killed animals that yielded positive results for the partial 16S rRNA gene were obtained from a sub-adult female and an adult male. In total, 35.29% (36/102; CI: 26.71–44.95%) tapirs (living or road-killed) presented positive results for hemotropic *Mycoplasma* sp. Results regarding positive samples, sampling dates, age/gender/location from tapirs, and GenBank accession numbers were summarized in **Table 2**.

Table 2. Identification, sampling dates, locations, gender/age, and Genbank accession number for each partial 16S rRNA-positive animals.

Animal ID	Samplingdate (DD/MM/YY)	Biome	Gender/Age	16S rRNA GenBank accession number	23S rRNA GenBank accession number	*RNAse P* GenBank accession number	*dnaK* GenBank accession number
KA-C	20/12/16	Cerrado	Male/adult	**OL985895**	NS	negative	negative
MO-P	08/12/14	Pantanal	Male/juvenile	**OL985896**	NS	negative	negative
MA-P-1**	28/07/13	Pantanal	Female/adult	**OL985902**	NS	negative	negative
GO-P	13/07/14	Pantanal	Male/sub-adult	**OL985897**	NS	negative	negative
JE-P-1**	06/12/17	Pantanal	Male/sub-adult	**OL985903**	NS	negative	negative
TD-P-1*	16/11/15	Pantanal	Male/sub-adult	**OL985900**	**OM022254**	NS	negative
SY-P	28/10/17	Pantanal	Female/adult	**OL985904**	NS	negative	negative
RA-P	04/05/14	Pantanal	Female/juvenile	NS	negative	negative	negative
CO-P	31/10/18	Pantanal	Male/sub-adult	NS	negative	negative	negative
CJO-P	06/07/14	Pantanal	Male/adult	NS	negative	negative	negative
CIO-P	23/07/13	Pantanal	Male/sub-adult	NS	NS	negative	negative
JA-P	05/05/14	Pantanal	Female/juvenile	**OL985905**	NS	negative	negative
LA-P	29/08/17	Pantanal	Female/juvenile	NS	negative	negative	negative
AA-P	22/10/18	Pantanal	Male/sub-adult	**OL985907**	**OM022255**	negative	negative
MU-P	22/10/13	Pantanal	Female/adult	NS	negative	negative	negative
IA-P	10/12/17	Pantanal	Male/adult	**OL985908**	negative	negative	negative
WM-P	05/12/14	Pantanal	Male/juvenile	**OL985909**	negative	NS	negative
SA-P	05/12/17	Pantanal	Female/juvenile	**OL985899**	**OM022256**	NS	negative
DA-P	25/08/18	Pantanal	Female/sub-adult	**OL985898**	**OM022257**	**OM317758**	negative
RTA-P	10/05/15	Pantanal	Female/adult	NS	negative	NS	negative
WE-P-1**	24/11/15	Pantanal	Female/sub-adult	**OL985901**	negative	NS	negative
VA-P-1*	01/09/18	Pantanal	Female/sub-adult	**OL985911**	negative	negative	negative
MIA-P	25/06/18	Pantanal	Female/sub-adult	**OL985912**	**OM022258**	**OM317759**	**OM339521**
GAO-P	19/06/18	Pantanal	Male/adult	**OL985913**	negative	NS	negative
AO-P	08/06/16	Pantanal	Male/juvenile	**OL985914**	**OM022259**	NS	negative
FA-P	18/06/18	Pantanal	Female/adult	**OL985915**	negative	NS	negative
MA-P-2**	19/05/15	Pantanal	Female/adult	NS	negative	negative	negative
ANO-C-2*	28/06/17	Cerrado	Male/adult	**OL985916**	negative	NS	negative
CNA-C-2*	19/09/18	Cerrado	Female/adult	**OL985917**	negative	NS	NS
SO-C-2*	09/02/17	Cerrado	Male/adult	**OL985918**	negative	negative	negative
FFO-P-2*	23/08/17	Pantanal	Male/adult	**OL985919**	negative	negative	negative
JO-P-2*	20/08/16	Pantanal	Male/sub-adult	**OL985920**	negative	negative	negative
DO-P-2*	25/06/18	Pantanal	Male/sub-adult	**OL985921**	**OM022260**	negative	negative
JE-P-2**	09/06/26	Pantanal	Male/sub-adult	NS	negative	negative	negative
BS-P	11/06/16	Pantanal	Male/sub-adult	**OL985922**	negative	negative	negative
TD-P-3*	18/06/16	Pantanal	Male/sub-adult	**OL985923**	NS	negative	negative

WE-P-2**	16/06/16	Pantanal	Female/sub-adult	**OL985924**	NS	negative	negative
WE-P-3**	15/12/16	Pantanal	Female/sub-adult	**OL985925**	NS	negative	negative
SAO-P-2*	20/10/13	Pantanal	Male/adult	**OL985926**	negative	negative	negative
NEC09-C	09/04/16	Cerrado	Female/sub-adult	NS	negative	negative	negative
NEC18-C	29/07/16	Cerrado	Male/adult	NS	negative	negative	negative

* Animal with more than one sample collected. Did not yield positive results for partial 16S rRNA in all samples.
** Animal with more than one sample collected. Positive results for partial 16S rRNA in all samples.
NS = Sequence presented expected size bands on electrophoresis but was not successfully sequenced.

In BLASTn analysis, 16S rRNA sequences obtained from 28 samples presented identity rates ranging from 97.20–98.20% with sequences of ‘*Candidatus* Mycoplasma haematoparvum’ from dogs from the USA and Italy (MH094850, AY383241), with query cover values of 99–100% and an E-value of 0.0. Meanwhile, two partial 16S rRNA gene sequences pre- sented identity rates of 94.99% with ‘*Candidatus* Mycoplasma haematobos’ from cattle from Cuba (MG948628) with 100% of query cover and an E-value of 0.0. Regarding partial 23S rRNA analysis, 18 samples (18/41; 43.90% CI: 29.89–58.96%) presented bands of the expected size. From these, seven samples that presented high intensity and unique bands on agarose gel electrophoresis were submitted to sequencing and deposited in the GenBank database (OM022254-OM022260). When amplifying a fragment of approximately 165 bp from the *RNAse P* gene, 12 samples (12/41; 29.27%; 17.61–44.48%) presented bands of the expected size. From this total, two samples were successfully sequenced and deposited in the GenBank database (OM317758-OM317759). Finally, two samples (2/41; 4.88%; CI: 1.35–16.14%) submitted to *dnaK*-based PCR protocol presented expected band sizes in agarose gel electrophoresis and one was successfully sequenced and deposited in the GenBank database (OM339521) (Table 2). Only one sample (ID: MIA-P) presented sequences for all four targeted genes. The BLASTn analysis results for each sequence obtained by am- plifying 23S rRNA, *RNAse P*, and *dnaK* genes in the present study are shown in Tables 3–5.

Table 3. BLASTn analysis of each partial hemoplasma 23S rRNA sequence obtained from free-ranging tapirs from the Brazilian biomes of Cerrado and Pantanal.

Animal ID	**16S rRNA GenBank accession number**	**BLASTn best hit**	**Host**	**Country**	**Query cover (%)**	**E-value**	**Identity (%)**	**Best hit GenBank accession number**
KA-C	OL985895	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	USA	100%	0.0	97.55%	AY383241
MO-P	OL985896	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	99%	0.0	97.93%	MH094850
MA-P-1**	OL985902	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	99%	0.0	97.46%	MH094850
GO-P	OL985897	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	99%	0.0	97.76%	MH094850
JE-P-1**	OL985903	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	100%	0.0	97.32%	MH094850
TD-P-1*	OL985900	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	USA	100%	0.0	97.20%	AY383241
SY-P	OL985904	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	100%	0.0	97.46%	MH094850
JA-P	OL985905	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	USA	100%	0.0	97.46%	AY383241
AA-P	OL985907	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	99%	0.0	98.02%	MH094850
IA-P	OL985908	*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	99%	0.0	98.20%	MH094850

WM-P	OL985909	*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	100%	0.0	98.08%	MH094850
SA-P	OL985899	*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	99%	0.0	97.82%	MH094850
DA-P	OL985898	‘*Candidatus* Mycoplasma haemobos’	*Bos taurus*	Cuba	100%	0.0	94.99%	MG948628
WE-P-1**	OL985901	*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	100%	0.0	97.44%	MH094850
VA-P-1*	OL985911	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	USA	99%	0.0	97.60%	AY383241
MIA-P	OL985912	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	100%	0.0	97.92%	MH094850
GAO-P	OL985913	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	USA	100%	0.0	97.39%	AY383241
AO-P	OL985914	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	USA	100%	0.0	97.75%	AY383241
FA-P	OL985915	*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	100%	0.0	98.17%	MH094850
ANO-C-2*	OL985916	*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	100%	0.0	97.81%	MH094850
CNA-C-2*	OL985917	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	USA	100%	0.0	97.20%	AY383241
SO-C-2*	OL985918	*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	100%	0.0	97.79%	MH094850

FFO-P-2*	OL985919	*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	100%	0.0	97.71%	MH094850
JO-P-2*	OL985920	*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	99%	0.0	97.92%	MH094850
DO-P-2*	OL985921	*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	99%	0.0	97.75%	MH094850
BS-P	OL985922	*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	100%	0.0	97.73%	MH094850
TD-P-3*	OL985923	*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	100%	0.0	97.29%	MH094850
WE-P-2**	OL985924	*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	100%	0.0	97.98%	MH094850
WE-P-3**	OL985925	*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	100%	0.0	97.96%	MH094850
SAO-P-2*	OL985926	‘*Candidatus* Mycoplasma haemobos’	*Bos taurus*	Cuba	100%	0.0	94.99%	MG948628

* Animal with more than one sample collected. Did not yield positive results for partial 16S rRNA in all samples.

** Animal with more than one sample collected. Positive results for partial 16S rRNA in all samples.

Table 4. BLASTn analysis of each partial hemoplasma RNAse P sequence obtained from free-ranging tapirs from the Brazilian biomes of Cerrado and Pantanal.

Animal ID	RNAse P GenBank accession number	BLASTn best hit	Query cover (%)	E-value	Identity (%)	Best hit GenBank accession number

DA-P	OM317758	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	95%	1e-34	96.88%	MH090015
MIA-P	OM317758	‘*Candidatus* Mycoplasma haematoparvum’	*Canis lupus familiaris*	Italy	98%	4e-29	93.62%	MH090015

Table 5. BLASTn analysis of each partial hemoplasma *dnaK* sequence obtained from free-ranging tapirs from the Brazilian biomes of Cerrado and Pantanal.

Animal ID	23S rRNA GenBank accession number	BLASTn best hit	Host	Country	Query cover (%)	E-value	Identity (%)	Best hit GenBank accession number
MIA-P	OM339521	‘*Candidatus* Mycoplasma erythrocervae’	Not informed	England	87%	5e-59	78.74%	KF`51050

3.3. Phylogenetic Inference

Phylogenetic trees were constructed for four partial gene fragments by Bayesian inference using 10^7 generations of MCMC (Monte Carlo Markov Chains) with two independent runs and 10% of burn-in (Figures 1–4). For the partial 16S rRNA gene analysis (Figure 1), a total size alignment of 909 bp was constructed using 99 homolog sequences and one outgroup (AB042061).

The best-fit model for this gene analysis was determined as F81+G. For the partial 23S rRNA gene analysis (**Figure 2**), an alignment with a total size of 720 bp was constructed using 41 homolog sequences and one outgroup (NR103037). The best-fit model for this gene analysis was determined as F81+G. For the partial *RNAse P* gene analysis (Figure 3), a total size alignment of 230 bp was constructed using 23 homolog sequences and one outgroup (U64878). The best-fit model for this gene analysis was determined as F81+I+G. For the partial *dnaK* gene analysis (Figure 4) a total size alignment of 622 bp was constructed using

19 homolog sequences and one outgroup (KJ690086). The best-fit model for this gene analysis was determined as F81+G.

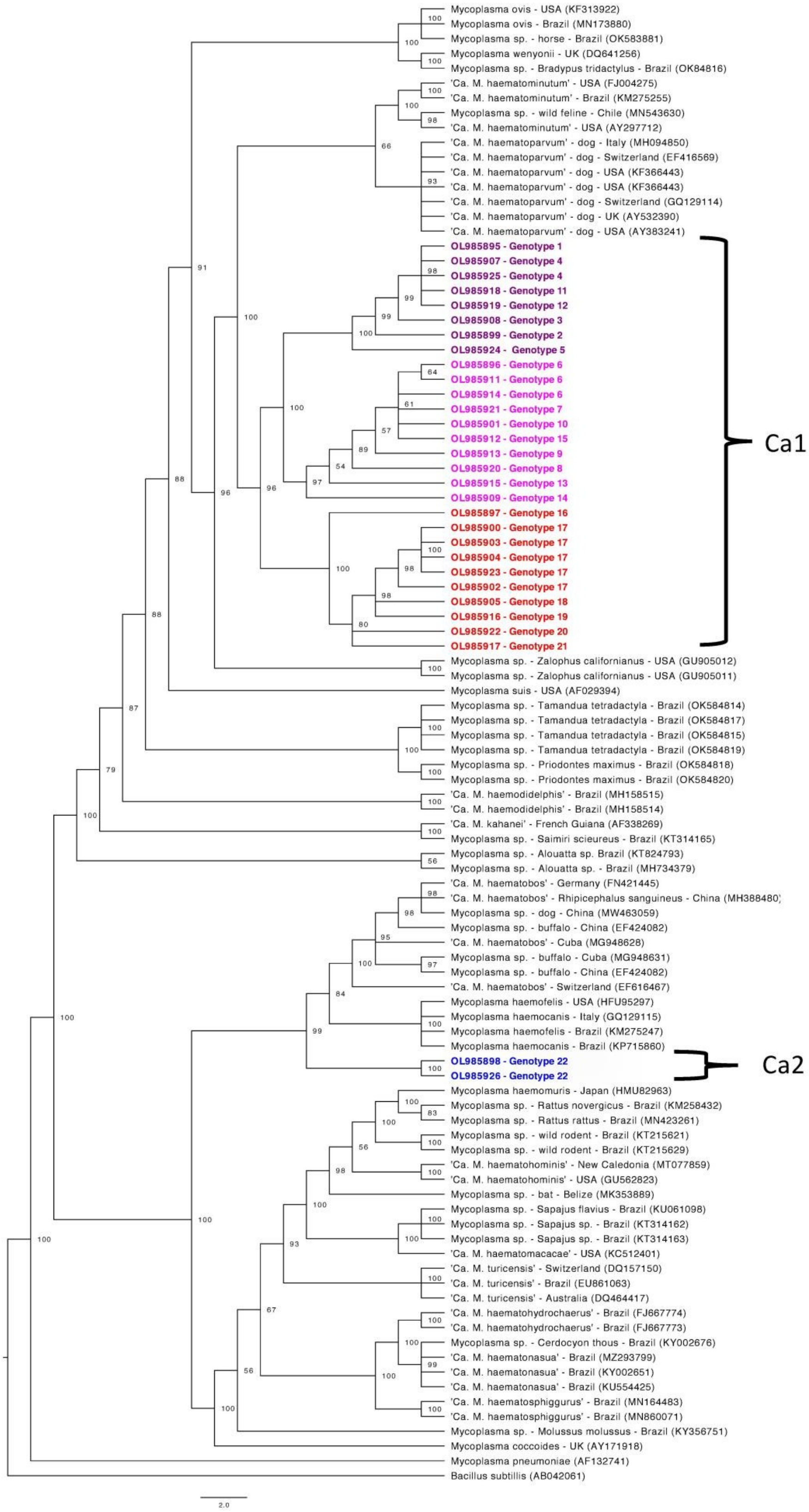

Figure 1. Phylogenetic tree based on partial 16S rRNA gene fragments of *Mycoplasma* sp. Tree was constructed by Bayesian Inference and a sequence from *Bacillus subtillis* (AB042061) was used as outgroup. Sequences obtained in the present study (Ca1 and Ca2) are highlighted in colors: Ca1 are highlighted in purple, pink and red colors, differentiating each subclade formed. Ca2 is highlighted in blue.

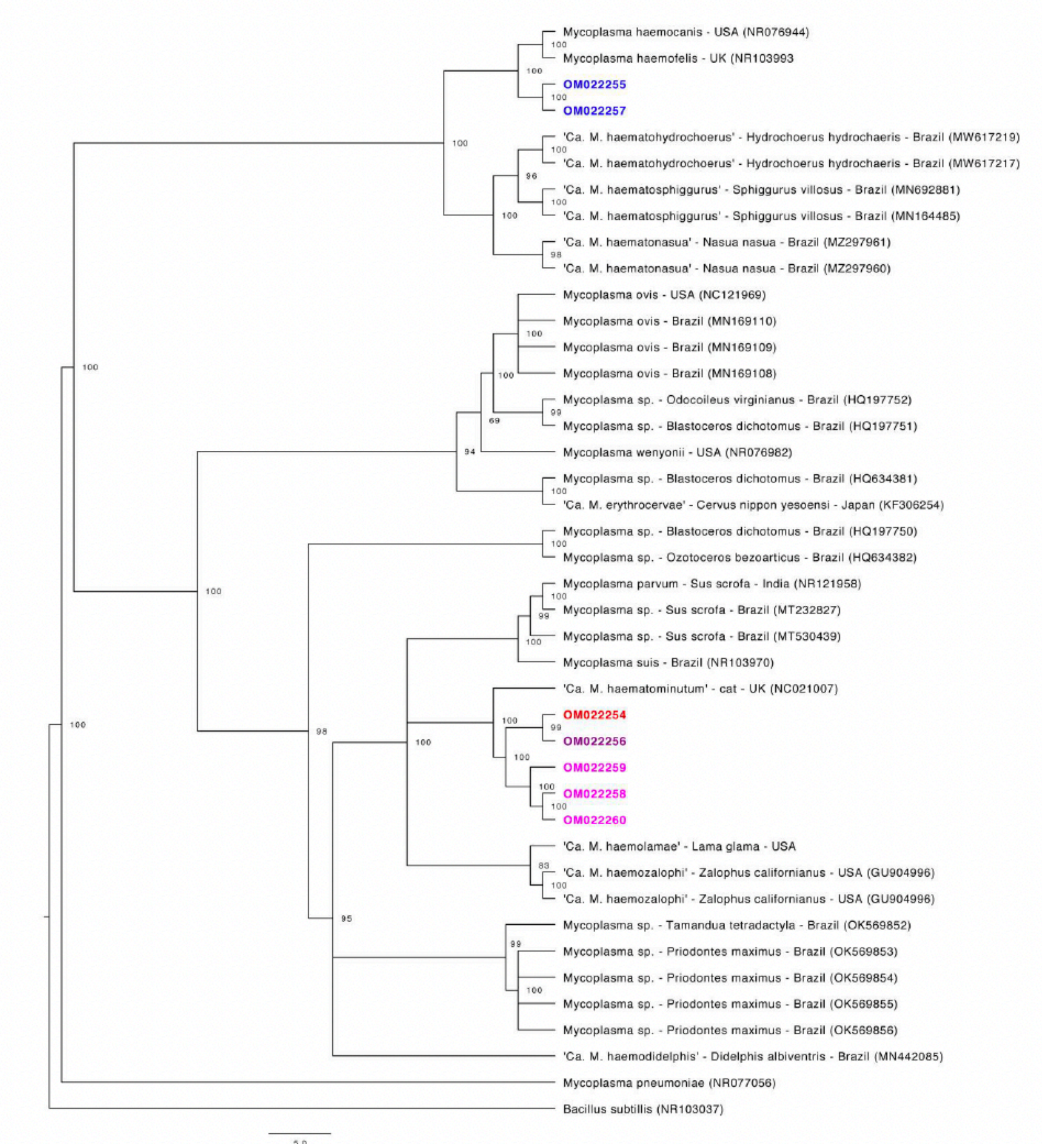

Figure 2. Phylogenetic tree based on partial 23S rRNA gene fragments of *Mycoplasma* sp. Tree was constructed by Bayesian Inference and a sequence from *Bacillus subtillis* (NR103037) was used as outgroup. Sequences obtained in the present study are highlighted in colors: sequences that fit in Ca1 by the 16S rRNa phylogeny are highlighted in purple, pink and red colors. Sequences that fit in Ca2 by the 16S rRNA phylogeny are highlighted in blue.

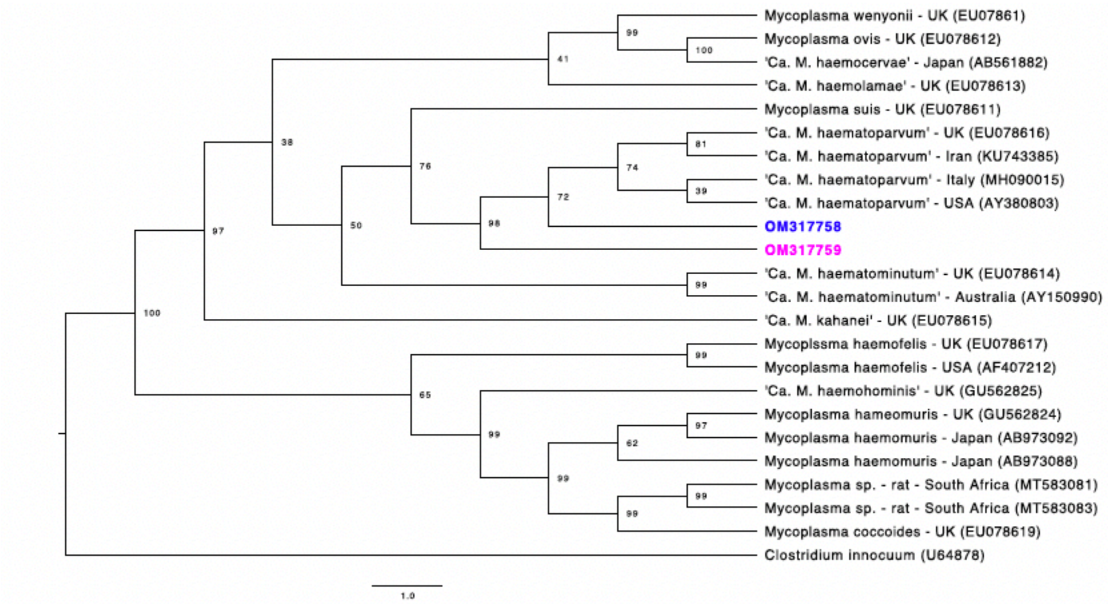

Figure 3. Phylogenetic tree based on partial *RNAse P* gene fragments of *Mycoplasma* sp. Tree was constructed by Bayesian Inference and a sequence from *Clostridium innocuum* (U64878) was used as outgroup. Sequences obtained in the present study are highlighted in colors: the sequence that fit in Ca1 by the 16S rRNA phylogeny is highlighted in pink. The sequence that fit in Ca2 by the 16S rRNA phylogeny is highlighted in blue.

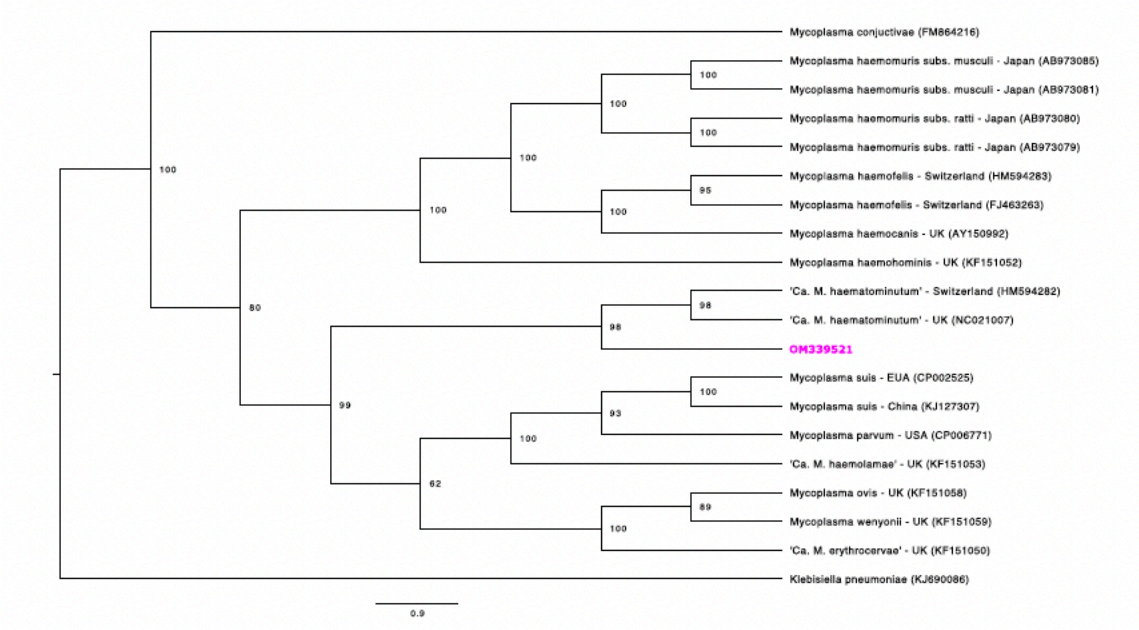

Figure 4. Phylogenetic tree based on partial *dnaK* gene fragments of *Mycoplasma* sp. Tree was constructed by Bayesian Inference and a sequence from *Klebisiella pneumonia* (KJ690086) was used as outgroup. Sequences obtained in the present study is highlighted in pink, once it fits in Ca1 by the 16S rRNA phylogeny.

Regarding the 16S rRNA phylogenetic analysis, our sequences were divided into two different major clades. The first clade was also sub-divided into three minor clades and positioned in the "*Mycoplasma suis* group", whereas the second clade comprised of only three sequences and was distantly positioned in the "*Mycoplasma haemofelis* group". These results suggest the occurrence of two distinct species occurring in sampled tapirs. In order to facilitate the understanding from now on these two clades will be identified as Ca1 and Ca2, respectively. Sequences that fitted in Ca1 on partial 16S rRNA-based phylogeny followed a similar pattern in other genes phylogenies, except for the *RNAse P* gene. In the phylogenetic tree based on the *RNAse P* gene, both obtained sequences were positioned in the "*M. suis* group", albeit each one was positioned in a different group on 16S rRNA phylogeny. Moreover, one sample (ID: AA-P) that was positioned in Ca1 on 16S rRNA-based phylogeny was found in Ca2 in the phylogenetic inference based on the partial 23S rRNA.

3.3. Genetic diversity assessment

Regarding the 16S rRNA genotype analysis, 22 different genotypes were identified among 30 sequences (**Figure 5**). Values of nucleotide diversity (π), polymorphism level (haplotype diversity - [dh]), number of haplotypes (h), average number of nucleotide differences (K) among the sequences obtained and the number of mutations between sequences from Ca1 and Ca2 were described in **Table 7**. 16S rRNA genotypes and sampling location of each obtained sequence are shown in **Supplementary Material 1 (Table S1)**. The map representing genotype distributions along the sampling area reinforced the geographical distance between genotypes identified in Pantanal and Cerrado areas (**Figure 6-8**).

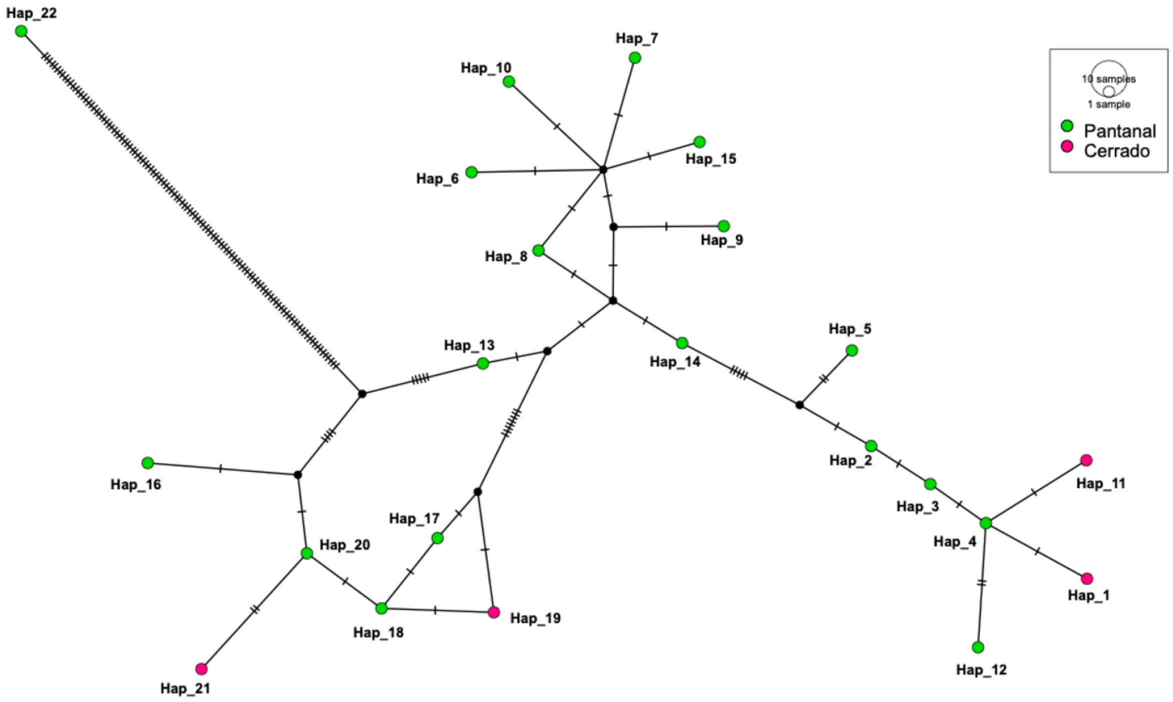

Figure 5: Genotype diversity among 16S rRNA gene sequences detected herein. Analysis were made using DnaSP6. Inference and graphic representation were made by TCS Network method on PopART software. Genotypes in blue were obtained from samples from tapirs in Pantanal regions meanwhile genotypes in green were obtained from samples from tapirs in Cerrado regions.

Table 6 Values obtained regarding genotype diversity by DnaSP software and based on partial hemoplasma 16S rRNA sequences detected in tapirs from the present study.

Nucleotide diversity (π)	**Genotype diversity (dh)**	**Number of haplotypes (h)**	**Average number of nucleotide differences between all sequences (K)**	**Average number of nucleotide differences between Ca1 and Ca2**		**Number of fixed differences between Ca1 and Ca2**
0,03112	0,966	22	24,21149	121,036	112	112

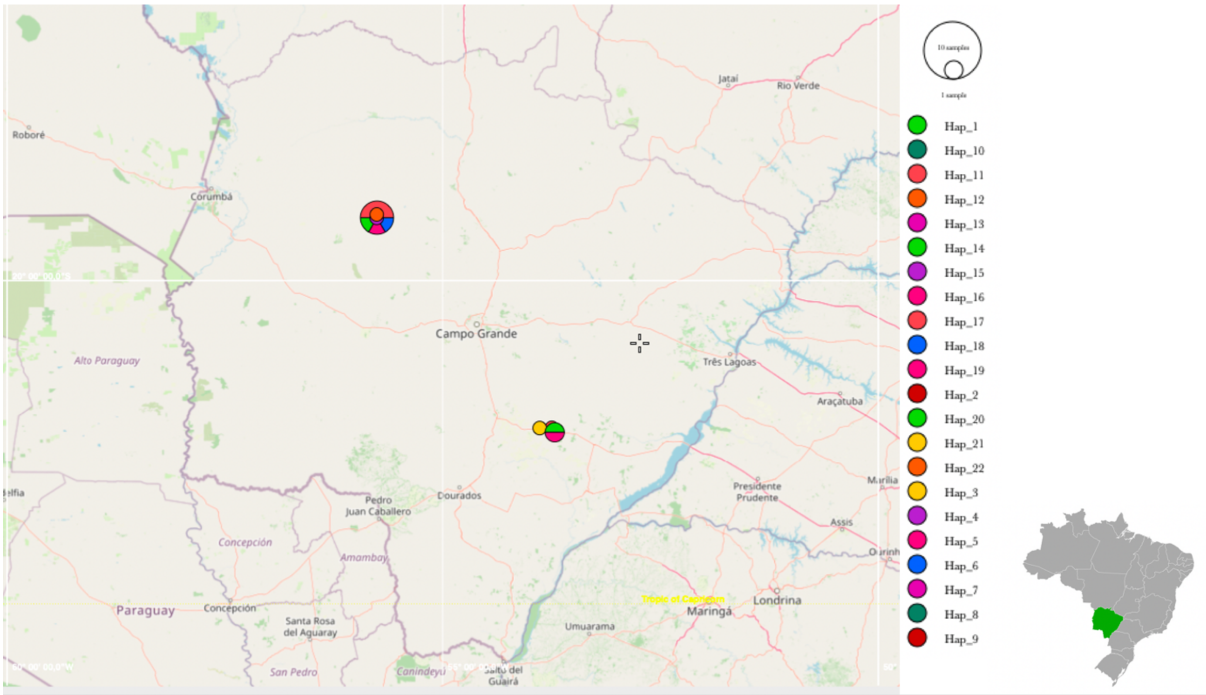

Figure 6. Representation of the genotype distribution along all sampling areas. Map was constructed using PopART software based on GPS coordinates data of each sampling.

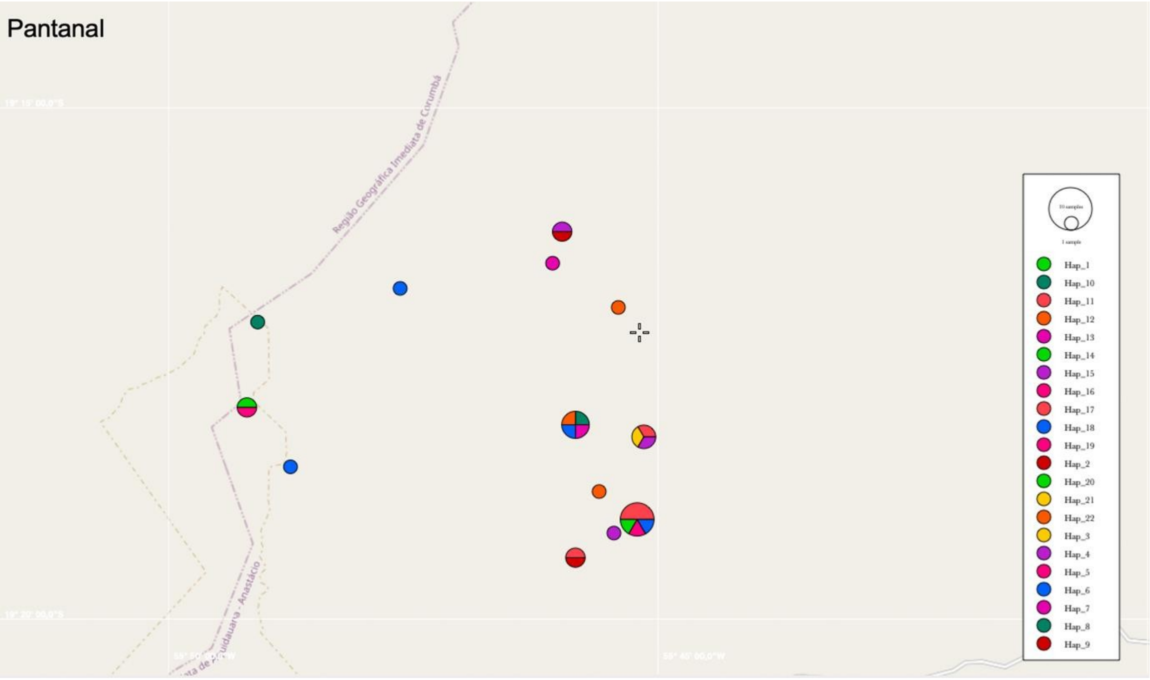

Figure 7. Representation of the genotype distribution of Pantanal biome areas. Map was constructed using PopART software based on GPS coordinates data of each sampling.

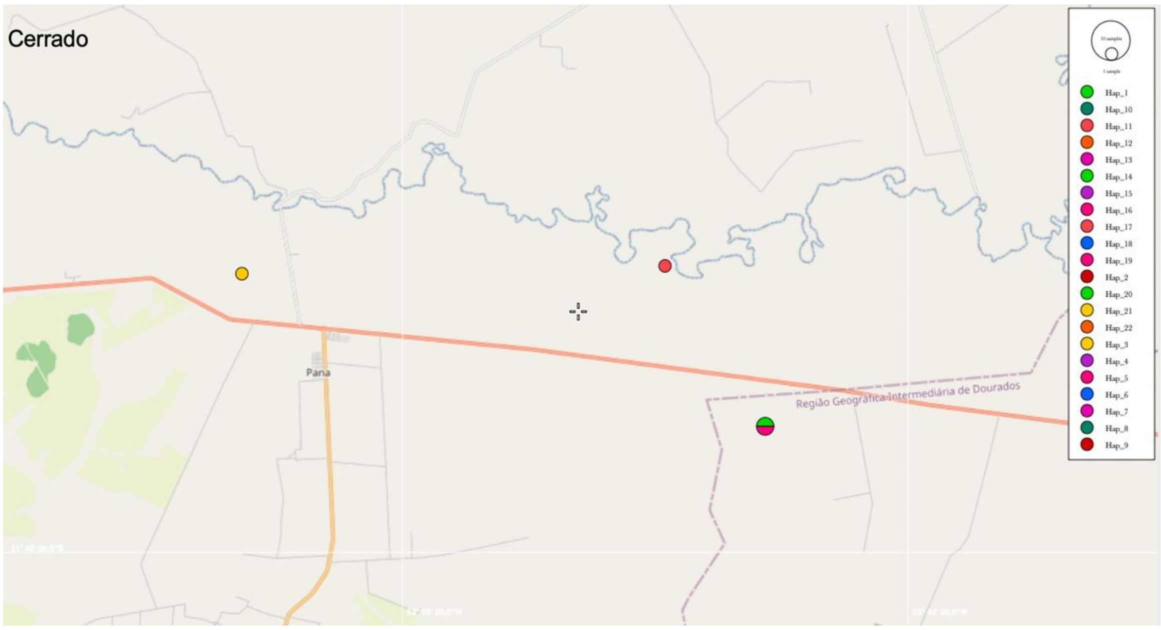

Figure 8. Representation of the genotype distribution of Cerrado biome areas. Map was constructed using PopART software based on GPS coordinates data of each sampling.

3.5. Distance Analysis by SplitsTree

The distance analysis performed by the neighbor-joining method and displayed by SplitsTree v4.14.6 demonstrated that Ca1 and Ca2 sequences were distinctly disposed among other hemotropic *Mycoplasma* species and Candidatus (Figure 9).

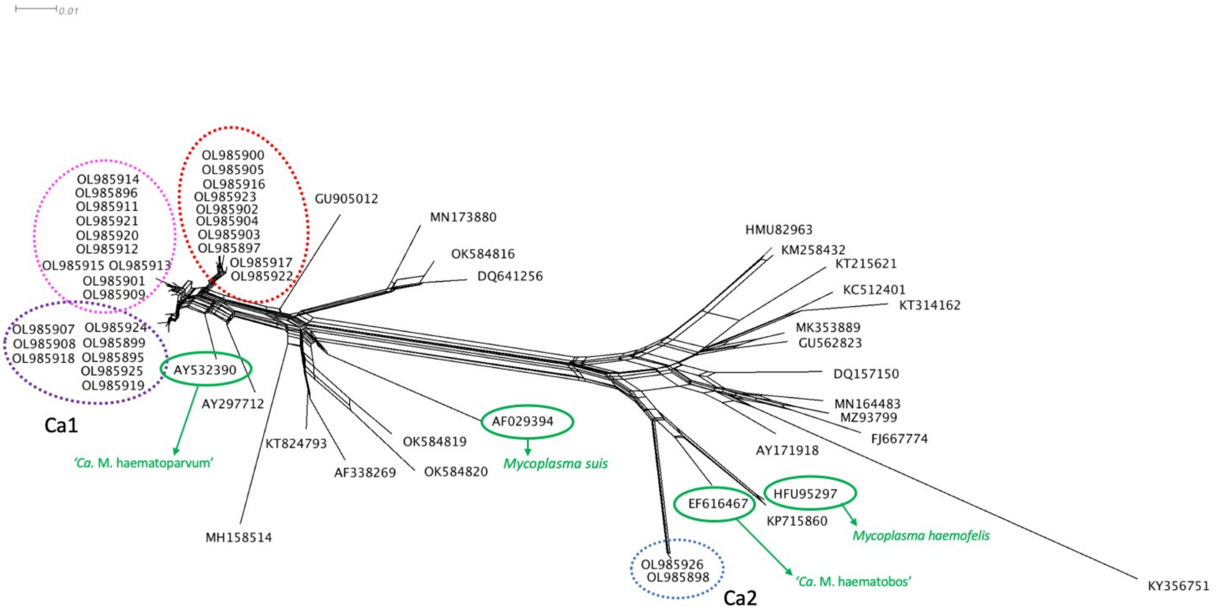

Figure 9. Distance analysis of 16S rRNA fragments from different *Mycoplasma* species was made using SplitsTree v4.14.6 software. Sequences from Ca1 and Ca2 are indicated in the tree. Regarding Ca1 sequences, colors from the Splitstree graph match with the subclades highlighted in 16S rRNA phylogeny (purple, pink and red). The Ca2 sequences are highlighted in blue. The species *M. suis* and *M. haemofelis* are highlighted in green. The species '*Ca*. M. haematoparvum' and '*Ca*. M. haematobos' were also highlighted in green to demonstrate their position compared to Ca1 and Ca2.

3.6. Distance Matrix Analysis

The heat map with the distance matrix from all partial 16S rRNA sequences detected herein corroborated with other achieved results from the present study and demonstrated a marked difference between sequences from Ca1 and Ca2 (Figure 10). The minimum and maximum divergence percentage rates found for all the sequences that fit in Ca1 were 0.0% and 2.92%, respectively. When comparing to the phylogenetic closest sequences (FJ004275, KM275255, MN543630, AY297712, MH094850, EF416569, GQ129112, KF366443, GQ129114, AY532390, AY383241), the minimum and maximum divergence found between Ca1 members and those sequences was 1.52% and 3.27%, respectively. When comparing the

Ca1 members and Ca2 members, the minimum and maximum divergence found was 15.23% and 16.22%, respectively. The minimum and maximum divergence percentage rates found for all members from Ca2 were 0.0%. When comparing to the phylogenetic closest sequences (FN421445, MG948628, MG948631, MH388480, MW463059, EF424082, EF616467, EF460765, HFU95297, GQ129115, KM275247, KP715860), the minimum and maximum divergence found between Ca2 members and those sequences was 5.12% and 7.23%, respectively. The distance matrix is available in **Supplementary Table S2**.

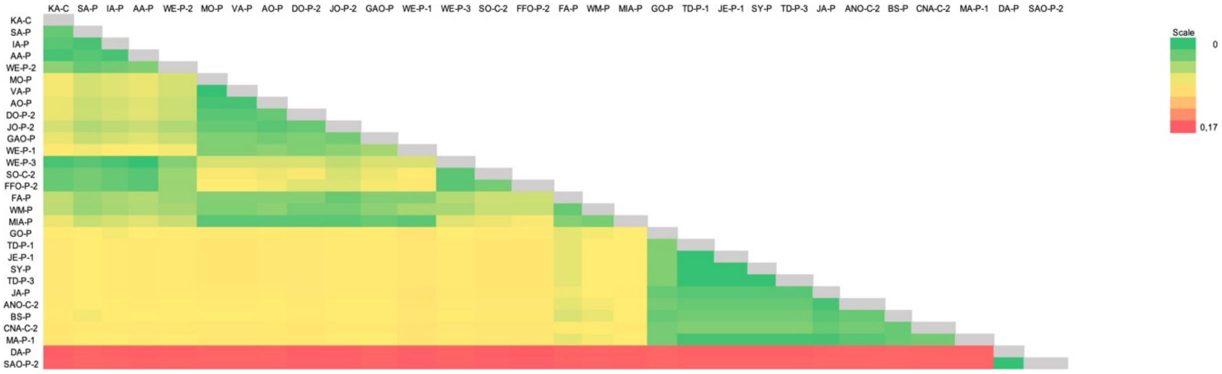

Figure 10. A heatmap constructed using the distance matrix based on *p*-value between all sequences obtained herein. The scale on the right demonstrates the color shade transition between obtained values.

3.7. Statistical Analysis

Statistical analysis results including OR (odds ratio) and *p*-value are summarized in Table 7. Regarding the analysis between location and positivity for hemoplasmas, the number of positive tapirs sampled in Pantanal wetlands was statistically higher when compared to those sampled in the Cerrado region (*p*-value = 0.0001). The other analyzed variables (gender and age) did not present statistically significant differences for this outcome (*p*-value > 0.05). The OR value demonstrated that tapirs from Pantanal were 5.64 times more likely to present positive results for the PCR protocol targeting the partial Mycoplasma spp. 16S rRNA gene used in the present study when compared to tapirs sampled in

Cerrado.

Table 7. Statistical analysis comparing the occurrence of Mycoplasma sp. in sampled tapirs and outcomes (gender, sampling location and age).

		16S rRNA *Mycoplasma* spp. PCR				
Variable		**+/n**	**(%)**	**OR**	**95% CI**	***P*-value**
Gender	Male	21/53	39.62	1.488	0.65-3.37	0.1707
	Female	15/49	30.61			
	Total	36/102				
Location	Pantanal	30/61	48.18	5.645	2.075-15.36	**0.0001721**
	Cerrado	6/41	14.63			
		36/102				
	Total					
Age	Sub-adult	20/47	42.22	1.806	0.79-4.10	0.09529
	Adult	16/55	29.09			
	Total	36/102				

+, Number of positive animals; n, number of samples; 95% CI, 95% confidence interval; OR, odd ratio. *P*-values <0.05 were considered statically significant and were highlighted in bold.

4. Discussion

We described herein the occurrence of at least two genetically distinct hemoplasma species occurring in free-ranging tapirs. Reports of *Candidatus* and novel species of these bacteria in Brazilian wild fauna are becoming common due to the growing use of molecular and genetic analysis tools. Based mainly on the partial 16S rRNA and 23S rRNA genes amplification, novel species of hemotropic *Mycoplasma* spp. infecting capybaras [22], hairy dwarf porcupines [23], opossums [24], and coatis [25] have been proposed. When analyzing the topologies found by partial 16S rRNA gene phylogeny from these reports, sequences from each novel *Candidatus* species were positioned separately in clades inside the "*Mycoplasma haemofelis* group", with satisfactory bootstrap values for species separation. In the present work, we detected different sequences from tapirs that fit in both the "*Mycoplasma haemofelis* group" (Ca2) and "*Mycoplasma suis* group" (Ca1) by partial 16S rRNA, 23S rRNA, and *dnaK* genes-based phylogeny, which was unprecedented for putative novel species of hemoplasmas reported in wild hosts from Brazil.

The 16S rRNA is considered the “gold standard” target gene for PCR assays aiming at detecting and identifying hemoplasmas and different pairs of primers have been used for its purpose [15]. Although this gene may be considered highly conserved, the 16S rRNA gene from bacterial species may exhibit considerable variations even on supposed conserved regions [53], which allow species differentiation. In the present study, all partial 16S rRNA sequences obtained for the Ca1 genotypes presented a similarity range of 97.20–98.20% with ‘*Candidatus* Mycoplasma haematoparvum’ detected in dogs from Italy and the USA (MH094850, AY383241) by BLASTn analysis. Meanwhile, the Ca2 genotypes demonstrated a similarity range of 94.99% with ‘*Candidatus* Mycoplasma haematobos’ detected in cattle from Cuba (MG948631). The *p*-distance values obtained by the distance matrix analysis showed that Ca1 members presented divergence rates of 0.0–2.92%, whereas the same comparison using the closest phylogenetic group and members of Ca1 presented rates of 1.52–3.27%. When using the 16S rRNA gene, a similarity rate of at least <97% is expected for bacteria belonging to different species. However, divergence rates <3% do not necessarily indicate that sequences belong to the same species [54]. Once phylogenetic analyses based on four molecular markers (16S rRNA, 23S rRNA, *RNAse P*, and *dnaK)* and 16S-based distance analyses strongly supported the differentiation between Ca1 and the closest hemoplasma species (‘*Ca*. M. haematoparvum’), it is likely that Ca1 represents a genetically distinct group of hemoplasmas.

The use of different genes for the molecular characterization of hemoplasmas is re- ported. Amplification of partial 23S rRNA gene was reported in phylogenetic studies of hemoplasmas identified in captive cervids from southern Brazil [55] and for the phyloge- netic study of *Mycoplasma ovis* infecting sheep from the same region [18]. Recently, this gene has also been used for the characterization of novel *Candidatus* species of hemoplasmas from wild animals in Brazil [22–25]. In the present study, partial 23S rRNA sequences that fit in Ca1 presented similarity percentages ranging from 89.59–91.50% with a sequence of ‘*Candidatus* Mycoplasma haematominutum’ from the UK (HE613254). Meanwhile, 23S rRNA sequences that fit in Ca2 presented similarity ranges of 90.20–90.57%

with a sequence of *Mycoplasma haemofelis* from the USA (NR103993). In fact, putative novel hemoplasma species may present lower similarity rates in BLASTn analysis for 23S rRNA sequences when compared to 16S rRNA sequences [22,55,56]. These differences may be explained by the fact that, although the 23S rRNA is considered as phylogenetically conserved as 16S rRNA, it presents a higher degree of sequence variability [57].

The *RNAse P* (*rnpB*) gene codifies the RNA subunit of endoribonuclease P with a length of approximately 400 bp [58]. Although only a few numbers of studies used phylogenetic trees of hemoplasmas using fragments of the *RNAse P* gene, our phylogeny analysis using this gene agreed with these studies, showing a separation of species among the "*Mycoplasma haemofelis* group" and "*Mycoplasma suis*/'*Ca*. M. haematominutum' group" [16,58]. However, both sequences obtained in the present study (OM317758-OM317759) were positioned in the "*Mycoplasma haemofelis* group", albeit these same two samples were positioned separately in the "*Mycoplasma haemofelis* group" and "*Mycoplasma suis* group" in all other analyzed targeted genes. Although it is expected that phylogenies using *RNAse P* fragments of hemoplasmas may present similar topologies to those using 16S rRNA [58], the fragments obtained herein are very short (~100 bp), precluding robust species differentiation.

Amplification and subsequent phylogenetic analysis of the *dnaK* gene have been demonstrated to be a useful tool for the separation of hemoplasmas from other Mollicute species [59] and also for the separation of subspecies of hemoplasmas [60]. This gene is responsible for coding a chaperon protein (heat shock protein 70) and is considered a great genetic marker for species differentiation, since it may present more variable regions within the sequences when compared to ribosomal fragments [59]. Unfortunately, only one sequence from the present study was successfully sequenced using the *dnaK* (OM339521) PCR protocol, and comparison with other sequences obtained from tapirs was not possible. However, this single sequence followed a similar topology pattern of 16S rRNA and 23S rRNA phylogenies, since it fitted on Ca1 in the "*Mycoplasma suis* group", reinforcing the phylogenetic position found for this putative novel hemoplasma species.

A high diversity of genotypes (*n* = 22) was found in the present work by analysis of 30 partial 16S rRNA sequences of hemoplasmas obtained from tapirs' samples. Richness on genotype diversity was also reported for hemoplasmas detected in bats from Brazil [56]. Although the genotypes found in tapirs from the Pantanal diverged from those found in tapirs from the Cerrado, a genetic proximity between these genotypes was demonstrated on the genotype network analysis. The occurrence of more than one genotype in the same region infecting wild hosts has already been reported in bats from Brazil [56]. Interestingly, one animal that was sampled at three different times (IDs: WE-P-1, WE-P-2, and WE-P-3) presented three different genotypes according to the time of sampling. Moreover, one animal (ID: AA-P), that was sampled only once, was positioned in Ca1 on 16S rRNA-based phylogeny and in Ca2 in the phylogenetic inference based on the partial 23S rRNA. These data suggest that tapirs may be susceptible to co-infections with different hemoplasma genotypes or species, with these co-infections occurring simultaneously or at different times.

Some animals that were sampled more than once presented positive samples in more than one sampling (IDs: MA-P-1, MA-P-2, JE-P-1, JE-P-2, WEP-1, WEP-2, WE-P3). Meanwhile, some animals that also were sampled more than once presented positive results for one sample only (IDs: TD-P-1, VA-P-1, ANO-C-2, CNA-C-2, SO-C-2, FFO-P-2, JO-P-2, DO-P-2, TD-P-3, SAO-P-2). These results may suggest either that tapirs may be maintained as chronically infected hosts or that hemoplasma bacteremia may be too low, precluding molecular detection. Chronic infection by hemoplasmas has been commonly reported in domestic species, such as pigs [61], cattle [62], and cats [63,64]. Although it is not possible to extend these findings for wildlife, chronic infection by hemoplasmas is appointed as a cause of unthriftiness in newborn piglets [65] and lower calf birth weight in cattle [62] which may raise a red flag in the context of species conservation.

Herein, gender and age were not associated with hemoplasma infection in the sampled tapirs. Although tapirs from Cerrado presented more health abnormalities when compared to tapirs from other biomes [65], we found that animals sampled in the Pantanal were 5.64 times more prone to be infected by hemoplasmas when compared to tapirs sampled in the Cerrado. In cats, population density may be associated as a risk factor for hemoplasma infection [66]. Some Pantanal sites and habitats were already reported as able to sustain high densities of tapir populations when these animals are not exposed to anthropic actions [67].

Tapirs from both Cerrado and Pantanal regions were found parasitized by *Amblyomma* spp. and Rhipicephalus microplus ticks. Although information regarding the transmission of hemoplasmas by ticks and other vectors is still lacking, *R. microplus* ticks were appointed as capable of transmitting '*Ca*. M. haematobos' for egg and larval stages and also to transmit this hemoplasma species for mice [68]. Future studies aiming at investigating the role of ticks in the transmission of hemoplasmas among tapirs are needed.

Mycoplasma haemofelis and 'Ca. M. haematominutum' DNA was detected in saliva and salivary glands of infected cats, suggesting that social interactions, such as aggression, may be related to hemoplasma infections [69]. Lowland tapirs were considered to be as solitary and show tolerance to other individuals not influenced by kinship [70]. More studies are necessary to elucidate the transmission routes for hemoplasmas among free-ranging lowland tapirs in Brazil.

The occurrence of anemia due to hemoplasma infection has been common in cats [15,19,71,72], pigs [61], cattle [73], sheep [74,75], and splenectomized dogs [76]. Regarding hemoplasma infection in wild animals, the occurrence of anemia has already been reported in reindeers (*Rangifer tarandus*) [77], non-human primates (*Sapajus flavius*) [17], and guignas (*Leopardus guigna*) [78]. Considering the occurrence of 35.29% (36/102; CI: 26.71–44.95%) infected individuals found in the present study, health assessment of infected animals may be useful to understand if hemoplasma-induced anemia is a threat for the largest land mammal species from Brazil.

Besides the assessment, for the first time, of the occurrence of hemotropic *Mycoplasma* among tapirs in two distinct Brazilian biomes, the present work showed high genetic diversity, and, at least, two genetically distinct hemoplasma species infecting these mammals. Accordingly, the obtained results reinforce the need for multi-locus and large-scale sequencing aiming at unraveling accurately the genetic diversity of hemoplasmas in wild animals.

5. Conclusions

At least two genetically distinct species of hemotropic *Mycoplasma* spp. occurs in free-ranging *T. terrestris* from the Pantanal and Cerrado regions in Brazil. The occurrence of hemoplasmas did not differ according to the gender or age of the sampled tapirs. Animals sampled in the Pantanal may be at a higher risk of becoming infected by hemoplasma when compared to those in the Cerrado. We propose that the two genetically divergent species found infecting tapirs from the present study represent putative novel *Candidatus* species and the names '*Candidatus* Mycoplasma haematoterrestris' and '*Candidatus* Mycoplasma haematotapirus' are proposed for the species found in Ca1 and Ca2, respectively.

Supplementary Materials: The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/microorganisms10030614/s1, Table S1: Hemoplasma-16S rRNA genotypes found in tapirs sampled in the present study according to sampling location.; Table S2: Distance matrix for partial 16S rRNA.

Author Contributions: Conceptualization, A.C.B.M. and M.R.A.; methodology, A.C.B.M., E.P.M., A.d.C.C., A.C.C. and M.R.A.; formal analysis, A.C.B.M.; investigation, A.C.B.M. and M.R.A.; re- sources, E.P.M., M.R.A. and R.Z.M.; data curation A.C.B.M., E.P.M. and M.R.A.; writing—original draft preparation, A.C.B.M. and M.R.A.; writing—review and editing, E.P.M. and M.R.A.; visualiza- tion, M.R.A.; supervision, M.R.A.; project administration, M.R.A.; funding acquisition, E.P.M., M.R.A. and R.Z.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by FAPESP (Foundation for Research Support of the State of São Paulo: #2018/02753-0; #2020/12037-0; #2019/26403-0) and CNPq (National Council for Scientific and Technological Development; Productivity Grant to MRA [CNPq Process #303701/2021-8]). LTCI's financial support comes from national and international agencies, including zoological institutions, foundations, private businesses, and private individuals

Institutional Review Board Statement: This study was approved by the Ethics Committee for Animal Experimentation of FCAV/UNESP (Faculty of Agricultural and Veterinary Sciences of the São Paulo State University) under protocol number 4558/20. The “Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO)” provided the required annual permits for the capture and immobilization of tapirs and collection of biological samples (SISBIO# 14,603).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: This study is part of Anna Mongruel’s PhD. She is carrying out her research at Programa de Pós-Graduação em Ciências Veterinárias—Universidade Estadual Júlio de Mesquita Filho (UNESP/Jaboticabal) and is sponsored by a fellowship from Foundation for Research Support of the State of São Paulo (FAPESP—Process number #2019/26403-0) at the the time of this study. Authors would like to thank the Lowland Tapir Conservation Initiative (LTCI)—Instituto de Pesquisas Ecológicas (IPÊ) for kindly providing the biological samples needed for the present study. The study of tapir health has been an important component of the long-term activities of the LTCI-IPÊ in Brazil. The LTCI has the institutional support from the International Union for Conservation of Nature (IUCN) Species Survival Commission (SSC) Tapir Specialist Group (TSG), Association of Zoos and Aquariums (AZA) Tapir Taxon Advisory Group (TAG), and European Association of Zoos and Aquariums (EAZA) Tapir Taxon Advisory Group (TAG).

Conflicts of Interest: The authors declare no conflict of interest.

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CAPÍTULO IV* – *Theileria terrestris* nov. sp.: A Novel *Theileria* in Lowland Tapirs (*Tapirus terrestris*) from Two Different Biomes in Brazil*

*Esse capítulo corresponde à publicação científica publicada no periódico Microorganisms 2022, 10, 2319. doi: https://doi.org/10.3390/microorganisms10122319.

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Abstract: The low-land tapir (*Tapirus terrestris*) is the largest wild terrestrial mammal found in Brazil. Although *T. terrestris* has been already reported as a host of hemoparasites, the occurrence and genetic identity of Piroplasmida agents in this species is still cloudy. Although it is reported that *Theileria equi*, an endemic equid-infective agent in Brazil, is occurring in lowland tapirs, these reports are probably misconceived diagnoses since they are solely based on small fragments of 18S rRNA that may not achieve accurate topologies on phylogenetic analyses. The present study aimed to detect and investigate the identity of *Theileria* spp. in tapirs from Pantanal and Cerrado biomes. Blood-DNA samples from tapirs were screened for a partial (~800 bp) 18S rRNA gene fragment from Piroplasmida and 64 (64/122; 52.46% CI: 43.66–61.11%) presented bands of expected size. Samples were submitted to different protocols for molecular characterization, including near-full length 18S rRNA gene (~1500 bp), and the *ema-1* gene from *T. equi*. Eight sequences were obtained for extended fragments (1182–1473 bp) from the 18S rRNA gene. Moreover, three sequences from partial *cox-1* and five from partial *hsp70* gene were obtained. None of the samples presented amplifications for the *ema-1* gene. Phylogenetic and distance analyses from the 18S rRNA sequences obtained demonstrated a clear separation from tapirs' *Theileria* spp. and *T. equi*. Phylogenetic analyses of *cox-1* and *hsp70* sequences obtained herein also showed a unique clade formed by tapir's *Theileria* spp. *Theileria terrestris* sp. nov. is positioned apart from all other *Theileria* species in 18S rRNA, *cox-1*, and *hps70* phylogenetic analyses. This novel proposed species represents a new Piroplasmida clade, yet to be characterized regarding biological features, vectors involved in the transmission cycles, additional vertebrate hosts, and pathogenicity.

Keywords: Piroplasmida; tapirs; Pantanal; Cerrado; theileriosis; wildlife

1. Introduction

Possibly, a larger number of parasites from Piroplasmida order may occur when compared to the number of vertebrate hosts that are capable of harboring these potential pathogens [1].

The low-land tapir (*Tapirus terrestris*) is the largest wild terrestrial mammal found in Brazil. Although *T. terrestris* has already been reported as a host of hemoparasites, such as *Trypanosoma* sp. [2] and hemoplasmas [3], the occurrence and genetic identity of Piroplasmida agents in this species is still incomplete.

In 2017, the molecular detection of piroplasms was reported in a single tapir from Mato Grosso do Sul State, Midwest Brazil. In this case, partial 18S rRNA (414 bp) amplification followed by sequencing and BLASTn analyses were conducted for nucleotide identity assessment, and BLASTn analysis indicated 98% identity with *Theileria equi* [4]. A few years later, T. equi was described as occurring in 11 Brazilian low land tapirs from the Amazon biome also based on the amplification of small fragments (392–475 bp) of the 18S rRNA gene and followed by maximum likelihood and Bayesian phylogenetic inferences [5].

The tick *Rhipicephalus microplus* is often associated as a vector of *T. equi* among horses in South America. However, due to decisive biological features, such as the fact that this tick species is a one-host tick and the lack of transovarilally transmission of *T. equi*, the role of *R. microplus* in *T. equi* cycle is still obscure [6]. Amblyomma sculptum has been reported infesting *T. equi*-positive horses in Brazil [7], including horses maintained in the Pantanal wetland areas [8]. However, A. sculptum was not able to transmit T. equi when feeding on infected horses under laboratory conditions [9]. Indeed, *A. sculptum* has been reported as a frequently found tick species in T. terrestris from Cerrado and Pantanal areas. *Rhipicephalus microplus* has also been reported in tapirs from these areas, albeit less frequently [10].

The present study aimed to investigate Piroplasmida in tapirs' blood samples from two Brazilian biomes, namely Cerrado and Pantanal, as well as to describe a new species of Theileria by assessing its phylogenetic positioning using large fragments of the 18S rRNA gene and additional molecular markers.

2. Materials and Methods

2.1 Sampling

Blood samples were collected from tapirs from Cerrado and Pantanal biomes (municipalities of Nova Andradina and Aquiadana, Mato Grosso do Sul State, Brazil). In total, 125 blood samples were collected from 94 living-tapirs: 61 (61/94; 64.89% CI: 54.83–73.78%) from Pantanal wetland areas, 33 (33/94; 35.11% CI: 26.22–45.17%) from Cerrado, and eight road-killed tapirs (8/125; 6.40% CI: 3.28–12.12%), also from Cerrado biome, that were sampled during necropsy procedures, summarizing 102 sampled individuals in both areas [3]. Fast-stained (Romanowsky-type stain) blood smears were made with collected blood samples from living tapirs for investigation of hemoparasites' inclusions in blood cells by light microscopy (Olympus BX43, Olympus Corporation, Tokyo, Japan) and images were captured using a lens' attached camera (Olympus DP73, Olympus Corporation, Tokyo, Japan) and computer software (CellSens, Olympus Corporation, Tokyo, Japan).

This study was approved by the Ethics Committee for Animal Experimentation of FCAV/UNESP (Faculty of Agricultural and Veterinary Sciences of the São Paulo State University) under protocol number 4558/20. The

“Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO)” provided the required annual permits for the capture and immobilization of tapirs and collection of biological samples (SISBIO# 14,603). All protocols for the capture, anesthesia, handling, and sampling of tapirs have been reviewed and approved by the Veterinary Advisors of the Association of Zoos and Aquariums (AZA)—Tapir Taxon Advisory Group (TAG), and the Veterinary Committee of the IUCN SSC Tapir Specialist Group (TSG). Further information about study areas and samplings is described elsewhere [3].

2.2. DNA Extraction and PCR Protocols for Mammals’ Endogenous Genes

DNA extraction was performed using a commercial kit (InstaGene™ Matrix, Biorad®, Hercules, CA, USA) and following the manufacturers’ instructions. In order to ensure success- ful DNA extraction, a conventional PCR for the mammal endogenous gene glyceraldehyde- 3-phosphate dehydrogenase (*gapdh*) (450 bp fragment) [11] was performed in all samples. Negative samples in the abovementioned PCR protocol were subjected to a second protocol targeting a 227 bp fragment of the *irpb* interphotoreceptor retinoid-binding protein (*irpb)* gene [12]. Samples that did not yield amplicons in either of the used PCR protocols were excluded from the subsequent analysis.

2.3. Conventional and Nested PCR Protocols for Piroplasmida Detection and Molecular Characterization

Initially, DNA samples from tapirs’ blood previously positive in the endogenous genes-based PCR protocols were submitted to a screening nested-PCR protocol aiming to amplify a ~800 bp fragment from the 18S rRNA gene of Piroplasmida [13]. Positive samples were then submitted to PCR protocols targeting fragments of different genes (*cox*-1 [14,15], *cox*-3 [16,17], *hsp70* [18], *cytb* [16,17], *b-tubulin* [19], and the intergenic spacer-1/ITS-1 [20]) from Piroplasmida as well as a fragment (396 bp) of *ema*-1 gene from *Theileria equi* [21]. Nucleotide sequences of primers and references used for the amplification of these genes are described in Table 1. Samples that yielded strong bands on electrophoresis for the partial 18S rRNA gene were submitted to two additional protocols for amplification of full-length 18S rRNA (~1500 bp) [22–

24]. All PCR were conducted in a final volume of 25uL. Detailed reagent concentrations and thermal conditions of all protocols used in the present study can be found in **Supplementary File S1**.

Table 1. Conventional and nested PCR protocols used in the present study.

Target gene	Primers	Fragment size (bp)	Reagents concentrations	Thermal conditions	References
18S rRNA	1st reaction: BTF1 (5’-GGCTCATTACAACAGTTATAG-3’) and BTR21 (5’-CCCAAAGACTTTGATTTCTCTC-3’); 2nd reation: BTF2 (5’-CCGTGCTAATTGTAGGGCTAATAC-3’) and BTR2 (5’- GGACTACGACGGTATCTGATCG-3’).	~800	1st reaction: 10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA. 2nd reaction: same concentrations and 1 uL from amplified product from 1st reaction as template.	1st round: Initial denaturation at 93°C for 03 min, followed by 45 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 20 sec and extension at 72° C for 30 sec, and final extension at 72° C for 07 min. 2nd round: same conditions but anneling at 62° C for 20 sec.	[13]
18S rRNA	Nbab_1F (5’-AAGCCATGCATGTCTAAGTATAAGCTTTT-3’) and 18Sapir (5’-GGATCACTCGATCGGTAGGAG-3’)	~1,500	10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA.	Initial denaturation at 95°C for 03 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 01 min and extension at 72° C for 01 min, and final extension at 72° C for 04 min.	[23,24]

18S rRNA	1st reaction: Piro0F (5'-GCCAGTAGTCATATGCTTGTGTTA-3') and Piro6R (5'-CTCCTTCCTYTAAGTGATAAGGTTCAC-3'); 2nd reaction: Piro1F (5'-CCATGCATGTCTWAGTAYAARCTTTTA-3') and Piro5.5R (5'-CCTYTAAGTGATAAGGTTCACAAAACTT-3')	~1,500	1st reaction: 10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA. 2nd reaction: same concentrations and 1 uL from amplified product from 1st reaction as template.	For both rounds: Initial denaturation at 95°C for 03 min, followed by 33 cycles of denaturation at 95°C for 01 min, annealing at 59°C for 01 min and extension at 72° C for 02 min, and final extension at 72° C for 10 min.	[22]
cox-1	1st reaction: Bab_for1 (5'-ATWGGATTYTATATGAGTAT-3') and Bab_Rev1 (5'- ATAATCWGGWATYCTCCTTGG- 3'), Bab_for2; 2nd reation: (5'-TCTCTWCATGGWTTAATTATGATAT-3') and Bab_Rev2 (5'-TAGCTCCAATTGAHARWACAAAGTG-3')	~924	1st reaction: 10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA. 2nd reaction: same concentrations and 1 uL from amplified product from 1st reaction as template.	1st round: Initial denaturation at 95°C for 01 min, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 48,1°C for 30 sec and extension at 72° C for 30 sec, and final extension at 72° C for 01 min. 2nd round: same conditions but anneling at 52° C for 30 sec.	[14,15]
cox-3	COX3F (5'-ACTGTCAGCTAAAACGTATC-3') and COX3R (5'-ACAGGATTAGATACCCTGG-3')	~600	10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA.	Initial denaturation at 94°C for 05 min, followed by 40 cycles of denaturation at 94°C for 20 sec, annealing at 55°C for 30 sec and extension at 68° C for 01 min, and final extension at 72° C for 07 min.	[16,17]

hsp70	hsp70F1 (5'- GAAGCACTGGCCHTTCAA-3') and hsp70R2 (5'-GBAGGTTGTTGTCCTTVGTCAT-3)	~740	10X Buffer, 50 mM MgCl$_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA.	Initial denaturation at 95°C for 05 min, followed by 35 cycles of denaturation at 95°C for 20 sec, annealing at 60°C for 30 sec and extension at 72° C for 30 sec, and final extension at 72° C for 07 min.	[18]
cytb	cytbF (5'-TTAGTGAAGGAACTTGACAGGT-3') and cytbR (5'-CGGTTAATCTTTCCTATTCCTTACG-3')	~1.000	10X Buffer, 50 mM MgCl$_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA.	Initial denaturation at 94°C for 05 min, followed by 49 cycles of denaturation at 95°C for 20 sec, annealing at 56°C for 30 sec and extension at 68° C for 45 sec, and final extension at 72° C for 07 min.	[16,17]
β-tubulina	Tubu-63F (5'-CAAATWGGYGCMAARTTYTGGGA-3') and Tubu-3F (5'-TCGTCCATACCTTCWCCSGTRTACCAGTG-3')	~1.200	10X Buffer, 50 mM MgCl$_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA.	Initial denaturation at 95°C for 05 min, followed by 30 cycles of denaturation at 94°C for 40 sec, annealing at 55°C for 1 min and extension at 72° C for 45 sec, and final extension at 72° C for 05 min.	[19]

ITS-1	1st reaction: ITS15C (5'-CGATCGAGTGATCCGGTGAATTA-3') and ITS13B (5'-GCTGCGTCCTTCATCGTTGTG-3'); 2nd reaction: (5'-AAGGAAGGAGAAGTCGTAACAAGG-3') and ITS15C (5'-TTGTGTGAGCCAAGACATCCA-3')	~450	1st reaction: 10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA. 2nd reaction: same concentrations and 1 uL from amplified product from 1st reaction as template.	1st round: Initial denaturation at 94°C for 01 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 01 min and extension at 72° C for 01 min, and final extension at 72° C for 05 min. 2nd round: same conditions but anneling at 49° C for 1 min.	[20]
ema-1	1st reaction: EMAE-F (5'-CCGCCCTTCACCTCGTTCTCAA-3') and EMAE-R (5'-TCTCGGCGGCATCCTTGACCTC-3'); 2nd reaction: EMAI-F (5'-CCGTCTCCGTTGACTTGGCCG-3') and EMAIR (5'- GGACGCGCTTGCCTGGAGCCT-3')	~396	1st reaction: 10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA. 2nd reaction: same concentrations and 1 uL from amplified product from 1st reaction as template.	For both rounds: 1st round: Initial denaturation at 94°C for 04 min, followed by 39 cycles of denaturation at 94°C for 01 min, annealing at 60°C for 01 min and extension at 72° C for 01 min, and final extension at 72° C for 04 min.	[21]

Products obtained in PCR assays were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide (Life Technologies™, Carlsbad, CA, USA) at 100 V/150 mA for 50 min, using 5 µL of amplified DNA per sample. The gels were imaged under ultraviolet light (ChemiDoc MP Imaging System, Bio Rad™, Hercules, CA, USA) using the Image Lab Software v4.1 (Biorad, Hercules, CA, USA). A map was constructed using QGis v. 3.26 software (http://qgis.org accessed on 26 October 2022) to illustrate the results obtained for partial 18S rRNA amplification from individual tapirs from each biome.

2.4 Sequencing, Sequence Analysis and Phylogeny

Amplified products were purified using a commercial kit (Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, WI, USA) and sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific™, Waltham, MA, USA) and ABI PRISM 310DNA Analyzer (Applied Biosystems™, Foster City, CA, USA) [25], at the “Centro de Recursos Biológicos e Biologia Genômica” (CREBIO, FCAV/UNESP, Jaboticabal, São Paulo, Brazil).

For sequencing of selected amplicons, the same primer pair from each PCR protocol or the primer pair from the 2° reaction of nested PCR assays were used, with exception for sequencing of extended 18S rRNA fragments. In the last case, primers used for sequencing [18,26] are described in Table 2. The obtained sequences were first submitted to a screening test using Geneious 11.1.3 software (http://www.geneious.com (accessed on 21 September 2021)) to evaluate the electropherogram quality and generate the consensus sequences. The BLASTn online program (National Center for Biotechnology Information, Bethesda, MD, USA) [27] was used to analyze the nucleotide sequences aiming to browse and compare with sequences from GenBank international database (http://www.ncbi.nlm.nih.gov/genbank (accessed on 21 September 2021)). Consensus sequences obtained in the current study and those retrieved from GenBank were aligned using the ClustalW software [28] via Bioedit v7.0.5.3 (http://www.mbio.ncsu. edu/BioEdit/bioedit.html, accessed on 3 July 2022) and also improved by an MAFFT alignment performed using GUIDANCE2 online server (http://ww.guidance.tau.ac.il, accessed on 3 August 2022) [29]. Phylogenetic inferences were based on Bayesian analysis via CIPRES online server (https://www.phylo.org/index.php/, accessed on 3 August 2022) [30]. The best-fit model was determined using jModeltest v2.1.6 via CIPRES online server (https://www.phylo.org/index.php/, accessed on 3 August 2022) [31].

Table 2. Set of primers used for the sequencing of near-full length 18S rRNA gene.

Primers	Primers	Annealing temperature	Primers Reference
Pair 1	Piro1F (5'- CCA TGC ATG TCT WAG TAY AAR CTT TTA-3') and Piro5.5R (5'- CCT YTA AGT GAT AAG GTT CAC AAA ACT T-3')	59°C	[26]
Pair 2	BabF2 (5'- CCG TGC TAA TTG TAG GGC TAA TAC A -3') and BabR2 (5'- GCT TGA AAC ACT CTA RTT TTC TCA A -3')	59°C	[18]
Single 1	Bab2F2 (5'- CTT TGA GAA ATT AGA GTG TTT -3')	59°C	Present study

Additionally, a distance-based analysis was performed using SplitsTree v4.14.6 (University of Tubingen, Tubingen, Germany) [32] to investigate the genetic relationship among extended 18S rRNA sequences detected in the present study and those previously deposited in GenBank.

3. Results

3.1. PCR for Endogenous Mammal's Genes

Out of 125 DNA samples extracted from blood samples, 122 samples (122/125; 97.60% CI: 93.18–99.18%) from 99 individuals successfully amplified the target endogenous genes. Three samples did not present bands of expected size for both protocols and were excluded from further PCR analyses. Regarding the animals that DNA from blood samples was submitted to further analyses; 61 (61/99; 61.62% CI: 51.77–70.59%) were sampled in Pantanal biome, whereas 38 (38/99; 38.38% CI: 29.41–48.23%) were sampled in Cerrado biome.

3.2. Screening Nested PCR for Piroplasmida Partial 18S rRNA Gene (~800 bp)

Out of 122 analyzed samples, 64 (64/122; 52.46% CI: 43.66–61.11%) showed expected band sizes on electrophoresis based on the 18S rRNA gene nested PCR. The 64 positive samples were obtained from 56 animals (56/99; 56.57% CI: 46.74–65.90%). Considering the prevalence at each biome, 41 positive animals were sampled in Pantanal (41/61; 67.21% CI: 54.72–77.66%) and 15 from the Cerrado (15/38; 39.47% CI: 25.60–55.28%). Among positive samples from Cerrado biome, four (4/15; 26.67%; CI: 10.90–51.95%) were collected from road-killed animals. From

Pantanal biome, six animals presented more than one positive sample during recaptures in different dates (in duplicates or triplicates), totalizing 48 (48/64; 75% CI: 63.18–83.99%) positive samples from this area. Regarding Cerrado biome, one animal presented more than one positive sample during recaptures in different dates (in duplicate), totalizing 16 (16/64; 25% CI: 16.01–36.82%) positive samples from this area.

The results described above are summarized in **Supplementary Files S2** and **S3**. The map (**Figure 1**) illustrates the number of tapirs that presented bands of expected sizes for the tested protocol.

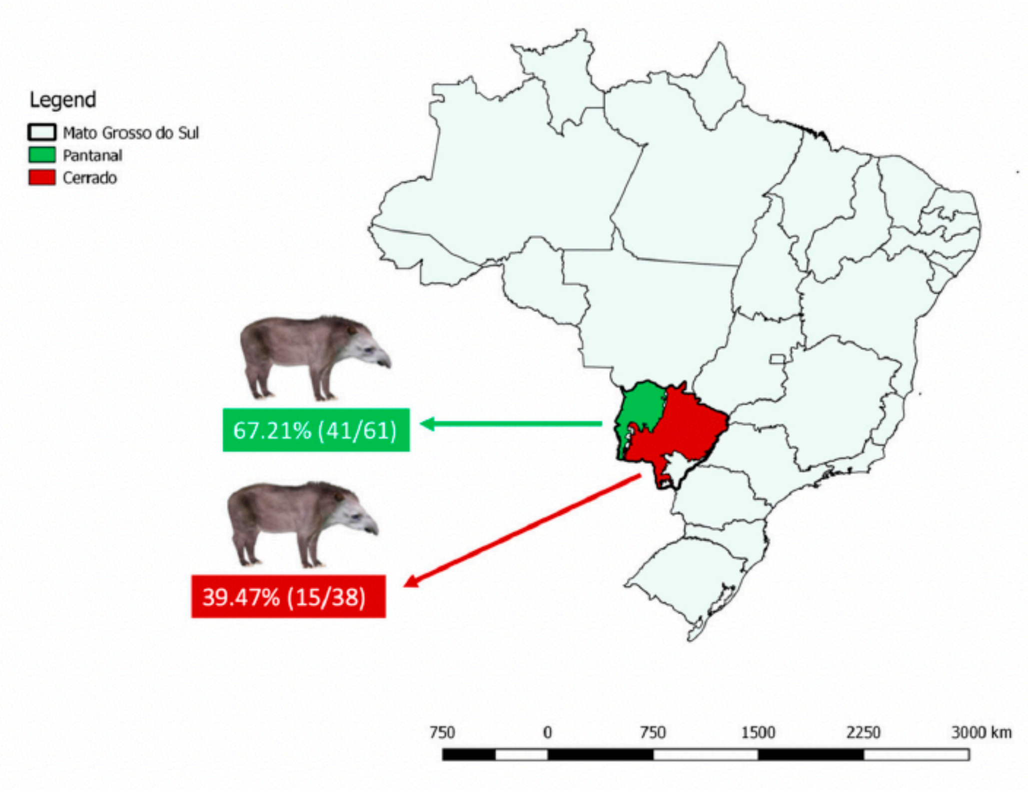

Figure 1. Number of tapirs from each biome that presented blood DNA samples with bands expected sizes on agarose gel electrophoresis when tested for partial Piroplasmida 18S rRNA by nested PCR analysis [13] in Mato Grosso do Sul State. This map was constructed using QGis 3.26 software (http://qgis.org, accessed on 26 October 2022).

3.3. Amplification of Molecular Markers for Additional Molecular Characterization of Piroplasmida

Twenty-one samples (21/64; 32.81% CI: 22.57–45.0%) presented fragments of different sizes for the *cox*-1 protocol and three sequences were successfully obtained. Although the protocol used a ~940 bp target fragment, the *cox*-1 sequences obtained from tapir ranged from 354 to 410 bp (GenBank access numbers OP169682, OP169600, OP169601). For the amplification of *hsp70* gene, 28 (28/64; 43.75%; CI: 32.29–55.91%) samples showed amplification of expected size and five sequences were successfully obtained with sizes ranging from 687 bp to 782 bp (GenBank access numbers (GenBank access numbers OP376711, OP169596-OP169599). Regarding the amplification of the Intragenic Spacer 1 (ITS-1), while 38 samples (38/64; 59.38% CI: 47.15–70.54%) presented amplification for the tested protocol, amplicons showed different size bands. Although different samples were chosen for purification and sequencing attempts, none of them were successfully purified and sequenced.

No sample presented amplification in the PCR protocols targeting the *cox*-3, *cytb*, *b-tubulin* genes, and *T. equi ema*-1 gene. BLASTn analyses results from the obtained sequences are described in **Supplementary File S4**.

3.4. Amplification of Extended Sequences from the Piroplasmida 18S rRNA Gene (~1500 bp)

Fifteen samples that presented strong amplification bands on electrophoresis for partial 18S rRNA (~800 bp) were sequenced to confirm identity and submitted to other two PCR protocols targeting an extended sequence of 18S rRNA gene (~1500 bp). Out of 15 samples tested, eight presented bands of the expected size for the first protocol [23,24]. Samples that did not yield bands of the expected size for the first protocol were then submitted to the second protocol [22] and additional four positive samples were obtained. All positive samples for both protocols (n = 12) were sequenced and eight sequences were successfully obtained, presenting sizes ranging from 1182 to 1473 bp (GenBank access numbers (OP023828-OP023835). BLASTn analyses results demonstrated that sequences obtained presented nucleotide identities ranging from 95.23% to 95.53% with *Theileria* spp. sequences from GenBank (**Supplementary File S4**).

3.5. Phylogenetic Analyses

For all three molecular markers described below, clades were identified according to
the phylogenetic study of Piroplasmida performed by Jalovecka et al. (2019) [33].

The extended 18S rRNA sequences obtained herein were subjected to Bayesian inference phylogeny analysis (**Figure 2**) with more 65 homologue sequences from GenBank database. Cardiosporidium cionae (GenBank access no. EU052685) was used as an outgroup. A total size of 1460 bp alignment was obtained and TrN+I+G was determined as the bestfit evolutionary model by Bayesian information criteria (BIC), using 10^7 generations of MCMC (Monte Carlo Markov Chain), two independent runs, and 10% of burn-in. Piroplasmida 18S rRNA sequences obtained from tapirs' blood samples in the present study formed a clade inside *Theileria* group, albeit separately from other *Theileria* species with high post-probability values (100).
Regarding phylogenetic analysis based on the *cox*-1 gene (Figure 3), a Bayesian inference phylogeny was performed with three sequences obtained in the present study and 21 homologous sequences retrieved from GenBank database. Plasmodium falciparum (AAP57966) was used as an external group. The nucleotide sequences were then transformed into amino acids using the ORFINDER software (https://www.ncbi.nlm.nih.gov/ orffinder/ (accessed on 10 February 2022)) and a total alignment of 259 amino acids was set up. The analysis was performed using the GTR+G evolutionary model, 10^7 generations of MCMC with two independent runs, and 10% burn-in. The sequences obtained for this fragment also grouped in a clade separated from other Piroplasmida species clades. The posteriori-probability value between lowland tapirs-associated *Theileria* and Equus group was 100.

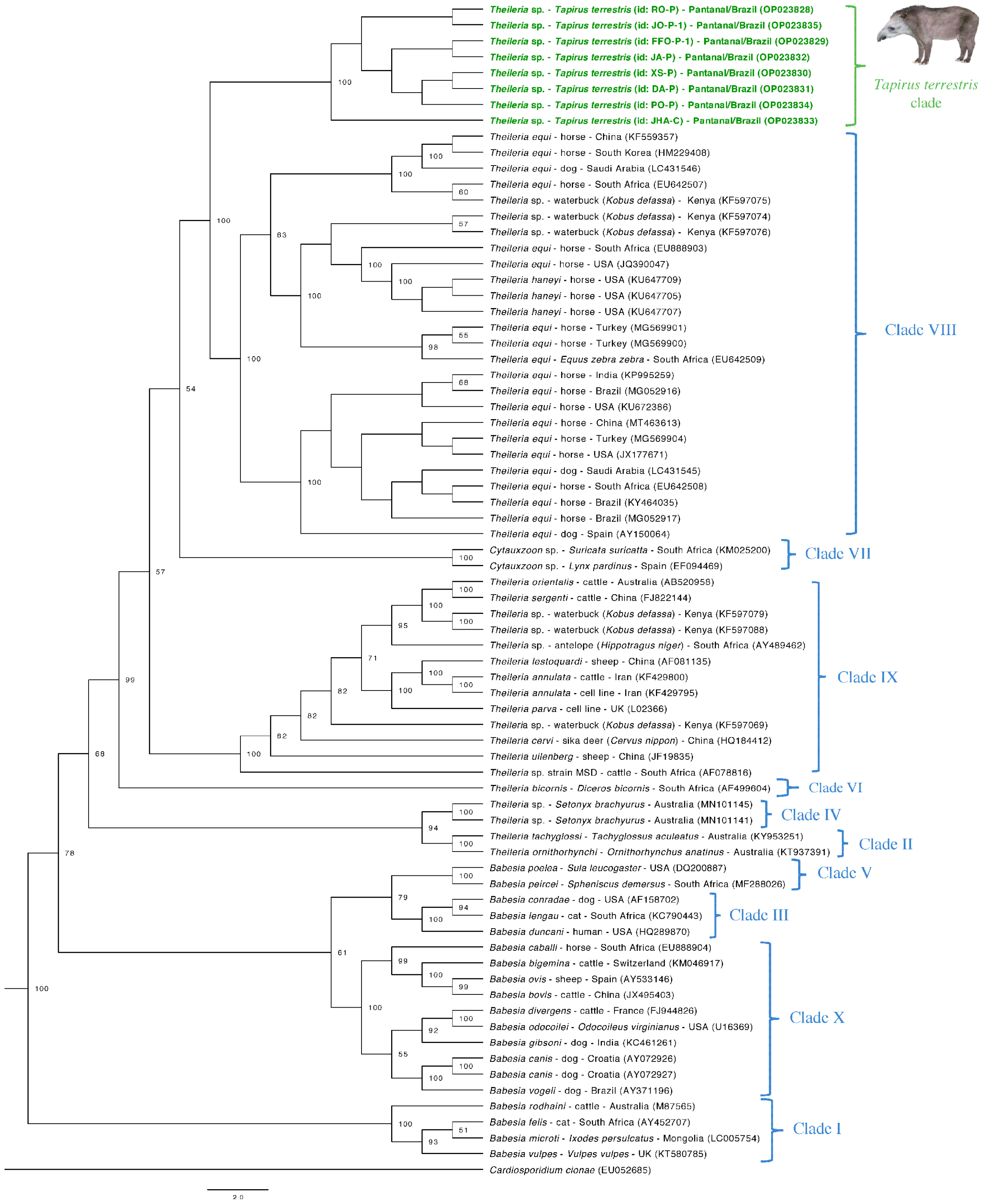

Figure 2. Phylogenetic tree based on the 18S rRNA gene from Piroplasmida. Tree was constructed by Bayesian inference using an alignment size of 1460 bp. Clades (I–X) were identified according to the phylogenetic study of Piroplasmida conducted by Jalovecka et al. (2019). Sequences from the present study are highlighted in

green. Post probability values >50 appear in tree. Identifications of the phylogenetic groups as proposed by Jalovecka et al. (2019) are indicated next to taxa or groups.

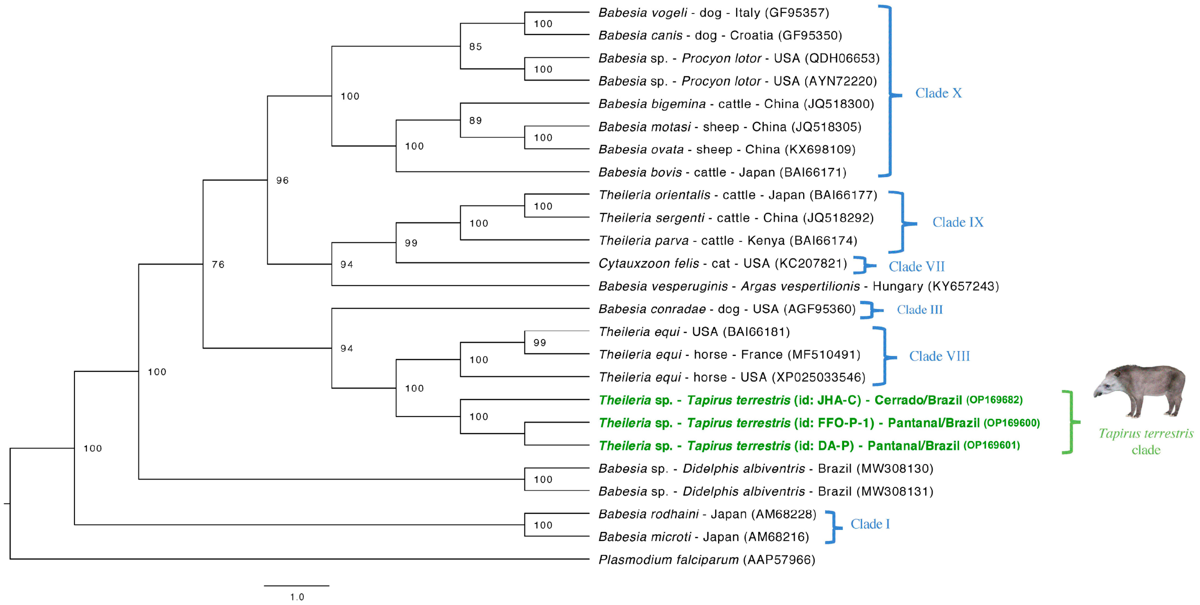

Figure 3. Phylogenetic tree based on partial *cox*-1 gene. Tree was constructed by Bayesian inference using an alignment size of 259 amino-acids. A sequence of *Plasmodium falciparum* (AAP57966) was used as outgroup. Sequences from the present study are highlighted in red. Post probability values >50 appear in tree. Clades (I, III, VII, VIII, IX, X) were identified according to the phylogenetic study of Piroplasmida conducted by Jalovecka et al. (2019) are indicated next to taxa or groups.

Phylogenetic analysis based on the five *hsp70* gene fragments obtained herein (**Figure 4**) was performed by Bayesian inference, with a total alignment of 690 bp, using 17 homologous sequences of the same gene and a sequence of *Cryptosporidium ratti* (MT507483) as an outgroup. The evolutionary model used for this analysis was F81+G, with 10^7 generations of MCMC, two independent runs, and 10% burn-in. Sequences from the present study grouped in a separate clade from other Piroplasmida species. This time, the *Theileria* obtained from lowland tapirs presented as closer related with *Theileria* sensu stricto and *Babesia* sensu stricto groups than with *Equus* group, with a high posteriori-probability value (100).

3.6. Distance Analysis by Splistree Software

A distance analysis was performed with a total alignment of 1460 bp of the Piroplas- mida 18S rRNA gene using Splistree with ‘Neighbor-net’ and ‘Uncorrected p-distance’ parameters (**Figure 5**). Taxons were identified according to the classification proposed by Jalovecka et al. (2019) [33]. Samples from the present study were circled in green. The extended sequences of 18S rRNA obtained herein formed a clearly separated group from *T. equi* group and other Piroplasmida species.

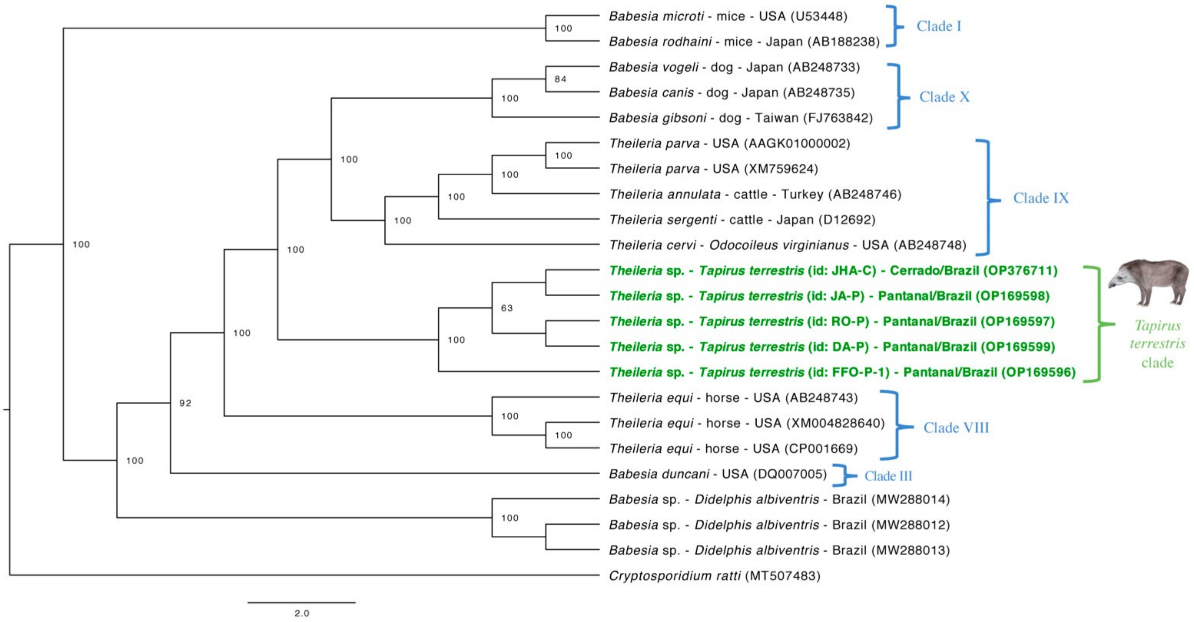

Figure 4. Phylogenetic tree based on partial *hsp70* gene. Tree was constructed by Bayesian inference using an alignment size of 690 bp. A sequence of *Cryptosporidium ratti* (MT507483) was used as outgroup. Sequences from the present study are highlighted in bold. Post probability values >50 appear in tree. Clades (I, III, VIII, IX, X) were identified according to the phylogenetic study of Piroplasmida conducted by Jalovecka et al. (2019) are indicated next to taxa or groups.

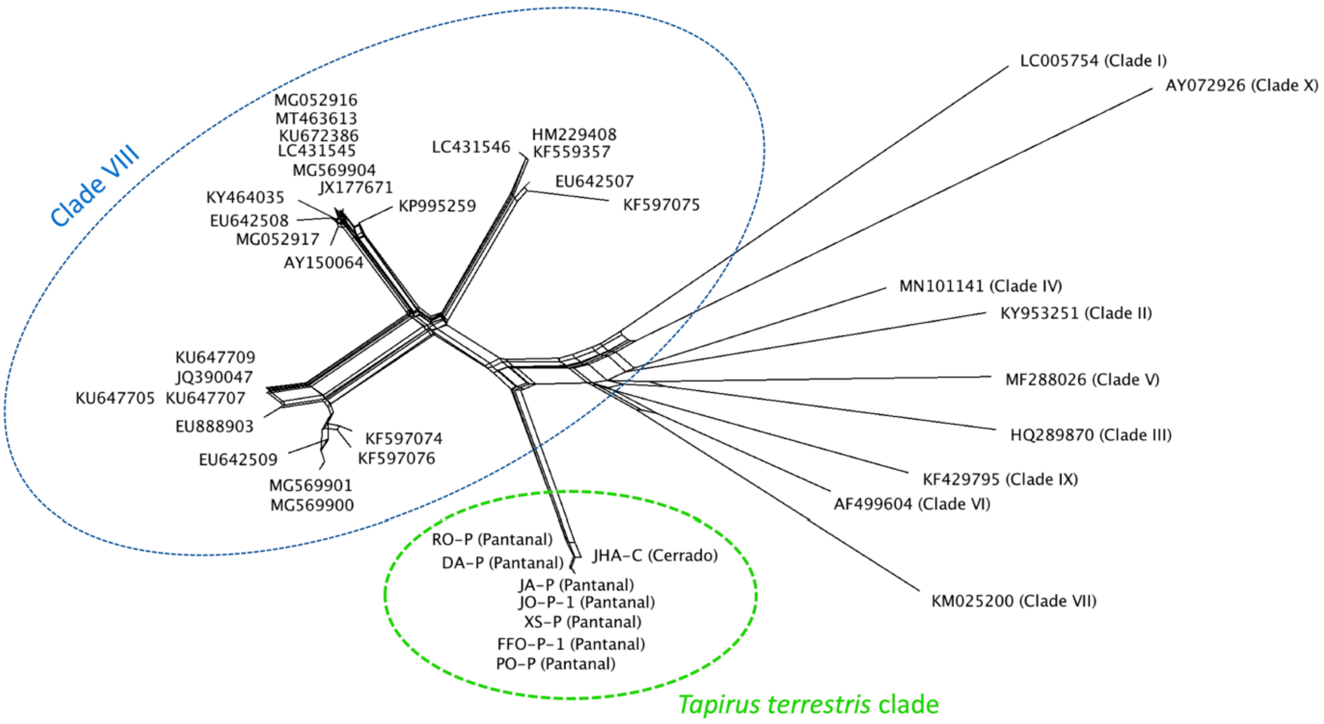

Figure 5. Splitstree network based on 'Neighbor-net' and 'Uncorrected-p' parameters, using an alignment of 1460 bp of the 18S rRNA. Identifications of the phylogenetic groups as proposed by Jalovecka et al. (2019) are indicated next to taxons or groups. Sequences from the present study are circled in green.

3.7. Blood Smears Analysis

Two blood smear samples from PCR-positive tapirs (ID: PO-P and FFO-P-1) presented inclusions suggestive of Piroplasmida infection in erythrocytes. Inclusions showed the classical forms of 'Maltese cross' (**Figure 6**), with the formation of tetrads. The length obtained for the tetrads was 1.42 µm.

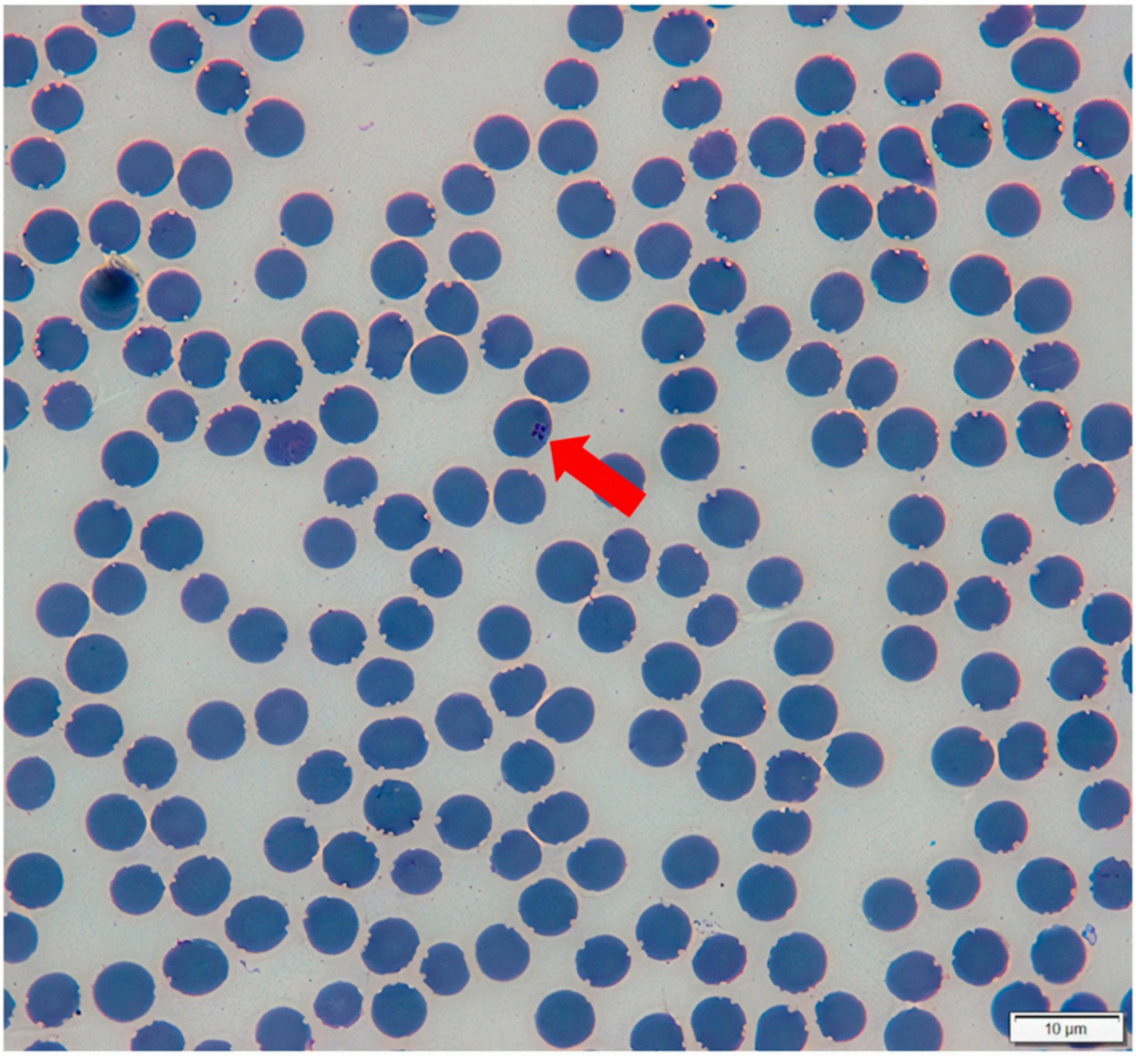

Figure 6. Maltese-Cross inclusions suggestive of *Theileria* spp. found in erythrocytes of *Tapirus terrestris* (indicated by the red arrow). Visualization was made by using a light microscope (1000×), during blood smear examination. The animal (ID: PO-P) was positive in a PCR protocol that amplify a fragment of approximately 1500 bp of the 18S rRNA gene from piroplasmids [23,24] in the present study.

3.8. Description of a New Species: Theileria terrestris nov.sp.

DESCRIPTION

Taxonomic review

Theileria terrestris n. sp.

Type-host: Lowland tapir *Tapirus terrestris* (Mammalia: Peryssodactila).

Type-locality: Pantanal and Cerrado Biomes, Mato Grosso do Sul State, Brazil. (19.301668883667233, 55.76404347032559; 21.583271686146354, 53.88629288988308).

Type-material: Holotype. A stained thin blood smear from a lowland tapir (*T. terrestris*– ID: PO-P) from Pantanal biome, containing the holotype (Figure 6) was deposited in the Vector-Borne Bioagents Laboratory of FCAV/UNESP (Jaboticabal, São Paulo, Brazil) in addition to genomic DNA samples extracted from the blood of lowland tapirs. Sample was collected and blood smear was made on 24 August 2018.

Vector: Unknow. It is assumed to be a local species of hard (Ixodida) tick. Representative sequences: GenBank accession numbers: OP023828-OP0232835 (18S rRNA); OP169682, OP169600 and OP169601 (*cox*-1); OP376711, OP169596-OP169599 (*hsp70*).

Etymology: The species is named after it was encountered in *Tapirus terrestris* hosts from Pantanal and Cerrado Biomes in Brazil.

ZooBank reference numbers: pub: CCF171BB-BE8C-4B98-ABCB-1BAB5E8CFAC7; act: 5674F76B-EA24-46E9-B6CE-ADE9A3819273.

Description: This *Theileria* organism shows the classical inclusion form inside erythrocytes from *T. terrestris*, with tetrads of merozoites forming a "Maltese-cross". It's location inside the erythrocyte can be described as more peripherical when compared to *T. equi* inclusions, with a length size found of 1.42 µm. On fast-stained blood smears, it shows a purple-blue coloration. The occurrence of inclusions of different forms or sizes is unknown.

4. Discussion

Analyses conducted herein suggested an evident genetic separation between *T. equi* and the lowland tapir-related *Theileria* sp. detected in the present study, based on the near full-length 18S rRNA gene of Piroplasmida. The phylogenetic tree constructed using Bayesian inference and a total alignment size of 1460 bp showed a separation supported by high post probabilities values (100) between Clade VIII (*Equus* group) and *T. terrestris* clade. A clear separation was also observed on distance analysis performed using SplitsTree software. These findings point out the existence of a potentially new *Theileria* species/phylogenetic group based on the Piroplasmida topology proposal [33]. The neighbor-joining tree constructed by Jalovecka et al. (2019) [33] with an alignment size of 1638 bp also achieved high bootstrap values (100) on separation of clades belonging to *Equus* group (Clade VIII), *Theileria* sensu stricto (Clade IX), and *Babesia* sensu stricto (Clade X) groups, which were the phylogenetically closest clades in their analysis. In our analysis, *Equus* group and *T. terrestris* clades fit more closely related to *Cytauxzoon* group (Clade VI) and distant from *Babesia* sensu stricto group. These differences may be due to the different sort of inferences used (neighbor-joining x Bayesian) as well as the fact that we included newly reported genotypes, which may influence the achieved topology. Indeed, *Theileria* and *Cytauxzoon* share some biological features. The sporozoites of both these representatives of the Theileriidae family are capable of invading hosts' leukocytes during the schizogony phase followed by the invasion of red blood cells [1].

Theileria sp. in free-ranging lowland tapirs was reported in post-mortem blood and spleen samples from road-killed animals from the Pantanal biome (Mato Grosso State) [34]. When fragments of approximately 740 bp were analyzed by Bayesian phylogenetic in- ference, these sequences (GenBank access no. MZ491096, MZ490586) clustered together in a clade with *Theileria* sp. previously reported in cats from Brazil (GenBank access no. KP410270-KP410273, KF970930) [35,36] and closely related to an *T. equi* clade [34]. Unfortunately, near-full length 18S rRNA from *Theileria* genotypes from cats are not available, precluding a better assessment on the phylogenetic positioning of these sequences within Piroplasmida clades. Indeed, 18S rRNA sequences of *Theileria* sp. obtained from tapirs with approximately 400 bp already fit in a clade separately from *T. equi* in Bayesian analysis [5], but extended sequences would be significant to highlight the phylogenetic relationship of *Theileria* sp. obtained from tapirs with those genotypes or species obtained from other wild animals from Brazil.

The equid-associated *T. equi* is considered endemic in Brazil [8] and a causative of red blood cell destruction [37]. *Theileria equi* DNA has also been reported in different vertebrate hosts, such as dogs [38], sheep [39] and zebras [40]. Considering its potential capacity to infect a different range of hosts species, sequences retrieved from non-equid hosts that present high percentages of similarity rates on BLASTn analysis may mislead to incorrect taxonomic prediction as *T. equi* when based solely on this similarity. Previously, occurrence of *T. equi* was reported in lowland tapirs from Pantanal [4] and Amazon [5] biomes. For both studies, percentages of identity computed by BLASTn analyses were reported as 98% when comparing partial 18S rRNA gene sequences (ranging from 392 to 475 bp) of *Theileria* spp. from lowland tapirs with homologue sequences from *T. equi*. Although this value could be considered high, taxonomic positioning based on 18S rRNA partial sequences shows lower accuracy when compared to those performed with (near) complete gene [41], and extended 18S rRNA sequences provide more resolutive phylogenetic positioning of Piroplasmida [17,42,43].

Regarding the other target genes evaluated herein, the topology obtained by the *cox*-1-based phylogenetic tree was very similar to those obtained with 18S rRNA, with both analyses agreeing when it comes to positioning the *T. terrestris*-clade closely to, albeit separated from, Clade VIII (post probability value of 94). The use of mitochondrial sequences, as the *cox*-1 gene fragments, is a useful tool to resolve Piroplasmida phylogenetic topologies [16]. Indeed, some studies performed the concatenated analysis with both 18S rRNA and *cox*-1 to achieve even more informative topologies [16,44]. Unfortunately, once we are working with clinical samples from wild animals, which were collected in-field, it is difficult to concatenate genes from a single sample in a trustworthy way, once the occurrence of genetic diversity of *Theileria* members is reported in animals from the same population [45–47].

The *hsp70* gene from Piroplasmida codifies the heat shock protein 70 proteins. When using fragments from this gene to phylogenetic assess the relationship between *T. terrestris*- clade and other Piroplasmida clades, we observed that tapirs' clade is now closely related to Clade IX (*Theileria* sensu stricto) and Clade X (*Babesia* sensu stricto) with high post- probabilities values (100). The pattern of evolution of a certain gene is an influent aspect on phylogenetic topologies [48]. Differences in the structure of the *hsp70* gene may reflect in distance between taxa once it codifies important functional proteins [49]. Even though the 18S rRNA is considered an important conservative gene for taxonomic positioning of Piroplasmida [33], the use of different nuclear and mitochondrial genes may answer questions about the evolutionary relationship among species from this Order.

Maltese-cross inclusion forms comprise four merozoites in one erythrocyte and are usual during the developmental stages of some Piroplasmida species [50]. This form has been described commonly in *Theileria* species, such as the horse-related species *T. equi, T. haneyi* [51], as well as in *T. parva* and in *Theileria* sp. from African waterbucks (*Kobus defassa*) [52]. Besides that, this inclusion form has also been reported for *Babesia microti* [50,53]. In the present study, Maltese-cross inclusions were found in the blood smear of one infected tapir. The length of the inclusion found in the blood smear of a *Theileria*-positive tapir from the present study was smaller (1.42 µm) than mean values reported for *T. equi* (1.88 µm) and larger than mean values of *T. haneyi* (1.15 µm) [51].

5. Conclusions

Phylogenetic analyses based on near-full length sequences of the 18S rRNA, *hsp70*, and *cox-1* genes supported the description of *Theileria terrestris* nov. sp. in tapir blood samples, which was positioned apart from all other *Theileria* species. *Theileria terrestris* nov. sp. represents a new Piroplasmida clade, yet to be characterized regarding biological features, vectors involved in the transmission cycles, additional vertebrate hosts, and pathogenicity.

Supplementary Materials: The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/microorganisms10122319/s1, File S1: Detailed reagent concentra- tions and thermal conditions of all protocols used in the present study.; File S2: Positive samples for detection of partial 18S rRNA from Piroplasmida: sample identification, biome where the animal was sampled, sampling date, gender and age.; File S3: List of tapirs that were sampled more than once and that presented at least one positive sample for Piroplasmida partial 18S rRNA amplification.; File S4: BLASTn analysis of the obtained gene fragments of Piroplasmida detected in lowland tapirs' blood samples.

Author Contributions: Conceptualization, A.C.B.M. and M.R.A.; methodology, A.C.B.M., E.P.M., A.d.C.C., A.C.C., L.P., B.C.B.R., J.F.S. and M.R.A.; formal analysis, A.C.B.M. and B.C.B.R.; investigation, A.C.B.M. and M.R.A.; resources, E.P.M., M.R.A. and R.Z.M.; data curation A.C.B.M., J.F.S. and M.R.A.; writing—original draft preparation, A.C.B.M. and M.R.A.; writing—review and editing, J.F.S. and M.R.A.; visualization, M.R.A.; supervision, M.R.A.; project administration, M.R.A.; funding acquisition, E.P.M., M.R.A. and R.Z.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by FAPESP (Foundation for Research Support of the State of São Paulo: #2019/24403-0; #2020/12037-0) and CNPq (National Council for Scientific and Technological Development; Productivity Grant to Marcos Rogério André [CNPq Process #303701/2021-8]). LTCI's financial support comes from national and international agencies, including zoological institutions, foundations, private businesses, and private individuals.

Data Availability Statement: GenBank (http://www.ncbi.nlm.nih.gov/genbank) accession num- bers: OP023828-OP0232835 (18S rRNA); OP169682, OP169600 and OP169601 (*cox*-1); OP376711, OP169596-OP169599 (*hsp70*).

Acknowledgments: This study is part of Anna Mongruel's thesis. She is carrying out her research at Programa de Pós-Graduação em Medicina Veterinária—Universidade Estadual Júlio de Mesquita Filho (UNESP/Jaboticabal) and is sponsored by a fellowship from Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP–Processo número 2019/26403-0) at the the time of this study. Authors would like to thank the Lowland Tapir Conservation Initiative (LTCI)–Instituto de Pesquisas Ecológicas (IPÊ) for kindly providing the biological samples needed for the present study. The study of tapir health has been an important component of the long-term activities of the LTCI-IPÊ in Brazil. The LTCI has the institutional support from the International Union for Conservation of Nature (IUCN) Species Survival Commission (SSC) Tapir Specialist Group (TSG), Association of Zoos and Aquariums (AZA) Tapir Taxon Advisory Group (TAG), and European Association of Zoos and Aquariums (EAZA) Tapir Taxon Advisory Group (TAG).

Conflicts of Interest: The authors declare no conflict of interest.

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CAPÍTULO V* – Molecular evidence of *Bartonella* spp. in wild lowland tapirs (*Tapirus terrestris*), the largest land mammals in Brazil*

*Esse capítulo corresponde à publicação científica publicada no periódico Comparative Immunology, Microbiology and Infectious Diseases 2023, 101, 102042. doi: https://doi.org/10.1016/j.cimid.2023.102042

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Abstract: The genus *Bartonella* (Hyphomicrobiales: Bartonellaceae) encompasses facultative intracellular α-proteobacteria that parasite erythrocytes and endothelial cells from a wide range of vertebrate hosts and can cause disease in animals and humans. Considering the large diversity of vertebrate species that may act as reservoirs and arthropod species that may be associated with *Bartonella* transmission, the exposure of animals and humans to these microorganisms is likely underestimated. The present study aimed to investigate the occurrence of *Bartonella* sp. in wild tapirs (*Tapirus terrestris*; Perissodactyla: Tapiridae) from two biomes in Brazil: Pantanal and Cerrado. Ninety-nine GPS-monitored wild tapirs were sampled in Pantanal (n = 61/99) and Cerrado (n = 38/99). A qPCR (quantitative real-time polymerase chain reaction) assay targeting the *nuoG* gene was used for the screening for *Bartonella* spp. DNA. Positive samples were additionally subjected to conventional PCR assays targeting five molecular markers (*ribC*, *gltA*, *rpoB*, *groEL*, ITS). Eight (8/99; 08,08%) animals were positive in the qPCR assay for *Bartonella* spp.: 7 from Cerrado (7/8; 87.5%) and 1 from Pantanal (1/8; 12.5%). The 5 *Bartonella ribC* sequences obtained from tapirs' _blood samples grouped together with *Bartonella henselae* obtained from cats, humans, wild felids and *Ctenocephalides felis* (Siphonaptera: Pulicidae*)* fleas. To the best of author's knowledge, this is the first report of *Bartonella* sp. in *Tapirus terrestris*. This finding contributes to the understanding of the occurrence of *B henselae* in wild mammals from Brazil as well as expands the knowledge regarding the potential vector-borne pathogens that may affect wild tapis from Cerrado and Pantanal biomes.

Keywords: Bartonellosis, Tapir, Wild animals, Brazil, Pantanal, Cerrado

1. Introduction

The genus *Bartonella* (Hyphomicrobiales: Bartonellaceae) encompasses facultative intracellular α-proteobacteria that parasite erythrocytes and endothelial cells from a wide range of vertebrate hosts and can cause disease in animals and humans [1,2]. Among the species that can be pathogenic for humans, *Bartonella henselae* has domestic cats (*Felis catus domesticus*; Carnivora: Felidae) as its main hosts [3,4]. This agent is the main causative agent of Cat Scratch Disease (CSD), which is usually characterized by regional lymphadenopathy and fever syndrome in humans [5].

While the human infection is transmitted by inoculation of viable *B. henselae* through cats' scratches or bites, the primary vector for the transmission of this agent among susceptible cats is the cat flea *Ctenocephalides felis* (Siphonaptera: Pulicidae) [6]. Additionally, ticks (*Ixodes ricinus* and *Rhipicephalus sanguineus*; Ixodida: Ixodidae) showed to be able to transmit experimentally *B. henselae* [7–9]. The occurrence of *Bartonella* sp. is well documented among wild animals. Rodents represent the main reservoir for distinct genotypes of *Bartonella* worldwide, such as in the USA [10], Japan [11], Poland [12], Switzerland [13], and Brazil [14,15], followed by bats [16,17]. Other wild mammals' groups, such as felids [18], canids [19] and ruminants [20] have also being reported as *Bartonella* hosts. Considering the large diversity of vertebrate species that may act as reservoirs and arthropod species that may be associated with *Bartonella* spp. transmission, the exposure of animals and humans to these microorganisms is likely underestimated [21]. The lowland tapir (*Tapirus terrestris*; Perissodactyla: Tapiridae) is the largest land mammal in Brazil and considered a species vulnerable for extinction, mainly due anthropic actions [22]. Although *Bartonella bacilliformis*, the etiological agent of Carrion Disease and "Verruga Peruana" in humans, was molecularly detected in *Amblyomma* spp. ticks that fed on in *Tapirus terrestris* in Peru [23], there is no report of *Bartonella* spp. in tapirs' blood or tissue samples so far. In light of this, the present study aimed to investigate the occurrence of *Bartonella* sp. in blood samples from wild lowland tapirs from Brazil.

2. Material and methods

2.1. *Sampling and DNA extraction*

The study was approved by the Ethics Committee for Animal Experimentation of FCAV/UNESP (Faculty of Agricultural and Veteri- nary Sciences of the São Paulo State University) under protocol number 4558/20. The "Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO)" provided the required annual permits for the capture and immobilization of tapirs and collection of biological samples (SISBIO# 14,603). All protocols for the capture, anesthesia, handling, and sampling of tapirs have been reviewed and approved by the Veterinary Advisors of the Association of Zoos and Aquariums (AZA) Tapir Taxon Advisory Group (TAG), and the Veterinary Committee of the IUCN SSC Tapir Specialist Group (TSG). Additional information about sampling procedures and study areas were described elsewhere [24].

Between 2013 and 2018, wild tapirs monitored by GPS collars (n=99) were sampled in two different biomes: Pantanal (n=61/99) and Cerrado (n=38/99) located in the Mato Grosso do Sul State, midwestern Brazil. For this purpose, three capture methods were used: box traps, pitfalls, and anesthetic darts. Tapirs captured in box traps and pitfalls were visually inspected before anesthesia to estimate body mass and observe general condition, posture and ability to move, behavior, signs of stress, and presence of lesions and scars. Lactating and pregnant tapirs were identified and immediately released. Animals were also identified regarding sex and age (adult = > 48 months old; sub-adult = < 48 months old). Blood samples were collected and stored at -20^{o}C until posterior analyses. DNA from blood was extracted using commercial kits (InstaGene™ _Matrix, Biorad®, Hercules, CA, US), and following the manufacturer's instructions. A map (**Fig. 1**) was constructed using QGIS v.3.26 software (http//: qgis.org) for visual representation of the biomes included in the present study.

2.2. Molecular assays

A conventional PCR (cPCR) for the mammal-endogenous gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) (450 bp) [25] was performed to ensure success in DNA extraction procedures. If any sample showed negative results in the abovementioned PCR protocol, it was subjected to an additional PCR protocol targeting a 227 bp fragment of the *irpb* (interphotoreceptor retinoid-binding protein) gene [26]. Samples that did yield amplicons in neither of the used PCR protocols were excluded from the subsequent analysis. A qPCR (quantitative real-time polymerase chain reaction) assay targeting the *nuoG* gene was used for the screening for *Bartonella* spp. DNA. The primers 5′-CAATCTTCTTTTGCTTCACC-3' 5′-TCAGGGCTTTATGTGAATAC-3' were used to amplify an 83 bp fragment from the *nuoG* gene of *Bartonella* spp., with the hydrolysis probe [6FAM] 5'-TTYGTCATTTGAACACG-3'[BHQ1]) [27]. Each DNA sample was evaluated in duplicates. Samples that presented differences in Cq values greater than 0.5 were retested in triplicate. For the construction of the standard curve of each reaction, serial dilutions were performed at different concentrations (2.0 × 107 to 2.0 × 101 copies) of a plasmid encoding an 83 bp fragment of the *nuoG* gene of *B. henselae* (pIDTSMART; Integrated DNA Technologies, Coralville, IA, USA). These plasmids were also used as positive controls. The number of plasmid copies was determined by the formula (XG/μL DNA/ [Plasmid Length (BP) x 660]) x 6. 22 x 10^{23} x plasmid copies/μL. The amplification efficiency (E) was calculated according to the slope of the standard curve using the formula E $10^{-1/slope}$ [28]. Ultra-purified sterilized water was used as a negative control for each reaction performed. The qPCR assays were carried out in a C1000-CFX96 thermocycler (BIORAD, Hercules, CA, USA). Positive samples in the qPCR assay were subjected to conventional (c)PCR assays targeting fragments targeting the *ribC* (420 bp) [29], *gltA* (750 bp) [30]) *rpoB* (800 bp) [31], *groEL* (752 pb) [31,32] genes and the intergenic-spacer region 16S-23S rRNA ITS (453–717 bp) [33,34] (**Table 1**). Ultra-purified sterilized water and *Bartonella machadoae* DNA [15] were used as negative and positive controls, respectively, in the conventional PCR assays.

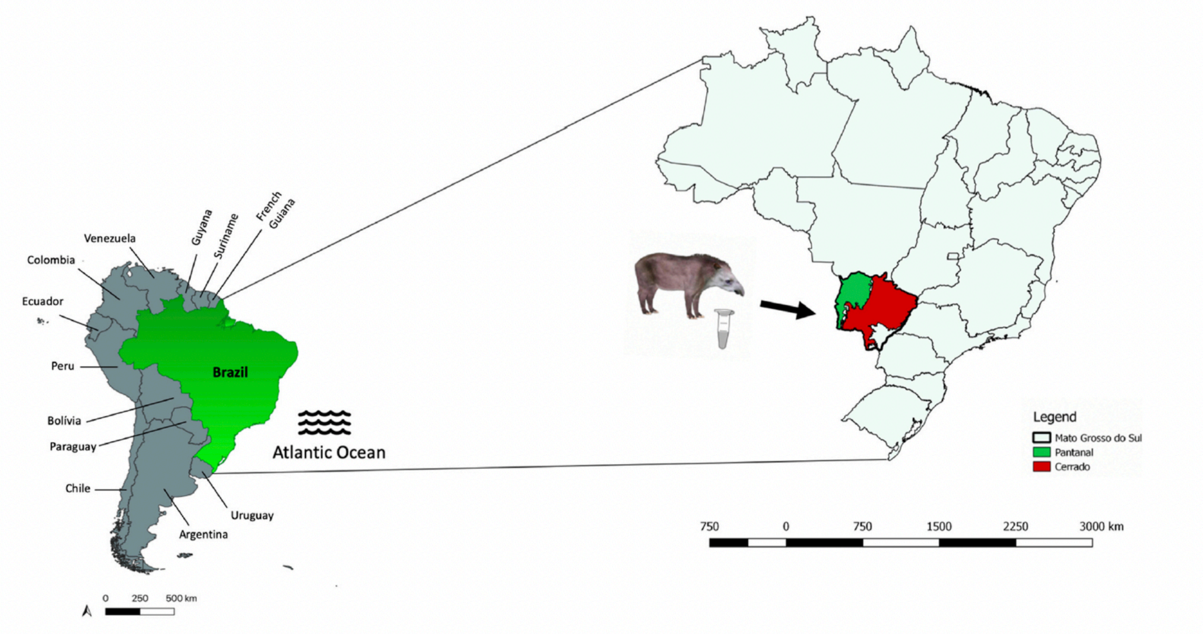

Fig. 1. Representative image of Brazil (highlighted in green within South America) and the location of Cerrado and Pantanal biomes within Mato Grosso do Sul State, Brazil, where *Tapirus terrestris* were sampled.

2.3. Sequencing and phylogenetic inference

Amplicons obtained in the cPCR assays were purified using a com- mercial kit (Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, WI, USA) and sequenced in both directions (forward and reverse) using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific™, Waltham, MA, USA) and ABI PRISM 3730 DNA Analyzer (Applied Biosystems™, Foster City, CA, USA) [35], at the "Centro de Estudos do Genoma Humano e Células-Tronco" (Instituto de Biociências, Universidade de São Paulo/USP, São Paulo, SP, Brazil).

A Bayesian Inference phylogenetic tree was constructed using the sequences obtained in the present study and homologous sequences retrieved from GenBank database (http://www.ncbi.nlm.nih.gov/ genbank). Sequences were first submitted to a screening test using Geneious 11.1.3 software to evaluate the electropherogram quality and generate consensus sequences. The BLASTn online program (National Center for Biotechnology Information, Bethesda, MD, USA) [36] was used to analyze the nucleotide sequences aiming to compare with se- quences deposited in GenBank. Consensus sequences obtained in the current study and those retrieved from GenBank were aligned using a MAFFT online software (https://mafft.cbrc.jp/alignment/server/). Phylogenetic inferences were based on Bayesian analysis via CIPRES online server (https://www.phylo.org/index.php/) [37]. The best-fit model was determined using jModeltest v2.1.6 via CIPRES online server (https://www.phylo.org/index.php) [38].

2.4. Statistical analysis

The Fisher-exact test was used to determine associations between variables (sex, biome and age) and the outcomes (positive or negative results on qPCR for *Bartonella* spp.). Odds ratio (OR), 95% confidence interval and *p* values were calculated for each variable. Results consid- ered significantly different when $p < 0.05$. Data were compiled and analyzed in Epi Info™ software (version 7.1.5, CDC).

3. Results

3.1. Molecular occurrence and characterization

All DNA blood samples successfully amplified at least one of the tested endogenous genes and were considered suitable for the further analysis. Eight (8/99; 08,08%) animals were positive in the qPCR for *Bartonella* spp.: 7 from Cerrado (7/8;

87.5%) and 1 from Pantanal region (1/8; 12.5%). The number of copies of a fragment of the *Bartonella nuoG* gene per microliter (duplicates showing $36\ 10^0$ and $36.4\ 10^0$) was possible to be estimated in only one sample. The Efficiency, R^2, Y-intercept and Slope values of the qPCR assays ranged from 91.3% to 103.4%, 0.996–0.998, 37.717–38.859 and - 3.244 to -3.550, respectively.

Regarding the conventional molecular assays for molecular charac- terization, 5 *ribC* sequences were obtained from tapirs sampled in Cerrado (Table 2). None of the other tested fragments were successfully amplified or sequenced. The *ribC* sequences obtained herein ranged from 307 to 318 bp and, despite the differences in length, were identical among each other. Results obtained in BLASTn analyses are described in Table 3.

3.2. Phylogenetic analysis

A phylogenetic tree was constructed based on a 314 bp alignment of the *ribC* gene (Fig. 2). A total of 54 sequences were used in the phylo- genetic reconstruction, including the 5 sequences obtained herein, 48 homologous sequences from GenBank database and one outgroup (*Brucellla melitensis*) (CP008750). The best-fit model chose was TPM1uf I G. The sequences obtained from tapirs' blood samples clustered together with *B. henselae* obtained from cats (KP822815, MW575369), humans (OM398559, AJ132928), wild felids (MT028357) and *C. felis* fleas (MZ395940) from different parts of the world.

Table 1. Description of each cPCR protocol used for molecular characterization of the samples positive in the qPCR for *Bartonella* spp. in the present study, with target gene, primers identification, primers sequence, fragment size, thermal conditions and references.

Molecular marker	Primer sequence		Thermal conditions	References
ribC	5'- TAACCGATATTGGTTGTGTTGAAG- '3 5'-TAAAGCTAGAAAGTCTGGCAACATAACG- '3	420	95°C for 10 min; 37 cycles: 95°C for 1min, 51°C for 1min and 72°C for 1min;	Johnson et al., 2003
gltA	5'- GGGACCAGCTCATGGTGGC- 3' 5'- AATGCAAAAAGAACAGTAAACA-3	750	95°C for 10 min; 35 cycles: 95°C for 20s, 51°C for 30s and 72°C for 2min; 72°C for 3 min	Norman et al., 1995

rpoB	5′-GCACGATTYG CATCATCATTTTCC-3′ 5′-CGCATT ATGGTCGTATTTGTCC-3′	900	94°C for 2 min; 40 cycles: 94°C for 45s, 52°C for 45s and 72°C for 45s; 72°C for 7 min	Paziewska et al., 2011
groEL	5′-GGAAAAAGTGGGCAATGAAG-3′ 5′-TCCTTTAACGGTCAACGCATT-3′	752	94°C for 2 min; 40 cycles: 94°C for 45s, 47°C for 45s and 72°C for 45s; 72°C for 7 min	Zeaiter et al., 2002; Paziewska et al., 2011
ITS 16S-23S rRNA	5'- CTTCAGATGATGATCCCAAGCCTTTTGGCG -3' 5'-GAACCGACGACCCCCTGCTTGCAAAGCA -3'	453-717	95°C for 2 min; 55 cycles: 94°C for 15s, 66°C for 15s and 72°C for 18s; 72°C for 1 min	Diniz et al., 2007; Maggi et al., 2008.

Table 2. Results for the detection of *Bartonella* spp. in blood DNA samples from *Tapirus terrestris*.

Animal ID	**Sex**	**Age**	**Biome**	**UTM Coordinate (X/Y – Z 22)**	**Municipality**	**Coordinates**	**qPCR *nuoG***	**qPCR Quantification**	**cPCR *ribC***
EIS-C	Female	Adult	Cerrado	200457/ 7606630	Nova Alvorada do Sul	***-53.8933672416*** ***-21.618250660786***	+	NQ	+
JHA-C	Female	Sub-adult	Cerrado	218819/ 7605201	Nova Andradina	***-53.716401401829*** ***-21.634138051133***	+	NQ	negative
SRE-C	Female	Adult	Cerrado	218819/ 7605201	Nova Andradina	***-53.716401401829*** ***-21.634138051133***	+	NQ	+
MRI-C	Female	Sub-adult	Cerrado	219089/ 7605787	Nova Andradina	***-53.71369641025*** ***-21.628892147049***	+	36×10^0 36.4×10^0	+
CNA-C	Female	Adult	Cerrado	202153/ 7611502	Nova Alvorada do Sul	***-53.8761314614*** ***-21.574572159156***	+	NQ	+
DDA-C	Female	Adult	Cerrado	239207/ 7598977	Nova Andradina	***-53.520566388891*** ***-21.693418862707***	+	NQ	+
LAS-C	Male	Sub-adult	Cerrado	234584/ 7601529	Nova Andradina	***-53.564806065231*** ***-21.669700064606***	+	NQ	negative
MDO-P	Male	Sub-adult	Pantanal	623867/ 7865799	Aquidauana	***-55.821072089916*** ***-19.297741709946***	+	NQ	negative

+ = positive sample; NQ = not possible to quantify the number of generated copies due to Monte Carlo effect.

Table 3 Results of the BLASTn analyses for the *Bartonella ribC* sequences obtained in the present study.

Sample ID	Gene	Fragment size (bp)	Accession number	BLASTn Best Match	Query cover	% identity	E-value
Tapir EIS-C	*ribC*	307	OR052091	*Bartonella henselae* – USA (CP020742)	100%	100%	3e-157
Tapir SRE-C	*ribC*	316	OR052094	*Bartonella henselae* – USA (CP020742)	100%	100%	3e-162
Tapir MRI-C	*ribC*	318	OR052095	*Bartonella henselae* – USA (CP020742)	100%	100%	3e-163
Tapir DDA-C	*ribC*	308	OR052093	*Bartonella henselae* – USA (CP020742)	100%	100%	9e-158
Tapir CNA-C-1	*ribC*	318	OR052092	*Bartonella henselae* – USA (CP020742)	100%	100%	3e-163

3.3. Statistical analysis

Results from statistical analysis including OR (*odds ratio*) and *p*-value were summarized on Table 4. The number of tapirs positive for *Barto- nella* spp. sampled in Cerrado biome was statistically higher when compared to those sampled in Pantanal (*p*-value 0.009565). Other variables (sex and age) did not present statistically significant differences for this outcome (*p*-value > 0,05). The OR value demonstrated that tapirs from Cerrado were 13.55 times more likely to present positive results in the qPCR protocol targeting a fragment of 83 bp fragment of the *nuoG* gene of *Bartonella* sp. when compared to tapirs sampled in Pantanal.

4. Discussion

Different species and genotypes of *Bartonella* have already been re- ported in wild animals from Brazil, mainly in bats [39–43] and rodents [14,43–45]. The present study reports, for the first time, the detection of *Bartonella* sp. in tapirs (*Tapirus terrestris*) and contributes to the knowl- edge regarding the occurrence of *Bartonella* genotypes in the wild fauna from Brazil.

The diagnosis and identification of *Bartonella* spp. is challenging [46]. An increased sensitivity on the detection of *Bartonella* sp. in do- mestic and wild animal blood samples by qPCR assays has been achieved by subjecting blood samples to enrichment liquid culture (BAPGM - *Bartonella* Alpha Proteobacteria Growth Medium) [15,47–49] for 7 days prior to DNA extraction. Studies that successfully cultivated *Bartonella* sp. from animal blood samples reported the use of fresh or frozen blood samples (maintained at least at 70^{O}C or liquid nitrogen) as the sort of samples for liquid culture [15,49,50]. However, such approach could not be performed in the present study. Therefore, the positivity for *Bartonella* spp. found herein might be underestimated.

Nevertheless, the use of molecular assays is still an import tool for the screening for *Bartonella* spp. in biological samples collected in the past and properly stored, especially those from wildlife, to shed some light on and monitor potential pathogens that these animals may carry [51,52]. Tapirs sampled in the present study were already reported as hosts for two different *Candidatus* species of hemotropic *Mycoplasma* [24] and a novel *Theileria* (*Theileria terrestris*; Piroplasmida: Theileriidae) [53]. In fact, one of the positive animals for *Theileria terrestris* [53] was also positive for *Bartonella* sp. (id: JHA-C), showing that coinfections by multiple vector-borne agents occur in wild tapirs.

Despite the efforts in performing a multi-locus sequencing approach aiming at achieving a more robust molecular characterization of the positive samples, only the riboflavin synthesis gene (*ribC*) was success- fully amplified and sequenced from five samples previously positive in the qPCR assay. The low levels of bacteremia in the samples tested herein, which hampered the estimation of quantification in 4/5 positive samples due the variation in amplification caused by the presence of low number of DNA copies (Monte Carlo effect) [54], might be a reason for the lack of amplification in the conventional PCR assays. The *ribC* gene is considered highly conserved among the available Bartonellae genomes and a useful amplification target since it is not present in the genome of vertebrates [29,55]. The *ribC*-based PCR protocol used herein is considered sensitive and specific for *Bartonella*, being capable of detecting at least 50 genome copies [29]. Additionally, *ribC* has been considered a highly discriminative molecular marker for *Bartonella* speciation [56].

The Pantanal wetland has been pointed out as an important scenario for the detection of different *Bartonella* species/genotypes in wild animals in Brazil. For instance, *B. machadoae* and *Bartonella harrusi* were first described in rodents and marsupials, respectively, in this biome [15, 48]. Nonetheless, the majority of positive samples in the present study were obtained from tapirs sampled in Cerrado biome (87.5%). The statistical analysis performed also demonstrated an increased risk of tapirs from Cerrado presenting positive samples for *Bartonella* by the qPCR protocol used herein. This finding diverges from previous results related to piroplasmid and hemoplasma infections obtained from the same tapir population, which demonstrated that rates of infection were higher in animals sampled in Pantanal [24,53].

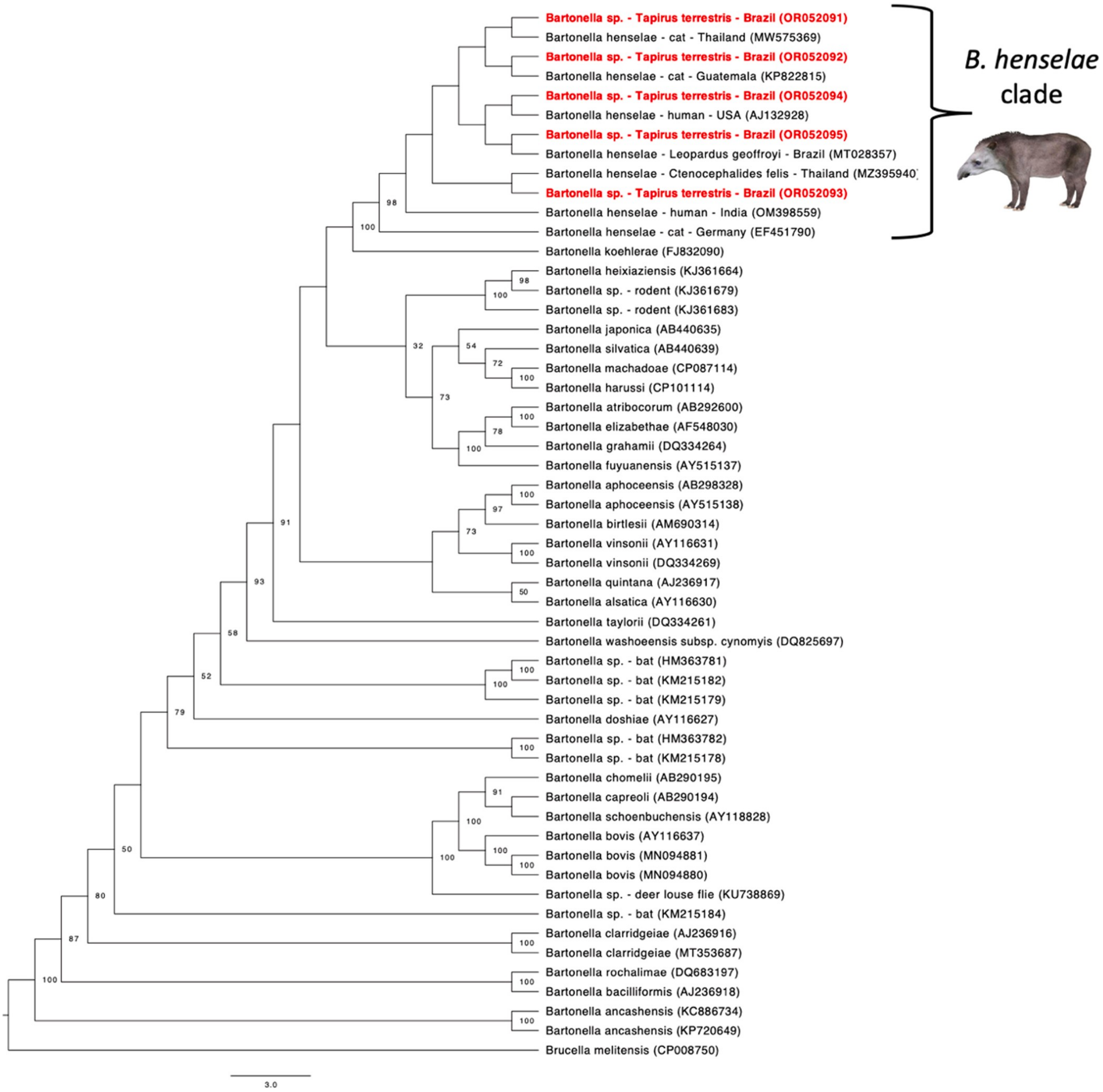

Fig. 2. Phylogenetic tree constructed based on a 314 bp alignment of the *Bartonella* spp. *ribC* gene. Only post probability values of > 50 were showed in the tree. A total of 54 sequences were used in the phylogenetic reconstruction and *Brucella melitensis* (CP008750) was used as outgroup. The best-fit model encountered was TPM1uf+I+G. Sequences from the present study are highlighted in red.

Table 4. Fisher-exact test was used to determine associations between variables (sex, biome and age) and the outcomes (positive or negative results on qPCR).

		qPCR for *Bartonella* sp.				
Variable		**+/n**	**(%)**	**OR**	**95% CI**	***P*-value**
Sex	Male	02/50	4	0.2986	0.05722 - 1.558	0.2556
	Female	06/49	12.24			
Biome	Cerrado	07/38	18.42	13.55	1.595 - 115.1	**0.009565**
	Pantanal	01/61	01.639			
Age	Adult	04/54	07.407	0.82	0.1931 - 3.482	0.9999999
	Sub-adult	04/45	08.889			

+, Number of positive animals; n, number of samples; 95% CI, 95% confidence interval.
P-values <0.05 were considered statically significant.

In the phylogenetic analysis based on the *ribC* gene, tapir-associated *Bartonella* grouped together with *B. henselae* sequences detected in different hosts. Although *B. henselae* has cats at its main hosts, there are reports of *B. henselae* in different animal species, such as horses [57], dogs [58–61], dromedary camels [62], mongooses [63,64], aquatic mammals [33,65] and birds [66]. Among the zoonotic species of *Bar- tonella*, *B. henselae* is the most reported infecting humans [67]. Once wild mammals have already been reported as reservoirs of zoonotic species of *Bartonella* [68,69], the use of molecular diagnostic tools may enhance the detection of different *Bartonella* genotypes in different host species, helping to understand and predict the emergence of zoonotic Bartonellae [67,70].

Although *Bartonella* species apparently present a host-specificity to certain mammalians hosts, *B. henselae* have been detected in a diverse range of hosts [70,71]. The blood-pathogens (piroplasmids and hemo- plasmas) previously detected in higher rates in tapir population from Pantanal [24,53] seemed to be, until now, tapir-related species and does not present, apparently, a wide range of vertebrate hosts, such as *B. henselae*. The increased contact between domestic and wild animals may facilitate the exchange of pathogens that were previously consid- ered to be host-specific [72]. In fact, tapirs from Cerrado have already been described as more exposed to environmental disturbances when compared to tapirs from Pantanal [73], which holds a tapir population reported as large and healthy [74]. Nevertheless, it is important to emphasize that the molecular prevalence reported herein might be underestimated due to the fact that samples were not pre-enrichened in BAPGM medium before qPCR analysis [49].

Although direct transmission of some *Bartonella* species between vertebrate hosts is suspected to occur through bites and/or scratches [75,76], and *B. henselae* has been already reported presenting relapsing patterns of bacteremia [46], the estimate bacteremia detected in tapirs herein appears to be low and an arthropod vector would likely be involved in such a transmission. Since *C. felis* fleas have not been re- ported parasitizing tapirs so far, the vectors involved in the transmission of *Bartonella* spp. to tapirs remains elusive. Even though the trans- mission of *B. henselae* by *I. ricinus* and *R. sanguineus* has been demon- strated experimentally [7–9], the real role of ticks in the transmission of Bartonellae deserves further investigation. *Rhipicephalus (Boophilus) microplus*, *Amblyomma coelebs*, *Amblyomma dubitatum*, *Amblyomma sculptum*, and *Amblyomma triste* (Ixodida: Ixodidae) ticks were reported infesting wild tapirs from Cerrado. Meanwhile, tapirs from Pantanal were found to be infested by *R. microplus*, *Amblyomma ovale*, *A. parvum*, and *A. sculptum* [77]. Interesting, *B. bacilliformis*, a specific human pathogen, was detected in *Amblyomma scalpturatum* (Ixodida: Ixodidae) and *A. ovale* ticks collected from tapirs in Peru [23]. More studies are necessary to understand the arthropod vector species involved in the transmission of *Bartonella* sp. to tapirs from Brazil.

5. Conclusion

To the best of author's knowledge, this is the first report of *B. henselae* in *Tapirus terrestris*. This finding contributes to the understanding of the occurrence of *Bartonella henselae* in wild mammals from Brazil as well as expands the knowledge regarding the potential vector-borne pathogens that may affect wild tapis from Cerrado and Pantanal biomes.

Ethics statement

The study was approved by the Ethics Committee for Animal Experimentation of FCAV/UNESP (Faculty of Agricultural and Veteri- nary Sciences of the Sao Paulo State University) under protocol number 4558/20. The “Instituto Chico Mendes de Conservaç ao da Biodiversidade (ICMBIO)” provided the required annual permits for the capture and immobilization of tapirs and collection of biological sam- ples (SISBIO# 14,603). All protocols for the capture, anesthesia, handling, and sampling of tapirs have been reviewed and approved by the Veterinary Advisors of the Association of Zoos and Aquariums (AZA) — Tapir Taxon Advisory Group (TAG), and the Veterinary Committee of the IUCN SSC Tapir Specialist Group (TSG).

Financing

This work was supported by FAPESP (Fundação de Amparo à Pes- quisa do Estado da São Paulo - Process #2022/08543–2) and CNPq (National Council for Scientific and Technological Development; PrOductivity Grant to MRA [CNPq Process #303701/2021–8]). This study is part of Anna Mongruel’s thesis. She is carrying out her research at Programa de Pós-Graduação em Medicina Veterinária—Universidade Estadual Júlio de Mesquita Filho (UNESP/Jaboticabal) and is sponsored by a fellowship from Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP–Process 2019/26403–0) at the time of this study. LTCI’s financial support comes from national and international agencies, including zoological institutions, foundations, private businesses, and private individuals.

Data linking

The datasets generated and analyzed during the current study are available in the NCBI GenBank Nucleotide platform (https://www.ncbi. nlm.nih.gov/genbank/) and can be accessed through accession numbers: OR052091, OR052092, OR052093, OR052094, OR052095.

Author contributions

Anna Claudia Baumel Mongruel: Conceptualization, Methodology, Visualization, Formal analysis, Writing – original draft, Writing – review & editing. Emília Patricia Medici: Methodology, Conceptualization, Visualization, Funding acquisition.

Ariel da Costa Canena: Methodol- ogy. Clara Morato Dias: Methodology, Formal analysis, Writing – original draft, Writing – review & editing. Rosangela Zacarias Machado: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. Marcos Rogério André: Conceptualization, Visualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study is part of Anna Mongruel's thesis. She is carrying out her research at Programa de Pós-Graduação em Medicina Veterinária - Universidade Estadual Júlio de Mesquita Filho (UNESP/Jaboticabal). She is sponsored by a fellowship from Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP – Processo número 2019/26403-0) at the time of this study. Authors would like to thank the Lowland Tapir Conservation Initiative (LTCI)–Instituto de Pesquisas Ecológicas (IPÊ) for kindly providing the biological samples needed for the present study. The study of tapir health has been an important component of the long term activities of the LTCI-IPÊ in Brazil. The LTCI has the institutional support from the International Union for Conservation of Nature (IUCN) Species Survival Commission (SSC) Tapir Specialist Group (TSG), Association of Zoos and Aquariums (AZA) Tapir Taxon Advisory Group (TAG), and European Association of Zoos and Aquariums (EAZA) Tapir Taxon Advisory Group (TAG).

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CAPÍTULO VI* – Molecular survey of vector-borne agents (Anaplasmataceae agents, *Hepatozoon* sp. and *Coxiella burnetii*) in lowland tapirs (*Tapirus terrestris*) from Brazil, with evidence of a putative novel Anaplasma genotype*

*Esse capítulo corresponde à publicação submetida ao periódico Scientific Reports

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Abstract: Although vector-borne agents have been detected in different species of wild animals, studies involving tapirs (*Tapirus terrestris),* the largest land mammals in Brazil, are scarce. The aim of the present study was to investigate the occurrence and molecular identity of Anaplasmataceae agents, *Coxiella burnetii* and *Hepatozoon* spp. in blood samples of wild *T. terrestris* from two different biomes (Cerrado and Pantanal) in Brazil. A total of 122 blood samples from 99 tapirs were analyzed. Sixty-one tapirs were sampled in Pantanal, whereas 38 in Cerrado biome. DNA was extracted from blood samples and subject to conventional and/or quantitative PCR assays for molecular screening and characterization of Anaplasmataceae (*Anaplasma*, *Ehrlichia*, and *Neorickettsia*), *C. burnetii* and *Hepatozoon* sp. None of the samples were positive for *Ehrlichia*, *C. burnetii* or *Hepatozoon* sp. Twenty-two samples (22/122; 18%)

amplified fragments from expected size for the *Anaplasma* 16S rRNA fragment tested herein. Out of these samples, 2 (9.1%) presented amplification for the *Anaplasma* ITS 23S-5S. Nine positive samples for the 16S rRNA assay were chosen for cloning and sequencing. Phylogenetically, distance and genotype analyses based on large fragments (>1,200 bp) of the 16S rRNA indicate that tapir-related *Anaplasma* and *Anaplasma odocoilei* are genetically similar but distinct species. Moreover, 31 (25.4%) samples were positive for *Neorickettsia* based on amplification of partial 16S rRNA. Phylogenetic assessment of three obtained sequences demonstrated relatedness with *Neorickettsia risticii*, the causative of Potomac fever in horses. This is the first report of *Neorickettsia* sp. and a putative novel genotype of *Anaplasma* sp. in tapirs.

Key-words: *Anaplasma, Neorickettsia*, wild animals, South America.

1. Introduction

Improvement in molecular methods, particularly those associated to 16S rRNA gene analysis, allowed the reclassification of specific bacterial species within the Anaplasmataceae family. Currently, this family comprises the genera *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia* [1]. Bacteria from *Anaplasma* and *Ehrlichia* genera are well described agents capable of infecting a wide range of mammalian and avian hosts. While *Anaplasma* species are reported to infect erythrocytes, platelets and leukocytes [2], *Ehrlichia* sp. endures on leukocytes [3]. Biological transmission by different Ixodidae tick species is the mainly route of transmission for these two genera [3].

Representatives of *Neorickettsia* genus are also related to animal and human infections. The species *Neorickettsia risticii* and *Neorickettsia findlayensis* are the causative of the Potomac horse fever [4,5], while the species *Neoricketssia sennetsu* is associated with the onset of human Sennetsu neoricketssiosis [6]. Moreover, *Neorickettsia helminthoeca* has been linked to salmon poisoning in dogs [7,8]. Differently from the genera described above, *Neorickettsia* transmission is mediated by trematode-borne interactions [1].

Another bacterium related to animal and human disease is *Coxiella burnetii*, a member from Coxiellaceae family and the causative of the worldwide reported zoonosis Q-fever [9]. A broad range of domestic animals are reported as hosts and potential infection sources for human beings, with ruminants playing a major role in the dispersion of the agent [10]. Transmission occurs mostly by inhalation or skin contact [9]. Although wild animals may not represent a significative source of transmission for humans, it is believed that wildlife has an important role in maintaining the pathogen in the environment, especially with the involvement of tick-related transmission [11,10].

Switching to the protozoan world, the genus *Hepatozoon* comprises apicomplexan hemoparasites that has vertebrates (mammals, amphibians, reptiles and birds) and invertebrates (arthropods) as intermediate and definitive hosts, respectively. Vertebrate hosts are infected through the ingestion of sporulated oocysts containing-infected invertebrates [12]. Hepatozoonosis is well described in dogs and caused by species *Hepatozoon canis*, that displays a mild disease as the most common outcome, and *Hepatozoon americanum*, an often-fatal infection [13].

Recently, different species of wild animals have been reported as hosts of vector-borne agents in Brazil. A notable number of *Anaplasma* and *Ehrlichia* genotypes/species have been detected in different host groups, such as birds [14,15,16,17), deer [18,19,20], wild canids [21,22,23], wild felids [21,22,24], coatis [22,25], rodents [26,27], wild boars [28], and xenarthrans [29]. Regarding *Neorickettsia* sp., the detection range of these agents in Brazilian wildlife is mostly concerned to bats [30,31] and coatis [32].

Coxiella burnetii has been molecularly detected only in rodents [33] and bats [34] in Brazil. However, serological evidence of exposure has already been reported in deer [35], one rodent (*Wiedomys pyrrhorhinos*) and one marsupial (*Didelphis albiventris*) [36]. On the other hand, *Hepatozoon* sp. has been described in wild canids [37,38], wild felids [37], rodents [39,40], bats [41], coatis [42], opossums [43,44], amphibians [45], and reptiles [46,47] in Brazil.

The lowland tapir (*Tapirus terrestris*) is considered the largest land mammal from Brazil, being found in different biomes in the country [48]. This mammal species has been recently reported as a host for different vector-borne agents, such as *Trypanosoma* sp. (*Trypanosoma terrestris*) [49], hemoplasmas (‘*Candidatus* Mycoplasma haematoterrestris’ and ‘*Candidatus* Mycoplasma haematotapirus’) [50], *Theileria* sp. (*Theileria terrestris*) [51], and *Bartonella* sp. [52]. The present study aimed to investigate the occurrence and molecular identity of Anaplasmataceae agents, *C. burnetii* and *Hepatozoon* spp. in blood samples from wild *T. terrestris* sampled in two different biomes in Brazil, namely Cerrado and Pantanal.

2. Material and Methods

2.1. Ethical aspects

The present study was approved by the Ethics Committee for Animal Experimentation of FCAV/UNESP (Faculty of Agricultural and Veterinary Sciences of the São Paulo State University) under protocol number 4558/20. The “Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO)” provided the required annual permits for the capture and immobilization of tapirs and collection of biological samples (SISBIO# 14,603). Followed protocols for capture, anesthesia, handling, and sampling of tapirs have been reviewed and approved by the Veterinary Advisors of the Association of Zoos and Aquariums (AZA)—Tapir Taxon Advisory Group (TAG), and the Veterinary Committee of the IUCN SSC Tapir Specialist Group (TSG). Tapir blood DNA samples from the present study were registered in the Brazilian National System for Management of Genetic Heritage and Associated Traditional Knowledge (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado – SISGEN) under register number AE4CC0C. Methods reported in the present study are in accordance with ARRIVE guidelines (http://aariveguidelines.org).

2.2. Sampling

Blood sampling of living (n = 94) and road-killed (n = 5) tapirs were performed for health assessment purposes. Considering all samples obtained and individuals sampled, a total of 122 blood samples from 99 tapirs were analyzed. From these animals, 61 (61/99; 61.6%) were sampled in Pantanal biome, whereas 38 (38/99; 38.4%) were sampled in Cerrado biome. Sampling of living animals was conducted during tapir anesthesia for GPS collars installation by professionals from the “Iniciativa Nacional para a Conservação da Anta Brasileira (INCAB-IPÊ)” (Lowland Tapir Conservation Initiative (LTCI-IPÊ). Additional information about study areas and sampling procedures were previously described [50].

2.3. DNA extraction and PCR protocols for mammals’ endogenous genes

DNA extraction was performed using a commercial kit (InstaGene™ Matrix, Biorad®, Hercules, CA, USA) and followed the manufacturers’ instructions. Aiming to ensure success in DNA extraction, a conventional PCR (cPCR) for the mammal-endogenous gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) (450 bp) was performed as previously described [53]. If any sample showed negative results in the abovementioned PCR protocol, it was subjected to an additional PCR protocol targeting a 227 bp fragment of the *irpb* (interphotoreceptor retinoid-binding protein) gene [54]. Samples that did yield amplicons in neither of the used PCR protocols were excluded from the subsequent analysis.

2.4. PCR assays for detection and characterization of Anaplasmataceae agents

The screening for the presence of *Anaplasma* DNA was performed using a cPCR assay targeting a 1,400 bp fragment of the 16S rRNA gene [55]. Additionally, DNA blood samples were subjected to a cPCR for *Ehrlichia* spp. targeting a 400 bp fragment of the *dsb* gene [56]. Positive samples for *Anaplasma* sp. were subjected to a quantitative real-time(q) PCR assay targeting the *msp-2* gene from *Anaplasma phagocytophilum* [57] and cPCR protocols targeting the *gltA* [58]*, msp5* [59,60]*, msp4* [61,62] genes, *groESL* operon [63], and 23S-5S intergenic region (ITS) [64] **(Table 1)**. *Ehrlichia canis* and *Anaplasma platys* [65] and ultra-purified water were used as positive and negative controls, respectively, in the cPCR assays. Serial dilutions of plasmids (pIDTSmart, IDT) encoding a fragment (122 pb) of the *msp-2* gene of *A. phagocytophilum* were used as positive controls in the qPCR protocol.

2.5. PCR assays for detection of Neorickettsia sp.

A nested (n)PCR protocol was performed to amplify a fragment of approximately 527 bp from the 16S rRNA [66]. Samples that yielded positive results in the 16S rRNA-based assay were subjected to addittional molecular assays targeting an approximately 569 bp from the 51-kDA (*p51*) gene and approximately 823 bp from the *groESL* operon [67] (**Table 1**). A DNA sample of *N. risticii* (kindly provided by Professor John Stephen Dumler, Uniformed Services University of the Health Sciences, Bethesda, MD, USA) was used as a positive control and distilled autoclaved ultrapure water (Nuclease-Free Water, Promega®, Madison, Wisconsin, United States) was used as a negative control in all reactions.

2.6. qPCR assay for detection of Coxiella burnetii

A qPCR assay was performed to assess the presence of *C. burnetii* DNA in blood samples from tapirs, based on the IS1111 gene element (approximately 295 bp) [68]. The qPCR reaction had a final volume of 10 μL, consisting of a mixture of 1 μL of DNA sample, 0.2 µM of each primer and hydrolysis probe, PCR buffer (PCRBIO Ultra Mix, PCR Biosystem, London, United Kingdom), and nuclease-free sterile water (Nuclease-Free Water, Promega®, Madison, Wisconsin, United States) q.s.p. 10 µL. Amplification reactions were carried out in a CFX96 Thermal Cycler (BioRad®, Hercules, California, United States). Protocol, hydrolysis probe and primers used are describe in **Table 1**. All samples were tested in duplicates. A sample know to be positive for *C. burnetii* (kindly provided by Professor Dr. Renato Arruda Mortara, Federal University of São Paulo, SP, Brazil) was used as a positive control, and distilled autoclaved ultrapure water (Nuclease-Free Water, Promega®, Madison, Wisconsin, United States) was used as a negative control.

Quantification of the target DNA copies/µL was performed using plasmids (IDT psmart, Integrated DNA Technologies®) containing the target sequence. Serial dilutions were made to create standards with different concentrations of plasmid DNA containing the target sequence (2.0 x 10^7 copies/µL to 2.0 x 10^2 copies/µL), in order to determine efficiency and correlation coefficient values of the reactions. The number of plasmid copies was determined according to the formula (Xg/µL DNA/ [plasmid size (bp) x 660]) x 6.022 x 10^23 x copies of the plasmid/µL. All analyses were conducted following the guidelines established by MIQE ("Minimum Information for Publication of Quantitative real-time PCR Experiments") [69].

2.7. Conventional PCR assay for Hepatozoon sp.

A nested PCR (nPCR) protocol was performed to amplify a fragment of approximately 1,120 base pairs from the 18S rRNA [70,71]. Primers and thermal conditions are described in **Table 1**. *Hepatozoon procyonis* DNA from naturally infected coatis [42] was used as a positive control, and distilled autoclaved ultrapure water (Nuclease-Free Water, Promega®, Madison, Wisconsin, United States) was used as a negative control.

Table 1. Description of each quantitative PCR (qPCR), nested (n), semi-nested (sn), and conventional (c) PCR protocols used in the present study with target gene, primers identification, primers sequence, fragment size, thermal conditions and references.

Assay: Agent (purpose)	Target gene/primers	Primer sequence	Size (bp)	Thermal conditions	Reference

qPCR: ***A. phagocytophilum*** **(characterization)**	*msp2* -903f -1024r	5′-AGTTTGACTGGAACACACCTGATC -3’ 5′-CTCGTAACCAATCTCAAGCTCAAC -3’ Hydrolysis probe (Taqman): 5′[FAM] (939p-TTAAGGACA ACTGCTTGTAGCTATGGAAG-GCA-[TAMRA]) 3'	122	50°C for 2 min, 95°C for 10 min, 40 cycles: 95°C for 15s and 60°C for 1 min	[57]
qPCR: ***Coxiella burnetii*** **(screening)**	*IS1111* -coxF -coxR	5’-CAAGAAACGTATCGCTGTGGC–3’ 5’–CACAGAGCCACCGTATGAATC–3’ Hydrolysis probe (Taqman): 5'FAM (259-287 CCGAGTTCGAAACAATGAGGGCTG-[TAMRA]) 3'.	295	50°C for 2 min, 95°C for 10 min, 45 cycles: 95°C for 15s and 58°C for 30s	[68]
cPCR: ***Anaplasma* spp.** **(screening)**	16S rRNA -AE1_F -AE1_R	5’-AAGCTTAACACATGCAAGTCGAA-3’ 5’-AGTCACTGACCCAACCTTAAATG-3’	1,400	94°C for 3 min; 35 cycles: 94°C por 30s, 59°C por 30s and 72°C por 30s; 72°C for 5 min	[55]
cPCR: ***Ehrlichia* spp.** **(screening)**	*dsb* -dsb-330 -dsb-728	5’-GATGATGTCTGAAGATATGAAACAAAT-3’ 5’-CTGCTCGTCTATTTTACTTCTTAAAGT-3’	409	95°C for 2 min; 50 cycles: 95°C for 30s, 55°C for 30s and 72°C for 1min; 72°C for 5 min	[56]
cPCR: ***Anaplasma* spp.** **(characterization)**	ITS—23S–5S -ITS2F -ITS2R	5′-AGGATCTGACTCTAGTAACGAG-3′ 5′-CTCCCATGTCTTAAGACAAAG-3′	300	94°C for 2 min, 35 cycles; 94°C for 30 s, 58 °C for 30 s, 72 °C for 1 min; 72 °C for 5 min	[64]
nPCR: **Anaplasmataceae** **(characterization)**	*gltA* -F4b -R1b -EHR-CS136F -EHR-CS778R	5’-CCAGGCTTTATGTCAACTGC-3’ 5’-CGATGACCAAAACCCAT-3’ 5’-TTYATGTCYACTGCTGCKTG-3’ 5’-GCNCCMCCATGMGCTGG-3’	561	95°C for 5 min; 40 cycles: 95°C for 30s, 55°C for 30s and 72°C for 1min; 72°C for 5 min 2º Round: same to the 1º but with 34 cycles.	[58]
nPCR: **Anaplasmataceae** **(characterization)**	*groESL* *-HS1a* *-HS6a* *-HS43* *-HSVR*	5’- AITGGGCTGGTAITGAAAT -3’ 5’- CCICCIGGIACIAIACCTTC -3’ 5’-AT(A/T)GC(A/T)AA(G/A)GAAGCATAGTC -3’ 5’- CTCAACAGCAGCTCTAGTAGC -3’	1,297	94°C for 1 min; 48°C for 2 min, 68°C for 1.5min; 37 cycles: 88°C for 1 min, 48°C for 2 min and 68°C for 1.5 min; 68°C for 5 min 2º Round: same to the 1º but using 55°C for annealing and 72°C for extension temperatures.	[63]
nPCR: ***Anaplasma* spp.** **(characterization)**	*msp5* -AMARMSP5 eF -AMARMSP5 eR -AMARMSP5 iF	5’- GCATAGCCTCCGCGTCTTTC -3’ 5’- TCCTCGCCTTGGCCCTCAGA -3’ 5’- TACACGTGCCCTACCGAGTTA -3’	458	94°C for 5 min; 34 cycles: 94°C for 1 min, 58°C for 1 min and 72°C for 1min; 72°C for 10 min 2º Round: same to the 1º	[59,60]

nPCR: ***Anaplasma* spp.** **(characterization)**	*msp4* -MSP4AP5 -MSP4AP3 -msp4f -msp4r	5’-ATGAATTACAGAGAATTGCTTGTAGG -3’ 5’-C TTAATTGAAAGCAAATCTTGCTCCTATG -3’ 5’-CTATTGGYGGNGCYAGAGT -3’ 5’-GTTCATCGAAAATTCCGTGGTA -3’	381	94°C for 3 min; 35 cycles: 94°C for 30s, 68°C for 1 min and 72°C for 1min; 72°C for 5 min 2º Round: 94°C for 5 min; 40 cycles: 94°C for 30s, 58°C for 30s and 72°C for 1min; 72°C for 5 min	[61,62]
nPCR: ***Neorickettsia* sp.** **(screening)**	16S rRNA -ER3-F -ER2-R -ER 3a-F -ER2a-R	5’-ATTTGAGAGTTTGATCCTGG -3’ 5’- GTTTTAAATGCAGTTCTTGG -3’5’-CTAGCGGTAGGCTTAAC-3’5’-CACACCTAACTTACGGG -3’	527	94°C for 5 min; 30 cycles: 94°C for 1min, 60°C for 2 min and 72°C for 1min30sec; 72°C for 7 min. 2º Round: same to the 1º	[66]
nPCR: ***Neorickettsia* sp.** **(characterization)**	*p51* -outerF -outerR -innerF -innerR	5'-GGATCGATAACTGCGATGCT-3' 5'-ACCGGCCTGACCACTAAAG-3' 5'-TCCTATAATGGCACCACTAGCG-3' 5'-CCATCCGCAGTAGAGTTTGAG-3'	569	94°C for 5 min; 30 cycles: 94°C for 1min, 45°C for 2 min and 72°C for 1min30sec; 72°C for 7 min. 2º Round: same to the 1º	[67]
nPCR: ***Neorickettsia* sp.** **(characterization**	*groESL* -outerF -outerR -innerF -innerR	5′-ACCAGGCTACCTCACAGGC-3′ 5′-TTGACCCTCGCATCAATG-3′ 5′-CACAAGTTGGTTCAATTTCTGC-3′ 5′-CCGAGATCTTCAACAGTAAGGC-3′	823	94°C for 5 min; 30 cycles: 94°C for 1min, 55°C for 2 min and 72°C for 1min30sec; 72°C for 7 min. 2º Round: same to the 1º	[67]
nPCR: ***Hepatozoon* sp.** **(screening)**	*18S rRNA* -HAM1 -HPF-2 -4558 -2733	5’-GCCAGTAGTCATATGCTTGTC-3’ 5’-GACTTCTCCTTCGTCTAAG-3’ 5’-GCTAATACATGAGAAAATCTCAA-3’ 5’-CGGATTAACCAGCAAAT-3’	1,120	95°C for 3 min; 40 cycles: 91°C for 1min, 56°C for 1 min and 72°C for 1min; 72°C for 7 min 2º Round: 94°C for 3 min; 40 cycles: 94°C for 1min, 55°C for 2min and 72°C for 2min; 72°C for 10 min	[70,71]

2.5. Cloning assays

In order to obtain large fragments from the *Anaplasma* sp. 16S rRNA gene, obtained amplicons were cloned. Cloning method was performed with the pGEM®-T Easy (Promega, Madison, WI, USA), following the manufacturer's recommendations. DNA and vector concentrations used in the binding reaction were determined to obtain an insert:vector ratio of 3:1. The DNA-binding reaction consisted of adding 40 ng of insert (amplicon), 5 µL buffer, 1 µL of pGEM-T Easy vector, 1 µL of T4 Ligase enzyme and sterilezed water q.s.p. Solutions were gently mixed and incubated at 4°C for 16 hours. Then, 50 µL of competent *Escherichia coli* DH5α cells (10^9–10^{10} CFU/ng DNA) was added to the 10 µl binding reaction. The mix was kept in ice for 30 min, followed by thermal shock transformation, where microtubes were placed into a water bath at 42°C for 2 min. Then, 100 µL of SOC (Super Optimal broth with Catabolite repression

– Tryptone; Yeast Extract; NaCl; KCl; MgCl2; MgSO4; glucose [ThermoFisher Scientific, Waltham, MA, USA]) was added, and cells were incubated at 37°C for 1.5 hour, under the agitation of 200 rpm. Subsequently, 250 µL of this medium was added to Petri plates containing agar LB (Luria Bertani medium - Tryptone; Yeast Extract; NaCl; distilled water q.s.p. [ThermoFisher Scientific, Waltham, MA, USA]) medium prepared with 100 µg/mL ampicillin, 40 µL X-gal (5-bromo-4-chloro-3-indolyl-β-Dgalactoside; 0.026%) and 20 µL IPTG (isopropylthio-β-galactoside; 0.82 mM). Plates were incubated at 37°C for approximately 20 hours. White bacterial colonies were transferred to tubes containing 5 ml of broth LB medium and incubated at 37°C for 20 hours, followed by plasmid DNA extraction using Wizard® Plus SV Minipreps DNA (Promega, Madison, WI, USA). Plasmid DNA samples were stored at −20°C.

2.6. *Sequencing, phylogeny and genetic diversity assessment*

Purification of amplified products from cPCR assays were performed using a commercial kit (Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, WI, USA). Purified amplicons and plasmids were sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific™, Waltham, MA, USA) and ABI PRISM 3730 DNA Analyzer (Applied Biosystems™, Foster City, CA, USA) [72], at the "Centro de Recursos Biológicos e Biologia Genômica - CREBIO" (Faculdade de Ciências Agrárias e Veterinárias/FCAV, UNESP, Jaboticabal, SP, Brazil).

For sequencing of selected amplicons directly obtained from cPCR assays, the same primer pair and respective annealing temperature from the cPCR protocol were used. Plasmid DNA samples extracted from the selected clones were sequenced using primers that flanked the multiple cloning site (M13) of the pGEM®-T Easy plasmid (Promega), which includes the target gene inserts. For this purpose, the M13-F (5′-GTAAAACGACGGCCAG-3′) and M13-R(5′-CAGGAAACAGCTATGAC-3′) [73] were used. In order to obtain trustworthy sequences of *Anaplasma* 16S rRNA, an internal pair of primers were specifically designed to amplify approximately 680 bp from the insert and were also utilized for sequencing purposes (AT-F2: 5'-AAGCCTGATCCAGCTATGCC-3' and AT-R2: 5'-CATCTCACGACACGAGCTGA-3'). Annealing temperature for both pairs (M13-F/M13-R and AT-F2/AT-R2) was 59^{O}C.

Obtained sequences were first submitted to a screening test using Geneious 11.1.3 software (hhtp://www.geneious.com) to evaluate the electropherogram quality and generate the consensus sequences. The BLASTn online program (National Center for Biotechnology Information, Bethesda, MD, USA [74] was used to analyze the nucleotide sequences aiming to browse and compare with sequences from GenBank international database (http://www.ncbi.nlm.nih.gov/genbank). Consensus sequences obtained in the current study and those retrieved from GenBank were aligned using a MAFFT online software (https://mafft.cbrc.jp/alignment/server/) (accessed on 3 February 2024). Phylogenetic inferences were based on Bayesian analysis via CIPRES online server (https://www.phylo.org/index.php/, accessed on 3 August 2023) [75]. The best-fit model was determined using jModeltest v2.1.6 via CIPRES online server (https://www.phylo.org/index.php/, accessed on 3 August 2023) [76]. Trees were recovered using FigTree v1.3.1. software [77].

Additionally, a pairwise distance matrix from the alignment of the *Anaplasma* 16S rRNA sequences detected in tapirs and those retrieved from Genbank was calculated using the *p*-distance model and included Transitions + Transversions substitutions with uniform rates. Analysis was performed using MEGAX software [78,79]. Data were transferred to a Microsoft Excel 2016 spreadsheet to construct the heat map according to the rates obtained on the pairwise distance matrix.

Genetic diversity assessment was performed for *Anaplasma* 16S rRNA sequences. The DnaSP program v5 [80] was used in order to calculate nucleotide diversity (π), polymorphism level (haplotype diversity - [dh]), number of haplotypes (h) and the average number of nucleotide differences (K) among the sequences obtained. Genotype network was constructed in PopART (http://popart.otago.ac.nz), using the TCS inference method [81]. Additionally, a distance-based analysis was performed using SplitsTree v4.14.6 (University of Tubingen, Tubingen, Germany) and NeighborNet method [82] to investigate the genetic relationship among sequences detected in the present study and those previously deposited in GenBank.

3. Results

3.1. Amplification of mammalian endogenous genes

All DNA blood samples described herein successfully amplified at least one of the tested endogenous genes and were considered suitable for further analysis.

3.2. Screening assays

Twenty-two samples (22/122; 18%) amplified fragments from expected size for *Anaplasma* spp. From these samples, 7 (7/38; 18.4%) were collected from animals in Cerrado biome and 15 (15/61; 24.6%) from Pantanal biome. None animal presented positive results for *Anaplasma* sp. in more than one sample.

Regarding the screening test for *Neorickettsia* sp., 31 (31/122; 25.41%) samples representing 29 animals (29/99; 29.3%) presented bands of expected size. Out of these 29 tapirs, ten were sampled in Cerrado biome (10/38; 34.48%) and 19 in Pantanal biome (19/61; 31.14%).

All samples presented negative results for *Ehrlichia* spp., *C. burnettii* and *Hepatozoon* sp. using the protocols tested herein.

3.3. Characterization assays

Regarding the characterization assays, two tapir blood samples (2/22; 9.1%) from Cerrado that amplified on the 16S rRNA protocol for *Anaplasma* sp. also presented amplification and were successfully sequenced for the *Anaplasma* sp. ITS 23S-5S rRNA. None of the other molecular markers tested herein for *Anaplasma* spp. or *Neorickettsia* spp. were successfully amplified.

3.4. Obtained sequences

Out of 22 samples that presented amplifications of expected sized for the *Anaplasma* 16S rRNA fragment, 9 (40.9%) were chosen for cloning. Selection was made based on intensity of electrophoresis bands and absence of nonspecific amplifications. These nine samples were successfully cloned and sequence sizes ranged from 1,083 to 1,376 bp. These sequences were originated from six animals from Pantanal (6/9; 66,7%) and three from Cerrado (3/9; 33,3%). When comparing the nearly-complete 16S rRNA gene sequences obtained herein with those deposited in GenBank database, all sequences from the present study presented identity rates of 99.27-99.68% with *Anaplasma odocoilei* obtained from white-tailed deer (*Odocoileus virginianus*) from the USA (NR118489), confirming the presence of DNA from *Anaplasma* spp. in these samples. Regarding the ITS 23S-5S, two sequences with sizes of 235 and 350 bp were obtained. When these sequences were compared with those deposited in GenBank database, identity rates of 92.40 and 94.60% with *A. platys* obtained from a dog (CP046391) were showed **(Table 2)**.

Three 16S rRNA *Neorickettsia* sequences ranging from 451-463 bp were obtained. These sequences represented two animals from Pantanal (2/3; 66,66%) and one from Cerrado (1/3; 33,33%). When comparing the *Neorickettsia* sp. 16S rRNA gene sequences obtained from tapirs with those deposited in GenBank database, all sequences from the present study presented identity rates of 100/% with *N. risticii* obtained from horse from Rio de Janeiro State, Brazil (MK760650), confirming the presence of DNA from *Neorickettsia* spp. in these samples (**Table 3**).

Table 2. Comparisons between *Anaplasma* sp. 16S rRNA and ITS sequences obtained herein from tapir blood samples and the best match with sequences deposited in GenBank database using the BLASTn software (accessed on 2024 January 14th).

Gene	Sample ID	Biome	bp	Best Match (access number)	Query Cover	E value	Identity	GenBank access number
16S rRNA	NAI-C	Cerrado	1,236	*Anaplasma odocoilei – Odocoileus virginianus* (NR118489)	100%	0.0	99.68%	PP100185
	JHA-C	Cerrado	1,336	*Anaplasma odocoilei – Odocoileus virginianus* (NR118489)	100%	0.0	99.40%	PP100189
	AN-C	Cerrado	1,083	*Anaplasma odocoilei – Odocoileus virginianus* (NR118489)	100%	0.0	99.54%	P100193
	SKI-P	Pantanal	1,269	*Anaplasma odocoilei – Odocoileus virginianus* (NR118489)	100%	0.0	99.37%	PP100188
	GO-P	Pantanal	1,376	*Anaplasma odocoilei –*	100%	0.0	99.27%	PP100191

				Odocoileus virginianus (NR118489)				
	AA-P	Pantanal	1,237	*Anaplasma odocoilei* – *Odocoileus virginianus* (NR118489)	100%	0.0	99.68%	PP100187
	PNA-P-1	Pantanal	1,327	*Anaplasma odocoilei* – *Odocoileus virginianus* (NR118489)	100%	0.0	99.32%	PP100192
	VA-P-2	Pantanal	1,329	*Anaplasma odocoilei* – *Odocoileus virginianus* (NR118489)	100%	0.0	99.62%	PP100190
	SAO-P-2	Pantanal	1,272	*Anaplasma odocoilei* – *Odocoileus virginianus* (NR118489)	100%	0.0	99.53%	PP100186
ITS	JHA-C	Cerrado	350	*Anaplasma platys* – *Canis lupus familiaris* (CP046391)	100%	5e-150	94.60%	PP158765
	NAI-C	Cerrado	236	*Anaplasma platys* – *Canis lupus familiaris* (CP046391)	100%	3e-93	92.40%	PP178638

Table 3. Comparisons between *Neorickettsia* sp. 16S rRNA sequences obtained herein from tapir blood samples and the best match with sequences deposited in GenBank database using the BLASTn software (accessed on 2024 February 4th).

Sample ID	Biome	bp	Best Match (access number)	Query Cover	E value	Identity	GenBank access number

FFO-P-1	Pantanal	456	*Neorickettsia ristícii – Equus caballus* (MK760560)	100%	0.0	100%	PP108262
MIA-P	Pantanal	451	*Neorickettsia ristícii – Equus caballus* (MK760560)	100%	0.0	100%	PP108263
CNA-C-2	Cerrado	463	*Neorickettsia ristícii – Equus caballus* (MK760560)	100%	0.0	100%	PP108264

3.5. *Phylogenetic analysis*

Phylogenetic trees for *Anaplasma* sp. were constructed based on the molecular markers obtained herein. For 16S rRNA fragments, a total size alignment of 1,259 bp was constructed and TVM+I+G was chosen as the best evolutionary model. Although nine sequences were obtained for this fragment in the present study, only eight were used once, one sequence (id: AN-C; GenBank access: P100193) presented a shorter length (1,083 bp) compared to the others (>1,200 bp). Sequences from the present study clustered together in a clade containing homologous sequences from *Anaplasma odocoilei* (NR118489, JX876644), *Anaplasma* sp. obtained from an *Amblyomma americanum* tick (U52514) and *'Ca*. Anaplasma capybara' (OR577133, OR577135) (**Figure 1**).

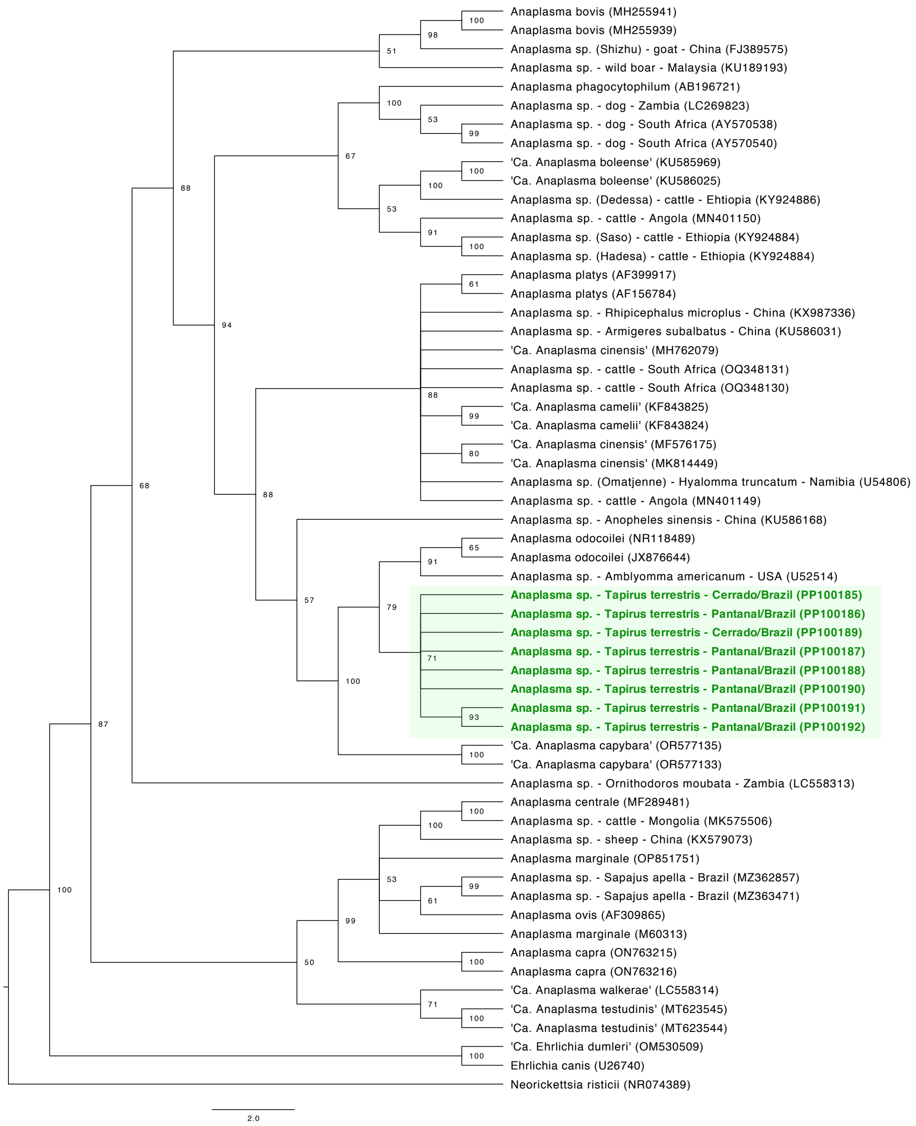

Figure 1. A Bayesian Inference tree constructed based on 16S rRNA gene with a total alignment size of 1,259 bp. Sequences from '*Candidatus* Ehrlichia dumleri' (OM530509), *Ehrlichia canis* (U26740) and *Neorickettsia risticii* (NR074389) were used as outgroups. Sequences obtained from *T. terrestris* were highlighted in green. Only post probabilities values of >50 were show.

Regarding the phylogenetic tree based on the ITS 23S-5S, an alignment of a total size of 339 bp was constructed and the best evolutionary model chosen was TPM3+G. The two sequences obtained herein clustered together with *A. platys* (CP046391) with a post-probability value of 74 (**Figure 2)**.

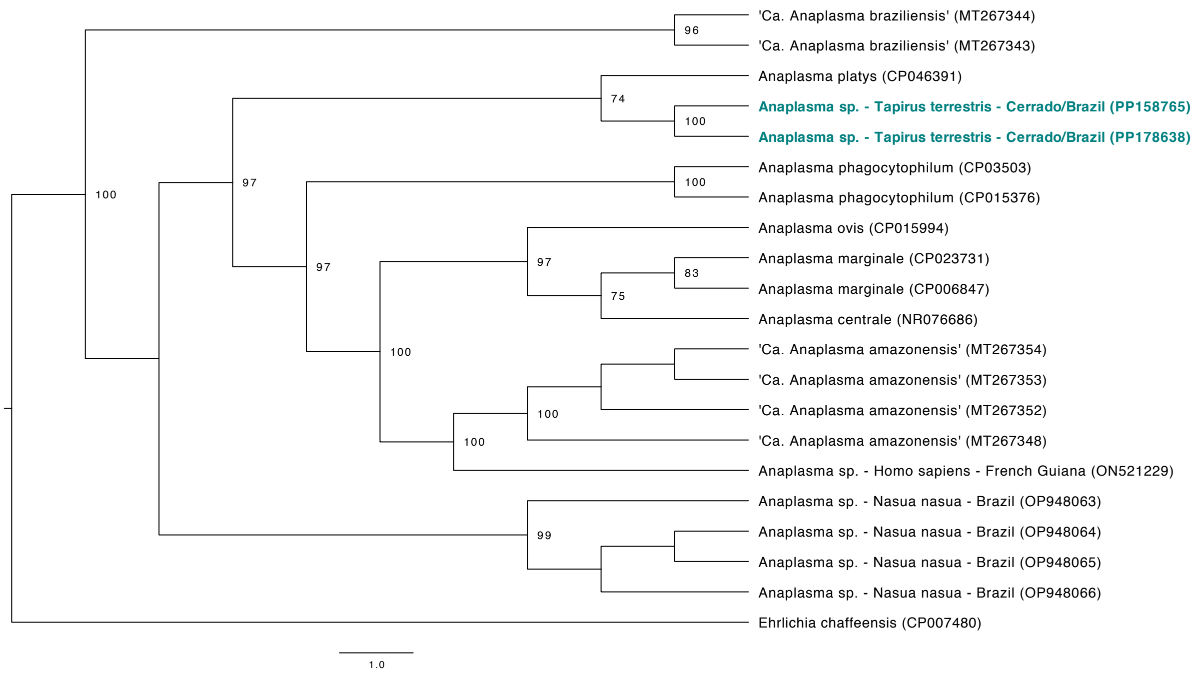

Figure 2. A Bayesian Inference tree constructed based on the ITS 23S-5S with a total alignment size of 339 bp. Sequences from *Ehrlichia cheffeensis* (CP007480) were used as outgroup. Sequences obtained from *T. terrestris* were highlighted in green. Only post probabilities values of >50 were show.

16S rRNA sequences obtained herein for *Neorickettsia* sp. were used to construct a Bayesian Inference phylogenetic tree based on alignment size of 455 bp. Nineteen (19) homologous sequences were retrieved from Genbank database and included in the analysis and the best evolutionary model chosen was K81+G. A sequence of *Ehrlichia canis* (U26740) was used as outgroup. Sequences from the present study clustered together in a monophyletic clade containing sequences of *N. risticii* and *Neorickettsia* sp. found in coatis and bats in Brazil (**Figure 3)**.

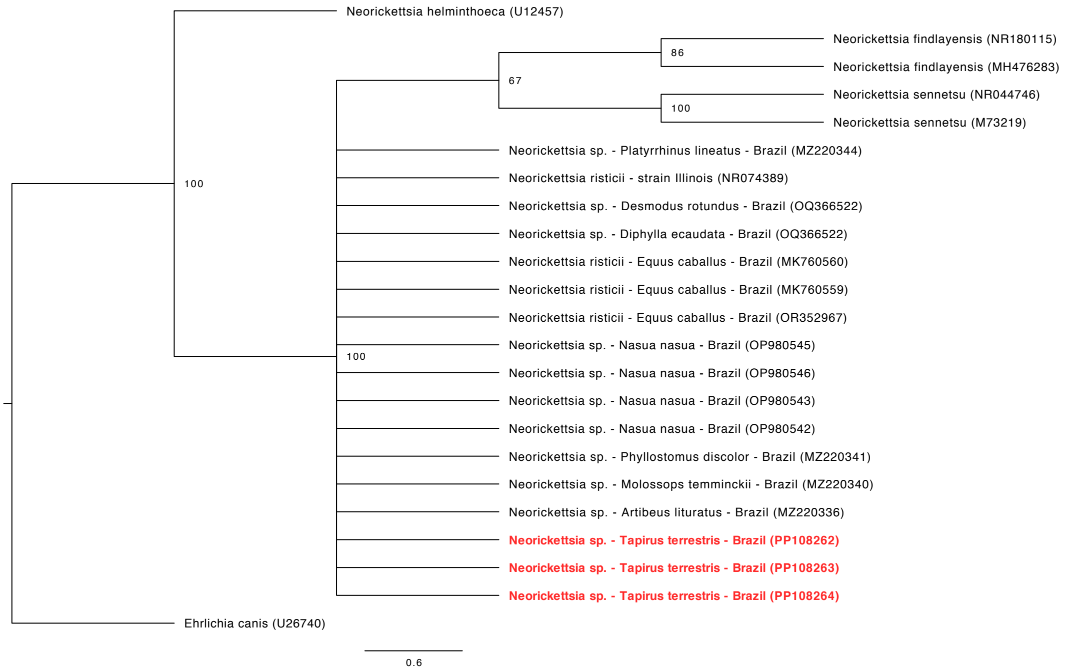

Figure 3. A Bayesian Inference tree constructed based on the *Neorickettsia* sp. 16S rRNA gene with a total alignment size of 455 bp. Sequences from *E. canis* (U26740) were used as outgroup. Sequences obtained from *T. terrestris* were highlighted in red. Only post probabilities values of >50 were show.

3.6. *Comparisons using p-distance matrix*

The eight 16S rRNA gene sequences from the present study used for *Anaplasma* phylogenetic assessment were also used for the construction of a *p*-distance matrix using a total alignment of 1,259 bp (**Supplementary file 1**). Six sequences (NR118489, JX8766440, U52514, KU586168, OR577133, OR577135) that were closely positioned to the *T. terrestris*-clade in the phylogenetic assessment performed herein were included in the *p*-distance analysis. Moreover, sequences from well-described species (MH255941, MF289481, AF309865, M60313, AB196721, AF156784) were also included for comparison purposes. Minimum and maximum divergence rates among the eight sequences from the present study included in this analysis were 0.00 and 0.32%, respectively. Considering the sequences that were most closely related to tapir-associated *Anaplasma* 16S RNA sequences in the phylogenetic assessment, minimum and maximum divergence rates between tapir sequences and *A. odocoilei* sequences (NR118489, JX876644) were 0.16 and 0.48%, respectively. Minimum and maximum divergence rates between tapir-associated *Anaplasma* 16S RNA and *Anaplasma* sp. obtained from *A. americanum* sequence were 0.40 and 0.63%, respectively. Minimum and maximum divergence rates between tapir-associated *Anaplasma* 16S RNA and *Anaplasma* sp. obtained from *A. sinensis* sequence were 0.73 and 0.97%, respectively All comparisons of divergence rates were described in **Table 4**.

Table 4. Maximum and minimum percentages of base differences per site between tapir-related *Anaplasma* 16S rRNA sequences and homologous sequences based on the *p*-distance matrix constructed.

	Minimum divergence	Maximum divergence
A. bovis (MH255941)	2.94%	3.45%
A. centrale (MF289481)	3.32%	3.69%
A. ovis (AF309865)	3.32%	3.69%
A. marginale (M60313)	3.17%	3.53%
A. phagocytophilum (AB196721)	2.04%	2.40%
A. platys (AF156784)	1.06%	1.68%
Anaplasma sp. (KU586168)	0.65%	0.97%
A. odocoilei (NR118489)	0.16%	0.48%
A. odocoilei (JX876644)	0.16%	0.48%
Anaplasma sp. (U52514)	0.40%	0.64%
Ca. A. capybara (OR577135)	0.65%	0.97%
Ca. A. capybara (OR577133)	0.78%	0.97%

3.7. *Genotype and SplitsTree analyses*

A genotype network was constructed based on sequences from the present study and those obtained that were closely clustered in the 16S rRNA phylogenetic tree. Based on our results, at least nine different genotypes are found. The eight sequences from the present study clustered in four different genotypes (**Figure 4**). The following values were found for nucleotide diversity (π) = 0.00423; polymorphism level (haplotype diversity—[dh]) = 0.879; number of haplotypes (h) = 9; and average number of nucleotide differences (K) = 4.802 Descriptions of each genotype found are displayed in **Table 5**.

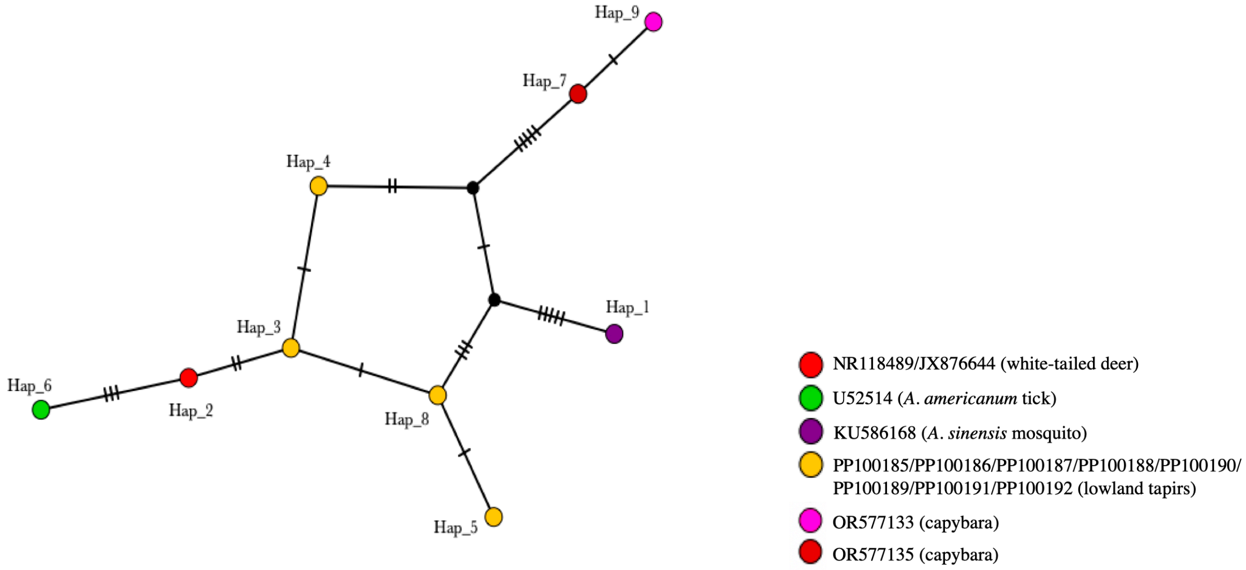

Figure 4. Genotype diversity among *Anaplasma* spp. 16S rRNA gene sequences detected in the present study and those closely related on phylogenetic inference. Analysis was made using DnaSP6. Inference and graphic representation were made by TCS network method on PopART software

Table 5. Descriptions of species, host, country and Genbank access number of sequences from each genotype.

Genotype (Hap)	Species	Host	Country	Genbank access number
Hap_1	*Anaplasma* sp.	*Anopheles sinensis*	China	KU586168
Hap_2	*Anaplasma* odocoilei	*Odocoileus virginianus*	USA	NR118489 JX876644
Hap_3	*Anaplasma* sp.	*Tapirus terrestris*	Brazil	PP100185 PP100186 PP100187 PP100188 PP100190
Hap_4	*Anaplasma* sp.	*Tapirus terrestris*	Brazil	PP100189
Hap_5	*Anaplasma* sp.	*Tapirus terrestris*	Brazil	PP100191

Hap_6	*Anaplasma* sp.	*Amblyomma americanum*	USA	U52514
Hap_7	'*Ca*. A. capybara'	*Hydrochoerus hydrochaeris*	Argentina	OR577135
Hap_8	*Anaplasma* sp.	*Tapirus terrestris*	Brazil	PP100192
Hap_9	'*Ca*. A. capybara'	*Hydrochoerus hydrochaeris*	Argentina	OR577133

The SplitsTree distance-based analysis was constructed based on an alignment of 1,259 bp. The alignment contained *Anaplasma* 16S rRNA sequences from the present study and those closely clustered on phylogenetic analysis. The analysis corroborates the phylogenetic inference and genotype network, demonstrating a separation between *Anaplasma* sp. from tapirs and *A. odocoilei*, and *Anaplasma* sp. from *A. americanum* (**Figure 5**).

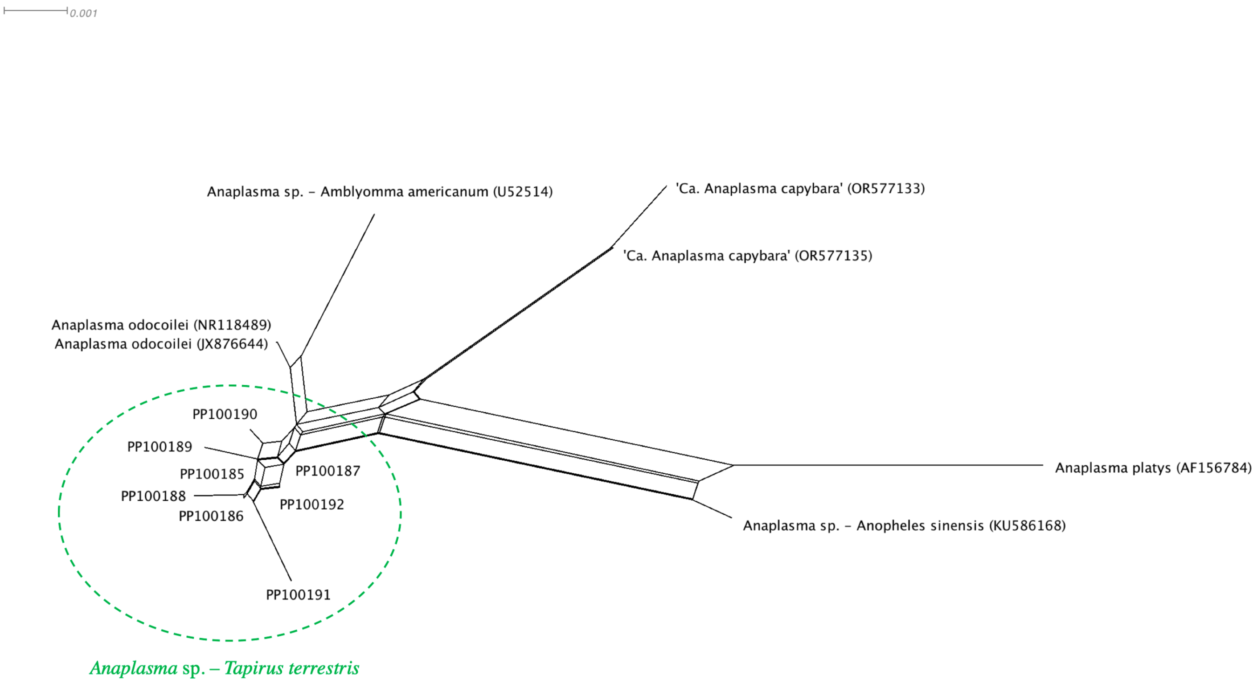

Figure 5. Distance analysis made by SplitsTree v. 4.14.6 software applying NeighborNet method based on a 1,259 bp alignment. Sequences from the present study are identified in green.

4. Discussion

We reported herein, for the first time, the occurrence of *Anaplasma* and *Neorickettsia* in wild lowland tapirs.

Detection of Anaplasmataceae agents has been reported in wildlife worldwide, especially with the advance of molecular techniques. However, caution is needed regarding the description of species and/or genotypes of *Anaplasma* sp. The use of short fragments from the 16S rRNA may result in the amplication of limited hypervariable regions, leading to a mislabeling in species assignation [83]. In the recent past, *Anaplasma* genotypes have been reported based on short-length 16S rRNA fragments (<700 bp) [15,16,22]. The amplification of short fragments does not imply the non-existence of these genotypes, or devaluation of the report, but rather precluded the possibility of a more reliable phylogenetic positioning.

However, obtaining larger fragments of amplicons remain challenging when using chain terminator sequencing. Limitations of Sanger sequencing relies on obtaining low-quality sequences for the first 15-40 bp due primer binding and the inability to obtain trustworthy nucleotide assignment for longer sequences (>900 bp) [84]. To address these issues, we chose to cloning the obtained *Anaplasma* 16S rRNA amplicons and utilizing primers that flanked the multiple cloning size for sequencing [73]. Moreover, an additional pair of primers, especially designed to amplify the inner region from the targeted fragment, was also needed to obtain reliable sequence quality. Certainly, resolving these limitations costs time and financial resources.

In the present study, the *Anaplasma* 16S rRNA sequences with lengths exceeding 1,200 bp were used for phylogenetic inference. Tapir-related sequences formed a clade closely positioned to *A. odocoilei* and *Anaplasma* sp. detected in *A. americanum*. Comparisons using the *p*-distance matrix corroborated the phylogenetic positioning of these sequences: the tapir-associated *Anaplasma* 16S rRNA sequences presented minimum and maximum divergence rates of 0.16-0.48% and 0.40-0.64% to *A. odocoilei* and *A. americanum*-*Anaplasma* related, respectively. *Anaplasma odocoilei* was previously described to infect platelets from *O. virginianus* in the USA, with evidence of infected deer presenting transient thrombocytopenia. Although transmission has not been proven yet, *A. americanum* is the suspected vector of *A. odocoilei* [85].

Sequences from the present study were also closely positioned to a novel *Candidatus* species described in capybaras from Argentina (OR577133, OR577135) but presenting strong separation value (post-probability = 100). The *p*-distance comparison exhibited higher minimum and maximum divergence rates for these sequences (0.65-1.04%).
A cloudy positioning resolution was found for an *Anaplasma* sp. sequence detected in *A. sinensis* mosquitoes in China (KU586168). Although the *p*-distance comparison presented lower rates of divergence (0.65-0.97%), this sequence was phylogenetically positioned more distantly to the tapir-associated *Anaplasma* than '*Ca*. A. capybara'. In fact, 16S rRNA similarities above 98.7% may be found for distinct species within the *Anaplasma* genus [83]. Genotype analysis and SplitsTree network also corroborated the uniqueness of the tapir-associated *Anaplasma* genotypes.

Phylogenetic inference of nearly-complete fragments from *Anaplasma* 16S rRNA formed two major clades: one containing species found to infect erythrocytes (*Anaplasma marginale*, *Anaplasma centrale* and *Anaplasma ovis*) and the other one containing species that primarily infect platelets and leukocytes (*A. platys, Anaplasma bovis*, and *Anaplasma phagocytophilum*), as previously demonstrated [83]. This topology was also observed in our phylogeny, with tapir-related sequences positioned in the platelet and leukocytes clade. A higher degree of intraspecies variance in the 16S rRNA gene is suggested for the species contained in this second clade, and species assignment should be carried out carefully [83]. In our analysis, *A. odocoilei* and *Anaplasma* sp. from tapirs appear to be closely related. In addition to marked differences concerning vertebrate host species, potential involved tick vectors (*A. americanum* does not occur in Brazil) and geographical location, our phylogenetic and distance analyses based on 16S rRNA gene indicate that tapir-related *Anaplasma* and *A. odocoilei* correspond to a genetically similar but different species.

Considering the limited capacity of 16S rRNA alone to assign *Anaplasma* species [83], we attempted to perform multi-locus sequencing of different target genes, but only sequences from the ITS 23S-5S were obtained. Analysis using the ITS 23S-5S has been demonstrated to exhibit a strong correlation with other *Anaplasma* targets for differentiation of novel genotypes [29]. The two sequences obtained herein were positioned closely to *A. platys* sequences, which is described as genetically related to *A. odocoilei* based on the *gltA* gene and *groESL* operon [85]. Unfortunately, there were no sequences of *A. odocoilei* ITS 23S-5S available in Genbank database to allow a comparison between the topology obtained with ITS 23S-5S and that one obtained with 16S rRNA gene.

Neorickettsia sp. was also found infecting lowland tapirs in the present study. This Anaplasmataceae agent has been reported in non-hematophagous bats [30] and coatis [32] from Mato Grosso do Sul state, the same state where the present study was conducted. Tapir-related sequences formed a monophyletic clade with other 14 homologous sequences of *N. risticii* detected in horses and *Neorickettsia* sp. detected in bats and coatis in Brazil. However, positioning based on the partial 16S rRNA should be carefully considered since a phylogenetic heterogenicity is reported for *N. risticii* [1].

Neorickettsia risticii is described to infect primarily the monocytes causing the Potomac horse fever (PHF), which courses with anorexia, diarrhea and colic in horses [86]. Molecular detection of *N. risticii* was reported in symptomatic horses from southern [87,88] and southeastern Brazil [89]. Transmission of *N. risticii* is presumably related to ingestion of infected metacercaria or snail-ridden pastures by naïve vertebrate hosts [1]. In southern Brazil, transmission was associated to cercariae from *Parapleurolophocercous cercariae* infecting snails from genus *Heleobia* [90]. Considering the taxonomic proximity between *Tapirus* and *Equus* genera, as Perissodactyla members, further investigations should be considered to evaluate if *Neorickettsia* sp. infection may represent a health concern for tapirs.

The present study did not find molecular evidence of infection by *Ehrlichia* sp., *C. burnetii* and *Hepatozoon* sp. using the PCR protocols described herein. Previously, *Ehrlichia* sp. was detected in wild animals in Mato Grosso do Sul State [91,92,18,22,29,43,25]. Although some studies reported the absence of *C. burnetii* DNA in blood and tissue samples from wild animals in Brazil [35,30,93,28], serological evidence of exposure is reported in deer [35] and small mammals [36], which may indicate that this pathogen circulates in wildlife and should be monitored. Moreover, blood samples may not represent the most sensitive sample for *C. burnetii* detection in tapirs. Remarkable frequencies of positive animals (>40%) were found when vaginal and rectal swabs from sheep were used for molecular diagnosis of *C. burnetii* employing the same qPCR protocol tested herein [94].

Hepatozoon DNA was not detected in a post-mortem molecular investigation in wild and captive *T. terrestris* from Cerrado and Pantanal regions [95]. Occurrence of *Hepatozoon* is mostly reported in reptiles and carnivores, with few reports of detection in ungulates [96]. Recently, carnivore-related *Hepatozoon* species was detected in spleen samples from wild *Rupicapra rupicapra* and *Capreolus capreolus* in Austria [97]. In Brazil, a 18S rRNA sequence phylogenetically related to *H. canis* was detected in the blood sample of one captive lowland tapir maintained in a wildlife rehabilitation center [98]. Besides ingestion of infected invertebrate hosts (e.g. ticks), predation is reported as an alternative transmission route between vertebrate hosts [12,99]. The lowland tapir is reported as prey for jaguars (*Panthera onca*) [100, 101], which can be parasitized by *Hepatozoon* sp. in Pantanal and Cerrado [102]. Considering this, it would be interesting to evaluate if tapirs could participate in transmission cycles of this apicomplexan in any way. However, we are still missing any evidence for it.

5. Conclusions

We described here the occurrence of a putative genotype of *Anaplasma* sp. in wilds tapirs from Pantanal and Cerrado. Phylogenetic, distance and genotype analyses based on large fragments from 16S rRNA and partial ITS 23S-5S suggest that this genotype is distinct albeit closely related to *A. odocoilei*. Amplification of different gene targets and whole genome sequencing would aid in the elucidation of the positioning of this tapir-related *Anaplasma*. Moreover, *Neorickettsia* sp. was also described, for the first time, in tapirs from both biomes. Putative vectors are still unknow.

Acknowledgments

This study is part of Anna Mongruel’s thesis. She is carrying out her research at Programa de Pós-Graduação em Medicina Veterinária—Universidade Estadual Júlio de Mesquita Filho (UNESP/Jaboticabal) and is sponsored by a fellowship from Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP– Process #2019/26403-0) at the time of this study. Authors would like to thank the Lowland Tapir Conservation Initiative (LTCI)–Instituto de Pesquisas Ecológicas (IPÊ) for kindly providing the biological samples needed for the present study. The study of tapir health has been an important component of the long-term activities of the LTCI-IPÊ in Brazil. The LTCI has the institutional support from the International Union for Conservation of Nature (IUCN) Species Survival Commission (SSC) Tapir Specialist Group (TSG), Association of Zoos and Aquariums (AZA) Tapir Taxon Advisory Group (TAG), and European Association of Zoos and Aquariums (EAZA) Tapir Taxon Advisory Group

(TAG). This work was supported by FAPESP - Process #2020/12037-0 and #2022/08543-2) and CNPq (National Council for Scientific and Technological Development; Productivity Grant to MRA - Process #303701/2021-8).

Data availability statement

The datasets generated and analyzed during the current study are available in the NCBI GenBank Nucleotide platform (https://www.ncbi.nlm.nih.gov/genbank/) and can be accessed through accession numbers: PP100185-PP100193, PP158765, PP178638, PP108262-PP108264.

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AUTHOR CONTRIBUTIONS

Anna Claudia Baumel Mongruel: Conceptualization, Methodology, Visualization, Formal analysis, Writing – original draft, Writing – review & editing. **Patricia Emília Medici:** Methodology, Conceptualization, Visualization, Funding acquisition. **Ariel da Costa Canena**: Methodology. **Amir Salvador Alabi Cordova**: Methodology. **Lorena Freitas das Neves:** Methodology. **Eliz de Oliveira Franco:** Methodology. **Rosangela Zacarias Machado**: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Marcos Rogério André**: Conceptualization, Visualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

ADDITIONAL INFORMATION

The authors declare no conflict of interest. This work was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado da São Paulo - Process #2020/12037-0 and #2022/08543-2) and CNPq (National Council for Scientific and Technological Development; Productivity Grant to MRA [CNPq Process #303701/2021-8]). This study is part of Anna Mongruel’s thesis. She is carrying out her research at Programa de Pós-Graduação em Medicina Veterinária—Universidade Estadual Júlio de Mesquita Filho (UNESP/Jaboticabal) and is sponsored by a fellowship from Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP–Process #2019/26403-0) at the time of this study. LTCI’s financial support comes from

national and international agencies, including zoological institutions, foundations, private businesses, and private individuals.

CAPÍTULO VII* - First molecular detection of *Borrelia* sp. in tapirs (*Tapirus terrestris*)*

*Esse capítulo corresponde à publicação científica publicada no periódico Veterinary Research Communications. http://doi.org/10.1007/s11259-024-10406-z.

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Received: 29 March 2024 / Accepted: 3 May 2024

Abstract

Borrelia theileri is a tick-borne spirochete causative agent of fever, apathy and reduced food consumption in cattle. Molecular diagnosis has expanded the understanding of *Borrelia theileri* with new hosts and geographical locations being described. The present study aimed to describe the first molecular detection of *B. theileri* in wild tapirs (*Tapirus terres- tris*) from South America. Blood DNA samples obtained from 99 tapirs sampled in Pantanal (n = 61) and Cerrado (n = 38) biomes were screened using a qPCR assay based on the 16 S rRNA gene of *Borrelia* sp. Positive samples in the qPCR assay were subjected to PCR assays to allow characterization of fragments from 16 S rRNA and *flaB* genes. Two (2/99; 2.0%) animals from Pantanal biome were positive in the qPCR and one sample presented bands of expected size for the *flaB* protocol. Amplicons from this sample were successfully cloned and sequenced. In the phylogenetic analysis, *Borrelia* sp. from *T. terrestris* grouped together with *B. theileri* sequences previously detected in *Rhipicephalus microplus* ticks and cattle from Minas Gerais State in Brazil, *Rhipicephalus geigyi* from Mali, and *R. microplus* and *Haemaphysalis sulcata* from Pakistan. This finding contributes to our knowledge regarding susceptible hosts species for *B. theileri*. More studies are necessary to understand the potential effects of *B. theileri* on tapir's health.

Keywords Borreliosis, PCR, Spirochetes, South America, Wildlife.

Introduction

Bacteria from the genus *Borrelia* are vector-borne spirochetes that can cause disease in animals and humans. Since the first borrelial description in the early 20th century, different species and strains, with distinct epidemiological and clinical aspects, have been reported. Currently, these organisms are divided into three main groups: (1) The Lyme group (LG): represented by the *Borrelia burgdorferi sensu lato* and transmitted exclusively by *Ixodes* ticks; (2) Relapsing fever group (RFG): represented by species mostly vectored by Argasidae ticks, with some species being trans- mitted by Ixodidae ticks or *Pediculus humanus* louse; and (3) Echidna-Reptile group (ERG): a recently described group, phylogenetically distinct from the former two, and transmitted by Ixodidae ticks (Margos et al. 2018; Trevisan et al. 2021a,b).

Some *Borrelia* species within the RFG group are known to be transmitted by hard ticks. *Borrelia theileri* is trans- mitted by *Rhipicephalus* sp. ticks and initially reported in Africa, Australia and South America (Theiler 1905; Callow 1967). Although this pathogen was first described in 1904 as a causative of fever, apathy and anorexia in cattle (Theiler 1904), it has been also reported in horses (Theiler 1904; Van Heerden and Reyers 1984) and small ruminants (Theiler 1904, 1905; Auoadi et al. 2017).

Molecular diagnosis has expanded the understanding of the epidemiology of *B. theileri* infections, allowing the description of new hosts and geographical locations for this agent. Up to now, this agent has been molecularly detected in raccoon dogs (*Nyctereutes procyonoide*) in Korea (Kang et al. 2018), impalas (*Aepyceros melampus*) in Zambia (Qiu et al. 2021) and rodents (*Rattus rattus*) in Thailand (Takhampunya et al. 2021). Additionally, genotypes closely related to *B. theileri* were detected in sika deer (*Cervus nippon*), wild boars (*Sus scrofa*), and one raccoon (*Procyon lotor*) in Japan (Furuno et al. 2017). Regarding tick vectors, DNA from *B. theileri* was detected in a pool of *Amblyomma* and *Rhipicephalus* (*Boophilus*) ticks in Ethiopia (Cutler et al. 2012) and in *Rhipicephalus* spp. ticks from Pakistan (Khan et al. 2023).

In South America, reports of *B. theileri* are restricted to ticks and cattle (Faccini-Martínez et al. 2022). Occurrence of this agent was molecularly confirmed in *Rhipicephalus microplus* collected from horses and cattle from southeastern Brazil (Yparraguirre et al. 2007; Cordeiro et al. 2018). Moreover, detection is also reported in cattle blood samples from southeastern, midwest and northern Brazil

(Paula et al. 2023; Figueiroa et al. 2023), Argentina (Morel et al. 2019) and Colombia (Ramires-Hernández et al. 2022). To date, there are no reports of *B. theileri* in wildlife in South America. The present study aimed to describe the molecu- lar detection of *B. theileri* in wild tapirs (*Tapirus terrestris*) from Pantanal region (Mato Grosso do Sul State) in Brazil.

Material and Methods

Sampling

Between 2013 and 2018, a total of 122 blood samples from free-living (*n* = 94) and road-killed (*n* = 5) wild tapirs were collected for health assessment purposes. Out of these animals, 61.6% (61/99) were sampled in Pantanal biome whereas 38.4% (38/99) were sampled in Cerrado biome (both biomes located in Mato Grosso do Sul State). All road-killed animals were sampled in Cerrado biome. Sampled animals were 49.5% (49/99) females and 50.5% (50/99) males, and 53.5% (53/99) adults (> 48 months old) and 46.5% (46/99) sub-adults (< 48 months old).

Sampling of free-living animals was performed during tapir anesthesia for the installation of GPS collars by professionals from the “Iniciativa Nacional para a Conservação da Anta Brasileira (INCAB-IPÊ)” (Lowland Tapir Conservation Initiative (LTCI-IPÊ). Detailed information about sampling procedures and study areas were described elsewhere (Mongruel et al. 2022a).

The study was approved by the Ethics Committee for Animal Experimentation of FCAV/UNESP (Faculty of Agricultural and Veterinary Sciences of the São Paulo State University) under protocol number 4558/20. The “Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO)” provided the required annual permits for the capture and immobilization of tapirs and collection of biological samples (SISBIO# 14,603). All protocols for the capture, anesthesia, handling, and sampling of tapirs have been reviewed and approved by the Veterinary Advisors of the Association of Zoos and Aquariums (AZA) — Tapir Taxon Advisory Group (TAG), and the Veterinary Committee of the IUCN SSC Tapir Specialist Group (TSG).

DNA extraction and amplification of mammals' endogenous gene

DNA extraction was performed individually on each tapir blood sample, without making pools, using a commercial kit (InstaGene™ Matrix, Biorad®, Hercules, CA, USA), following the manufacturers' instructions. Conventional PCR (cPCR) assays for the mammalian endogenous genes glyc- eraldehyde-3-phosphate dehydrogenase (*gapdh*) (450 bp) (Birkenheuer et al. 2003) and interphotoreceptor retinoid- binding protein (*irpb*) (227 bp) (Ferreira et al. 2010) were performed to ensure DNA recovery from blood samples. Samples that did not yield amplicons in either of the PCR protocols were excluded from the subsequent analysis.

Molecular assay

Screening of positive samples was performed using a quantita- tive real-time (qPCR) assay based on the 16 S rRNA gene from *Borrelia* sp., with a detection limit of approximately 10–20 copies of the fragment (Parola et al. 2011). Each DNA sample was independently evaluated in duplicates and samples that presented differences in Cq values higher than 0.5 were retested in triplicate. For the construction of the standard curve of each reaction, serial dilutions were performed at different concen- trations (2.0×10^7 to 2.0×10^1 copies) of a plasmid encoding a conservative fragment of the 16 S rRNA gene from *Borrelia* sp. (pIDTSMART; Integrated DNA Technologies, Coralville, IA, USA). These plasmids were also used as positive controls. The number of plasmid copies was determined by the formula (XG/µL DNA/ [Plasmid Length (BP) × 660]) × 6. 22×10^{23} × plasmid copies/µL. The amplification efficiency (E) was calcu- lated according to the slope of the standard curve using the for- mula $E= 10^{-1/\text{slope}}$ (Bustin et al. 2009). Ultra-purified sterilized water (Nuclease-Free Water, Promega®, Madison, Wisconsin, United States) was used as a negative control for each reac- tion performed. The qPCR assays were carried out in a C1000- CFX96 thermocycler (BIORAD, Hercules, CA, USA).

Positive samples in the qPCR assay were subjected to cPCR assays to allow characterization of fragments from 16 S rRNA (Marti Ras et al. 1996) and *flaB* (flagellin) genes (Stromdahl et al. 2003). A positive sample for *Borrelia anserina* (Ataliba et al. 2007) was used as positive control and ultra-purified sterilized water (Nuclease-Free Water, Promega®, Madison, Wisconsin, United States) was used as a negative control for all reactions. Fragment sizes, primer sequences, qPCR hydrolysis probes and annealing temperature used herein are described in Table 1.

Cloning and sequencing assays

Amplicons obtained in the cPCR assays were cloned using pGEM®-T Easy System (Promega, Madison, WI, USA), following the manufacturer's recommendations. DNA and vector concentrations used in the ligation reaction were determined to obtain an insert: vector ratio of 3:1. The DNA binding reaction consisted of adding 40 ng of insert (ampli- con), 5 µL buffer, 1 µL of pGEM-T Easy vector, 1 µL of T4 Ligase enzyme and sterilized water q.s.p. Solutions were kindly mixed and incubated at 4°C for 16 h. Then, 50 µL of competent *Escherichia coli* DH5α cells (10^9–10^{10} CFU/ ng DNA) was added to the 10 µL ligation reaction. The mix was kept in ice for 30 min, followed by thermal shock, where microtubes were placed into a water bath at 42 °C for 2 min. Then, 100 µL of SOC (Super Optimal broth with Catabolite repression – Tryptone; Yeast Extract; NaCl; KCl; MgCl2; MgSO4; glucose [ThermoFisher Scientific, Waltham, MA, USA]) was added, and cells were incubated at 37 °C for 1.5 h, under the agitation of 200 rpm. Subsequently, 250 µL of this medium was added to Petri plates containing agar LB (Luria Bertani medium - Tryptone; Yeast Extract; NaCl; distilled water q.s. [ThermoFisher Scientific, Waltham, MA, USA]) medium prepared with 100 µg/mL ampicillin,
40 µl X-gal (5-bromo-4-chloro-3-indolyl-β-Dgalactoside; 0.026%) and 20 µL IPTG (isopropylthio-β-galactoside; 0.82 mM). Plates were incubated at 37 °C for approximately 20 h.

Colonies of bacteria containing the inserts (white colonies) were transferred to tubes containing 5 mL of broth LB medium and incubated at 37 °C for 20 h, followed by plasmid DNA extraction using Wizard® Plus SV Minipreps DNA (Promega, Madison, WI, USA). Purified plasmids were sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific™, Waltham, MA, USA) and ABI PRISM 3730 DNA Analyzer (Applied Biosystems™, Foster City, CA, USA) (Sanger et al. 1977), at the "Centro de Recursos Biológi- cos e Biologia Genômica - CREBIO" (Faculdade de Ciências Agrárias e Veterinárias/FCAV, UNESP, Jaboticabal, SP, Brazil). For sequencing, primers GTAAAACGACGGCCAG-3′) and M13-R(5′-CAGGAAACAGCTATGAC-3′) flanking the multiple cloning site (M13) of the pGEM®-T Easy plasmid (Promega), which includes the target gene inserts, were used (Lau et al. 2010).

Table 1 Description of gene and size fragment, primer sequences, annealing temperature and reference of the qPCR and cPCR assays used in the present study

Gene and total fragment size		Primer	Sequence	Annealing temperature (ºC)	Reference
16 S rRNA (qPCR) 148 bp		Bor16S3-F	5’-AGCCTTTAAAGCTTCGCTTGTAG-3’	60	
		Bor16S3-R	5’-GCCTCCCGTAGGAGTCTGG-4’		Parola et al. 2011
		Probe Bor16S3P	[6FAM] CCGGCCTGAGAGGGTGAACGG		
16 S rRNA (cPCR) 1489 bp	1st Round	FD3 (F)	5’-AGAGTTTGATCCTGGCTTAG-3’	55	
		T50 (R)	5’-GTTACGACTTCACCCTCCT-3’		
	2nd Round	FD3 (F)	5’-AGAGTTTGATCCTGGCTTAG-3’	56	
		16s-1 (R)	5’-TAGAAGTTCGCCTTCGCCTCTG-3’		Marti Ras et al. 1996
	3rd Round	16s-2 (F)	5’-TACAGGTGCTGCATGGTTGTCG-3’	56	
		T50 (R)	5’-GTTACGACTTCACCCTCCT-3’		
	4th Round	Rec4 (F)	5’-ATGCTAGAAACTGCATGA-3’	54	
		Rec9 (R)	5’-TCGTCTGAGTCCCCATCT-3’		
flaB (cPCR) 665 bp		FlaRL (F)	5’-GCAATCATAGCCATTGCAGATTGT-3’	55	Stromdahl et al. 2003
		FlaLL (R)	5’-ACATATTCAGATGCAGACAGAGGT-3’		

Phylogenetic reconstruction

Obtained sequences were first submitted to a screening test using Geneious 11.1.3 software (hhtp://www.geneious. com) to evaluate the electropherogram quality and gener- ate the consensus sequences. The BLASTn online program (National Center for Biotechnology Information, Bethesda, MD, USA (Altschul et al. 1990) was used to analyze the nucleotide sequences aiming to browse and compare with sequences from GenBank international database (https:// www.ncbi.nlm.nih.gov/genbank/*).* Consensus sequences obtained in the current study and those retrieved from GenBank were aligned using a MAFFT online software (https:// mafft.cbrc.jp/alignment/server/) (accessed on 3 March 2024). Phylogenetic inferences were based on Maximum Likelihood (ML) which was carried out using the IQ-TREE on ACCESS tool via CIPRES portal (https://www.phylo. org, accessed on 3 March 2024) (Miller et al. 2010) and for both best-fit model (following Bayesian Inference Criteria) determination and tree reconstruction. Clades' support was evaluated using bootstrap analysis of 1000 repetitions. Trees were recovered and edited using FigTree v1.3.1. software (Rambaut 2010).

Results

All DNA blood samples successfully amplified at least one of the tested endogenous genes and were considered suit- able for the further analysis. Two (2/99; 2.0%) animals from Pantanal biome were positive in the qPCR for *Bor- relia* spp. The Efficiency, R^2, Y-intercept and Slope values of the qPCR assays ranged from 92.3 to 99.8%, 0.997 to 1.000, 36.612 to 38.896 and − 3.349 to -3.520, respectively. Quantification values obtained for positive samples were described in Table 2.

The two positive samples in the qPCR assay were negative for the cPCR 16 S rRNA assay tested herein. Regarding the *flab* assay, only one sample (animal ID WE-P-1) amplified bands of expected size. Amplicons from this sample were successfully cloned and sequenced. A sequence of 593 bp from *Borrelia* spp. *flaB* gene was obtained and compared with sequences deposited in the GenBank database using the BLASTn software. The tapir-related sequence showed identity values of 99.66% (query cover: 99%; E-value: 0.0) with a sequence from *B. theileri* detected in *R. microplus* collected from a horse in Brazil (EF141022), 99.65% (Query cover: 96%; E-value: 0.0) with *B. theileri* from *R. microplus* collected from cattle in Colombia (PP262609) and 98.65% (Query cover: 100%; E-value: 0.0) with *B. theileria* from cattle in Goiás State, Brazil (ON191583). The sequence identified in the present study was deposited in the GenBank database under access number PP259188. Results from the molecular assays conducted here were summarized in Table 2.

A ML tree was constructed based on a total alignment of 615 bp containing 37 homologue *flaB* sequences and TPM3 + F + G4 as an evolutionary model. Sequences of representatives from LG (*B. burgdorferi* – DQ016625) and REG (*Borrelia turcica* - AB109246; *Borrelia tachyglossi* - KY586966) were used as outgroups. Sequence obtained from *T. terrestris* grouped with *B. theileri* sequences detected in *R. microplus* ticks in Brazil (MG601737, EF141022), cattle from Brazil (OQ344270, OQ344269, OQ344268, ON191583), *R. geigyi* from Mali (KF569936), *R. microplus* from Pakistan (OR574986), *R. microplus* from cattle in Colombia (PP262609) and *Haemaphysalis sulcata* from Pakistan (OR574985). The *B. theileri*-clade presented a separation in two minor sub-clades with high bootstrap values (100) (Fig. 1).

Although 2/99 tapirs showed positive results in the qPCR protocol for *Borrelia* spp., amplification and sequencing of additional genes, and consequently confirmation of the agent, were possible for only one (1.01%; 1/99) positive tapir.

Table 2 Information (ID and location) of tapirs positive for *Borrelia* sp. and results obtained in qPCR for 16 S rRNA gene and cPCR for *flaB* gene.

Animal ID	Location	Sex/Age	qPCR 16 S rRNA	qPCR Quantification (fragment of 16 S rRNA copy numbers/µL)	Quantification cycle (Cq)	cPCR flaB
WE-P-1	Pantanal	Female/sub-adult	+	1.020×10^1 9.412×10^0	32.86 32.98	+
NAO-P	Pantanal	Male/adult	+	1.358×10^0 1.673×10^0	38.43 38.11	negative

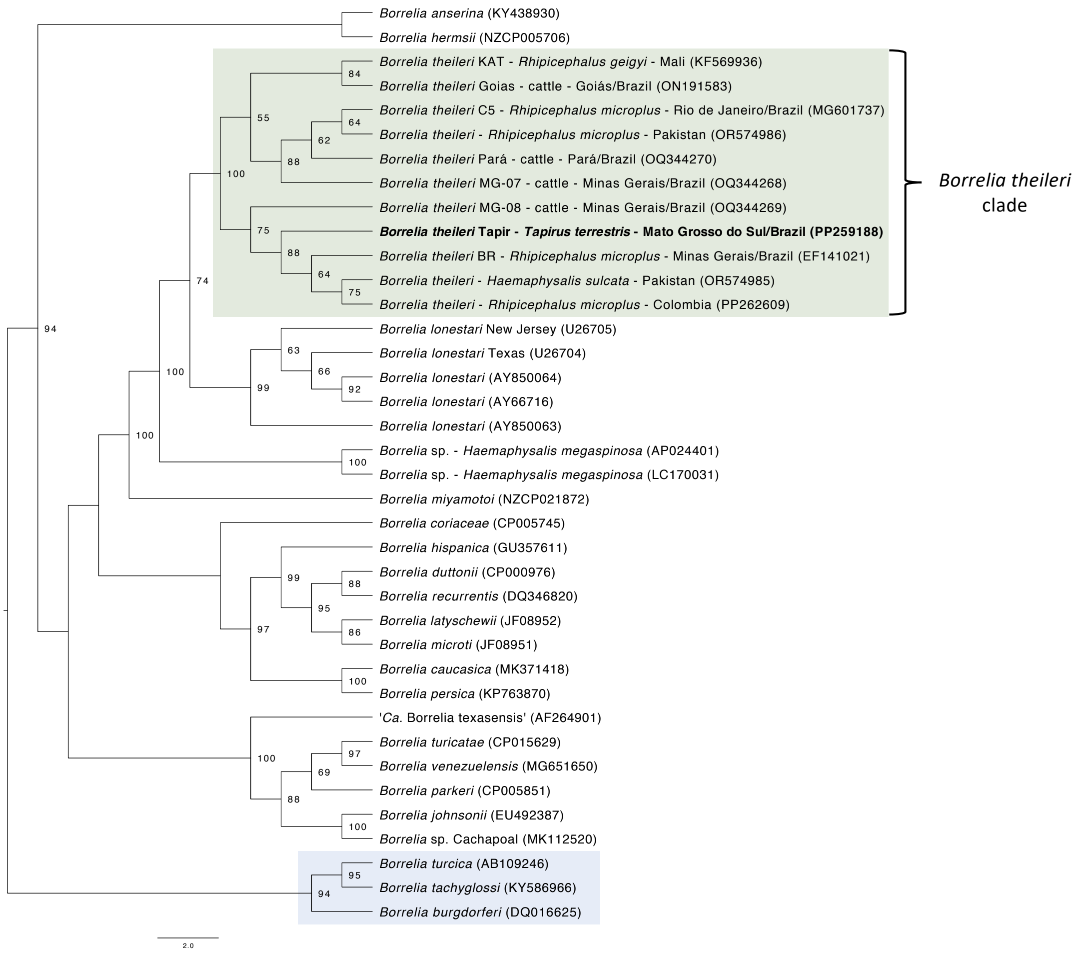

Fig. 1 A Maximum Likelihood tree constructed based on a 615 bp fragment of the *flaB* gene and TPM3u + F + G evolutionary model. Sequence from the present study is highlighted in bold. The clade formed containing *Borrelia theileri* sequences is highlighted in green. Sequences of *B. burgdorferi* (DQ016625), *B. turcica* (AB109246) and *B. tachyglossi* (KY586966) were used as outgroups (highlighted in blue). Only bootstraps values > 50 are shown.

Discussion

We described herein the first evidence of *B. theileri* occurring in a wild animal in Brazil. The lowland tapir is considered the largest terrestrial mammal from Brazil and the last representative of Neotropical mega-herbivores (Thoisy et al. 2010). Although this mammal species is currently found in four different biomes in the country, it is classified as vulnerable for extinction due factors linked to human activities (e.g. illegal hunting, wildfires, road-kills and land competition) (Medici et al. 2012).

Recently, lowland tapirs have been reported as hosts for different blood-borne and vector-borne agents, including *Trypanosoma terrestris* (Acosta et al. 2013), '*Candidatus* Mycoplasma haematoterrestris', '*Candidatus* Mycoplasma haematotapirus' (Mongruel et al. 2022a), piroplasmids (Gonçalves et al. 2020; Silva et al. 2021; Mongruel et al. 2022b) and *Bartonella* sp. (Mongruel et al. 2023). This is the first detection of *Borrelia* spp. in animals of the genus *Tapirus*. Although serological studies conducted in Germany evidenced the exposure to *B. burgdorferi* sensu lato (s.l.) in captive *T. terrestris* (Stoebel et al. 2003), the occur- rence of cross-reactivity between antibodies produced by *B. theileri* and whole-cell *B. burgdorferi* antigens is described (Rogers et al. 1999) and must be considered during the interpretation of serological results using crude antigens. In this scenario, evidence of *B. theileri* exposure may be misinterpreted. In South America, the molecular screening of *B. burgdorferi* s.l. in *Amblyomma* spp. ticks collected from Andean tapirs (*Tapirus pinchaque*) from Ecuador resulted in negative results (Pesquera et al. 2015).

Molecular detection of *B. theileri* in Brazil has only been reported previously in cattle and *R. microplus* ticks. The 1.01% prevalence found in the present study is within the range reported by previous studies, including 1.0- 1.52% for cattle (Paula et al. 2023; Figueiroa et al. 2023), and 0.25-2.0% for *R. microplus* collected from horses and cattle (Yparraguirre et al. 2007; Cordeiro et al. 2018). Furthermore, attempts to characterize additional target genes through cPCR was successful for only one positive sample and one molecular marker (*flaB*). These results might be due the low levels of bacteremia found in the sampled animals, evidenced by the low quantification values estimated by the qPCR assay.

Clinical manifestations associated with *B. theileri* infection are usually non-specific. Classically, this pathogen has been described as a causative of fever (Theiler 1904) and other non-specific clinical signs, such as apathy, rough hair coat, and pallor of mucous membranes in cattle (Sharma et al. 2000; Cordeiro et al. 2018). A study with cattle from Cameroon demonstrated a statically significant correlation between *B. theileri* infection and anemia (Abanda et al. 2019). Moreover, cattle presenting reduced milk produc- tion and food consumption were found to be positive for *B. theileri* in Brazil (Figueiroa et al. 2023). However, animals with subclinical infection have also been reported (Paula et al. 2023). Co-infection with *B. theileri* and other vector-borne agents have been reported reported favoring clinical alterations (Abanda et al. 2019; Figueiroa et al. 2023). The *B. theileri*-positive blood sample from the present study also amplified fragments from hemotropic *Mycoplasma* spp. (hemoplasmas) 16 S rRNA (Mongruel et al. 2022a), but the presence of clinical signs due to this coinfection is unknown.

The tick species *R. microplus* is described as the primary vector for *B. theileri* in South America (Faccini-Martínez et al. 2022). In fact, *R. microplus* collected from cattle and horses from Minas Gerais (Yparraguirre et al. 2007) and Rio de Janeiro (Cordeiro et al. 2018) states and infested cattle from Goiás (Paula et al. 2023), Minas Gerais and Pará states (Figueiroa et al. 2023) were reported to be infected by *B. theileri* in Brazil. Moreover, this tick species has also been reported infesting tapirs from Pantanal, as a consequence of land sharing between tapirs and cattle (Labruna et al. 2021). The phylogenetic analysis confirmed the identity of the sequence detected within the *B. theileri* clade. Moreover, this clade was divided in two minor clades, with high support value (100): one containing *B. theileri* strain C5 (MG601737) and related sequences, and other one containing *B. theileri* strain BR (EF1401022) and related sequences, including the sequence obtained from a tapir. The sequence from strain *B. theileri* C5 was obtained from an engorged female *R. microplus* hemolymph. The tick was collected from apparently healthy bovines maintained in Rio de Janeiro State, Brazil (Cordeiro et al. 2018). Sequence from *B. theileri* BR strain was obtained from *R. microplus* collected from a horse in Minas Gerais State, Brazil (Ypar- raguirre et al. 2007). This pattern of two major clades being formed was also observed in phylogenetic reconstructions inferred before using this same gene fragment (Paula et al. 2023; Figueiroa et al. 2023; Khan et al. 2023). More studies are necessary to understand if there are any differences among strains regarding the division of the *B. theileri* clade observed here.

We report the first documented occurrence of *B. theileri* in a wild tapir from South America. This finding contributes to our knowledge regarding susceptible hosts species for *B. theileri*. More studies are necessary to understand the potential effects of *B. theileri* on tapir's health.

Acknowledgements This study is part of Anna Mongruel's PhD the- sis. She is carrying out her research at Programa de Pós-Graduação em Medicina Veterinária—Universidade Estadual Júlio de Mesquita Filho (UNESP/Jaboticabal) and is sponsored by a fellowship from Funda- ção de Apoio à Pesquisa do Estado de São Paulo (FAPESP– Process #2019/26403-0) at the time of this study. Authors would like to thank the Lowland Tapir Conservation Initiative (LTCI)–Instituto de Pes- quisas Ecológicas (IPÊ) for kindly providing the biological samples needed for the present study. The study of tapir health has been an important component of the long-term activities of the LTCI-IPÊ in Brazil. The LTCI has the institutional support from the International Union for Conservation of Nature (IUCN) Species Survival Commission (SSC) Tapir Specialist Group (TSG), Association of Zoos and Aquariums (AZA), Tapir Taxon Advisory Group (TAG), and European Association of Zoos and Aquariums (EAZA). This work was supported by FAPESP - Process #2020/12037-0 and #2022/08543-2) and CNPq (National Council for Scientific and Technological Development; Productivity Grant to MRA - Process #303701/2021-8).

Author contributions Authors Contribution statement Anna Claudia Baumel Mongruel: Conceptualization, Methodology, Investigation, Formal analysis, Writing – Original Draft, Writing – Review & Editing. Emília Patrícia Medici: Methodology, Conceptualization, Re- sources, Writing – Review & Editing. Ariel da Costa Canena: Methodology, Writing – Review & Editing. Rosangela Zacarias Machado: Investigation, Writing – Review & Editing. Keith Clay: Methodology, Resources, Writing – Review & Editing, Supervision. Marcelo Bahia Labruna: Writing – Review & Editing. Marcos Rogério André: Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision.

Funding Anna Mongruel is sponsored by a fellowship from Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP – Process #2019/26403-0) at the time of this study. This work was supported by FAPESP - Process #2020/12037-0 and #2022/08543-2) and CNPq (National Council for Scientific and Technological Development; Productivity Grant to MRA - Process #303701/2021-8). LTCI's financial support comes from national and international agencies, including zoological institutions, foundations, private businesses, and private individuals.

Data availability: The dataset generated and analyzed during the current study is available in the NCBI GenBank Nucleotide platform (https://www.ncbi.nlm.nih.gov/genbank/) and can be accessed through accession number: PP259188.

Declarations

Ethics approval The study was approved by the Ethics Committee for Animal Experimentation of FCAV/UNESP (Faculty of Agricultural and Veterinary Sciences of the São Paulo State University) under protocol number 4558/20. The "Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO)" provided the required annual permits for the capture and immobilization of tapirs and collection of biological samples (SISBIO# 14,603). All protocols for the capture, anesthesia, handling, and sampling of tapirs have been reviewed and approved by the Veterinary Advisors of the Association of Zoos and Aquariums (AZA) — Tapir Taxon Advisory Group (TAG), and the Veterinary Committee of the IUCN SSC Tapir Specialist Group (TSG). Tapir blood DNA samples from the present study were registered in the Brazilian National System for Management of Genetic Heritage and Associated Traditional Knowledge (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado – SIS- GEN) under register number AE4CC0C.

Competing interests The authors declare no competing interests.

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CAPÍTULO VIII* – Investigating the blood bacterial microbiome of wild lowland tapirs (*Tapirus terrestris*)*

*Esse capítulo corresponde à publicação que será submetida ao periódico Veterinary Microbiology

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Abstract: Microbiome studies targeting hypervariable regions from the 16S rRNA gene are suitable for investigations of different interactions between animals and their associated microbes. Although many studies have focused on the gut microbiome of vertebrate hosts, microbiome assessment of blood samples are scarce despite the variety of blood-borne pathogens in vertebrates. The overarching goal of the present study was to investigate the bacterial community present in blood samples from 79 living and 07 road-killed wild tapirs sampled in two different biomes in midwestern Brazil, namely Pantanal and Cerrado. Animals were identified regarding condition (living or dead), sex (female or male), age (adult, sub-adult or juvenile) and site (Pantanal or Cerrado). Fragments from the V3-V4 hypervariable region of the bacterial 16S rRNA were obtained from 86 samples and four negative controls. After identification of contaminants and non-identified sequences, a total frequency of

14,523,219 sequences, divided in 2,448 ASVs were found. When considering all samples (obtaining from living and road-killed animals), alpha and beta diversity only presented significant differences for condition. When only samples from living animals (79/86; 91.86%) were analyzed, alpha diversity differed significantly by site but not beta diversity. Several potential vector-borne bacterial pathogens were found in tapir blood in the present study, such as Anaplasmataceae, *Bartonella* and *Borrelia* sp. Additionally, evidence of transient translocation of microbial communities from other parts of the body to the bloodstream was also observed. Amplification of bacterial 16S rRNA from blood samples of wild *T. terrestris* provided information about the diversity of blood-borne microbiota of lowland tapirs. Moreover, NGS demonstrated to be a useful tool for the screening of potential vector-borne pathogens in this species that is currently classified as vulnerable to extinction.

Key-words: Brazilian tapir, NGS, wild animals, microbiota

1.Introduction

Research focusing on interactions between microorganisms and animal hosts has increased significantly in the last decades, including studies that investigates the host's microbiome. The microbiome is defined as a characteristic microbial community that occupies a well-defined habitat with characteristic physico-chemical properties, which can include eukaryotic hosts as a macroecosystem in which the microbiome is integrated. The microbiome is not limited to the microorganisms involved, but also encompasses their specific ecological niches (Berg et al., 2020). The relationship between a certain microbial community and its host is described as one of the most complex and intimate biological communities (Christian et al., 2015).

Studies on microbiomes are increasing in part due to the growing access to new technologies such as next generation sequencing (NGS) and computational tools for bioinformatic analyses (Cullen et al., 2020). The use of NGS is particularly advantageous given that it can identify non-cultivable species, especially when there are no prior assumptions regarding the bacterial community present in a certain biological system (Galan et al., 2016). Both whole genome sequencing (WGS) and sequencing of informative regions from the 16S ribosomal (rRNA) gene can be used for microbial identification. In fact, rRNA genes are considered universal and conservative targets useful for bacteria discovery and identification (Petrosino et al., 2019).

In non-domesticated wild animals, microbiome studies are often linked to efforts focused on species conservation. The study of oral and/or gut microbiota of critically endangered species, such as amphibians (Mann et al., 2021), rhinoceros (Gibson et al., 2019) and birds (Ran et al., 2021; West et al., 2022) has been used for elucidating how dietary and environmental changes may affect microbiome composition and host health. Moreover, surveillance of pathogens in these animals is fundamental for identifying or preventing the emergence of infectious diseases in humans and domestic and wild animals (Galan et al., 2016). Although many studies have focused on the gut microbiome of vertebrate hosts (Gibson et al., 2019; Mann et al., 2021; Ran et al., 2021; West et al., 2022; Jonge et al., 2022; Tang et al, 2022), microbiome assessments on blood samples are scarce (Gavish et al., 2014; Fronton et al., 2023).

The genus *Tapirus* (Family Tapiridae, Order Perissodactyla) comprises four described species. One species (*Tapirus indicus*, Desmarest, 1819) occurs in Asia while the other three occur in Central and South America (*Tapirus pinchaque* [Roulin, 1829], *Tapirus terrestris* [Linnaeus, 1758], and *Tapirus bairdii* [Gill, 1865]) (Ashley et al., 1996, García et al., 2012). Currently, the species *T. indicu*s, *T. pinchaque,* and *T. bairdii* are considered endangered, while *T. terrestris* is considered vulnerable to extinction (Traeholt et al., 2016; Lizcano et al., 2016; García et al., 2016; Varela et al., 2019). The lowland tapir (*Tapirus terrestris*, Linnaeus, 1758; Family Tapiridae, Order Perissodactyla) can be found in different countries from South America, including Argentina, Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Peru, Suriname and Venezuela (Varela et al., 2019). Despite the fact that this species can be found in four biomes throughout Brazil (Pantanal, Cerrado, Amazon and Atlantic Forest), it is classified as vulnerable throughout the country by assessing the risk of extinction in consequence mainly due human actions (such as hunting, domestic livestock production, wild fires and road-kills) (Medici et al., 2012).

The overarching goal of the present study was to investigate the bacterial community present in blood samples from wild tapirs sampled in the Pantanal and Cerrado biomes in midwestern Brazil by amplification and sequencing of V3-V4 hypervariable region of the bacterial 16S rRNA. Our results add to our understanding of the blood microbiome of wild mammals and vulnerable species.

2. Material and methods

2.1. Sampling

From 2013 to 2018, blood samples from wild *T. terrestris* were collected from 94 living and 8 road-killed individuals, totaling 126 samples (some living animals were sampled more than once at different times). Out of this, 78 (79/126; 61.90%) blood samples were collected from 61 tapirs (61/94; 64.89%) in the Pantanal wetlands and 40 samples (40/126; 31.74%) were collected from 33 tapirs (33/94; 35.11%) from the Cerrado biome. Additionally, several road-killed (8/126; 6.34%) tapirs were sampled in Cerrado biome, for a total of 41 individuals from this biome. Both biomes and study areas are located in Mato Grosso do Sul State, central-western Brazil (**Figure 1)**.

Blood sampling was performed during tapir anesthesia for the installation of GPS collars by professionals from the "Iniciativa Nacional para a Conservação da Anta Brasileira (INCAB-IPE)" (Lowland Tapir Conservation Initiative (LTCI-IPE)). Samples (up to 2 mL each) were maintained in -20°C freezer until further analysis. All procedures were approved by the Ethics Committee for Animal Experimentation of FCAV/UNESP (Faculty of Agricultural and Veterinary Sciences of the São Paulo State University) under protocol number 4558/20. The "Instituto Chico Mendes de Conservção da Biodiversidade (ICMBIO)" provided the required annual permits for the capture and immobilization of tapirs and collection of biological samples (SISBIO# 14,603).

All protocols for the capture, anesthesia, handling, and sampling of tapirs have been reviewed and approved by the Veterinary Advisors of the Association of Zoos and Aquariums (AZA)— Tapir Taxon Advisory Group (TAG), and the Veterinary Committee of the IUCN SSC Tapir Specialist Group (TSG). Tapir blood DNA samples from the present study were registered in the Brazilian National System for Management of Genetic Heritage and Associated Traditional Knowledge (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado – SISGEN) under register number AE4CC0C.

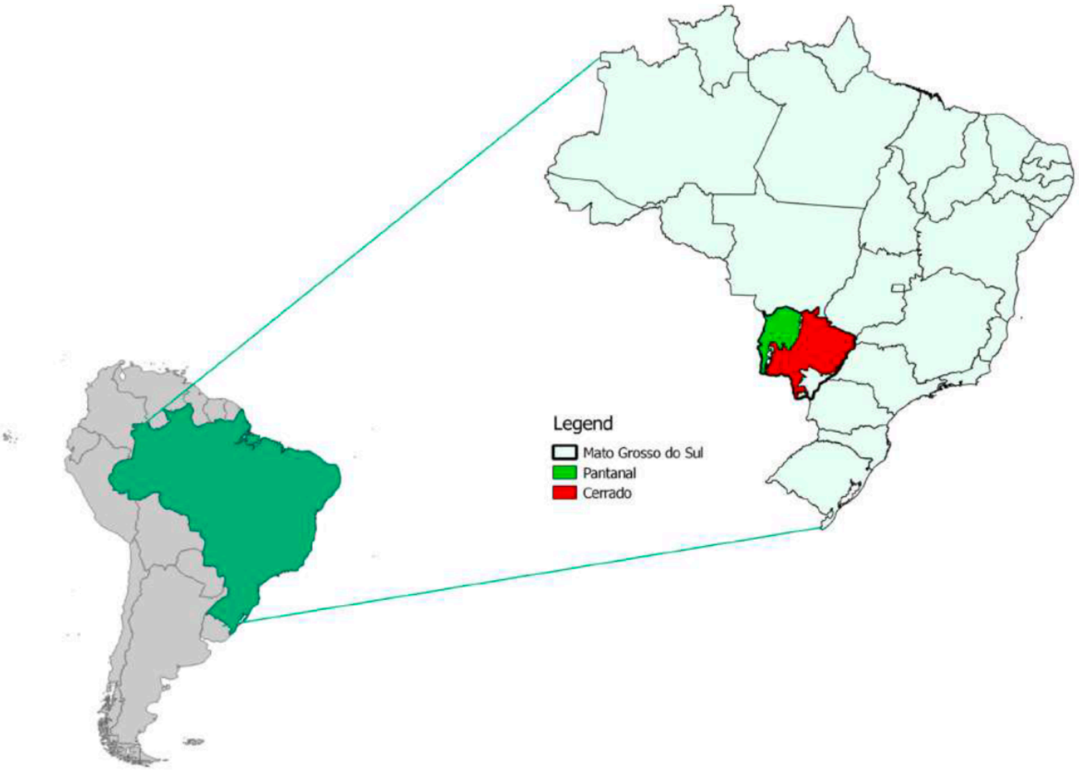

Figure 1. Map of Brazil (highlighted in South America) showing the location of Pantanal and Cerrado biomes within Mato Grosso do Sul State.

2.2. DNA extraction

DNA extraction procedures were performed at the Vector-Borne Bioagents Laboratory, UNESP, Jaboticabal (Brazil), using a commercial kit (DNeasy Blood and Tissue Kit Mini Spin, Qiagen, Hilden, Germany), and following manufacturers' instructions. The kit was opened and used exclusively for processing the samples from the present study. In order to monitor potential contaminations, DNA extraction procedures were performed in six different rounds and two negative controls were included in each round (totaling 12 negative controls). One negative control from each round contained only reagents from the extraction kit (without any blood sample). The other negative control of each round comprised ultra-purified sterilized water (Nuclease-Free Water, Promega®, Madison, Wisconsin, United States) in addition to extraction kit reagents. Prior to each extraction round, the working bench was cleaned with three different solutions in the following order: enzymatic detergent (Zymedet

Gold, Prolink, Guapiaçu, SP, Brazil), 70% ethanol, and 10% sodium hypochlorite (bleach). Additionally, the entire room was exposed to UV light for 15 minutes prior DNA extraction. DNA concentration of each sample was measured using a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and samples with a minimum concentration of 12 ng of DNA/sample (average volume of DNA per sample of 38 µL) were selected for sequencing.

2.3. Sequencing

The DNA samples were shipped from Brazil to New Orleans (Louisiana, US) (U.S. FWS permit #MAPER5230668). Library preparation of the 16S rRNA V3-V4 regions were performed at the LSU Translational Genomics Core using Illumina^{TM} MiSeq equipment (San Diego, California, United States). The primer sequences used to amplify an approximately 450 pb fragment of the bacterial 16S rRNA gene were Bakt_314F 5'- CCTACGGGNGGCWGCAG –'3 and Bakt_805R 3'- GACTACHVGGGTATCTAATCC -5' (Herlemann et al., 2011). Illumina sequencing adapters and dual-index barcodes were added using full complement of Nextera XT indices (Illumina, Inc. San Diego, CA, USA), following the instructions proposed by the Illumina protocol (Illumina, 2013). The pooled final DNA library was sequenced using paired 300-bp reads that overlap in their ends, generating high quality, full-length reads for the targeted V3 and V4 regions. Run outputs typically exceeded 20 million reads and, considering 96 indexed samples, more than 100,000 reads per sample were expected to be produced, which is commonly recognized as adequate for metagenomic surveys (Illumina, 2013).

2.4. Bioinformatics data processing

Demultiplexed raw fastaq files were processed using QIIME2 (Quantitative Insights into Microbial Ecology) (Boylen et al., 2019). Sequence trimming, primer removal, denoising, and singleton removals were performed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) implemented in QIIME2. The classifier was trained using the Greengenes 13_9 97% OTU database (McDonald et al., 2012) to classify sequences. Removal of contaminants was performed using the package microDecon on R software (McKnigth et al., 2019) and the plugin "filtering" on QIIME2 based on previous reports of contaminants present in low biomass microbiome studies

(Paniagua-Voirol et al., 2021). Data compilation and graphics were performed using Microsoft Excel software.

2.5. Diversity and statistical analyses

Diversity measures and statistical significance were calculated using the plugin "diversity" on QIIME2 (Bolyen et al., 2019). Diversity analyses were divided in two sections: 1. All samples (living and road-killed animals); and 2. Living animals only. For both sections, rarefaction curves were constructed in order to avoid any bias caused by variations in sequencing depth. For alpha diversity, both richness and evenness were calculated using Shannon's metrics and statistical significance was computed using Kruskall-Wallis (significance was considered when p-value < 0.05). For beta diversity, the Principal Coordinate Analysis (PCoA) was used to construct an ordination plot, together with Weighted Unifrac, for the calculation of dissimilarity of the microbial communities. Community analyses, using Non-metric multidimensional scaling (NMDS) (Clarke, 1993), were also performed using the package “vegan” v2.6-2 (Oksanen et al., 2013) and package “phyloseq” v.1.4 (McMurdie and Holmes, 2013) on R software. Moreover, ASVs from families related with vector-borne agents were compared using BLASTn tool (https://blast.ncbi.nlm.nih.gov/) in order to verify similarities between the obtained sequences and those deposited in GenBank database.

3. Results

3.1. Obtained sequences

From 89 tapir blood DNA samples and 12 negative controls subjected to NGS, fragments from the V3-V4 hypervariable region of the bacterial 16S rRNA were successfully obtained from 86 samples (86/89; 96.62%) and four (4/12; 33.33%) negative controls.

Regarding the negative controls that generated sequences, two were composed of reagents and water and the remaining two of reagents only. Regarding the samples that were successfully sequenced, 46 were obtained in Pantanal biome and 40 were obtained in Cerrado biome. **Table 1** describes the total number of samples obtained for each category (site, sex and age) from all samples combined. Moreover, samples of living animals only were described separately.

Table 1. Description of samples successfully sequenced with percentages from each category. Samples were divided into two groups: all samples (living and road-killed animals) and samples from living animals only.

Category		**All samples (living and road-killed animals)**	**Samples from living animals only**
Site	Pantanal	46 (46/86; 53.49%)	46 (46/79; 58.22%)
	Cerrado	40 (40/86; 46.51%)	33 (33/79; 41.77%)
Sex	Female	39 (39/86; 45.34%)	36 (36/79; 45.57%)
	Male	47 (47/86; 54.66)	43 (43/79; 54.43%)
Age	Adult	44 (44/86; 51.16%)	40 (40/79; 50.63%)
	Sub-adult	33 (33/86; 38.37%)	31 (31/79; 39.24%)
	Juvenile	09 (9/86; 10.46%)	08 (8/79; 10.12%)
Total		86	79

3.2. Primer removal, denoising and filtering of the obtained sequences

Primers were successfully removed from all sequence reads. Forward and reverse sequences were trimmed in lengths of 260 bp and 199 bp, respectively, and an overlap of 15 bp was obtained after construction of the contigs. A total of 31,769 amplicon sequence variants (ASVs) were identified, with a total of 32,555,385 sequences obtained and a mean value per sample (MSD) of 361,727. After identifying and filtering mitochondrial and unidentified sequences from the database, the total number of ASVs dropped to 5,367, with a total of 30,525,022 sequences and an MSD of 339,167. A table with the frequency of each ASV per sample was created and exported to R software for analysis using the microDecon package. The package identified and removed contaminant sequences, based on sequences and their frequencies found in negative controls and cross-contamination. Also, negative controls were removed from any further analyses. The number of total ASVs then dropped to 5,216 with 17,574,253 total sequences and an MSD of 204,352. For removal of additional contaminants, the dataset was initially filtered based on a list of taxa reported as contaminants in microbiome studies of low biomass samples. Moreover, taxa found in the present study were also manually reviewed for the identification of potential contaminants. The list of removed ASVs by microDecon and all key-words included in Qiime2 filtering command are presented in **Supplementary File 1** and **Supplementary File 2**, respectively. After filtering out contaminants, the final total number of ASVs obtained was 2,448 with a total of 14,523,219 sequences and an MSD of 168,875.

3.3. ASV diversity

Representatives from phyla Pseudomonadota, Bacillota, Mycoplasmatota, Bacterioidota and Actinomycetota were the dominant taxa present in the analysis. For a better visualization of the most frequent taxa found herein, the 10 most frequent taxa (top 10) were highlighted and compared to the remaining found taxa in each group (all samples, alive animals, road-killed animals). Pie charts were used to represent the frequency distribution (**Figure 2).** A taxonomic bar plot (**Figure 3**) was also generated to illustrate the relative frequency of the 20 most frequent ASVs detected in all samples after the identification and removal of contaminant sequences. Sequences were identified until taxonomic rank number 5 (Family). Samples in the taxonomic bar plot are identified as numbers. A sheet coitaining the identification of samples regarding sex, age, biome and condition is avaible in **Supplementary File 3**.

Aeromonadaceae and Anaplasmataceae were the two most frequent bacterial groups found when all samples were analyzed together. The top 10 most frequent ASVs represented 90% of all ASVs found in all samples. Aeromonadaceae and Anaplasmataceae were also the two most frequent taxa groups found in samples from living animals. The top 10 ASVs represents 93% of all ASVs found in these samples. The top 10 from all samples and living animals' samples were very similar. By contrast, Lactobacillaceae was the most frequent taxonomic group found in samples from road-killed animals. The top 10 ASVs represented 47% of all ASVs found in road-killed samples. The composition of the top 10 ASVs from road-killed animals' samples was different than the top 10 composition found when all samples were analyzed together and when samples from living animals only were analyzed. When compared with samples from living animals, the second most frequent taxa group (Anaplasmataceae) did not appear in the top 10 in road-killed animal samples. The most frequent taxa group for living animal samples (Aeromonadaceae) appeared as the 9th most frequent taxa group for road-killed animal samples.

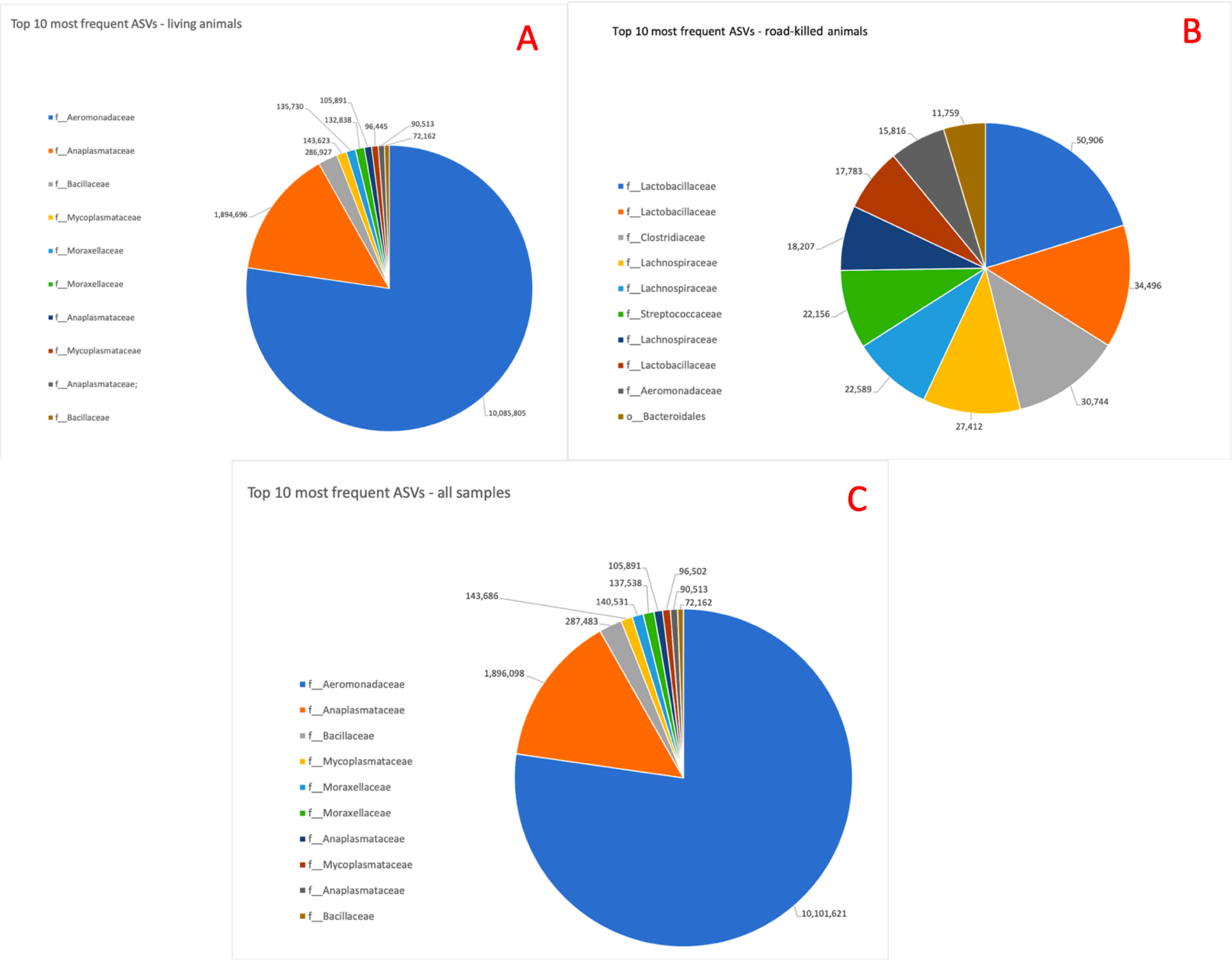

Figure 2. Representation of the top 10 ASVs from samples of living animals (A), road-killed animals (B) and all samples (living and road-killed) (C).

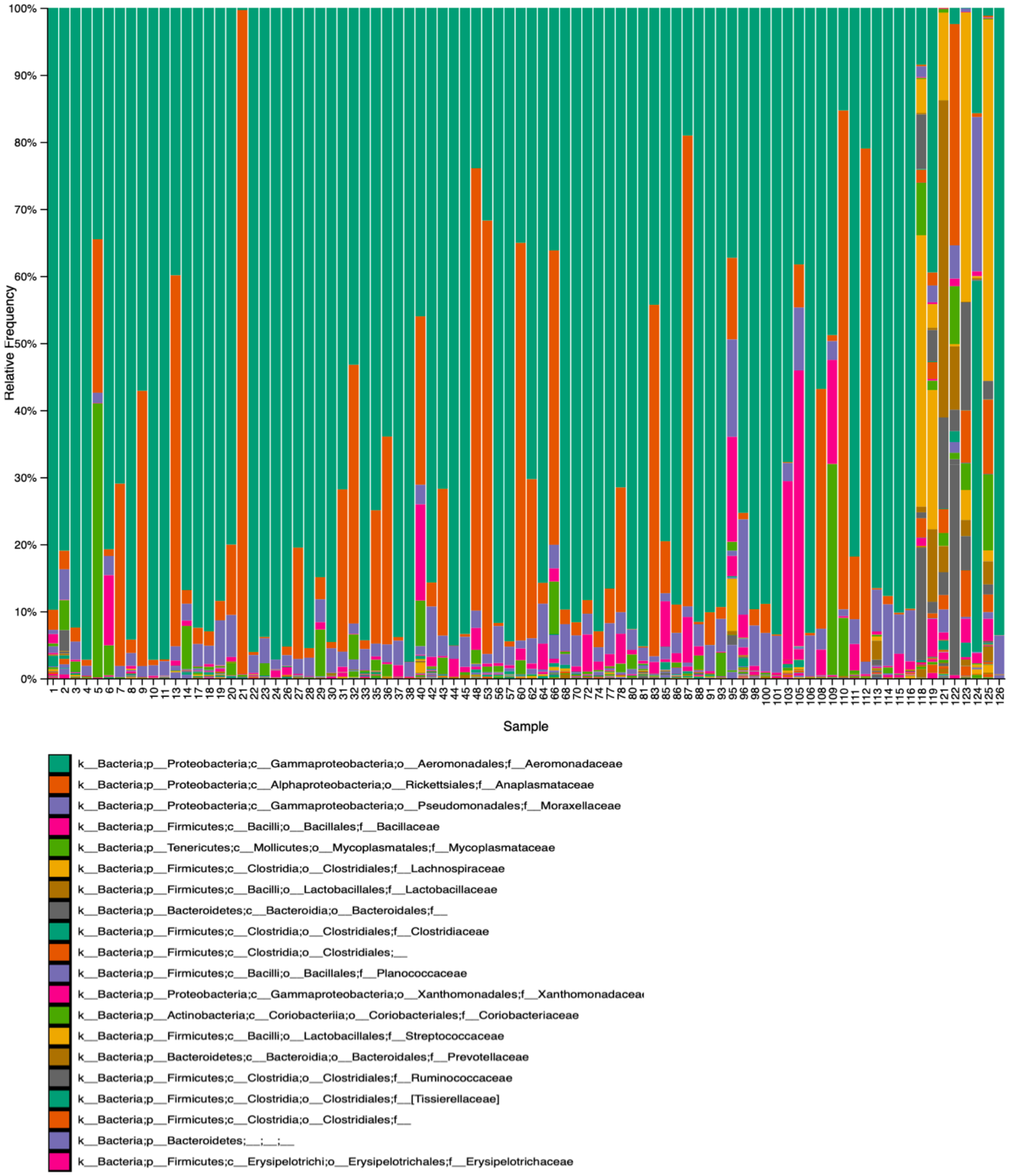

Figure 3. Taxonomic bar plot demonstrating the relative frequency of the 20 most frequent identified taxa in analyzed samples (living and road-killed tapirs).

3.4. Diversity analysis of all samples - alpha diversity

The maximum sample depth obtained for this analysis was 459,082. The rarefaction value of 83,109 was chosen for alpha diversity measurements once it retained 40.06% of the total frequency of ASVs (5,871,630) in 81.40% (n=70) of the samples at the specified sample depth. Shannon's entropy metric was chosen to evaluate both species richness and evenness. From all variables analyzed (site, sex, age and condition), the only statistically significant variable was condition (living or road-killed animals) (*p*-value = 0.003565). The bar plot for this result is showed in **Figure 4**, together with the obtained *p*-value (**Table 2**). The variables sex, age and site were not statistically significant for the Shannon's metric. Based on the obtained result, the number of different organisms and how evenly distributed these organisms are in terms of abundance within the samples were significantly different between samples from living and road-killed animals.

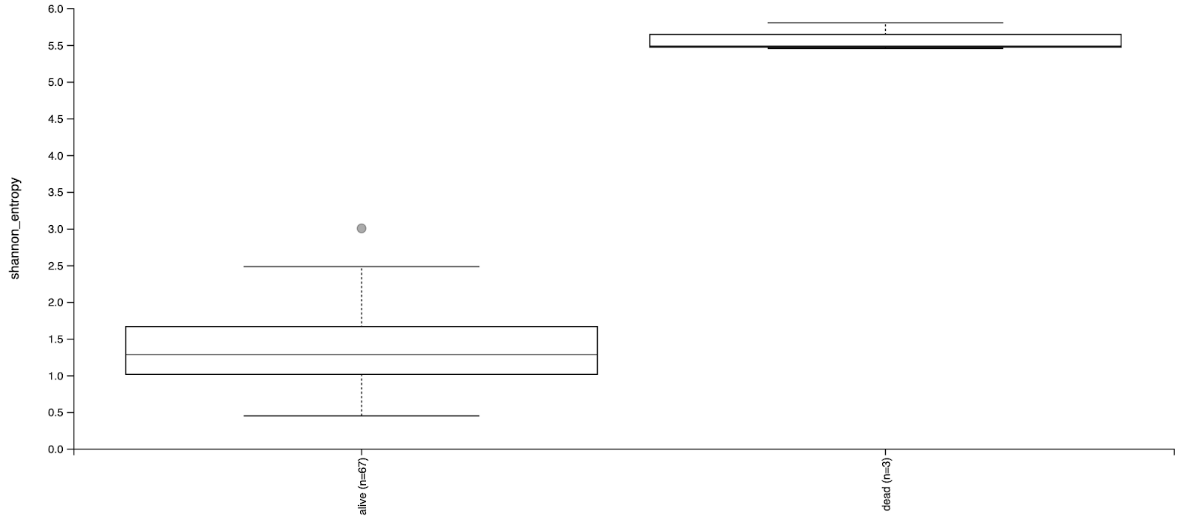

Figure 4. Shannon's metric bar plots comparing the obtained values of alpha diversity between blood samples of living and road-killed animals.

Table 3. Statistical analysis comparing the Shannon index for alpha diversity between blood samples obtained from living and road-killed tapirs (values were considered significant when *p*-value < 0.05).

Group 1	Group 2	H	*p*-value	q-value
Living (n=67)	Road-killed (n=3)	8.492958	0.003565	0.003565

3.4.2. Diversity analysis of all samples - beta diversity

The same rarefaction point (83,109) was used for beta diversity analysis. The Principal Coordinate Analysis (PCoA) was used together with Weighted Unifrac for the calculation of distances for the communities in beta diversity analysis. **Figure 5** demonstrates the distances regarding the variable sex. Statistical analysis of beta diversity was performed using the Permutational Multivariate Analysis of Variance test (PERMANOVA) to calculate if distances were significantly different among groups. For this analysis, the only statistically significant difference was found for condition (living vs. road-killed) (*p*-value = 0.001) (**Figure 6, Table 3**). The variables sex, age and site did not show statistically significant differences.

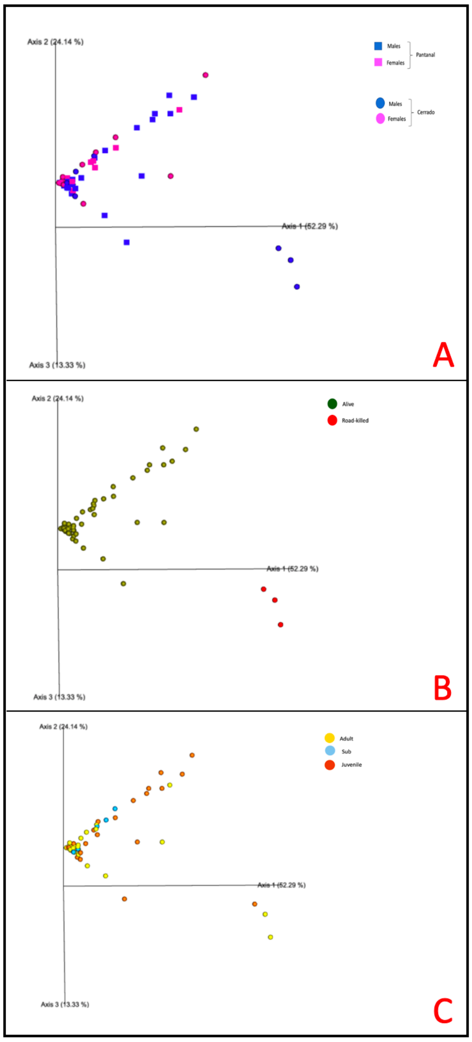

Figure 5. Weighted Unifrac distances plot for the distribution of the analyzed samples. Samples from females are highlighted in pink whereas samples from males are highlighted in blue. **A**: Samples from Pantanal are represented by squares whereas

samples from Cerrado are represented by spheres; **B**: Samples from living animals are highlighted in green while samples from road-killed animals are highlighted in red; **C**: Samples from adult animals are highlighted in yellow, samples from sub-adult animals are highlighted in blue, and samples from juvenile animals are highlighted in orange.

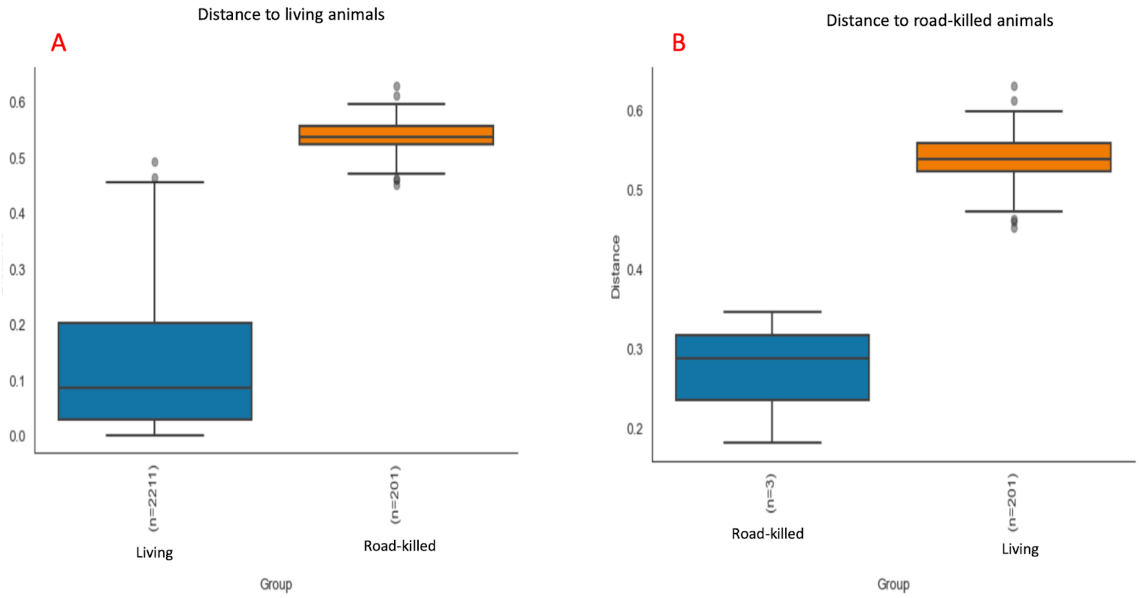

Figure 6. Bar plots comparing the distances between bacterial communities found in blood samples of living and road-killed animals. Plot A computes the distances of communities found in blood samples within living animals and between living and road-killed animals. Plot B computes the distances of communities found in blood samples within road-killed animals and between road-killed animals and living animals.

Table 3. Statistical analysis comparing the distances calculated using Pairwise PERMANOVA test between bacterial communities found in blood samples obtained from living and road-killed tapirs.

Group 1	**Group 2**	**Sample size**	**Permutations**	**pseudo-F**	***p*-value**	**q-value**
Living	Road-killed	70	999	47.457612	0.001	0.001

3.5. Diversity analysis of living animals' blood samples – alpha diversity

As described above, the composition of bacterial communities presented in blood samples from road-killed animals appeared to be significantly different from those obtained from blood samples from living animals. It is likely that this difference results from decomposition and bacterial proliferation in road-killed animals. For this reason, sequences from road-killed animals were filtered out and additional analysis were performed with samples from only live animals to test if the other variables may influence the alpha and beta diversity metric. A total of 13,986,663 sequences were retrieved from 79 samples, representing 1,527 ASVs. The MSD value obtained was 117,046 with minimum and maximum sample depth values of 10,116 and 459,082, respectively.

3.6. Diversity analysis of living animals' blood samples – alpha diversity

The maximum number of sequences per sample obtained in this analysis was 459,082. The rarefaction value of 89,178 was chosen for alpha diversity measurements once it retained 40.81% of the total frequency of ASVs (5,707,392) in 81.01% (n=64) of the samples at the specified sample depth. The Shannon's entropy metric was chosen to evaluate both species richness and evenness in this analysis. From all variables analyzed (site, sex and age), the only one exhibiting statistical significance for alpha diversity was site (*p*-value = 0.027459). The bar plot for this result is showed in **Figure 7**, together with the obtained *p*-value (**Table 4**). The variables sex or age did not show statistically significant differences for Shannon metric.

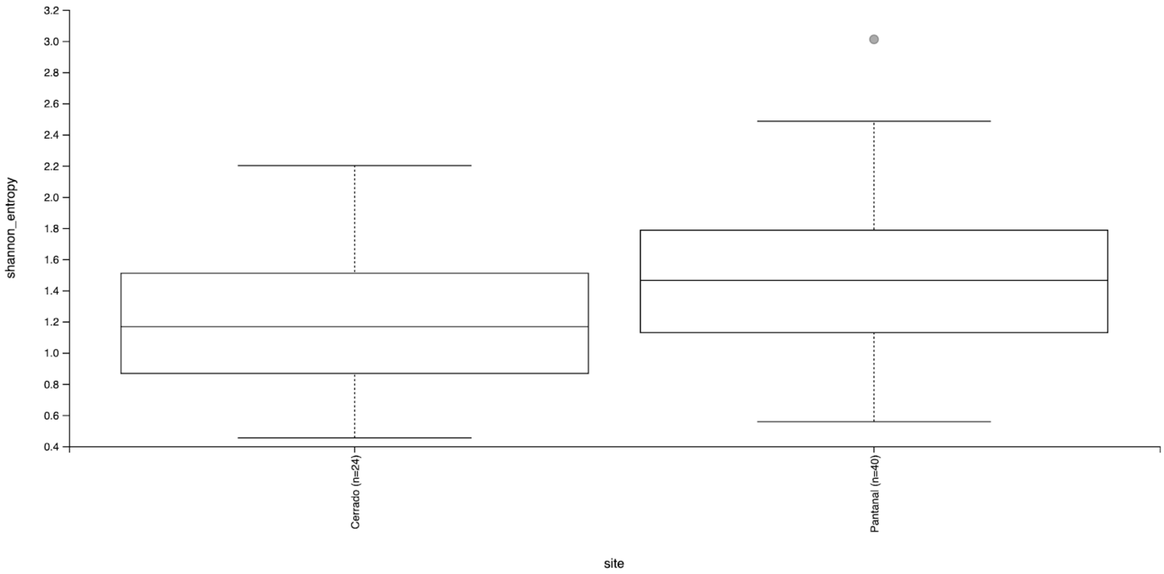

Figure 7. Shannon entropy bar plots comparing the obtained values of alpha diversity between the bacterial community found in blood samples of animals sampled in Cerrado or Pantanal biome.

Table 4. Statistical analysis based on the Shannon metric for alpha diversity between the bacterial community found in blood samples from living and road-killed tapirs (values were considered significant when *p*-value < 0.05).

Group 1	Group 2	H	*p*-value	q-value
Cerrado (n=24)	Pantanal (n=40)	4.861731	0.027459	0.027459

3.7. Diversity analysis of living animals' blood samples – beta diversity

The same rarefaction point (83,109) was used for beta diversity analysis. The Principal Coordinate Analysis (PCoA) was used as ordination approach together with Weighted Unifrac for the calculation of distances of the communities in beta diversity analysis. **Figure 8** demonstrate the distance regarding the variable sex. Statistical analysis was performed using the Permutational Multivariate Analysis of Variance test (PERMANOVA) to calculate if distances were significant among groups. For this analysis, none of the variables (sex, age or site) showed statistically significant differences (*p*-value <0.05).

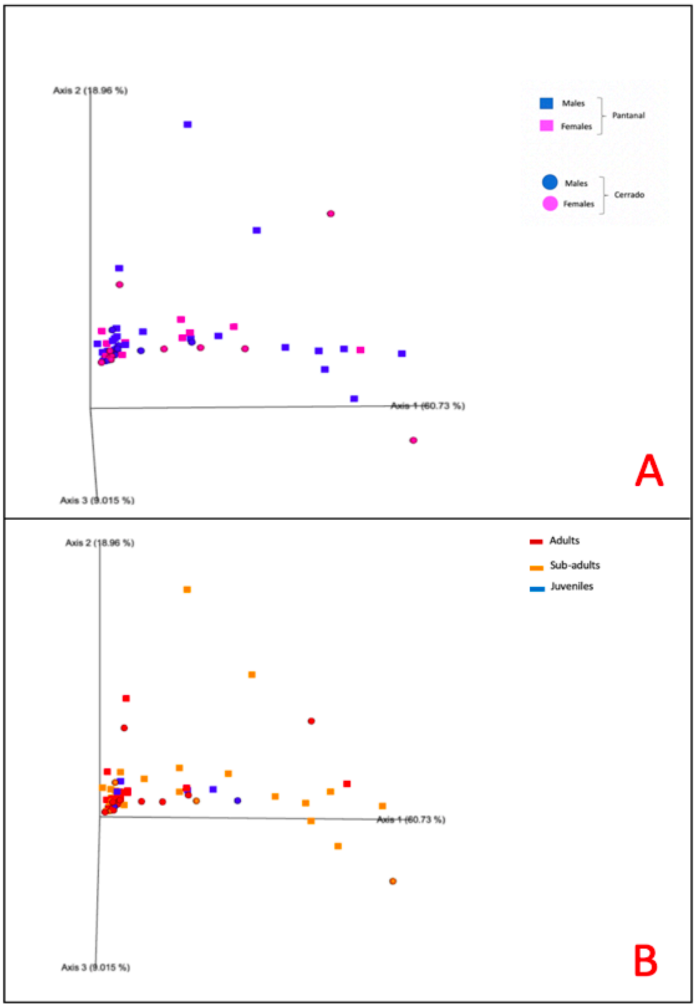

Figure 8. Weighted Unifrac distances plot for the distribution of the bacterial community found in living animals' blood samples. **A**: Samples from females are highlighted in pink whereas samples from males are highlighted in blue. **B**: Samples from adult animals are highlighted in red, samples from sub-adult animals are highlighted in orange and samples from juvenile animals are highlighted in blue. Samples from Pantanal are represented by squares whereas samples from Cerrado are represented by circles.

3.8. Diversity analysis of living animals' blood samples - Non-metrical multidimensional scaling (NMDS)

A NMDS analysis was performed to analyze the distribution of points and community composition based on the relative frequencies of ASVs in each sample and the variables using Bray-Curtis dissimilarity and Hellinger standardization, on vegan package, and the ordiellipse function on R software (**Figure 9**). All generated ellipses were largely overlapping, demonstrating that there is no correlation among variables and beta-diversity, which corroborates with the results obtained in the previous analyses. The stress metric obtained from the analysis was 0.1468507, which is within a satisfactory rate (<0.2), and the best solution was repeated 1 time in 26 tries.

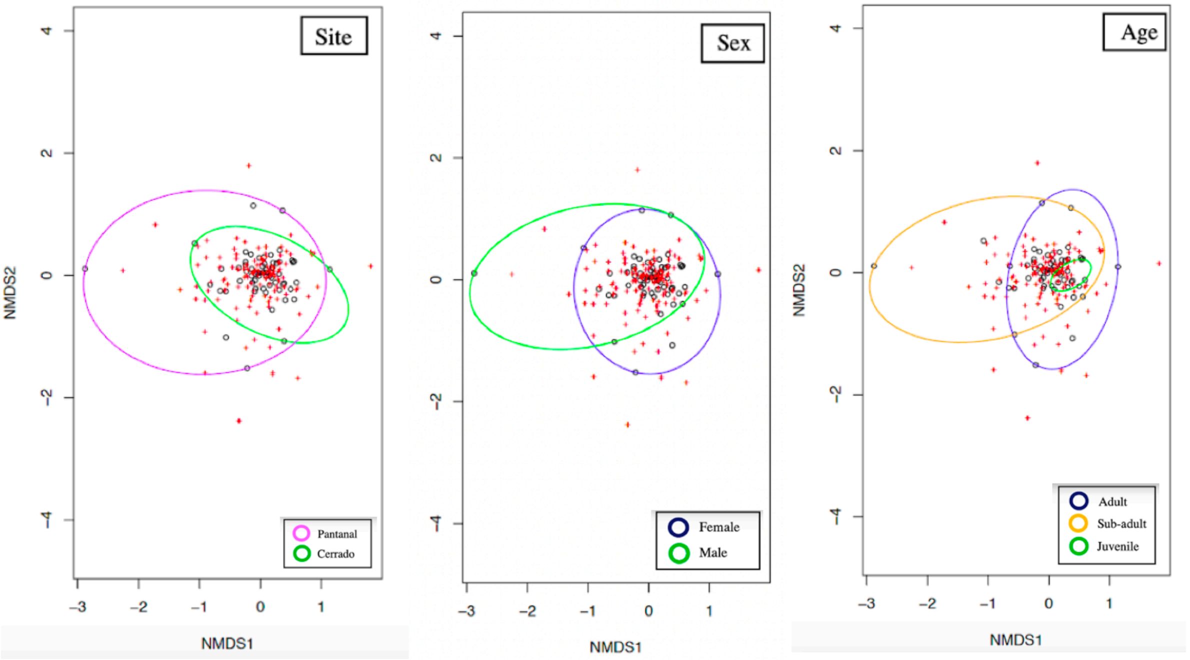

Figure 9. NMDS analysis performed using Bray-Curtis dissimilarity metric and Hellinger standardization on R software. The ellipses generated based on each variable (site, sex and age) seemed to collapse, indicating no differences among the groups.

5. Discussion

We report the first information about the blood bacterial microbiome of lowland tapirs, a species considered vulnerable to extinction in Brazil. Initially, sequencing recovered 32,555,385 sequences, 31,769 ASVs, and MSD of 361,726 for a dataset of 86 samples and four negative controls. After identification and removal of contaminant sequences, these counts dropped to a total frequency of 14,523,219 sequences, 2,448 ASVs, and MSD of 168,874.6 for the dataset of 86 samples. In comparasion, a study using blood samples from camels (*Camelus dromedarius*) from Sudan described the recovery of a total of 403,680 sequences, 3,772 ASVs, and an average of 21,246 sequences per sample (MSD). After the identification and filtering out contaminant sequences, 333,054 sequences, 2,372 ASVs, and an MSD of 18,503 for the dataset of 18 camels' blood samples was found (Mohamed et al., 2021). Comparing both studies, Mohamed et al. (2021) obtained a drop of 17.5%, 37.12% and 12.92% for total frequency, number of ASVs and MSD, respectively, after the removal of contaminant sequences. In our study, we obtained a drop of 55.39%, 92.3% and 53.32% for total frequency, number of ASVs and MSD, respectively, after the removal of contaminant sequences. In our study, we obtained higher sequence counts before and after the identification of contaminants. These differences may be explained by the difference in the number of analyzed samples and also by the fact that we accounted for sequences obtained from negative controls in our study, allowing the subsequent elimination of higher rates of contaminants.

Different taxa related to know vector-borne pathogens were found in the present study. Bacteria from the Anaplasmataceae family represented a large portion of the taxa found in all samples and in living animal samples, indeed comprising the second most frequent ASV. Similar NGS-approach study using camels' blood samples also reported high frequency of Anaplasmataceae (Mohammed et al., 2021). Recognized and novel genotypes of Anaplasmataceae have been molecularly detected and reported in different groups of wild vertebrates from Brazil, such as wild canids and felids (André et al., 2010; André et al., 2012; André, 2018), coatis (*Nasua nasua*) (Perles et al., 2022), anteaters and sloths (Calchi et al., 2020), deer (Sacchi et al., 2012; Mongruel et al., 2017a; Soares et al., 2017) and birds (Machado et al., 2012; Werther et al., 2017; Mongruel et al., 2017b; Sacchi et al., 2021). Considering such diversity of Anaplasmataceae in wildlife from Brazil, microbiome studies targeting variable regions of the 16S rRNA can act as important tools to reveal the diversity of members from this family and how they differ among host species (Makgabo et al., 2023). Sequences from the family Bartonellaceae were also found in the present study. Recently, a *Bartonella henselae*-like genotype, was described in the same studied population in the present study, based on amplification of the *nuoG* and *ribC* genes (Mongruel et al., 2023). *Bartonella henselae* is the primary causative agent of Cat Scratch Disease in humans (Florin et al., 2008).

Sequences from family Spirochaetaceae were found in the present study. Spirochaetaceae comprises helical bacteria that can be found as free-living or host-associated bacteria. Certain strains pose pathogenic threats to humans and animals, such as species from the genus *Borrelia* (Paster and Dewhirst, 2000). When one Spirochaetaceae sequence that was found in two tapirs was compared with sequences deposited in the GenBank database using the BLASTn software (https://blast.ncbi.nlm.nih.gov/), there was 100% identity with sequences of *Borrelia theileri* previously detected in ruminants (GenBank accession numbers (MN621894, MN621893, MN619805). *Borrelia* spirochetes are causative of diseases in humans and animals and can be transmitted by hard (Ixodidae) and soft (Argasidae) ticks (Trevisan et al., 2021). One of the best-known pathogenic representatives from the genus is *Borrelia burgdorferi*, the causative agent of Lyme disease, a multisystemic zoonosis (Lopes et al., 2017), but occurrence of this pathogen remains uncertain in Brazil (Faccini-Martínez et al., 2021). The *Borrelia* 16S rRNA sequence found here showed strong resemblance to sequences from *B. theileri*, part of the Relapsing Fever Group (RFG) *Borrelia* that are transmitted by hard ticks, specifically by *Rhipicephalus* spp. (Martins et al., 1996; Faccini-Martínez et al., 2022), which have previously been reported infesting tapirs in Pantanal and Cerrado regions (Labruna et al., 2021). In Brazil, *B. theileri* has been described in *Rhipicephalus microplus* from southern (Martins et al., 1996) and southeastern (Yparraguirre et al., 2007; Cordeiro et al., 2018) regions of the country. Recently, *B. theileri* was molecularly diagnosed causing subclinical infection in a cow from the mid-west region of Brazil (Paula et al., 2023). Further investigations are needed to elucidate if the *Borrelia* species found in tapirs is indeed *B. theileri*. Nonetheless, this finding contributes to the knowledge regarding the distribution and potential hosts of RFG organisms in Brazil.

Previous studies have reported that Pseudomonadota (synonym: Proteobacteria), Actinomycetota (synonym: Actinobacteria), Bacillota (synonym: Firmicutes) and Bacterioidota (synonym: Bacterioidetes) phyla are the most prevalent in the human blood microbiome (Goraya et al., 2022). In our study, using lowland tapirs' blood samples, Pseudomonadota, Bacillota, Mycoplasmatota (synonym: Tenericutes), Bacterioidota and Actinomycetota were the dominant phyla found. Most of the Pseudomonadota found here were represented by Gammaproteobacteria and Alphaproteobacteria. Pseudomonadota are one of the most abundant bacterial phyla and is considered to cause intestinal and extraintestinal diseases (Rizzatti et al., 2017). In the microbiome analysis of *Amblyomma scalpturatum* and *Amblyomma ovale* ticks collected from *T. terrestris* in Peru, species from *Acinetobacter* genus, a member of Pseudomonadota, was the most abundant taxa found (Rojas-Jaimes et al., 2022).

Bacillota and Bacterioidota include bacteria linked to the digestion of fiber and polysaccharides, respectively. One previous study that compared microbiome gut composition of captive and wild bharals (*Pseudois nayaur*) reported a higher frequency of Bacillota in wild animals, but a lower frequency of Bacterioidota, which was linked to differences in dietary habits between these two feeding categories (Chi et al., 2019). Moreover, an increase in dietary fiber composition has also been linked to an increased Bacillota: Bacterioidota ratio in monogastric animals (Molist et al., 2012). Wild lowland tapirs' diet is composed of large portions of plant fibers and smaller proportions of fruits (Chalukian et al., 2013), which would corroborate with the higher prevalence of Firmicutes compared to Bacterioides found in the present study.

The phylum Mycoplasmatota was represented by their single family Mycoplasmataceae in the present study. Hemoplasmas (hemotropic *Mycoplasma* spp.) are small pleomorphic bacteria from the Mycoplasmataceae which attach to the surface of erythrocytes of different mammalian hosts and may be a causative agent of anaemia (Messick, 2004). The occurrence of two different *Candidatus* species of hemoplasmas was previously reported from the same studied tapir population as in the present project (Mongruel et al., 2022). Another major group found was Actinomycetota, which comprise a large number of bacteria related to pathogens of animals and humans, but also as gut commensals (Barka et al., 2015). This phylum also comprises the largest number of bacteria from the human skin microbiota (Goraya et al., 2022). In the present study, Actinomycetota was mostly composed of Coriobacteriales and Bifidobacteriales, which have been described as commensal gut microorganisms (Gupta et al., 2017). Further, Actinomycetota was also described as part of the blood microbiome from healthy human adults (Paisse et al., 2016).

The genus *Tapirus* comprises four species, namely *T. terrestris* (the lowland tapir), *Tapirus bairdii* (Baird's tapir), *Tapirus pinchaque* (mountain tapir) and *Tapirus indicus* (malayan tapir) (García et al., 2012). To the best of our knowledge, the present work presents the first microbiome analysis of blood samples from the genus *Tapirus* based on the amplification of V3-V4 region of the bacterial 16S rRNA. A previous study of the fecal microbiome of *T. bairdii* using similar techniques has been recently published, in which the fecal microbiome of Baird's tapirs was dominated by Bacillota, Bacterioidota, Pseudomonadota, Kiritimatiellaeota, and Spirochaetota

(Yanez-Montalvo et al., 2021). In the present study, the blood bacterial microbiome of lowland tapirs was dominated by phylum Pseudomonadota, Bacillota, Mycoplasmatota, Bacterioidota and Actinomycetota. Also, Spirochaetota appeared in our study as the 7th most prevalent phylum. Considering the composition of Pseudomonadota, *T. bairdii*'s fecal microbiome revealed a vast majority of Gammaproteobacteria, albeit with a small portion of Alphaproteobacteria (Yanez-Montalvo et al., 2021). In the present study, the dominant phyla were Gammaproteobacteria, mostly represented by Aeromonaceae and Moraxellaceae, but also a large portion of Alphaproteobacteria, which was represented by Anaplasmataceae. These differences are expected considering the different types of biological samples used (feces vs. blood). In our study we worked with blood samples, which allowed the detection of blood-borne bacteria, such as those from family Anaplasmataceae. Moreover, gut-related bacteria are expected to be found in higher prevalence in fecal samples.

For many years, blood was believed to be a sterile environmental and the presence of microorganisms in the blood was an indication of infection. However, with the advancement of molecular and sequencing techniques, the occurrence of a "healthy blood microbiome" has been discussed (Castillo et al., 2019; Goraya et al., 2022). While some studies describe the existence of human blood microbiota (Paisse et al., 2016; D'Aquila et al., 2021; Goroya et al., 2022), a recent investigation reported the absence of conclusive evidence of a common blood microbiome in humans. Instead, the study suggested that bacterial DNA in blood may be linked to transient translocation of microbial communities from other body sites into the bloodstream (Tan et al., 2023). Dysbiosis is defined as a change in the composition of commensal communities compared to the composition found in healthy individuals. The origin source of a dysbiosis is an interruption of the homeostasis that might be produced by inflammation, infection, metabolic diseases, or external interventions (e.g. use of antibiotics) (Petersen and Round, 2014). Indeed, although it is known that circulating bacterial DNA can be found in blood, they need to be cultivable to be considered viable (Whittle et al., 2019). However, the presence of these bacterial DNA fragments in blood may be related to translocation from the original site within the host and can act as an interesting biomarker for the microbiome of other body systems (Whittle et al., 2019).

The necrobiome of clotted blood samples from road-killed tapirs was also performed. The organic matter available after the death of a host continues the interaction between the host and its microbiota, making host decomposition the last stage of symbiosis (Preiswerk et al., 2018). The composition of the bacterial communities between living and dead animals was very different and this was expected due to the decomposition process. Regarding diversity analyses for all samples, the condition (living or dead) was the only variable that was statistically significant for both the alpha and beta diversity (p-value < 0.05) metrics used here.

Moreover, the top 10 ASVs from live and dead animal blood samples exhibited clearly different compositions. For live animals, Aeromonaceae and Anaplasmataceae families were dominant members of the top 10 components. By contrast, dead animals presented a more diversified group of ASVs, with Lactobacillaceae and Clostridiaceae families representing the most prevalent taxa. In the initial phases of the decomposition, depletion of oxygen allows the propagation of anaerobic bacteria, such as Clostridiaceae, derived from the gastrointestinal tract (Carter et al., 2007). Similarly, Lactobacillaceae represent a varied group of lactic-acid producing bacteria found in the gastrointestinal tract of humans and animals (Huynh et al., 2023) and may also proliferate during early stages of decomposition (Hauther et al., 2015).

Regarding the diversity analysis for live animal blood samples, the Shannon's entropy metric (alpha diversity) was significantly different between sites. Meanwhile, beta diversity metrics did not exhibit differences for any of the analyzed variables. Also, the NMDS analysis generated overlapping ellipses, demonstrating the lack of clearly separated groups based on the analyzed variables. Populations of wild tapirs from Cerrado and Pantanal biomes presents different preservation status. For example, the tapir populations from the Cerrado biome are more exposed to environmental disturbances (Fernandes-Santos et al., 2020). An earlier study reported that Pantanal biome maintains a tapir population considered to be healthier and larger due to the existence of more preserved areas (Medici, 2011). However, in recent times, the Pantanal has experienced significant destruction by wildfires, with an estimated loss over 20% of its areas to the fires (LASA/UFRJ, 2020), affecting at least 65 million native vertebrates (Berlinck et al., 2021). Considering that the bacterial communities found here only showed differences for alpha diversity when considering sampling site, it might reflect that tapirs from Pantanal may contain slightly different taxa, albeit in low frequencies. In this scenario, we hypothesize that tapir populations from both biomes share the dominant bacterial community members.

Reporting potential contaminants is an important contribution for metagenomic studies (Weyrich et al., 2018). Blood samples are considered low microbial biomass samples and are easily contaminated by contaminant DNA and/or cross-contamination during the processing of samples (Eisenhofer et al., 2019). Indeed, even though recommended precautions to avoid contamination were taken during the DNA extraction process (Galan et al., 2016), the number of ASVs dropped by 45.61% (from 5,367 to 2,448) after the steps of identification and filtering out contaminants. It is worth noting that samples from the present project were initially collected to perform health assessment of wild tapirs, but specific sampling procedures for microbiome analysis (Eisenhofer et al., 2019) were not taken, which might have allowed field contamination. Moreover, well-to-well contamination during sequencing processes has also been reported (Olomu et al., 2020). Even though a large number of ASVs were eliminated after the filtering steps, we do not discard that some contaminant sequences might have remained, including those present in high frequencies. ASVs identified as contaminants and removed in the present study were reported (**Supplementary File 1**).

In the present study, we found different microbial taxa that may be related to the microbiota of distinct anatomical sites but also to arthropod-borne infections. Here, amplification and NGS of bacterial 16S rRNA from blood samples of wild *T. terrestris* provided information about the diversity of bacterial agents found in lowland tapirs and potential vector-borne pathogens for this species.

6. Conclusions

The phyla Pseudomonadota, Bacillota, Mycoplasmatota, Bacterioidota and Actinomycetota were the dominant taxa present in the analysis of the microbiome of living and road-killed lowland tapirs using amplification and NGS of a fragment of approximately 400 pb from the V4-V3 regions of the bacterial 16S rRNA. Although it is not possible to state that it was derived from a dysbiosis situation, frequent phylum found herein matched with taxa described as commensal for the gut or skin microbiome in other mammal or *Tapirus* species.

The composition of the most frequent ASVs between samples from living and road-killed animals differed extensively as expected due to the process of decomposition in road-killed animals. For living tapirs, diversity analyses suggest that the dominant community members are likely shared among tapirs from all groups.

Sequences from different vector-borne bacteria were found in the present study, mostly represented by Anaplasmataceae and Mycoplasmataceae families. Evidence of potential zoonotic agents, such as *Borrelia* sp. and *Bartonella* sp., was also found.

Acknowledgments

This study is part of Anna Mongruel's thesis. She is carrying out her research at Programa de Pós-Graduação em Medicina Veterinária—Universidade Estadual Júlio de Mesquita Filho (UNESP/Jaboticabal) and is sponsored by a fellowship from Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP– Process #2019/26403-0 and #2022/13016-1) at the time of this study. Authors would like to thank the Lowland Tapir Conservation Initiative (LTCI)–Instituto de Pesquisas Ecológicas (IPÊ) for kindly providing the biological samples needed for the present study. The study of tapir health has been an important component of the long-term activities of the LTCI-IPÊ in Brazil. The LTCI has the institutional support from the International Union for Conservation of Nature (IUCN) Species Survival Commission (SSC) Tapir Specialist Group (TSG), Association of Zoos and Aquariums (AZA) Tapir Taxon Advisory Group (TAG), and European Association of Zoos and Aquariums (EAZA) Tapir Taxon Advisory Group (TAG). This work was supported by FAPESP - Process #2020/12037-0 and #2022/08543-2) and CNPq (National Council for Scientific and Technological Development; Productivity Grant to MRA - Process #303701/2021-8).

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CAPÍTULO IX – Resumo dos resultados obtidos

1. Resultados gerais dos ensaios de PCR e qPCR

O presente estudo teve como objetivo investigar a ocorrência molecular e diversidade genética de agentes das famílias Mycoplasmataceae, Babesiidae, Theileriidae, Bartonellaceae, Anaplasmataceae, Coxiellaceae, Hepatozoidae e Borreliaceae em amostras de sangue de antas (*Tapirus terrestris*) de vida livre nos biomas Cerrado e Pantanal, no estado do Mato Grosso do Sul. Ainda, realizou-se também a caracterização do microbioma bacteriano do sangue desses animais.

Das 122 amostras testadas, 104 (104/122; 85,2%) apresentaram resultados positivos para pelo menos um agente testado. Tais amostras representam 87 animais (87/99; 87,9%). Dos animais do Cerrado, 29 (29/38; 76,3%) apresentaram resultado positivo para pelo menos um agente. Dos animais do Pantanal, 58 (58/61; 95,1%) apresentaram resultado positivo para pelo menos um agente. Ainda, todas as amostras de animais mortos por atropelamento aqui testadas (n=5) foram positivas para pelo menos um agente.

Todos os animais amostrados no presente trabalho foram negativos nos ensaios moleculares utilizados para a pesquisa de DNA de *Ehrlichia* spp., *Hepatozoon* spp. e *Coxiella burnetii*. Ainda, 18 amostras (18/122; 14,8%), representando 12 animais (12/99; 12,1%) não obtiveram resultados positivos para nenhum agente testado. Desses animais, nove (9/12; 75%) foram amostrados no Cerrado e três (3/12; 25%) no Pantanal.

2. Detecção de hemoplasmas

Em relação à detecção molecular de hemoplasmas, 41 amostras (41/122; 33,6%) apresentaram resultados positivos no protocolo de PCR convencional baseada no gene 16S rRNA utilizado como triagem. Tais amostras representam 36 (36/99; 36,4%) animais. Desses animais, 30 (30/36; 83,3%) foram amostrados em região de Pantanal e 06 (06/36; 16,7%) em região de Cerrado. Ainda, duas amostras positivas (02/36; 5,5%) foram coletadas de animais mortos.

Das amostras positivas, foram obtidas 30 sequências do gene 16S rRNA, sete sequências do gene 23S rRNA, duas sequências do gene *RNAse*-P e uma sequência do gene *dnaK*, confirmando a presença de *Mycoplasma* spp. nas amostras. As análises filogenéticas, de genótipos e de distância realizadas, baseadas no gene 16S rRNA, demonstraram a ocorrência de duas potenciais novas espécies, distintas entre si, ocorrendo nesses animais, denominadas '*Ca*. M. haematoterrestris' (ocorrendo em animais do Pantanal e Cerrado) e '*Ca*. M. haematotapirus' (ocorrendo em animais do Pantanal).

A análise filogenética da sequência do gene 16S rRNA obtida de um animal (AA-P) demonstrou ocorrência de '*Ca*. M. haematoterrestris', enquanto análise filogenética da sequência obtida pela amplificação do gene 23S rRNA, desse mesmo animal e amostra, demonstrou a ocorrência de '*Ca*. M. haematotapirus', o que pode sugerir a ocorrência de co-infecção entre as duas espécies *Candidatus* nesse hospedeiro. Ainda, alguns animais foram positivos para '*Ca*. M. haematoterrestris' em amostras coletadas em datas diferentes (média de seis meses de diferença), sugerindo a ocorrência de animais cronicamente infectados, como já relatado em outras espécies de hemoplasmas, ou que vieram a se re-infectar com o agente.

Embora análise estatística realizada não tenha mostrado diferença significativa entre sexo e idade dos animais quanto à positividade para hemoplasmas, maior número de animais amostrados no Pantanal quando comparado àquele do Pantanal foi positivos nos ensaios de triagem para micoplasmas hemotrópicos. Esse foi o primeiro relato da detecção de hemoplasmas em *T. terrestris*. A forma de transmissão e as consequências da infecção para para a saúde das antas ainda são desconhecidas.

3. Detecção de piroplasmídeos

Em relação à detecção de piroplasmídeos, 64 (64/122; 52,46%) amostras foram consideradas positivas no protocolo de nested PCR baseado no gene 18S rRNA utilizado como triagem. Tais amostras representam 56 animais (56/99; 56,6%), sendo 41 animais do Pantanal (41/56; 73,2%) e 15 animais do Cerrado (15/56; 26,8%). Ainda, quatro amostras de animais positivos foram coletadas de animais mortos (4/56; 7,1%).

Dentre as amostras positivas, foram obtidas oito sequências do gene 18S rRNA, cinco sequências do gene *hsp70* e três sequencias do gene *cox*-1. As análises filogenéticas de fragmentos longos obtidos do gene 18S rRNA (>1.100 pb), demonstraram a formação de um clado separado das demais espécies pertencentes à ordem Pirioplasmida. Tal clado posicionou-se próximo à *Theileria equi*. As análises de distância realizadas, assim como as análises filogenéticas baseadas nos demais genes, corroboraram com esse achado. Ainda, inclusões intraeritrocitárias em formato de cruz-de-Malta, forma essa já descrita para agentes Theileriidae, foram observadas em um animal positivo (resultado confirmado por sequenciamento). Adicionalmente, todos os animais mostraram-se negativos na PCR para *Theileria equi* (*ema*-1).

Considerando tais achados, uma nova espécie de piroplasmídeo foi proposta: *Theileria terrestris* nov. sp. Alguns animais foram positivos em amostras coletadas em datas diferentes, sugerindo a ocorrência de animais cronicamente infectados, como já relatado em outras espécies de *Theileria* spp. O presente estudo demonstrou a importância da utilização de fragmentos longos do gene 18S rRNA para uma melhor resolução taxonômica de espécies do gênero *Theileria*. Os carrapatos vetores envolvidos na transmissão de *Theileria terrestris* e as consequências da infecção para a saúde das antas ainda são desconhecidos.

4. Detecção de *Bartonella* sp.

Em relação à detecção de *Bartonella* sp., oito amostras (08/122; 6,55%), representando oito animais (8/99; 8,1%), foram positivas no protocolo de triagem utilizando PCR quantitativa baseada no gene *nuoG*. Desses animais, sete (07/08; 87,5%) foram amostrados em área de Cerrado e apenas um (01/08; 12,5%) no Pantanal. Dessas amostras, foram obtidas cinco sequências do gene *ribC*, todas de animais do Cerrado. Nenhuma amostra de animais mortos foi positiva.

Na análise filogenética do gene *ribC*, as sequências detectadas em antas agruparam-se no mesmo clado que *Bartonella henselae*, agente zoonótico responsável pela Doença da Arranhadura do Gato, já descrito em diferentes espécies animais. Embora a análise estatística não tenha mostrado diferenças significativas entre as variáveis sexo e idade dos animais quanto à positividade para *Bartonella* spp., maior número de animais amostrados no Cerrado quando comparado àquele do Pantanal foi positivo no protocolo de triagem para *Bartonella* spp.

Os níveis de bacteremia de *Bartonella* spp. na corrente sanguínea de seus hospedeiros vertebrados são considerados baixos. Visando melhorar a sensitividade do ensaio, a cultura de amostras de sangue fresco (ou mantidas em congelamento a -80ºC) em meio enriquecido BAPGM por sete dias, previamente à extração de DNA e qPCR, é sugerida para a detecção de *Bartonella* spp. Entretanto, considerando que as amostras do presente trabalho não foram coletadas com tal intuito, não foi possível utilizar de tal estratégia. Consequentemente, é possível que uma subestimação da real ocorrência de *Bartonella* sp. nesses animais tenha ocorrido no presente trabalho, considerando a baixa sensibilidade da estratégia diagnóstica utilizada. Esse foi o primeiro relato de *Bartonella* sp. em antas. O vetor envolvido e as consequências para a saúde das antas ainda são desconhecidos

5. Detecção de *Anaplasma* sp.

Em relação à detecção de *Anaplasma* sp., 22 amostras (22/122; 18%), representando 22 animais (22/99; 22,2%) foram positivas no protocolo de triagem utilizando PCR convencional baseado no gene 16S rRNA. Dessas amostras, sete foram obtidas no Cerrado (07/22; 31,8%) e 15 (15/22; 68,2%) no Pantanal. Nenhuma amostra de animal morto foi positiva. Das amostras positivas foram obtidas novas sequências do gene 16S rRNA (>1.000 pb) e duas sequências do espaço intergênico (ITS) 23S-5S.

Para as análises filogenéticas e de distância baseadas no gene 16S rRNA, oito sequências foram utilizadas pois apresentaram tamanho adequado (>1.200 pb). Em tais análises, as sequências obtidas de antas demonstraram posicionamento distinto das demais sequências do gênero *Anaplasma*, sugerindo a ocorrência de uma nova espécie ou genótipo nesses animais. As sequências do gene 16S rRNA obtidas no presente trabalho posicionaram-se próxims à *A. odocoilei*, um agente descrito em veado-da-cauda-branca (*O. virginianus*) na América do Norte. Considerando a natureza conservada do gene 16S rRNA entre as espécies do gênero *Anaplasma*, o sequenciamento *multi-locus* é importante para a determinação de novas espécies.

Entretanto, não foi possível obter sequências de outros alvos gênicos no presente estudo apesar de inúmeras tentativas.

O presente trabalho descreve, pela primeira vez, a ocorrência de *Anaplasma* sp. em antas, com evidências da ocorrência de um novo genótipo ou espécie nesses animais. O vetor envolvido e as consequências para a saúde das antas ainda são desconhecidos

6. Detecção de *Neorickettsia* sp.

Em relação à detecção de *Neorickettsia* spp., 31 amostras (31/122; 25.4%) representando 29 animais (29/99; 29.3%) foram positivas para o protocolo de triagem utilizando nested PCR convencional baseada no gene 16S rRNA. Dentre os animais positivos, 10 foram amostrados em região de Cerrado (10/29; 34,5%) e 19 em região de Pantanal (19/29; 65,5%). Dentre as amostras positivas, foram obtidas três sequências do gene 16S rRNA de *Neorickettsia* sp. Na análise filogenética, tais sequências formaram um clado monofiléitco com *N. risticii* e sequências de *Neorickettsia* sp. obtidas de morcegos e quatis no Brasil. *Neorickettsia risticii* é causadora da Febre do Cavalo de Potomac, uma doença que acomete equinos no Brasil. Embora exista plausabilidade nesse achado, considerando a proximidade taxonômica entre antas e equinos, deve-se observar os resultados com cautela considerando que uma grande heterogenicidade é descrita para o gene 16S rRNA dentre as espécies do gênero *Neorickettsia*. Outrossim, o fragmento do gene 16S rRNA sequenciado apresentou pequeno tamanho, impossibilitando análises filogenéticas mais robustas.

O presente trabalho descreve, pela primeira vez, a ocorrência de *Neorickettsia* sp. em antas. O vetor envolvido e as consequências para a saúde das antas ainda são desconhecidos.

7. Detecção de *Borrelia theileri*

Em relação à detecção de *Borrelia* spp., duas amostras (02/122; 1,6%), representando dois animais (02/99; 02%) do Pantanal, foram positivas no ensaio de

PCR quantitativa baseada no gene 16S rRNA utilizado como triagem. Dessas amostras, uma sequência do gene *flaB* foi obtida. A análise filogenética mostrou que a sequência obtida foi posicionada em um clado com sequências de *B. theileri*, próximo a sequências obtidas de *R. microplus* no Brasil e Colômbia, bovinos no Brasil e carrapatos da espécie *H. sulcata* no Pasquistão.

Esse foi o primeiro relato da detecção de *B. theileri* em um animal selvagem no Brasil. Anteriormente, esse agente só havia sido detectado no país, por meio de técnicas moleculares, em carrapatos *R. microplus* e bovinos. Em bovinos, esse agente é relatado como responsável por causar febre, apatia e queda na produtividade. A detecção de tal agente em antas sugere a ocorrência de *spill-over* entre animais domésticos e selvagens. O vetor envolvido e as consequências para a saúde das antas ainda são desconhecidos

8. Microbioma bacteriano do sangue de antas

Em relação à caracterização do microbioma bacteriano do sangue, por meio da amplificação de regiões hipervariáveis do gene bacteriano 16S rRNA, 86 amostras foram utilizadas, sendo 40 delas (40/46; 46,5%) de animais do Cerrado e 46 (46/86; 53,5%) de animais do Pantanal. Ainda, sete amostras (7/86; 8,1%) obtidas de animais mortos foram analisadas, assim como quatro controles negativos. Após identificação dos contaminantes e sequências não identificadas, uma frequência total de 14.523.219 sequências, divididas em 2.448 ASVs, foi observada. Os filos Pseudomonadota, Bacillota, Mycoplasmatota, Bacterioidota e Actinomycetota foram os táxons dominantes presentes na análise.

Quando consideradas todas as amostras (obtidas de animais vivos e mortos), a diversidade alfa e beta apresentou diferenças significativas apenas para a variável condição (vivo ou morto), não sendo observadas diferenças para as variáveis sexo, idade e local. As diferenças encontradas entre as comunidades bacterianas das amostras de animais vivos e mortos eram esperadas, devido ao processo de decomposição dos animais mortos. Quando apenas amostras de animais vivos foram analisadas, a diversidade alfa mostrou significância estatística para o local, porém não foi observada diferença para a diversidade beta. Para os animais vivos, as análises de diversidade sugerem que os membros dominantes das comunidades bacterianas provavelmente são compartilhados entre antas de todos os grupos.

DNA de patógenos transmitidos por vetores foram detectados no sangue das antas, tais como agentes Anaplasmataceae, *Bartonella* spp. e *Borrelia* spp. Além disso, também foram observadas evidências de translocação transitória de comunidades microbianas de outros locais para a corrente sanguínea. Embora não seja possível afirmar que tais achados caracterizam uma situação de disbiose, os filos mais frequentes aqui encontrados corresponderam a táxons descritos como comensais para o microbioma intestinal ou da pele em outras espécies de mamíferos ou animais do gênero *Tapirus*.

As amostras de sangue são consideradas amostras de baixa biomassa microbiana e são facilmente contaminadas por DNA exógeno e/ou contaminação cruzada durante o processamento das amostras. Embora tenham sido tomadas as precauções recomendadas para evitar contaminação durante o processo de extração de DNA, o número de ASVs caiu em 45,61% (de 5.367 para 2.448) após as etapas de identificação e filtragem de contaminantes. Vale ressaltar que amostras do presente trabalho foram coletadas inicialmente com a intenção de realizar avaliação de saúde dos animais, mas não foram realizados procedimentos de coleta específicos para análise de microbioma, o que pode ter permitido contaminação em campo. Mesmo que um grande número de ASVs tenha sido eliminado após as etapas de filtragem, não pode ser descartado que algumas sequências contaminantes possam ter permanecido, inclusive aquelas presentes em altas frequências.

O presente trabalho relatou o primeiro ensaio com objetivo de caracterizar o microbioma bacteriano do sangue de *T. terrestris*, utilizando sequenciamento de nova geração. Ainda, tal ensaio demonstrou ser uma ferramenta útil para o rastreio de potenciais agentes patogênicos transmitidos por vetores nesta espécie considerada vulnerável à extinção.

9. Co-infecções identificadas pelos ensaios moleculares de triagem

A ocorrência de co-infecção, envolvendo dois ou mais patógenos, baseando-se nos ensaios de triagem, foi observada em 49 amostras (40/122; 40,1%). Tais amostras representaram 41 animais, sendo 31 do Pantanal (31/99; 31,3%) e 10

(10/99; 10,1%) do Cerrado. Dentre os animais co-infectados do Pantanal, 12 (12/31; 38,7%) eram adultos, 15 (15/31; 48,4%) sub-adultos e 04 (04/31; 12,9%) juvenis. Ainda, 20 foram machos (20/31; 54,5%) e 11 (11/31; 35,5%) fêmeas. Já em realação aos animais do Cerrado, dois (02/10; 20%) foram classificados como adultos, quatro (4/10; 40%) sub-adultos e quatro (04/10; 40%) como juvenis. Em relação ao sexo desses animais, quatro foram machos (04/10; 40%) e seis fêmeas (06/10; 60%). Ainda, dentre os animais co-infectados, dois eram animais mortos por atropelamento (02/41; 4,87%).

As co-infecções observadas estão listadas abaixo:

- *Anaplasma* spp. + *Neorickettsia* spp. = 02
- *Anaplasma* spp. + *Bartonella* spp. = 01
- *Anaplasma* spp. + hemoplasmas = 03
- Hemoplasmas + *Neorickettsia* spp. = 02
- Piroplasmas + *Anaplasma* spp. = 07
- Piroplasmas + *Borrelia* spp. = 01
- Piroplasmas + hemoplasmas = 13
- Piroplasmas + *Neorickettsia* spp. = 06
- Piroplasmas + hemoplasmas + Borrelia spp. = 01
- Hemoplasmas + *Anaplasma* spp. + *Neorickettsia* sp. = 01
- Piroplasmas + hemoplasmas + *Neorickettsia* spp. = 06
- Piroplasmas + *Anaplasma* spp. + *Bartonella* spp. = 01
- Piroplasmas + *Anaplasma* spp. + hemoplasmas = 01
- Piroplasmas + *Anaplasma* spp. + *Neorickettsia* spp. = 03
- Piroplasmas + hemoplasmas + *Anaplasma* spp. + *Neorickettsia* spp. = 01

Alguns animais que foram amostrados em duplicatas ou triplicatas, em datas diferentes (n=7), apresentaram co-infecção em mais de uma amostra. Tais animais estão listados no tabela abaixo (**Tabela 1**):

Tabela 1. Descrição das amostras de um mesmo animal (coletadas em datas diferentes) que obtiveram resultados positivos, contendo o bioma de origem, a identificação da amostra, data de coleta e a positividade observada nos protocolos de triagem.

Bioma	**ID da amostra**	**Data da coleta**	**Co-infecção observada**
Cerrado	SOC-C-1	11/04/2018	Piroplasmas + *Neorickettsia* spp.
	SO-C-2	09/02/2017	Piroplasmas + hemoplasmas
Pantanal	FFO-P-1	29/08/2018	Piroplasmas + hemoplasmas
	FFO-P-2	23/08/2017	Piorplasmas + hemoplasmas + *Neorickettsia* sp.
	VA-P-1	01/09/2017	Piroplasmas + hemoplasmas
	VA-P-2	19/08/2017	Piroplasmas + *Anaplasma* spp.
	JE-P-1	06/12/2017	Hemoplasmas + Anaplasma spp.
	JE-P-2	09/06/2016	Piroplamas + hemoplamas
	TD-P-2	17/12/2014	Piroplasmas + Anaplasma spp.
	TD-P-3	18/06/2016	Piroplasmas + hemoplasmas
	SAO-P-2	20/20/2013	Piroplasmas + hemoplasmas + *Anaplasma* spp.
	SAO-P-3	11/05/2015	Piroplasmas + *Anaplasma* spp.

WE-P-1	14/11/2015	Piroplasmas + hemoplasmas + *Borrelia* spp.
WE-P-2	16/06/2016	Piroplasmas + hemoplasmas
WE-P-3	15/12/2016	Piroplasmas + hemoplamas + *Neorickettsia* sp.

10. Co-infecções confirmadas por sequenciamento

Co-infecções confirmadas por sequenciamento foram observadas em 10 amostras (10/122; 8,2%), representando 10 animais (10/99; 10,1%). Desses animais, dois (02/10; 20%) eram do Cerrado e oito (08/10; 80%) do Pantanal. Dentre os animais co-infectados do Pantanal, dois (02/08; 25%) eram adultos, cinco (05/08; 62,5%) sub-adultos e um (01/08; 12,5%) juvenil. Ainda, quatro eram machos (04/08; 50%) e quatro (04/08; 50%) fêmeas. Já em realação aos animais do Cerrado, os dois animais co-infectados foram representados por um adulto (01/02; 50%) e um juvenil (01/02; 50%). Em relação ao sexo desses animais, os dois eram fêmeas (02/02; 100%). Não foram observadas co-infecções confirmadas em mais de uma amostra do mesmo animal. A **Tabela 2** descreve cada co-infecção confirmada por sequenciamento observada.

As co-infeções confirmadas por sequenciament observadas estão listadas abaixo:

- '*Ca*. M. haematotapirus' + *Anaplasma* sp. = 01
- '*Ca*. M. haematoterrestris' + *Anaplasma* sp. = 01
- '*Ca*. M. haematoterrestris' + *Borrelia theileri* = 01
- '*Ca*. M. haematoterrestris' + *Neorickettsia* sp. = 02
- '*Ca*. M. hematoterrestris' + '*Ca*. M. haematotapirus' + *Anaplasma* sp. = 01

- *Theileria terrestris* + '*Ca*. M. haematotapirus' = 01
- *Theileria terrestris* + '*Ca*. M. haematoterrestris' = 01
- *Theileria terrestris* + *Anaplasma* sp. = 01
- *Theileria terrestris* + *Neorickettsia* sp. = 01

Tabela 2. Descrição dos animais que obtiveram co-infeção confirmada por sequenciamento, contendo o bioma de origem do animal, identificação da amostra, sexo e idade do animal e a co-infecção observada.

Bioma	ID da amostra	Sexo/idade	Co-infecção observada
Cerrado	JHA-C	Fêmea/juvenil	*Theileria terrestris* + *Anaplasma* sp.
	CNA-C-2	Fêmea/adulta	'*Ca*. M. haematoterrestris' + *Neorickettsia* sp.
Pantanal	SAO-P-2	Macho/adulto	'*Ca*. M. haematotapirus' + *Anaplasma* sp.
	MIA-P	Fêmea/sub-adulta	'*Ca*. M. haematoterrestris' + *Neorickettsia* sp.
	GO-P	Macho/sub-adulto	'*Ca*. M. haematoterrestris' + *Anaplasma* sp.
	JA-P	Fêmea/sub-adulta	*Theileria terrestris* + '*Ca*. M. haematoterrestris'
	AA-P	Macho/sub-adulto	'*Ca*. M. haematoterrestris' + '*Ca*. M. hematotapirus' + *Anaplasma* sp.

	DA-P	Fêmea/sub-adulta	*'Ca*. M. haematotapirus' + *Theileria terrestris*
	WE-P-1	Fêmea/sub-adulta	*'Ca*. M. haematoterrestris' + *Borrelia theileri*
	FFO-P-1	Macho/adulto	*'Ca*. M. haematoterrestris' + *Neorickettsia* sp.

CAPÍTULO X – Considerações finais

O presente trabalho demonstrou que antas de vida livre do Pantanal e Cerrado, no Mato Grosso do Sul, podem ser infectadas por diferentes hemoparasitos. De acordo com estudos anteriores, os dois biomas diferem em relação às características climáticas, de vegetação e densidade populacional de antas. A ocorrência de '*Ca*. M. haematoterrestris', *Theileria terrestris*, *Bartonella* sp., *Anaplasma* sp. e *Neorickettsia* sp. foi observada em animais tanto do Pantanal quanto do Cerrado. A detecção de '*Ca*. M. haematotapirus' e *Borrelia theileri* foi observada somente em animais do Pantanal, enquanto um genótipo de *Bartonella* sp. filogeneticamente próximo a *Bartonella henselae* foi observado somente em animais do Cerrado. Futuros estudos são necessários para caracterizar o genótipo de *Bartonella* sp. que circula em antas no Pantanal.

Alguns dos agentes aqui detectados e caracterizados demonstram uma possível especificidade para esse hospedeiro vertebrado, considerando o alto número de animais positivos e o ineditismo da detecção, como é o caso dos hemoplasmas (*'Ca*. M. haematoterrestris e *'Ca*. M. hematotapirus') e do piroplasmídeo *Theileria terrestris*. Embora sejam necessários mais estudos para confirmar esta hipótese, a ocorrência de outros hemoparasitos em antas poderia estar relacionada a um mecanismo de *spill-over* (transmissão de agentes entre animais domésticos e selvagens), considerando seu histórico de descrição em outras espécies animais e o baixo número de antas positivas. Como exemplo, poderíamos citar a detecção de *Borrelia theileri*, um agente até então descrito somente em bovinos e carrapatos na América do Sul. Esse é um achado que abre portas para perspectivas futuras considerando o compartilhamento de habitat entre animais domésticos e selvagens no Pantanal, visto que a pecuária extensivista é uma atividade econômica bastante presente dentro desse bioma.

Além disso, *Bartonella henselae*, agente zoonótico que tem felinos como principais reservatórios, foi detectado em antas no Cerrado. Tal agente já foi descrito em diferentes espécies animais e, com base nos resultados aqui obtidos, a anta pode representar mais um hospedeiro. Estudos anteriores descrevem o Cerrado como um bioma bastante degradado, cuja população de antas é descrita como mais exposta às consequências das ações antrópicas comparativamente às populações de outros biomas.

As infecções por hemoparasitos aqui descritas estão relacionadas, provavelmente, à transmissão por vetores artrópodes (para piroplasmídeos, *Anaplasma* sp., *Bartonella* spp. e *Borrelia theileri*) e trematódeos cujas metacercárias podem ser encontradas em moluscos (*Neorickettsia* sp.). Para os hemoplasmas, pode-se considerar também a possibilidade de transmissão via uterina e interações agressivas entre hospedeiros, como já descrito, em estudos anteriores, para espécies deste grupo. Ainda é necessário elucidar quais espécies de vetores estão associados aos agentes aqui descritos. Nas análises estatísticas realizadas, não foram encontradas associações com o sexo ou a idade dos animais, somente com o bioma. Animais do Pantanal apresentaram um maior risco para infecção por hemoplasmas, enquanto os animais do Cerrado apresentaram um maior risco para infecção por *Bartonella* sp.

A análise do microbioma do sangue de antas mostrou-se útil para a detecção de DNA de hemoparasitas em amostras nas quais os ensaios de PCR não mostraram resultados positivos, considerando uma maior sensibilidade do sequenciamento de nova geração. As principais comunidades bacterianas encontradas no microbioma do sangue são compartilhadas entre todos os grupos de antas estudados. Adicionalmente, essa análise identificou táxons relacionados a outros sistemas (como o trato gastrointestinal) no sangue de antas. Futuramente, tais achados poderão representar opções de marcadores biológicos para a avaliação de saúde dessa espécie animal.

Embora não seja possível, até o presente momento, determinar quais as consequências da infecção pelos inúmeros parasitos aqui detectados para a saúde das antas, a descrição desta grande diversidade de agentes potencialmente patogênicos contribui para o conhecimento acerca dos agentes infecciosos que circulam em antas de vida livre. Futuros trabalhos objetivando correlacionar dados de saúde e avaliação hematológica e bioquímica sérica dos animais com a positividade para os hemoparasitos aqui detectados são necessários, a fim de contribuir para ações que visem a conservação dessa espécie considerada vulnerável à extinção.

APÊNDICES

APÊNDICE A – Material suplementar citado no Capítulo III

Expanding the Universe of Hemoplasmas: Multi-Locus Sequencing Reveals Putative Novel Hemoplasmas in Lowland Tapirs (*Tapirus terrestris*), the Largest Land Mammals in Brazil*

*Material corresponde à publicação científica publicada no periódico Microorganisms 2022, 10, 614. doi: https://doi.org/10.3390/microorganisms10030614

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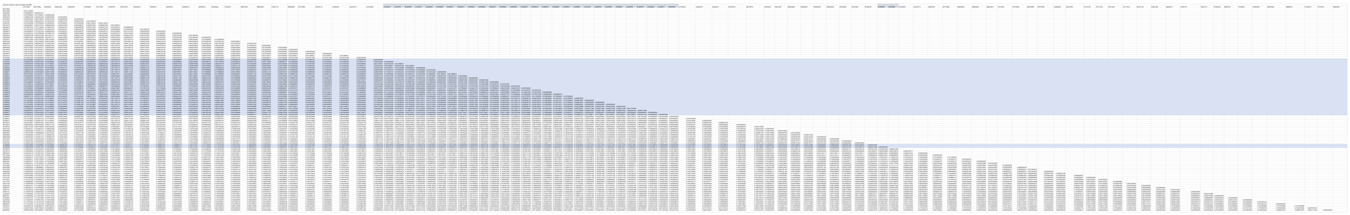
Table S1: Hemoplasma-16S rRNA genotypes found in tapirs sampled in the present study according to sampling location.

Genotypes	16S rRNA Sequences (GenBank accession numbers)	Location
1	OL985895	Cerrado
2	OL985899	Pantanal
3	OL985908	Pantanal
4	OL985907 OL985925	Pantanal
5	OL985924	Pantanal
6	OL985896 OL985911 OL985914	Pantanal
7	OL985921	Pantanal
8	OL985920	Pantanal
9	OL985913	Cerrado
10	OL985901	Pantanal
11	OL985918	Cerrado
12	OL985919	Pantanal
13	OL985915	Pantanal
14	OL985909	Pantanal
15	OL985912	Pantanal
16	OL985897	Pantanal
17	OL985903 OL985900 OL985904 OL985923 OL985902	Pantanal
18	OL985905	Pantanal
19	OL985916	Cerrado
20	OL985922	Pantanal
21	OL985917	Cerrado
22	OL985898 OL985926	Pantanal

Table S2*: Distance matrix for partial 16S rRNA



*Para melhor visualização, por favor acesse: https: //www.mdpi.com/article/10.3390/microorganisms10030614/s1

APÊNDICE B – Material suplementar do Capítulo IV

Theileria terrestris nov. sp.: A Novel Theileria in Lowland Tapirs (*Tapirus terrestris*) from Two Different Biomes in Brazil*

* Material corresponde à publicação científica publicada no periódico Microorganisms 2022, 10, 2319. doi: https://doi.org/10.3390/microorganisms10122319.

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Supplementary File 1. Detailed reagent concentrations and thermal conditions of all protocols used in the present study.

Target gene	Primers	Fragment size (bp)	Reagents concentrations	Thermal conditions	References
18S rRNA	1st reaction: BTF1 (5’-GGCTCATTACAACAGTTATAG-3’) and BTR21 (5’-CCCAAAGACTTTGATTTCTCTC-3’); 2nd reation: BTF2 (5’-CCGTGCTAATTGTAGGGCTAATAC-3’) and BTR2 (5’-GGACTACGACGGTATCTGATCG-3’).	~800	1st reaction: 10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA. 2nd reaction: same concentrations and 1 uL from amplified product from 1st reaction as template.	1st round: Initial denaturation at 93° C for 03 min, followed by 45 cycles of denaturation at 95° C for 30 sec, annealing at 58° C for 20 sec and extension at 72° C for 30 sec, and final extension at 72° C for 07 min. 2nd round: same conditions but	[13]

				anneling at 62° C for 20 sec.	
18S rRNA	Nbab_1F (5’-AAGCCATGCATGTCTAAGTATAAGCTTTT-3’) and 18Sapir (5’-GGATCACTCGATCGGTAGGAG-3’)	~1,500	10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA.	Initial denaturation at 95° C for 03 min, followed by 35 cycles of denaturation at 94° C for 30 sec, annealing at 60° C for 01 min and extension at 72° C for 01 min, and final extension at 72° C for 04 min.	[23,24]
18S rRNA	1st reaction: Piro0F (5’-GCCAGTAGTCATATGCTTGTGTTA-3’) and Piro6R (5’-CTCCTTCCTYTAAGTGATAAGGTTCAC-3’); 2nd reaction: Piro1F (5’-CCATGCATGTCTWAGTAYAARCTTTTA-3’) and Piro5.5R (5’-CCTYTAAGTGATAAGGTTCACAAAACTT-3’)	~1,500	1st reaction: 10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA. 2nd reaction: same concentrations and 1 uL from amplified product from	For both rounds: Initial denaturation at 95° C for 03 min, followed by 33 cycles of denaturation at 95° C for 01 min, annealing at 59° C for 01 min and extension at 72° C for 02 min, and final extension at 72° C for 10 min.	[22]

			1st reaction as template.		
cox-1	1st reaction: Bab_for1 (5'-ATWGGATTYTATATGAGTAT-3') and Bab_Rev1 (5'- ATAATCWGGWATYCTCCTTGG-3'), Bab_for2; 2nd reation: (5'-TCTCTWCATGGWTTAATTATGATAT-3') and Bab_Rev2 (5'-TAGCTCCAATTGAHARWACAAAGTG-3')	~924	1st reaction: 10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA. 2nd reaction: same concentrations and 1 uL from amplified product from 1st reaction as template.	1st round: Initial denaturation at 95° C for 01 min, followed by 35 cycles of denaturation at 95° C for 15 sec, annealing at 48,1° C for 30 sec and extension at 72° C for 30 sec, and final extension at 72° C for 01 min. 2nd round: same conditions but anneling at 52° C for 30 sec.	[14,15]
cox-3	COX3F (5'-ACTGTCAGCTAAAACGTATC-3') and COX3R (5'-ACAGGATTAGATACCCTGG-3')	~600	10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA.	Initial denaturation at 94° C for 05 min, followed by 40 cycles of denaturation at 94° C for 20 sec, annealing at 55° C for 30 sec and extension at 68° C for 01 min, and	[16,17]

				final extension at 72° C for 07 min.	
hsp70	hsp70F1 (5'-GAAGCACTGGCCHTTCAA-3') and hsp70R2 (5'-GBAGGTTGTTGTCCTTVGTCAT-3)	~740	10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA.	Initial denaturation at 95° C for 05 min, followed by 35 cycles of denaturation at 95° C for 20 sec, annealing at 60° C for 30 sec and extension at 72° C for 30 sec, and final extension at 72° C for 07 min.	[18]
cytb	cytbF (5'-TTAGTGAAGGAACTTGACAGGT-3') and cytbR (5'-CGGTTAATCTTTCCTATTCCTTACG-3')	~1.000	10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA.	Initial denaturation at 94° C for 05 min, followed by 49 cycles of denaturation at 95° C for 20 sec, annealing at 56° C for 30 sec and extension at 68° C for 45 sec, and final extension at 72° C for 07 min.	[16,17]

β-tubulin	Tubu-63F (5'-CAAATWGGYGCMAARTTYTGGGA-3') and Tubu-3F (5'-TCGTCCATACCTTCWCCSGTRTACCAGTG-3')	~1.200	10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA.	Initial denaturation at 95° C for 05 min, followed by 30 cycles of denaturation at 94° C for 40 sec, annealing at 55° C for 1 min and extension at 72° C for 45 sec, and final extension at 72° C for 05 min.	[19]
ITS-1	1st reaction: ITS15C (5'-CGATCGAGTGATCCGGTGAATTA-3') and ITS13B (5'-GCTGCGTCCTTCATCGTTGTG-3'); 2nd reaction: (5'-AAGGAAGGAGAAGTCGTAACAAGG-3') and ITS15C (5'-TTGTGTGAGCCAAGACATCCA-3')	~450	1st reaction: 10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA. 2nd reaction: same concentrations and 1 uL from amplified product from 1st reaction as template.	1st round: Initial denaturation at 94° C for 01 min, followed by 35 cycles of denaturation at 94° C for 30 sec, annealing at 52° C for 01 min and extension at 72° C for 01 min, and final extension at 72° C for 05 min. 2nd round: same conditions but	[20]

				anneling at 49° C for 1 min.	
ema-1	1st reaction: EMAE-F (5'-CCGCCCTTCACCTCGTTCTCAA-3') and EMAE-R (5'-TCTCGGCGGCATCCTTGACCTC-3'); 2nd reaction: EMAI-F (5'-CCGTCTCCGTTGACTTGGCCG-3') and EMAIR (5'- GGACGCGCTTGCCTGGAGCCT-3')	~396	1st reaction: 10X Buffer, 50 mM $MgCl_2$, 10 mM dNTP, primers at 10 mM, 5U/ µL Platinum Taq DNA Polymerase (Life Technologies®, Carlsbad, California, USA), ultra-purified water and 5 µL from template DNA. 2nd reaction: same concentrations and 1 uL from amplified product from 1st reaction as template.	For both rounds: 1st round: Initial denaturation at 94° C for 04 min, followed by 39 cycles of denaturation at 94° C for 01 min, annealing at 60° C for 01 min and extension at 72° C for 01 min, and final extension at 72° C for 04 min.	[21]

Supplementary File 2. Positive samples for detection of partial 18S rRNA from Piroplasmida: sample identification, biome where the animal was sampled, sampling date, gender and age.

ID	Biome	Sampling date	Gender	Age
SO-C-1	Cerrado	11 April 2018	Male	Adult
RER-C	Cerrado	15 March 2016	Male	Sub-adult
NAI-C	Cerrado	03 May 2017	Female	Sub-adult
ANO-C-1	Cerrado	19 October 2016	Male	Adult
JHA-C	Cerrado	08 April 2016	Female	Sub-adult
CBI-C-1	Cerrado	23 February 2017	Female	Adult
ISA-C	Cerrado	16 March 2016	Female	Adult
AN-C	Cerrado	25 May 2017	Male	Sub-adult
CRO-C	Cerrado	25 June 2017	Male	Sub-adult
TI-C	Cerrado	12 March 2016	Male	Adult
PO-P	Pantanal	24 August 2018	Male	Sub-adult

SKI-P	Pantanal	14 June 2018	Male	Adult
MA-P-1	Pantanal	28 July 2013	Female	Adult
JO-P-1	Pantanal	27 October 2014	Male	Sub-adult
CO-P	Pantanal	23 July 2013	Male	Sub-adult
CRA-P-1	Pantanal	21 July 2013	Female	Adult
JA-P	Pantanal	05 May 2014	Female	Sub-adult
XS-P	Pantanal	21 August 2017	Male	Sub-adult
IA-P	Pantanal	10 December 2017	Male	Adult
COS-P	Pantanal	08 December 2017	Male	Sub-adult
SIA-P	Pantanal	05 December 2017	Female	Sub-adult
DA-P	Pantanal	25 August 2018	Female	Sub-adult
NAO-P	Pantanal	20 June 2016	Male	Adult
RTA-P	Pantanal	10 May 2015	Female	Adult
RCK-P	Pantanal	21 October 2014	Male	Adult
WEP-P-1	Pantanal	14 November 2015	Female	Sub-adult
HRY-P	Pantanal	22 August 2016	Male	Sub-adult
VA-P-1	Pantanal	01 September 2018	Female	Sub-adult
FPE-P	Pantanal	17 December 2014	Male	Adult
RO-P	Pantanal	25 June 2018	Male	Sub-adult
LRO-P	Pantanal	10 December 2017	Male	Adult
SCO-P	Pantanal	08 December 2016	Male	Adult
MLE-P	Pantanal	03 July 2014	Female	Sub-adult
BEN-P	Pantanal	16 August 2016	Male	Adult
GIL-P	Pantanal	27 October 2017	Male	Sub-adult
FFO-P-1	Pantanal	29 August 2018	Male	Adult
MIA-P	Pantanal	25 June 2018	Female	Sub-adult
DHO-P-1	Pantanal	26 October 2017	Male	Sub-adult
CHA-P-2	Pantanal	22 October 2018	Female	Sub-adult
NLA-P	Pantanal	25 June 2018	Female	Adult
KIN-P	Pantanal	12 July 2014	Female	Adult
PNA-P	Pantanal	15 November 2015	Female	Adult
GAO-P	Pantanal	19 June 2018	Male	Adult
INA-P	Pantanal	27 August 2018	Female	Adult
MCI-P	Pantanal	02 July 2015	Female	Adult
GLA-P	Pantanal	06 July 2014	Female	Sub-adult
CNA-C-2	Cerrado	19 September 2018	Female	Adult
SO-C-2	Cerrado	09 February 2017	Male	Adult
FFO-P-2	Pantanal	23 August 2017	Male	Adult
LNA-P-2	Pantanal	11 August 2018	Female	Adult
VA-P-2	Pantanal	19 August 2017	Female	Sub-adult
DHO-P-2	Pantanal	25 June 2018	Male	Sub-adult
JE-P-2	Pantanal	09 June 2016	Male	Sub-adult
BRS-P	Pantanal	11 June 2016	Male	Sub-adult

TD-P-2	Pantanal	17 December 2014	Male	Sub-adult
TD-P-3	Pantanal	18 June 2016	Male	Sub-adult
WE-P-2	Pantanal	18 June 2016	Female	Sub-adult
WE-P-3	Pantanal	15 December 2016	Female	Sub-adult
SAO-P-2	Pantanal	20 October 2013	Male	Adult
SAO-P-3	Pantanal	11 May 2015	Male	Adult
NEC09-C	Cerrado	04 April 2016	Female	Sub-adult
NEC07-C	Cerrado	10 March 2016	Female	Sub-adult
NEC15-C	Cerrado	20 July 2016	Male	Adult
NEC24-C	Cerrado	18 November 2016	Female	Adult

Supplementary File 3. List of tapirs that were sampled more than once and that presented at least one positive sample for Piroplasmida partial 18S rRNA amplification.

Animal ID	Biome	Sample ID	Sampling dates	Partial 18S rRNA PCR
SO-C	Cerrado	SO-C-1	11 April 2018	Positive
		SO-C-2	09 February 2017	Positive
ANO-C	Cerrado	ANO-C-1	19 October 2016	Positive
		ANO-C-2	28 July 2017	Negative
CBI-C	Cerrado	CBI-C-1	23 February 2017	Positive
		CBI-C-2	11 October 2018	Negative
CNA-C	Cerrado	CNA-C-1	05 April 2016	Negative
		CNA-C-2	19 September 2018	Positive
CHA-P	Pantanal	CHA-P-1	22 August 2017	Negative
		CHA-P-2	22 October 2018	Positive
SAO-P	Pantanal	SAO-P-1	29 October 2017	Negative
		SAO-P-2	20 October 2013	Positive
		SAO-P-3	11 May 2015	Positive
MA-P	Pantanal	MA-P-1	28 July 2013	Positive
		MA-P-2	19 May 2015	Negative
JE-P	Pantanal	JE-P-1	06 December 2017	Negative
		JE-P-2	09 June 2016	Positive
TD-P	Pantanal	TD-P-1	16 November 2015	Negative
		TD-P-2	17 December 2014	Positive
		TD-P-3	18 June 2016	Positive
JO-P	Pantanal	JO-P-1	27 October 2014	Positive
		JO-P-2	20 August 2016	Negative
CRA-P	Pantanal	CRA-P-1	21 July 2013	Positive
		CRA-P-2	18 October 2014	Negative
LNA-P	Pantanal	LNA-P-1	29 August 2017	Negative
		LNA-P-2	11 August 2016	Positive
WE-P	Pantanal	WE-P-1	14 November 2015	Positive
		WE-P-2	16 June 2016	Positive
		WE-P-3	15 December 2016	Positive
VA-P	Pantanal	VA-P-1	01 September 2018	Positive
		VA-P-2	19 August 2017	Positive
FFO-P	Pantanal	FFO-P-1	29 August 2018	Positive
		FFO-P-2	23 August 2017	Positive
PNA-P	Pantanal	PNA-P-1	15 November 2015	Positive
		PNA-P-2	25 August 2017	Negative
DHO-P	Pantanal	DHO-P-1	26 October 2017	Positive

	DHO-P-2	25 June 2018	Positive

Supplementary File 4. BLASTn analysis of the obtained gene fragments of Piroplasmida detected in lowland tapirs' blood samples.

Gene Fragment (size)	**Sample ID**	**GenBank accession number**	**Biome**	**BLASTn Best Match**	***Query cover***	***E-value***	**Similarity rate**
hsp70 (782 bp)	JHA-C	OP376711	Cerrado	*Theileria equi* – Mongolia (AB248743)	99%	0.0	81.69%
hsp70 (771bp)	JA-P	OP169598	Pantanal	*Theileria equi* – Mongolia (AB248743)	100%	0.0	81.17%
hsp70 (687 bp)	DA-P	OP169599	Pantanal	*Theileria equi* – Mongolia (AB248743)	99%	0.0	81.49%
hsp70 (695 bp)	RO-P	OP169597	Pantanal	*Theileria equi* – Mongolia (AB248743)	99%	0.0	81.36%
hsp70 (694 bp)	FFO-P-1	OP169596	Pantanal	*Theileria equi* – Mongolia (AB248743)	99%	0.0	81.07%
cox1 (410 bp)	JHA-C	OP169682	Cerrado	*Babesia bigemina* – China (JQ518300)	88%	$1,00^{-46}$	77.11%
cox1 (354 bp)	DA-P	OP9601	Pantanal	*Babesia bigemina* – China (JQ518300)	95%	$6,00^{-39}$	76.45%
cox1 (357 bp)	FFO-P-1	OP9600	Pantanal	*Babesia bigemina* – China (JQ518300)	94%	$1,00^{-36}$	76.09%
18S rRNA (1,401 bp)	JHA-C	OP023833	Cerrado	*Theileria* sp. – Kenia (KF597074)	100%	0.0	95.23%
18S rRNA (1,518 bp)	JA-P	OP023832	Pantanal	*Theileria equi* – Brazil (MG052902)	100%	0.0	95.33%
18S rRNA (1,348 bp)	XS-P	OP023830	Pantanal	*Theileria equi* - Israel (MN611344)	100%	0.0	95.48%

18S rRNA (1,213 bp)	DA-P	OP023831	Pantanal	*Theileria equi* - Chile (MT463613)	100%	0.0	95.31%
18S rRNA (1,182 bp)	RO-P	OP023828	Pantanal	*Theileria equi* - Israel (MN611344)	100%	0.0	95.53%
18S rRNA (1,308 bp)	FFO-P-1	OP023829	Pantanal	*Theileria equi* – USA (CP001669)	100%	0.0	95.40%
18S rRNA (1,473 bp)	PO-P	OP023834	Pantanal	*Theileria equi* – USA (CP001669)	100%	0.0	95.40%
18S rRNA (1,352 bp)	JO-P-1	OP023835	Pantanal	*Theileria equi* – Israel (MN611343)	100%	0.0	95.44%

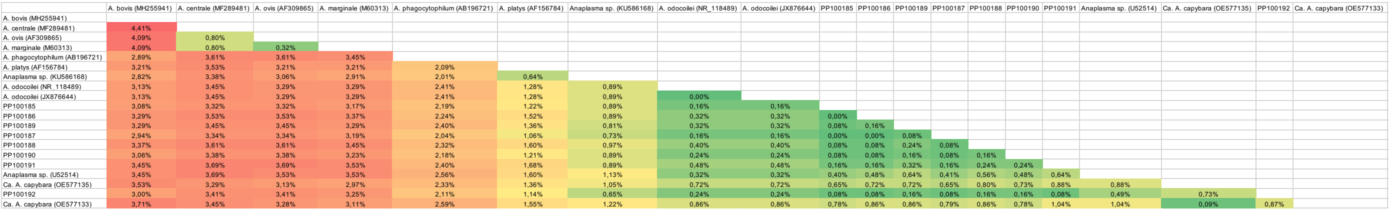
APÊNDICE C – Material suplementar citado no Capítulo VI

Molecular survey of vector-borne agents (Anaplasmataceae agents, *Hepatozoon* sp. and *Coxiella burnetii*) in lowland tapirs (*Tapirus terrestris*) from Brazil, with evidence of a putative novel *Anaplasma* genotype*

* Material corresponde à publicação científica subtmetida ao periódico Scientific Reports.

Anna Claudia Baumel Mongruel[1]; Emília Patrícia Medici[2,3,4]; Ariel Costa Canena[2]; Amir Salvador Alabi Cordova[1]; Lorena Freitas das Neves[1]; Eliz de Oliveira Franco[1]; Rosangela Zacarias Machado[1]; Marcos Rogério André[1]*

Supplementary File 1. Estimates of Evolutionary Divergence between Sequences. The number of base differences per site from between sequences are shown. This analysis involved 20 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1259 positions in the final matrix.



	A. bovis (MH255941)	A. centrale (MF289481)	A. ovis (AF309865)	A. marginale (M60313)	A. phagocytophilum (AB196721)	A. platys (AF156784)	Anaplasma sp. (KU586168)	A. odocoilei (NR_118489)	A. odocoilei (JX876644)	PP100185	PP100186	PP100189	PP100187	PP100188	PP100190	PP100191	Anaplasma sp. (U52514)	Ca. A. capybara (OE577135)	PP100192	Ca. A. capybara (OE577133)
A. bovis (MH255941)																				
A. centrale (MF289481)	4,41%																			
A. ovis (AF309865)	4,09%	0,80%																		
A. marginale (M60313)	4,09%	0,80%	0,32%																	
A. phagocytophilum (AB196721)	2,89%	3,61%	3,61%	3,45%																
A. platys (AF156784)	3,21%	3,53%	3,21%	3,21%	2,09%															
Anaplasma sp. (KU586168)	2,82%	3,38%	3,06%	2,91%	2,01%	0,64%														
A. odocoilei (NR_118489)	3,13%	3,45%	3,29%	3,29%	2,41%	1,28%	0,89%													
A. odocoilei (JX876644)	3,13%	3,45%	3,29%	3,29%	2,41%	1,28%	0,89%	0,00%												
PP100185	3,08%	3,32%	3,32%	3,17%	2,19%	1,22%	0,89%	0,16%	0,16%											
PP100186	3,29%	3,53%	3,53%	3,37%	2,24%	1,52%	0,89%	0,32%	0,32%	0,00%										
PP100189	3,29%	3,45%	3,45%	3,29%	2,40%	1,36%	0,81%	0,32%	0,32%	0,08%	0,16%									
PP100187	2,94%	3,34%	3,34%	3,19%	2,04%	1,06%	0,73%	0,16%	0,16%	0,00%	0,00%	0,08%								
PP100188	3,37%	3,61%	3,61%	3,45%	2,32%	1,60%	0,97%	0,40%	0,40%	0,08%	0,08%	0,24%	0,08%							
PP100190	3,06%	3,38%	3,38%	3,23%	2,18%	1,21%	0,89%	0,24%	0,24%	0,08%	0,08%	0,16%	0,08%	0,16%						
PP100191	3,45%	3,69%	3,69%	3,53%	2,40%	1,68%	0,89%	0,48%	0,48%	0,16%	0,16%	0,32%	0,16%	0,24%	0,24%					
Anaplasma sp. (U52514)	3,45%	3,69%	3,53%	3,53%	2,56%	1,60%	1,13%	0,32%	0,32%	0,40%	0,48%	0,64%	0,41%	0,56%	0,48%	0,64%				
Ca. A. capybara (OE577135)	3,53%	3,29%	3,13%	2,97%	2,33%	1,36%	1,05%	0,72%	0,72%	0,65%	0,72%	0,72%	0,65%	0,80%	0,73%	0,88%	0,88%			
PP100192	3,00%	3,41%	3,41%	3,25%	2,11%	1,14%	0,65%	0,24%	0,24%	0,08%	0,08%	0,16%	0,08%	0,16%	0,16%	0,08%	0,49%	0,73%		
Ca. A. capybara (OE577133)	3,71%	3,45%	3,28%	3,11%	2,59%	1,55%	1,22%	0,86%	0,86%	0,78%	0,86%	0,86%	0,79%	0,86%	0,78%	1,04%	1,04%	0,09%	0,87%	

APÊNDICE D – Material suplementar citado no Capítulo VIII

Unraveling the blood bacterial microbiome of wild lowland tapirs (*Tapirus terrestris*)*

*Esse capítulo corresponde à publicação que será subtmetida ao periódico Veterinary Microbiology.

Anna Claudia Baumel Mongruel[1], Emilia Patricia Medici[2,3,4], Rosangela Zacarias Machado[1], Keith Clay[5], Marcos Rogério André[1]

	ASV _ID	
1	03ad66b39cec401d1036891ca8c262cc	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__[Chromatiaceae]; g__Rheinheimera
2	04ba9e3f4a32f7a515e7c6e616d3dc5f	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
3	04be702b895fe5ed0568344daf564276	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Planococcaceae; g__Staphylococcus; s__saprophyticus
4	05fafcc76412edec77c53f7dce960fc5	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Propionibacteriaceae; g__Propionibacterium; s__granulosum
5	08135439d312c5bdb27840a374c9fa92	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Acinetobacter; s__
6	0a1f5ed70dcb37a86924dbd5fde981af	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Enhydrobacter; s__aerosaccus
7	0aa6767415c3c1d08d147c80b16ec596	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Acinetobacter; s__
8	0edc5ff275d55237c682d5bcb45b0e19	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
9	11c192ca47293df7d3c26138fd796fe1	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__Flavobacterium; s__frigidarium
10	13f7a1ef880f3b73eeffa18951ffe96f	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus; s__equi
11	14cb24789ad29bdddf97a42ca4c6dfce	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
12	16fe9b7db2d3563076c6e8a24abddb0f	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
13	18731d80f49ed91b1eb1c865c2ce41ca	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales
14	1f4bbbbfa2e2e5a5878d05c1233dae43	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae; g__Caldimonas; s__taiwanensis
15	1fa3c26d2bac5466a32336205841c9e1	k__Bacteria; p__Actinobacteria; c__Coriobacteriia; o__Coriobacteriales; f__Coriobacteriaceae; g__Paraeggerthella; s__hongkongensis
16	2015380aad94e031f9a2cf0e01ba03dd	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
17	20ac467db592c5f2150163c7967d8a74	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Leuconostocaceae; g__Weissella
18	21097c058b705302ecd7802c443528f0	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Microbacteriaceae
19	21b6ee8ac084654120e056cb2dd381b7	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
20	2272b2d9f186d6ad24563cc0dcaf3765	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
21	248d28bb07fd2d5fa5922de2371a21a4	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
22	255ddf004f2234a0064c14af08344de1	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
23	262259af1422a097100b3380887e9d04	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae
24	26750b51a2edd93e5d3ab98b36662317	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
25	26cf4b3a1d8ec76ae515ab623941648b	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__hamsteri
26	2739134975e692f1a0bee23916119749	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__[Chromatiaceae]; g__Rheinheimera
27	28dab638e3e969901d92af3ed9353bf6	k__Bacteria; p__OD1
28	29f5fc3922c18e5e8025d2cdeb5dedf8	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
29	2a1dbaa7b0d0dc33362e64e4c13e3818	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae; g__Herbaspirillum
30	2e119ce67894fe00027176e52f17a225	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
31	311223ac9943dee24dbaebb6bc1a304e	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae
32	31c5d6d689fe24ec7157fa1c0783fbd4	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Methylobacteriaceae
33	32ab1812bd3770f4b7f6aad7273783da	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae
34	35deff07f7a8db58bfb12827dba1158a	k__Bacteria; p__Bacteroidetes; c__[Saprospirae]; o__[Saprospirales]
35	362480cdff6780038f4bd6fcacdb2bf1	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
36	3835e304a2d4a518f6e249be5e14f50a	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Corynebacteriaceae; g__Corynebacterium; s__pilosum
37	3a8afb7663f6d93d5ae4db4795a84d27	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Acinetobacter; s__
38	3ae56d41fc8731dec40c08f286b36716	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae
39	3c3c05b27064591a63bdabfe89ee6022	k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Porphyromonadaceae; g__Macellibacteroides; s__fermentans
40	3cebcd5992609cda278e47ab7376fe83	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
41	3f1db669424e77cab5735a17bcb27e59	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria
42	4394468b49611f78b96b64d72449fab5	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae
43	4429157a05e174a4eba7af3128dd0c64	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Oxalobacteraceae
44	4502317a578382c4547acb32ff4574d6	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
45	454a7f1535c0cbd3b5d3f31d3d9a9480	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
46	45893b0ffe9ee24172c0ce0933fa305f	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
47	4674dea25f9deb23b7be1d8d4d7cb144	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria
48	4a14dee1a8cc9d0a813528b5bcc0ed8e	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Xanthomonadales; f__Sinobacteraceae; g__Panacagrimonas; s__perspica
49	4b595f7827967ef0eff53497f5d849ab	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Corynebacteriaceae; g__Corynebacterium; s__pilosum
50	4c9939a93db7b14a028245155492ac8e	k__Bacteria; p__OD1
51	4f16c9a7a80c3c9cc90a31da039e3989	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
52	513bf19be2924761125550a901a313cc	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
53	517225c482bb65ad7c3407d1e45cf489	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae
54	5539341edb94af8d5138ae0193366c62	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
55	570979eb5a8ef3a00af9339c650d1a00	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
56	572a0665979c2c6a45616619930514f6	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
57	5bcd69e7e6afaae2c17463a8d00391d0	k__Bacteria; p__Bacteroidetes; c__[Saprospirae]; o__[Saprospirales]; f__Chitinophagaceae
58	5beee790c3f7d29e9b0808faa064f187	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
59	5cb5319c5fd3426bd95d3641ff186662	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
60	5e125599ee89ac3260cc4024ea8b4724	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
61	607210555620dd5a1df145d2c8876d73	k__Bacteria; p__Chlamydiae; c__Chlamydiia; o__Chlamydiales
62	62611dedc42201591d0c89973f903dde	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Planococcaceae; g__Staphylococcus; s__saprophyticus
63	630c90c1a33f9d34869c153442022884	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
64	6312ccac35ef76b6cd6136041c541f3d	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae
65	65144636814a18e49450d44233d6e814	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
66	66abdc6d88690803ca7ef1ce089e5f0c	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
67	6774b77a918f788ccec66d24dedb2973	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria
68	68e8cb9a10ff6e7476a705adb4fac4e7	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
69	6ae4104372af1a36292b2bd613c38927	k__Bacteria; p__Proteobacteria; c__Deltaproteobacteria; o__Myxococcales; f__Polyangiaceae
70	6fb421a1c5aa43eeb0ab888d6c080bc7	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
71	7272c51d83268a0131ee950b06b8239b	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales; f__Anaplasmataceae; g__Anaplasma; s__
72	7330ee02d391259df0295ae2b5d7cf21	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae
73	78a0bcfac38c80548dca6efacab0b2c6	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
74	78e2ddd93f90ebe4fc536f9ad057f4a5	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
75	7ab99091ebbddaeb2650f11761a51479	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
76	7aff7bbcb23a694a29fdc20d42b0af83	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__Flavobacterium; s__frigidarium
77	7b3e30e983fce82edc4e3f3b40ebbe47	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
78	7ba23cc85c3a44a4b32f2b234e3e1dd2	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Microbacteriaceae
79	7e4e6676a435751d2ab10295065f5cd4	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
80	81b62f43536add84f73af476071594c4	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__[Weeksellaceae]; g__Elizabethkingia; s__
81	876ed260be5f69590ce1242534b48054	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Planococcaceae; g__Staphylococcus; s__saprophyticus
82	892a20bbdc3ce599dc0c5d9f0866c352	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales
83	89ad88b91e12fcce5d5790fd77000a57	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__[Weeksellaceae]
84	8b1b32d9af60d57158e71d22b1f843be	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
85	8b61f5e4967530a115aff8a360cc5842	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
86	8bd941f369146b21668cf4aebfd00ac6	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__[Weeksellaceae]; g__Elizabethkingia; s__
87	8be473aa64c27225df77865b4f345666	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
88	90075165fc9ab9a8057e2113d942d7e6	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__[Chromatiaceae]; g__Rheinheimera
89	90f895dbdb04327995b0f4dd78e575fb	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
90	982fdc802f340a9f69032d1ae49a7386	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__Flavobacterium; s__frigidarium
91	9ad0d25560a9d3a0b750c36b7aad8392	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
92	9af351833167aa882a6ce2fdafaf6f3e	k__Bacteria; p__OD1; c__; o__; f__; g__; s__
93	a1c6668670cc737922f8e4334b64267f	k__Bacteria; p__Bacteroidetes; c__Sphingobacteriia; o__Sphingobacteriales; f__Sphingobacteriaceae
94	a1d3220a20ac98af61a2e091539e69a5	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
95	a4cd6152db4ba6371bd3d35fcda62a19	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus; s__equi
96	a7e222a52a943c1de5d92c61464a3f07	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
97	a7f4f37e18a72851ae88e97b45c1276c	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
98	acfd2b263f99cde32d98fc4df36539ee	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales
99	adaee256a34ff7012e77d1a26d82b874	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Micrococcaceae; g__Rothia; s__terrae
100	ae67b413f957a845132c368fd32d1fce	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales
101	aeb12f61d5193dbf1123ace2381bd3d2	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Acinetobacter; s__
102	b6dcdf54545737c71d980b191de75e3a	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
103	babc009e4ac52b2c6b9a63837da6c2a5	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
104	bdd73509000af1a93b2927ff1ba4fed9	k__Bacteria; p__Bacteroidetes; c__Cytophagia; o__Cytophagales
105	c27ad79848adcd406bf90804cb13ec9b	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae
106	c2a2f35f1ae2a41d88121662b03d83e6	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
107	c41af3ba7aeabdb1886cf01eddb79139	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Sphingomonadales; f__Sphingomonadaceae; g__Sphingomonas; s__suberifaciens
108	c428e029aad3404d18c9fcfd795dc508	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
109	c49cc7c2c45bd7a87913453e515ea14f	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Acinetobacter; s__
110	c4a5d68c1391a83b4bab19e5812a7904	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Corynebacteriaceae; g__Corynebacterium; s__pilosum
111	c533a557f90737bbc8bccbbc981a8c63	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus
112	c55a571614ca92c4b5875935df08f257	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae
113	c64d0b194420756c348758b5e64f855e	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Rhizobiaceae; g__Rhizobium; s__daejeonense
114	c6510e50c8d7d2c9c539b82845c4df5b	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
115	c68781248b41691661cf9f194719be06	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae
116	ca65437e6163b04f99baf2731c1f0a0a	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae
117	cbcfd3f7e2b5be34fc29899e7f0e319f	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
118	cd3909b22b87684aafd93fcb66630f52	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Acinetobacter; s__
119	ce35ab9691c1ec5db18a78bf12aa0bc5	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae
120	cf547e363a5572e57e68c5566f429996	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
121	d3829c53b66db828d6bff7f21c6dad28	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus; s__equi
122	d521e757593957df103c8faa80e5950e	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Acinetobacter; s__
123	d6971310164f968a778a7d83d88c19a5	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Actinomycetaceae; g__Actinomyces; s__hyovaginalis
124	d6dffb454cbb0b0c87367b03432c2b05	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae
125	dc1b41b34f1a9f7cbd2d4272b6e7bf51	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
126	dcba46b787795490df1667d483068d6c	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae
127	debbd2518f89bd208ea561ccac858d00	k__Bacteria; p__Verrucomicrobia; c__Opitutae; o__Opitutales; f__Opitutaceae
128	dfb87f858fd7f121bdb797690faced5c	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales; f__Rhizobiaceae
129	e11e824b7530998773a8908ab96a8cd8	k__Bacteria; p__OD1
130	e256943a9c23f573c73878576ddc18e3	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Micrococcaceae; g__Arthrobacter
131	e277a74232d5b221ca58c64797ae674a	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Rhodocyclales; f__Rhodocyclaceae; g__Petrobacter; s__succinatimandens
132	e800d52e2b60d3de7885f60243bb6e0b	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Planococcaceae
133	e9243fff2a19040bb29074393cfc3935	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
134	e95b972d78d4466655a9d6d84b8c4453	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
135	ea20cbcfceb2a1d9a7dd6a4f1f51ad77	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Pseudomonadaceae; g__Pseudomonas
136	ec098ad12ef2923b449a01762462578b	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae
137	efcae378b99455f9632a9cede24c1176	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
138	f326908d7207821ffdb47e46357abfac	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
139	f445684720dc694918e46dcb9a958503	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
140	f5f998a060cbe1d99a7e4494c188b70f	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales
141	f6094ee5aa97f36d4449c147d524dcd8	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Acinetobacter; s__
142	f6896f4964f294b605c73a6d6de6683d	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
143	f72e007caf0154c2851b91d8396d676f	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales
144	f91087040dbc65ffe7d038920443fc56	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pseudomonadales; f__Moraxellaceae; g__Acinetobacter; s__
145	fa3652eba79d0a64026ed0edc5c3390e	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
146	faedcc957861632b6d3d7be71642ac41	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
147	fccf5e3406daf30c18d7122afea31316	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae
148	fd327273995619fb5d70b358dffee040	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__ACK-M1; g__Planktophila; s__limnetica
149	fdfb39789f959d409b8f1895c1d18164	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
150	ffa1234367dc498e33448a07a3453637	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Aeromonadales; f__Aeromonadaceae; g__Aeromonas
151	ffabea3d960100f92a000051cb63bd52	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae

Supplementary File 1: List of contaminant ASVs identified by microDecon software. ASVs listed below were removed from all samples.

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qiime taxa filter-table --i-table tabelakitome1.qza --i-taxonomy taxonomy-kitome1.qza --p-mode contains --p-include p__ --p-exclude 'Afipia,Aquabacterium,Asticcacaulis,Aurantimonas,Beijerinckia,Bosea,Bradyrhizobium,Brevundimonas,Caulobacter,Craurococcus,Devosia,Hoeflea,Mesorhizobium,Methylobacterium,Novosphingobium,Ochrobactrum,Paracoccus,Pedomicrobium,Phyllobacterium,Rhizobium,Roseomonas,Sphingobium,Sphingomonas,Sphingopyxis,Acidovorax,Azoarcus,Azospira,Burkholderia,Comamonas,Cupriavidus,Curvibacter,Delftia,Duganella,Herbaspirilluma,Janthinobacterium,Kingella,Leptothrixa,Limnobacter,Massilia,Methylophilus,Methyloversatilis,Oxalobacter,Pelomonas,Polaromonas,Ralstonia,Schlegelella,Sulfuritalea,Undibacterium,Variovorax,Acinetobactera,Enhydrobacter,Enterobacter,Escherichia,Nevskiae,Pseudomonas,Pseudoxanthomonas,Psychrobacter,Stenotrophomonasa,Xanthomonas,Aeromicrobium,Arthrobacter,Beutenbergia,Brevibacterium,Corynebacterium,Curtobacterium,Dietzia,Geodermatophilus,Janibacter,Kocuria,Microbacterium,Micrococcus,Microlunatus,Patulibacter,Propionibacterium,Rhodococcus,Tsukamurella,Abiotrophia,Bacillus,Brevibacillus,Brochothrix,Facklamia,Paenibacillus,Streptococcus,Chryseobacterium,Dyadobacter,Flavobacteriumd,Hydrotalea,Niastella,Olivibacter,Pedobacter,Wautersiella,Deinococcus,Actinomadura,Intrasporangiaceae,Calothrix,Tolypothrix,Thermogemmatisporaceae,Gaiella,Sphingosinicella,Solirubrobacter,Methylosarcina,Nostocoida,Rheinheimera,Chromatiaceae,Chroococcidiopsis,Leifsonia,Nostocaceae,Nostocoida,Papillibacter,Rhodospirillales,Thermoleophilia,Solibacterales,Mucilaginibacter,Saprospiraceae,Millisia,Deltaproteobacteria,Pirellulaceae,Leptotrichiaceae,Blastococcus,Scytonema,Cystobacter,Legionella,Methylotenera,Rhodopila,Chloronema,Armatimonadaceae,Rhodospirillaceae,Polyangium,Scytonema,Actinomycetales,Xanthobacter,Denitromonas,Marvinbryantia,Chthonomonadales,Koribacteraceae,Methanocorpusculaceae,Desulfuromonas,Sporosarcina,Truepera,Granulicella,Thioalkalivibrio,Desulfurellale,Blastococcus,Ornithinimicrobium,Cellvibrio,Sandarakinorhabdus,Chloroflexi,OD1,Verrucomicrobia,Thermicanus,Macellibacteroides,Siphonobacter,Petrobacter,SR1,Alysiella,Turneriella,Halobacteriales,Prosthecobacter,Porphyrobacter,TA18,Phycisphaerae,Flavobacterium,Cyanobacteria,Cytophagaceae,Rhodothermales,Halolamina,Alcanivorax,Phycisphaeraceae,Haloferula,Chloracidobacteria,Rhodobacter,Lishizhenia,Planctomycetes,Vibrio,Leptolyngbya,Ferruginibacter,Gemmatales,Trachelomonas,rimae,Chroococcales,TM6,Pedosphaerales,Anaerosinus,Gemmatimonadetes,Chloracidobacteria,Phycisphaerales,Acetonema,Algoriphagus,Terrimonas,Nitrospira,Ferruginibacter,Thermomonas,Verrucomicrobia,Spirochaeta,Acidobacteria,TM7,Gemmatimonadales,Gulbenkiania,Cytophaga,Synechococcaceae,Planctomyces,Rhodobacteraceae,Anaerorhabdus,Succinispira,Sphingomonadaceae,Xenococcaceae,Flavobacterium,RFP12,Rhodocyclaceae,Albidovulum,Chitinophagacea,Nonlaben,Cytophagales,Ilumatobacter,Nostocophycideae,Planctomycetia,Sphingomonadales,Gloeocapsopsis,Bryocella,Filimonas,Agitococcus,Terriglobus,sharmana,Methylacidiphilales,Nostocales,Microthrixaceae,Lentisphaeria,Phycisphaerae,Pseudogulbenkiania,Acidobacteriaceae,Lysobacter,Rhizobium,Methylobacteriaceae,Blattabacteriaceae,Agrobacterium,Lachnoanaerobaculum,Elusimicrobia,Aquitalea,GN02,Synergistales,Simkania,Sulfuricurvum,Hyphomicrobium,Defluviitalea, Marinomonas,Mariprofundaceae,OP11,Asteroleplasma,Persicivirga,Planifilum,Amorphomonas,Fimbriimonas,Selenomonas,Pleomorphomonas,Sphingobacterium,Nitrosospira,Alkanindiges,Thermovum,Alteromonadaceae,Sphingobacterium,OPB56,Aquitalea,SHA-37,OPB41,OP3' --o-filtered-table tabelakitome2.qza
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Supplementary File 2: Qiime2 fitering command used for filtering of contaminant taxa, based on reports of contaminants in microbiome studies. Every ASV idenfied with the taxa included in the command were excluded.

number	sex	age	site	dead_or_living
numeric	categorical	categorical	categorical	categorical
1	Male	adult	Cerrado	living
2	Male	subadult	Cerrado	living
3	Male	adult	Cerrado	living
4	Female	adult	Cerrado	living
5	Female	adult	Cerrado	living
6	Female	adult	Cerrado	living
7	Female	subadult	Cerrado	living
8	Male	adult	Cerrado	living
9	Female	juvenile	Cerrado	living
10	Female	adult	Cerrado	living
11	Female	adult	Cerrado	living
13	Male	subadult	Cerrado	living
14	Male	juvenile	Cerrado	living
17	Female	adult	Cerrado	living
18	Female	adult	Cerrado	living
19	Female	adult	Cerrado	living
20	Male	adult	Cerrado	living
21	Female	subadult	Cerrado	living
22	Female	subadult	Cerrado	living
23	Female	adult	Cerrado	living
24	Male	subadult	Cerrado	living
26	Male	adult	Cerrado	living
27	Female	adult	Cerrado	living
28	Female	juvenile	Cerrado	living
29	Male	subadult	Cerrado	living
30	Female	adult	Cerrado	living
31	Male	adult	Cerrado	living
32	Male	subadult	Cerrado	living
33	Male	subadult	Cerrado	living
35	Female	subadult	Pantanal	living
36	Male	juvenile	Pantanal	living
37	Male	adult	Pantanal	living
38	Female	adult	Pantanal	living
40	Male	subadult	Pantanal	living
42	Male	adult	Pantanal	living
43	Female	adult	Pantanal	living
44	Male	subadult	Pantanal	living
45	Male	subadult	Pantanal	living
48	Male	subadult	Pantanal	living
53	Male	subadult	Pantanal	living
56	Male	subadult	Pantanal	living
57	Female	adult	Pantanal	living
60	Male	subadult	Pantanal	living
62	Male	juvenile	Pantanal	living
64	Male	juvenile	Pantanal	living
66	Female	juvenile	Pantanal	living
68	Male	adult	Pantanal	living
70	Male	adult	Pantanal	living
72	Male	subadult	Pantanal	living
74	Male	adult	Pantanal	living
77	Male	adult	Pantanal	living
78	Female	subadult	Pantanal	living
80	Male	subadult	Pantanal	living
81	Male	adult	Pantanal	living
83	Male	subadult	Pantanal	living
85	Female	adult	Pantanal	living
86	Female	adult	Pantanal	living
87	Female	adult	Pantanal	living
88	Male	adult	Pantanal	living
91	Female	subadult	Pantanal	living
93	Male	juvenile	Pantanal	living
95	Female	adult	Pantanal	living
96	Female	adult	Pantanal	living
98	Female	adult	Cerrado	living
100	Female	adult	Cerrado	living
101	Female	adult	Cerrado	living
103	Male	adult	Pantanal	living
105	Female	adult	Pantanal	living
106	Male	subadult	Pantanal	living
108	Female	subadult	Pantanal	living
109	Male	subadult	Pantanal	living
110	Male	subadult	Pantanal	living
111	Male	subadult	Pantanal	living
112	Male	subadult	Pantanal	living
113	Male	subadult	Pantanal	living
114	Female	subadult	Pantanal	living
115	Female	subadult	Pantanal	living
116	Male	adult	Pantanal	living
118	Female	subadult	Cerrado	dead
119	Female	juvenile	Cerrado	dead
121	Male	adult	Cerrado	dead
122	Male	adult	Cerrado	dead
123	Male	adult	Cerrado	dead
124	Female	adult	Cerrado	dead
125	Male	subadult	Cerrado	dead
126	Female	adult	Cerrado	living

Supplementary File 3: Data regarding sex, age, biome and condition from each tapir sample from the present study.

APÊNDICE E - Resultados gerais obtidos no presente trabalho

Numero da amostra	data	SIGLA	Sexo	Idade	Local	Rodovia	Piroplasmas	Coxiella	Hemoplasmas	Hepatozoon	Bartonella	Ehrlichia (dsb)	Anaplasma	Neorickettsia	Borrelia (qPCR)
1	11/04/18	SO-C-1	Macho	adulto	Cerrado	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
2	08/12/14	RER-C	Macho	sub	Cerrado	-	**POSITIVO**	neg	neg	neg	neg	neg	**POSITIVO**	neg	neg
3	10/03/16	LOU-C-1	Macho	adulto	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
4	03/04/17	PAA-C	Femea	adulto	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
5	16/03/16	SNE-C	Femea	adulto	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
6	07/04/16	EIS-C	Femea	adulto	Cerrado	-	neg	neg	neg	neg	**POSITIVO**	neg	neg	neg	neg
7	03/05/17	NAI-C	Femea	sub	Cerrado	-	**POSITIVO**	neg	neg	neg	neg	neg	**POSITIVO**	neg	neg
8	19/10/16	ANO-C-1	Macho	adulto	Cerrado	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
9	04/08/16	JHA-C	Femea	juvenil	Cerrado	-	**POSITIVO**	neg	neg	neg	**POSITIVO**	neg	**POSITIVO**	neg	neg
10	23/02/17	CBI-C-1	Femea	adulto	Cerrado	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
11	16/03/17	ISA-C	Femea	adulto	Cerrado	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
12	05/04/16	CNA-C-1	Femea	adulto	Cerrado	-	neg	neg	neg	neg	**POSITIVO**	neg	neg	neg	neg
13	09/02/17	AEL-C	Macho	sub	Cerrado	-	neg	neg	neg	neg	neg	neg	**POSITIVO**	**POSITIVO**	neg
14	25/05/17	AN-C	Macho	juvenil	Cerrado	-	**POSITIVO**	neg	neg	neg	neg	neg	**POSITIVO**	**POSITIVO**	neg
15	28/07/16	SU-C	Femea	adulto	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
16	16/09/17	SFA-C	Femea	adulto	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
17	15/09/17	DDA-C	Femea	adulto	Cerrado	-	neg	neg	neg	neg	**POSITIVO**	neg	neg	neg	neg
18	26/07/16	ZCA-C-1	Femea	adulto	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
19	22/04/17	SRE-C	Femea	adulto	Cerrado	-	neg	neg	neg	neg	**POSITIVO**	neg	neg	neg	neg
20	21/03/17	SDO-C	Macho	adulto	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
21	27/10/16	EOA-C	Femea	sub	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
22	29/04/17	DNA-C	Femea	sub	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
23	02/05/17	ZGY-C	Femea	adulto	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
24	27/04/17	LIS-C	Macho	sub	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
25	13/10/16	MRI-C	Femea	juvenil	Cerrado	-	neg	neg	neg	neg	**POSITIVO**	neg	**POSITIVO**	neg	neg
26	12/10/16	KA-C/KKA-C	Macho	adulto	Cerrado	-	neg	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
27	24/06/17	ANN-C	Femea	adulto	Cerrado	-	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg	neg
28	26/07/16	BLA-C	Femea	juvenil	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
29	26/06/13	CRO-C	Macho	sub	Cerrado	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
30	12/02/17	ZFA-C	Femea	adulto	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
31	12/03/16	TI-C	Macho	adulto	Cerrado	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
32	11/02/17	ZIO-C	Macho	sub	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
33	29/06/17	LAS-C	Macho	sub	Cerrado	-	neg	neg	neg	neg	**POSITIVO**	neg	neg	neg	neg
34	27/08/17	ANA-P	Femea	adulto	Pantanal	-	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg	neg
35	22/08/17	CHA-P-1	Femea	sub	Pantanal	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
36	19/05/15	THO-P	Macho	juvenil	Pantanal	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
37	29/10/17	SAO-P-1	Macho	adulto	Pantanal	-	neg	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
38	13/11/15	DRA-P-1	Femea	adulto	Pantanal	-	neg	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
39	17/08/16	WLY-P	Macho	adulto	Pantanal	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
40	24/08/18	PO-P	Macho	sub	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	**POSITIVO**	**POSITIVO**	neg
41	08/12/14	MO-P/MNO-P	Macho	juvenil	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
42	14/06/18	SKI-P	Macho	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	**POSITIVO**	neg	neg
43	28/07/13	MA-P-1	Femea	adulto	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
44	25/07/17	MDO-P	Macho	sub	Pantanal	-	neg	neg	neg	neg	**POSITIVO**	neg	neg	neg	neg
45	18/12/13	TCO-P	Macho	sub	Pantanal	-	neg	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
46	13/07/14	GO-P	Macho	sub	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	**POSITIVO**	neg	neg
47	20/08/14	DUD-P	Femea	adulto	Pantanal	-	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg	neg
48	06/12/17	JE-P-1	Macho	sub	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	**POSITIVO**	**POSITIVO**	neg
49	16/11/15	TD-P-1	Macho	sub	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
50	28/10/17	SY-P	Femea	adulto	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
51	15/08/16	PYE-P	Macho	adulto	Pantanal	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
52	04/05/14	RA-P	Femea	juvenil	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
53	31/10/18	CO-P	Macho	sub	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	**POSITIVO**	neg	neg
54	27/10/14	JO-P-1	Macho	sub	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
55	06/07/14	CJO-P	Macho	adulto	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
56	23/07/13	CIO-P/CO-P	Macho	sub	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	**POSITIVO**	**POSITIVO**	neg
57	21/07/13	CRA-P-1	Femea	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
58	05/05/14	JA-P	Femea	juvenil	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
59	29/08/17	LA-P/LNA-P-1	Femea	adulto	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
60	22/10/18	AA-P	Macho	sub	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	**POSITIVO**	neg	neg
61	22/10/13	MU-P	Femea	adulto	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
62	21/08/17	XS-P	Macho	juvenil	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	**POSITIVO**	neg	neg
63	10/12/17	IA-P	Macho	adulto	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
64	08/12/17	COS-P	Macho	juvenil	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
65	05/12/14	WM-P	Macho	juvenil	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	neg	**POSITIVO**	neg
66	05/12/17	SA-P/SIA - P	Femea	juvenil	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	**POSITIVO**	neg
67	25/08/18	DA-P	Femea	sub	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
68	20/06/16	NAO-P	Macho	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	**POSITIVO**
69	10/05/15	RTA-P	Femea	adulto	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	**POSITIVO**	neg
70	21/10/14	RCK-P	Macho	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
71	14/11/15	WE-P-1	Femea	sub	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	neg	**POSITIVO**
72	22/08/16	HRY-P	Macho	sub	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
73	01/09/18	VA-P-1	Femea	sub	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
74	17/12/14	FPE-P	Macho	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
75	25/06/18	RO-P	Macho	sub	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	**POSITIVO**	neg	neg
76	10/12/17	LRO-P	Macho	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
77	08/12/16	SCO-P	Macho	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
78	03/07/14	MLE-P	Femea	sub	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
79	16/08/16	BEN-P	Macho	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
80	27/10/17	GIL-P	Macho	sub	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
81	29/08/18	FFO-P-1	Macho	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
82	25/06/18	MIA-P	Femea	sub	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	**POSITIVO**	neg
83	26/10/17	DHO-P-1	Macho	sub	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
84	22/10/18	CHA-P-2	Femea	sub	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
85	25/06/18	NLA-P	Femea	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
86	12/07/14	KIN-P	Femea	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
87	15/11/15	PNA-P-1	Femea	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	**POSITIVO**	**POSITIVO**	neg
88	19/06/18	GAO-P	Macho	adulto	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
89	27/08/18	INA-P	Femea	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
90	02/07/15	MCI-P	Femea	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
91	06/07/14	GLA-P	Femea	sub	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
92	22/10/14	DJO-P	Femea	adulto	Pantanal	-	neg	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
93	08/06/16	AO-P	Macho	juvenil	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
94	18/06/18	FA-P	Femea	adulto	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
95	18/10/14	CRA-P-1	Femea	adulto	Pantanal	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
96	19/05/15	MA-P-2	Femea	adulto	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
97	28/06/17	ANO-C-2	Macho	adulto	Cerrado	-	neg	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
98	14/11/17	ZCA-C-2	Femea	adulto	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
99	19/09/17	LOU-C-2	Macho	adulto	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
100	19/09/18	CNA-C-2	Femea	adulto	Cerrado	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	**POSITIVO**	neg
101	11/10/18	CBI-C-2	Femea	adulto	Cerrado	-	neg	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
102	09/02/17	SO-C-2	Macho	adulto	Cerrado	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
103	23/08/17	FFO-P-2	Macho	adulto	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	**POSITIVO**	neg
104	25/08/17	PNA-P-2	Femea	adulto	Pantanal	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
105	11/08/16	LNA-P-2	Femea	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
106	20/08/16	JO-P-2	Macho	sub	Pantanal	-	neg	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
107	28/07/13	DRA-P-2	Femea	adulto	Pantanal	-	neg	neg	neg	neg	neg	neg	neg	neg	neg
108	19/08/17	VA-P-2	Femea	sub	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	**POSITIVO**	neg	neg
109	25/06/18	DO-P-2/DHO-P-2	Macho	sub	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
110	09/06/16	JE-P-2	Macho	sub	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
111	11/06/16	BS-P/BRS-P	Macho	sub	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
112	17/12/14	TD-P-2	Macho	sub	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	**POSITIVO**	neg	neg
113	18/06/16	TD-P-3	Macho	sub	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
114	16/06/16	WE-P-2	Femea	sub	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
115	15/12/16	WE-P-3	Femea	sub	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	**POSITIVO**	neg
116	20/10/13	SAO-P-2	Macho	adulto	Pantanal	-	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	**POSITIVO**	neg	neg
117	11/05/15	SAO-P-3	Macho	adulto	Pantanal	-	**POSITIVO**	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
118	23/05/16	Nec 09	Fêmea	sub	Cerrado	BR267	**POSITIVO**	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
119	10/03/16	Nec 07	Fêmea	juvenil	Cerrado	BR267	**POSITIVO**	neg	neg	neg	neg	neg	neg	**POSITIVO**	neg
120	13/10/16	Nec 21	Macho	adulto	Cerrado	BR267	-	-	-	-	-	-	-	-	-
121	22/10/16	Nec 22	Macho	adulto	Cerrado	BR267	-	-	-	-	-	-	-	-	-
122	20/07/16	Nec 15	Macho	adulto	Cerrado	MS040	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
123	29/07/16	Nec 18	Macho	adulto	Cerrado	BR267	neg	neg	**POSITIVO**	neg	neg	neg	neg	neg	neg
124	18/11/16	Nec 24	Fêmea	adulto	Cerrado	BR267	**POSITIVO**	neg	neg	neg	neg	neg	neg	neg	neg
125	10/11/16	Nec 23	Macho	sub	Cerrado	BR267	-	-	-	-	-	-	-	-	-

Figura 01: Resultados das triagens para cada amostra. As amostras marcadas em vermelho representam amostras de DNA que não amplificaram genes endógenos e, portanto, não foram submetidas aos ensaios para pesquisa de hemoparasitos.

Numero da amostra	data	SIGLA	Sexo	Idade	Local	Rodovia	Piroplasmas	Piro Sequenciadas	Coxiella	Hemoplasmas	Hemo Sequenciadas	Hepatozoon	Bartonella	Barto Sequenciadas	Ehrlichia (dsb)	Anaplasma	Ana Sequenciadas	Neorickettsia	Neo Sequenciadas	Borrelia (qPCR)	Borrelia Sequenciadas
1	11/04/18	SO-C-1	Macho	adulto	Cerrado	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
2	08/12/14	RER-C	Macho	sub	Cerrado	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	POSITIVO	-	neg		neg	
3	10/03/16	LOU-C-1	Macho	adulto	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
4	03/04/17	PAA-C	Femea	adulto	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
5	16/03/16	SNE-C	Femea	adulto	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
6	07/04/16	EIS-C	Femea	adulto	Cerrado	-	neg	-	neg	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg		neg	
7	03/05/17	NAI-C	Femea	sub	Cerrado	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	POSITIVO	SEQUENCIADA	neg		neg	
8	19/10/16	ANO-C-1	Macho	adulto	Cerrado	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
9	04/08/16	JHA-C	Femea	juvenil	Cerrado	-	POSITIVO	SEQUENCIADA	neg	neg	-	neg	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg		neg	
10	23/02/17	CBI-C-1	Femea	adulto	Cerrado	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
11	16/03/17	ISA-C	Femea	adulto	Cerrado	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
12	05/04/16	CNA-C-1	Femea	adulto	Cerrado	-	neg	-	neg	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg		neg	
13	09/02/17	AEL-C	Macho	sub	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	POSITIVO	-	POSITIVO		neg	
14	25/05/17	AN-C	Macho	juvenil	Cerrado	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	POSITIVO	SEQUENCIADA	POSITIVO		neg	
15	28/07/16	SU-C	Femea	adulto	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
16	16/09/17	SFA-C	Femea	adulto	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
17	15/09/17	DDA-C	Femea	adulto	Cerrado	-	neg	-	neg	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg		neg	
18	26/07/16	ZCA-C-1	Femea	adulto	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
19	22/04/17	SRE-C	Femea	adulto	Cerrado	-	neg	-	neg	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg		neg	
20	21/03/17	SDO-C	Macho	adulto	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
21	27/10/16	EOA-C	Femea	sub	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
22	29/04/17	DNA-C	Femea	sub	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
23	02/05/17	ZGY-C	Femea	adulto	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
24	27/04/17	LIS-C	Macho	sub	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
25	13/10/16	MRI-C	Femea	juvenil	Cerrado	-	neg	-	neg	neg	-	neg	POSITIVO	SEQUENCIADA	neg	POSITIVO	-	neg		neg	
26	12/10/16	KA-C/KKA-C	Macho	adulto	Cerrado	-	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
27	24/06/17	ANN-C	Femea	adulto	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	POSITIVO	-	neg		neg	
28	26/07/16	BLA-C	Femea	juvenil	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
29	26/06/13	CRO-C	Macho	sub	Cerrado	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
30	12/02/17	ZFA-C	Femea	adulto	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
31	12/03/16	TI-C	Macho	adulto	Cerrado	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
32	11/02/17	ZIO-C	Macho	sub	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
33	29/06/17	LAS-C	Macho	sub	Cerrado	-	neg	-	neg	neg	-	neg	POSITIVO	-	neg	neg	-	neg		neg	
34	27/08/17	ANA-P	Femea	adulto	Pantanal	-	neg	-	neg	neg	-	neg	neg	-	neg	POSITIVO	-	neg		neg	
35	22/08/17	CHA-P-1	Femea	sub	Pantanal	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
36	19/05/15	THO-P	Macho	juvenil	Pantanal	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
37	29/10/17	SAO-P-1	Macho	adulto	Pantanal	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
38	13/11/15	DRA-P-1	Femea	adulto	Pantanal	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
39	17/08/16	WLY-P	Macho	adulto	Pantanal	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
40	24/08/18	PO-P	Macho	sub	Pantanal	-	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg	POSITIVO	-	POSITIVO		neg	
41	08/12/14	MO-P/MNO-P	Macho	juvenil	Pantanal	-	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
42	14/06/18	SKI-P	Macho	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	POSITIVO	SEQUENCIADA	neg		neg	
43	28/07/13	MA-P-1	Femea	adulto	Pantanal	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
44	25/07/17	MDO-P	Macho	sub	Pantanal	-	neg	-	neg	neg	-	neg	POSITIVO	-	neg	neg	-	neg		neg	
45	18/12/13	TCO-P	Macho	sub	Pantanal	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
46	13/07/14	GO-P	Macho	sub	Pantanal	-	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	POSITIVO	SEQUENCIADA	neg		neg	
47	20/08/14	DUD-P	Femea	adulto	Pantanal	-	neg	-	neg	neg	-	neg	neg	-	neg	POSITIVO	-	neg		neg	
48	06/12/17	JE-P-1	Macho	sub	Pantanal	-	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	POSITIVO	-	POSITIVO		neg	
49	16/11/15	TD-P-1	Macho	sub	Pantanal	-	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
50	28/10/17	SY-P	Femea	adulto	Pantanal	-	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
51	15/08/16	PYE-P	Macho	adulto	Pantanal	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
52	04/05/14	RA-P	Femea	juvenil	Pantanal	-	neg	-	neg	POSITIVO	-	neg	neg	-	neg	neg	-	neg		neg	
53	31/10/18	CO-P	Macho	sub	Pantanal	-	neg	-	neg	POSITIVO	-	neg	neg	-	neg	POSITIVO	-	neg		neg	
54	27/10/14	JO-P-1	Macho	sub	Pantanal	-	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
55	06/07/14	CJO-P	Macho	adulto	Pantanal	-	neg	-	neg	POSITIVO	-	neg	neg	-	neg	neg	-	neg		neg	
56	23/07/13	CIO-P/CO-P	Macho	sub	Pantanal	-	POSITIVO	-	neg	POSITIVO	-	neg	neg	-	neg	POSITIVO	-	POSITIVO		neg	
57	21/07/13	CRA-P-1	Femea	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
58	05/05/14	JA-P	Femea	juvenil	Pantanal	-	POSITIVO	SEQUENCIADA	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
59	29/08/17	LA-P/LNA-P-1	Femea	adulto	Pantanal	-	neg	-	neg	POSITIVO	-	neg	neg	-	neg	neg	-	neg		neg	
60	22/10/18	AA-P	Macho	sub	Pantanal	-	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	POSITIVO	SEQUENCIADA	neg		neg	
61	22/10/13	MU-P	Femea	adulto	Pantanal	-	neg	-	neg	POSITIVO	-	neg	neg	-	neg	neg	-	neg		neg	
62	21/08/17	XS-P	Macho	juvenil	Pantanal	-	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg	POSITIVO	-	neg		neg	
63	10/12/17	IA-P	Macho	adulto	Pantanal	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
64	08/12/17	COS-P	Macho	juvenil	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
65	05/12/14	WM-P	Macho	juvenil	Pantanal	-	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	POSITIVO		neg	
66	05/12/17	SA-P/SIA - P	Femea	juvenil	Pantanal	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	POSITIVO		neg	
67	25/08/18	DA-P	Femea	sub	Pantanal	-	POSITIVO	SEQUENCIADA	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
68	20/06/16	NAO-P	Macho	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		POSITIVO	
69	10/05/15	RTA-P	Femea	adulto	Pantanal	-	POSITIVO	-	neg	POSITIVO	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
70	21/10/14	RCK-P	Macho	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
71	14/11/15	WE-P-1	Femea	sub	Pantanal	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		POSITIVO	SEQUENCIADA
72	22/08/16	HRY-P	Macho	sub	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
73	01/09/18	VA-P-1	Femea	sub	Pantanal	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
74	17/12/14	FPE-P	Macho	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
75	25/06/18	RO-P	Macho	sub	Pantanal	-	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg	POSITIVO	-	neg		neg	
76	10/12/17	LRO-P	Macho	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
77	08/12/16	SCO-P	Macho	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
78	03/07/14	MLE-P	Femea	sub	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
79	16/08/16	BEN-P	Macho	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
80	27/10/17	GIL-P	Macho	sub	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
81	29/08/18	FFO-P-1	Macho	adulto	Pantanal	-	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO	SEQUENCIADA	neg	
82	25/06/18	MIA-P	Femea	sub	Pantanal	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	POSITIVO	SEQUENCIADA	neg	
83	26/10/17	DHO-P-1	Macho	sub	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
84	22/10/18	CHA-P-2	Femea	sub	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
85	25/06/18	NLA-P	Femea	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
86	12/07/14	KIN-P	Femea	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
87	15/11/15	PNA-P-1	Femea	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	POSITIVO	SEQUENCIADA	POSITIVO		neg	
88	19/06/18	GAO-P	Macho	adulto	Pantanal	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
89	27/08/18	INA-P	Femea	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
90	02/07/15	MCI-P	Femea	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
91	06/07/14	GLA-P	Femea	sub	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
92	22/10/14	DJO-P	Femea	adulto	Pantanal	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
93	08/06/16	AO-P	Macho	juvenil	Pantanal	-	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
94	18/06/18	FA-P	Femea	adulto	Pantanal	-	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
95	18/10/14	CRA-P-1	Femea	adulto	Pantanal	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
96	19/05/15	MA-P-2	Femea	adulto	Pantanal	-	neg	-	neg	POSITIVO	-	neg	neg	-	neg	neg	-	neg		neg	
97	28/06/17	ANO-C-2	Macho	adulto	Cerrado	-	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
98	14/11/17	ZCA-C-2	Femea	adulto	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
99	19/09/17	LOU-C-2	Macho	adulto	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
100	19/09/18	CNA-C-2	Femea	adulto	Cerrado	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	POSITIVO	SEQUENCIADA	neg	
101	11/10/18	CBI-C-2	Femea	adulto	Cerrado	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
102	09/02/17	SO-C-2	Macho	adulto	Cerrado	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
103	23/08/17	FFO-P-2	Macho	adulto	Pantanal	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	POSITIVO		neg	
104	25/08/17	PNA-P-2	Femea	adulto	Pantanal	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
105	11/08/16	LNA-P-2	Femea	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
106	20/08/16	JO-P-2	Macho	sub	Pantanal	-	neg	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
107	28/07/13	DRA-P-2	Femea	adulto	Pantanal	-	neg	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
108	19/08/17	VA-P-2	Femea	sub	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	POSITIVO	SEQUENCIADA	neg		neg	
109	25/06/18	DO-P-2/DHO-P-2	Macho	sub	Pantanal	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
110	09/06/16	JE-P-2	Macho	sub	Pantanal	-	POSITIVO	-	neg	POSITIVO	-	neg	neg	-	neg	neg	-	neg		neg	
111	11/06/16	BS-P/BRS-P	Macho	sub	Pantanal	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
112	17/12/14	TD-P-2	Macho	sub	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	POSITIVO	-	neg		neg	
113	18/06/16	TD-P-3	Macho	sub	Pantanal	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
114	16/06/16	WE-P-2	Femea	sub	Pantanal	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	neg		neg	
115	15/12/16	WE-P-3	Femea	sub	Pantanal	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	neg	-	POSITIVO		neg	
116	20/10/13	SAO-P-2	Macho	adulto	Pantanal	-	POSITIVO	-	neg	POSITIVO	SEQUENCIADA	neg	neg	-	neg	POSITIVO	SEQUENCIADA	neg		neg	
117	11/05/15	SAO-P-3	Macho	adulto	Pantanal	-	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
118	23/05/16	Nec 09	Fêmea	sub	Cerrado	BR267	POSITIVO	-	neg	POSITIVO	-	neg	neg	-	neg	neg	-	neg		neg	
119	10/03/16	Nec 07	Fêmea	juvenil	Cerrado	BR267	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	POSITIVO		neg	
120	13/10/16	Nec 21	Macho	adulto	Cerrado	BR267	-	-	-	-	-	-	-	-	-	-	-	-		-	
121	22/10/16	Nec 22	Macho	adulto	Cerrado	BR267	-	-	-	-	-	-	-	-	-	-	-	-		-	
122	20/07/16	Nec 15	Macho	adulto	Cerrado	MS040	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
123	29/07/16	Nec 18	Macho	adulto	Cerrado	BR267	neg	-	neg	POSITIVO	-	neg	neg	-	neg	neg	-	neg		neg	
124	18/11/16	Nec 24	Fêmea	adulto	Cerrado	BR267	POSITIVO	-	neg	neg	-	neg	neg	-	neg	neg	-	neg		neg	
125	10/11/16	Nec 23	Macho	sub	Cerrado	BR267	-	-	-	-	-	-	-	-	-	-	-	-		-	

Figura 02: Amostras apresentando co-infecção. As amostras marcadas em cinza representam co-infeções identificadas nos ensaios de triagem. As amostras marcadas em roxo representam co-infeções confirmadas com sequenciamento genético. As amostras marcadas em vermelho representam amostras de DNA que não amplificaram genes endógenos e, portanto, não foram submetidas aos ensaios para pesquisa de hemoparasitos.