UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CÂMPUS DE JABOTICABAL

CARACTERIZAÇÃO MORFOLÓGICA, BIOQUÍMICA, MOLECULAR E FENOTÍPICA DE ISOLADOS DE *Bartonella* spp. DE ROEDORES E MARSUPIAIS NO PANTANAL BRASILEIRO, COM DESCRIÇÃO DE *Bartonella machadoae* sp. nov E *Bartonella harrusi* sp. nov.

Renan Bressianini do Amaral

Biólogo

2022

UNIVERSIDADE ESTADUAL PAULISTA - UNESP

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CARACTERIZAÇÃO MORFOLÓGICA, BIOQUÍMICA, MOLECULAR E FENOTÍPICA DE ISOLADOS DE *Bartonella* spp. DE ROEDORES E MARSUPIAIS NO PANTANAL BRASILEIRO, COM DESCRIÇÃO DE *Bartonella machadoae* sp. nov E *Bartonella harrusi* sp. nov.

Discente: Renan Bressianini do Amaral

Orientador: Prof. Dr. Marcos Rogério André

Coorientadora: Rosangela Zacarias Machado

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Microbiologia Agropecuária.

2022

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Caracterização morfológica, bioquímica, molecular e fenótipica de isolados de BARTONELLA SPP. Dde roedores e marsupiais no pantanal Brasileiro , com descrição de BARTONELLA MACHADOAE sp. nov e BARTONELLA HARRUSI sp. nov. / Renan Bressianini do Amaral. -- Jaboticabal, 2022

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Orientadora: Marcos Rogério André

Coorientadora: Rosangela Zacarias Machado

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Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: CARACTERIZAÇÃO MORFOLÓGICA, BIOQUÍMICA, MOLECULAR E FENOTÍPICA DE ISOLADOS DE *Bartonella* spp. DE ROEDORES E MARSUPIAIS NO PANTANAL BRASILEIRO, COM DESCRIÇÃO DE *Bartonella machadoae* sp. nov. E *Bartonella harrusi* sp. nov.

AUTOR: RENAN BRESSIANINI DO AMARAL
ORIENTADOR: MARCOS ROGÉRIO ANDRÉ
COORIENTADORA: ROSANGELA ZACARIAS MACHADO

Aprovado como parte das exigências para obtenção do Título de Doutor em MICROBIOLOGIA AGROPECUÁRIA, pela Comissão Examinadora:

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Ananda Müller P.

Profa. Dra. ANANDA MÜLLER PEREIRA (Participaçao Virtual)
Ross University School of Veterinary Medicine / Basseterre/Ilha de São Cristovão/Caribe

Jaboticabal, 27 de maio de 2022

DADOS CURRICULARES DO AUTOR

Renan Bressianini do Amaral – nascido na cidade de Ribeirão Preto, São Paulo, em 12 de maio de 1994. Formado em Ciências Biológicas (Bacharelado) pela Universidade Estadual Paulista “Júlio de Mesquita Filho” – Faculdade de Ciências Agrárias e Veterinárias – FCAV/UNESP, Jaboticabal, São Paulo, no ano de 2015. Durante a graduação, foi bolsista PROPE (2014-2015) e FAPESP (Processo 2015/03262-1) trabalhando com pesquisas na área de Parasitologia sob a orientação do Prof. Dr. Marcos R. André. Ingressou no curso de Pós-Graduação em Microbiologia Agropecuária na FCAV/UNESP em março de 2016, com bolsa da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) até agosto de 2016 e Bolsa FAPESP (Processo número 2016/12574-0) até fevereiro de 2018. Ingressou no curso de Doutorado em Microbiologia Agropecuária na Universidade Estadual Paulista “Júlio de Mesquita Filho” – Faculdade de Ciências Agrárias e Veterinárias – FCAV/UNESP, Campus de Jaboticabal, em março de 2018, inicialmente com bolsa da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e, depois, beneficiado pela bolsa da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP Processo número 2018/19672-2).

Epígrafe

“Os livros não são feitos para que alguém acredite neles, mas para serem submetidos à investigação. Quando consideramos um livro, não devemos perguntar o que diz, mas o que significa.”

Umberto Eco

DEDICATÓRIA

Aos meus pais, Márcia dos Reis Bressianini do Amaral e

Teotônio Firmino do Amaral

por todo apoio, amor e compreensão.

AGRADECIMENTOS

Aos meus pais Marcia e Teotônio, e minha irmã Juliana pelo apoio incondicional, todos os dias.

Aos meus amigos, Adryelle Moreira, Matheus Panosso, Kayo Garcia e minha namorada Amanda Segalla, por todo apoio durante esse período difícil vivemos.

Aos meus companheiros do Laboratório de Imunoparasitologia, Livia Perles, Rafaela Beraldo, Mariele de Santi, Victória Valente, Ana Claudia Calchi, Anna Cláudia Mongruel, Ana Carolina, Ricardo Bassini e Caroline Secato pela amizade, ensinamentos compartilhados e colaboração no trabalho apresentado. Em especial aos meus colegas Matheus Santana pelo auxílio nas coletas e Luiz Ricardo, Maria Eduarda e Clara Morato, pela colaboração na obtenção dos isolados de *Bartonella* spp. de roedores e marsupiais.

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Agradeço também à Dra. Cláudia Maria Toffanelli Fiorillo do Laboratório de Microscopia Eletrônica da FCAV/UNESP e Beatriz Mauricio do Laboratório de Biologia Celular do Instituto Butantan, pela assistência na realização da técnica de microscopia eletrônica de varredura.

À Coordenação do Programa de Pós-Graduação em Microbiologia

Agropecuária.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela bolsa de estudo concedida no primeiro semestre do programa.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pelo Auxílio à Pesquisa (Processo: 2015/14896-1) e à bolsa de Doutorado concedida (Processo: 2018/19672-2).

E, em especial, gostaria de agradecer a Profa. Darci Barros-Battesti, à minha coorientadora, Profa. Dra. Rosangela Zacarias Machado, e ao meu orientador Prof. Dr. Marcos Rogério André, sempre estando presentes compartilhando conhecimento, conselhos e apoiando mesmo em um dos momentos mais difíceis pelo qual passei.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

Sumário

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

C E R T I F I C A D O

Certificamos que o projeto de pesquisa intitulado **"Isolamento, genotipagem e sequenciamento genômico de *Bartonella* spp. em pequenos mamíferos no Pantanal Sul-matogrossense"**, protocolo nº 010099/18**,** 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 10 de outubro de 2018.

Vigência do Projeto	15/10/2018 a 05/02/2022
Espécie / Linhagem	Ordem Rodentia / Ordem Didelphimorphia
Nº de animais	-
Peso / Idade	Variável
Sexo	Variável
Origem	Captura de vida livre

Jaboticabal, 10 de outubro de 2018.

Profª Drª Fabiana Pilarski
Coordenadora – CEUA

Autorização para atividades com finalidade científica

Número: 64204-1	Data da Emissão: 08/10/2018 11:47:55	Data da Revalidação*: 08/10/2019

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

Dados do titular

Nome: Marcos Rogério André	CPF: 302.435.148-59
Nome da Instituição: UNESP JABOTICABAL	CNPJ: 48.031.918/0012-87

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Revisão de Literatura	08/2018	02/2022
2	Análise das sequências - Filogenia molecular	01/2020	07/2021
3	Discussão dos resultados e redação final	06/2021	02/2022
4	Colheita das amostras	09/2018	03/2021
5	Clonagem e seqüenciamento dos amplicons positivos	01/2020	12/2020
6	PCR para os agentes sob estudo	12/2018	10/2021
7	Sequenciamento do genoma completo de Bartonella spp.	01/2019	07/2020

Equipe

#	Nome	Função	CPF	Nacionalidade
1	Wesley Arruda Gimenes Nantes	Mestrando - captura	056.176.551-08	Brasileira
2	Renan Bressianini do Amaral	Doutorando - captura e processamento das amostras	418.312.778-59	Brasileira
3	Heitor Miraglia Herrera	Pesquisador colaborador	444.869.871-87	Brasileira
4	Rosangela Zacarias Machado	Pesquisadora colaboradora	125.043.436-04	Brasileira

Observações e ressalvas

1	A autorização não eximirá o pesquisador da necessidade de obter outras anuências, como: I) do proprietário, arrendatário, posseiro ou morador quando as atividades forem realizadas em área de domínio privado ou dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso; II) da comunidade indígena envolvida, ouvido o órgão indigenista oficial, quando as atividades de pesquisa forem executadas em terra indígena; III) do Conselho de Defesa Nacional, quando as atividades de pesquisa forem executadas em área indispensável à segurança nacional; IV) da autoridade marítima, quando as atividades de pesquisa forem executadas em águas jurisdicionais brasileiras; V) do Departamento Nacional da Produção Mineral, quando a pesquisa visar a exploração de depósitos fossilíferos ou a extração de espécimes fósseis; VI) do órgão gestor da unidade de conservação estadual, distrital ou municipal, dentre outras.
2	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
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4	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.

Este documento foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

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Autorização para atividades com finalidade científica

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Dados do titular

Nome: Marcos Rogério André	CPF: 302.435.148-59
Nome da Instituição: UNESP JABOTICABAL	CNPJ: 48.031.918/0012-87

Observações e ressalvas

5	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
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Outras ressalvas

1	As armadilhas utilizadas para captura de mamíferos deverão ser vistoriadas pelo menos duas vezes ao dia (matutino e vespertino) para minimizar a morte devido à hipo ou hipertermia. Não está autorizada a coleta, transporte de fêmeas grávidas ou em processo de amamentação.	CGPEQ

Locais onde as atividades de campo serão executadas

#	Descrição do local	Município-UF	Bioma	Caverna?	Tipo
1	Fazenda Nhumirim da Embrapa Pantanal	Corumbá-MS	Pantanal	Não	Fora de UC Federal

Atividades X Táxons

#	Atividade	Táxon	Qtde.
1	Captura de animais silvestres in situ	Oecomys	-
2	Coleta/transporte de amostras biológicas in situ	Oecomys	-
3	Coleta/transporte de espécimes da fauna silvestre in situ	Oecomys	20
4	Coleta/transporte de amostras biológicas in situ	Didelphis	-
5	Coleta/transporte de espécimes da fauna silvestre in situ	Didelphis	20
6	Captura de animais silvestres in situ	Didelphis	-
7	Coleta/transporte de espécimes da fauna silvestre in situ	Gracilinanus	20
8	Coleta/transporte de amostras biológicas in situ	Gracilinanus	-
9	Captura de animais silvestres in situ	Gracilinanus	-
10	Coleta/transporte de espécimes da fauna silvestre in situ	Monodelphis	20

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Dados do titular

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Atividades X Táxons

#	Atividade	Táxon	Qtde.
11	Coleta/transporte de amostras biológicas in situ	Monodelphis	-
12	Captura de animais silvestres in situ	Monodelphis	-
13	Coleta/transporte de espécimes da fauna silvestre in situ	Clyomys	20
14	Coleta/transporte de amostras biológicas in situ	Clyomys	-
15	Captura de animais silvestres in situ	Clyomys	-
16	Coleta/transporte de espécimes da fauna silvestre in situ	Thrichomys	30
17	Coleta/transporte de amostras biológicas in situ	Thrichomys	-
18	Captura de animais silvestres in situ	Thrichomys	-
19	Coleta/transporte de espécimes da fauna silvestre in situ	Thylamys	20
20	Coleta/transporte de amostras biológicas in situ	Thylamys	-
21	Captura de animais silvestres in situ	Thylamys	-

Materiais e Métodos

#	Tipo de Método (Grupo taxonômico)	Materiais
1	Amostras biológicas (Outros mamíferos)	Fragmento de tecido/órgão, Ectoparasita, Sangue, Outras amostras biológicas(Saliva)
2	Método de captura/coleta (Outros mamíferos)	Armadilha tipo gaiola com atração por iscas (¿Box Trap/Tomahawk/Sherman¿)

Destino do material biológico coletado

#	Nome local destino	Tipo destino
1	UNESP JABOTICABAL	Outro

Autorização para atividades com finalidade científica

Número: 64204-1	Data da Emissão: 08/10/2018 11:47:55	Data da Revalidação*: 08/10/2019

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Dados do titular

Nome: Marcos Rogério André	CPF: 302.435.148-59
Nome da Instituição: UNESP JABOTICABAL	CNPJ: 48.031.918/0012-87

Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de Amostra	Qtde.	Data

* Identificar o espécime do nível taxonômico possível.

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 A835492

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

Número do cadastro:	**A835492**
Usuário:	**Marcos Rogério André**
CPF/CNPJ:	**302.435.148-59**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie
Clyomys laticeps
Oecomys mamorae
Thrichomys fosteri
Thylamys macrurus
Monodelphis domestica
Polygenis bohlsi
Gamasellus lativentralis
Gigantolaelaps mattogrossensis
Gigantolaelaps oudemansi
Amblyomma sculptum
Amblyomma ovale
Bartonella machadoae
Bartonella harrusi

Título da Atividade:	**CARACTERIZAÇÃO MORFOLÓGICA, BIOQUÍMICA, MOLECULAR E FENOTÍPICA DE ISOLADOS DE Bartonella SPP. DE ROEDORES E MARSUPIAIS NO PANTANAL BRASILEIRO, COM DESCRIÇÃO DE Bartonella machadoae sp. nov. E Bartonella harrusi sp. nov.**

Equipe

Marcos Rogério André	**Campus de Jaboticabal**
Rosangela Zacarias Machado	**Unesp FCAV**
Heitor Miraglia Herrera	**UCDB**
Renan Bressianini do Amaral	**Unesp FCAV**

Parceiras Nacionais

03.226.149/0001-81 / Universidade Católica dom Bosco

Resultados Obtidos

Divulgação de resultados em meios científicos ou de comunicação

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Data do Cadastro:	**13/10/2022 22:46:05**
Situação do Cadastro:	**Concluído**

CARACTERIZAÇÃO MORFOLÓGICA, BIOQUÍMICA, MOLECULAR E FENOTÍPICA DE ISOLADOS DE *Bartonella* SPP. DE ROEDORES E MARSUPIAIS NO PANTANAL BRASILEIRO, COM DESCRIÇÃO DE *Bartonella machadoae* sp. nov. E *Bartonella harrusi* sp. nov.

Resumo – O gênero *Bartonella* compreende alphaproteobactérias Gram-negativas intracelulares facultativas e fastidiosas capazes de infectar uma grande variedade de mamíferos e transmitidas por artrópodes vetores. Embora roedores representem importantes reservatórios de *Bartonella* ao redor do mundo, poucos são os estudos acerca da caracterização genômica de espécies de *Bartonella* comumente encontradas neste táxon. Por outro lado, *Bartonella* spp. foi detectada em marsupiais e/ou ectoparasitas associados somente na Austrália e Estados Unidos. O presente estudo teve como objetivo caracterizar molecular, morfológica, bioquímica e fenotipicamente espécies de *Bartonella* isoladas de amostras de sangue de roedores e marsupiais de vida livre amostrados no Pantanal brasileiro, a maior área úmida da América do Sul. Para tanto, foram capturados 61 animais (59 roedores e 2 marsupiais) em dezembro de 2018 e 76 (70 roedores e 6 marsupiais) em fevereiro de 2019, totalizando 137 pequenos mamíferos amostrados, dos quais 129 (94,4 %) roedores e oito (5,6%) marsupiais. Dentre os 129 roedores amostrados, 79 (61,2%) animais pertenciam à espécie *Thrichomys fosteri*, quatro (3,1%) à espécie *Clyomys laticeps*, e 46 (35,7%) à espécie *Oecomys mamorae*. Dentre os oito marsupiais capturados, 5 (62,5%) pertenciam à espécie *Thylamys macrurus* e 3 (37,5%) à espécie *Monodelphis domestica*. Todas as amostras biológicas foram submetidas à qPCR para *Bartonella* spp. baseada no gene *nuoG*, cultura líquida de pré-enriquecimento e cultura sólida em ágar chocolate. *Bartonella* spp. foi detectada em 24% dos roedores amostrados e em quatro dos oito marsupiais amostrados. Uma cepa isolada de amostra de sangue de *T. fosteri* e uma cepa isolada de amostra de sangue de *T. macrurus* que se mostraram intimamente relacionadas ao complexo *Bartonella vinsonii* por meio de análises filogenéticas (Máxima Verossimilhança) e de distância (SplitsTree) baseadas em diversos marcadores moleculares, foram selecionadas para sequenciamento do genoma completo, utilizando para tal abordagem híbrida em plataformas de sequenciamento Illumina NovaSeq e Nanopore. As cepas isoladas de *T. fosteri* (strain 56A) e *T. macrurus* (117A) exibiram um genoma circular de 2,7 Mbp e 2,35 Mbp, com um conteúdo C + G médio de 39% e 38,8%, e codificação de 2.239 e 2.013 genes, respectivamente. Além disso, exibiram uma identidade nucleotídica média (ANI) de 85% com espécies de *Bartonella* do grupo *vinsonii* e de 91% entre si. Análise filogenômica baseada em 291 genes codificantes para proteínas e compartilhados entre genomas de 53 espécies de *Bartonella,* posicionou ambas as cepas em um clado intimamente relacionado a *Bartonella vinsonii* subsp. *vinsonii*, *B. vinsonii* subsp. *berkhoffii* e *B. vinsonii* subsp. *arupensis*, com alto valor de suporte de clado, apoiando a separação destas cepas em duas espécies diferentes, agora formalmente denominadas *Bartonella machadoae* nov. sp. (isolada de *T. fosteri*) e *Bartonella harrusi* nov. sp. (isolada de *T. macrurus*). *Bartonella machadoae* sp. nov. e *Bartonella harrusi* sp. nov. foram caracterizadas como pequenos bastonetes capnofílicos, microaerofílicos e aeróbios com ausência de pili e flagelos. Em conclusão, descrevemos as características bioquímicas, morfológicas, fenotípicas e genômicas de *Bartonella machadoae*, uma nova espécie isolada de amostras de sangue de roedor da espécie *T. fosteri*, e de *Bartonella harrusi,* uma nova espécie isolada de amostras de sangue de marsupiais da espécie *T. macrurus,* no Pantanal brasileiro.

Palavras-chave: bartonelose; Marsupialia; Rodentia; filogenômica; WGS; classificação taxonômica

MORPHOLOGICAL, BIOCHEMICAL, MOLECULAR AND PHENOTYPICAL CHARACTERIZATION OF *Bartonella* spp. ISOLATES FROM RODENTS AND MARSUPIALS IN THE BRAZILIAN PANTANAL, WITH DESCRIPTION OF *Bartonella machadoae* sp. nov AND *Bartonella harrusi* sp. nov.

Abstract - The genus *Bartonella* comprises facultative and fastidious intracellular Gram-negative alphaproteobacteria capable of infecting a wide variety of mammals and transmitted by arthropod vectors. Although rodents represent important reservoirs of *Bartonella* around the world, there are few studies on the genomic characterization of *Bartonella* species commonly found in this taxon. On the other hand, *Bartonella* spp. has been detected in marsupials and/or associated ectoparasites only in Australia and the United States. The present study aimed to characterize molecularly, morphologically, biochemically, and phenotypically *Bartonella* isolated from blood samples of free-living rodents and marsupials sampled in the Brazilian Pantanal, the largest wetland in South America. For this purpose, 61 animals (59 rodents and 2 marsupials) were captured in December 2018 and 76 (70 rodents and 6 marsupials) in February 2019, totaling 137 small mammals sampled, of which 129 (94.4%) were rodents and eight were (5.6%) marsupials. Among 129 rodents sampled animals, 79 (61.2%) were *Thrichomys fosteri*, four (3.1%) *Clyomys laticeps*, 46 (35.7%) were *Oecomys mamorae*. Among the eight marsupials, 5 (62,5%) were *Thylamys macrurus* and 3 (37,5%) were *Monodelphis domestica*. Biological samples were submitted to a qPCR for *Bartonella* based on the *nuoG* gene, pre-enrichment liquid culture and solid culture on chocolate agar. *Bartonella* spp. detected in 24% of sampled rodents and in 4 out of 8 sampled marsupials. A strain isolated from a blood sample of *T. fosteri* and a strain isolated from a blood sample of *T. macrurus,* which shown to be closely related to the *Bartonella vinsonii* complex by phylogenetic (Maximum Likelihood) and distance (SplitsTree) analyzes based on several molecular markers, were selected for whole genome sequencing, using for such a hybrid approach in Illumina NovaSeq and Nanopore sequencing platforms. The isolated strains of *T. fosteri* (strain 56A) and *T. macrurus* (117A) exhibited a circular genome of 2.7 Mbp and 2.35 Mbp, with an average C + G content of 39% and 38.8%, and encoding 2.239 and 2.013 genes, respectively. In addition, they exhibited a mean nucleotide identity (ANI) of 85% with *Bartonella* species of the *vinsonii* group and 91% with each other. Phylogenomic analysis based on 291 protein-coding genes shared between genomes of 53 *Bartonella* species positioned both strains in a clade closely related to *Bartonella vinsonii* subsp. *vinsonii*, *B. vinsonii* subsp. *berkhoffii* and *B. vinsonii* subsp. *arupensis*, with high clade support value, supporting the separation of these new strains into two different species, now formally named *Bartonella machadoae* nov. sp. (isolated from *T. fosteri*) and *Bartonella harrusi* nov. sp. (isolated from *T. macrurus*). *Bartonella machadoae* sp. nov. and *Bartonella harrusi* sp. nov. were characterized as small capnophilic, microaerophilic and aerobic rods with the absence of pili and flagella. In conclusion, we describe the biochemical, morphological, phenotypic and genomic characteristics of *Bartonella machadoae*, a new species isolated from *T. fosteri* rodent blood samples, and *Bartonella harrusi*, a new species isolated from *T. macrurus* marsupial blood samples in the Brazilian Pantanal.

KeyWords: bartonellosis; Marsupialia; Rodentia; phylogenomics; WGS; taxonomic classification

Capítulo 1 - CONSIDERAÇÕES INICIAIS

1. INTRODUÇÃO

O bioma Pantanal é caracterizado por uma ampla diversidade de espécies de vertebrados e invertebrados. Essa região sofre inundações sazonais, favorecendo interações ecológicas e padrões de diversidade de espécies que não são observadas em nenhum outro bioma no Brasil (Alho & Sabino, 2011). Uma vez que cerca de 75% das doenças infecciosas emergentes compreendem zoonoses, das quais a maioria é transmitida por vetores artrópodes (Breitschwerdt et., 2014), esse bioma torna-se um ambiente propício para a ocorrência de diversas zoonoses.

Entre essas zoonoses encontram-se as bartonelloses, enfermidades causadas por bactérias do gênero *Bartonella.* Tais agentes compreendem bactérias Gram-negativas, intracelulares facultativas e fastidiosas, classificadas na sub-classe das α -2-Proteobacterias (Chomel et al., 2006; Gutiérrez et al., 2020). Este grupo de organismos é caracterizado por infectar uma grande variedade de vertebrados, sendo em sua maioria mamíferos, causando diversas manifestações clínicas dependendo do hospedeiro e espécie de *Bartonella* envolvida (Boulouis et al., 2001).

Recentemente, nosso grupo de pesquisa detectou genótipos de *Bartonella* associados a roedores selvagens amostrados no Pantanal sul-matogrossense. Pelo menos dois genótipos ou espécies de *Bartonella* spp. circulantes entre esses roedores foram encontrados: enquanto um deles mostrou-se filogeneticamente relacionado a *Bartonella* sp. previamente detectado em roedores das famílias Cricetidae e Echimyidae(Favacho et al., 2015; Rozental et al., 2017), o outro mostrou-se mais próximo de genótipos detectados em ectoparasitos coletados de roedores (de Sousa et al., 2018; Schott et al., 2020).

Essa diversidade genotípica de *Bartonella* spp., tanto em roedores quanto em seus ectoparasitas, implica em desafios epidemiológicos e de diagnóstico, uma vez que técnicas convencionais atualmente mostrarem-se incapazes de distinguir espécies filogeneticamente próximas (Buffet et al., 2013; Scola et al., 2003).

Por outro lado, embora estudos tenham objetivado investigar a ocorrência de *Bartonella* spp. em marsupiais e seus ectoparasitas nos estados de Pernambuco (Fontalvo et al., 2017), Mato Grosso do Sul (de Sousa et al., 2018) e Rio de Janeiro (Gonçalves et al., 2020), tais agentes ainda não foram detectados neste grupo de mamíferos.

O presente estudo buscou contribuir com dados a respeito da ocorrência de *Bartonella* spp. em roedores e marsupiais selvagens amostrados no bioma Pantanal, por meio de ensaios microbiológicos e moleculares. Adicionalmente, caracterizar morfológica, bioquímica, fenotípica e molecularmente isolados de *Bartonella* de espécies de roedores e marsupiais. Por fim, o presente estudo buscou contribuir no que diz respeito ao perfil proteico funcional de membros do gênero *Bartonella* por meio de análises dos genomas completos de integrantes deste grupo de bactérias depositados nos bancos de dados.

2. REVISÃO DE LITERATURA

2.1. Gênero *Bartonella*

2.1.1. Taxonomia

O gênero *Bartonella* engloba bactérias Gram-negativas intracelulares facultativas, as quais parasitam principalmente eritrócitos e células endoteliais, além de macrófagos, monócitos e células dendríticas (Eicher and Dehio, 2012; Musso et al., 2001). Sua distribuição é cosmopolita e pode ser transmitida principalmente por

vetores artrópodes hematófagos, como pulgas, carrapatos, mosquitos, moscas e piolhos (Breitschwerdt and Kordick, 2000; Chomel et al., 2009). Atualmente estão classificadas no Filo Proteobacteria, Classe Alphaproteobacteria, Ordem Rhizobiales e Família Bartonellaceae (Chomel et al., 2006; Pitassi et al., 2015).

Anteriormente a 1993 o gênero *Bartonella* era reconhecido por apresentar somente uma espécie, *Bartonella bacilliformis* (STRONG, 1915), agente causador da Febre de Oroya e Verruga Peruana (Minnick et al., 2014; STRONG, 1915). BRENNER et al. (1993), baseando-se em estudos moleculares de sequências de DNA da subunidade 16S rRNA, observaram relações filogenéticas entre *Rochalimaea quintana* e *B. bacilliformis*. Propuseram, assim, a transferência de *R. quintana*, pertencente à família Rickettsiaceae, para a família Bartonellaceae, mantendo as duas espécies no gênero *Bartonella*, devido a este ser o mais antigo. A partir daí, mais três espécies do antigo gênero *Rochalimaea* foram renomeadas como *B. vinsonii, B. henselae* e *B. elizabethae* (Birtles et al., 1995; Brenner et al., 1993; Gutiérrez et al., 2015). De forma similar, bactérias do gênero *Grahamella*, detectadas em sua maioria em pequenos mamíferos, também foram agrupadas ao gênero *Bartonella*, sendo renomeadas como *B. grahamii*, *B. taylorii*, *B. doshiae* (Birtles et al., 1995; Gutierrez et al., 2015).

Atualmente, segundo o site https://lpsn.dsmz.de/genus/bartonella acessado em março de 2022, o gênero *Bartonella* compreende 37 espécies validadas e com seu genoma anotado, em alguns casos contendo mais de uma subespécie. Adicionalmente, mais de 31 "*Candidatus*" já foram descritos, porém ainda não validados, a partir de isolados de seres humanos, animais domésticos e selvagens (Angelakis & Raoult, 2014; Breitschwerdt et al., 2010; Kaewmongkol et al., 2011; Okaro et al., 2017).

Este gênero compreende bactérias bacilares ou cocobacilares, podendo ter ou não pili e flagelo, dependendo da espécie. Seu tamanho pode variar entre 1 a 2 µm e com genomas variáveis entre 1,45 a 2,90 Mb, respectivamente (Bermond et al., 2002; Gutiérrez et al., 2020; Li et al., 2015; Mullins et al., 2015).

2.1.2. Características microbiológicas de *Bartonella* spp.

O gênero *Bartonella* é caracterizado por bastonetes pleomórficos Gram-negativos que não se coram de forma adequada pelo Gram, mas sim pela coloração de Gimenez (Okaro et al., 2017). Estas bactérias são fastidiosas, com crescimento lento e com ampla necessidade nutricional, sendo necessário para seu isolamento diversos aminoácidos, ácidos graxos, meios de cultura com características cinéticas enzimáticas específicas, além de requerer nutrientes do grupamento heme encontrado em eritrócitos. Seu crescimento ocorre entre 35 a 37ºC (variando de acordo com a espécie) com 5% de CO_2 (Okaro et al., 2017).

2.1.3. Bartoneloses

O gênero *Bartonella* spp. compreende organismos adaptados ao parasitismo em mamíferos, sendo isoladas de uma grande variedade de espécies de canídeos, roedores, ruminantes, felinos, quirópteros, entre outros. Sua transmissão acontece por contato direto, por meio de arranhões e mordidas de animais, ou veiculadas por diversos artrópodes vetores (pulgas, carrapatos, mosquitos, piolhos, carrapatos e moscas hematófagas) (Deng et al., 2018). Uma vez transmitidos a um hospedeiro susceptível causam uma bacteremia intraeritrocítica de longa duração, o que aumenta as chances de transmissão desses agentes por vetores artrópodes a outros hospedeiros (Maggi et al., 2011). Em seus hospedeiros reservatórios esta bacteremia intraeritrocítica duradoura aparentemente não causa prejuízos, porém

quando *Bartonella* spp. infectam acidentalmente hospedeiros não habituais podem gerar manifestações clínicas (Deng et al., 2018; Mosepele et al., 2012; Raoult, 2007).

As diversas espécies de mamíferos parasitados, domésticos e selvagens, tais como ruminantes, cães, gatos, roedores e humanos, podem atuar como reservatórios para diferentes espécies de *Bartonella* spp. (Deng et al., 2012). Tal diversidade de hospedeiros implica em desafios epidemiológicos, clínicos e de diagnóstico, tanto para a Medicina Veterinária quanto para a Medicina Humana (Breitschwerdt et al., 2010).

Dentre as várias espécies de *Bartonella* parasitas de seres humanos, aquelas mais frequentemente associadas às infecções são *B. henselae*, *B. quintana* e *B. bacilliformis* (Angelakis and Raoult, 2014). Na **Tabela 1** encontram-se as espécies identificadas por métodos moleculares, publicadas e validadas pelo “International Code of Nomenclature of Prokaryotes” (ICNP) em seres humanos (Krügel et al., 2022).

Tabela 1. Espécies de *Bartonella* identificadas por métodos moleculares em mamíferos.

Espécie	Reservatório	Infecção em humanos	Referências
Bartonella vinsonii	Roedores e canideos	Sim	(Brenner et al., 1993)
Bartonella tribocorum	Roedores	Sim	(Heller et al., 1998)
Bartonella taylorii	Roedores	-	(Birtles et al., 1995)
Bartonella talpae	Toupeira	-	(Birtles et al. 1995)
Bartonella silvatica	Roedores	Sim	(Inoue et al., 2010)
Bartonella senegalensis	Não identificado	-	(Mediannikov et al. 2014)
Bartonella schoenbuchensis	Ruminantes	Sim	(Dehio et al., 2001)
Bartonella rochalimae	Canídeos	Sim	(Eremeeva et al., 2007)
Bartonella rattaustraliani	Roedores	-	(Gundi et al., 2009)
Bartonella quintana	Humano	-	(Brenner et al. 1993)
Bartonella queenslandensis	Roedores	-	(Gundi et al. 2009)
Bartonella peromysci	Roedores	-	(Birtles et al. 1995)
Bartonella pachyuromydis	Roedores	-	(Sato et al., 2013)
Bartonella krasnovii	Roedores	Sim	(Gutiérrez et al., 2020)
Bartonella kosoyi	Roedores	-	(Gutiérrez et al., 2020)
Bartonella koehlerae	Felinos	Sim	(Droz et al. 2000)
Bartonella japonica	Roedores	-	(Inoue et al., 2010)
Bartonella jaculi	Roedores	-	(Sato et al., 2013)
Bartonella henselae	Felinos	Sim	Brenner et al. 1993
Bartonella heixiaziensis	Roedores	-	(Li et al., 2015)
Bartonella grahamii	Roedores	Sim	Birtles et al. 1995
Bartonella fuyuanensis	Roedores	-	(Li et al., 2015)
Bartonella florencae	Musaranho	-	(Mediannikov et al. 2014)
Bartonella elizabethae	Roedores	Sim	(Brenner et al. 1993)
Bartonella doshiae	Roedores	Sim	(Birtles et al. 1995)
Bartonella coopersplainsensis	Roedores	-	(Gundi et al. 2009)
Bartonella clarridgeiae	Felinos	Sim	(Lawson and Collins 1996)
Bartonella chomelii	Ruminantes	-	(Maillard et al., 2004)
Bartonella capreoli	Ruminantes	-	(Bermond et al., 2002)
Bartonella callosciuri	Roedores	-	(Sato et al., 2013)
Bartonella bovis	Ruminantes	-	(Bermond et al., 2002)
Bartonella birtlesii	Roedores	-	(Bermond et al. 2000)
Bartonella bacilliformis	Humanos	Sim	(Strong et al. 1915)
Bartonella apis	Abelha	-	(Kešnerová et al. 2016)
Bartonella ancashensis	Humanos	Sim	(Mullins et al., 2015)
Bartonella alsatica	Coelhos	Sim	(Heller et al., 1999)
Bartonella acomydis	Roedores	-	(Sato et al., 2013)

Fonte: https://lpsn.dsmz.de/genus/bartonella

2.1.4. Infecções por *Bartonella* spp.

A infecção por *Bartonella* spp. em humanos é caracterizada por uma fase inicial de colonização, a qual é controlada pelo sistema imunológico, causando manifestações clínicas locais caracterizadas por linfadenopatia, as quais geralmente estão associadas a *B. henselae*, *B. quintana* e *B. alsatica* (Schülein et al., 2001; Jacomo et al., 2002; Minnick and Battisti, 2009; Safont et al., 2014). Esta relação entre as espécies de *Bartonella* com seus hospedeiros ainda não está bem esclarecida. Até o momento sabe-se que tais bactérias atingem a corrente sanguínea após colonização inicial em células endoteliais, onde invadem eritrócitos para replicação intracelular (Schülein et al., 2001; Merrell and Falkow, 2004). Esta fase eritrocítica é caracterizada por uma febre, a qual é conhecida como febre de Oroya (na infecção por *B. bacilliformis*) ou febre das trincheiras (na infecção por *B. quintana*). Neste estágio, essas bactérias são transportadas e podem colonizar secundariamente tecidos vasculares, baço e o fígado. Algumas espécies buscam regiões mais frias do corpo, como vasos periféricos da pele; como exemplo, pode-se citar a Verruga Peruana, a qual é causada por *B. bacilliformis* e *B. ancashensis,* e angiomatose bacilar, causada por *B. henselae* (Minnick and Battisti, 2009; Mullins et al., 2015). A circulação de *Bartonella* spp. na corrente sanguínea pode durar semanas e cursar de forma assintomática, o que favorece e facilita a transmissão por artrópodes nas diversas comunidades de mamíferos (Billeter et al., 2008; Chomel et al., 2009).

2.1.5. Diagnóstico de infecções por *Bartonella* spp.

Devido suas características microbiológicas (bactérias fastidiosas) e curso das infecções em seus hospedeiros, caracterizados por baixa bacteremia, o diagnóstico das infecções por *Bartonella* spp. é desafiador. De fato, não há uma técnica “padrão

ouro” utilizada para o diagnóstico (Álvarez-Fernández et al., 2018).

Até o presente momento existem várias abordagens de diagnóstico utilizadas, dentre as quais técnicas moleculares de PCR convencional, tempo real e *digital droplet* PCR, isolamento e cultivo celular, além de técnicas sorológicas (Reação de Imunofluorescência Indireta, Ensaios Imunoenzimáticos e Western Blotting), histopatológicas e imunoistoquímicas. As referidas técnicas apresentam graus variáveis de especificidade e sensibilidade (Maggi et al., 2020; Neupane et al., 2018; Okaro et al., 2017).

Como elencado anteriormente nas características microbiológicas de *Bartonella* spp., o método de isolamento e cultura requerem meios específicos, sendo o mais utilizado o meio de cultura líquido enriquecido com composição base para crescimento de células de inseto (BAPGM) (meio de crescimento eficiente para todas as espécies de *Bartonella*), seguido de plaqueamento em ágar chocolate ou sangue à temperatura de 35 a 37ºC com 5% de CO_2. Este ensaio demonstra alta sensibilidade desde que amostras sejam coletadas e armazenadas de forma adequada, a fim de manter a viabilidade de bactérias (Duncan et al., 2007; Maggi and Breitschwerdt, 2005; Pultorak et al., 2013).

Os métodos sorológicos (RIFI e ELISA) mostram alta relevância quando consideramos o papel desses métodos no diagnóstico de *B. henselae* (causadora da doença da arranhadura do gato), pois se tratam de métodos rápidos para confirmação de exposição de *Bartonella* spp. nos diversos hospedeiros (Lashnits et al., 2018; Norman et al., 1995). A RIFI é o método sorológico mais utilizado para o diagnóstico de infecções por *Bartonella*, embora imunoensaios enzimáticos (ELISA) também sejam utilizados (Giladi et al., 2001; Jost et al., 2018). Porém é necessário ressaltar a dificuldade na utilização da RIFI por se tratar de uma técnica subjetiva e apresentar

limitação devido a sororreatividade cruzada entre as diversas espécies desse gênero, além de ser relatada reatividade cruzada com *Coxiella burnetii* e *Chlamydia pneumoniae*, limitando a sensibilidade e especificidade (Drancourt et al., 1995; Lashnits et al., 2018; Okaro et al., 2017; Scola et al., 2003; Shapira et al., 2021).

Adicionalmente, a abordagem molecular (PCR convencional e em tempo real) é utilizada na ampla maioria dos trabalhos publicados, devido sua praticidade e uma menor demanda de tempo, pois requer menos trabalho e tempo no diagnóstico (Gutiérrez et al., 2015). Entretanto, a baixa bacteremia pode resultar em dificuldades na detecção do DNA do agente. Afim de resolver esse problema associado à baixa bacteremia, o desenvolvimento da técnica de *Digital Droplet* PCR mostrou-se uma tecnologia promissora para o diagnóstico, devido à alta confiabilidade e sensibilidade, sendo capaz de detectar quantidades de até 0,001 pg/uL de DNA bacteriano (Maggi et al., 2020).

Além disso, alta diversidade genotípica de *Bartonella* spp. implica em desafios epidemiológicos e de diagnóstico, uma vez que a análise do gene 16S rRNA que outrora eram utilizadas para discriminar espécies de *Bartonella* (Dauga et al., 1996), atualmente mostra-se incapaz de distinguir espécies filogeneticamente próximas (Buffet et al., 2013; Scola et al., 2003). Com o objetivo de tentar solucionar este desafio, La Scola et al. (2003) propuseram a realização da caracterização molecular de *Bartonella* spp. utilizando simultaneamente diversos genes “housekeeping”, por uma técnica chamada de “Multi-Locus Sequence” (MLS), a fim de determinar a diversidade de espécies de *Bartonella* spp. em animais domésticos e selvagens e seus ectoparasitas.

Atualmente, com a diminuição nos custos e avanços na tecnologia dos chamados “sequenciamentos de nova geração” (NGS), as técnicas anteriormente

utilizadas mostram, comparativamente, menor acurácia na discriminação de espécies filogeneticamente relacionadas. Neste sentido, a análise de seis a oito marcadores moleculares vem sendo substituída pela obtenção do genoma completo de isolados de *Bartonella*. Como consequência, estudos filogenômicos baseados na análise de centenas de genes proporcionam maior confiabilidade na definição de espécies. Por fim, tendo em vista a disponibilidade de coleções de genomas procarióticos, as abordagens bioinformáticas tornam-se ferramentas robustas para traçar relações taxonômicas e filogenéticas entre esses organismos (Krügel et al., 2022).

2.2. *Bartonella* spp. em roedores

A ordem Rodentia é considerada a mais abundante e diversificada entre as espécies de mamíferos, compreendendo uma riqueza de espécies de aproximadamente 40% de todos os mamíferos (Huchon et al., 2002). A associação entre roedores e *Bartonella* é de grande importância, pois muitas espécies do gênero *Bartonella* possuem roedores como seus reservatórios naturais (Gutiérrez et al., 2015). Nos roedores, estas bactérias causam uma bacteremia persistente e subclínica que pode durar meses (Maggi et al., 2012). Essa bacteremia persistente também facilita o compartilhamento de diferentes espécies e genótipos de *Bartonella* spp. pelo mesmo hospedeiro, além de favorecer diversos eventos de recombinação (Buffet et al., 2013).

Atualmente existem 22 espécies/subespécies de *Bartonella* relatadas em roedores, das quais nove foram incriminadas como agentes zoonóticos em humanos (*B. doshiae, B. elizabethae, B. grahamii, B. rochalimae, B. tamiae, B. tribocorum, B. vinsonii* subsp. *arupensis*, *B. washoensis* e *B. krasnovii*), causando diferentes manifestações clínicas.

A distribuição geográfica e prevalência das espécies de *Bartonella* em membros da ordem Rodentia estão estritamente relacionadas com a distribuição das espécies de roedores e vetores, pois algumas espécies de *Bartonella* são altamente adaptadas a certas espécies de roedores que habitam um bioma específico, enquanto outras são generalistas, possuindo assim uma distribuição global (Buffet et al., 2013).

Embora atualmente novas espécies vêm sendo isoladas e caracterizadas de roedores ou seus ectoparasitas (Dahmana et al., 2020), a falta de dados genômicos e fenotípicos adequados de várias amostras detectadas levam a uma lista extensa de espécies com *status* taxonômico incerto (Gutiérrez et al., 2020; KOSOY et al., 2018).

Bartonella spp. vêm sendo relatadas em roedores em todo o mundo, com ocorrência variando de 2% a 90% dependendo da localidade amostrada, estrutura populacional, temperatura, entre outros fatores bióticos e abióticos (Bai et al., 2013; Gutiérrez et al., 2015; Kosoy et al., 2018).

No Brasil, poucos são os estudos realizados no que diz respeito à ocorrência e caracterização molecular de espécies de *Bartonella* em roedores. Neste sentido, já foi reportado o isolamento de *B. queenslandensis* (5/26) e *B. tribocorum* (1/26) em roedores sinantrópicos amostrados em Salvador, Bahia (Costa et al., 2014).

Além disso, genótipos de *Bartonella* filogeneticamente relacionados a *B. coopersplainsensis* foram detectados molecularmente em roedores sinantrópicos (2/55) do estado de Santa Catarina, sul do Brasil (Gonçalves et al., 2020).

No que diz respeito a roedores selvagens, Favacho et al. (2015) detectaram DNA de *Bartonella* sp. filogeneticamente relacionado a *B. vinsonii* subsp. *arupensis* (18/42) em roedores selvagens amostrados no estado do Mato Grosso do Sul.

Nosso grupo de pesquisa detectou DNA de *Bartonella* sp. (117/457) em roedores da família Cricetidae, Echimyidae e Muridae em cinco biomas brasileiros.

Neste estudo foram detectados genótipos de *Bartonella* intimamente relacionados a sequências previamente detectadas em roedores dessa mesma família nos E.U.A. e Peru. As sequências obtidas mostraram-se filogeneticamente relacionadas ao complexo de espécies de *Bartonella vinsonii* (Gonçalves et al., 2016).

Na Mata Atlântica no estado do Rio de Janeiro, ocorrência molecular de 17,6% (23/131) para *Bartonella* spp. foi relatada em roedores selvagens da família Cricetidae. A filogenia pelo método de Máxima Verossimilhança baseada no gene *gltA* apontou a presença de dois genótipos: enquanto um deles mostrava-se intimamente relacionado com o grupo de *B. vinsonii*, semelhantemente ao descrito no trabalho de Gonçalves et al. (2016), o segundo foi posicionado proximamente a *B. doshiae* (Rozental et al., 2017).

Nosso grupo de pesquisa encontrou uma positividade de 32% (35/110) para *Bartonella* spp. em roedores selvagens amostrados no Pantanal sul-matogrossense (de Sousa et al., 2018). Pelo menos dois genótipos ou espécies de *Bartonella* spp. circulam entre roedores na região estudada: enquanto um deles mostrou-se filogeneticamente relacionado a *Bartonella* sp. previamente detectado em roedores nos EUA e Brasil (Gonçalves et al., 2016), o outro foi posicionado em um clado contendo sequências de *B. alsatica, B. pachyuromidis, B. birtlesii, B. acomydis, B. silvat*ica e *B. callosciuri*. Ainda, pulgas da espécie *Polygenis (polygenis) bohlsi bolshi* foram incriminadas como possíveis vetores de *Bartonella* spp. entre roedores selvagens no Pantanal sul-matogrossense (de Sousa et al., 2018).

Gonçalves-Oliveira et al. (2020) detectaram *Bartonella* spp. no estado do Rio de Janeiro em oito roedores da subfamília Sigmodontinae (sendo cinco da espécie *Akodon cursor* e três da espécie *Nectomys squamipes*). A filogenia posicionou todas as amostras detectadas com sequências previamente descritas foi pelo nosso grupo

de pesquisa, as quais mostraram-se próximas ao complexo *B. vinsonii*. Uma amostra de baço de um roedor da espécie *A. cursor* mostrou-se positiva para *Bartonella* nos ensaios de PCR baseados em fragmentos dos genes *ftsZ* e *groEL,* mostrando-se intimamente relacionada com *B. rochalimae*.

Um genótipo relacionado às espécies de *Bartonella* do complexo *vinsonii* e próximo dos genótipos detectados anteriormente em roedores no Brasil também foi detectado em pulgas associadas a roedores, das espécies *Polygenis (P.) bohlsi bohlsi*, *Polygenis occidentalis occidentalis*, *Polygenis platensis* e *Craneopsylla minerva minerva*, coletados de roedores pertencentes às famílias Echimyidae e Cricetidae no bioma Pantanal, no estado do Mato Grosso do Sul, e nos biomas Pampa e Mata Atlântica, no Estado do Rio Grande do Sul (de Sousa et al., 2018; Schott et al., 2020).

2.3. *Bartonella* spp. em marsupiais

Marsupiais são mamíferos pertencentes à Infraclasse Marsupialia e caracterizam-se por serem vivíparos, com finalização do desenvolvimento no marsúpio (Reis et al., 2005). No Brasil são encontradas 58 espécies diferentes, a maioria de porte pequeno e com hábitos principalmente arborícolas. As diferentes espécies dessa Infraclasse podem ser encontradas em todos os biomas brasileiros (https://www.icmbio.gov.br/portal/faunabrasileira/estado-de-conservacao/2802-mamiferos-marsupiais) – acessado em março de 2022. Embora *Bartonella* spp. sejam amplamente estudadas em roedores e quirópteros, poucos são os estudos acerca da detecção molecular e/ou isolamento de *Bartonella* em marsupiais. De fato, até o momento apenas uma espécie de *Bartonella*, denominada *Bartonella australis*, foi isolada em cangurus (*Macropus giganteus*) na Austrália (Fournier et al., 2007).

Ainda no continente australiano, *Bartonella* sp. filogeneticamente relacionada com *B. tamiae* foi detectada em carrapatos *Ixodes tasmani* coletados de coalas (*Phascolarctos cinereus*) (Vilcins et al., 2009). Adicionalmente, '*Candidatus* Bartonella bandicootii' e ´*Candidatus* Bartonella woyliei´ foram detectados em pulgas (*Pygiopsylla tunneyi*) de "bandicoots" (*Perameles bougainville*) e em pulgas (*Pygiopsylla hilli*) e carrapatos (*Ixodes australiensis*) coletados de "woylies" (*Blettongia penicillata*), respectivamente (Kaewmongkol et al., 2011).

Nas Américas, *Bartonella* spp. filogeneticamente relacionadas com *B. henselae* e *B. clarridgeiae* foram detectadas molecularmente em *Ctenocephalides felis* coletadas de gambás da espécie *Didelphis virginiana* nos EUA (Nelder et al., 2009; Reeves et al., 2005). No Brasil ainda mais escassos são os estudos sobre a ocorrência de *Bartonella* spp. em marsupiais. De fato, apenas três trabalhos foram encontrados na literatura acerca da detecção de DNA de *Bartonella* spp., via ensaios de PCR convencional e em tempo real, a partir de amostras de sangue, com ausência de detecção dos agentes sob estudo (Fontalvo et al., 2017; de Sousa et al., 2018; Gonçalves et al., 2020).

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Capítulo 2 - *Bartonella machadoae* sp. nov. isolated from wild rodents in the Pantanal wetland

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Abstract

It has been estimated that 75% of emerging infectious diseases comprise zoonoses, whose majority have free-living animals as reservoirs and are mainly transmitted by arthropod vectors. Although rodents represent important *Bartonella* reservoirs, there are few studies on the genotypic characterization of *Bartonella* species commonly found in this taxon and from different Brazilian biomes. Therefore, the present study aimed to investigate the occurrence, isolate and molecularly, morphologically and phenotypically characterize a new *Bartonella* species infecting free-living rodents sampled in the Brazilian Pantanal, the largest wetland in South America. For this purpose, 129 free-living rodents (79 *Thrichomys fosteri*, 4 *Clyomys laticeps*, and *Oecomys mamorae*) were captured. While blood samples were collected from 57 *T. fosteri*, 4 *C. laticeps* and 32 *O. mamorae*; spleen samples were collected from 22 *T. fosteri* and 14 *O. mamorae*. Blood and spleen samples were submitted to a qPCR for *Bartonella* spp. targeting the *nuoG* gene, using DNA samples extracted directly from blood/spleen, after passage in pre-enrichment liquid culture, and from colonies obtained from solid culture on chocolate agar. Combining all techniques, occurrence of 24.8% for *Bartonella* sp. was found among the sampled rodents. One *Bartonella* isolate (strain 56A) obtained from a *T. fosteri*'s blood sample was closely related to the *Bartonella vinsonii* complex and selected for Whole Genome Sequencing (WGS) hybrid approach using Illumina NovaSeq and Nanopore sequencing platforms. This strain exhibits a circular 2.7 Mbp genome with an average C+G content of 39% and encoding to 2,239 genes. In the phylogenomics based on 291 shared protein-coding genes, this strain was positioned in a unique clade, closely related to *Bartonella vinsonii* subsp. *vinsonii*, *B. vinsonii* subsp. *berkhoffii* and *B. visonii* subsp. *arupensis.* An Average Nucleotide Identity of 85% was found between the obtained isolate and *Bartonella* species belonging to *B. vinsonii* complex. These findings supported the

separation of this strain, now formally named as *Bartonella machadoae* sp. nov., from the *Bartonella vinsonii* complex. In addition, *Bartonella machadoae* sp. nov. was characterized by capnophilic, microaerophilic and aerobic small rods with absence of pili and flagella. Phylogenetic and distance analyses based on five concatenated molecular markers suggest that *Bartonella machadoae* may parasite rodents from different Brazilian biomes. In conclusion, we described biochemical, phenotypic and genomic characteristics of *Bartonella machadoae* nov. sp. isolated from blood samples of *T. fosteri* rodents from the Brazilian Pantanal.

Keys words: bartonellosis; Rodentia; biochemical characterization; phylogenomics; WGS

1. Introduction

The bacteria of the genus *Bartonella* encompass facultative intracellular Gram-negative bacteria, which mainly parasitize erythrocytes and endothelial cells, as well as macrophages, monocytes and dendritic cells (Musso et al., 2001; Eicher and Dehio, 2012). These bacteria are cosmopolitan and can be transmitted mainly by hematophagous arthropod vectors, such as fleas, ticks, mosquitoes, flies and lice (Breitschwerdt and Kordick, 2000; Chomel and Karsten, 2010). They are currently classified into the Phylum Proteobacteria, Class Alphaproteobacteria, Order Rhizobiales and Family Bartonellaceae (Chomel et al., 2006; Gutiérrez et al., 2020).

Currently, according to the List of Prokaryotic names with Standing in Nomenclature database (https://lpsn.dsmz.de/genus/bartonella) - accessed in November 2021 -, the genus *Bartonella* comprises 37 different species, from which some are represented by more than one subspecies. Additionally, more than 31 *Candidatus* species have also been described but not yet validated (Breitschwerdt et al., 2010; Kaewmongkol et al., 2011; Angelakis and Raoult, 2014; Okaro et al., 2017).

Rodentia, considered the most abundant and diversified order of the Mammalia class, comprises 40% of all mammals species richness (Huchon et al., 2002). Rodents are the natural reservoirs for different *Bartonella* species (Gutiérrez et al., 2015). For instance, 22 species/subspecies of *Bartonella* have been reported in rodents; nine of which have been incriminated as zoonotic agents in humans (*B. doshiae*, *B. elizabethae*, *B. grahamii*, *B. rochalimae*, *B. tamiae*, *B. tribocorum*, *B. vinsonii* subsp. *arupensis*, *B. washoensis* and *B. krasnovii*), causing different clinical manifestations.

Although new *Bartonella* species have being isolated and characterized from rodents and/or their ectoparasites (Dahmana et al., 2020), the lack of adequate

genomic and phenotypic data from several isolates leads to an extensive list of strains with uncertain taxonomic status (Kosoy et al., 2018; Gutiérrez et al., 2020).

In Brazil, few studies have been performed regarding the occurrence and molecular characterization of *Bartonella* species in rodents. For instance, *B. queenslandensis* and *B. tribocorum* were isolated from blood samples of synanthropic *Rattus norvegicus* in Salvador, Bahia, northeastern Brazil (Costa et al., 2014). In addition, *Bartonella* genotypes phylogenetically related to *B. coopersplainsensis* were molecularly detected in synanthropic rodents from the state of Santa Catarina, south Brazil (Gonçalves et al., 2020). In wild rodents, *Bartonella* genotypes phylogenetically related to *Bartonella* species belonging to *B. vinsonii* complex were molecularly detected in the states of Mato Grosso do Sul, Mato Grosso, Rio Grande do Norte, Piauí, Ceará, Santa Catarina, São Paulo, Minas Gerais, Goiás Tocantins and Rio de Janeiro (Favacho et al. 2015; Gonçalves et al., 2016; Rozental et al., 2017; de Sousa et al., 2018). This genotype has also been detected in rodent-associated fleas, namely *Polygenis* (*P*.) *bohlsi bohlsi*, *Polygenis occidentalis occidentalis*, *Polygenis platensis* and *Craneopsylla minerva minerva,* collected from rodents belonging to the Echimyidae and Cricetidae families in the Pantanal biome, in the state of Mato Grosso do Sul, and in the biomes of Pampa and Atlantic Forest, in the State of Rio Grande do Sul (de Sousa et al., 2018; Schott et al., 2020).

The present study aimed to investigate the occurrence, isolate and molecularly, morphologically and phenotypically characterize *Bartonella* sp. in wild rodents sampled in the Pantanal biome, a wetland in central-western Brazil.

2. Material and Methods

2.1. Ethical Statement

Procedures and management protocols involving wild rodents were approved by the “Institute Chico Mendes for Conservation of Biodiversity” (SISBIO number 64204) and by the “Ethics Committees on Animal Use of the School of Agricultural and Veterinarian Sciences” (FCAV/UNESP) (protocol number #11794).

2.2. Sampled animals and studied area

Wild rodents were captured during two expeditions in the Pantanal of the state of Mato Grosso do Sul, central-western Brazil, more specifically in the “Alegria” farm, Nhecolândia region (-19.050399; -56.675623): one in December 2018 and the second in February 2019, using a seven-night sampling effort. In each expedition, rodents were captured using live-traps (Tomahawk and Shermann), arranged in transects with capture stations distributed every 10 meters. The traps were baited with a mixture of oats and peanut butter. The traps were opened in the late afternoon and checked out in the following morning. The captured animals were chemically immobilized with a combination of 10% ketamine hydrochloride (100 mg/mL) and 1% acepromazine (10 mg/mL) in a 9:1 ratio, using a dose of 0.1 mL/100 g intramuscularly. If the death of the anesthetized animal did not occur after the collection of whole blood, euthanasia was performed by administrating 19.1% potassium chloride intracardiac (2 mL / kg). The taxonomic identification of the sampled rodents was based on external morphology (Bonvicino et al., 2008). Subsequently, blood samples stored into DNAse/RNAse-free microtubes containing ethylene diaminetetraacetic acid (EDTA) and spleen samples stored in DNAse/RNAse-free microtubes were kept in liquid nitrogen cylinders and, and once in the laboratory, in a freezer at -80 °C (**Figure 1**).

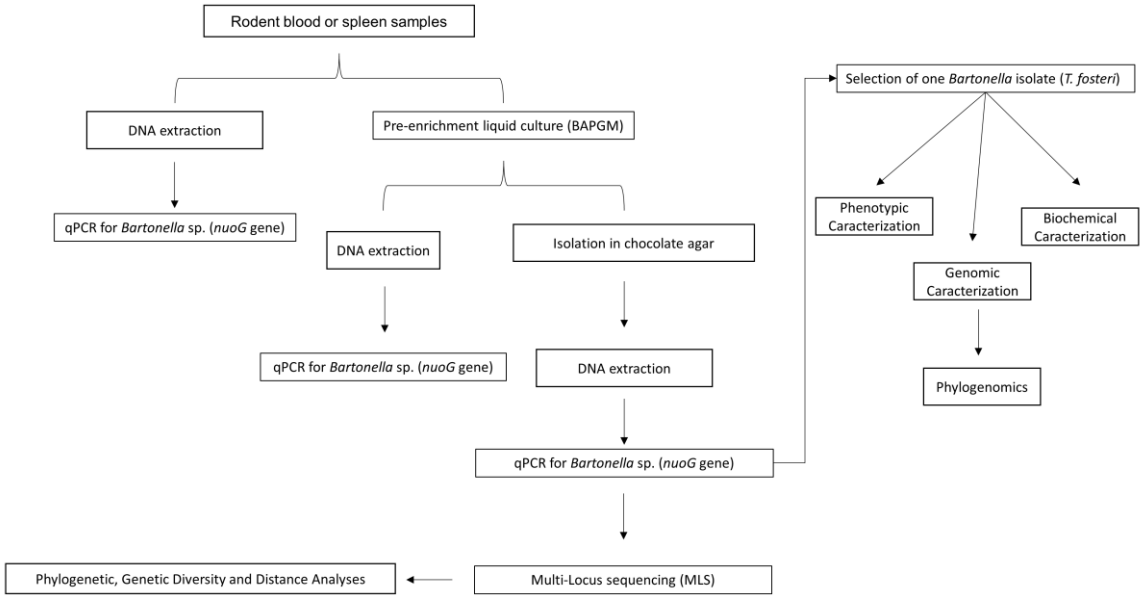

Figure 1. Flow chart of the microbiological and molecular analyses applied in this study for isolation and characterization of *Bartonella machadoae* sp. nov.

2.3. *Bartonella* isolation

A pre-enrichment liquid medium used was based on that previously described as *Bartonella*-Alphaproteobacteria Growth Medium. For this purpose, 500 mL of IPL-41 Insect Medium (Sigma-Aldrich, St. Louis, Missouri, USA) was supplemented with 0.055 mg of NAD (Sigma- Aldrich, St. Louis, Missouri, USA), 0.69 mg of NADP (Sigma-Aldrich, St. Louis, Missouri, USA), 1.1 mg of ATP (Sigma-Aldrich, St. Louis, Missouri, USA), 1.1 mg sodium pyruvate (Sigma-Aldrich, St. Louis, Missouri, USA), and 1.1 g yeast extract (Sigma-Aldrich, St. Louis, Missouri, USA). Amino acid supplementation was performed by adding 35.1 mg of L-arginine HCl, 8.66 mg of L-cystine HCl, 11.64 mg of L-histidine, 14.6 mg of L-isoleucine and L-leucine each, 20.14 mg L-lysine, 4.16 mg L- methionine, 9.02 mg L-phenylalanine, 13,02 mg of L-threonine, 2.7 mg of L-

tryptophan, 12 mg of L-tyrosine 2Na $2H_2O$, and 13 mg of L-valine (Dinâmica, Indaiatuba, São Paulo, Brazil).

After the supplementation, the medium pH was adjusted to 6.2 and subsequently sterilized through a 0.2 µM filter (Corning, Corning, New York, United States). After filtration, the medium was also supplemented with 55 mL of defibrinated sheep blood (previously tested negative by qPCR for *Bartonella* sp.) and adjusted to a final concentration of 11% vol / vol. Rodent spleen samples were prepared for pre-enrichment liquid culture by maceration and homogenization 200 µL of PBS (pH 7.2). Homogenized spleen samples and 200 µL of blood samples were homogenized to 2 mL of medium containing defibrinated sheep blood, placed into culture flasks (20 cm^2, model 430639, Corning, Corning, New York, United States) and incubated for seven days, under constant movement, at 35ºC with 5% CO_2 in a CO_2 / O_2 Water Jacketed incubator (NuAire) Plymouth, Massachusetts, USA) (Duncan et al., 2007; Maggi and Breitschwerdt, 2005; Furquim et al., 2021). Two flasks of negative controls were also added for each batch of 20 tested samples: one containing only the pre-enrichment medium and another one containing both the pre-enrichment medium and defibrinated sheep blood.

After seven days, 200 µL of the liquid culture were submitted to DNA extraction using the Illustra Tissue & Cells genomic Prep Mini Spin Kit, following the manufacturer's recommendations. Additionally, 200 µL of the content of each bottle were plated onto enriched chocolate agar (Laborclin, Pinhais, Paraná, Brazil) and kept at 35ºC with 5% CO_2 for up to 60 days in a CO_2 / O_2 Water Jacketed incubator (NuAire, Plymouth, Massachusetts, USA), and observed daily. Three negative controls were added for each batch of tested samples: two corresponding to the negative controls previously used in the pre-enrichment liquid culture and another one represented by a

chocolate agar plate without any sample. Colonies suggestive of *Bartonella* were collected and submitted to DNA extraction by the boiling method (Keim et al., 2000) and qPCR assay for *Bartonella* sp. based on the *nuoG* gene (André et al., 2016). Colonies molecularly confirmed to belong to *Bartonella* sp. were then submitted to five passages until pure isolated cultures were obtained.

2.4. Quantitative real-time PCR assay (qPCR) for *Bartonella* sp. based on the *nuoG* (nicotinamide adenine dinucleotide dehydrogenase gamma subunit) gene

The quantitative PCR (qPCR) protocol based on the *nuoG* gene (André et al., 2016) was used to detect and quantify *Bartonella* sp. DNA (number of copies of a fragment of *nuoG* gene/µL) in rodent blood/spleen samples, rodent blood/spleen samples added to BAPGM-based liquid culture and colonies isolates from chocolate agar plates. The qPCR assays were performed in 10 µL final volume reaction mixtures, containing 1 µL of DNA sample, 1.2 µM of each primer F-Bart (5'-CAATCTTCTTTTGCTTCACC-3'), R-Bart (5'- TCAGGGCTTTATGTGAATAC-3') and hydrolysis probe TexasRed-5'-TTY GTCATTTGAACACG-3' [BHQ2a-Q]-3', Master Mix 2x buffer (GoTaq™ Probe qPCR Master Mix, Promega Corporation, Madison, USA) and ultra-pure sterilized water (Nuclease-Free Water, Promega Corporation, Madison, USA) q.s.p. 10 µL. The amplification conditions were 95˚C for 3 minutes followed by 40 cycles at 95˚C for 10 seconds and 52.8˚C for 30 seconds. PCR amplifications were conducted in low-profile multiplate unskirted PCR plates (BioRad™, CA, USA), using a CFX96 Thermal Cycler (BioRad™, CA, USA). Standard curves were constructed with serial dilutions of plasmid DNA (pIDTSMART—Integrated DNA Technologies) ($1.0x10^7$ to $1.0x10^0$ copies/µL), which encoded an 83 bp *Bartonella henselae nuoG*

gene fragment. The number of plasmid copies was determined by (Xg/µL DNA/ [plasmid length in bp x 660]) x 6.022 x 10^{23} x plasmid copies/µL (André et al., 2016).

All DNA samples were initially tested in duplicates. All duplicates whose Cq difference was higher than 0.5 were re-tested in triplicates. Amplification efficiency (E) was calculated from the slope of the standard curve in each run using the following formula ($E = 10^{-1/slope}$). The standard curves generated by 10-fold dilutions were used to determine the amount of DNA that could be detected with 95% of sensitivity (André et al., 2016).

2.5. Multi-locus sequencing for *Bartonella* sp.

To perform the molecular characterization, isolates that showed to be positive in the qPCR for *Bartonella* sp. were submitted to previously described conventional PCR assays targeting five molecular markers, namely 16S rRNA (400 bp) (Dauga et al., 1996), *gltA* (750 bp) (Norman et al., 1995), the intergenic spacer region 16S-23SrRNA ITS (453-717bp) (Maggi and Breitschwerdt, 2005), *groEL* (752 bp) (Paziewska et al., 2011), and *ftsZ* (600 bp) (Paziewska et al., 2011). *Bartonella henselae* previously detected in a cat sampled in southeastern Brazil (Furquim et al., 2021) and sterilized ultrapure water (Nuclease-Free Water, Promega™, Madison, Wisconsin, USA) were used as positive and negative controls, respectively. The amplicons obtained in cPCR assays were submitted to ethidium bromide (Life Technologies, Carlsbad, California, United States)-stained agarose gels (1%) electrophoresis, at 100 V and 150 mA for 50 min. The gels were imaged under ultraviolet light (ChemiDoc MP Imaging System; Bio Rad, Hercules.

2.6. Concatenated phylogenetic analysis based on the 16S rRNA, *gltA*, *groEL*, *ftsZ* genes and ITS region

Amplified products from conventional PCR assays were purified using the ExoSAP- IT (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific™, Waltham, MA, USA) and the ABI PRISM 310 DNA Analyzer (Applied Biosystems™, Foster City, CA, USA) (Sanger et al., 1977). The primers used in the sequencing reactions were previously described in the conventional PCR assays for *Bartonella* sp. The sequences obtained were first submitted to a screening test using Phred-Phrap software version 23 (Ewing and Green, 1998; Ewing et al., 1998) to evaluate the electropherogram quality and to obtain consensus sequences from the alignment of the sense and antisense sequences. The BLAST program (Altschul et al., 1990) was used to analyze the sequences of nucleotides (BLASTn), in order to browse and compare with sequences from an international database (GenBank) (Benson et al., 2016. The consensus sequences obtained in this study and those retrieved from GenBank were aligned using the Clustal/W software (Thompson et al., 1994) via Bioedit v. 7.0.5.3 (Hall, 1999). Phylogenetic inference was based on Maximum Likelihood (ML) method. The Maximum-likelihood (ML) analysis was inferred with the W-IQ-Tree tool available online (http://iqtree.cibiv.univie.ac.at/) (Trifinopoulos et al., 2016; Nguyen et al., 2015) using 1,000 bootstrapping replicates. The best evolution model was selected by the program jModelTest2 (version 2.1.6) on XSEDE (Darriba et al., 2012). Tree was edited in Treegraph 2.0.56–381 beta (Stover and Muller, 2010).

2.7. Genetic diversity and genealogy of *Bartonella* sp.

For genetic diversity analysis, the obtained 16S rRNA, *gltA*, *groEL*, *ftsZ,* and the intergenic spacer region 16S-23S rRNA (ITS) sequences obtained from *Bartonella* isolates were aligned and used to calculate the nucleotide diversity (π), polymorphism level (diversity of haplotypes [dh], number of haplotypes [h] and the average number of nucleotide differences [K]), using DnaSP v5 software (Librado and Rozas, 2009). The nucleotide sequences were aligned with sequences previously deposited in the GenBank database and submitted to distance analysis based on Split-Network, using the programs Population Analysis with Reticulate Trees (popART) and SplitsTree v4.11.3 (Huson and Bryant, 2006; Leigh and Bryant, 2015), respectively.

2.8. Phenotypic characterization of isolated *Bartonella* sp.

For the phenotypic characterization, one *Bartonella* isolate (strain 56A) from *T. fosteri* blood sample was seeded onto chocolate agar plates (Novamed) and incubated at 35 °C in duplicates, under the following conditions: (i) aerobic (i.e. standard incubator); (ii) capnophilic (i.e. 5% CO_2) in a CO_2 incubator (Thermo Forma II Water Jacket CO2 Incubator, Thermo Scientific); (iii) microaerophilic (i.e. 5 a 15% O_2, 10% % CO_2) using Microaerobac® bags (Probac); and (iv) anaerobic (i.e. <3% O_2, 4 a 10% CO_2) using Anaerobac® bags (Probac).

2.9. Light microscopy and Scanning Electron Microscopy (SEM)

The *Bartonella* strain 56A was submitted to light microscopy and scanning electron microscopy (SEM). For light microscopy, the isolate was stained with Gram stain set (Hy-labs) and visualized using 100× objectives. For SEM, a *Bartonella* isolated from a *T. fosteri* blood sample (strain 56A) was collected from chocolate agar

plate and fixed in a solution containing 2% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M cacodylate buffer for 1 hour, and then washed three times in phosphate buffer. Thereafter, 1% osmium tetroxide was added and the solution was incubated for 30 minutes. The bacteria were then dehydrated through a graded ethanol series (50–100%), and dried with a critical point dryer (EMS 850). After the critical point, the sample was attached to metal stubs, and coated with gold (5 nm) on a sputtering Danton Vacuum Desk II. Finally, the bacterial cells were visualized by SEM. Samples were analyzed and photographed under a Zeiss Evo 10 scanning electron microscope (10–20 kV).

2.10. Biochemical and metabolic characterization

For biochemical and metabolic characterization, the *Bartonella* strain isolated from *T. fosteri* (strain 56A) was tested with the BBL Crystal Enteric/Nonfermenter (BD Becton Dickinson), API 20E and NF III (Probac) identification systems, following the manufacturers' instructions. The enzymatic catalase reaction was carried out with hydrogen peroxide (3%).

2.11. Whole genome sequencing of *Bartonella* isolated from *Thrichomys fosteri* (strain 56A) and genome annotation

Genomic DNA extraction from a *Bartonella* isolate (strain 56A) grown onto chocolate agar plates was performed using commercial DNeasy® Blood & Tissue kit (QIAGEN), following the manufacturers' instructions. For genome assembly, the genomic DNA sample was sequenced by Illumina Novaseq 6000 (PE 150). Pair-end FASTQ files were obtained, with a total of 6,474,816 and 6,730,260 reads, which were trimmed for adapters. Potential human sequence contaminants were mapped using

Bowtie2 using human reference genome (Langmead and Salzberg, 2012). Additionally, FASTQ files were also obtained from MinION run (SQK-LSK109 kit, Oxford Nanopore), totaling 67.115 reads, with read length median of 14,083 pb. Reads were trimmed for low quality and adapters using NanoFilt and Porechop, respectively (Wick, 2017). All trimmed Illumina and Nanopore reads were then used for hybrid assembly using MaSuRCa assembler v4.0.4 and default parameters (Zimin et al., 2017). The assembled genome was automatically annotated with the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016).

2.12. Phylogenomic analyses

The assembled genome of the new *Bartonella* isolate (strain 56A) and 53 complete *Bartonella* genomes retrieved from the NCBI RefSeq database (O'Leary et al., 2016) were used for the comparative analysis. Two-hundred ninety one shared genes were retrieved using the roary tool (Page at al., 2015). These genes were aligned with MAFFT (Katoh and Standley, 2013) and further used for the phylogenomic analyses.

The ML analysis was inferred with the W-IQ-Tree tool (Nguyen et al., 2015; Trifinopoulos et al., 2016) using 1,000 bootstrapping replicates. The trees were edited in Treegraph 2.0.56–381 beta (Stöver and Müller, 2010). *Brucella abortus* and *Brucella ceti* (GenBank accession number: CP007681; NC_022905) were used as an outgroup.

The average nucleotide identities between *Bartonella machadoae* sp. nov. and *Bartonella vinsonii* complex species (*B. vinsonii* subsp. *vinsonii*, *B. vinsonii* subsp. *arupensis* and *B. vinsonii* subsp. *berkhoffii*) were calculated with OrthoANIu 1.2 (Lee et al., 2016).

3. Results

3.1. Occurrence of *Bartonella* DNA in rodents' blood and spleen samples

Fifty-nine rodents were captured in December 2018 and 70 rodents in February 2019. Among the 129 rodents sampled, 79 (61.2%) belonged to the species *Thrichomys fosteri*, four (3.1%) to the species *Clyomys laticeps*, and 46 (35.7%) to the species *Oecomys mamorae*. Blood samples were collected from 57 *T. fosteri*, 4 *C. laticeps* and 32 *O. mamorae*. Spleen samples were collected from 22 *T. fosteri* and 14 *O. mamorae*. All 129 biological samples (93 blood and 36 spleen samples) were submitted to the qPCR for *Bartonella* sp. based on the *nuoG* gene. Fifteen out of 129 (11.6%) rodents (5/57 from *T. fosteri* and 10/46 from *O. mamorae*) were positive in the qPCR for *Bartonella* sp. All 15 positive samples were represented by rodents' blood samples. None of rodent spleen sample was positive in the qPCR for *Bartonella* sp. The number of copies of a fragment of *Bartonella nuoG* gene fragment per microliter ranged from 2.91 x 10^1 to 1.08 x 10^3. The efficiency, R^2, slope and Y-intercept of the reactions ranged from 91.4% to 103.9%, 0.995 to 0.999, -3.547 to -3252 and 38.571 to 40, respectively.

3.2. Isolation of *Bartonella* sp. on agar chocolate

Out of the 129 pre-enrichment liquid cultures obtained from rodent biological samples (93 blood and 36 spleen samples), 31 (24.03%; 18 blood samples from *T. fosteri* and 13 blood samples from *O. mamorae*) were positive in the qPCR for *Bartonella* sp. The copy number of the *nuoG* gene fragment per microliter ranged from 4.11x10^0 to 2.16x10^7. The efficiency, R^2, slope and Y-intercept of the reactions ranged from 91.4% to 103.9%, 0.995 to 0.999, -3.547 to -3252 and 38.571 to 40, respectively. Sixteen qPCR-negative rodent blood samples were positive in the

qPCR for *Bartonella* sp. after been submitted to liquid culture. All 15 blood samples that were qPCR-positive for *Bartonella* spp. were also positive in the qPCR from the liquid culture. The quantification of a fragment of the *nuoG* gene per microliter increased in 12 qPCR-positive blood samples when submitted to liquid culture.

Out of 31 qPCR-positive liquid culture samples (18 for *T. foste*ri and 13 for *O. mamorae*), 14 (45.16%) showed the presence of *Bartonella* colonies on chocolate agar plates, whose molecular identity was confirmed by qPCR. Only one sample negative in the qPCR for *Bartonella* from both DNA extracted directly from blood and liquid culture generated an isolate onto chocolate agar plate (**Table 1**).

Table 1. Blood samples from rodents captured in the Brazilian Pantanal and submitted to qPCR for *Bartonella* sp. based on the *nuoG* gene (with quantification of a fragment of the targeted gene copy numbers / µL), liquid culture in BAPGM pre-enrichment medium and isolation onto chocolate agar plates.

Rodent species	**qPCR for *Bartonella* spp. (*nuoG*)**		
	Blood	**Liquid Culture (BAPGM)**	**Solid Culture (chocolate agar)**
Oecomys mamorae	Neg	$4{,}41 \times 10^{2}$	Neg
Thrichomys fosteri	Neg	$1{,}04 \times 10^{1}$	Neg
Thrichomys fosteri	Neg	$1{,}7 \times 10^{1}$	**Pos**
Oecomys mamorae	$8{,}63 \times 10^{2}$	$3{,}42 \times 10^{6}$	**Pos**
Thrichomys fosteri	$1{,}47 \times 10^{3}$	$1{,}69 \times 10^{6}$	**Pos**
Oecomys mamorae	$2{,}04 \times 10^{2}$	$2{,}16 \times 10^{7}$	**Pos**
Thrichomys fosteri	Neg	$4{,}1 \times 10^{1}$	Neg
Thrichomys fosteri	Neg	$2{,}30 \times 10^{5}$	**Pos**
Oecomys mamorae	$2{,}91 \times 10^{1}$	$2{,}45 \times 10^{5}$	**Pos**
Thrichomys fosteri	Neg	$1{,}4 \times 10^{1}$	Neg
Thrichomys fosteri	Neg	$4{,}11 \times 10^{0}$	Neg
Thrichomys fosteri	Neg	Neg	**Pos**
Thrichomys fosteri	Neg	$1{,}25 \times 10^{1}$	Neg
Oecomys mamorae	$1{,}35 \times 10^{2}$	$2{,}32 \times 10^{2}$	Neg
Oecomys mamorae	$7{,}20 \times 10^{2}$	$4{,}21 \times 10^{4}$	Neg
Thrichomys fosteri	$1{,}15 \times 10^{2}$	$2{,}79 \times 10^{5}$	**Pos**
Oecomys mamorae	Neg	$1{,}69 \times 10^{1}$	Neg
Thrichomys fosteri	Neg	$3{,}48 \times 10^{5}$	Neg
Thrichomys fosteri	Neg	$8{,}30 \times 10^{3}$	Neg
Thrichomys fosteri	$3{,}62 \times 10^{2}$	$1{,}85 \times 10^{5}$	**Pos**
Oecomys mamorae	$1{,}08 \times 10^{3}$	$2{,}23 \times 10^{6}$	Neg
Thrichomys fosteri	$1{,}32 \times 10^{2}$	$2{,}16 \times 10^{6}$	**Pos**
Thrichomys fosteri	Neg	$3{,}74 \times 10^{4}$	**Pos**
Thrichomys fosteri	$3{,}48 \times 10^{1}$	$2{,}25 \times 10^{7}$	**Pos**
Oecomys mamorae	Neg	$3{,}91 \times 10^{2}$	Neg
Thrichomys fosteri	Neg	$2{,}94 \times 10^{2}$	Neg
Oecomys mamorae	$2{,}3 \times 10^{2}$	$1{,}06 \times 10^{6}$	**Pos**
Thrichomys fosteri	Neg	$4{,}82 \times 10^{4}$	Neg
Oecomys mamorae	$9{,}45 \times 10^{2}$	$2{,}93 \times 10^{6}$	**Pos**
Oecomys mamorae	$8{,}11 \times 10^{0}$	$1{,}92 \times 10^{5}$	Neg
Oecomys mamorae	$2{,}29 \times 10^{2}$	$1{,}37 \times 10^{5}$	**Pos**
Thrichomys fosteri	Neg	$9{,}57 \times 10^{0}$	Neg

By combining the results obtained by qPCR performed directly from blood/spleen DNA samples with the molecular results obtained from both liquid and

solid cultures, an overall occurrence of 24.8% for *Bartonella* sp. was found among the sampled rodents.

3.3. Multi-locus sequencing and phylogenetic analysis for *Bartonella* sp.

BLASTn analysis demonstrated that the sequences of *Bartonella* sp. corresponding to five targeted genes amplified from isolates of bartonellae from rodent blood samples were strictly related to sequences previously described in rodents from Brazil and also to those species belonging to *Bartonella vinsonii* complex (**Table 2**).

Table 2. BLASTn results for five molecular target gene sequences of 11 *Bartonella* isolates obtained from blood samples of rodents (six from *T. foste*ri and five from *O. mamorae*).

Target gene/ intergenic region	Species	Host	Locality	Identity	Query cover	E-value	GenBank Accession Number
16S rRNA	*Bartonella vinsonii* subsp. *arupensis*	Human	USA	99%	100%	0	NR_104902
gltA	*Bartonella* sp.	*Oecomys mamorae*	MS, Brazil	100%	100%	0	KF021604
groEL	*Bartonella* sp.	*Hylaeamys* sp.; *Akodon* sp.	CE and MA, Brazil	99%	100%	0	KX086734; KX086737
ftsZ	*Bartonella* sp.	*Thrichomys fosteri*	MS, Brazil	100%	100%	0	KY273626
ITS	*Bartonella vinsonii*	not available	not available	92%	25%	$2x10^{-99}$	LR134529

The concatenated phylogenetic tree based on the sequences of five molecular markers (16S rRNA, *gltA*, *groEL*, *ftsZ*, and ITS) of 11 *Bartonella* isolates (five from *O. mamorae* and six from *T. fosteri*) and inferred by Maximum Likelihood (ML) analysis and the TVM+F+I evolutionary model formed a single and monophyletic cluster. The

sequences from *Bartonella* isolates of the present study were positioned together with sequences previously detected in *O. mamorae* rodents from Pantanal, state of Mato Grosso do Sul (KX578719, KX827420), and in a larger clade with several sequences of *Bartonella* sp. detected in rodents from the Brazilian states of Maranhão, Ceará and Rio de Janeiro (KX086721, KX270253, MN613442, KX270232, MN613441, KX270248), sequences detected in rodents sampled in Peru (KF021604), and sequences detected in *Polygenis (P.) bohlsi bohlsi* fleas collected from rodents in the Pantanal, state of Mato Grosso do Sul (KY304482, KY304483). Additionally, this clade was phylogenetically related to the clade of *Bartonella* sp. detected in bats and Streblidae bat flies (*Strebla guajiro*) (MG654770, KY356753), and *Bartonella* sp. detected in rodents of the Cricetidae family in Peru and U.S.A. (C4-phy, R-phy2, C1-phy) (**Figure 2**).

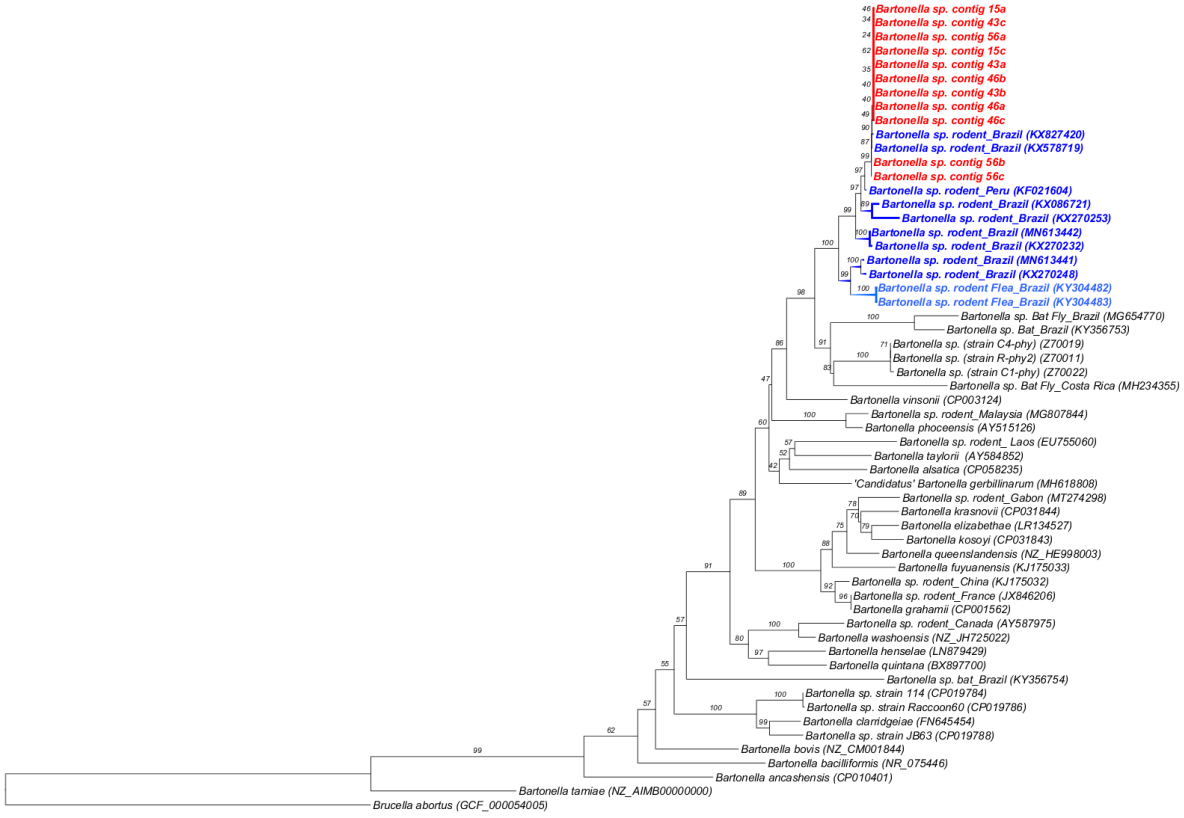

Figure 2. Concatenated phylogenetic analysis within the genus *Bartonella* based on the 16S rRNA, *gltA*, *ftsZ*, *groEL* genes and 16S-23S intergenic region (ITS). The

phylogenetic tree was inferred using the Maximum Likelihood method and the TVM+F+I evolutionary model. The sequences amplified in this study are highlighted in red. The numbers in each branch correspond to the boostrap values accessed with 1,000 replicates. *Brucella* abortus was used as an outgroup.

3.4. Diversity analyses

Sequences representing the same gene/intergenic region obtained from five molecular markers (16S rRNA, *gltA, groEL, ftsZ*, and ITS) from 11 rodent isolates (six from *T. fosteri* and five from *O. mamorae*) were identical to each other. The genealogical analysis of nucleotide sequences by Network, using the Splitstree v4.11.3 program (Huson and Bryant, 2006), corroborates the results obtained by the phylogenetic analysis, forming three relevant clusters. Cluster #1 positioned *Bartonella* sequences obtained from isolates from blood cultures of *T. fosteri* and *O. mamorae,* together with sequences previously detected in rodents from Brazil, in the states of Mato Grosso do Sul, Maranhão, Ceará, and Rio de Janeiro (KX578719, KX578720, KX086721, KX270253, MN613442, KX270232, MN613441, KX270248), *Bartonella* sequences detected in rodents from Peru (KF021604), and sequences previously detected in rodent-associated fleas in the Pantanal wetland (MKY304482; KF021604). While Cluster #2 contained sequences from *Bartonella* sp. previously detected in rodents from Peru, Cluster #3 contained sequences of *Bartonella* sp. previously detected in bats and associated ectoparasites from Brazil (MG654770, KY356753) (**Figure 3**).

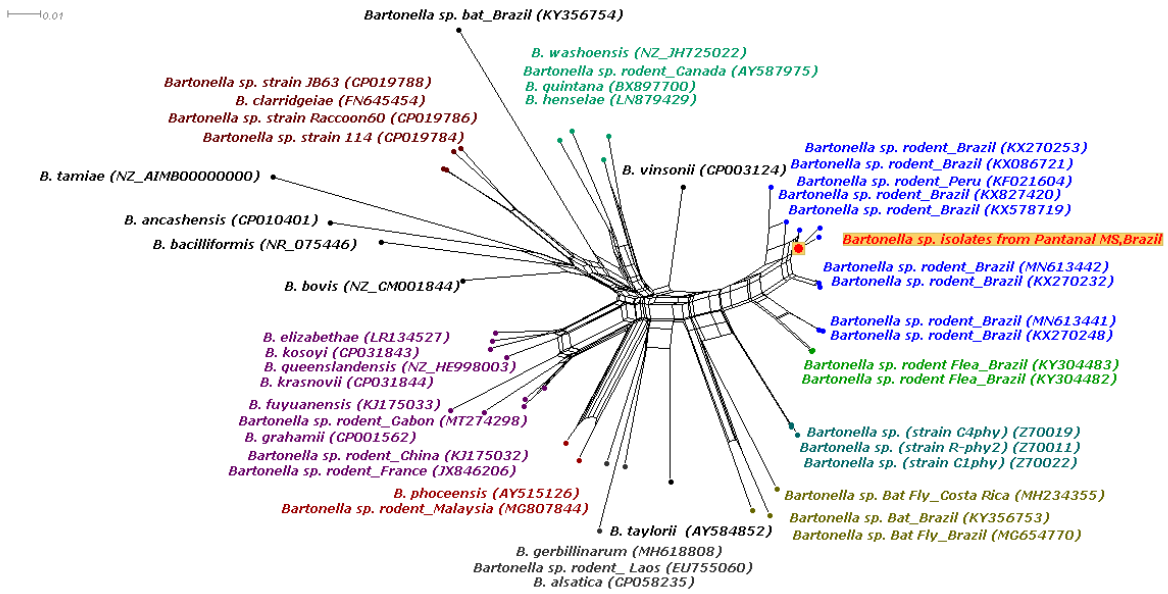

Figure 3. Neighbor-Net analysis of DNA sequences from the 16S rRNA, *gltA*, *ftsZ*, *groEL* genes and 16S-23S intergenic region (ITS) of *Bartonella* sp. isolated from wild rodents (*Thrichomys fosteri* and *Oecomys mamorae*) sampled in the Brazilian Pantanal in the present study when compared with closely related *Bartonella* sequences previously deposited in GenBank.

3.5. Phenotypic and morphological characterization of isolated *Bartonella* sp.

The *Bartonella* strain 56A isolated from the blood sample of a *T. fosteri* rodent comprised of Gram-negative rods (1–2.0 µm length and 0.3–0.6 µm width) without flagella and pili. The bacteria showed to be capnophilic, microaerophilic and aerobic, since no growth was obtained under anaerobic conditions (**Table 3**). At SEM, bacteria appeared as small rods without polar pili and flagella, forming compact aggregates of bacteria (**Figure S3 and Figure 4**). In addition, we observed the lack of adhesin (*BadA*) and flagellum (*FlaA* and *FlaB*)-related genes, by analysing the genome of the obtained strain (**Table 3**).

Table 3. Phenotypic and Genomic features of *Bartonella machadoae* sp. nov.

Phenotypic characterization	
Gram staining	Negative
Morphology	Rods
Length	1–2.0 µm - 0.3–0.6 µm
Growth conditions	capnophilic, microaerophilic and aerobic
Genomic characterization	
Estimated completeness	99.84%
Total size	2.708.600 pb
%C+G	39%
Genes (total)	2,511
CDSs (total)	2,459
Genes (coding)	2,239
CDSs (with protein)	2,239
Genes (RNA)	52
rRNAs	2, 2, 2 (5S, 16S, 23S)
complete rRNAs	2, 2, 2 (5S, 16S, 23S)
tRNAs	42
ncRNAs	4
Pseudo Genes (total)	220
CDSs (without protein)	220
Pseudo Genes (ambiguous residues)	0 of 220
Pseudo Genes (frameshifted)	113 of 220
Pseudo Genes (incomplete)	114 of 220
Pseudo Genes (internal stop)	58 of 220
Pseudo Genes (multiple problems)	53 of 220
Flagellum genes (*FlagA* and *FlagB*)	absent
Adhesin genes (*BadA*)	absent

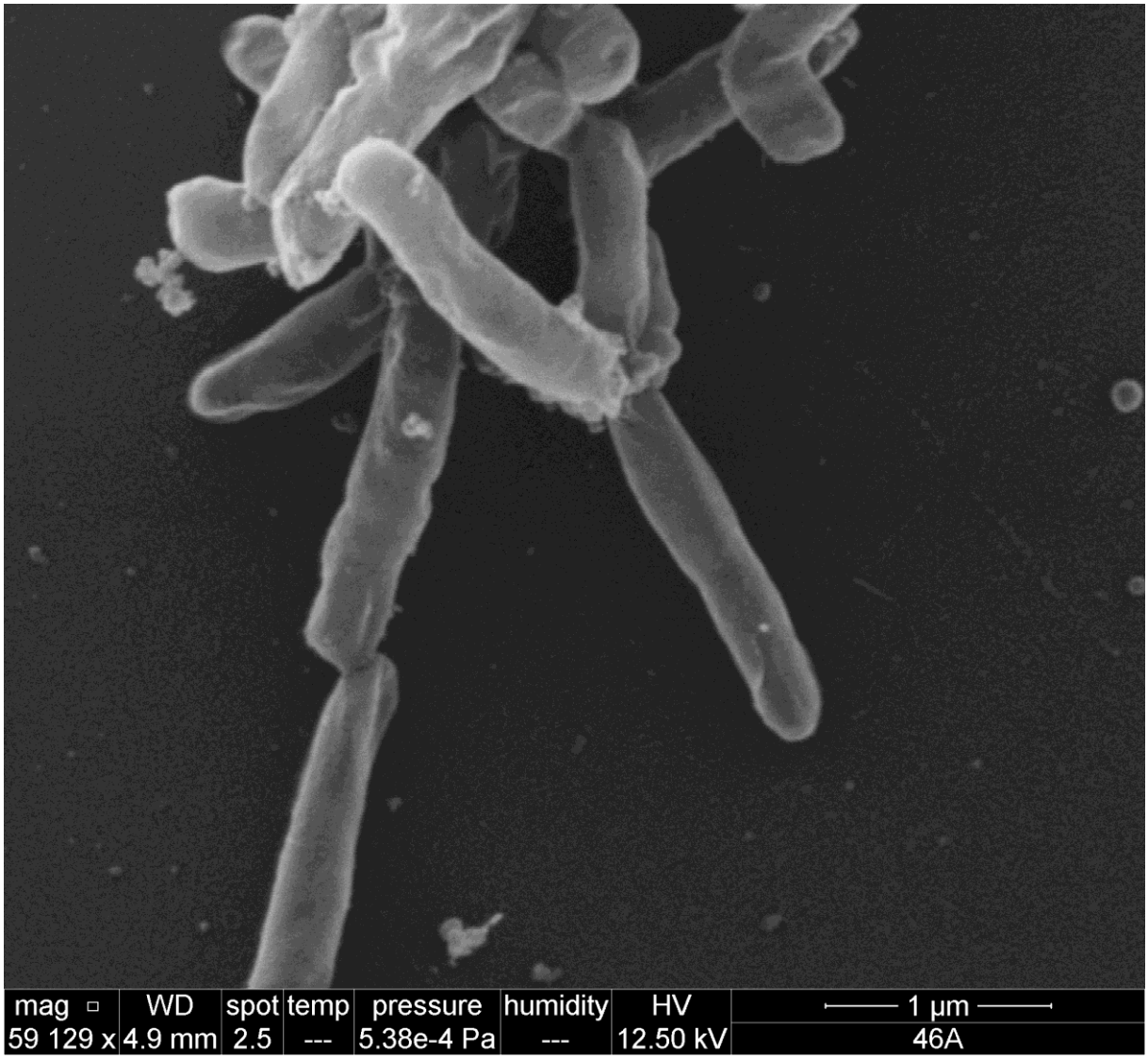

Figure 4. Scanning electron microscopy SEM) of *Bartonella* strain isolated from blood samples of *Thrichomys fosteri* (*Bartonella machadoae* sp. nov.) showing absence of flagellum and pili. A 1 µM scale bar is shown in the image.

3.6. Biochemical and metabolic characterization

The *Bartonella* strain 56A obtained from the blood sample of a *T. fosteri* rodent was negative for oxidase, catalase, urease, indole production, nitrate reduction and acetoin production (Voges-Proskauer reaction). It was inert for all carbohydrates tested (glucose, mannitol, Inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose). It was negative for arylamidase activities of the amino acids arginine, lysine, tryptophan. Moreover, it was negative for esculin, DNAse activity, β-D-

galactosidase nitrate and McConkey growth. Also, it showed sensitivity to Polymyxin B.

3.7. Phylogenomic Analysis

Since the sequences representing the same gene/intergenic region of five targeted molecular markers (16S rRNA, *gltA*, *ftsZ*, *groEL*, and ITS) from 11 isolates of rodent (six from *T. fosteri* and five from *O. mamorae*) were 100% identical to each other, and grouped together in the phylogenetic and distance analyses, an isolate obtained from *T. fosteri* (strain 56A) was chosen for being submitted to Whole Genome Sequencing. The genome was assembled into a circular chromosomal contig of 2.70 Mbp. The genomic characteristics of *Bartonella* sp. strain 56A are described in **Table 3**. When the mean complete genome nucleotide identity (ANI) was calculated in comparison to those species phylogenetically related, ANI of 85% was found with those species belonging to *B. vinsonii* complex (**Figure 5**). The phylogenomic analysis was based on 291 protein-coding genes shared among 53 complete genomes of *Bartonella* species with validly published names and some *Bartonella* not yet named. The phylogenomics positioned *Bartonella machadoae* sp. nov. in a clade closely related to *Bartonella vinsonii* subsp. *vinsonii*, *B. vinsonii* subsp. *berkhoffii* and *B. vinsonii* subsp. *arupensis,* albeit separated with 100% bootstrap **(Figure 6)**.

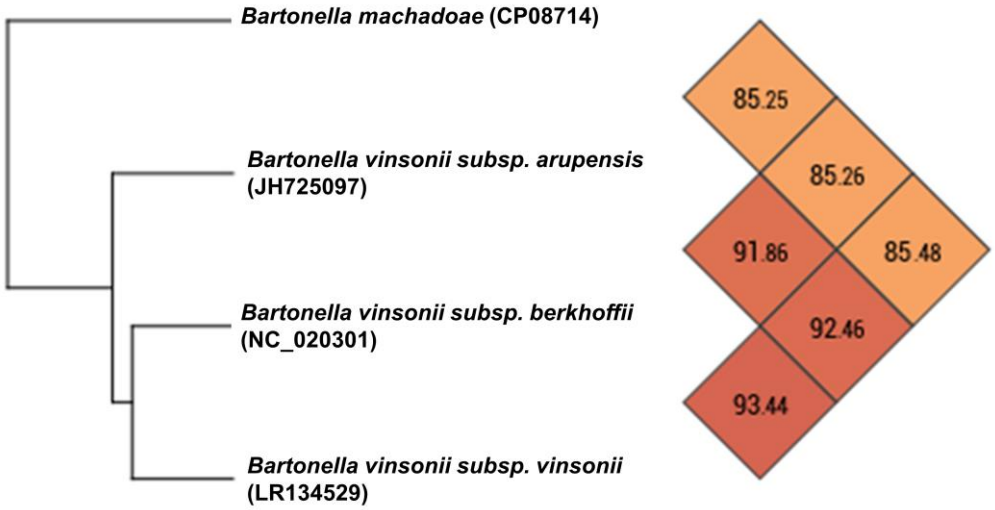

Figure 5. Heatmap generated with OrthoANI values calculated between *Bartonella vinsonii* complex and *Bartonella* sp. isolated in present study using OAT software.

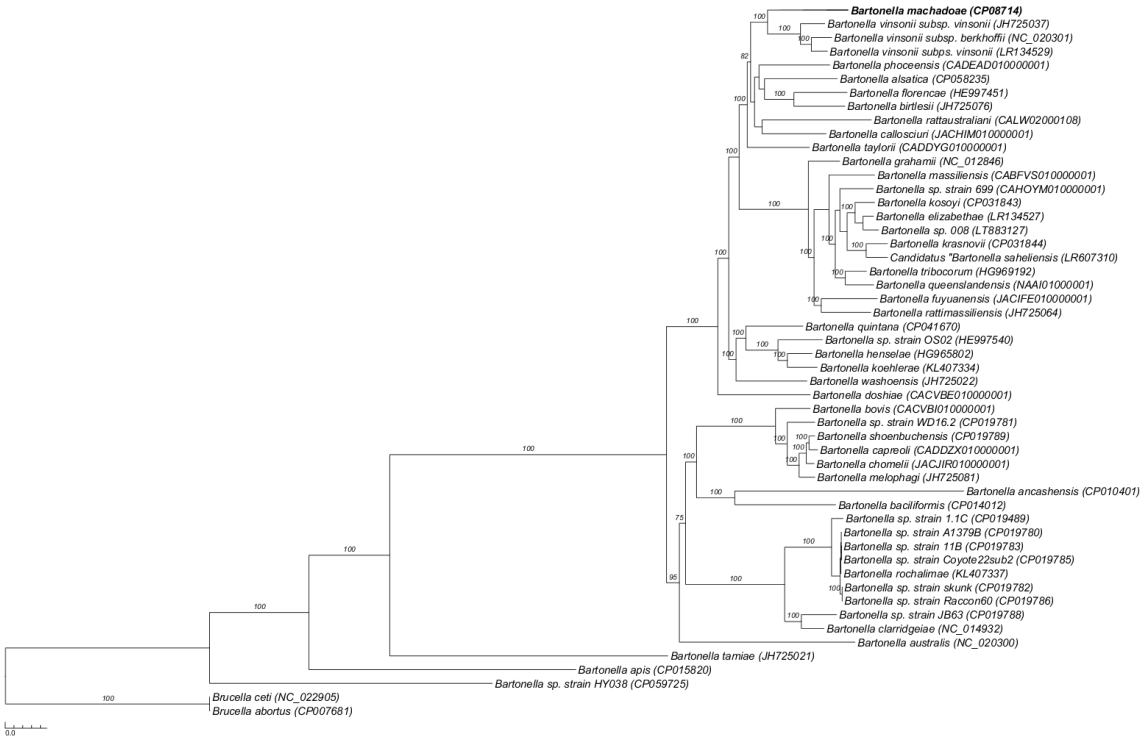

Figure 6. Phylogeny based on the complete genome of 53 *Bartonella* isolates. Phylogenetic tree of species of the genus *Bartonella* based on 291 shared protein-coding genes. Bootstrap values are shown in nodes. *Brucella abortus* and *Brucella ceti* were used as an outgroup.

4. Discussion

The present work aimed to contribute to the identification, molecular, morphological and phenotypic characterization of *Bartonella* sp. in wild rodents in the Pantanal, state of Mato Grosso do Sul, central-western Brazil. Taking into account the overall positivity for *Bartonella* sp. by qPCR based on the *nuoG* gene from blood/spleen samples, liquid and solid cultures, occurrence of 24.8% (32/129) was found among sampled rodents. Previously, our research group found positivity for *Bartonella* sp. of 32% (35/110) in wild rodents sampled in the Pantanal, state of Mato Grosso do Sul, using a qPCR assay based on the *nuoG* gene for screening spleen samples (de Sousa et al., 2018). If liquid and solid cultures had not been used, the present study would have found an occurrence of 11.62% (15/129). Duncan et al. (2007) highlighted the advantages of using the BAPGM pre-enrichment medium ("liquid culture") aiming at increasing the diagnostic sensitivity and successful isolation of *Bartonella* sp. in mammal biological samples. These authors found a positivity of 4.7% for *Bartonella* sp. by qPCR performed directly from dog blood DNA samples; after pre-enrichment, positivity of 8.1% was obtained, thus increasing the success in detecting and isolating *Bartonella* on solid media. Recently, our research group has also achieved an increased sensitivity in detecting *Bartonella* in blood samples from cats in southeastern Brazil using the same diagnostic platform (Furquim et al., 2021).

Regarding phenotypic characteristics, the strain 56A of *Bartonella* sp. isolated from a blood sample of *T. fosteri* showed optimal growth under increased CO_2 conditions, presenting aerobic, capnophilic and microaerophilic features, showing rounded and smooth colonies (1-2 mm) after 4 days of plating onto chocolate agar. On the other hand, the strain 56A of *Bartonella* sp. demonstrated an inability to grow under anaerobic conditions. Such growth characteristics have already been reported in other

species of the genus *Bartonella*, such as in *B. elizabethae*, *B. henselae* (strain Houston-3), *B. clarridgeiae*, *B. quintana* and *B. vinsonii* subsp. *vinsonii* (Clarridge et al., 1995). On the other hand, *B. kosoy*i and *B. krasnovii* isolated from *Rattus rattus* and *Synosternus cleopatrae* fleas collected from *Gerbillus andersoni*, respectively, did not grow in strictly aerobic and anaerobic conditions (Gutiérrez et al., 2020).

Bacteria of the genus *Bartonella* are bacillary or coccobacillary, and may or may not have pili and flagellum, depending on the involved species (Bermond et al., 2002; Li et al., 2015; Mullins et al., 2015; Gutiérrez et al., 2020). The *Bartonella* sp. isolated in the present study (strain 56A) did not present pili or flagella by SEM. This finding was confirmed by the lack of adhesin (*BadA*) and flagellum (*FlaA* and *FlaB*)-related genes when analysing the genome of the obtained strain. Similarly, other rodent-associated *Bartonella* species, such as *B. vinsonii* (Breitschwerdt et al., 1995), *B. japonica*, *B. silvatica* (Inoue et al., 2010) and *B. heixianziensis* (Li et al., 2015), also lack flagellum and pili. On the other hand, flagella have already been described in several species of this genus, such as *B. bacilliformis*, *B. schoenbuchensis*, *B. capreoli, B. chomelii*, *B. clarridgeiae* and *B. rochalimae*. Furthermore, the presence of pilli has been described in *Bartonella* species associated with rodents, such as *B. tribocorum* (Heller et al., 1998), *B. alsatica* (Heller et al., 1999), *B. acomydis*, *B. jaculi*, *B callosciuri*, B*. pachyuromydis* (Sato et al., 2013), *B. fuyuanensis* (Li et al., 2015), *B. kosoyi* and *B. krasnovii* (Gutiérrez et al., 2020).

Sequencing of *Bartonella*-16S rRNA amplicons obtained from 11 rodent blood samples isolates showed high identity (99%) with *B. vinsonii* subsp. *arupensis*, corroborating previous studies that aimed to molecularly detect *Bartonella* in Echimyidae and Cricetidae rodents from Brazil (Favacho et al., 2015; Gonçalves et al., 2016; de Sousa et al., 2018). Although 16S rRNA gene fragments have been used in

the past to discriminate *Bartonella* species (Dauga et al., 1996), it is known that they are unable to distinguish closely related species (Scola et al., 2003; Buffet et al., 2013). In order to solve this problem, Scola et al. (2003) proposed the molecular characterization of *Bartonella* sp. targeting simultaneously several “housekeeping” genes, aiming at determining the diversity of *Bartonella* species in domestic and wild animals and associated ectoparasites. This need was clearly evidenced when we checked the BLAST identity of the sequences obtained in the present work based on different molecular markers when compared with *Bartonella* sequences from the *B. vinsonii* complex deposited in Genbank. In this sense, while genes (such as *groEL* and 16S rRNA) presented identity values for *Bartonella* of the *B. vinsonii* complex a little above the discriminatory cutoff point, *gltA* and *ftsZ* genes presented values below the point proposed by Scola et al. (2003), generating inconsistency in the possible distinction of new species (**Table 4**).

Table 4. Cutoff point for the description of a new species proposed by La Scola et al. (2003) based on the percentage (%) of identity with *Bartonella* species of the *B. vinsonii* complex deposited in GenBank.

Target gene/ intergenic region	**Identity**	**Query cover**	**E-value**	**Cutoff point (La Scola et al., 2003)**
16S rRNA	99%	100%	0.0	98.3%
gltA	92.73%	99%	0.0	93.6%
groEL	93.52%	99%	0.0	92.6%
ftsZ	91.15%	100%	0.0	94.4%
ITS	92%	25%	$2x10^{-99}$	93.9%

However, the separation of the isolates obtained in the present study from *Bartonella* species belonging to the *B. vinsonii* complex was better evidenced in the concatenated phylogenetic (ML method) and distance (Splistree) analyzes for the different molecular markers (*gltA*, *ftsZ groEL*, 16S rRNA, and ITS). In these analyses, the rodent-associated isolates obtained in the present study were positioned in a

separated clade, grouping only with *Bartonella* genotypes previously detected in Echimyidae and Cricetidae rodents (Gonçalves et al., 2016; de Sousa et al., 2018; Rozental et al., 2017; Gonçalves-Oliveira et al., 2020; Muller et al., 2020), albeit closely related to *Bartonella* genotypes detected in bats and associated ectoparasites (Ikeda et al., 2017; 2020; do Amaral et al., 2018; Gonçalves-Oliveira et al., 2020).

Since the sequences representing the same gene/intergenic region of five targeted molecular markers (16S rRNA, *gltA*, *ftsZ*, *groEL*, and ITS) from 11 isolates of rodent (six from *T. fosteri* and five from *O. mamorae*) were 100% identical to each other, one could suggest that all isolates belong to the same species of *Bartonella*. The ANI value = 85% of the *Bartonella* strain 56A isolated in the present work when compared to species of the *B. vinsonii* complex supported the hypothesis that this isolate is a new species, since a AIN value of 95% is considered as a cutoff for the differentiation of bacterial species (Goris et al., 2007; Jain et al., 2018; Gutiérrez et al., 2020). The phylogenomic analysis based on 291 protein-coding genes corroborated the aforementioned results, placing the *Bartonella* strain isolated in the present study in a single clade, phylogenetically associated with *Bartonella* species of the *B. vinsonii* group, under 100% support of separation. This solid genomic evidence supports the description of a new species of *Bartonella* sp. in wild rodents from Brazil. Thus, we named this species *Bartonella machadoae* sp. nov. in honor of the Brazilian researcher Rosangela Zacarias Machado, an Emeritus Professor of Veterinary Parasitology at the Faculty of Agrarian and Veterinary Sciences, Júlio de Mesquita Filho University, Jaboticabal, Brazil, for her extensive contribution to the study of arthropod-borne hemoparasites.

Despite the fact that vectors of the new species described herein are still unknown, fleas play an extremely important role in the transmission cycles of

Bartonella species among rodents. Indeed, in addition to harboring a high diversity of *Bartonella* genotypes, fleas show great efficiency in the transmission of bartonellae among this group of mammals (Brinkerhoff et al., 2010; Morick et al., 2013; Gutiérrez et al., 2015).

Indeed, de Sousa et al. (2018) detected *Bartonella* DNA in *Polygenis (Polygenis) bohlsi bolshi* fleas collected from wild rodents in the Brazilian Pantanal. Additionally, *Bartonella* sp. was molecularly detected in fleas (namely *Polygenis occidentalis occidentalis*, *Polygenis platensis* and *Craneopsylla minerva minerva*) collected from Echimyidae and Cricetidae rodents in the Pampa and Atlantic Forest biomes, in the State of Rio Grande do Sul, southern Brazil (Schott et al., 2020). Interestingly, the *Bartonella* genotypes detected in fleas from southern Brazil were closely related to the *Bartonella* genotype previously detected in rodent species from several Brazilian biomes. In the present study, the *Bartonella* genotypes previously detected in rodents and associated ectoparasites formed a monophyletic clade with *Bartonella machadoae* sp. nov., leading us to raise the hypothesis that siphonapterans may act as vectors of *Bartonella machadoae* sp. nov. among wild rodents in different Brazilian biomes.

Finally, taking into account that the *Bartonella* strain found in the present study (strain 56A) showed to be strictly related to species belonging to *B. vinsonii* complex, further studies about its zoonotic potential should be carried out. Several studies have associated infection by *Bartonella* species belonging to *B. vinsonii* complex with clinical manifestations in humans in the U.S.A., United Kingdom, France, Russia and Thailand. The symptoms described were diverse and characterized by fever, confusion, difficulty of walking, facial numbness, fatigue, endocarditis, acute chest pain and neurological signs. Most of the clinical cases were reported in people with direct contact with

animals, such as veterinarians, livestock producers and pet owners (Welch et al., 1999; Roux et al., 2000; Fenollar et al., 2005; Breitschwerdt et al., 2010; Bai et al., 2012).

4.1. Description of *Bartonella machadoae* sp. nov.

The bacterial cells of this new proposed species are rods (1–2.0 µm length and 0.3–0.6 µm width), without flagella and pili, and showing excellent growth in capnophilic conditions (5% CO_2) at 35ºC. Colonies grown onto chocolate agar are smooth and circular. The time required for growth to reach 1.0–2.0 mm colonies is 4 days at 35ºC. They were negative for oxidase, catalase, urease, indole production, acetoin production and nitrate reduction. These features were similar to those found in the biochemical profiles of *Bartonella* species from the *B. vinsonii* complex (Welch et al., 1994). However, differences were found with other *Bartonella* species: while *B. krasnovii* showed to be able to ferment sucrose, citrate and arabinose, *Bartonella kosoyi* was able to ferment sorbitol, melibiose, rhamnose and glucose, as well as showed positive reaction for arginine (Gutiérrez et al., 2020). On the other hand, *B. machadoae* sp. nov. was not able to ferment the aforementioned carbohydrates. The type strain (56A) carried a single circular chromosome of 2.7 MB, with an average G+C DNA content of 39 mol%. The most closely related species of the genus *Bartonella* with a valid published name is *B. vinsonii* by genetic composition (85% ANI pair). *Bartonella machadoae* sp. nov. (strain 56A) is stored at the Laboratory of Immunoparasitology, Department of Pathology, Reproduction and Unique Health, Faculty of Agrarian and Veterinary Sciences/Universidade Estadual Paulista, UNESP, Jaboticabal, SP, Brazil.

5. Conclusion

Bartonella machadoae sp. nov. was described as a new species isolated from *T. fosteri*'s blood sample in the Brazilian Pantanal. The diagnostic platform combining *nuoG*-based qPCR results from blood samples, blood samples in pre-enrichment liquid culture and suggestive colonies in chocolate agar plates proved to be efficient in detecting a new species of *Bartonella* in rodents. The phylogenomic analysis based on 291 genes and an ANI value of 85% obtained by whole genome sequencing of the *Bartonella* isolate from the rodent *T. fosteri* supported the separation of *Bartonella machadoae* sp. nov. from *Bartonella* species of the *B. vinsonii* complex. The results of the phylogenetic and distance analyzes based on the concatenation of five molecular markers suggest that *Bartonella machadoae* sp. nov. may be spread in rodents from different Brazilian biomes

6. Data availability

The data that support the findings of this study are openly available in National Center for Biotechnology Information at https://www.ncbi.nlm.nih.gov/, reference number genome BioProject: PRJNA778951, genome accession numbers: CP08714. All sequences obtained from *Bartonella* isolates were deposited in the GenBank international database under the following accession numbers: *gltA*: OL441244-OL441254; *groEL*: OL441255-OL441265; *ftsZ*: OL441266-OL441276; 16S rRNA: OL423101-OL423111; ITS: OL423157-OL423167.

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8. Supplementary Material.

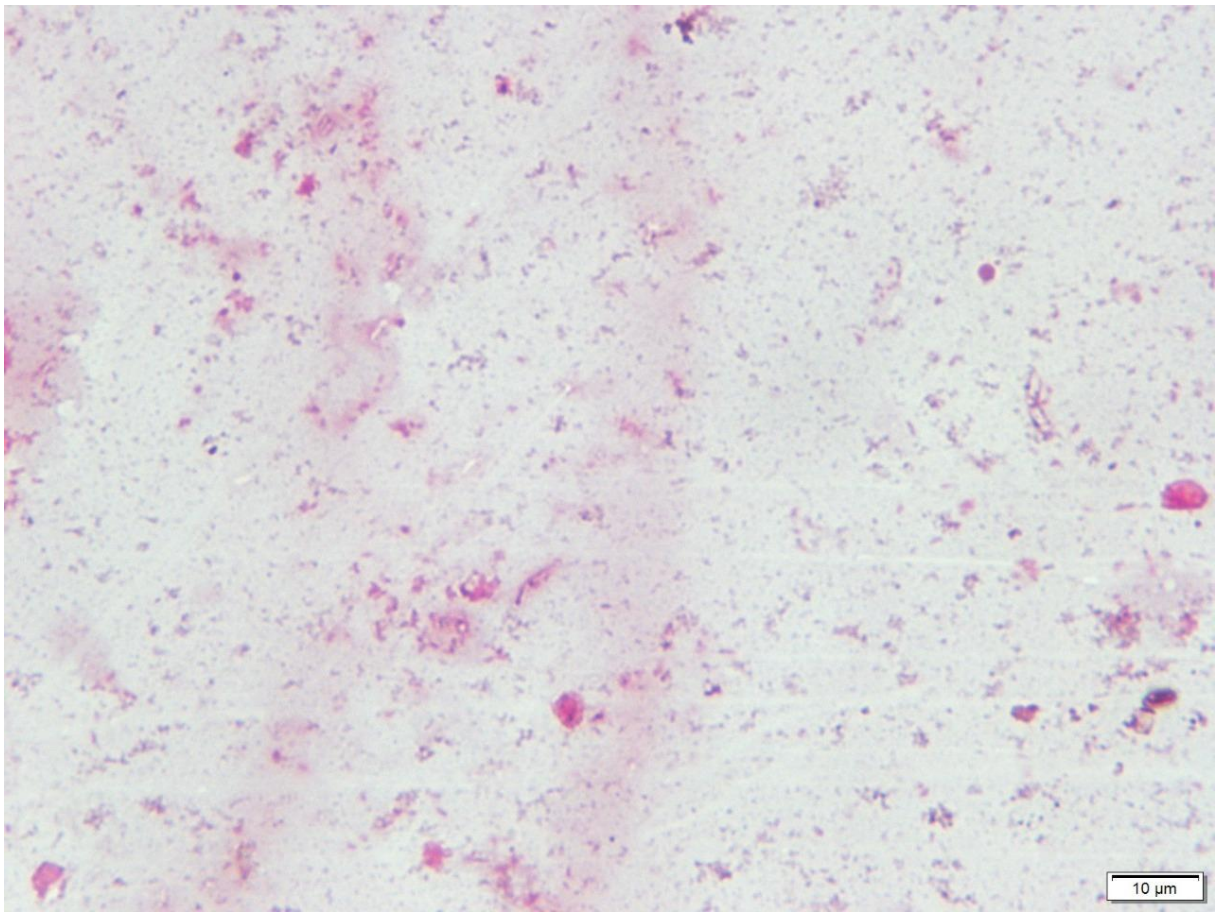

Figure S3. Light microscopy of Gram-staining of *Bartonella machadoae* sp. nov. (strain 56A).

Capítulo 3 - First report of *Bartonella* spp. in marsupials from Brazil, with description of *Bartonella harrusi* sp. nov. and a new proposal for taxonomic reclassification of species of the genus *Bartonella*

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Abstract

The genus *Bartonella* (Rhizobiales: Bartonellaceae) encompasses facultative intracellular Gram-negative alphaproteobacteria that parasitize mainly erythrocytes and endothelial cells, as well as macrophages, monocytes and dendritic cells. Although they can infect numerous mammal species and arthropod vectors worldwide, reports of *Bartonella* infection in marsupials are scarce. In fact, such agents have only been detected in marsupials and/or associated ectoparasites in Australia and the United States of America until the present moment. The present study aimed to isolate and characterize molecularly, morphologically and phenotypically *Bartonella* infecting free-living marsupials sampled in the Brazilian Pantanal, the largest wetland in South America. Two marsupials were captured in December 2018 and six marsupials in February 2019, totaling eight small mammals sampled: 5 (62.5%) *Thylamys macrurus* and 3 (37.5%) *Monodelphis domestica*. All blood samples were submitted to qPCR for *Bartonella* spp. based on the *nuoG* gene, pre-enrichment liquid culture and chocolate agar solid culture. *Bartonella* sp. was isolated from 3 *T. macrurus* and one *M. domestica*. One *Bartonella* isolate obtained from *T. macrurus* blood sample (strain 117A) that showed to be closely related to *Bartonella vinsonii* complex and *Bartonella machadoae* was selected for Whole genome sequencing using a hybrid approach based on Illumina NovaSeq and Nanopore sequencing platforms. This strain showed a genome of 2.35 Mbp, with an average C + G content of 38.8%, coding for 2,013 genes, and a 29 kb plasmid with an average C + G content of 34.5%. In addition, this strain exhibited an average nucleotide identity (ANI) of 85% with *Bartonella* species belonging to the *B. vinsonii* group and 91% with *B. machadoae*. Phylogenomic analysis based on 291 protein coding genes shared by genomes of 53 *Bartonella* species positioned this strain closely to *B. machadoae*. This new isolated species was named *Bartonella harrusi* sp. nov., which was characterized as small capnophilic, microaerophilic and aerobic rods with absence of pili and flagella. In conclusion, the present work describes the biochemical, phenotypic and genomic characteristics of *Bartonella harrusi*, a new species isolated from *T. macrurus* blood samples of the Brazilian Pantanal. Finally, a review of the taxonomic classification of members of the genus *Bartonella* is proposed, based on the ANI values accessed by whole genome sequencing analyses.

Key-Words: bartonelosis; Marsupialia; biochemical characterization; phylogenomics; WGS; taxonomic classification

1. Introduction

The *Bartonella* genus comprise Gram-negative, intracellular facultative and fastidious α-2-Proteobacteria (Chomel et al., 2006; Gutiérrez et al., 2020). These bacteria are known to infect a wide range of vertebrates worldwide, mostly mammals, and may cause different clinical manifestations depending on the host and species of *Bartonella* involved (Boulouis et at., 2005). These agents are transmitted by hematophagous arthropods (fleas, ticks, mosquitoes, flies and lice) (Breitschwerdt et al., 2000; Chomel et al., 2010.

Currently this genus comprises 37 species, among which some are represented by more than one subspecies, validated according to the "List of Prokaryotic names with Standing in Nomenclature database" (https://lpsn.dsmz.de/genus/bartonella) - accessed in June 2022 (Breitschwerdt et al., 2010; Kaewmongkol et al., 2014; Angelakis etal ., 2014; Okaro et al., 2017; do Amaral et al., 2022) . The vast majority of validated *Bartonella* species were submitted to biochemical, phenotypic and molecular characterization by DNA-DNA hybridization techniques or Whole Genome Sequencing (List of Prokaryotic names with Standing in Nomenclature database). In view of the availability of collections of prokaryotic genomes, bioinformatics approaches become robust tools to trace taxonomic and phylogenetic relationships between organisms.

Marsupials are viviparous mammals belonging to the Infraclass Marsupialia with completion of development in the marsupial (Yadav, 1971). In Brazil, 58 species of marsupials have been described, most of them are small in size and with mainly arboreal habits, and can be found in all Brazilian biomes (https://www.icmbio.gov.br/portal/faunabrasileira/estado-de-conservacao/2802-mamiferos-marsupiais) - accessed in June 2022. Although *Bartonella* spp. is widely

studied in felids, rodents and bats, there are few studies on the detection, molecular characterization and/or isolation of *Bartonella* in marsupials. In fact, to date, only one species of *Bartonella* (*Bartonella australis*) has been isolated from kangaroos (*Macropus giganteus*) in Australia (Fournier et al., 2007). Also, *Bartonella* sp. phylogenetically related to *B. tamiae* was detected in *Ixodes tasmani* ticks collected from koalas (*Phascolarctos cinereus*) in Australia (Vilcins et al., 2008). Additionally, *'Candidatus* Bartonella bandicootii' and *'Candidatus* Bartonella woyliei´ were detected in fleas (*Pygiopsylla tunneyi*) of "bandicoots" (*Perameles bougainville*) and fleas (*Pygiopsylla hilli*) and ticks (*Ixodes australiensis*) collected from woylies (*BIettongia penicillata*), respectively (Kaewmongkol et al., 2011). In the Americas, *Bartonella* spp., closely related to *B. henselae* and *B. clarridgeiae*, were molecularly detected in *Ctenocephalides felis* fleas collected from opossums (*Didelphis virginiana*) in the USA (Reeves et al., 2005; Nelder et al., 2009). In Brazil, scarce are the studies on the occurrence of *Bartonella* spp. in marsupials. In fact, only three studies were found in the literature regarding the molecular detection of *Bartonella* spp. in this group of mammals. Based on conventional and real-time PCR assays, *Bartonella* DNA was not found in marsupials' blood samples in the states of Mato Grosso do Sul and Rio de Janeiro (de Sousa et al., 2018; Gonçalves et al., 2020; Gonçalves-Oliveira et al., 2020).

The present study aimed to detect and characterize, using microbiological, molecular and bioinformatics analyses, *Bartonella* sp. in blood samples from wild marsupials sampled in the Pantanal biome, a flooded area in central-western Brazil. Additionally, a new isolate of *Bartonella* from *Thylamys macrurus* was characterized, using morphological, biochemical, phenotypic and molecular characters. Finally, by using bioinformatics analyses of whole genomes *Bartonella* species deposited in international databases, the present study aimed to contribute to the re-assessment of

separation of *Bartonella* species using ANI values, as well as to infer differences in the metabolic profile of this group of agents.

2. Material and Methods

2.1. Ethics Statement

The procedures and management protocols involving capture and blood sampling of wild marsupials were approved by the "Chico Mendes Institute for Biodiversity Conservation" (SISBIO number 64204) and "Ethics Committee on Animal Use of the Faculty of Agrarian and Veterinary Sciences" (FCAV/UNESP) (protocol number #11794).

2.1. Area of study and collection of samples

Wild marsupials were captured during two expeditions in the Pantanal of the state of Mato Grosso do Sul, central-western Brazil, more specifically in the "Alegria" farm, Nhecolândia region (-19.050399; -56.675623): one in December 2018 and the second in February 2019. The procedures performed to obtain biological samples of small mammals were described in a previous work (do Amaral et al., 2022).

Blood samples collected from marsupials were stored in DNAse/RNAse-free microtubes containing ethylenediaminetetraacetic acid (EDTA) and kept in liquid nitrogen cylinders. Once in the laboratory they were stored in a freezer -80 °C (**Figure 1**).

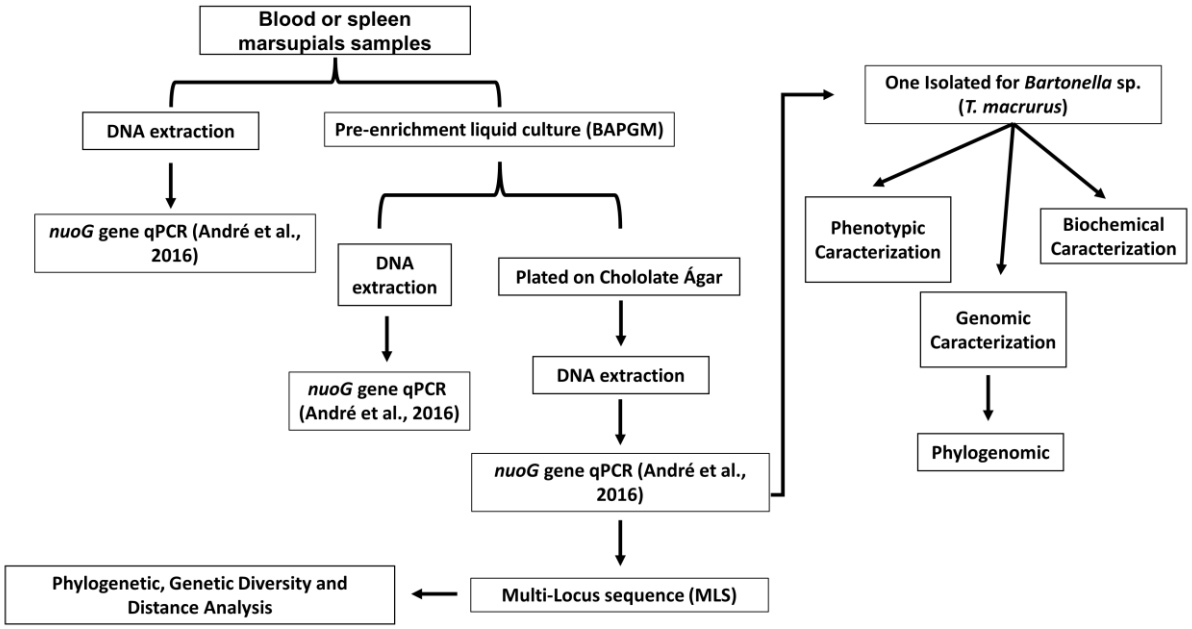

Figure 1. Flowchart of the microbiological and molecular analyzes used in this study for the isolation and characterization of *Bartonella harrusi* sp. nov.

2.2. *Bartonella* isolation

A pre-enrichment liquid medium previously described as *Bartonella*-Alphaproteobacteria Growth Medium (BAPGM) was used in the present study. Briefly, 500 mL of IPL-41 insect medium (Sigma-Aldrich, St. Louis, Missouri, USA) were supplemented as previously described (Maggi et al., 2005; Duncan et al., 2007; do Amaral et al., 2022). Two vials of negative controls were also added: one containing only the pre-enrichment medium and the other one containing both the pre-enrichment medium and sheep defibrinated blood (Furquim et al., 2021; do Amaral et al., 2022).

After seven days, 200 µL of liquid culture was submitted to DNA extraction with the Illustra Tissue & Cells genomic Prep Mini Spin kit, following the manufacturer's recommendations. Additionally, 200 µL of the contents of each bottle were sown onto

enriched chocolate agar (Laborclin, Pinhais, Paraná, Brazil) and kept at 35ºC with 5% CO_2 for up to 60 days in a CO_2/O_2 Water Jacketed incubator (NuAire, Plymouth, Massachusetts, USA), and observed daily. Three negative controls were used: two corresponding to the negative controls previously used in the liquid culture of pre-enrichment and the other represented by a chocolate agar plate without any biological sample. Smooth and circular colonies suggestive of *Bartonella* were collected and submitted to DNA extraction by the boiling method (Keil et al., 2000) and qPCR assay for *Bartonella* sp. based on the *nuoG* gene (André et al., 2015). Colonies molecularly confirmed as belonging to the genus *Bartonella* were then submitted to five passages until pure isolates were obtained.

2.3. Quantitative real-time PCR assay (qPCR) for *Bartonella* sp. based on the *nuoG* gene (nicotinamide adenine dinucleotide dehydrogenase gamma subunit) and conventional PCR (PCR) for the mammalian endogenous gene

DNA from marsupial blood samples was extracted using the InstaGene™ Matrix kit (Bio-Rad Laboratories, São Paulo, Brazil), according to the manufacturer's recommendations. A PCR assay based on the mammalian endogenous glyceraldehyde gene 3-phosphate dehydrogenase (*gapdh*) (Birkenheuer et al., 2003) was used to verify the absence of inhibitors in DNA samples extracted. A quantitative PCR protocol (qPCR) based on the *nuoG* gene André et al., 2015) was used to detect and quantify *Bartonella* sp DNA. (number of copies of a 83 bp fragment of *nuoG* gene/µL) in marsupial blood samples, marsupial blood samples added to BAPGM-based liquid culture, and colonies isolated from chocolate agar plates. PCR assays were conducted on PCR plates low profile multiplate (BioRad™, CA, USA), using

CFX96 thermocycler (BioRad™, CA, USA). The standard curves were constructed with serial dilutions of plasmid DNA (pIDTSMART—Integrated DNA Technologies) ($1.0x10^7$ to $1.0x10^0$ copies/µL), which encoded an 83 pb fragment of *Bartonella henselae nuoG* gene. The number of copies of the plasmid was determined by (Xg/µL DNA/[plasmid length in pb x 660]) x 6.022 x 10^{23} x plasmid/µL copies (André et al., 2016). All DNA samples were initially tested in duplicates. All duplicates whose difference in Cq (Cycle of quantification) values was higher than 0.5 were retested in triplicates.

2.4. Multilocus analysis for *Bartonella* sp.

For molecular characterization, isolates that were positive in the qPCR for *Bartonella* sp. were submitted to previously described conventional PCR assays based on six molecular markers: 16S rRNA (400 bp) (Dauga et al., 1996), *gltA* (750 bp) (Norman et al., 1995), the 16S-23SrRNA ITS intergenic spacer region (453-717pb) Maggi et al., 2005, *groEL* (752pb) (Paziewska et al., 2011), and *ftsZ* (600pb) (Paziewska et al., 2011), and *rpoB* (800bp) (Paziewska et al., 2011). *Bartonella henselae* previously detected in a cat sampled in southeastern Brazil (Furquim et al., 2021) and sterile ultrapure water (Nuclease-Free Water, Promega™, Madison, Wisconsin, USA) were used as positive and negative controls, respectively. The amplicons obtained in the conventional PCR assays were electrophoresed in agarose gels (1%) stained with ethidium bromide (Life Technologies, Carlsbad, California, United States) at 100 V and 150 mA for 50 min. The gels were photographed under ultraviolet light (ChemiDoc MP Imaging System; Bio Rad, Hercules).

2.5. Concatenated phylogenetic analysis based on the 16S rRNA, *gltA*, *groEL*, *ftsZ*, *rpoB* genes and ITS intergenic region

Products amplified in conventional PCR assays were purified using ExoSAP-IT (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific™, Waltham, MA, USA) and the ABI PRISM 310 DNA Analyzer (Applied Biosystems™, Foster City, CA, USA) (Sanger et al., 1977). The primers used in the sequencing reactions were the same used in the conventional PCR assays for *Bartonella* sp. The sequences obtained were first submitted to a screening test using the Phred-Phrap software version 23 (Ewing et al., 1998a; Ewing et al., 1998b) to assess the quality of the electropherogram and to obtain consensus sequences from the alignment of the forward and reverse sequences.

The BLAST program (BLASTn) (Altschul et al., 1990) was used to compare the obtained nucleotide sequences with those from the international database (GenBank) (Benson et al., 1990). The consensus sequences obtained in this study and those retrieved from GenBank were aligned using the Clustal/W software (Thompson et al., 1994) via Bioedit v. 7.0.5.3 (Hall, 1999). Phylogenetic analysis was based on the Maximum Likelihood (ML) method and inferred with the W-IQ-Tree tool available online (http://iqtree.cibiv.univie.ac.at/) (Nguyen et al., 2015; Trifinopoulos et al., 2016) using 1,000 bootstrap replicates. The best evolutionary model was selected by the jModelTest2 program (version 2.1.6) in XSEDE (Darriba et al., 2012). The tree was edited in Treegraph 2.0.56–381 beta (Stöver et al., 2010).

In addition, nucleotide sequences were aligned with sequences previously deposited in GenBank and subjected to Split-Network-based distance analysis using SplitsTree v4.11.3 software (Huson et al., 2006).

2.6. Phenotypic characterization of *Bartonella* sp.

For phenotypic characterization, a *Bartonella* isolated from a blood sample of *Thylamys macrurus* (strain 117A) was seeded on chocolate agar plates (Novamed) and incubated at 35°C in duplicates, under the following conditions: (i) aerobic (i.e. , standard incubator); (ii) capnophilic (i.e., 5% CO_2) in a CO_2 incubator (Thermo Forma II Water Jacket CO2 Incubator, Thermo Scientific); (iii) microaerophilic (i.e. 5 to 15% O_2, 10% % CO_2) using Microaerobac® bags (Probac); and (iv) anaerobic (i.e., <3% O_2, 4 to 10% CO_2) using Anaerobac® bags (Probac).

2.7. Light microscopy and scanning electron microscopy (SEM)

Bartonella strain 117A was submitted to light microscopy and scanning electron microscopy (SEM). For light microscopy, the isolate was stained with Gram (Hy-labs) and visualized using 100x objectives. For SEM, the *Bartonella* isolate (strain 117A) was collected from the chocolate agar plate and fixed in a solution containing 2% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M cacodylate buffer for 1 hour and then washed three times in phosphate buffer. Then, 1% osmium tetroxide was added and the solution was incubated for 30 minutes. The bacteria were then dehydrated through a graded ethanol series (50-100%) and dried with a critical point dryer (EMS 850). After the critical point, the sample was fixed in metal stubs, and coated with gold (5 nm) in a Danton Vacuum Desk II. Finally, bacterial cells were visualized by SEM. Samples were analyzed and photographed using a Zeiss Evo 10 scanning electron microscope (10–20 kV).

2.8. Biochemical and metabolic characterization

For biochemical and metabolic characterization, *Bartonella* strain 117A isolated from *T. macrurus* was tested with the identification systems BBL Crystal Enteric/Nonfermenter (BD Becton Dickinson), API 20E and NF III (Probac), following the manufacturers' instructions. The enzymatic catalase reaction was carried out with hydrogen peroxide (3%).

2.9. Growth curve

To perform the growth curve of the *Bartonella* isolated from marsupial (strain 117A), 10 colony forming units (CFU) were removed from chocolate agar plates and deposited into 500 mL of supplemented BAPGM medium. The inoculum was then maintained at 35°C with 5% CO_2 in a CO_2/O_2 culture incubator (Water Jacketed, NuAire). Every 6 hours, 100 µL were collected for 8 days, totaling 32 collections. This volume was subjected to a 9:1 serial dilution in supplemented BAPGM medium (10^{-1} to 10^{-6}). One hundred microliters of each of the six dilutions were plated onto chocolate agar plates with a Drigalski loop. Bacterial quantification was performed using the standard plate counting method, determining the number of colony forming units (CFU/mL). Each plate was stored at 35°C with 5% CO_2 in a CO_2/O_2 culture incubator (Water Jacketed, NuAire). The incubation period of the plates was 72 hours and, after this period, colony forming units per mL (CFUs/mL) were counted.

2.10. Whole genome sequencing of *Bartonella* isolated from blood samples of *Thylamys macrurus* (strain 117A) and genome annotation

The genomic DNA extraction from the *Bartonella* isolate of *T. macrurus* (strain 117A) previously grown on chocolate agar plates was performed using the commercial

kit DNeasy® Blood & Tissue (QIAGEN), following the manufacturers' instructions. For genome assembly, the genomic DNA sample was sequenced by Illumina Novaseq 6000 (PE 2x150bp). A total of 5,871,200 paired-end reads were obtained. These reads were trimmed with Trimmomatic (Bolger et al., 2014), In addition, FASTQ files were also obtained from MinION run (kit SQK-LSK109, Oxford Nanopore), totaling 118,191 reads, with an average read length of 5,471 bp. Readings were clipped to low quality and adapters using NanoFilt and Porechop, respectively (Wicket al., 2017). All trimmed Illumina and Nanopore reads were then used for hybrid assembly using the MaSuRCa v4.0.4 assembler with default parameters (Zimin et al., 2017). The assembled genome was automatically annotated with the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016).

2.11. Phylogenomic analyses

The assembled genome of the new *Bartonella* isolate (strain 117A) and 53 complete *Bartonella* genomes retrieved from the NCBI RefSeq database (O'Leary et al., 2016) were used for the comparative analysis. For this purpose, 291 shared genes were retrieved using the roary tool (Page et al., 2015). These genes were aligned with MAFFT [44] and later used for phylogenomic analyses.

ML analysis was inferred with the W-IQ-Tree tool (Nguyen et al., 2015; Trifinopoulos et al., 2016) using 1,000 bootstrapping replicates. The trees were edited in Treegraph 2.0.56–381 beta (Stöver et al., 2010). *Brucella abortus* and *Brucella ceti* (GenBank accession number: CP007681; NC_022905) were used as an outgroup.

The average nucleotide identities (ANI) between *Bartonella harrusi* sp. nov., *Bartonella machadoae* and the species of the *Bartonella vinsonii* complex (*B. vinsonii* subsp. *vinsonii*, *B. vinsonii* subsp. *arupensis* and *B. vinsonii* subsp. *berkhoffii*) were

calculated with OrthoANIu 1.2 (Lee et al., 2016). In addition, an ANI analysis was performed between the nucleotide sequence of the plasmid found in *Bartonella harrusi* compared with eight other plasmid sequences from *Bartonella* species deposited in GenBank.

2.12. Taxonomic reclassification of *Bartonella* species based on ANI

The whole genome consensus sequence of the new *Bartonella* isolate (strain 117A) and the 216 whole genomes of *Bartonella* species retrieved from the NCBI RefSeq database (O'Leary et al., 2016) were submitted to the dRep pipeline (Olm et al., 2017). After filtering by length, the completeness and contamination of the sequences were analyzed using CheckM (Parks et al., 2015). Primary and secondary clustering was performed using pairwise MASH, in order to calculate the ANI. As ANI cutoff parameters, 95% and 97% were used for primary and secondary clustering, respectively, aining at separating species and assigning objective taxonomic classifications to be applied to all available genomes of *Bartonella* species.

2.13. Search for active carbohydrate enzymes (CAZy) families

A search for the occurrence of carbohydrate active enzymes (CAZy) families was performed. For this purpose, the GenomeSet described in the section above was submitted to the dbCAN database for automated annotation of active carbohydrate enzymes (Huang et al., 2018) using HMMER 3 via the HMMER tool available online (https://www. kbase.us/) (Wheeler et al., 2013).

2.14. "*In silico*" analysis of the metabolic profile and ribosomal and polymerase phylogenetic markers for *Bartonella* species

The assembled whole genome of the new *Bartonella* isolate (strain 117A) and 53 whole genomes of *Bartonella* species retrieved from the NCBI RefSeq database (O'Leary et al., 2016) were aligned by the RASTtk (Rapid Annotations using Subsystems Technology) pipeline (Aziz et al., 2008). After annotation, a GenomeSet was generated, which was used to analyze the metabolic profile and search for phylogenetic markers via the HMMER tool available online (https://www.kbase.us/) (Wheeler et al., 2013).

3. Results

3.1. Occurrence of *Bartonella* sp. in marsupial blood samples

Two marsupials were captured in December 2018 and 6 marsupias in February 2019. Among the eight marsupials sampled, five (62.5%) animals belonged to the species *Thylamys macrurus* and three (37.5%) to the species *Monodephis domestica*. All eight marsupial blood DNA samples were positive in the PCR for the endogenous mammalian *gapdh* gene. None of the eight DNA samples extracted from marsupial blood was positive in the qPCR for *Bartonella* sp. based on the *nuoG* gene. The efficiency, R^2, slope and intercept on the Y axis of the reactions ranged from 91.4% to 103.9%, 0.995 to 0.999, -3.547 to -3252 and 38.571 to 40, respectively (**Table 1**).

Table 1. Blood samples from marsupials captured in the Brazilian Pantanal and subjected to a qPCR for *Bartonella* sp. based on the *nuoG* gene (with quantification of the target gene fragment/µL), liquid culture in BAPGM pre-enrichment medium and isolation on chocolate agar plates.

Marsupial species (ID)	qPCR for *Bartonella* spp. (number of *nuoG* copies/µL)		
	Blood	**Liquid culture (BAPGM)**	**solid culture (chocolate agar)**
Thylamys macrurus **(#22)**	Neg	Neg	Neg
Thylamys macrurus **(#36)**	Neg	Neg	Neg
Monodelphis domestica **(#70)**	Neg	Neg	Neg
Monodelphis domestica **(#78)**	Neg	$1.39x10^5$	**Pos**
Thylamys macrurus **(#79)**	Neg	Neg	Neg
Monodelphis domestica **(#96)**	Neg	2.16×10^7	**Pos**
Thylamys macrurus **(#116)**	Neg	$1.37x10^5$	**Pos**
Thylamys macrurus **(#117)**	Neg	$5.9x10^2$	**Pos**

3.2. Isolation of *Bartonella* sp. in chocolate agar

Out of the eight marsupial blood samples submitted to pre-enrichment liquid BAPGM culture, four (50%; two *T. macrurus* blood samples and two *M. domestica* blood samples) were positive in the qPCR for *Bartonella* sp. The number of copies of the *nuoG* gene fragment per microliter ranged from $5.9x10^2$ to $2.93x10^6$. The efficiency, R^2, slope and intercept on the Y axis of the reactions ranged from 91.4% to 103.9%, 0.995 to 0.999, -3.547 to -3252 and 38.571 to 40, respectively.

Out of the four qPCR-positive liquid culture samples, three (45.16%) (two *T. macrurus* blood samples and one *M. domestica* blood sample) showed the presence of *Bartonella* colonies on chocolate agar plates, whose identity was confirmed by qPCR for *Bartonella* spp. based on the *nuoG* gene (**Table 1**).

3.3. Multilocus and phylogenetic analyses for *Bartonella* sp.

BLASTn analysis demonstrated that the different target genes of the *Bartonella* colonies isolated from marsupials showed high identities to *B. machadoae* and *Bartonella* genotypes previously reported in ectoparasites collected from rodents from Brazil (**Table 2**).

Table 2. BLASTn results for six target gene sequences from four *Bartonella* isolates obtained from marsupial blood samples (three from *T. macrurus* and one from *M. domestica*).

Target gene	Species	Host	Locality	Identity	Query cover	"E-value"	GenBank Accession Number
16S rRNA	*Bartonella* sp. strain R-phy1	Não descrito	France	99.16%	100%	$1x10^{-179}$	Z70005
gltA	*Bartonella* sp.	*Polygenis (P.) bohlsi bohlsi*	Mato Grosso do Sul, Brazil	100%	100%	0	KY304483
groEL	*Bartonella* sp.	*Hylaeamys* sp.; *Akodon* sp.	Ceará and Maranhão, Brazil	100%	97.46%	0	KX086735
ftsZ	*Bartonella* sp.	*Polygenis (P.) bohlsi bohlsi*	Mato Grosso do Sul, Brazil	100%	100%	0	KY304486
rpoB	*B. machadoae*	*Trichomys fosteri*	Mato Grosso do Sul, Brazil	95.54%	99%	0	CP087114
ITS	*B. machadoae*	*Trichomys fosteri*	Mato Grosso do Sul, Brazil	91.77%	32%	$1x10^{-52}$	CP087114

The phylogenetic tree based on the sequences of the 16S rRNA*, gltA, groEL, ftsZ, rpoB* genes and ITS intergenic region of three *Bartonella* isolates from one *T. macrurus* individual (strains 117A, 117B and 117C) and one from *M. domestica* (strain 78A) inferred by the Maximum Likelihood method (ML) and evolutionary model TVM+F+I formed a single and monophyletic cluster. The sequences of *Bartonella* isolated from marsupials were positioned together with sequences previously detected in *Polygenis (P.) bohlsi bohlsi* fleas collected from rodents in the Pantanal, state of Mato Grosso do Sul (KY304482, KY304483) and in a larger clade with several

sequences of *Bartonella* sp. previously detected in *Oecomys mamorae* rodents in the Brazilian Pantanal, state of Mato Grosso do Sul (KX578719, KX827420), rodents from the Brazilian states of Maranhão, Ceará and Rio de Janeiro (KX086721, KX270253, MN613442, KX270232, MN613441, KX270248), rodent-associated genotypes from Peru (KF021604) and *B. machadoae* (CP08714). Furthermore, this clade was closely related to the clade of *Bartonella* genotypes detected in bats and Streblidae bat flies (*Strebla guajiro*) (MG654770, KY356753), and *Bartonella* sp. previously detected in Cricetidae rodents from Peru and USA (C4-phy, R-phy2, C1-phy) (**Figure 2**).

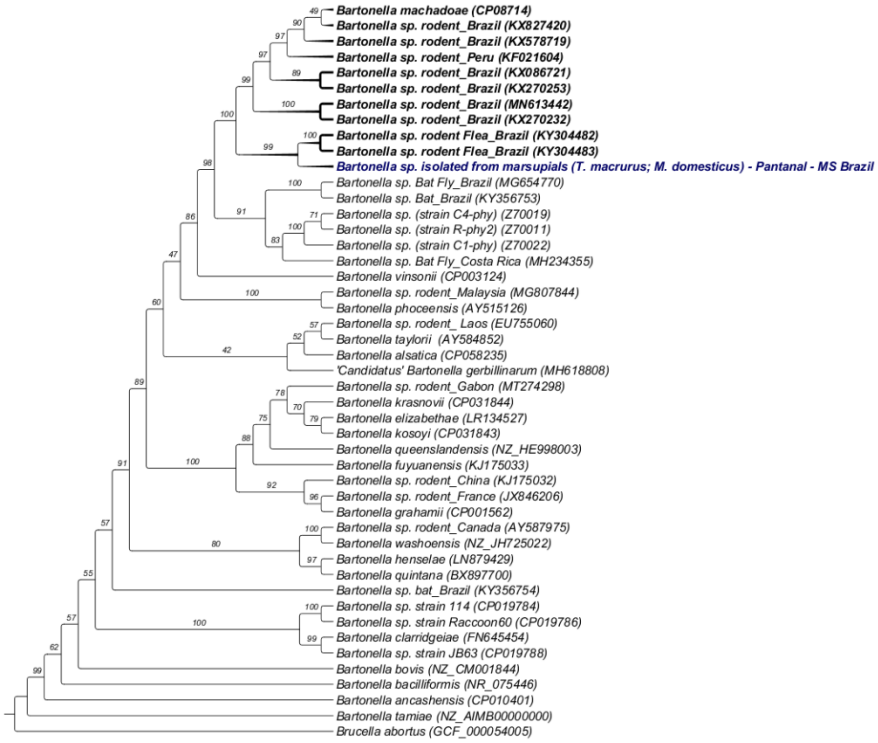

Figure 2. Concatenated phylogenetic analysis within the genus *Bartonella* based on the 16S rRNA*, gltA, ftsZ, groEL, rpoB* genes and 16S-23S rRNA intergenic region (ITS). The phylogenetic tree was inferred using the Maximum Likelihood method and the evolutionary model TVM+F+I. The sequences obtained in this study are highlighted in red. The numbers in each branch correspond to the bootstrap values accessed with 1,000 replicates. *Brucella abortus* was used as an outgroup.

3.4. Diversity analyses

The nucleotide sequences (16S rRNA*, gltA, groEL, ftsZ, rpoB,* and ITS) obtained from four *Bartonella* isolates from two marsupials, one from the *T. macrurus* species (strains 117A, 117B and 117C) and one from the *M. domestica* species (strain 78A), were identical to each other. The genealogical analysis of the nucleotide sequences by Network, using the program Splitstree v4.11.3 [37], showed a similar result to that obtained by the phylogenetic analysis, forming three relevant groups. The Cluster #1, consisting of sequences from *Bartonella* isolates of *T. macrurus* and *M. domestica*, was related to *Bartonella* genotypes previously detected in rodent-associated fleas in the Pantanal (MKY304482; KF021604). The Cluster #2 consisted of *Bartonella* genotypes previously detected in rodents from the Brazilian states of Mato Grosso do Sul, Maranhão, Ceará and Rio de Janeiro (KX578719, KX578720, KX086721, KX270253, MN613442, KX270232, MN613441, KX270248), rodents from Peru (KF021604) and *B. machadoae*. The Cluster #3 contained *Bartonella* genotypes previously detected in rodents from Peru and the USA (**Figure 3**).

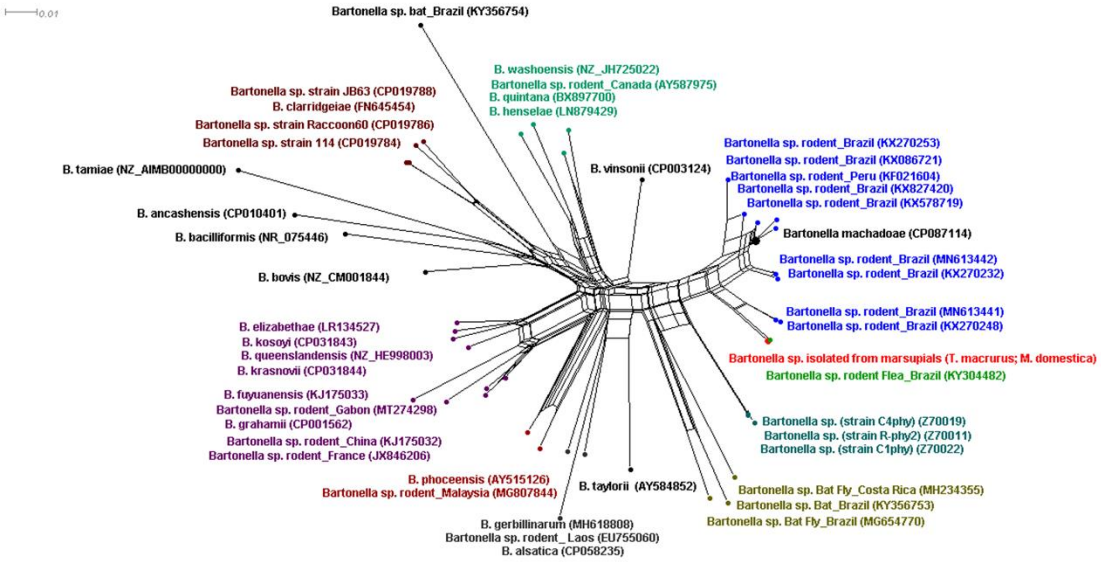

Figure 3. Neighbor-Net analysis of DNA sequences from the 16S rRNA, *gltA, ftsZ, groEL, rpoB* genes and 16S-23S intergenic region (ITS) of *Bartonella* sp. isolated from wild marsupials (*T. macrurus* strain 117A 117B and 117C and *M. domestica* strain 78A) sampled in the Brazilian Pantanal in the present study when compared to closely related *Bartonella* sequences previously deposited in GenBank.

3.5. Phenotypic and morphological characterization of *Bartonella* sp.

The *Bartonella* 117A strain isolated from a blood sample of *T. macrurus* was characterized by Gram-negative rods 1–2.0 µm long and 0.3–0.6 µm wide, without flagella and pili. The bacteria proved to be capnophilic, microaerophilic and aerobic, since there was no growth under anaerobic conditions. In SEM, the bacteria appeared as small rods without polar pili and flagella, forming compact aggregates of bacteria (**Figure S1 and Figure 4**).

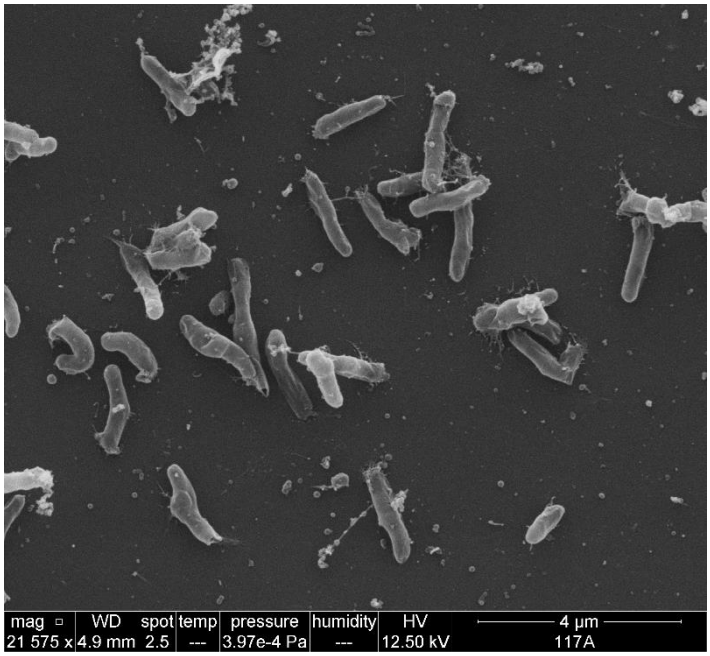

Figure 4. Scanning electron microscopy (SEM) of *Bartonella* strain 117A isolated from a blood sample of *T. macrurus* (*Bartonella harrusi* sp. nov.) showing absence of flagellum and pili. A 1 µM scale bar is shown in the image.

3.6. Biochemical and metabolic characterization

Bartonella strain 117A obtained from the blood sample of *T. macrurus* was negative for oxidase, catalase, urease, indole production, nitrate reduction and acetoin production (Voges-Proskauer reaction). It was inert for all tested carbohydrates (glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose) and negative for arginine, lysine and tryptophan arylamidase activities. Furthermore, it was negative for esculin, DNAse activity, β-D-galactosidase nitrate and McConkey growth. It showed sensitivity to Polymyxin B. 3.6.

3.7. Growth curve

The *B. harrusi* growth curve showed moderately slow growth in the supplemented BAPGM medium. The bacteria entered into the logarithmic phase 30 hours after the inoculum preparation and maintained an average quantification of 10^7 CFU/mL until the 90 hours of the experiment. After this period, the bacteria entered the decline phase (**Figure 5**).

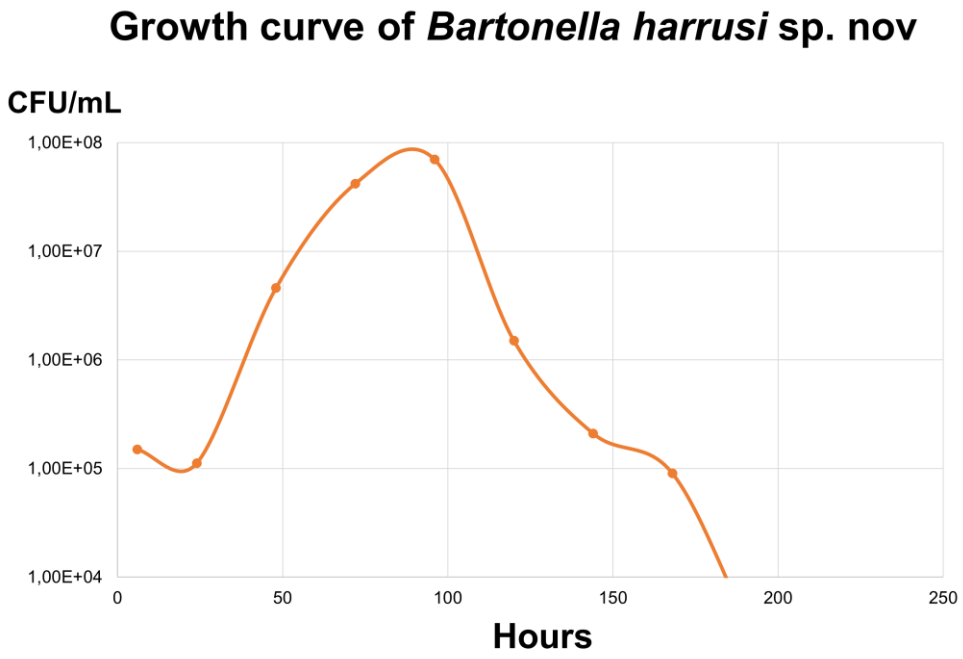

Figure 5. Graph of the growth curve of *Bartonella harrusi* sp. nov. representing the collection hours (X axis) by the quantification of CFU/mL (Y axis).

3.8. Phylogenomic analysis

The genome was assembled into a 2.23 Mbp circular chromosome and a 29 Kbp circular plasmid. The genomic characteristics of *Bartonella* strain 117A are described in **Table 3**. The whole genome ANI of strain 117A was calculated in relation to phylogenetically related species: ANI values of 85% and 91.98% were found when compared to *Bartonella* species belonging to the *B. vinsonii* complex (*B. vinsonii* subsp. *vinsonii*, *B. vinsonii* subsp. *arupensis* and *B. vinsonii* subsp. *berkhoffii*) and *B.*

machadoae, respectively (**Figure 6**). In the ANI analysis of the plasmid sequences of *Bartonella* species available in GenBank, *Bartonella* strain 117A showed 81.45% when compared to *Bartonella krasnovii*, which was the highest value found. Plasmids of *Bartonella* sp. strain HY038 and the pMS plasmid from *B. schoenbuchensis* did not show any phylogenetic relationship with plasmids found in other *Bartonella* species (**Figure 7**).

Table 3. Phenotypic and genomic characteristics of *Bartonella harrusi* sp. nov.

Phenotypic characterization	
Gram	Negative
Morphology	rods
Size	1–2.0 µm - 0.3–0.6 µm
Growing conditions	Capnophilic, microaerophilic and aerobic
Plasmid characterization	
Total size	29.892 pb
%C+G	34,5%
Genomic characterization	
estimated completeness	99.35%
Total size	2.235.184 pb
%C+G	38,8%
Genes (total)	2,013
CDSs (total)	1,961
Genes (coding)	1,737
CDSs (with proteins)	1,737
Genes (RNA)	44
rRNAs	2, 2, 2 (5S, 16S, 23S)
rRNAs completes	2, 2, 2 (5S, 16S, 23S)
tRNAs	44
ncRNAs	4
Pseudo Genes (total)	224
CDSs (without proteins)	224
Pseudo Genes (ambiguous residues)	0 of 224
Pseudo Genes (frameshift)	144 of 224
Pseudo Genes (incomplete)	83 of 224
Pseudo Genes (stop internal)	68 of 224
Pseudo Genes (multiple problems)	63 of 224
Flagellum genes (*FlagA* and *FlagB*)	Absent
Adhesin genes (*BadA*)	Absent

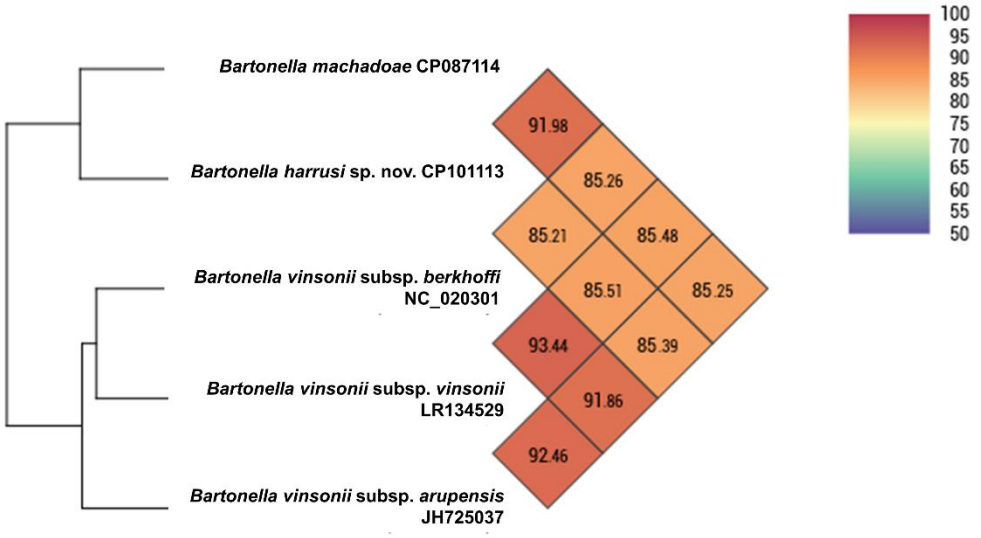

Figure 6. Heat map generated with OrthoANI values calculated between A: *Bartonella harrusi* sp. nov. isolated from a blood sample of *T. macrurus*, *B. machadoae*, species of the *B. vinsonii* complex.

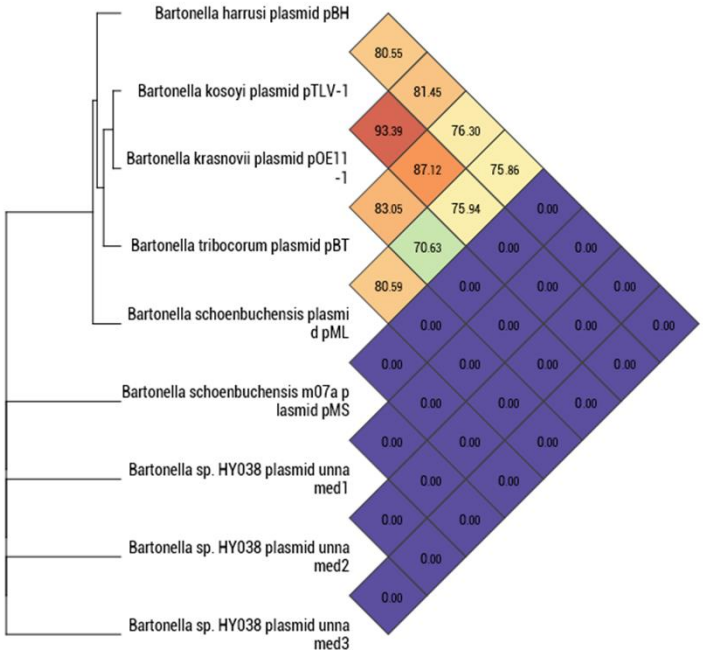

Figure 7. Heat map generated with OrthoANI values calculated between the plasmids found in *Bartonella* species and the plasmid found in *Bartonella harussi* sp. nov. isolated in the present study using the OAT software.

The phylogenomic analysis was based on 291 protein-coding genes shared by 53 whole genomes of *Bartonella* species with validly published names and some *Bartonella* species not yet named. Based on this analysis, *Bartonella* strain 117A was positioned in a clade closely related to *B. machadoae* and *Bartonella* species belonging to the *B. vinsonii* complex, albeit separated with 100% bootstrap (**Figure 8**).

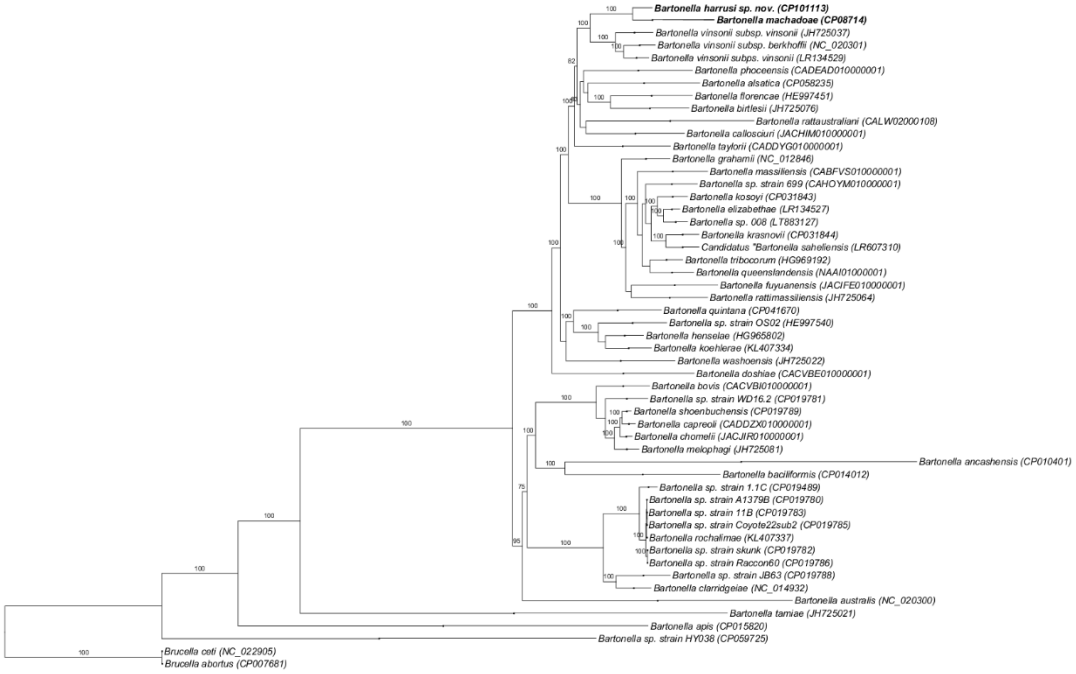

Figure 8. Phylogeny based on the complete genome of 53 species of *Bartonella*, using 293 coding genes shared between these species. Bootstrap values are shown under nodes. *Brucella abortus* and *Brucella ceti* were used as an outgroup.

3.9. Use of average nucleotide identity (ANI) to separate species of the genus *Bartonella*

The *Bartonella* genomic sequences available in Genbank were analyzed for completeness and contamination in order to determine the quality of the genomes for use in ANI-based taxonomic classification. Among the 217 genomes available, only the genome of *'Candidatus* Bartonella saheliensis' (LR607310) presented low quality

both for completeness (below 70%) and percentages of contamination (above 15%) (**Figures 9A and 9B**), and it was therefore removed from subsequent analyses. Regarding the mean nucleotide identity (ANI) in general, most *Bartonella* species showed ANI values between 75 and 90%. However, ANI values lower than 70% were found for *B. tamiae*, *B. apis* and *Bartonella* sp. strain HY038, species considered more diversified within this genus, when compared to the other *Bartonella* species analyzed (**Figure S2**). Furthermore, it was possible to observe ANI values greater than 95% among the available *Bartonella* genomes, which were divided into 50 different groups (**Figure S2** and **Table S1**). Among these groups, 20 were represented by a single sequence and 30 had more than one sequence with a variation between 95 and 100%. Genomes with more than one sequence were subdivided using cutoff points 95 < ANI < 97%. Eighteen groups presented ANI above 97% when compared to the other genomes; 12 genomes had one to four sequences with variation between 95 to 97% of ANI, and they were reclassified as subspecies (**Table S1**).

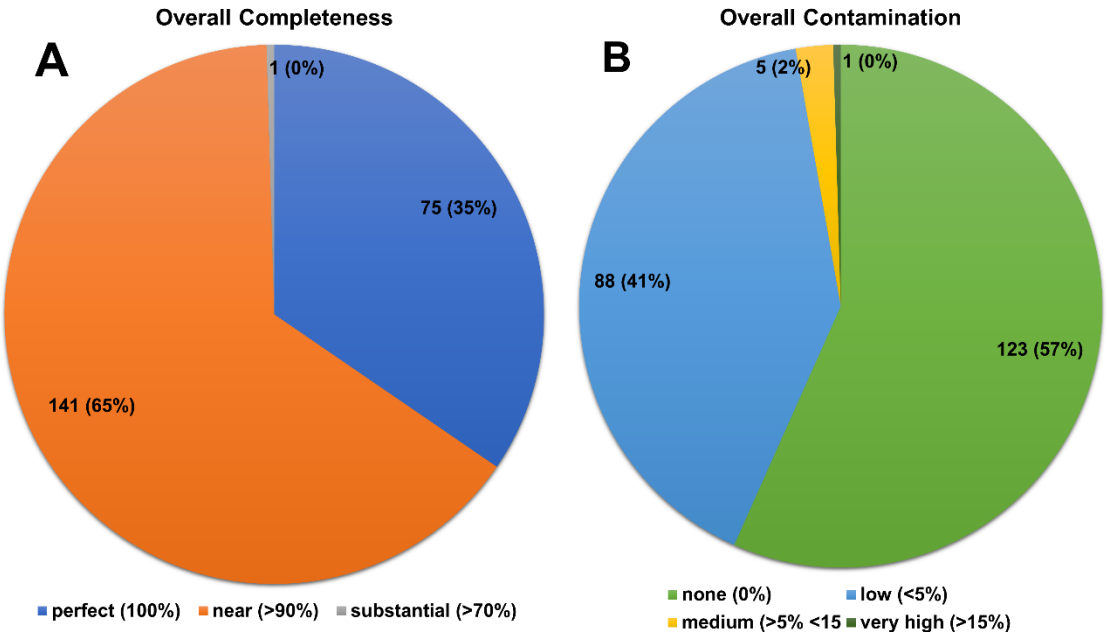

Figure 9. Pie chart showing in A the completeness of the 217 analyzed genomes of *Bartonella* spp. and in B the level of contamination of the genomes used for the analysis of ANI.

3.10. *"In silico"* analysis of metabolic profiles, ribosomal and polymerase phylogenetic markers for species of the genus *Bartonella*

The analysis of the metabolic profile for species of the genus *Bartonella* using the genome available in Genbank showed similar profiles: all species analyzed (n=53) have the capacity to oxidize hydrogen and oxygen, as well as to fix carbon. Some *Bartonella* species (strain 117A, *B. machadoae*, *B. tribocorum*, *B. schoenbuchensis*, *B. massiliensis*, *B. capreoli*, *B. ancashensis*, *B. bovis*, *B. bacilliformis*, *B. rattimassiliensis*, and *B. melophagi*) were not able to oxidize sulfur because they did not have related genes. The most diverse species found were *Bartonella* sp. strain HY038 and *Bartonella apis*, which presented in their genomes genes related to nitrogen fixation, oxidation of urea, selenium, arsenic and halogenated compounds, a profile that was not observed in any other *Bartonella* species. Interestingly, *Bartonella*

sp. strain HY038, *Bartonella apis* and *Bartonella tamiae* showed genes related to the use of C1 compounds (**Figure S3**).

Regarding the profile of ribosomal phylogenomic markers and polymerases, only punctual differences were found. In fact, none of the analyzed species showed considerable differences (**Figure S4**).

3.11. Search for active carbohydrate enzymes (CAZy) families

When analyzing genes related to groups of enzymes Carbohydrate Esterases (CAZy), enzymes classified into families 24, 94 and 103 of the group of glycoside hydrolases enzymes, families 19, 29, 30, 51 and 84 of the group of glycoside transferases, and family 11 of the group of carbohydrate transferases were found in all analyzed genomes (**Figure S5**). However, genes related to the production of family 3 of enzymes of the glycoside hydrolases group were found only in some *Bartonella* species belonging to lineages 1 (*B. tamiae, B. apis, B. ancachensis, Bartonella* sp. strain HY038), 2 (*B. bovis, B. schoenbuchensis, B. chomelii*) and 3 (*B. clarridgeiae, B. rochalimae, Bartonella* spp. strains Coyote22, stunk WD16.2 and 1-1C JB63). The Family 4 of the group of glycoside transferases was found only in a few species, including *B. machadoae* recently isolated from rodents in the same region where the present study was performed, but not in *Bartonella* sp. strain 117A described in the present study (**Figure S5**).

Interestingly, although family 24 of glycoside hydrolases was found in the genome of the 53 *Bartonella* species analyzed, there was a difference in the number of occurrences depending on the analyzed genome, with variations from 1 to 12 genic regions (**Figure S5**).

4. Discussion

The present work aimed to contribute to the diagnosis, molecular, morphological and phenotypic characterization of *Bartonella* spp. in wild marsupials from the Pantanal, state of Mato Grosso do Sul, central-western Brazil.

There are few works in the literature that have investigated the role of marsupials as reservoirs of *Bartonella* spp. For instance, Fournier et al. (2007) isolated and described *Bartonella australis* in a kangaroo (*Macropus giganteus*) in Australia. *Bartonella* DNA was also detected in *Ixodes tasmani* ticks collected from koalas (*Phascolarctos cinereus*) (Vilcins et al., 2009) in Australia. Additionally, *'Candidatus* Bartonella bandicootii' and *'Candidatus* Bartonella woyliei' were molecularly detected in fleas (*Pygiopsylla tunneyi*) from bandicoots (*Perameles bougainville*) and in fleas (*Pygiopsylla hilli*) and ticks (*Ixodes australiensis*) collected from woylies (*BIettongia penicillata*), respectively (Kaewmongkol et al., 2011). When it comes to Brazil, *Bartonella* DNA was not detected in marsupials (*Thylamys macrurus*, *Gracilinanus agilis*, *Monodelphis domestica* and *Didelphis albiventris*) in the Pantanal of Mato Grosso do Sul (de Sousa et al., 2018). This finding might be associated with the low *Bartonella* bacteremia, which might have hampered the detection of this agent by qPCR directly from the blood. *Bartonella* bacteremia in marsupials sampled by (de Sousa et al., 2018) was probably below the limit of detection of the qPCR technique based on the *nuoG* gene. Similar results were found herein when marsupial blood DNA samples were submmited to qPCR assays. In the present study, *Bartonella* DNA was found in marsupial blood samples only after passage in BAPGM liquid medium (pre-enriched liquid culture) and isolation of *Bartonella* spp. on chocolate agar. The present work brings the first detection and isolation of *Bartonella* in marsupials in the Americas.

The use of BAPGM pre-enrichment medium and isolation on chocolate agar brings greater sensitivity in the diagnosis of *Bartonella* spp., as previously reported. Duncan et al. (2007), when using the abovementioned diagnostic platform for *Bartonella* spp. in dog blood samples, found a positivity of 8.1%; on the other hand, when they performed molecular diagnosis directly from blood DNA samples, a positivity of 4.7% was found. Our research group has been using this diagnostic platform and showing the advantages of using the “liquid medium” for successful diagnosis and isolation of *Bartonella henselae* in blood samples from cats (Furquim et al., 2021). More recently, using the same diagnostic platform used in the present study, we reported the diagnosis and isolation of *Bartonella machadoae* from rodent blood samples sampled in the Pantanal of Mato Grosso do Sul (do Amaral et al., 2022).

Regarding the phenotypic characteristics, strain 117A of *Bartonella* sp. isolated from a blood sample of *T. macrurus* showed microbiological and morphological characteristics identical to the *B. machadoae* recently described by our research group (do Amaral et al., 2022), showing excellent growth under conditions of increased CO_2. Additionally, this strain showed aerobic, capnophilic and microaerophilic characteristics. It was characterized by rounded and smooth colonies (1-2 mm) after 4-5 days of plating on chocolate agar. Similarly to *B. machadoae*, strain 117A was not able to grow under anaerobic conditions. Such growth characteristics are similar to those reported in other species of the genus *Bartonella*, such as in *B. elizabethae*, *B. henselae* (Houston-3 strain), *B. clarridgeiae*, *B. quintana* and *B. vinsonii* subsp. *vinsonii* (Clarridge et al., 1995). In contrast, *B. kosoyi* and *B. krasnovii* isolated from *Rattus rattus* and *Synosternus cleopatrae* fleas collected from *Gerbillus andersoni*, respectively, did not grow under strictly aerobic and anaerobic conditions (Gutiérrez et al., 2015).

Bartonella strain 117A was negative for oxidase, catalase, urease, indole production, acetoin production and nitrate reduction. These characteristics were similar to those found in the biochemical profiles of *Bartonella* species of the *B. vinsonii* complex (Welch et al., 1999) and *B. machadoae* (do Amaral et al., 2022). However, differences were found with other *Bartonella* species: while *B. krasnovii* was able to ferment sucrose, citrate and arabinose, *Bartonella kosoyi* was able to ferment sorbitol, melibiose, rhamnose and glucose, in addition to showing a positive reaction to arginine (Gutiérrez et al., 2020). On the other hand, but similarly to *B. machadoae, Bartonella harrusi* sp. nov. was not able to ferment the above mentioned carbohydrates (do Amaral et al., 2022).

Regarding the bacterial growth curve, the results of the present study showed discrepancies between the growth curves of the two isolated species: while *B. machadoae* (unpublished data) required a shorter time to enter into the logarithmic phase and presented a higher peak of CFU/mL, remaining longer in the stationary phase, the *Bartonella* strain 117A needed more time to reach the highest CFU/mL count, in addition to remaining in the stationary phase for a shorter time. When compared with other species of the genus, *B. henselae* (using supplemented RPMI 1640 medium) and *B. quintana* (using DS2 medium formulated to favor cell growth of insect cells) showed curves similar to that presented by *Bartonella* strain 117A, both requiring a longer time to enter into the logarithmic phase and, similarly to the present work, they did not remain in a stationary phase for a long time (Chenoweth et al., 2004).

Bartonellae isolated in the present study did not show pili or flagella at SEM, which is confirmed by the absence of genes encoding adhesin (*BadA*) and flagella (*FlaA* and *FlaB*). The absence of pili or flagellum is shared with other rodent-associated *Bartonella* species, such as *B. vinsonii* (Breitschwerdt et al., 1995), *B. japonica, B.*

silvatica (Inoue et al., 2010), *B. heixianziensis* (Li et al., 2015), and *B. machadoae* (do Amaral et al., 2022). Differently, *B. bacilliformis, B. schoenbuchensis, B. capreoli, B. chomelii, B. clarridgeiae* and *B. rochalimae* have flagella. The presence of pilli has been described in rodent-associated *Bartonella* spp., such as *B. tribocorum* (Heller et al., 1998), *B. alsatica* (Heller et al., 1998), *B. acomydis, B. jaculi, B callosciuri*, *B. pachyuromydis* (Sato et al., 2013), *B. fuyuanensis* Li et al., 2015), *B. kosoyi* and *B. krasnovii* (Gutiérrez et al., 2020).

With regard to multilocus analysis (MLSA), do Amaral et al. (2022) demonstrated that, due to the high genotypic diversity of *Bartonella* species described in different hosts, previously used criteria, such as those proposed by la Scola et al. (2003), have less accuracy and precision for discrimination of strictly related *Bartonella* species. In the present work, it was possible to observe a lack of resolution of the phylogeny based on six molecular markers, which was able to separate *Bartonella* sp. isolated from marsupials in the present work from *Bartonella* species belonging to *B. vinsonii* complex, but not to clearly separate them from *B. machadoae*, a rodent-associated *Bartonella* species recently described in the same region where the present study was performed (do Amaral et al., 2022) (**Figure 2; Table 4**).

Table 4. Percentage (%) of nucleotide identity between *Bartonella harrusi* sp. nov. and *B. machadoae* according to different molecular markers. The cut-off values proposed by La Scola et al. (2003) for description of new *Bartonella* species for each molecular marker are shown.

Target gene region	Identity	Query cover	E-value	Cut-off Point (La Scola et al., 2003)
16S rRNA	100%	100%	0.0	98.3%
gltA	94.53%	100%	0.0	93.6%
groEL	96.09%	100%	0.0	92.6%
ftsZ	92.84%	100%	0.0	94.4%
rpoB	95.54%	99%	0.0	92.8%
ITS	91.77%	32%	$1x10^{-52}$	93.9%

Phenotypic and biochemical analyzes are shown to be inefficient in the discrimination of new species of *Bartonella* due to very similar biochemical profiles, with rare exceptions in more diversified species, as shown in the present study by “in silico” analyses. Multilocus analysis based on 6-8 molecular markers has been replaced by WGS analysis, due to greater ease and more accessible values of WGS of *Bartonella* isolates. In fact, phylogenomic analyzes based on hundreds of genes provide higher reliability in the definition of species, in addition to allowing the inference of biochemical profiles without the use of time-consuming and laborious techniques (do Amaral et al., 2022; Krügel et al., 2022).

In the present study, bioinformatics analysis applied to whole genomes of *Bartonella* species deposited in databases allowed us to find a relative diversity in genes related to CAZy families, such as the presence of family 3 in some *Bartonella* species and the variation in the amount of occurrences of the family 24 of glycosyl hydrolases, enzymes known as β-N-acetylglucosaminidase and muramidase, respectively, and related to the degradation of chitin-based carbohydrates (Matano et al., 2016). More studies related to CAZy families should be conducted, since chitinases may play a role on interaction of bartonellae and the peritrophic membrane of

ectoparasites, enabling bartonella mobility and favoring transmission. Furthermore, it was possible to identify genes related to family 4 glycosyltransferase enzymes in some species of *Bartonella*. Considering that such enzymes are associated with the accumulation of glycogen for the survival of the agent in the environment, they may be involved in the maintenance of some *Bartonella* species in flea and lice feces (Bejarano et al., 2020). Interestingly, although no genes related to this family of enzymes were found in the genome of *Bartonella* strain 117A isolated herein, such genes were found in the genome of *B. machadoae*, which was recently isolated from *Trichomys fosteri'* blood samples in the same region where the present study was performed (do Amaral et al., 2022). Thus, the role of this enzyme family in the survival and transmission of *Bartonella* species between their vertebrate and invertebrate hosts should be further investigated.

Although the number of species and Candidatus for the genus *Bartonella* has increased in the last years, especially those associated with small mammals, many of them have not been fully characterized and, quite often, with insufficient information for reliable determination of new species and subspecies (Krügel et al., 2022). In addition to demonstrating the inability to distinguish phylogenetically close species by criteria previously used, the present work proposes a review of the taxonomic classification of species of the genus *Bartonella* by using ANI values obtained from whole genomes. ANI values below 95% are used as a cut-off point for determining new species (Goris et al., 2007; Jain et al., 2018; Gutiérrez et al., 2020). Based on this criterion, it is possible to separate the genus *Bartonella* into 50 different species (**Table S1**). In addition, we were able to solve taxonomic issues in several subgroups of the genus *Bartonella*. For instance, the taxa *Bartonella apis* and *Bartonella tribocorum* are composed of two and three different species, respectively. *Bartonella* subspecies

belonging to the *Bartonella vinsonii* complex actually comprise three different species (*Bartonella vinsonii*, *Bartonella arupensis* and *Bartonella berkhoffii*, since they have an ANI value below 95% among themselves. On the other hand, *B. schoenbuchensis, Bartonella chomelii, B. capreoli,* and *B. melophagi* should be grouped as subspecies of *B. schoenbuchensis* (species described first among the four cited) (Bermond et al., 2002), since they show ANI values above 95% among themselves. Finally, based on the *Bartonella* species (*B. henselae, B. quintana* and *B. bacilliformis*) with the highest number of genomes deposited in the database, the variation of ANI values within the same species ranged from 97 to 100%. Therefore, we propose a cut-off point of 95 < ANI < 97% for the classification of new *Bartonella* subspecies (**Table S1**).

The present work brings the first description of *Bartonella* in marsupials outside the Australian continent. We propose that it be named *Bartonella harrusi* sp. nov., in honor of Israeli researcher Shimon Harrus, professor at The Hebrew University of Jerusalem, Koret School of Veterinary Medicine, for his extensive contribution to the study of members of the *Bartonella* genus.

Although the vectors involved in the transmission of this new *Bartonella* species, siphonapterans may act as possible vectors of this agent. Indeed, *gltA* sequences from *B. harrusi* sp. nov. showed 100% identity with *Bartonella* genotypes previously detected in rodent-associated fleas (*Polygenis (Polygenis) bohlsi bolshi*, *Polygenis occidentalis occidentalis*, *Polygenis platensis* and *Craneopsylla minerva minerva)* in the biomes of Pantanal (state of Mato Grosso do Sul), Pampa and Atlantic Forest, in the state of Rio Grande do Sul (de Sousa et al., 2018; Schott et al., 2020). Interestingly, the *Bartonella* genotypes detected in fleas from central-western and southern Brazil were found in regions where the circulation of *B. machadoae*, a species recently described in rodents, was also reported (do Amaral et al., 2022). Recently,

Santana et al. (Santana et al., 2022) detected *B. machadoae* in *Amblyomma sculptum* ticks collected from wild boars (*Sus scrofa*) in southeastern Brazil. Thus, it is necessary to evaluate the diversity of hosts, vectors and the zoonotic potential of *B. machadoae* and *B. harrusi*, considering that both are closely related to *Bartonella* species belonging to *B. vinsonii* complex, which have already been shown to have zoonotic potential in several regions in the world (Welch et al., 1999; Roux et al., 2000; Fenollar et al., 2005; Breitschwerdt et al., 2010; Bai et al., 2012; do Amaral et al., 2022)

4.1. Description of *Bartonella harrusi* sp. nov.

The bacterial cells of this new proposed species are rods (1–2.0 µm long and 0.3–0.6 µm wide), without flagella and pili, showing excellent growth under capnophilic conditions (5% CO_2) at 35ºC. Colonies grown on chocolate agar are smooth and circular. The time required for growth to reach colonies of 1.0–2.0 mm is 4 days at 35°C. They are negative for oxidase, catalase, urease, indole production, acetoin production and nitrate reduction. They are unable to ferment the carbohydrates sorbitol, melibiose, rhamnose and glucose, albeit showed a positive reaction to arginine. The type strain (117A) carried a single circular chromosome of 2.23 MB, with an average G+C DNA content of 38.8% by mol. and a 29,892 bp circular plasmid with an average G+C content of 34.5%. The closest species with a valid published name is *B. machadoae* (91.98% ANI). *Bartonella harrusi* sp. nov. (strain 117A) is stored at the Laboratory of Immunoparasitology, Department of Pathology, Reproduction and Single Health, Faculty of Agrarian and Veterinary Sciences/Universidade Estadual Paulista, UNESP, Jaboticabal, SP, Brazil.

5. Conclusion

The present work presents the first description of *Bartonella* in marsupials from the Americas. *Bartonella harrusi* sp. nov. was isolated from blood samples of *T. macrurus* in the Pantanal of Mato Grosso do Sul. The diagnostic platform combining qPCR results based on the *nuoG* gene from blood samples in pre-enrichment liquid culture and chocolate agar isolation proved to be efficient in detecting *Bartonella* in marsupials. Phylogenomic analysis based on 291 genes and an ANI value of 91.98% obtained by WGS of *Bartonella* 117A allowed the separation of *Bartonella harrusi* sp. nov. from *Bartonella machadoae*. Bioinformatics analysis applied to complete genomes should be used for classification and taxonomic review of members of the genus *Bartonella* based on ANI values.

6. Data Availability Statement

The data that support the findings of this study are openly available in National Center for Biotechnology Information at https://www.ncbi.nlm.nih.gov/, reference number genome BioProject: PRJNA854809, genome accession numbers: CP101113-CP101114.

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8. Material Suplementar

Table S1. Taxonomic reclassification of the *Bartonella* species based on the 95% cut-off point assessed by the Average Nucleotide Identity (ANI) value. *Bartonella* species with a new proposed taxonomic status are red-marked. n/a: species that did not have ANI variation below the 97% cut-off.

Species on Genbank	Strain	Accession number	Lineage (based on cut off = 95%)	Subcluster (cut off = 97%)	New proposed classification
Bartonella bovis	CIP 106692	CACVBI010000001	1	subsp. 1	*Bartonella bovis* subsp. *1*
Bartonella bovis	91-4	CM001844	1	subsp. 1	*Bartonella bovis* subsp. *1*
Bartonella bovis	BbUM	MWVG01000001	1	subsp. 2	*Bartonella bovis* subsp. *2*
Bartonella bovis	m02	KB915624	1	subsp. 3	*Bartonella bovis* subsp. *3*
Bartonella sp.	WD12.1	MUBG01000001	2	n/a	*Bartonella* sp. nov. linage 2
Bartonella schoenbuchensis	CCUG 50783	CADDYD010000001	3	subsp. 1	*Bartonella schoenbuchensis* subsp. *schoenbuchensis*
Bartonella schoenbuchensis	R1	CP019789	3	subsp. 1	*Bartonella schoenbuchensis* subsp. *schoenbuchensis*
Bartonella schoenbuchensis	m07a	KB915627	3	subsp. 1	*Bartonella schoenbuchensis* subsp. *schoembuchensis*
Bartonella chomelii	DSM 21431	NZ_JACJIR010000001	3	subsp. 1	*Bartonella schoenbuchensis* subsp. *schoembuchensis*
Bartonella capreoli	DSM 21569	CADDZX010000001	3	subsp. 2	*Bartonella schoenbuchensis* subsp. *capreoli*
Bartonella melophagi	K-2C	JH725081	3	subsp. 3	*Bartonella melophagi* subsp. *melophagi*
Bartonella sp.	WD16.2	CP019781	4	n/a	*Bartonella* sp. nov. linage 4
Bartonella bacilliformis	USM-LMMB	LQWW01000001	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	USM-LMMB	LQXX01000001	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	ATCC 35685D-5	CP014012	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	INS	AMQK01000001	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	KC583	CP000524	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	Ver075	KL503822	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	CAR600-02	KL503826	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	VAB9028	KL503815	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	CUSCO5	KL503795	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	Cond044	KL503799	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	Peru38	KL503808	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	Hosp800-02	KL503779	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	Heidi San Pedro600-02	KK097685	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	Peru-18	KK097680	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	Ver097	KK097689	5	subsp. 1	*Bartonella bacilliformis* subsp. *1*
Bartonella bacilliformis	Aust/NH1	KL503802	5	subsp. 2	*Bartonella bacilliformis* subsp. *2*
Bartonella australis	NCTC12898	CP003123	6	n/a	*Bartonella australis*
Bartonella elizabethae	BeUM	LR134527	7	subsp. 1	*Bartonella elizabethae* subsp.. *1*
Bartonella elizabethae		LFMF01000001	7	subsp. 1	*Bartonella elizabethae* subsp.. *1*

Bartonella elizabethae	F9251 = ATCC	JADB01000001	7	subsp. 1	*Bartonella elizabethae* subsp.. *1*
Bartonella elizabethae	F9251	JH725033	7	subsp. 1	*Bartonella elizabethae* subsp.. *1*
Bartonella elizabethae	Re6043vi	JH725139	7	subsp. 1	*Bartonella elizabethae* subsp.. *1*
Bartonella elizabethae	F9251 = ATCC	CADEAC010000001	7	subsp. 1	*Bartonella elizabethae* subsp.. *1*
Bartonella sp.	.008	LT883127	7	subsp. 2	*Bartonella elizabethae* subsp. *2*
Bartonella krasnovii	B60_6	CP093035	8	subsp. 1	*Bartonella krasnovii* subsp. *1*
Bartonella krasnovii	B84_4	CP093038	8	subsp. 1	*Bartonella krasnovii* subsp. *1*
Bartonella krasnovii	84B_2	CP093046	8	subsp. 1	*Bartonella krasnovii* subsp. *1*
Bartonella krasnovii	75A_1b	CP093040	8	subsp. 1	*Bartonella krasnovii* subsp. *1*
Bartonella krasnovii	90B_7	CP093044	8	subsp. 1	*Bartonella krasnovii* subsp. *1*
Bartonella krasnovii	51A_7	CP093043	8	subsp. 1	*Bartonella krasnovii* subsp. *1*
Bartonella krasnovii	B9_5	CP093039	8	subsp. 1	*Bartonella krasnovii* subsp. *1*
Bartonella krasnovii	75A_4b	CP093042	8	subsp. 1	*Bartonella krasnovii* subsp. *1*
Bartonella krasnovii	87B_3	CP093041	8	subsp. 1	*Bartonella krasnovii* subsp. *1*
Bartonella krasnovii	B1_2	CP093037	8	subsp. 1	*Bartonella krasnovii* subsp. *1*
Bartonella krasnovii	B71	CP093034	8	subsp. 1	*Bartonella krasnovii* subsp. *1*
Bartonella krasnovii	B35_1_2	CP093033	8	subsp. 1	*Bartonella krasnovii* subsp. *1*
Bartonella krasnovii	B51_4	CP093036	8	subsp. 1	*Bartonella krasnovii* subsp. *1*
Bartonella krasnovii	OE 1-1	CP031844	8	subsp. 2	*Bartonella krasnovii* subsp. *2*
Bartonella krasnovii	36C_7b	CP093045	8	subsp. 3	*Bartonella krasnovii* subsp. *3*
Candidatus Bartonella saheliensis	.077	LR607310	9	n/a	*Bartonella saheliensis* sp. nov.
Bartonella grahamii	ATCC 700132	JACX01000001	10	subsp. 1	*Bartonella grahamii* subsp. *1*
Bartonella grahamii	NCTC12860	UFTD01000005	10	subsp. 1	*Bartonella grahamii* subsp. *1*
Bartonella grahamii	as4aup	CP001562	10	subsp. 2	*Bartonella grahamii* subsp. *2*
Bartonella grahamii	A7JPB	CACVBG010000001	10	subsp. 3	*Bartonella grahamii* subsp. *3*
Bartonella grahamii	A1JPB	CACVBJ010000001	10	subsp. 4	*Bartonella grahamii* subsp. *4*
Bartonella tribocorum	L103	CADDYI010000001	11	n/a	*Bartonella* sp. nov. linage *11*
Bartonella tribocorum	L103	NJGE01000001	11	n/a	*Bartonella* sp. nov. linage *11*
Bartonella tribocorum	CIP 105476	AM260525	12	n/a	*Bartonella tribocorum*
Bartonella tribocorum	BM1374166	CADDYK010000001	12	n/a	*Bartonella tribocorum*
Bartonella kosoyi	Tel Aviv	CP031843	13	n/a	*Bartonella kosoyi*
Bartonella tribocorum	C635	NJPP01000001	14	n/a	*Bartonella* sp. nov. linage *14*
Bartonella tribocorum	C635	CADDYJ010000001	14	n/a	*Bartonella* sp. nov. linage *14*
Bartonella queenslandensis	AUST/NH15	HE997969	15	subsp. 1	*Bartonella queenslandensis* subsp. *1*
Bartonella queenslandensis	BqUM	NZ_NAAI01000001	15	subsp. 2	*Bartonella queenslandensis* subsp. *2*
Bartonella massiliensis	OS09	CABFVS010000001	16	n/a	*Bartonella massiliensis*
Bartonella rattimassiliensis	15908	CALY02000093	17	n/a	*Bartonella rattimassiliensis*
Bartonella rattimassiliensis	15908	JH725064	17	n/a	*Bartonella rattimassiliensis*
Bartonella fuyuanensis	DSM 100694	JACIFE010000001	18	n/a	*Bartonella fuyuanensis*
Bartonella koehlerae	C-29	KL407334	19	n/a	*Bartonella koehlerae*
Bartonella koehlerae	CCUG 50773	CADEAH010000001	19	n/a	*Bartonella koehlerae*
Bartonella henselae	BM1374164	CACVBL010000001	20	n/a	*Bartonella henselae*

Bartonella henselae	BM1374163	CACVBD010000001	20	n/a	*Bartonella henselae*
Bartonella henselae	BM1374165	CACVBK010000001	20	n/a	*Bartonella henselae*
Bartonella henselae	ATCC49882 T var-1	CP072903	20	n/a	*Bartonella henselae*
Bartonella henselae	ATCC49882 T	CP072902	20	n/a	*Bartonella henselae*
Bartonella henselae	Marseille	CP072904	20	n/a	*Bartonella henselae*
Bartonella henselae	88-64	CP072899	20	n/a	*Bartonella henselae*
Bartonella henselae	FR96/BK38	CP072898	20	n/a	*Bartonella henselae*
Bartonella henselae	G-5436	CP072900	20	n/a	*Bartonella henselae*
Bartonella henselae	FR96/BK3	CP072897	20	n/a	*Bartonella henselae*
Bartonella henselae	FDAARGOS_ 1462	CP082885	20	n/a	*Bartonella henselae*
Bartonella henselae	Berlin-I	CP072901	20	n/a	*Bartonella henselae*
Bartonella henselae	623-125	BLJS01000001	20	n/a	*Bartonella henselae*
Bartonella henselae	804-29	BLJT01000001	20	n/a	*Bartonella henselae*
Bartonella henselae	Houston-I	CP020742	20	n/a	*Bartonella henselae*
Bartonella henselae	A242	LOAF01000001	20	n/a	*Bartonella henselae*
Bartonella henselae	A121	LOAC01000001	20	n/a	*Bartonella henselae*
Bartonella henselae	F1	LOAI01000001	20	n/a	*Bartonella henselae*
Bartonella henselae	A244	LOAG01000001	20	n/a	*Bartonella henselae*
Bartonella henselae	FR96/BK3	CP072897	20	n/a	*Bartonella henselae*
Bartonella henselae	A112	LOAB01000001	20	n/a	*Bartonella henselae*
Bartonella henselae	A235	LOAE01000001	20	n/a	*Bartonella henselae*
Bartonella henselae	A71	LOAA01000001	20	n/a	*Bartonella henselae*
Bartonella henselae	A233	LOAD01000001	20	n/a	*Bartonella henselae*
Bartonella henselae	A20	LNZX01000001	20	n/a	*Bartonella henselae*
Bartonella henselae	Houston-1	LRIJ02000001	20	n/a	*Bartonella henselae*
Bartonella henselae	A76	LNZZ01000001	20	n/a	*Bartonella henselae*
Bartonella henselae	A74	LNZY01000001	20	n/a	*Bartonella henselae*
Bartonella henselae	MVT02	LN879429	20	n/a	*Bartonella henselae*
Bartonella henselae	BM1374163	HG965802	20	n/a	*Bartonella henselae*
Bartonella henselae	BM1374165	HG969191	20	n/a	*Bartonella henselae*
Bartonella henselae	Houston-1	BX897699	20	n/a	*Bartonella henselae*
Bartonella henselae	Zeus	KL411698	20	n/a	*Bartonella henselae*
Bartonella henselae	JK 53	KL411717	20	n/a	*Bartonella henselae*
Bartonella henselae	JK 41	KI911811	20	n/a	*Bartonella henselae*
Bartonella henselae	JK 42	KI911798	20	n/a	*Bartonella henselae*
Bartonella henselae	JK 50	KI911793	20	n/a	*Bartonella henselae*
Bartonella henselae	JK 51	KI911786	20	n/a	*Bartonella henselae*
Bartonella senegalensis	OS02	NZ_HE997540	21	n/a	*Bartonella senegalensis*
Bartonella callosciuri	DSM 28538	JACHIM010000001	22	n/a	*Bartonella callosciuri*
Bartonella phoceensis	CIP107707	CADEAD010000001	23	n/a	*Bartonella phoceensis*
Bartonella quintana	CCUG 45777	CADDYB010000001	24	n/a	*Bartonella quintana*
Bartonella quintana	NCTC12899	LS483373	24	n/a	*Bartonella quintana*
Bartonella quintana	G1712	CP091505	24	n/a	*Bartonella quintana*
Bartonella quintana	G1713	CP091504	24	n/a	*Bartonella quintana*
Bartonella quintana	KorN	CP041670	24	n/a	*Bartonella quintana*

Bartonella quintana	MF1-1	AP019773	24	n/a	*Bartonella quintana*
Bartonella quintana	RM-11	CP003784	24	n/a	*Bartonella quintana*
Bartonella quintana	Toulouse	BX897700	24	n/a	*Bartonella quintana*
Bartonella quintana	JK 68	KL446932	24	n/a	*Bartonella quintana*
Bartonella quintana	JK 39	KL446941	24	n/a	*Bartonella quintana*
Bartonella quintana	JK 56	KL446928	24	n/a	*Bartonella quintana*
Bartonella quintana	JK 67	KL446937	24	n/a	*Bartonella quintana*
Bartonella quintana	JK 63	KL411737	24	n/a	*Bartonella quintana*
Bartonella quintana	JK 31	KL411732	24	n/a	*Bartonella quintana*
Bartonella quintana	JK 19	KL411729	24	n/a	*Bartonella quintana*
Bartonella quintana	BQ2-D70	KI911820	24	n/a	*Bartonella quintana*
Bartonella quintana	JK 7	KI911831	24	n/a	*Bartonella quintana*
Bartonella quintana	JK 73	KI911825	24	n/a	*Bartonella quintana*
Bartonella quintana	JK 73rel	KI911823	24	n/a	*Bartonella quintana*
Bartonella quintana	JK 12	KI911828	24	n/a	*Bartonella quintana*
Bartonella washoensis	NCTC13399	UAQI01000043	25	n/a	*Bartonella washoeensis*
Bartonella washoensis	085-0475	JH725101	25	n/a	*Bartonella washoeensis*
Bartonella washoensis	Sb944nv	JH725022	25	n/a	*Bartonella washoeensis*
Bartonella machadoae	56A	CP087114	26	n/a	*Bartonella machadoae*
Bartonella harrusi sp. nov.	117A		27	n/a	*Bartonella harrusi* sp. nov.
Bartonella vinsonii subsp. *arupensis*	ATCC 700727	CADEAE010000001	28	n/a	*Bartonella arupensis*
Bartonella vinsonii subsp. *arupensis*	Pm136co	JH725043	28	n/a	*Bartonella arupensis*
Bartonella vinsonii subsp. *arupensis*	OK-94-513	JH725037	28	n/a	*Bartonella arupensis*
Bartonella vinsonii	NCTC12905	LR134529	29	n/a	*Bartonella vinsonii*
Bartonella vinsonii subsp. *vinsonii*	CIP 103738	CADEAJ010000001	29	n/a	*Bartonella vinsonii*
Bartonella vinsonii subsp. *berkhoffii*	Winnie	CP003124	30	n/a	*Bartonella berkhoffii*
Bartonella vinsonii subsp. *berkhoffii*	ATCC	JACY01000001	30	n/a	*Bartonella berkhoffii*
Bartonella vinsonii subsp. *berkhoffii*	Tweed	KB915630	30	n/a	*Bartonella berkhoffii*
Bartonella vinsonii subsp. *berkhoffii*	CIP 104960	CADEAK010000001	30	n/a	*Bartonella berkhoffii*
Bartonella doshiae	CCUG 50770	CACVBH010000001	31	n/a	*Bartonella doshiae*
Bartonella doshiae	A14JPB	CACVBC010000001	31	n/a	*Bartonella doshiae*
Bartonella doshiae	BM1374167	CACVBF010000001	31	n/a	*Bartonella doshiae*
Bartonella doshiae	SRS24	CACVBE010000001	31	n/a	*Bartonella doshiae*
Bartonella doshiae	NCTC12862	UFTF01000002	31	n/a	*Bartonella doshiae*
Bartonella doshiae	BM1374167	CCBL010000001	31	n/a	*Bartonella doshiae*
Bartonella doshiae	DSM 102055	JACHEI010000001	31	n/a	*Bartonella doshiae*
Bartonella doshiae	NCTC 12862 = ATCC	JAGY01000001	31	n/a	*Bartonella doshiae*
Bartonella doshiae	NCTC 12862	JH725094	31	n/a	*Bartonella doshiae*
Bartonella birtlesii	IBS 325	CM001557	32	n/a	*Bartonella birtlesii*
Bartonella birtlesii	E4	KE007216	32	n/a	*Bartonella birtlesii*
Bartonella birtlesii	E11	KE007210	32	n/a	*Bartonella birtlesii*

Bartonella birtlesii	E7	KE007202	32	n/a	*Bartonella birtlesii*
Bartonella birtlesii	LL-WM9	JH725076	32	n/a	*Bartonella birtlesii*
Bartonella florencae	R4	HE997451	33	n/a	*Bartonella florencae*
Bartonella alsatica	IBS 382T	CACVBB010000001	34	n/a	*Bartonella alsatica*
Bartonella alsatica	CIP 105477	CP058235	34	n/a	*Bartonella alsatica*
Bartonella alsatica	IBS 382	JH725020	34	n/a	*Bartonella alsatica*
Bartonella sp.	B191	LR736368	35	subsp. 1	*Bartonella* sp. nov. linage *35* subsp. *1*
Bartonella sp.	DB5-6	JH725114	35	subsp. 2	*Bartonella* sp. nov. linage *35* subsp. *2*
Bartonella taylorii	A12JPB	CADDYH010000001	36	subsp. 1	*Bartonella taylorii* subsp. *1*
Bartonella taylorii	SRS19	CADDYE010000001	36	subsp. 1	*Bartonella taylorii* subsp. *1*
Bartonella taylorii	8TBB	JH725050	36	subsp. 1	*Bartonella taylorii* subsp. *1*
Bartonella taylorii	SRS10	CADDYG010000001	36	subsp. 2	*Bartonella taylorii* subsp. *2*
Bartonella taylorii	SRS29	CADEAG010000001	36	subsp. 3	*Bartonella taylorii* subsp. *3*
Candidatus Bartonella raoultii	.094	JAIFRO010000010	37	n/a	*Bartonella raoultii* sp. nov.
Bartonella rattaustraliani	AUST/NH4	CALW02000108	38	n/a	*Bartonella rattaustraliani*
Bartonella tamiae	Th307	JH725021	39	n/a	*Bartonella tamiae*
Bartonella tamiae	Th239	JH725147	39	n/a	*Bartonella tamiae*
Bartonella sp.	HY038	CP059725	40	n/a	*Bartonella* sp. linage *40*
Bartonella sp.	B10834G3	JACFOM010000009	41	n/a	*Bartonella* sp. linage *41*
Bartonella sp.	B10834H15	JACFOL010000031	41	n/a	*Bartonella* sp. linage *41*
Bartonella sp.	W8125	JACFRR010000008	41	n/a	*Bartonella* sp. linage *41*
Bartonella apis	BBC0122	CP015625	41	n/a	*Bartonella* sp. linage *41*
Bartonella sp.	B10834G6	JACFOX010000008	42	subsp. 1	*Bartonella* sp. linage *42* subsp. *1*
Bartonella sp.	W8099	JACFSN010000007	42	subsp. 1	*Bartonella* sp. linage *42* subsp. *1*
Bartonella apis	PEB0122	LXYU01000009	42	subsp. 2	*Bartonella* sp. linage *42* subsp. *2*
Bartonella apis	PEB0150	LXYS01000009	42	subsp. 2	*Bartonella* sp. linage *42* subsp. *2*
Bartonella apis	PEB0149	LXYT01000007	42	subsp. 2	*Bartonella* sp. linage *42* subsp. *2*
Bartonella sp.	W8098	JACFOW010000007	42	subsp. 3	*Bartonella* sp. linage *42* subsp. *3*
Bartonella sp.	W8152	JACFSP010000008	42	subsp. 3	*Bartonella* sp. linage *42* subsp. *3*
Bartonella sp.	W8151	JACFSO010000007	42	subsp. 3	*Bartonella* sp. linage *42* subsp. *3*
Bartonella sp.	W8167	JACFSQ010000017	42	subsp. 3	*Bartonella* sp. linage *42* subsp. *3*
Bartonella apis	BBC0244	CP015821	43	n/a	*Bartonella apis*
Bartonella sp.	BBC0178	CP015820	43	n/a	*Bartonella apis*
Bartonella apis	M0190	JACFOS010000003	43	n/a	*Bartonella apis*
Bartonella sp.	M0187	JACFOR010000010	43	n/a	*Bartonella apis*
Bartonella sp.	W8122	JACFOO010000008	43	n/a	*Bartonella apis*
Bartonella sp.	W8097	JACFON010000008	43	n/a	*Bartonella apis*
Bartonella sp.	P0291	JACFOQ010000001	43	n/a	*Bartonella apis*
Bartonella sp.	M0176	JACFOP010000004	43	n/a	*Bartonella apis*
Bartonella sp.	M0193	JACFOV010000087	43	n/a	*Bartonella apis*
Bartonella sp.	M0192	JACFOU010000003	43	n/a	*Bartonella apis*
Bartonella sp.	M0191	JACFOT010000007	43	n/a	*Bartonella apis*
Bartonella sp.	M0280	JACFSJ010000008	43	n/a	*Bartonella apis*
Bartonella sp.	M0177	JACFOY010000001	44	n/a	*Bartonella* sp. nov. linage *44*

Bartonella sp.	M0283	JACFSK010000001	45	n/a	*Bartonella* sp. nov. linage *45*
Bartonella sp.	JB15	CP019787	46		*Bartonella* sp. nov. linage *46*
Bartonella sp.	JB63	CP019788	46		*Bartonella* sp. nov. linage *46*
Bartonella clarridgeiae	73	FN645454	47	n/a	*Bartonella clarridgeiae*
Bartonella clarridgeiae	ATCC 51734	JADC01000009	47	n/a	*Bartonella clarridgeiae*
Bartonella sp.	114	CP019784	48	subsp. 1	*Bartonella rochalimae* subsp. *1*
Bartonella sp.	11B	CP019783	48	subsp. 1	*Bartonella rochalimae* subsp. *1*
Bartonella sp.	Raccoon60	CP019786	48	subsp. 1	*Bartonella rochalimae* subsp. *1*
Bartonella sp.	CDC_skunk	CP019782	48	subsp. 1	*Bartonella rochalimae* subsp. *1*
Bartonella sp.	A1379B	CP019780	48	subsp. 1	*Bartonella rochalimae* subsp. *1*
Bartonella rochalimae	ATCC BAA-1498	KL407337	48	subsp. 1	*Bartonella rochalimae* subsp. *1*
Bartonella sp.	1-1C	CP019489	48	subsp. 2	*Bartonella rochalimae* subsp. *2*
Bartonella sp.	Ga0114335_11	MUYE01000001	49	n/a	*Bartonella* sp. nov. linage *49*
Bartonella ancashensis	20.00	CP010401	50	n/a	*Bartonella ancashensis*

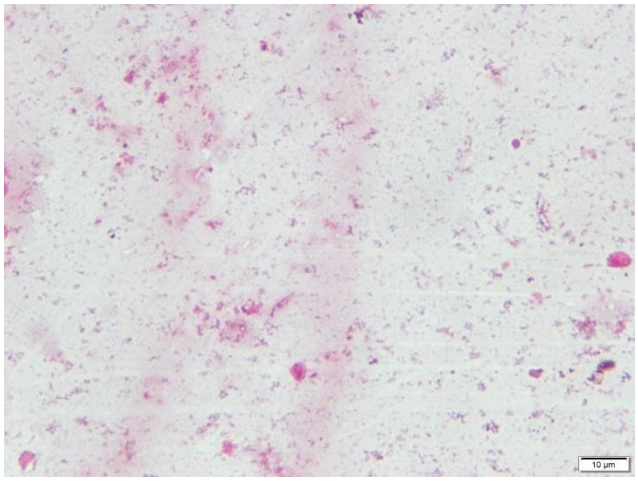

Figure S1. Gram-staining of *Bartonella harrusi* sp. nov. (strain 117A).

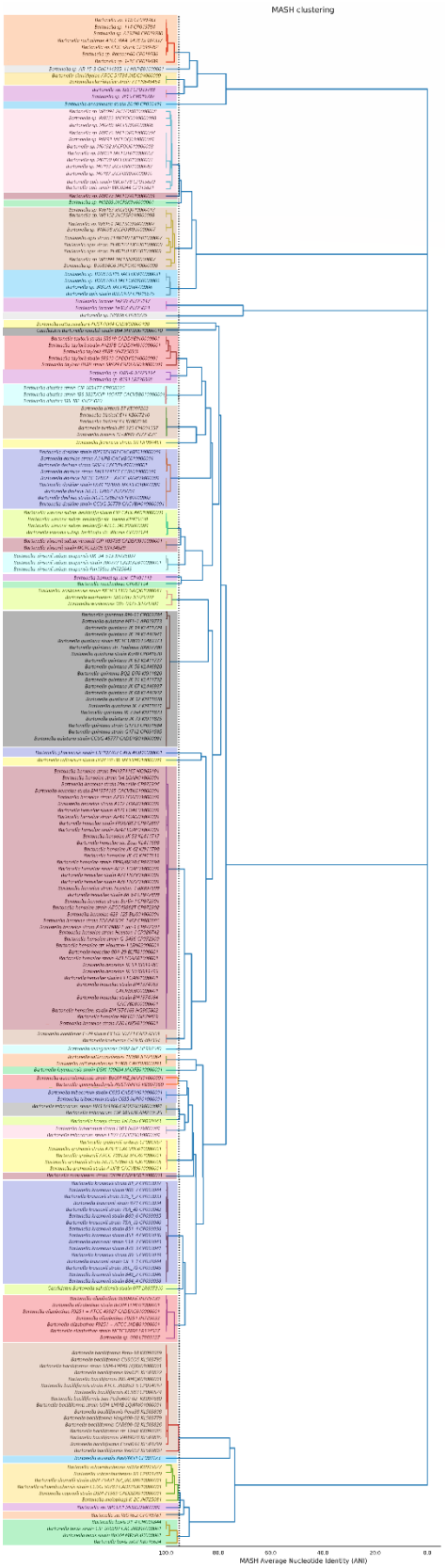
MASH clustering
100.0
80.0
60.0
40.0
20.0
0.0
MASH Average Nucleotide Identity (ANI)

Figure S2. Dendrogram created from the dRep analysis of all genomes deposited in GenBank using the 95% ANI value (average nucleotide identity) as a cut-off point by obtaining 50 different strains.

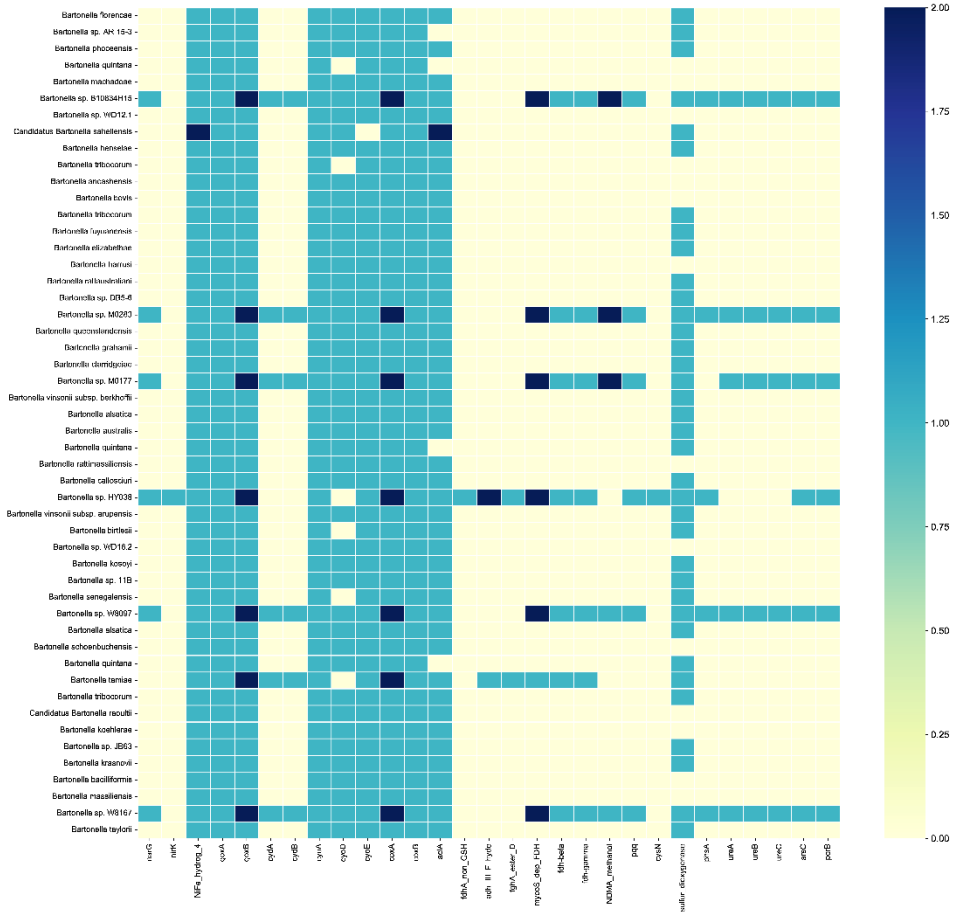

Figure S3. Heat map generated in the search for genes related to metabolic profiles using the genomes of *Bartonella* species available in GenBank.

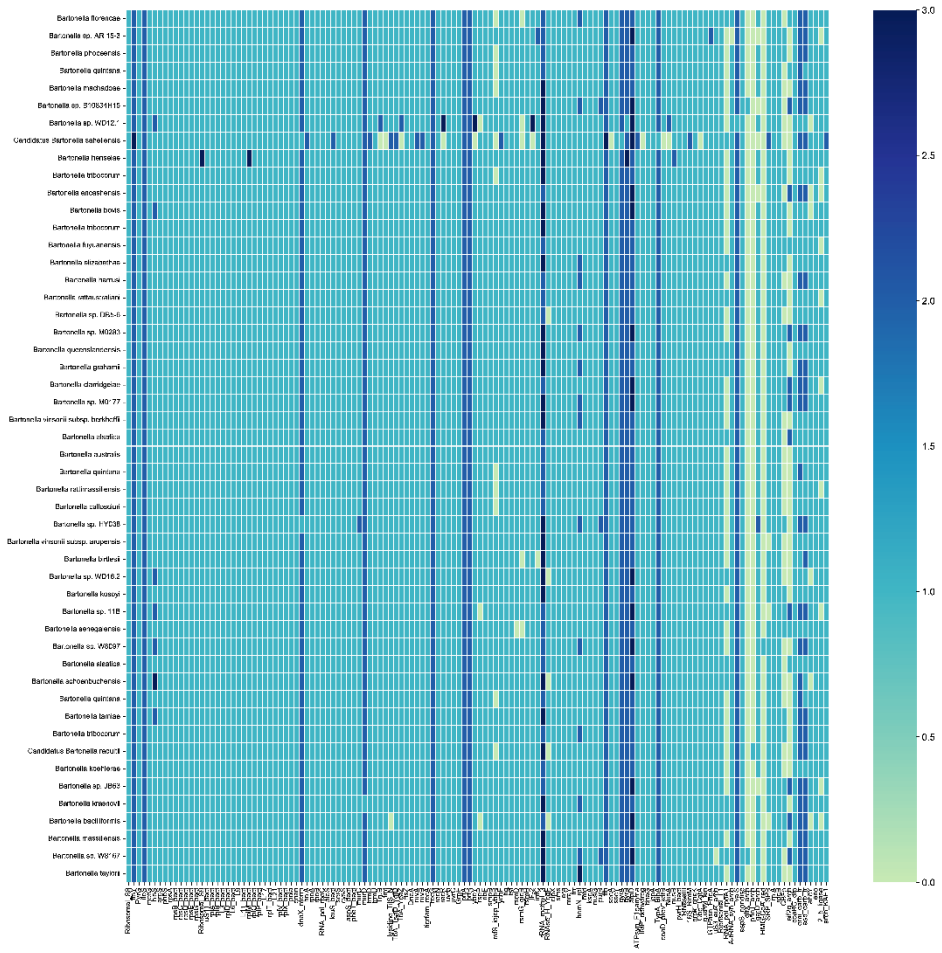

Figure S4. Heat map generated in the search for genes related to ribosomal and polymerase phylogenomic markers for species of the genus *Bartonella.*

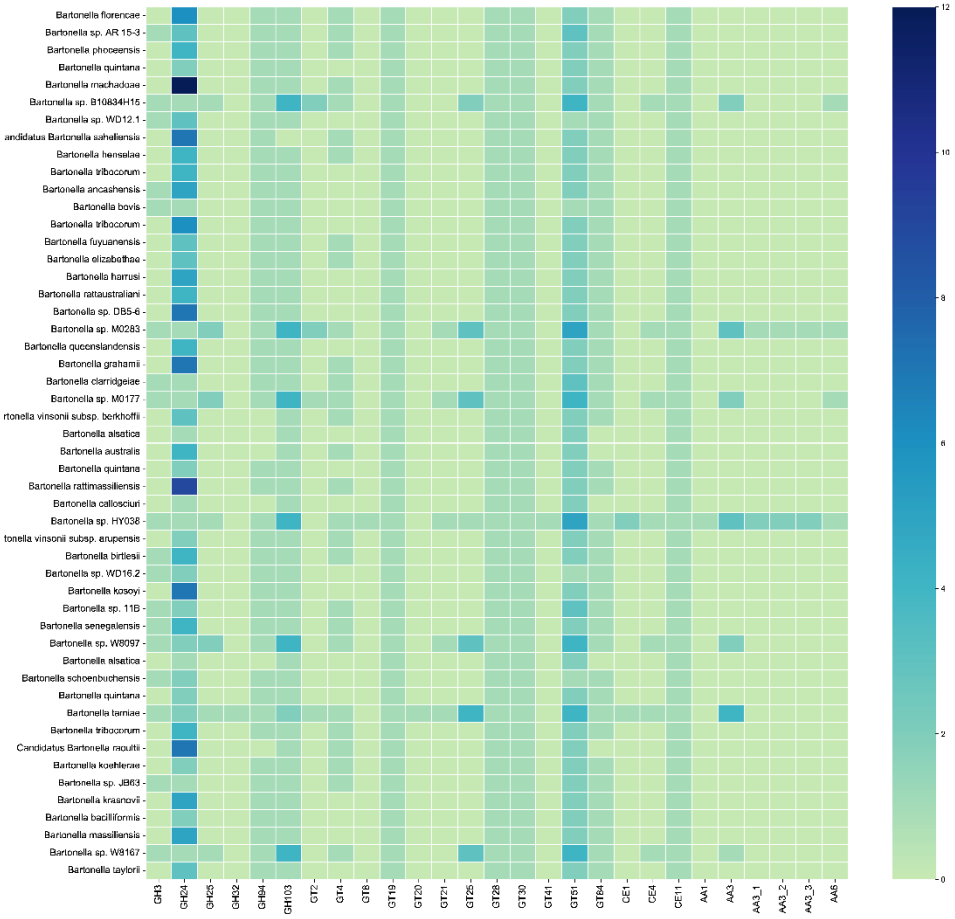

Figure S5. Heat map generated in the search for families of active carbohydrate enzymes (CAZy) for species of the genus *Bartonella.*