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Microorganismos transmitidos por garrapatas en la fauna silvestre de Chile: nuevos agentes de los géneros *Anaplasma*, *Borrelia* y *Babesia*

Tesis para optar el grado de Doctora en Ciencias Veterinarias

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Dedicatoria

Este trabajo está dedicado a la memoria del profesor Daniel González Acuña, quien confió en mí desde el principio de mi viaje académico en este país. Su apoyo y confianza fueron fundamentales en mi camino.

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TABLA DE CONTENIDOS

RESUMEN

Las garrapatas son vectores de virus, bacterias y protozoarios. Estos agentes infecciosos (AI) se mantienen en complejos ciclos que involucran a vertebrados silvestres. En este contexto, los roedores y los ciervos desempeñan un papel esencial en el mantenimiento de las poblaciones de garrapatas y en los ciclos enzoóticos de microorganismos de los géneros *Anaplasma*, *Borrelia* y *Babesia*, que tienen el potencial de transmitirse a los humanos o animales domésticos, lo que puede dar lugar a brotes o epidemias. Por ello, el monitoreo de la fauna silvestre y la identificación de vectores de AI son fundamentales para comprender sus ciclos e identificar posibles amenazas y factores de riesgo para la conservación de la biodiversidad, así como para la salud humana y animal. Esto es especialmente relevante en los actuales escenarios de cambios climáticos. Aunque la implementación de las técnicas moleculares ha incrementado los informes sobre estos AI en Sudamérica, el conocimiento actual sigue siendo limitado. Por lo tanto, el objetivo de esta investigación fue describir la diversidad genética, relaciones evolutivas y potenciales vectores y hospederos reservorios de especies de *Anaplasma*, *Borrelia* y *Babesia* en roedores cricétidos (Rodentia: Cricetidae), el ciervo *Pudu puda* (Artiodactyla: Cervidae) y dos garrapatas, *Ixodes stilesi* (Ixodida: Ixodidae) y *Ornithodoros octodontus* (Ixodida: Argasidae), en Chile. Los resultados de este estudio revelaron que las secuencias de ADN obtenidas en cricétidos, *P. puda* y garrapatas de *Anaplasma*, *Borrelia* y *Babesia*, representan nuevas genoespecies. Esta investigación marca un hito al constituir el primer registro de infección por *Borrelia* spp. y *Babesia* spp. en roedores en Chile, así como por *Babesia* sp. y *Anaplasma* sp., en *P. puda*. Estos mamíferos podrían ser potenciales hospederos reservorios de los nuevos genotipos reportados. Además, se ha evidenciado que *O. octodontus*, una garrapata asociada a roedores nativos del género *Octodon,* puede transmitir una *Borrelia* sp. a cobayas bajo condiciones de laboratorio. A pesar de estos avances, los ciclos epidemiológicos de *Anaplasma* spp., *Borrelia* spp. y *Babesia* spp. detectados en esta investigación continúan siendo desconocidos.

ABSTRACT

Ticks serve as vectors of viruses, bacteria, and protozoa. These infectious agents (IA) are maintained in complex cycles involving wild vertebrates. In this context, rodents and deer play an essential role in sustaining tick populations and the enzootic cycles of microorganisms from the genera *Anaplasma*, *Borrelia*, and *Babesia*, which have the potential to be transmitted to humans or domestic animals, potentially causing outbreaks or epidemics. For this reason, monitoring wildlife and identifying the vectors of IA is crucial for comprehending their cycles and pinpointing potential threats and risk factors in biodiversity conservation, as well as in human and animal health. This is particularly relevant in the current scenarios of climate changes. While the implementation of molecular techniques has led to increased reports on these IA in South America, our current knowledge remains limited. Thus, this research aimed to describe the genetic diversity, evolutive relationships, and potential vectors and reservoir hosts of *Anaplasma*, *Borrelia*, and *Babesia* species in cricetid rodents (Rodentia: Cricetidae), *Pudu puda* (Artiodactyla: Cervidae), and two ticks, *Ixodes stilesi* (Ixodida: Ixodidae) and *Ornithodoros octodontus* (Ixodida: Argasidae), in Chile. The results of this study reveal that the genetic sequences obtained in cricetid, *P. puda* and ticks from *Anaplasma*, *Borrelia*, and *Babesia* represent new genospecies. This research marks a milestone by constituting the first record of *Borrelia* spp. and *Babesia* spp. infection in rodents in Chile, as well as *Babesia* sp. and *Anaplasma* sp. in *P. puda*. These mammals could be potential reservoirs of the reported new genotypes. Furthermore, it has been demonstrated that *O. octodontus*, a tick associated with native rodents of the genus *Octodon*, can transmits a *Borrelia* sp. to guinea pigs under laboratory conditions. Despite these advancements, the epidemiological cycles of *Anaplasma* spp., *Borrelia* spp., and *Babesia* spp. detected in this research remains unknown.

INTRODUCCIÓN GENERAL

Las garrapatas son los artrópodos vectores con la capacidad de transmitir la mayor diversidad de agentes infecciosos (AI) (Wikel, 2018) como virus, bacterias y protozoos, los cuales se desarrollan en ciclos de vida complejos y dinámicos que depende de la historia natural de los vectores, los hospedadores y el ambiente abiótico y biótico (Baneth, 2014; Pfäffle et al., 2013).

Una garrapata se considera un vector competente cuando adquiere, mantiene y transmite con éxito a un AI (Sonenshine y Mather, 1994). Los AI son adquiridos por la garrapata durante la alimentación, a partir de la sangre infectada de un hospedero vertebrado (Nuttall, 2023). Una vez dentro de la garrapata, los AI deben superar barreras tisulares que incluyen el intestino medio, el hemocele y las glándulas salivales o el ovario en los casos de transmisión transovárica (Hajdušek et al., 2013). Durante este recorrido, los AI eluden o modulan las defensas inmunes innatas de la garrapata (Boulanger y Wikel, 2021; Hajdušek et al., 2013). Dentro de los mecanismos de defensa celulares y humorales se encuentran moléculas antimicrobianas defensivas que producen los hemocitos, el epitelio del intestino medio y las glándulas salivales (Boulanger y Wikel, 2021).

Los AI que se establecen en las glándulas salivares de la garrapata son transmitidos a los hospederos vertebrados a través de la saliva durante la alimentación del artrópodo (Reuben Kaufman, 2010). La saliva no solo facilita la sujeción al hospedador sino que también farmacomodula su respuesta inmunitaria mediante la liberación de moléculas analgésicas, anti-hemostáticas, anti-inflamatorias, inmunomoduladoras y de vasoconstricción (Nuttall, 2019). La regulación inmunitarias es aprovechada por los AI para transmitirse e infectar al hospedero vertebrado, la denominada “transmisión asistida por saliva” (Nuttall, 2023).

Para que ocurra una infección —la invasión, desarrollo o multiplicación de un AI en el hospedero vertebrado— (OMS, 2023; García et al., 2010), el AI debe eludir tanto la respuesta inmune innata como la adaptativa del hospedero. La inmunidad innata constituye la primera línea defensiva e incluye barreras físicas como la piel y las membranas mucosas de tracto respiratorio y gastrointestinal,

así como células fagocitarias —fagocitos, neutrófilos, células dendríticas y monocito/macrofágos— y factores solubles que oponizan y eliminan a los AI o potencian la acción de anticuerpos en la respuesta adaptativa (Boulanger y Wikel, 2021; García et al., 2010; Lambris et al., 2008; Ricklin et al., 2010).

La respuesta inmune adaptativa es altamente especifica y diferencia estructuras específicas de los AI; por ejemplo, los anticuerpos producidos por las células B neutralizan y marcan a los AI para su eliminación (respuesta humoral), mientas que las células T destruyen las células infectadas por patógenos y regulan otras células del sistema inmunológico, así como también ayudan en la activación de las células B para la producción de anticuerpos (respuesta celular) (Boulanger y Wikel, 2021; García et al., 2010; Lambris et al., 2008; Ricklin et al., 2010).

Las enfermedades transmitidas por garrapatas (TBD, por su sigla en inglés) son frecuentes en medicina humana y veterinaria, su diagnóstico y tratamiento son limitados, y prevenir estas enfermedades es complicado debido a la compleja cadena de transmisión entre hospedadores y garrapatas, en un entorno siempre cambiante (Dantas-Torres et al., 2012). En ese sentido, la perturbación antropogénica de las redes ecológicas, que afectan la dinámica poblacional de los hospederos vertebrados y las garrapatas, puede influir en la tasa de transmisión de las TBDs (Baneth, 2014).

Las enfermedades emergentes transmitidas por garrapatas circulan entre los animales silvestres antes de ser reconocidas como causantes de enfermedades clínicas en humanos (Baneth, 2014; Dantas-Torres et al., 2012). Debido a esto, conforme se descubren nuevos AI transmitidos por garrapatas, es crucial entender cómo se propagan entre sus hospederos, así como monitorear AI nuevos y conocidos (Goodman et al., 2005). Asimismo, es fundamental conocer las especies de garrapatas que actúan como vectores, los vertebrados que sirven de hospederos a los AI en una región específica, las enfermedades que causan y las fases del ciclo de vida de la garrapata que están involucradas. Estos elementos no solo pueden orientar la investigación y desarrollo de pruebas diagnósticas más específicas y efectivas; también son claves para mejorar la comprensión de la epidemiología de las TBDs, identificar áreas de riesgo

potencial de infección y comprender las dinámicas de transmisión. Asimismo, contribuyen a las estrategias de diagnóstico y tratamiento óptima, especialmente en pacientes con síntomas inespecíficos en áreas endémicas de las TBDs o donde ocurren las garrapatas que las trasmiten (Madison-Antenucci et al., 2020).

En los ciclos de transmisión y mantenimiento de las bacterias de los géneros de *Anaplasma* y *Borrelia*, así como de los protozoarios del género *Babesia*[1] se reconoce la participación fundamental de los roedores, ciervos y sus garrapatas como hospederos de estos agentes infecciosos (Atif, 2016; Cutler, 2015; e et al., 2017; Fanelli, 2021; Han et al., 2015; Hrazdilová et al., 2020; Kurokawa et al., 2020).

La diversificación evolutiva de los hospederos y garrapatas es la principal fuerza impulsora en la filogenia de estos AI transmitidos por garrapatas (AITG); esto se debe a que las relaciones filogenéticas de los AITG dependen de las adaptaciones moleculares divergentes que surgen debido a las presiones selectivas diferentes ejercidas por los hospederos y vectores con los que interactúan (Jalovecka et al., 2019; Oppler et al., 2022; Rar et al., 2021). Esta influencia se manifiesta en las estrechas relaciones filogenéticas de los AITG que están asociados a vectores y hospederos que son filogenéticamente cercanos (Hrazdilová et al., 2020; Jalovecka et al., 2019; Oppler et al., 2022; Rar et al., 2021).

Aunque en Chile existen los entornos epidemiológicos adecuados, es decir, la presencia de garrapatas, roedores y venados que podrían permitir la ocurrencia de los ciclos de estos agentes, actualmente se desconoce si estos agentes infecciosos ocupan este nicho ecológico en el país. Es así como el estudio de *Anaplasma*, *Borrelia* y *Babesia* en mamíferos silvestres en Chile está en sus primeras etapas de desarrollo, con investigación principalmente en las regiones del centro y sur del país (Di Cataldo et al., 2022, 2020; Llanos-Soto and González-Acuña, 2019).

Asimismo, las investigaciones sobre AITG se centran principalmente en las garrapatas, dejando de lado a los hospedadores, por ejemplo, mamíferos

[1]Se profundiza más sobre los ciclos epidemiológicos de los agentes bacterianos y protozoos pesquisados en esta sección en cada capítulo referido a cada uno de estos agentes.

(Ivanova et al., 2014; Muñoz-Leal et al., 2019b, 2019a; Thompson et al., 2021). Rodentia es el orden de mamíferos más diverso de Chile, con 69 especies, siendo la familia Cricetidae (cricétidos) la que alberga la mayor riqueza con 38 especies (Ministerio del Medio Ambiente, 2018). En particular, en Chile la mayoría de las garrapatas positivas a agentes infecciosos se han relacionado con cricétidos (Ivanova et al., 2014; Muñoz-Leal et al., 2019b, 2019a). Además, se ha encontrado que los cricétidos pueden ser más proclives que otros roedores para albergar patógenos emergentes con potencial zoonótico (Han et al., 2015).

Por otra parte, *Pudu puda* ha sido el único ciervo reportado con garrapatas infectadas con AITG en Chile (Verdugo et al., 2017). Este ciervo es generalmente parasitado por las garrapatas *Ixodes taglei* e *Ixodes stilesi* (Nava et al., 2017). A pesar de que este cérvido y sus garrapatas podrían estar involucrados en los ciclos de *Anaplasma*, *Borrelia* y *Babesia*, de manera similar a otras especies de ciervos parasitados por *Ixodes* spp. en el hemisferio norte (Atif, 2016; Cutler, 2015; Cutler et al., 2017; Fanelli, 2021; Han et al., 2015; Hrazdilová et al., 2020; Kurokawa et al., 2020), hasta ahora solo se ha investigado y detectado *Borrelia* en *I. stilesi* (Verdugo et al., 2017).

En Chile, la mayoría de las detecciones de *Borrelia* se han realizado en garrapatas del género *Ornithodoros* (Ivanova et al., 2014; Muñoz-Leal et al., 2019b, 2019a; Thompson et al., 2021); sin embargo, aún no se han realizado estudios experimentales que confirmen la competencia de estas garrapatas como vectores de estas espiroquetas. Las espiroquetas del grupo de borrelias de fiebre recurrente (GFR) del género *Borrelia* proliferan en ciclos enzoóticos en los que participan *Ornithodoros* spp. (Argasidae) y roedores, principalmente (Barbour and Gupta, 2021).

Para comprobar competencia vectorial frente a estas bacterias, en Brasil se han implementado modelos experimentales utilizando roedores nativos para alimentar garrapatas del género *Ornithodoros* y detectar espiroquetas en sangre de los roedores infectados (Muñoz-Leal et al., 2018; Oliveira et al., 2023). La implementación de este tipo de estudios en el país es fundamental para conocer cuáles de las 11 especies de *Ornithodoros* presentes en Chile (González-Acuña

and Guglielmone, 2005; Muñoz-Leal et al., 2023, 2020, 2016) podrían ser vectores competentes para las especies de *Borrelia*.

Considerando lo expuesto, el objetivo principal de esta investigación fue describir la diversidad genética, relaciones evolutivas y potenciales vectores y hospederos de *Anaplasma*, *Borrelia* y *Babesia* en roedores cricétidos, *P. puda* y dos especies de garrapatas (*I. stilesi* y *O. octodontus*). Para ello, se llevó a cabo una búsqueda de estos agentes en roedores de la familia Cricetidae, en *P. puda* y en *I. stilesi* que parasitaban estos ciervos. Además, en el caso de *Borrelia*, se evaluó la competencia vectorial de la garrapata *O. octodontus* utilizando conejillos de india (*Cavia porcellus*) como modelo experimental.

Hipótesis del trabajo

1. Los roedores cricétidos, el ciervo *Pudu puda* y la garrapata *Ixodes stilesi* en Chile son hospederos potenciales de *Anaplasma*, *Borrelia* y *Babesia.*
2. Las secuencias de ADN de los microorganismos identificados en roedores cricétidos, *P. puda* y en *I. stilesi* presentan similitudes filogenéticas significativas con secuencias de microorganismos previamente identificados en otras especies de roedores, ciervos y sus respectivas garrapatas.
3. La garrapata *Ornithodoros octodontus* actúa como vector de *Borrelia* sp. del grupo Fiebre recurrente.

Objetivos

Objetivo general I

Pesquisar y evaluar la diversidad genética y posición filogenética de microorganismos transmitidos por garrapatas (*Anaplasma*, *Borrelia* y *Babesia*) en hospederos silvestres (roedores cricétidos, el ciervo *Pudu puda* y la garrapata *Ixodes stilesi*) en Chile.

Objetivo general II

Evaluar la competencia vectorial de garrapatas de la familia Argasidae presentes en Chile en la transmisión de *Borrelia*.

Objetivos específicos

1. Pesquisar el ADN de microorganismos de los géneros *Anaplasma*, *Borrelia* y *Babesia* en roedores (Cricétidos), el ciervo *Pudu puda* y la garrapata *Ixodes stilesi* en Chile y caracterizar molecularmente los agentes infecciosos encontrados.
2. Inferir la posición filogenética de los microorganismos detectados y su relación con agentes transmitidos por garrapatas previamente identificados.
3. Evaluar la competencia vectorial de la garrapata *Ornithodoros octodontus* en la transmisión de borrelias del grupo de fiebre recurrente en *Cavia porcellus.*

Estructura del documento

Los resultados de este estudio se han organizado para su presentación en cinco manuscritos incorporados en tres capítulos que incluyen un resumen y los artículos científicos que se desprenden de los objetivos desarrollados, adicionalmente se incluyen las conclusiones generales de la tesis. Los dos primeros capítulos corresponden a las hipótesis y objetivos específicos 1 y 2, y se centran en la detección, caracterización molecular e inferencia filogenética de *Anaplasma*, *Borrelia* y *Babesia* en roedores, el ciervo *P*. *puda* e *I*. *stilesi* en Chile; resultados que se encuentran publicados en revistas científicas internacionales indexadas en Web of Science. El tercer capítulo corresponde a la hipótesis y objetivo específico 3, y se enfoca en la evaluación experimental de la competencia vectorial de una especie de garrapata blanda (*O*. *octodontus*) en la transmisión de *Borrelia* sp. en el roedor *C. procellus*. Este estudio ya ha sido aceptado para su publicación a la revista "Experimental and Applied Acarology". Los artículos científicos incluidos en cada capítulo se detallan a continuación:

CAPÍTULO I: Estudio de *Anaplasma*, *Borrelia* y *Babesia* en roedores cricétidos de Chile.

Thomas, R; **Santodomingo, AM**; Muñoz-Leal, S; Silva-de la Fuente, MC; Llanos-Soto, S; Moreno-Salas, L; González-Acuña, D. Rodents as potential reservoirs for *Borrelia* spp. in northern Chile. **Rev Braz J Vet Parasitol** 2020; 29(2): e000120. https://doi.org/10.1590/S1984-29612020029

Santodomingo, A; Thomas, R; Quintero-Galvis JF, Echeverry-Berrio, D; Silva-de la Fuente, M; Moreno-Salas, L; Muñoz-Leal, S. Apicomplexans in small mammals from Chile, with the first report of the *Babesia microti* group in South American rodents. **Parasitol Res** 2022; 121(3): 1009-1020. https://doi.org/10.1007/s00436-022-07452-4

CAPÍTULO II: Estudio de *Anaplasma*, *Borrelia* y *Babesia* en el ciervo *Pudu puda* e *Ixodes stilesi* en el sur de Chile.

Santodomingo, A; Robbiano, S; Thomas, R; Parragué-Migone, C; Cabello-Stom, J; Vera-Otarola, F; Valencia-Soto, C; Moreira-Arce, D; Moreno, L; Hidalgo-Hermoso, E; Muñoz-Leal, S. A search for piroplasmids and spirochetes in threatened pudu (*Pudu puda*) and associated ticks from Southern Chile unveils a novel *Babesia* sp. and a variant of *Borrelia chilensis*. **Transbound Emerg Dis** 2022. https://doi.org/10.1111/tbed.14743

Santodomingo, A; Thomas, R; Robbiano, S et al. Wild deer (*Pudu puda*) from Chile harbor a novel ecotype of *Anaplasma phagocytophilum*. **Parasites Vectors** 2023; 16, 38. https://doi.org/10.1186/s13071-023-05657-9.

CAPÍTULO III: Transmisión experimental de una nueva *Borrelia* relacionada con *Ornithodoros octodontus* (Ixodida: Argasidae) en Chile

Experimental transmission of a novel relapsing fever group *Borrelia* harbored by *Ornithodoros octodontus* (Ixodida: Argasidae) in Chile. Submitted to **Exp Appl Acarol**.

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CAPÍTULO I: Estudio de *Anaplasma*, *Borrelia* y *Babesia* en roedores cricétidos de Chile

Resumen

En los ciclos de transmisión y mantenimiento de las especies de *Anaplasma*, *Borrelia* y *Babesia*, se reconoce la participación fundamental de los roedores. En el hemisferio norte, se ha identificado a diferentes especies de roedores como hospederos de estos agentes infecciosos. Sin embargo, en el caso de los roedores sudamericanos que albergan estos parásitos, la información disponible es escasa. A pesar de la relevancia de los roedores para el mantenimiento de las infecciones por *Anaplasma*, *Borrelia* y *Babesia* en otros lugares, su papel como reservorios no se ha investigado exhaustivamente en Chile. Probablemente se deba a que la mayoría de los estudios previos han priorizado el análisis de las garrapatas en lugar de explorar posibles reservorios vertebrados silvestres.

Los mamíferos silvestres de Chile son principalmente del orden Rodentia, dentro de este grupo, la familia Cricetidae se destaca como la que alberga la mayor riqueza. Los cricétidos pueden presentar una mayor propensión que otros tipos de roedores a albergar agentes emergentes con potencial zoonótico. En este contexto, es crucial identificar los reservorios vertebrados de estos agentes para comprender sus ciclos de transmisión en los ecosistemas chilenos.

Con el objetivo de ampliar el conocimiento sobre la diversidad genética, relaciones evolutivas de especies de *Anaplasma*, *Borrelia* y *Babesia* en roedores cricétidos en Chile, en este estudio, se llevó a cabo un análisis molecular para investigar la presencia de estos agentes infecciosos en la sangre y los órganos (bazo e hígado) de 420 roedores cricétidos pertenecientes a 13 especies, recolectados en 22 localidades de Chile. Ninguno de los roedores cricétidos analizados resulto positivo a *Anaplasma.* Mientras que, a partir de tres ejemplares pertenecientes a dos especies de roedores (*Phyllotis xanthopygus* y *Oligoryzomys longicaudatus*), se obtuvieron tres genotipos de *Borrelia* para el gen *flaB* y dos para IGS. Los análisis filogenéticos, inferidos utilizando métodos

bayesianos (IB) y de máxima verosimilitud (MV), revelaron que las secuencias generadas en este estudio se agrupan con *Borrelia* spp. del grupo de fiebre recurrente (FR) y grupo borreliosis de Lyme (BL).

Los resultados de las filogenias BI y MV para IGS también indican que las secuencias de *Borrelia* sp. A10 y *Borrelia* sp. A44 representan taxones nuevos y están estrechamente relacionadas con una espiroqueta (*Borrelia* sp. 95325, HM583797) encontrada de un *Ornithodoros* sp. Recolectado en Bolivia. (ver artículo Rodents as potential reservoirs for *Borrelia* spp. In northern Chile).

Por otro lado, las secuencias del gen *flaB* de *Borrelia*, recuperadas de la sangre de *O. longicaudatus* (*Borrelia* sp. A53), se ramificaron como genotipos independientes dentro del grupo BL y se relacionaron con *B. chilensis* (CP009910), una genoespecie previamente reportada en *Ixodes stilesi*.

Los análisis filogenéticos realizados con secuencias derivadas de los genes 18S y citocromo c oxidasa (COI) proporcionaron evidencia de la presencia de una *Babesia* sp. (*Babesia* sp. LC77), perteneciente al grupo *Babesia microti*, en *P. darwini*. Además, se identificó un genotipo nuevo de *Babesia* (*Babesia* sp. LC87) en *P. darwini* y *Abrothrix jelskii*. Estos hallazgos, en conjunto con registros previos de *B. microti* en Bolivia, sugieren que *B. microti* podría estar subrepresentada en América del Sur y que quizás circulan cepas patógenas para los humanos que aún no han recibido suficiente atención.

En forma interesante, *Babesia* sp. LC87 se agrupa filogenéticamente con una serie de babesias recientemente descritas asociadas con murciélagos y roedores de Brasil. Es relevante destacar que las secuencias obtenidas son relativamente cortas, y análisis basados en secuencias más extensas de 18S podrían conducir a una topología de árbol diferente. No obstante, estos resultados son respaldados por filogenias derivadas de estudios anteriores, lo que sugiere la existencia de un linaje sudamericano de *Babesia* spp. (ver artículo Apicomplexans in small mammals from Chile, with the first report of the *Babesia microti* group in South American rodents)

Este informe constituye el primer registro de infección por *Borrelia* spp. y *Babesia* spp. en roedores en Chile. Los resultados sugieren que los roedores *P. xanthopygus* y *O. longicaudatus* así como *P. darwini* y *A. jelskii,* podrían desempeñar un papel como potenciales reservorios de estos genotipos de *Borrelia* y *Babesia* respectivamente en ecosistemas naturales de Chile. No obstante, se requieren investigaciones futuras para determinar la competencia de estos roedores en el mantenimiento de las infecciones causadas por estos agentes. Además, es esencial investigar si otras especies de pequeños mamíferos también participan en los ciclos enzoóticos de estos microorganismos.

A continuación, se presentan dos artículos que detallan los resultados publicados de este capítulo. Estos artículos corresponden al análisis inicial realizado en las muestras de roedores del norte y centro del país. Sin embargo, es importante señalar que las muestras del sur, obtenidas posteriormente, arrojaron resultados negativos para todos los agentes infecciosos, por lo que se excluyeron de la publicación. Asimismo, cabe destacar que solo los resultados de *Babesia* que se encuentran en el artículo titulado "Apicomplexans in small mammals from Chile, with the first report of the *Babesia microti* group in South American rodents" son los que se abordan en esta tesis. Los resultados relacionados con *Hepatazoon* y Sarcocystidae corresponden a una tesis de doctorado que se desarrolló simultáneamente a ésta. Las evidencias sobre *Hepatozoon* y Sarcocystidae se incluyeron en la misma publicación debido a que se obtuvieron a partir de las mismas muestras.

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Original Article

Rodents as potential reservoirs for *Borrelia* spp. in northern Chile

Roedores como potenciais reservatórios de *Borrelia* spp. no norte do Chile

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Abstract

Small mammals play an essential role in the transmission and maintenance cycles of *Borrelia* spirochetes. In Chile, recent studies have characterized novel *Borrelia* genotypes in ticks collected from small mammals, a fact that suggests these vertebrates are hosts for spirochetes from this genus. Considering this evidence, the goal of this study was to determine the presence of *Borrelia* DNA in small mammals inhabiting northern Chile. In winter of 2018, 58 small mammals were captured in five localities. Blood samples were collected from rodents and DNA was extracted to determine the presence of *Borrelia* DNA by PCR targeting the *flaB* gene and *rrs–rrlA* intergenic spacer (IGS). From three individuals (5%), belonging to two rodent species of Cricetidae family (*Phyllotis xanthopygus* and *Oligoryzomys longicaudatus*), we retrieved three *flaB* and two IGS *Borrelia* genotypes. Phylogenetic analyses performed with both Maximum Likelihood and Bayesian inferences showed that our sequences grouped with homologous genotypes from the relapsing fever and Lyme borreliosis groups. Our findings suggest that *P. xanthopygus* and *O. longicaudatus* rodents may play a role as reservoirs for borrelial spirochetes in Chile.

Keywords: *Borrelia*, infectious diseases, small mammals, reservoirs, rodent, Chile.

Resumo

Pequenos mamíferos possuem um papel essencial na transmissão e manutenção de espiroquetas do gênero *Borrelia*. No Chile, estudos recentes têm descrito novos genótipos de *Borrelia* em carrapatos, parasitando pequenos mamíferos. Isso sugere que esses vertebrados podem atuar como possíveis reservatórios dessas espiroquetas. Considerando-se essa evidência, o objetivo deste estudo foi determinar a presença de DNA de *Borrelia* em pequenos mamíferos da região norte do Chile. Durante o inverno de 2018, 58 pequenos mamíferos foram capturados em cinco localidades. Amostras de sangue obtidas a partir dos indivíduos capturados foram submetidas à extração de DNA e ensaios de PCR, para a detecção de *Borrelia* spp. baseados no gene *flaB* e espaçador intergênico *rrs–rrlA* (IGS). A partir de três espécimes (5%) pertencentes a duas espécies de roedores da família Cricetidae (*Phyllotis xanthopygus* e *Oligoryzomys longicaudatus*) obtiveram-se três genótipos de *Borrelia* para o gene *flaB* e dois para IGS. Análises filogenéticas inferidas, usando-se os métodos Bayesiano e de Máxima Verossimilhança, indicaram que as sequências geradas neste estudo agrupam-se com borrelias do grupo da Febre Recorrente e Borreliose de Lyme. Os achados deste estudo sugerem que roedores *P. xanthopygus* e *O. longicaudatus* poderiam atuar como possíveis reservatórios para *Borrelia* spp. no Chile.

Palavras-chave: *Borrelia*, doenças infecciosas, pequenos mamíferos, reservatórios, roedor, Chile.

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Introduction

In Chile, studies on bacterial infections in small mammals have been performed mostly in the central and southern regions of the country (Müller et al., 2018; Llanos-Soto & González-Acuña, 2019). In the northern region of Chile, research regarding bacterial infection on mammals consists in three surveys focusing on *Escherichia* and *Salmonella* of marine vertebrates (*Otaria flavescens*) (Salinas et al., 2010; Sturm et al., 2011; Toro et al., 2015). Data on vector-borne bacterial pathogens transmitted by mites or ticks are absent for this region of the country.

Rodentia is among the most diverse mammal order in Chile, with 69 species distributed along the country (MMA, 2018). This group of vertebrates plays an important role in the maintenance and propagation of tick-borne pathogens (bacterial, protozoan and viral) in urban and natural environments (Llanos-Soto & González-Acuña, 2019). Globally, rodents act as hosts for tick populations and serve as reservoirs for zoonotic pathogenic agents, such as *Borrelia* species (Cutler, 2015; Cutler et al., 2017).

Spirochetes in the genus *Borrelia* merge their transmission cycles with vertebrates and their associated ticks in wild ecosystems (Kurtenbach et al., 1995; Talagrand-Reboul et al., 2018). For instance, in the Northern Hemisphere, human-pathogenic *Borrelia burgdorferi* sensu lato (s.l.) spirochetes use rodents and hard ticks (Acari: Ixodidae) of the *Ixodes ricinus* species complex as reservoirs and vectors, respectively, and are the causative agents of Lyme borreliosis (LB) in humans (Donahue et al., 1987; Kurtenbach et al., 1995; Hazler & Ostfeld, 1995; Rauter & Hartung, 2005). Moreover, small mammals and some species of soft ticks (Acari: Argasidae) of the *Ornithodoros* genus maintain natural foci of relapsing fever (RF) borreliae in tropical and subtropical ecosystems in both hemispheres (Talagrand-Reboul et al., 2018).

Despite their importance for the maintenance of *Borrelia* infections elsewhere, the role of rodents as sylvatic reservoirs for this genus of spirochetes has not been properly addressed in Chile, since most studies have focused on vectors rather than potential vertebrate reservoirs. For instance, only one valid genospecies, *Borrelia chilensis,* has been identified in *Ixodes stilesi* ticks collected from the environment, and from long-tailed pygmy rice rats (*Oligoryzomys longicaudatus*) (Ivanova et al., 2014). In this case, although ticks were infected with *B. chilensis*, this does not necessarily mean that *O. longicaudatus* were carrying the spirochetes, since positive nymphs could have acquired the bacterium through a previous blood meal (Guttman et al., 1996). A similar scenario encompasses the recent findings of novel *Borrelia* genotypes in *Ixodes sigelos* s.l. group, and an *Ornithodoros* sp. closely related with *Ornithodoros atacamensis* in northern Chile (Muñoz-Leal et al., 2019a, b), for which their associated hosts, i. e. rodents of genus *Phyllotis*, were not assessed for *Borrelia* infection (Muñoz-Leal et al., 2019a). In this context, to elucidate the identity of vertebrate reservoirs for *Borrelia* is still a crucial step to understand transmission cycles of these bacteria in Chilean ecosystems. In this study, we aimed to assess the role of rodents and marsupials from northern Chile as potential reservoirs for *Borrelia* spp. through molecular analyses performed in blood obtained from these mammals.

Material and Methods

Study area

This study surveyed rodents in five localities belonging to hyper-arid hydrographic regions from northern Chile (Figure 1) during July (Austral winter) of 2018. Hyper-arid hydrographic region in Chile is characterized by having an annual precipitation and potential evapotranspiration ratio <0.05; the annual precipitation does not exceeds 100 mm, presenting an annual water deficit higher than 1200 mm; and dryness prevails throughout the year with a short peak of humidity that lasts one month (MMA, 2018).

Sample collection

Rodents and marsupials were captured using Sherman-like traps. Eighty traps remained active during two consecutive nights (10 hours per night) in each locality, and were placed along four parallel lines distanced approximately 100 m from each other, with 20 traps per line (spaced 10 m between each other). Animal handling was performed according to protocols used in field and laboratory studies on rodents (Herbreteau et al., 2011). Fifty microliters of blood were collected from each captured rodent through puncture of the caudal ventral vein and stored in sterile tubes with 96% ethanol (Sigma-Aldrich®). All rodents were identified to the species level using a taxonomic guide (Iriarte, 2008), and after blood collection, were released in the same place of capture.

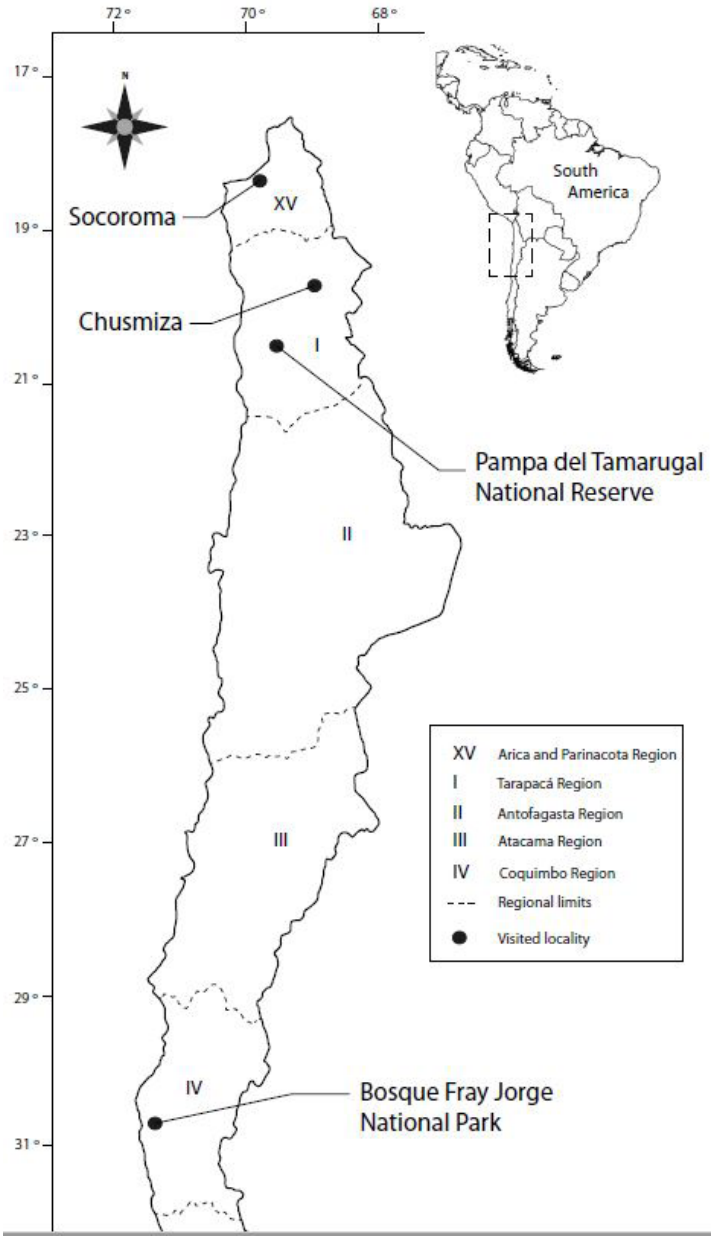

Figure 1. Map of northern Chile with the locations (black dots) were small mammals were captured.

Authorization for small mammal captures was granted by the Servicio Agrícola y Ganadero (SAG; Resolution N° 1532/2019 and 9071/2018). Field work in national parks and reserves was authorized by the Corporación Nacional Forestal (CONAF; Permits 39/2018; 67/2019; 05/2018; 76/2018; 66/2018). All procedures were approved and carried out according to the Bioethics Committee of the School of Veterinary Sciences, Universidad de Concepción (Form CBE-19-2017).

DNA extraction and gene amplification

DNA was extracted from blood using the DNeasy Blood & Tissue Kit (QUIAGEN, GERMANY). Forty microliters of Buffer AE (10 mM Tris-Cl; 0.5 mM EDTA, pH 9.0) were used to suspend the final DNA yield. To test successful extractions, and rule out the presence of PCR inhibitors, DNA quantity (concentration) and quality (purity and integrity) of DNA was assessed by A_{260} / A_{280} absorbance (A) in each sample using an Epoch™ Microplate Spectrophotometer. Samples with an A_{260} / A_{280} DNA ratio ranging between 1.6–2.0 were considered pure, and suitable for PCR amplification (Khare et al., 2014). Additionally, samples were tested through conventional PCR targeting endogenous *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) gene as an internal control following Birkenheuer et al. (2003).

Successfully extracted samples were screened for *Borrelia* flagellin gene (*flaB)*, and *rrs–rrlA* intergenic spacer (IGS) through nested PCR protocols, using primers listed in Table 1. Reactions were performed into a final volume of 25 µL containing 12.5 µL of Dream Taq Green PCR Master Mix (Thermo Scientific, USA), 1 µL of each primer (10 pmol), 2 µL of DNA for conventional PCR, 1 µL of the product for nested rounds, and ultra-pure water to complete the final volume of the mix. Amplicons were verified in 1.5% agarose gels stained with SYBR Safe (Life Technologies/Thermo Fisher Scientific, Carlsbad, CA), and visualized through UV light. Positive samples were purified and sequenced in both directions at AUSTRAL-omics (Valdivia, Chile).

Table 1. List of primers used for the detection and characterization of *Borrelia* DNA in this study.

Target	Primer	Sequence	Expected length (bp)	Reference
Flagelin gene fragments (*flaB*)	FlaLL	5´-ACATATTCAGATGCAGACAGAGGT-3´	658	(Stromdahl et al., 2003)
	FlaRL	5´-GCAATCATAGCCATTGCAGATTGT-3´		
	FlaLS	5´-AACAGCTGAAGAGCTTGGAATG-3´	354	
	FlaRS	5´-CTTTGATCACTTATCATTCTAATAGC-3´		
rrs–rrlA intergenic spacer (IGS)	IGS-F	5´-GTATGTTTAGTGAGGGGGGTG-3´	987	(Bunikis et al., 2004)
	IGS-R	5´-GGATCATAGCTCAGGTGGTTAG-3´		
	IGS-Fn	5´-AGGGGGGTGAAGTCGTAACAAG-3´	945	
	IGS-Rn	5´-GTCTGATAAACCTGAGGTCGGA-3´		

Phylogenetic analyses

The obtained sequences were edited with ProSeq Version (V) 3 (Filatov, 2009), compared using BLASTn (https://blast.ncbi.nlm.nih.gov), and aligned with records from the NCBI database using the ClustalW algorithm (Thompson et al., 1994) implemented in MEGA 7.0 (Kumar et al., 2016). Alignments for *flaB* gene and IGS were used to construct both Bayesian Inference (BI) and Maximun Likelihood (ML) trees with MrBayes 3.2.2. (Ronquist et al., 2012), and IQ-TREE v1.6.12 (Nguyen et al., 2015), respectively. We chose these methods because they are based on models of molecular evolution (Huelsenbeck et al., 2001; Felsenstein, 2004). Evolutionary models for BI were selected using MrBayes 3.2.2, employing the option "lset nst=mixed rates=gamma", and the Bayesian Information Criterion (BIC) (Schwarz, 1978). ModelFinder (Kalyaanamoorthy et al., 2017) with the option "-m MFP+MERGE", and the BIC were employed to select best evolutionary models for the ML analysis (Schwarz, 1978).

BI was performed with two independent tests of 10^7 generations, running four MCMC chains, sampling trees every 1000 generations, and discarding the first 25% as burn-in. MCMC chain correlation was confirmed with Tracer v1.7.1 (Rambaut et al., 2018). Statistical support of internal nodes was evaluated employing Bayesian posterior probabilities (BPPs) and considering values ≥0.70 as strong support (Huelsenbeck & Ronquist, 2001). The ML analysis was carried using rapid hill-climbing and stochastic disturbance methods, evaluating the robustness of the inferred tree with 1000 pseudo-replicates of ultrafast bootstrapping. We used the criteria of Minh et al. (2013) to evaluate the ultrafast bootstrap: values <70% were considered non-significant statistical support; values between 70-94% as moderately significant; and values ≥ 95% as highly significant.

Results

Positive animals and PCR

A total of 58 small mammals belonging to 12 species in the families Cricetidae, Muridae and Didelphidae were captured (Table 2). Although DNA purity obtained after measurements of A_{260} / A_{280} absorbance ratio was optimal in 56/58 of the samples (97%), five samples (including the two with low A_{260} / A_{280} absorbance ratio) were negative after GAPDH gene PCR and excluded from further analyses. Three out of 53 rodents (5%) were positive for *Borrelia flaB* screening, and two of these samples were positive for IGS (4%) (Table 2).

Table 2. Number and identity of small mammals captured in Northern Chile. Specimens positive for *Borrelia* by PCR assays are highlighted in bold. Abbreviations: BFJNP, Bosque Fray Jorge National Park; Ch, Chusmiza; Par, Parinacota; PTNR, Pampa del Tamarugal National Reserve; Soc, Socoroma.

Order	Family	Species	Locality	Geographic coordinates	No. Positive/ No. Capture
Didelphimorphia	Didelphidae	*Thylamys elegans*	BFJNP	30°39'07.07"S, 71°41'09.44"W	0/2
Didelphimorphia	Didelphidae	*Thylamys pallidor*	Soc	18°16'44.30"S, 69°35'28.40"W	0/1
Rodentia	Cricetidae	*Abrothrix andinus*	Soc	18°12'00.00"S, 69°16'00.12"W	0/1
Rodentia	Cricetidae	*Abrothrix andinus*	Par	18°12'00.00"S, 69°16'00.12"W	0/4
Rodentia	Cricetidae	*Abrothrix berlepschii*	Par	18°12'00.00"S, 69°16'00.12"W	0/1
Rodentia	Cricetidae	*Abrothrix berlepschii*	Soc	18°16'44.30"S, 69°35'28.40"W	0/4
Rodentia	Cricetidae	*Abrothrix jelskii*	Par	18°12'00.00"S, 69°16'00.12"W	0/1
Rodentia	Cricetidae	*Abrothrix longipilis*	BFJNP	30°39'07.07"S, 71°41'09.44"W	0/1
Rodentia	Cricetidae	*Abrothrix olivacea*	BFJNP	30°39'07.07"S, 71°41'09.44"W	0/3
Rodentia	**Cricetidae**	***Oligoryzomys longicaudatus***	**BFJNP**	**30°39'07.07"S, 71°41'09.44"W**	**1/1**
Rodentia	Cricetidae	*Phyllotis limatus*	Soc	18°16'44.30"S, 69°35'28.40"W	0/1
Rodentia	Cricetidae	*Phyllotis magister*	Soc	18°16'44.30"S, 69°35'28.40"W	0/1
Rodentia	Cricetidae	*Phyllotis xanthopygus*	Ch	19°41'01.27"S, 69°11'53.05"W	0/4
Rodentia	Cricetidae	*Phyllotis xanthopygus*	PTNR	20°28'14.03"S, 69°40'25.16"W	0/4
Rodentia	Cricetidae	*Phyllotis xanthopygus*	Par	18°12'00.00"S, 69°16'00.12"W	0/5
Rodentia	**Cricetidae**	***Phyllotis xanthopygus***	**Soc**	**18°16'44.30"S, 69°35'28.40"W**	**2/16**
Rodentia	Muridae	*Mus musculus*	Soc	18°16'44.30"S, 69°35'28.40"W	0/2
Rodentia	Muridae	*Rattus rattus*	PTNR	20°28'14.03"S, 69°40'25.16"W	0/1
Total					**3/53**

Three different genotypes were obtained for *flaB* gene. Two *flaB* sequences of 304 bp (99.67% of identity between them) were retrieved from blood of two *Phyllotis xanthopygus* collected in Socoroma (named as *Borrelia* sp. A10 and *Borrelia* sp. A44). BLASTn comparisons revealed that *flaB* sequences for *Borrelia* sp. A10 and *Borrelia* sp. A44 were 97.48% (271/278 bp, 91% query cover, 0 gap, 5e-130 E-value) and 97.12% (270/278 bp, 91% query cover, 0 gap, 2e-128 E-value) identical with *Borrelia* sp. 95325 (HM583797) characterized from undetermined *Ornithodoros* sp. from Bolivia (Parola et al., 2011), respectively. On the other hand, a different genotype of *flaB* gene (307 bp) was obtained from one *O. longicaudatus* captured in Bosque Fray Jorge National Park. After BLASTn comparisons, this sequence (named as *Borrelia* sp. A53) was 98.70% (303/307 bp, 94% query cover, 0 gap, 3e-151 E-value) identical to *Borrelia* spp. characterized from ticks belonging to *I. sigelos* group in Chile (MH187987 and MH178397; Muñoz-Leal et al., 2019a). The sequences of *flaB* from *Borrelia* sp. A10, A44, and A53 were deposited in GenBank under accession numbers MN596012, MN596013, and MN596014, respectively.

Two different IGS sequences were obtained from the same samples of *P. xantophygus* positive for *flaB* gene. Sequences were 96.49% identical between them and 94.36% identical (202/214 bp, 41% query cover, 3 gaps, 8e-85 E-value) to *Borrelia* sp. TM (DQ000283; referred as "cf. *Borrelia crocidurae*" amplified from ticks). IGS sequences from *Borrelia* sp. A10 and A44 were deposited in Genbank under accession numbers MN598782 and MN598783, respectively.

Phylogenetic analyses

Overall, BI and ML phylogenetic trees for *flaB* and IGS depicted similar and well-supported logic topologies, grouping *Borrelia* spp. into LB and RF groups. In particular, phylogenetic analyses for *flaB* gene positioned our sequences within a clade with *Borrelia* sp. 95325 (HM583797) having statistically significant support (BPP=1

in Figure 2A, and Bootstrap=100 in Figure 2B). For the BI analysis, the clade composed by our sequences and *Borrelia* sp. 95325 formed a clade with high statistical support (BPP=0.98 in Figure 2A) with *Borrelia latyschewii* (JF708952), *Borrelia microti* (JF708951), *Borrelia duttonii* (NC011229), *Borrelia recurrentis* (CP000993), *Borrelia crocidurae* (CP004267), *Borrelia hispanica* (MF432465), and *Borrelia persica* (NZAYOT01000225). However, the internal relations of this clade were undefined. On the other hand, with high support (Bootstrap=97 in Figure 2B), the ML analysis supported *Borrelia* sp. A10 and A44 as independent branches into a monophyletic group with *Borrelia* sp. 953225 (HM583797). On the other hand, and with high statistical support (BPP= 0.84 in Figure 2A, and Bootstrap= 90 in Figure 2B), *Borrelia* sp. A53 clustered with *Borrelia* genotypes characterized from ticks belonging to the *I. sigelos* group (MH187987, MH178397), with *B. chilensis* VA1 (CP009910), and *Borrelia* sp. ISIG1 (KX417768) obtained from *I.* cf. *neuquenensis,* and *I. sigelos* from Argentina as sister groups (Figure 2).

With high statistical support (BBP= 1 in Figure 3A, and Bootstrap= 96 in Figure 3B), phylogenetic analyses for IGS sequences showed that *Borrelia* sp. A10 and *Borrelia* sp. A44 form an independent clade related to *Borrelia* spp. belonging to the relapsing fever group (Figure 3).

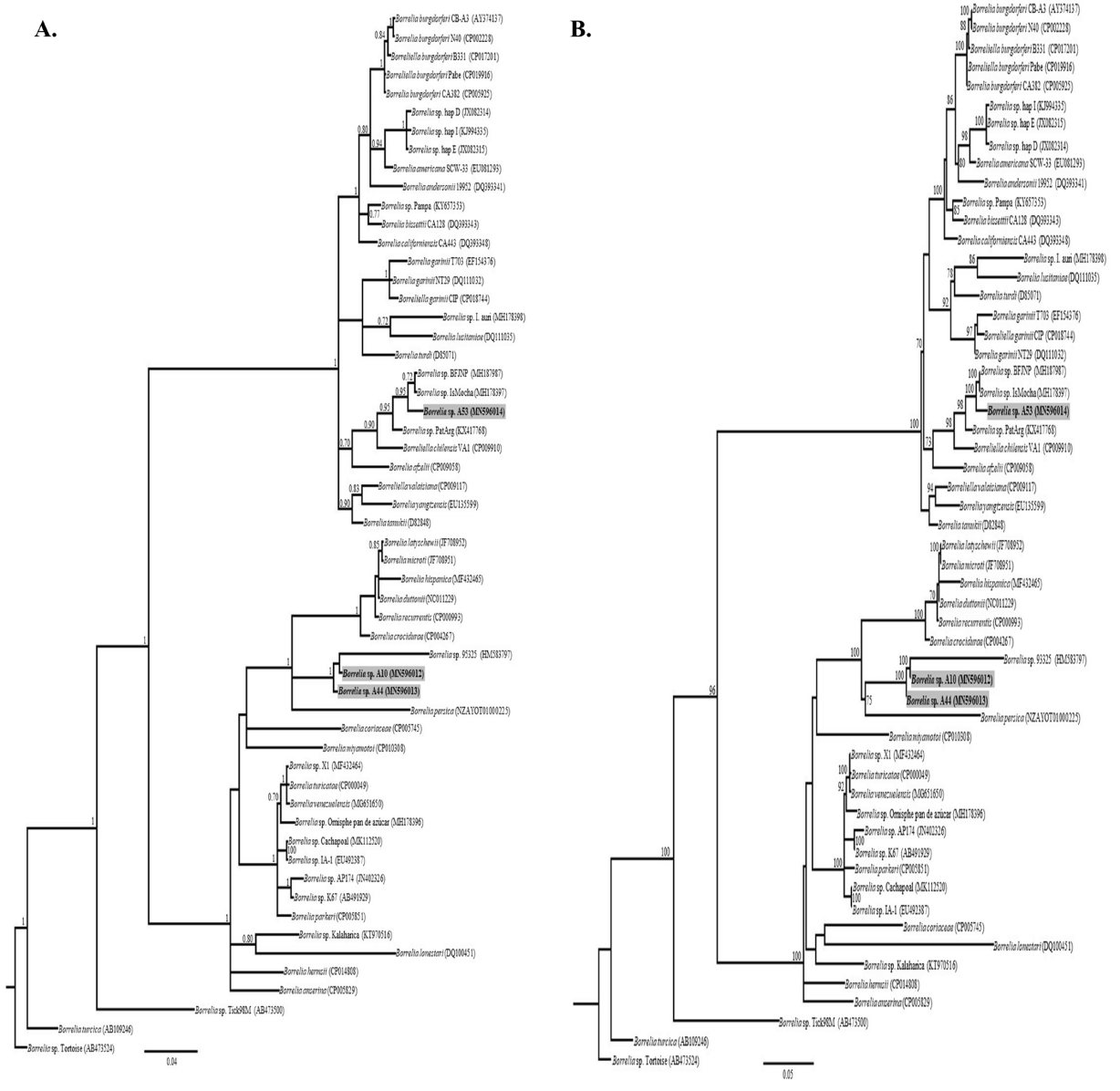

Figure 2. Bayesian (A) and Maximum Likelihood (B) phylogenetic trees for *Borrelia flaB* gene (alignment length, 657 bp). Otherwise omitted, numbers above branches represent BPP and bootstrap values ≥ 0.70 and ≥ 70%, respectively. GenBank accession numbers for the sequences included in the analyses are embedded in each tree. The position of *Borrelia* sp. A10, A44, and A53 are highlighted in bold with a gray background.

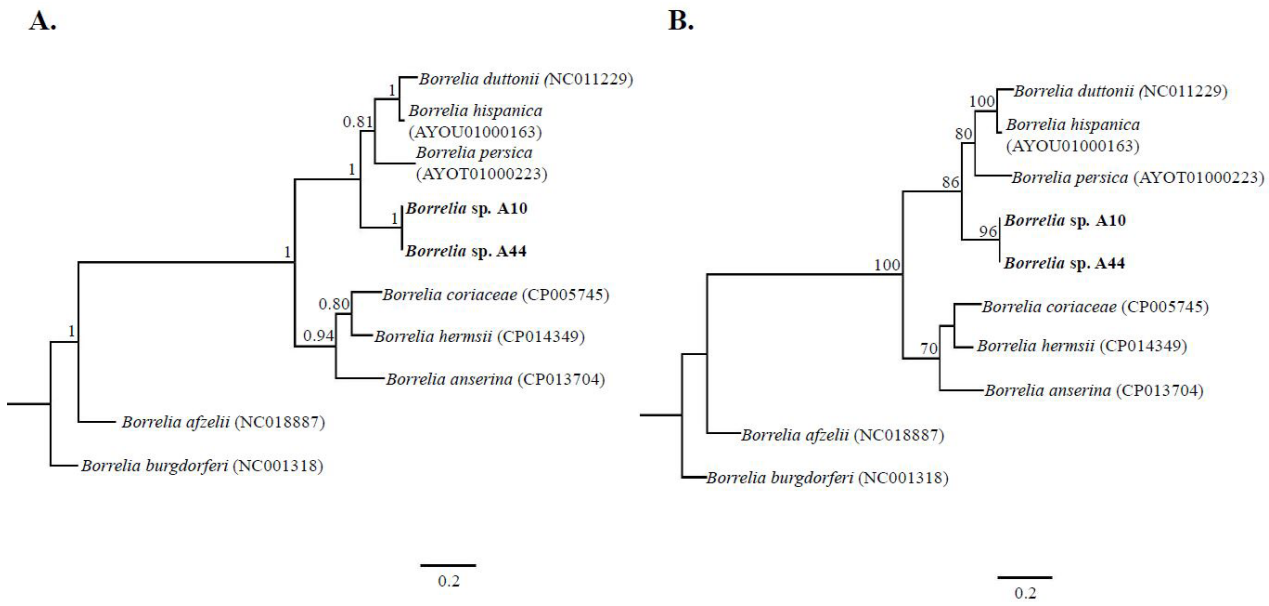

Figure 3. Bayesian (A) and Maximum Likelihood (B) phylogenetic trees for *Borrelia* IGS sequences gene (alignment length, 713 bp). Otherwise omitted, numbers above branches represent BPP and bootstrap values ≥ 0.70 and ≥ 70%, respectively. GenBank accession numbers for the sequences included in the analyses are embedded in each tree. The position of *Borrelia* sp. A10, and A44 are highlighted in bold with a gray background.

Discussion

The identification of wild vertebrate reservoirs implicated in the maintenance of pathogenic agents should be considered a permanent task in scientific research (Karesh et al., 2012). Rodents are important sylvatic reservoirs, as at least 217 out of 2777 known species harbor 66 zoonotic agents (Han et al., 2015). Considering this scenario, and the recent detection of *Borrelia* spp. in rodent-associated ticks in Chile, we aimed to assess the presence of *Borrelia* DNA in 12 species of small mammals (ten rodents and two marsupials) from this country. We detected DNA of *Borrelia* spp. belonging to the LB and RF groups in two cricetid rodents, namely *P. xanthopygus* and *O. longicaudatus*, respectively. While cricetid rodents (i. e. *Peromyscus leucopus*) have been previously reported as competent reservoirs for *Borrelia burgdorferi* sensu stricto (s.s.) in North America (Levine et al., 1985; Hofmeister et al., 1999; Bunikis et al., 2004), high prevalence for *Borrelia burgdorferi* s.l. have been reported in synanthropic murid rodents (*Mus musculus* and *Rattus rattus*) too (Solís-Hernández et al., 2016). In our study, synanthropic *M. musculus* were negative to *Borrelia* detection, a fact that could be attributed to the small sample of this species that was analyzed.

Apart from rodents, we assessed blood from marsupials, but with negative results. Nevertheless, *Borrelia*-like spirochetes have already been isolated from opossums (Marsupialia) in the United States (Hanson, 1970), so the role of Chilean marsupials as hosts for *Borrelia* spp. should not be discarded. Studies focusing on small mammals and enzootic cycles of borrelial spirochetes have been performed only for Northern (Levine et al., 1985; Hofmeister et al., 1999; Bunikis et al., 2004) and Central American species (Solís-Hernández et al., 2016). Whether rodents may act as reservoirs for *Borrelia* in South America remained unknown until the current study.

Parola et al. (2011) detected a RF *Borrelia* sp. (*Borrelia* sp. 95325, HM583797) in an undetermined *Ornithodoros* sp. collected in Bolivia. Remarkably, the *flaB* gene sequence retrieved by Parola et al. (2011) formed an independent clade with the sequences obtained in our study (*Borrelia* sp. A10 and *Borrelia* sp. A44), suggesting a close phylogenetic relationship. The phylogenetic resolution of the *flaB* gene has been useful to define lineages in the genus *Borrelia* (Fukunaga et al., 1996). In agreement with this fact, our BI and ML analyses indicated with high support that the detected genotypes constitute putatively new species (Figure 2). According to these results, the BI and ML phylogenies for IGS also point that the sequences of *Borrelia* sp. A10 and *Borrelia* sp. A44 constitute novel taxa, related to RF borreliae (Figure 3). To date, only one rodent parasitized by an *Ornithodoros* sp. has been proposed as putative reservoir for a RF *Borrelia* sp. in Chile (Muñoz-Leal et al., 2019b). However, the sequences detected in this study differ from *Borrelia* genotypes previously detected in soft ticks from this country (Muñoz-Leal et al., 2019a).

Different genotypes of *flaB* gene belonging to the LB group have been reported in Chile, namely *Borrelia* sp. Navarino (MH178398), characterized from *Ixodes auritulus* collected in the bird a *Troglodytes musculus*, and

several genotypes related to *B. chilensis* from ticks of the *I. sigelos* group (MH178397, MH187987, CP009910; Ivanova et al.2014; Muñoz-Leal et al., 2019a). Even though those studies have made valuable contributions to the understanding of the diversity of *Borrelia* in Chile, evidence of mammal hosts acting as reservoir for these agents was previously non-existent. In this study, sequences of *Borrelia flaB* gene retrieved from blood of *O. longicaudatus* (*Borrelia* sp. A53) branched as independent genotypes within the LB group of borreliae (Figure 2). Remarkably, *Borrelia* sp. A53 was also related to *B. chilensis* (CP009910), a genospecies previously reported in *I. stilesi* parasitizing the same rodent species in Southern Chile (Ivanova et al., 2014). Moreover, genotypes of *Borrelia* detected in larvae and nymphs of *I. sigelos* s.l. collected on *P. darwini* and *Octodon degus* (Muñoz-Leal et al., 2019a) clustered with *Borrelia* sp. A53 as well. This fact suggests that LB genotypes of *Borrelia* associated with rodents could constitute a monophyletic group related to *B. chilensis*. A similar hypothesis pointing a natural group of borreliae infecting rodents has been proposed through phylogenetic analyses using *Borrelia* genotypes detected in rodent-associated ticks in Southern Argentina (Sebastian et al., 2016).

To our knowledge, this is the first report of infection by *Borrelia* spp. in small mammals from Chile and South America, and the first isolation of DNA from these spirochetes in *P. xanthopygus* and *O. longicaudatus*. Our results suggest that these rodents may act as potential reservoirs for novel *Borrelia* genotypes in natural ecosystems of northern Chile. However, future studies are needed to further determine the competence of these rodents in maintaining *Borrelia* infections, and to investigate if other species of small mammals participate in the enzootic cycles of these spirochetes as well.

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PROTOZOOLOGY - ORIGINAL PAPER

Apicomplexans in small mammals from Chile, with the first report of the *Babesia microti* group in South American rodents

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Abstract

Small mammals play an essential role as disseminators of pathogens because they reach high population densities and have ubiquitous distributions. In the Northern Hemisphere rodents are well recognized as reservoirs for tick-borne bacteria of the Anaplasmataceae family and also apicomplexan protozoans. In contrast, South American rodents hosting these microorganisms have been rarely identified. In this study, we collected blood from rodents and marsupials in northern Chile and screened for Anaplasmataceae bacteria and apicomplexan protozoa. Overall, 14.7% of the samples were positive for *Babesia*, *Hepatozoon*, and Sarcocystidae using conventional PCR assays targeting the structural 18S rRNA locus (18S). Phylogenetic analyses performed with amplicons derived from 18S and cytochrome c oxidase (COI) gene provided evidence of a *Babesia* sp. belonging to the *Babesia microti* group in *Phyllotis darwini*, and a novel *Babesia* genotype in *P. darwini* and *Abrothrix jelskii*. Furthermore, four novel genotypes of *Hepatozoon* retrieved from *Abrothrix olivacea*, *P. darwini*, and *Oligoryzomys longicaudatus*, formed independent lineages within a clade that includes additional *Hepatozoon* spp. detected in South American rodents. Moreover, an incidental finding of a previously detected apicomplexan, herein designated as Sarcocystidae sp., was recorded in *Thylamys* opossums with a high prevalence, indicating a possible specific association with these mammals. Phylogenetic analysis of Sarcoystidae sp. clearly demonstrated its relatedness to apicomplexans detected in Australian marsupials. Our results expand the range of mammals hosting tick-borne apicomplexans in South America, highlight a novel clade consisting of South American babesias, and report for the first time the *B. microti* group infecting rodents in the region.

Keywords South American *Babesia* group · *Babesia microti* group · *Hepatozoon* · Sarcocystidae · Small mammals · Chile

Introduction

Small mammals are recognized hosts of bacteria of the Anaplasmataceae family and of apicomplexans with importance in veterinary and public health (Goodman et al. 2005; Perles et al. 2019). Due to their ubiquity, high population densities, and implications in food webs, small mammals play an important role in the dissemination of pathogens (Han et al. 2015). However, knowledge in relation to whether these vertebrates naturally carry pathogenic tick-borne bacteria or protozoans in South America is still emerging and requires further study.

The Anaplasmataceae family of bacteria includes intracellular obligate gram-negative alphaproteobacteria that infect ticks, and some strains cause disease in humans (Rar and Golovljova 2011). While pathogenic species of *Anaplasma*, *Ehrlichia*, and "*Candidatus* Neoehrlichia" are known to infect wild rodents in the Northern Hemisphere

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(Rar and Golovljova 2011), significantly less is known regarding vertebrate hosts of these bacteria in Southern latitudes of the globe. In particular, research on tick-borne bacterial pathogens is still incipient in Chile.

The Apicomplexa are obligate parasites with more than 6,000 described species, some of which may cause severe disease to their hosts and provoke significant economic losses worldwide (Votýpka et al. 2017). Decades ago, the genus *Babesia* attracted the attention of scientists because of a clinical disease that affected humans and domestic animals (Homer et al. 2000; Schreeg et al. 2016). In this context, it is currently known that species in the *Babesia microti* complex are zoonotic agents in Asia, Europe, and North America, with rodents carrying pathogenic strains in some cases (Goethert 2021). Although *B. microti* has never been detected in wild animals in South America, a *B. microti*-like agent was identified using molecular methods in humans from Bolivia (Gabrielli et al. 2016), suggesting that these parasites may represent neglected pathogens in the region.

Animals can also develop illness when infected with apicomplexan parasites such as species in the genus *Hepatozoon* (Merino et al. 2009; de Sousa et al. 2017; Perles et al. 2019). *Hepatozoon* spp. are intraerythrocytic and intraleukocytic parasites with a heteroxenous life cycle that involves vertebrates and invertebrates as intermediate and definitive hosts, respectively (Smith 1996). In South America, studies based on the detection of the *Hepatozoon* 18S rRNA structural locus (18S hereafter) suggested that in nature, these parasites use rodents and other small mammals as intermediate and paratenic hosts (Smith 1996; Merino et al. 2009; Wolf et al. 2016; Muñoz-Leal et al. 2019; Perles et al. 2019; Alabí et al. 2021). *Hepatozoon* spp. are transmitted to vertebrates through the ingestion of infected ectoparasites (Smith 1996). Interestingly, ticks of the genus *Ixodes* associated with rodents have be suggested as potential vectors in Chile (Muñoz-Leal et al. 2019).

Chilean small mammals are represented by 72 species: 67 belong to the order Rodentia and five to Marsupialia (D'elía et al. 2020; Mejías et al. 2021). Although the role of Chilean rodents and marsupials as reservoirs of pathogenic microorganisms is still obscure, DNA of Anaplasmataceae, *Bartonella*, *Borrelia*, *Hepatozoon*, and *Mycoplasma* has been detected previously (Merino et al. 2009; Müller et al. 2018, 2020; Muñoz-Leal et al. 2019; Thomas et al. 2020; Alabí et al. 2020, 2021). Therefore, it seems likely that widening the repertoire of species submitted to molecular analyses searching for those agents would contribute to the understanding of eco-epidemiological cycles. To address this objective, we targeted DNA of bacterial and apicomplexan agents in a range of small mammals captured in northern Chile.

Material and methods

Study area and collection of samples

Collections were performed during July of 2018 (Austral winter) in seven localities of northern Chile roughly between latitudes 19 and 31°S as follows: Parinacota and Socoroma (Arica and Parinacota Region), Enquelga, Chusmiza, and Pampa del Tamarugal National Reserve (PTNR) (Tarapacá Region), Llanos de Challe National Park (LCNP), and Bosque Fray Jorge National Park (BFJNP) (Atacama Region and Coquimbo regions, respectively) (Fig. 1). This area of northern Chile is characterized by constant dryness along the year, with small peaks of humidity in summer (Luebert and Pliscoff 2017), and includes the core of the Atacama Desert, the driest ecosystem on earth (Clarke 2006).

To capture rodents and marsupials, we set an average of 113 Sherman-like traps baited with oats along transects that remained active for two nights per locality (100 h in total), totalizing 1139 traps/night. Animals were manipulated as recommended by Sikes (2016). Briefly, we used a 300 g scale (Pesola) to weight the animals, and an intraperitoneal injection with a ketamine (60 mg/kg)-xylazine (3 mg/kg) solution to restrain them (Carpenter and Marion 2018). Approximately 20 µL of blood was obtained by nicking the caudal vein. Samples were preserved in sterile tubes with 96% ethanol (Sigma-Aldrich). Animals were identified morphologically in situ using a taxonomic guide (Patton et al. 2015), and released at their capture sites after recovering from anaesthesia.

DNA extraction and gene amplification

Total genomic DNA was extracted from blood samples employing the DNeasy Blood & Tissue Kit (QIAGEN, Germany), and eluted in 40 µL of Buffer AE (10 mM Tris–Cl; 0.5 mM EDTA, pH 9.0). Successful DNA extractions were checked through a conventional PCR targeting the mammalian *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) gene (Birkenheuer et al. 2003). Positive samples were subsequently screened for Anaplasmataceae (16S rRNA), Piroplasmida spp. (18S and COI), and *Hepatozoon* spp. (18S) DNA using conventional PCR protocols. A touchdown PCR to amplify a fragment of the mammalian *cytb* gene was used to genetically identify positive animals (Leite and Patton 2002). Primers and PCR thermal conditions used in this study are provided in Table 1.

Each PCR reaction was performed by adding 2 µL genomic DNA into a mix of 2.5 µL DreamTaq Buffer, 0.5 µL dNTPs (0.2 mM), 0.3 µL DreamTaq Polymerase,

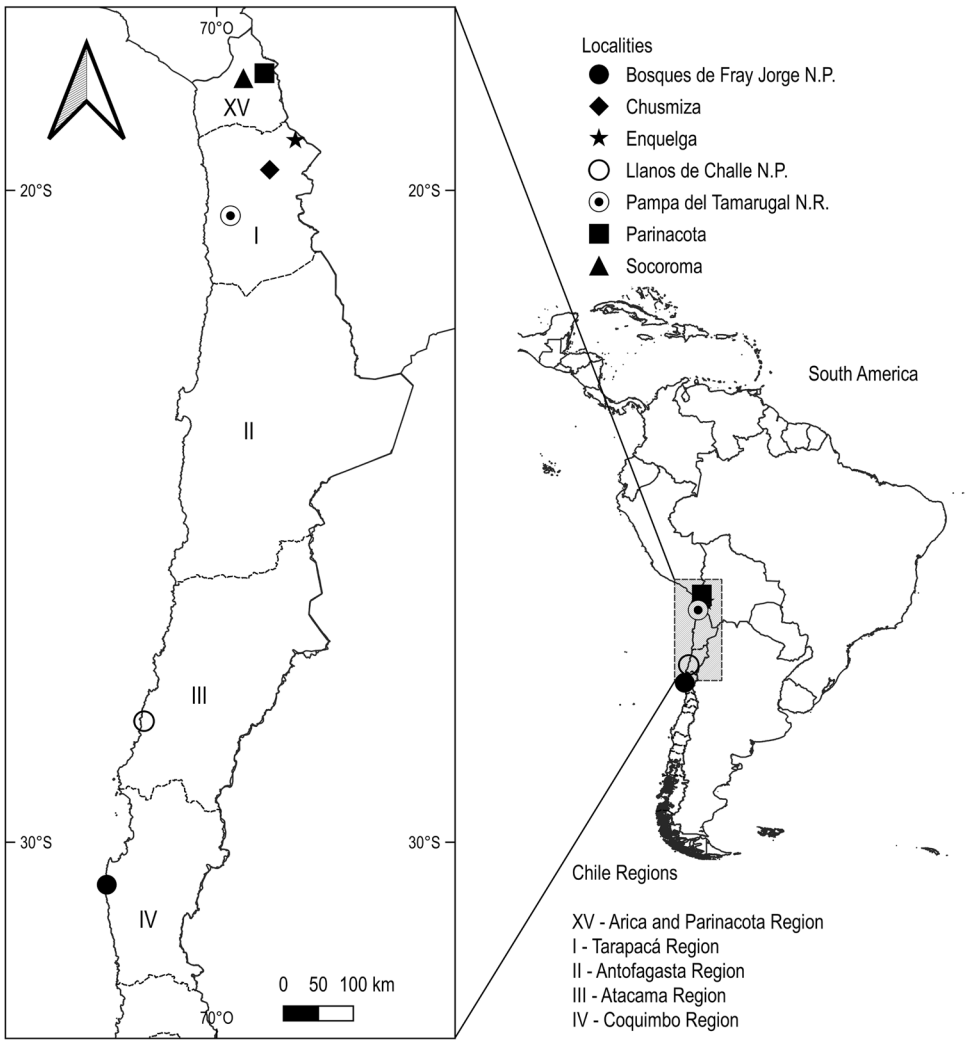

Fig. 1 Map of Northern Chile with the localities where collections were performed, highlighted with black and white symbols. Administrative regions: I, Tarapacá; III, Atacama; IV, Coquimbo; XV, Arica and Parinacota. Abbreviations: NP, National Park; NR, National Reserve

1 µL of each primer (10 pmol), and 17.7 µL of ultra-pure water. Amplicons were submitted to electrophoresis into 2% agarose gels, stained with SYBR Safe (Life Technologies/Thermo Fisher Scientific, Carlsbad, CA), and visualized by UV transillumination. Products of the expected size were purified and sequenced in both directions at the AUSTRAL-*omics* facility, in Universidad Austral de Chile (Valdivia, Chile). Positive controls included DNA of *Ehrlichia canis*, *Hepatozoon canis*, and *Babesia canis* previously obtained from infected dog blood.

Phylogenetic analyses

Sequences were quality-checked and edited with Geneious Prime® version (v) 2021.2.2 (www.geneious.com). Pairwise comparisons were performed with BLASTn (https://blast.ncbi.nlm.nih.gov), and similar sequences were downloaded from GenBank (https://www.ncbi.nlm.nih.gov) to construct alignments with MAFFT (Katoh and Standley 2013).

Phylogenies were built using Bayesian Inference (BI) and Maximum Likelihood (ML) methods with MrBayes 3.2.2. (Ronquist et al. 2012) and IQ-TREE v 1.6.12 (Nguyen et al. 2015), respectively. BI best evolutionary models were selected using the MrBayes command "lset nst = mixed rates = gamma" (Huelsenbeck 2004; Ronquist et al. 2012). Two independent tests of 10^7 generations and four MCMC chains were run, with sampling of trees every 1,000 generations removing the first 25% as burn-in. Tracer v 1.7.1 was used to confirm the effective sample size values (ESS) and the correlation of Markov chains (Rambaut et al. 2018). Bayesian posterior probabilities (BPP) with values ≥ 0.70 were considered to represent strong statistical support (Huelsenbeck and Rannala 2004). ModelFinder was used to select best nucleotide substitution model for ML analyses (Kalyaanamoorthy et al. 2017). We used rapid hill-climbing and stochastic disturbance methods to evaluate the robustness of the inferred tree with 1,000 ultrafast bootstrapping pseudo-replicates. Ultrafast bootstrap values ≥ 70%, between 70 and 94%, and > 95% were interpreted as low, medium,

Table 1 Primers and thermal conditions for PCR were used for the detection and genetic characterization of Anaplasmataceae, Piroplasmida, *Hepatozoon*, and mammals

Organism	Gene	Primer	Sequence	T_m (C°)	Expected length (bp)	Reference
Anaplasmataceae	16S rRNA	EHR 16SD	GGTACCYACAGAAGAAGTCC	55.0	345	Parola et al. 2000
		EHR16SR	TAGCACTCATCGTTTACAGC			
Hepatozoon	18S rRNA	HEP144-169	GGTAATTCTAGAGCTAATACA TGAGC	50.0	600–800	Almeida et al. 2012
		HEP743-718	ACAATAAAGTAAAAAACA YTTCAAAG			
Piroplasmida	18S rRNA	BAB2 143–167	CCGTGCTAATTGTAGGGCTAA TACA	58.0	349	Almeida et al. 2012
		BAB2 694–667	GCTTGAAACACTCTARTTTTC TCAAAG			
	COI	COI-F	GGAAGTGGWACWGGWTGG AC	55.0	1,080	Schreeg et al. 2016
		COI-R	TTCGGTATTGCATGCCTTG			
Mammal	*gapdh*	gapdh F	CCTTCATTGACCTCAACT ACAT	52	400	Birkenheuer et al. 2003
		gapdh R	CCAAAGTTGTCATGGATGACC			
	cytb	MVZ05	CGAAGCTTGATATGAAAAACC ATCGTTG	*60.0–51.0 phase 1	800	Leite and Patton 2002
		MVZ16	AAATAGGAARTATCAYTCTGG TTTRAT	50.0 phase 2		

*Touchdown PCR. The annealing temperature was programmed to reduce to one degree per cycle (60–51 °C) for 11 cycles in phase one and a total of 35 cycles in phase 2

and strong statistical support values respectively (Minh et al. 2013).

Results

A total of 102 small mammals belonging to 10 species and three families were captured (Table 2). The production of amplicons of the expected size for *gapdh* corroborated successful DNA extractions in all samples. While PCR screenings for Anaplasmataceae yielded negative results, 15/102 (14.7%), samples were positive for apicomplexan DNA (Table 2). Sequences of mammalian *cytb* were obtained for 13/15 positive animals, confirming the identities of five species of rodents and one marsupial (Table S1). GenBank accession numbers for nucleotide sequences generated in this study are available in Table S2.

Babesia

Two genotypes of *Babesia* 18S were obtained (*Babesia* sp. LC87, and *Babesia* sp. LC77, hereafter). The *Babesia* sp. LC87 genotype (537 bp) was obtained from two specimens of *Phyllotis darwini* (Rodentia: Cricetidae) and two *Abrothrix jelskii* (Rodentia: Cricetidae) in LCNP and Parinacota, respectively. BLASTn comparisons for this genotype yielded an identity of 94.8% (439/463 bp, 83% query cover, 7 gaps, 0 *E* value) with *Theileria* sp. ex *Damaliscus lunatus* clone TS23-6 (HQ179765), amplified from the common tsessebe (*Damaliscus lunatus*, Artiodactyla: Bovidae) in South Africa (Brothers et al. 2011). The *Babesia* sp. LC77 genotype (546 bp) was retrieved from a single specimen of *P. darwini* captured at LCNP. BALSTn comparisons for *Babesia* sp. LC77 revealed an identity of 98.6% (489/496 bp, 90% query cover, 0 gaps, 0 *E* value) with *B. microti* isolate Kv21 (MG062780), detected in *Ixodes persulcatus* from Russia (Livanova et al. 2018).

ML and BI phylogenetic reconstructions for 18S showed 11 discrete phylogenetic clades (Fig. 2). With strong support for *Babesia* sp. LC87 clustering into a monophyletic clade with a *Babesia* sp. detected in *Thrichomys pachyurus* (Rodentia: Echymidae) and a *Babesia* sp. detected in *Phyllostomus discolor* (Chiroptera: Phyllostomidae) from Brazil (clade XI, Fig. 2). In contrast, the *Babesia* sp. LC77 genotype is grouped within the *B. microti* complex, branching basally to several strains of this group detected in the Northern Hemisphere (clade I, Fig. 2).

Amplicons of COI target (922 bp) were obtained only in the sample from which *Babesia* sp. LC77 was retrieved, showed an identity of 86.7% (794/916 bp, 98% query cover, 6 gaps, 0 *E* value) with *B. microti* strain RI (LN871600) isolated from humans (Cornillot et al. 2012). In accordance with this genetic similarity, ML and BI

Table 2 Sampled small mammal species, localities of collection, and PCR assays. The GenBank accession numbers are indicated for the positive samples. Every sequence was obtained from a different specimen. Abbreviations: *BFJNP*, Bosque Fray Jorge National Park; *LCNP*, Llanos de Challe National Park; *PTNR*, Pampa del Tamarugal National Reserve

Order	Family	Species	Locality	Geographical coordinates (latitude, longitude)	Positive specimens/ total	*Babesia*	*Hepatozoon*	Sarcocystidae
Didelphimorpha	Didelphidae	*Thylamys elegans*	BFJNP	– 30.652003, – 71.685947	2/2			MW881038[b]
		Thylamys pallidior	Socoroma	– 18.264076, – 69.602025	1/1			MW881038[b]
Rodentia	Cricetidae	*Abrothrix* sp.	Parinacota	– 18.203307, – 69.268336	0/6			
		Abrothrix sp.	Socoroma	– 18.264076, – 69.602025	0/6			
		Abrothrix jelskii	Parinacota	– 18.203307, – 69.268336	2/3	MW881037 [b]		
		Abrothrix sp.	BFJNP	– 30.652003, – 71.685947	0/3			
		Abrothrix olivacea	BFJNP	– 30.652003, – 71.685947	1/4		MW881032[b]	
		Oligoryzomys longicauidatus	BFJNP	– 30.652003, – 71.685947	0/1			
		Oligoryzomys longicauidatus	Socoroma	– 18.264076, – 69.602025	1/1		MW881033[b]	
		Phyllotis darwini	BFJNP	– 30.652003, – 71.685947	4/10		MW881034[b]	
		Phyllotis darwini	LCNP	– 28.196424, – 71.043979	4/24	MW883578[a] MW881036[b]	MW881035[b]	
		Phyllotis sp.	Chusmiza	– 19.683803, – 69.188193	0/5			
		Phyllotis sp.	Enquelga	– 19.228963, – 68.804700	0/1			
		Phyllotis sp.	Parinacota	– 18.203307, – 69.268336	0/5			
		Phyllotis sp.	PTNR	– 20.470566, – 69.673656	0/6			
		Phyllotis sp.	Socoroma	– 18.264076, – 69.602025	0/21			
	Muridae	*Mus musculus*	Socoroma	– 18.264076, – 69.602025	0/2			
		Rattus rattus	PTNR	– 20.470566, – 69.673656	0/1			
Total					15/102			

[a]COI gene partial GenBank accession number

[b]18S gene partial GenBank accession numbers

phylogenies for COI confirmed that *Babesia* sp. LC77 belongs to the *B. microti* group (clade I; Fig. 3).

Hepatozoon

Four genotypes of *Hepatozoon* 18S were identified. Three identical sequences were recovered from three different specimens of *P. darwini* captured in BFJNP. A consensus of 544 bp designated as *Hepatozoon* sp. BFJ69 represents those sequences. *Hepatozoon* sp. BFJ69 was identical to *Hepatozoon* sp. isolate HepIxo-281 (MH174345), detected in ticks of the *Ixodes sigelos* group collected at the same locality (Muñoz-Leal et al. 2019). A second genotype of 544 bp (*Hepatozoon* sp. LC82 hereafter) was obtained from two specimens of *P. darwini* collected in BFJNP and LCNP. BLASTn comparisons of this second haplotype yielded 99.8% of identity with *Hepatozoon* sp. isolate HepIxo-284 (MH174344), obtained from the same *Ixodes* ticks at BFJNP (Muñoz-Leal et al. 2019).

Two additional *Hepatozoon* genotypes of 566 and 577 bp were recovered from *Abrothrix olivacea* (Rodentia: Cricetidae) collected in BFJNP (*Hepatozoon* sp. BFJ7 hereafter) and *Oligoryzomys longicaudatus* (Rodentia: Cricetidae) from Socoroma (*Hepatozoon* sp. Soc48 hereafter), respectively. *Hepatozoon* sp. BFJ7 showed an identity of 99.8% (565/566 bp, 100% query cover, 0 gaps, 0 *E* value) with

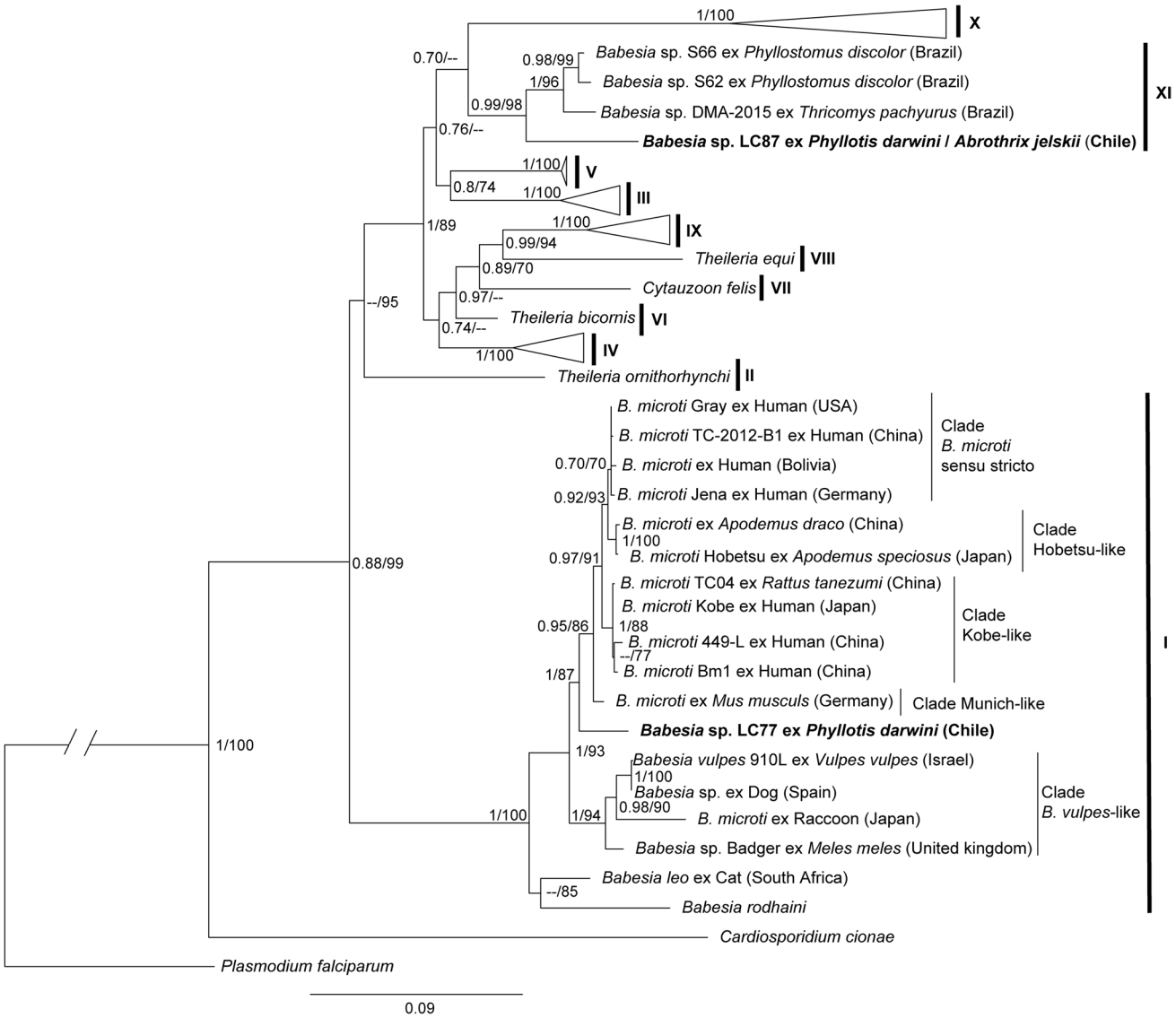

Fig. 2 Bayesian and Maximum Likelihood phylogenies for a subset of Piroplasmida spp. inferred using an alignment (1,721 bp) of the gene encoding 18S rDNA. Calculated substitution models for BI and ML were M_{125}, M_{191}, M_{134}, M_{200}, M_{136}, M_{40}, and M_{189}, and TIM3 + F + R6, respectively. Best models were chosen using the Bayesian Information Criterion (BIC) (Schwarz 1978). Values of Bayesian Posterior probability/ML Bootstrap are indicated above or below main branches. The position of *Babesia* spp. characterized in the present study is highlighted in bold. Roman numbers indicate phylogenetic lineages of the Piroplasmida order according to Jalovecka et al. (2019) and Ikeda et al. (2021); I: *B. microti* group, II: Monotremata group, III: Western group, IV: Marsupialia group, V: Percei group, VI: Rhinocerotidae group, VII: *Cytauxzoon*, VII: *Equus* group, IX: *Theileria* sensu stricto, X: *Babesia* sensu stricto, XI: *Babesia* spp. detected in South American mammals

Hepatozoon sp. AS7 (FJ719819) detected in *Abrothrix sanborni* (Rodentia: Cricetidae) from Chiloé Island, in southern Chile (Merino et al. 2009). On the other hand, *Hepatozoon* sp. Soc48 showed an identity of 98.6% (569/577 bp, 100% query cover, 1 gap, 0 *E* value) with *Hepatozoon* sp. HepIxo-281 (MH174345) recovered from ticks of the *I. sigelos* group in BFJNP (Muñoz-Leal et al. 2019).

Phylogenetic analyses performed for *Hepatozoon* 18S yielded a logic topology, separating the genus into two large clades: one composed by *Hepatozoon* spp. detected in amphibians, small mammals, reptiles, and ticks, and a second one conformed by species detected in canids. The genotypes characterized herein formed four independent lineages within a large clade composed by *Hepatozoon* spp. detected in South American rodents and their ticks (Fig. 4).

Sarcocystidae

Interestingly, the primers targeting DNA of Piroplasmida order amplified a 577-bp fragment of 18S that upon

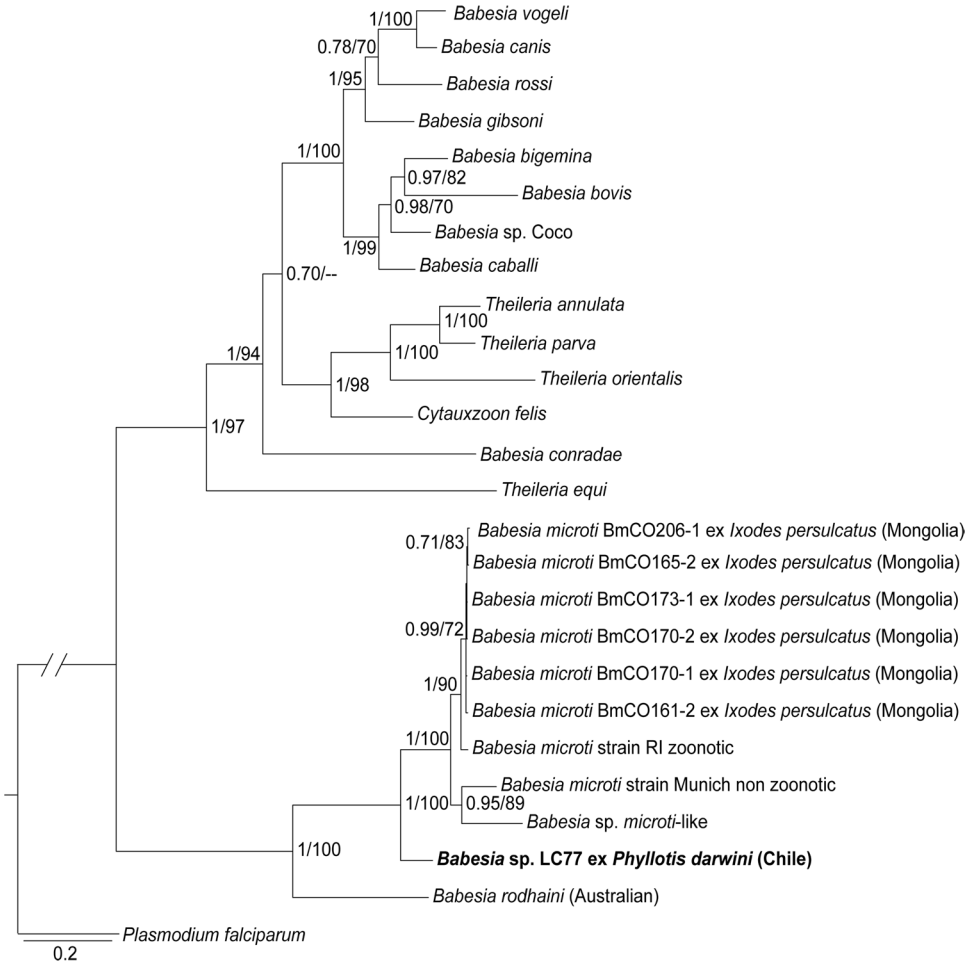

Fig. 3 Bayesian Inference and Maximum Likelihood phylogenies for a subset of Piroplasmida spp. inferred using an alignment (1,401 bp) of the gene encoding cytochrome *c* oxidase I (COI). Calculate substitution models for BI and ML were M_{136}, M_{40}, M_{125}, M_{191}, and M_{198} (part1), M_{93}, M_{184}, and M_{155} (part2), M_{201}, M_{162}, M_{200}; M_{189}, M_{203}, M_{134}, M_{138}, and M_{198} (part3), and TN + F + I + G4 (part1), TVM + F + R3 (part2), GTR + F + I + G4 (part3), respectively. Best models were chosen using the Bayesian Information Criterion (BIC) (Schwarz 1978). Values of Bayesian Posterior probability/ML Bootstrap are indicated above each branch. The position of *Babesia* spp. characterized in the present study is highlighted in bold

BLASTn analyses did not match any piroplasmids species. The amplicons were produced form two specimens of *Thylamys elegans* (Didelphimorphia: Didelphidae) and a single specimen of *Thylamys pallidior* (Didelphimorphia: Didelphidae), captured at BFJNP and Socoroma, respectively. Sequences obtained from the two species of *Thylamys* were identical and showed 100% sequence similarity with a undetermined apicomplexan denominated as Sarcocystidae sp. TE1 (577/577, 100% query cover, 0 gaps, 0 *E* value, EU443095), detected in the blood of *T. elegans* captured in Chile (Merino et al. 2010). Considering the high similarity with the sequence reported by Merino et al. (2010), and that the identity of these apicomplexans remains unsolved, we opted to designate it as Sarcocystidae sp. BFJ35 until additional genes can be analysed.

From a phylogenetic point of view, Sarcocystidae sp. BFJ35 formed a monophyletic clade with sequences of sarcocystids detected in the Australian marsupials *Petaurus australis* (Diprotodontia: Petauridae) and *Acrobates pygmaeus* (Diprotodontia: Acrabatidae) (Fig. 5) (Zhu et al. 2009; Holz et al. 2020). Collectively, the undetermined apicomplexan parasites detected in *Acrobates*, *Petaurus*, and *Thylamys* form an independent lineage within the Sarcocystidae family.

Discussion

To elucidate the role of wild mammals in the tick-host-microorganism reservoir system is important for recognizing areas where outbreaks of tick-borne pathogens might occur in nature (Mills 1998). Although previous research in Chile looking for tick-borne Anaplasmataceae identified "*Candidatus* Neoehrlichia chilensis" in *Abrothrix* sp., *Mus musculus* (Rodentia: Murinae), and *Ixodes* ticks collected upon *P. darwini* and *Octodon degus* (Rodentia: Octodontidae) (Müller et al. 2018; Muñoz-Leal et al. 2019), in our study, none of the tested animals was positive. Importantly, we used blood samples to perform the screenings whereas previous studies extracted DNA from the spleen to detect these agents (Müller et al. 2018), likely increasing the sensitivity of the assays. While our samples were negative for Anaplasmataceae, we detected novel genotypes of *Babesia* and *Hepatozoon* in rodents and expanded the distributional

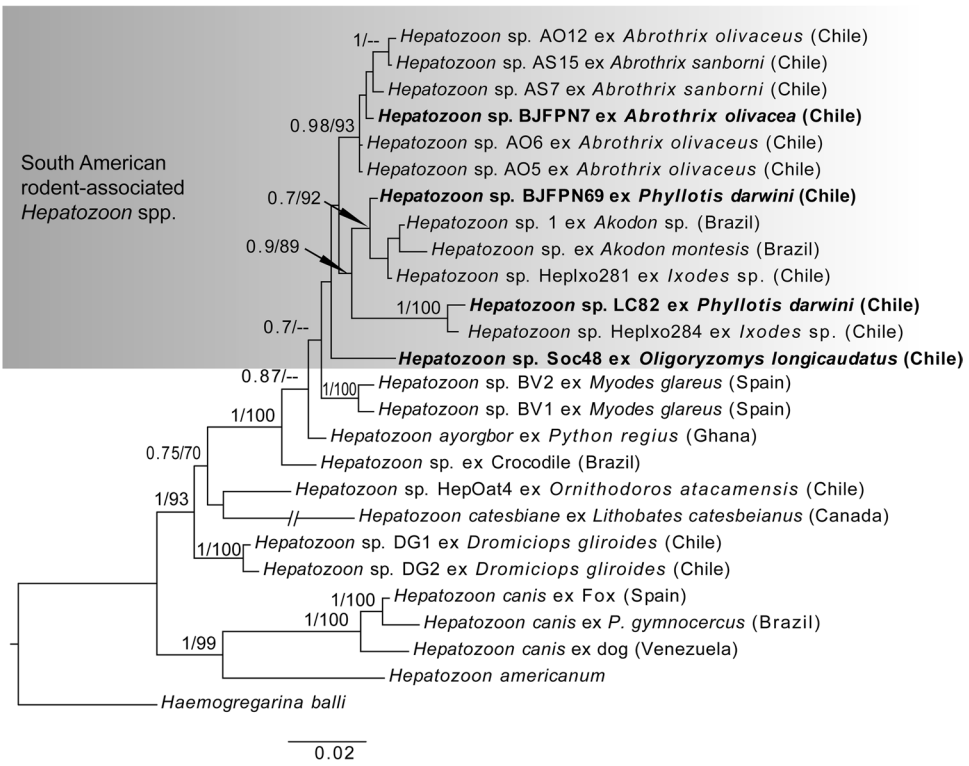

Fig. 4 Bayesian and Maximum Likelihood phylogenies for a subset of *Hepatozoon* spp. inferred using an alignment (1,662 bp) of the gene encoding 18S rRNA gene. Calculate substitution models for BI and ML were M_{85}, M_{15}, M_{177}, M_{147}, M_{134}, and M_{179}, and HKY + F + G4, respectively. Best models were chosen using the Bayesian Information Criterion (BIC) (Schwarz 1978). Values of Bayesian Posterior probability/ML Bootstrap are indicated above, below, or arrowing major branches. The position of *Hepatozoon* spp. characterized in the present study is highlighted in bold

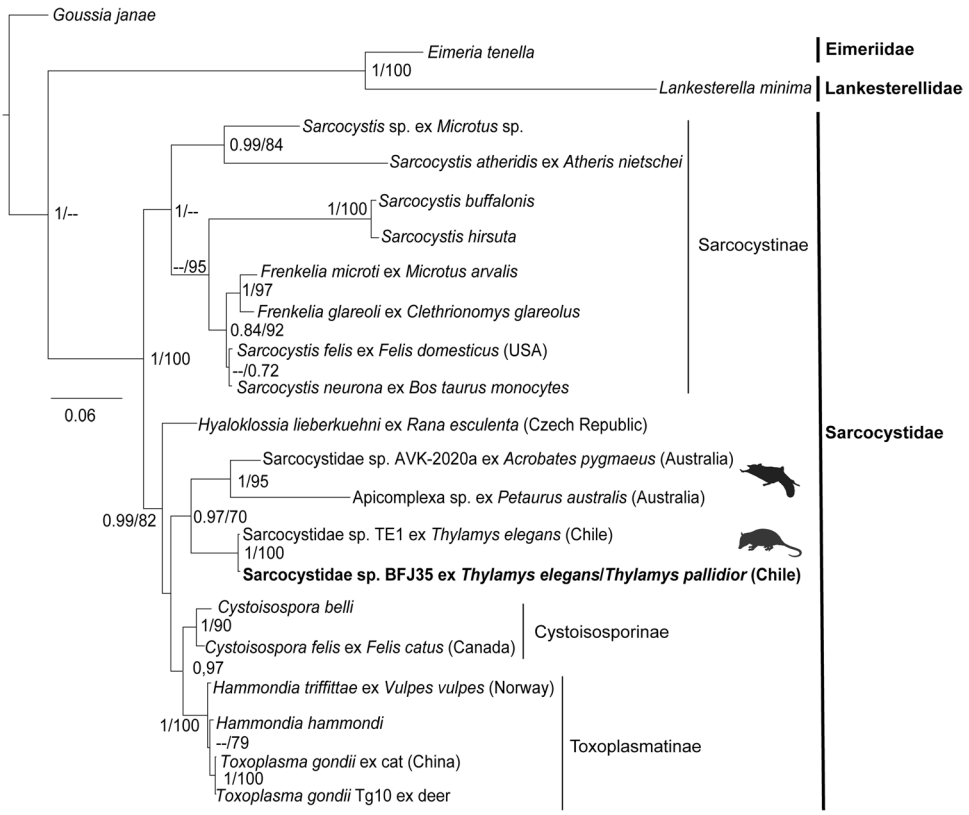

Fig. 5 Bayesian and Maximum Likelihood phylogenies for a subset of apicomplexan protozoans. inferred using an alignment (1,992 bp) of the gene encoding 18S rRNA. Calculate substitution models for BI and ML were M_{40}, M_{134}, M_{136}, M_{162}, M_{138}, and M_{125}, and TIM2 + F + R3, respectively. Best models were chosen using the Bayesian Information Criterion (BIC) (Schwarz 1978). Values of Bayesian Posterior probability/ML Bootstrap are indicated above, below, or arrowing major branches. The position of Sarcocystidae sp. BFJ35 characterized in the present study is highlighted in bold

and host range of an apicomplexan of the Sarcocystidae family that infects marsupials.

Babesia

Babesia species are transmitted by ticks and merge their life cycles with wild mammals that maintain the infection in nature (Karshima et al. 2021). The *B. microti* group is of medical importance in the Northern Hemisphere, and five clades currently define its diversity (Goethert and Telford 2003). Human pathogenic strains belong to *B. microti* sensu stricto (Goethert and Telford 2003), and are represented mostly by Eurasian and North American isolates (Goethert 2021). Remarkably, a *Babesia* sp. 99% identical with *B. microti* sensu stricto detected in blood from inhabitants of the Bolivian Chaco (Gabrielli et al. 2016) represents the sole genetic evidence of this blood parasite in South America. Despite being a human pathogen, the identity of the vector or vertebrate reservoir of this agent is yet to be elucidated. Herein, we characterized amplicons derived from the 18S and COI of a *Babesia* sp. (*Babesia* sp. LC77) belonging to the *B. microti* group in *Phyllotis* rodents from Chile, suggesting that these mammals could act as a reservoir. Our results, combined to the record of Gabrielli et al. (2016) from Bolivia, indicate that the *B. microti* group is likely underrepresented in South America, and that neglected human-pathogenic strains circulate in the region.

The role of rodents as hosts for *Babesia* spp. has been barely assessed in South American ecosystems. However, *Babesia* spp. have been detected in *T. pachyurus* (Wolf et al. 2016), *Thrichomys fosteri* (de Sousa et al. 2018), *Rattus norvegicus*, and *Oligoryzomys nigripes* from Brazil (Gazeta et al. 2004). Herein, we characterized an additional genotype, *Babesia* sp. LC87, detected in *P. darwini* and *A. jeslkii*, therefore adding novel hosts for these tick-borne apicomplexans in the continent. Interestingly, *Babesia* sp. LC87 clusters phylogenetically within a recently described array of babesias associated with bats and rodents from Brazil (Wolf et al. 2016; Ikeda et al. 2021) (Fig. 2). It is important to note that we are aware that our sequences are relatively short, and that analyses employing longer sequences of 18S might yield a different tree topology. However, the phylogenies derived from previous studies do mirror our analyses (de Sousa et al. 2018; Ikeda et al. 2021), and support the occurrence of a South American lineage of *Babesia* spp. (Fig. 2). Although the vectors of these agents in the region remain unidentified, it is well known that *Ixodes* ticks transmit *Babesia* in the Northern Hemisphere (Karshima et al. 2021). In Chile, the *Ixodes sigelos* group of ticks are common parasites of *Abrothrix* and *Phyllotis* rodents (Muñoz-Leal et al. 2019; Landaeta-Aqueveque et al. 2021), so attempts to understand the ecoepidemiology of *Babesia* sp. LC77 and LC87 should consider those ticks as potential vectors.

Hepatozoon

Ten years ago, Merino et al. (2009) reported an *Hepatozoon* sp. in *A. olivacea* and *A. sanborni* at Chiloé island. Recently, *Ixodes* ticks collected upon *Abrothrix longipilis* (Rodentia; Cricetidae) and *P. darwini* were positive to this apicomplexan as well (Muñoz-Leal et al. 2019). Herein, we report *P. darwini* as new host for *Hepatozoon* in Chile, therefore expanding the distribution of agents of this genus within the country. Collectively, these results point that rodent of genera *Abrothrix*, *Oligoryzomys*, and *Phyllotis* are common intermediate hosts for *Hepatozoon* along Chilean ecosystems. Importantly, the four genotypes of *Hepatozoon* characterized in this study were retrieved from cricetid rodents, and clustered into a large monophyletic group including sequences retrieved from other rodents of this family and their ticks (Fig. 4a). This fact supports the hypothesis that cricetid rodents and their ectoparasites could maintain enzootic cycles of a natural group of *Hepatozoon* spp. in the region (Muñoz-Leal et al. 2019). Although the detection of *Hepatozoon* in ticks feeding on small mammals does not demonstrate any transmission capacity (Giannelli et al. 2013), these ectoparasites should not be ruled out as potential vectors (Muñoz-Leal et al. 2019).

Recently, Alabí et al. (2021) reported DNA of *Hepatozoon* in synanthropic rodents (*M. musculus* and *R. rattus*) and *O. longicaudatus* from Valdivia, in southern Chile. Given that the 18S sequences generated by Alabí et al. (2021) corresponded to a different region of the locus, we were unable to include them in our phylogenies. However, according to our phylogenetic inferences (Fig. 4), and those of Alabí et al. (2021), discrete lineages of *Hepatozoon* would be associated with specific genera of native rodents, likely denoting events of coevolution (Poulin and Keeney 2008). A similar pattern of association between *Hepatozoon* spp. and their rodent hosts was also reported by Merino et al. (2009), yet this trend seems to be an exception, since *Hepatozoon* species are considered by some authors to be rather generalist parasites with low specificity for their vertebrate hosts (Smith 1996; Telford et al. 2001). The fact that in Chile, *Hepatozoon* species appear to be associated with specific genera of rodents is intriguing and suggests that the evolutionary history of these mammals may be shaping the diversity of the parasites (Hoberg and Brooks 2010). To test this hypothesis, future research should aim to retrieve complete *Hepatozoon* 18S sequences together with data for fast-evolving loci such as COI, and target previously unstudied rodent species.

Sarcocystidae

The detection of apicomplexans in mouse opossums of genus *Thylamys* is an incidental finding that reflects the low

specificity exhibited by the primers used in this study to amplify 18S of piroplasmid species. Moreover, our findings are not a novelty in Chile. In fact, Merino et al. (2010) reported an undescribed apicomplexan species related to the Sarcocystidae family in *T. elegans*. Although the number of *Thylamys* analysed in our study was low ($n=3$), it is noteworthy that all the specimens were positive. In this context, the previous detection of a Sarcocystidae sp. in Chilean opossums also showed a high prevalence of infection (Merino et al. 2010). In contrast, all rodents ($n=99$) sampled in our study were negative to this apicomplexan, indicating a degree of specificity for *Thylamys* opossums. Indeed, specific associations between Sarcocystidae parasites and their hosts have been reported to occur in other ecosystems (Šlapeta et al. 2003).

The relatedness of Sarcocystidae sp. BFJ35 genotype detected in Chilean opossums with apicomplexans from Australian marsupials is particularly interesting (Fig. 5), and suggests that a common ancestor that infected marsupials diverged with them 45–50 million years ago during the Eocene era, after the split of Antarctica and Australia (Nilsson et al. 2004; Merino et al. 2010). It is pertinent to note that there is evidence that this novel apicomplexan taxon can disseminate and cause severe liver, spleen, and lung infection, and as such, it would represent a threat to marsupial populations with conservation issues (Holz et al. 2020). In the meantime, the Sarcocystidae sp. associated with Australian and Chilean marsupials remains as an understudied taxon that clearly deserves more attention (Duszynski 2016).

Parasites of the Sarcocystidae family have heteroxenous life cycles involving carnivores as definitive hosts, and other vertebrates such as marsupials, as intermediate hosts (Votýpka et al. 2017). Oocysts of these parasites are released into the environment through faeces and reach novel hosts after ingestion of contaminated food or water (Votýpka et al. 2017; Holz et al. 2020). While the ecology of Sarcocistidae sp. BFJ35 is still unclear, the study of owls and foxes that usually prey on *Thylamys* spp. (Jaksic Andrade 1993; Carevic et al. 2013; Valladares Faúndez et al. 2018) should shed light on the definitive hosts of this novel apicomplexan species in Chile.

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Author contribution Adriana M. Santodomingo: conceptualization, data curation, methodology, formal analysis, investigation, writing—original draft, and writing—review and editing. Richard S. Thomas: conceptualization, formal analysis, investigation, methodology, and writing—review and editing. Julian F. Quintero-Galvis: formal analysis, methodology, and review and editing. Diana M. Echeverry-Berrio: methodology, writing—original draft, and writing—review and editing. María C. Silva-de la Fuente: methodology, and review and editing. Lucila Moreno-Salas: formal analysis, investigation, project administration, review and editing. Sebastián Muñoz-Leal: conceptualization, data curation, methodology, resources, formal analysis, investigation, project administration, writing—original draft, and writing—review and editing. All authors read and approved the final manuscript.

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Declarations

Ethics approval Procedures performed in this study were verified and approved by the Bioethics Committee of School of Veterinary Sciences, Universidad de Concepción (Form CBE-19–2017). Captures of small mammals and field work in national parks and reserves were authorized by the Servicio Agrícola y Ganadero (SAG; Resolution No. 1532/2019 and 9071/2018), and the Corporación Nacional Forestal (CONAF; Permits 39/2018; 67/2019; 05/2018; 76/2018; 66/2018), respectively.

Competing interests The authors declare no competing interests.

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SUPPORTING INFORMATION

Table S1. BLASTn results of *cytb* sequences obtained from positive small mammals.

species/sample code	Accession code NCBI	BLASTn result
Abrothrix olivacea BFJ7	MW883579	98.86% (783/792, 98% query cover, 0 gaps, 0 E-value) identical with *Akodon olivaceus* FMNH 132309 (AF027305)
Oligoryzomys longicaudatus Soc48	MW883580	100% (720/720, 100% query cover, 0 gaps, 0 E-value) identical with *Oligoryzomys longicaudatus* isolate NK96860 (KR822254)
Phyllotis darwini BFJ66	MW883581	99.59% (722/725, 100% query cover, 0 gaps, 0 E-value) identical with *Phyllotis darwini* voucher MSB 69977 (U86820)
Phyllotis darwini BFJ67	MW883582	99.73% (727/729, 100% query cover, 0 gaps, 0 E-value) identical with *Phyllotis darwini* voucher MSB 69977 (U86820)
Phyllotis darwini BFJ68	MW883583	99.34% (754/759, 100% query cover, 0 gaps, 0 E-value) identical with *Phyllotis darwini* voucher MSB 69977 (U86820)
Phyllotis darwini BFJ69	MW883584	99.33% (743/748, 100% query cover, 0 gaps, 0 E-value) identical with *Phyllotis darwini* voucher MSB 69977 (U86820)
Phyllotis darwini LC82	MW883585	99.72% (724/726, 100% query cover, 0 gaps, 0 E-value) identical with

		Phyllotis darwini voucher MSB 69977 (U86820)
Phyllotis darwini LC87	MW883586	100% (667/667, 100% query cover, 0 gaps, 0 E-value) identical with *Phyllotis darwini* voucher MSB 69977 (U86820)
Abrothrix jelskii Par46	MW883587	98.62% (717/727, 100% query cover, 0 gaps, 0 E-value) identical with *Abrothrix jeslkii* (KY753932)
Abrothrix jelskii Par55	MW883588	98.69% (756/766, 99% query cover, 0 gaps, 0 E-value) identical with *Abrothrix jeslkii* (KY753932)
Phyllotis darwini LC77	MW883589	99.85% (665/666, 100% query cover, 0 gaps, 0 E-value) identical with *Phyllotis darwini* voucher MSB 69977 (U86820)
Phyllotis darwini LC95	MW883590	99.86% (690/691, 100% query cover, 0 gaps, 0 E-value) identical with *Phyllotis darwini* voucher MSB 69977 (U86820)
Thylamys elegans BFJ4	MW883591	99.84% (612/613, 100 query cover, 0 gaps, 0 E-value) identical with *Thymalys elegans* UCK23 (KP994525)
Thylamys elegans BFJ35	MW883592	99.85% (678/679, 100 query cover, 0 gaps, 0 E-value) identical with *Thymalys elegans* UCK23 (KP994525)

Table S2. GenBank accession numbers of the sequences used for Piroplasmida, *Hepatozoon* and Apicomplexa phylogenetic trees. Sequences obtained in this study are highlighted in bold.

Sequence ID	COI GenBank accession number	18S GenBank accession number
Babesia		
Babesia caballi		Z15104
Babesia bigemina		HQ840960
Babesia divergens		AJ439713
Babesia sp. S66		MT002356
Babesia sp. S62		MT002348
Babesia sp. DMA-2015		KP757840
Babesia sp. LC87		**MW881037**
Babesia peircei		MF288026
Babesia poelea		DQ200887
Babesia duncani		HQ289870
Babesia conradae		AF158702
Theileria sp. ex *D. lunatus*		HQ179765
Theileria parva		L02366
Theileria orientalis		HM538266
Theileria equi		EU642511
Cytauzoon felis		L19080
Theileria bicornis		MF536661
Theileria sp. TB102		MN101139
Theileria sp. TB105		MN101137
Theileria ornithorhynchi		KT937390
B. microti Gray		AY693840
B. microti TC-2012-B1		KF410824
B. microti ex Human (Bolivia)		KT318132
B. microti Jena		EF413181
B. microti ex *Apodemus draco*		KY649345
B. microti Hobetsu		AB050732
B. microti TC04		KY649339
B. microti Kobe		AB032434
B. microti 449-L		JQ609304
B. microti Bm1		KX008034
B. microti ex *Mus musculs* (Germany)		AB071177
***Babesia* sp. LC77**		**MW881036**
Babesia vulpes 910L		KJ871351
Babesia sp. ex Dog		AY534602
B. microti ex Raccoon		AB197940
Babesia sp. Badger		KX528553
Babesia leo		AY452708
Babesia rodhaini		M87565
Cardiosporidium cionae		EU052685
Plasmodium falciparum		JQ627152
Babesia vogeli	KC207825	
Babesia canis	KC207822	
Babesia rossi	KC207823	
Babesia gibsoni	AB499087	
Babesia bigemina	AB499085	
Babesia sp. Coco	KC207824	

Babesia bovis	AB499088
Babesia caballi	AB499086
Theileria annulata	NW_001091933
Theileria parva	AB499089
Theileria orientalis	AB499090
Theileria equi	AB499091
Cytauxzoon felis	KC207821
Babesia conradae	KC207826
Babesia microti strain RI zoonotic	LN871600
Babesia microti strain Munich non zoonotic	AB624353
***Babesia* sp. LC77**	**MW883578**
Babesia sp. *microtilike*	KC207827
Babesia rodhaini	AB624357
Plasmodium falciparum	AY283019
Babesia microti BmCO206-1	LC005806
Babesia microti BmCO165-2	LC005786
Babesia microti BmCO173-1	LC005796
Babesia microti BmCO170-2	LC005792
Babesia microti BmCO170-1	LC005791
Babesia microti BmCO161-2	LC005783
Hepatozoon	
Hepatozoon sp. AO12 Chile	FJ719815
Hepatozoon sp. AS15 Chile	FJ719816
Hepatozoon sp. AS7 Chile	FJ719819
***Hepatozoon* sp. BJF7**	**MW881032**
Hepatozoon sp. AO6 Chile	FJ719817
Hepatozoon sp. AO5 Chile	FJ719818
Hepatozoon sp. 1 *Akodon* sp. Brazil	KU667308
Hepatozoon sp. *Akodon montesis* Brazil	MH111416
Hepatozoon sp. HepIxo-281 Chile	MH174345
***Hepatozoon* sp. BFJ69**	**MW881034**
***Hepatozoon* sp. LC82**	**MW881035**
Hepatozoon sp. HepIxo-284 Chile	MH174344
***Hepatozoon* sp. Soc48**	**MW881033**
Hepatozoon sp. BV2 *Myodes glareus* Spain	AY600625
Hepatozoon sp. BV1 *Myodes glareus* Spain	AY600626
Hepatozoon ayorgbor Ghana	EF157822
Hepatozoon sp. *Crocodilus* Brazil	KY684007
Hepatozoon sp. HepOat4 Chile	MH174343
Hepatozoon catesbiane	AF130361
Hepatozoon sp. DG1 Chile	FJ719813
Hepatozoon sp. DG2 Chile	FJ719814
Hepatozoon canis Spain	AY150067
Hepatozoon canis Brazil	AY461376
Hepatozoon canis	DQ439543
Hepatozoon americanum	AF176836
Haemogregarina balli	HQ224959
Sarcocystidae	
Goussia janae	GU479644
Eimeria tenella	U67121
Lankesterella minima	AF080611
Sarcocystis sp.	U97524
Sarcocystis atheridis	AF120114
Sarcocystis buffalonis	AF017121

Sarcocystis hirsuta	AF017122
Frenkelia microti	AF009244
Frenkelia glareoli	AF009245
Sarcocystis felis	AY656815
Sarcocystis neurona	U07812
Hyaloklossia lieberkuehni	AF298623
Sarcocystidae sp. AVK-2020a	MT560683
Apicomplexa sp.	FJ012144
Sarcocystidae sp. TE1	EU443095
Sarcocystidae sp. BFJ35	MW881038
Cystoisospora belli	JX025652
Cystoisospora felis	KT184364
Hammondia triffittae	GQ984222
Hammondia hammondi	AH008381
Toxoplasma gondii	KX008004
Toxoplasma gondii Tg10	KX008033

CAPÍTULO II: Estudio de *Anaplasma*, *Borrelia* y *Babesia* en el ciervo *Pudu puda* e *Ixodes stilesi* en el sur de Chile

Resumen

Existen especies de garrapatas identificadas como vectores de diversos agentes infecciosos que tienen un impacto económico en la salud pública y veterinaria debido a las enfermedades que causan. Estas garrapatas, junto con los agentes infecciosos que transmiten, se encuentran distribuidos por todo el mundo y dependen de animales silvestres para completar sus ciclos de vida. Los cérvidos desempeñan un papel fundamental como hospederos de garrapatas, y aunque se ha determinado que son refractarios a infección por ciertos agentes, como *Borrelia*, actúan como hospederos reservorio de otros microorganismos, tales como *Babesia* y *Anaplasma.*

En Chile, los bosques templados son el hábitat del pudú (*Pudu puda*), una especie de ciervo clasificada como Casi Amenazada, hospedador importante de los adultos de las garrapatas duras (Ixodidae) *Ixodes stilesi* e *Ixodes taglei*. *Ixodes stilesi* en su etapa adulta se alimenta de *P. puda*, mientras que las ninfas lo hacen, además de *P. puda,* de *Oligoryzomys longicaudatus*. Esta especie de garrapata se ha encontrado únicamente en Chile, específicamente en las regiones del Maule y Los Ríos. Además de los datos sobre distribución geográfica y asociación a hospederos, no existe información sobre su ecología.

El objetivo de esta investigación fue ampliar el conocimiento sobre la diversidad genética y relaciones evolutivas de especies de *Anaplasma*, *Borrelia* y *Babesia* en pudúes e *I. stilesi* en Chile. En este estudio, se llevaron a cabo análisis genéticos con el fin de detectar la presencia de *Anaplasma, Borrelia* y *Babesia* en la sangre de 55 pudúes del sur de Chile, así como en 20 garrapatas *I. stilesi* recolectadas de estos animales. Se logró detectar ADN de *Anaplasma* tanto en los cérvidos como en *I. stilesi.* Con base en la divergencia promedio de secuencias parciales de *groEL* y en filogenias respaldadas para *rrs*, *gltA* y *groEL*, se identificó una nueva genovariante de *A. phagocytophilum* asociada con pudúes e *I. stilesi*. Para esta genovariante, se propone el nombre "Patagonia".

En consecuencia, esta genovariante ha sido clasificada como el ecotipo V (grupo 8) de *A. phagocytophilum*, marcando el primer registro de un ecotipo de este complejo de especies en América del Sur.

En contraste, no se amplificó ADN de *Borrelia* en la sangre de pudúes, lo cual no es inesperado, dado que en otros lugares se ha documentado que los cérvidos no son hospederos competentes para estas espiroquetas. Sólo se detectó una garrapata positiva, y el análisis genético reveló una variante nueva de *B. chilensis*, ya que dos loci *flaB* y *pepX* difirieron 0,93% y 0,18% respectivamente, en comparación con la cepa tipo (VA1) de la especie. (ver artículo A search for piroplasmids and spirochetes in threatened pudu (*Pudu puda*) and associated ticks from Southern Chile unveils a novel *Babesia* sp. and a variant of *Borrelia chilensis*).

Así mismo, tanto las garrapatas como la sangre de los pudúes, se detectó ADN de *Babesia*. En este estudio, se caracteriza una nueva genoespecie de *Babesia*, nombrada como *Babesia* sp. pudui asociada con los cérvidos y garrapatas. La investigación incluyó el cálculo de la divergencia genética para los genes 18S rRNA, COI y *cytb*, así como análisis filogenéticos que mostraron claramente que las secuencias obtenidas formaban un linaje discreto dentro de *Babesia* sensu stricto. En particular, *Babesia* sp. pudui se agrupó dentro de un clado relacionado con babesias de ungulados, lo que refuerza la idea de que las *Babesia* spp. que parasitan a estos mamíferos conforman un grupo monofilético (ver artículo A search for piroplasmids and spirochetes in threatened pudu (*Pudu puda*) and associated ticks from Southern Chile unveils a novel *Babesia* sp. and a variant of *Borrelia chilensis*).

La detección del ADN de *A. phagocytophilum* "Patagonia" y *Babesia* sp. pudui no confirma de manera concluyente el papel de pudúes e *I. stilesi* en la epidemiología de estos agentes ni su impacto clínico en la salud del pudú. No obstante, considerando que los cérvidos se reconocen como los principales hospederos reservorios de *Anaplasma* spp. y *Babesia* spp., y que *P. puda* es el único venado nativo que actualmente habita en las áreas en las que se registraron los casos positivos para estos agentes infecciosos, junto con la

evidencia de coespeciación entre cérvidos y babesias, es razonable plantear que *P*. *puda* podría actuar como hospedero reservorio de *A. phagocytophilum* cepa "Patagonia" y *Babesia* sp. pudui (ver artículo Wild deer (*Pudu puda*) from Chile harbor a novel ecotype of *Anaplasma phagocytophilum*).

Además, considerando el papel de *Ixodes* spp. como vectores de *Anaplasma* spp. y *Babesia* spp. en los cérvidos del hemisferio norte, es importante mencionar que *I. stilesi,* que comúnmente parasita a los pudúes, podría ser un vector potencial para los agentes detectados en este estudio. Sin embargo, esta hipótesis deberá ser confirmada mediante estudios experimentales. Mientras tanto, los ciclos epidemiológicos de *A. phagocytophilum* cepa "Patagonia" y *Babesia* sp. pudui siguen siendo desconocidos.

A continuación, se presentan los dos artículos que detallan los resultados de este capítulo.

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WILEY

ORIGINAL ARTICLE

A search for piroplasmids and spirochetes in threatened pudu (*Pudu puda*) and associated ticks from Southern Chile unveils a novel *Babesia* sp. and a variant of *Borrelia chilensis*

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Abstract

Cervids are important hosts for ticks and although they are refractory to some tick-borne agents such as *Borrelia*, they do act as reservoirs for others such as *Babesia*. *Babesia* and *Borrelia* are commonly transmitted by *Ixodes* spp. associated with deer, and most of the knowledge on their biological cycles comes from northern latitudes of the globe. In this study, we performed genetic screenings to detect tick-borne agents in blood and *Ixodes stilesi* ticks collected from an insular population of threatened pudu (*Pudu puda*), a pygmy deer species that inhabits temperate rainforests of southern South America. Inferred by phylogenetic analyses for 18S rRNA, COI and *cytb* genes, our results unveiled a novel genospecies of *Babesia* (*Babesia* sp. pudui) genetically related to *Babesia odocoilei*, a species that infects *Odocoileus virginianus* deer in North America. Although blood of the deer was negative for *Borrelia* infection, multilocus sequencing typing performed in one *I. stilesi* tick revealed the occurrence of a novel genetic variant of *Borrelia chilensis*, differing 0.93% and 0.18% in *flaB* and *pepX* genes with the type of strain for the species, respectively. Such a genetic divergence could be the result of thousands of years of isolation because of recent glaciation events that separated pudus and their tick populations at Chiloé Island approximately 437,000 years ago. The finding of a *Babesia* sp. has no precedents for wild and domestic ungulates in Chile and shows a novel piroplasmid that must be considered now on in rehabilitation centres and zoos that attend pudu deer. Further research is now necessary to confirm pathogenic roles.

KEYWORDS
Babesia odocoilei, *Babesia* sp. pudui, *Borrelia chilensis*, Chile, *Ixodes stilesi*, Southern Pudu

1 | INTRODUCTION

Ticks and their transmitted agents are globally distributed and involve wild vertebrates in order to complete their biological cycles (Sonenshine & Roe, 2014). A great number of tick species are cosmopolitan vectors of infectious microorganisms that impact economically because they cause disease in humans and animals, especially in the countries of the Southern Hemisphere (Boulanger et al., 2019). In particular, tick-borne piroplasmids of the genus *Babesia* and *Borrelia* spirochetes are emerging in South America, as recent discoveries gradually uncover their diversity (Ikeda et al., 2021; Muñoz-Leal et al., 2020; Santodomingo et al., 2022; Weck et al., 2022). To recognize tick-borne diseases of wild threatened animals is important from a conservation viewpoint, since pathogens can exacerbate under stressful situations (Mathieu et al., 2018; Penzhorn, 2006).

Cervids are parasitized by ticks of the genus *Ixodes* worldwide (Guglielmone et al., 2014) and constitute reservoirs for tick-transmitted agents. For instance, in North America, the white-tailed deer *Odocoileus virginianus* hosts adults of *Ixodes scapularis* that transmit *Babesia odocoilei* and *Borrelia burgdorferi* sensu lato (Bbsl) spirochetes (i.e. *B. burgdorferi* sensu stricto, *Borrelia mayonii*) (Eisen, 2020; Fanelli, 2021; Martínez-García et al., 2021). In Eurasia, *Ixodes ricinus* and *Ixodes persulcatus* transmit *Babesia divergens*, *Babesia venatorum* and *Babesia capreoli* (Fanelli, 2021). Moreover, *I. ricinus* and *I. persulcatus* can harbour multiple *Borrelia* species, such as *Borrelia afzelii*, *B. burgdorferi* s.s. or *Borrelia garinii* (Eisen, 2020). Importantly, while cervids are considered incompetent hosts for Bbsl (Kurokawa et al., 2020), they do act as reservoirs of *Babesia* spp. (Fanelli, 2021). Indeed, it has been recently proposed that ungulate-associated babesias form a monophyletic group (Hrazdilová et al., 2020).

Apicomplexans of the genus *Babesia* dwell inside erythrocytes, and with more than 100 described species, they are considered one of the most common haemoparasites infecting mammals (Antunes et al., 2017; Jalovecka et al., 2019). Noteworthy, the genus *Babesia* is paraphyletic and currently composed of the *Babesia* sensu stricto (s.s.), the Western *Babesia*, the *microti* and *peircei* groups (Jalovecka et al., 2019). Moreover, a fifth group associated with South American micromammals (i.e. rodents and bats) has been recently proposed (Ikeda et al., 2021; Santodomingo et al., 2022). Some *Babesia* spp. are considered zoonotic pathogens (i.e. *B. capreoli*, *B. divergens*, *Babesia duncani*, *Babesia microti*, *B. odocoilei*, inter alia) (Karshima et al., 2022; Scott et al., 2021; Yabsley & Shock, 2013), and infection by these parasites is normally subclinical in wild animals but causes a malaria-like disease in humans (Penzhorn, 2006; Vannier et al., 2008). However, stressors such as concurrent disease, poor nutrition, rutting season, calving, high population density and transportation of captive wild animals may lead to fatal infections in wild species (Mathieu et al., 2018; Penzhorn, 2006). The diversity of piroplasmids expands mainly because of the use of genetic tools for their identification (Barbosa et al., 2019; Greay et al., 2018; Hrazdilová et al., 2020; Ikeda et al., 2021; Santodomingo et al., 2022), and because the search for these parasites includes nowadays a growing number of previously unexplored wildlife species (Schnittger et al., 2022). From a genetic viewpoint, the use of nuclear (18S rRNA) and mitochondrial (COI) loci is considered of enough resolution to infer deep phylogenetic relationships (Schnittger et al., 2012, 2022) and to separate closely related taxa (Hrazdilová et al., 2020; Schreeg et al., 2016) within the genus *Babesia*, respectively.

Bbsl is a complex of species that includes the agents of Lyme disease in humans and distributes mainly along northern latitudes of the globe (Kurokawa et al., 2020). However, they are not absent in South America, as shown by the isolation and multilocus sequencing typing (MLST) of *Borrelia* genospecies from Brazil and Chile (Ivanova et al., 2014; Muñoz-Leal et al., 2020; Weck et al., 2022). For instance, *Borrelia chilensis*, a basal representative of the group, is harboured by the tick *Ixodes stilesi* in Chile, with native cervids and rodents likely implicated in its transmission cycles.

The southern pudu (*Pudu puda*), referred as pudu in this study, is a cervid species that occurs in South American temperate rainforests of Chile and Argentina (roughly between 35° and 46° S), and is currently classified as a near-threatened species (Silva-Rodriguez et al., 2016). Pudus are commonly parasitized by the tick *I. stilesi* (Nava et al., 2017). Although some *Ixodes* spp. have shown to harbour multiple species of *Borrelia*, only *B. chilensis* has been detected in *I. stilesi* (Ivanova et al., 2014; Verdugo et al., 2017). In this study, we were able to analyse blood and ticks collected upon pudus from Valdivian forests in Southern Chile. The fact that piroplasmids have never been identified in cervids from southern South America prompted us to perform genetic screenings seeking *Babesia* DNA. Moreover, as we were provided with pudu ticks collected on an island, we hypothesized that geographical isolation would prompt the detection of a novel *Borrelia* variant or species.

2 | MATERIAL AND METHODS

2.1 | Blood samples and tick identification

Between July 2017 and March of 2022, blood of rescued pudus from 35 localities were collected at the Centro de Conservación Chiloé Silvestre, Nal Bajo, in Chiloé Island (−41.839786, −73.936015) and Cerefas Universidad San Sebastián, Puerto Montt (−41.469628, −72.907159) (Figure 1). Ticks were collected on different cervids from which blood was obtained. Blood samples and ticks were preserved in sterile tubes with 99.8% ethanol. The taxonomic keys of Nava et al. (2017) and original descriptions of Neotropical Ixodidae (Guglielmone et al., 2010; Keirans et al., 1976) were used to identify ticks with the aid of a NexiusZoom (EVO) Stereo Microscope (Euromex, Holland). The identity of ticks that were positive to genetic screenings for *Babesia* and *Borrelia* was further confirmed by sequencing a fragment of the tick mitochondrial 16S rRNA gene. References for primers and thermal conditions used in this study are indicated in Table S1. A map showing the localities where pudus came from was built with QGIS 3.18.1-Zürich (https://www.gnu.org/licenses).

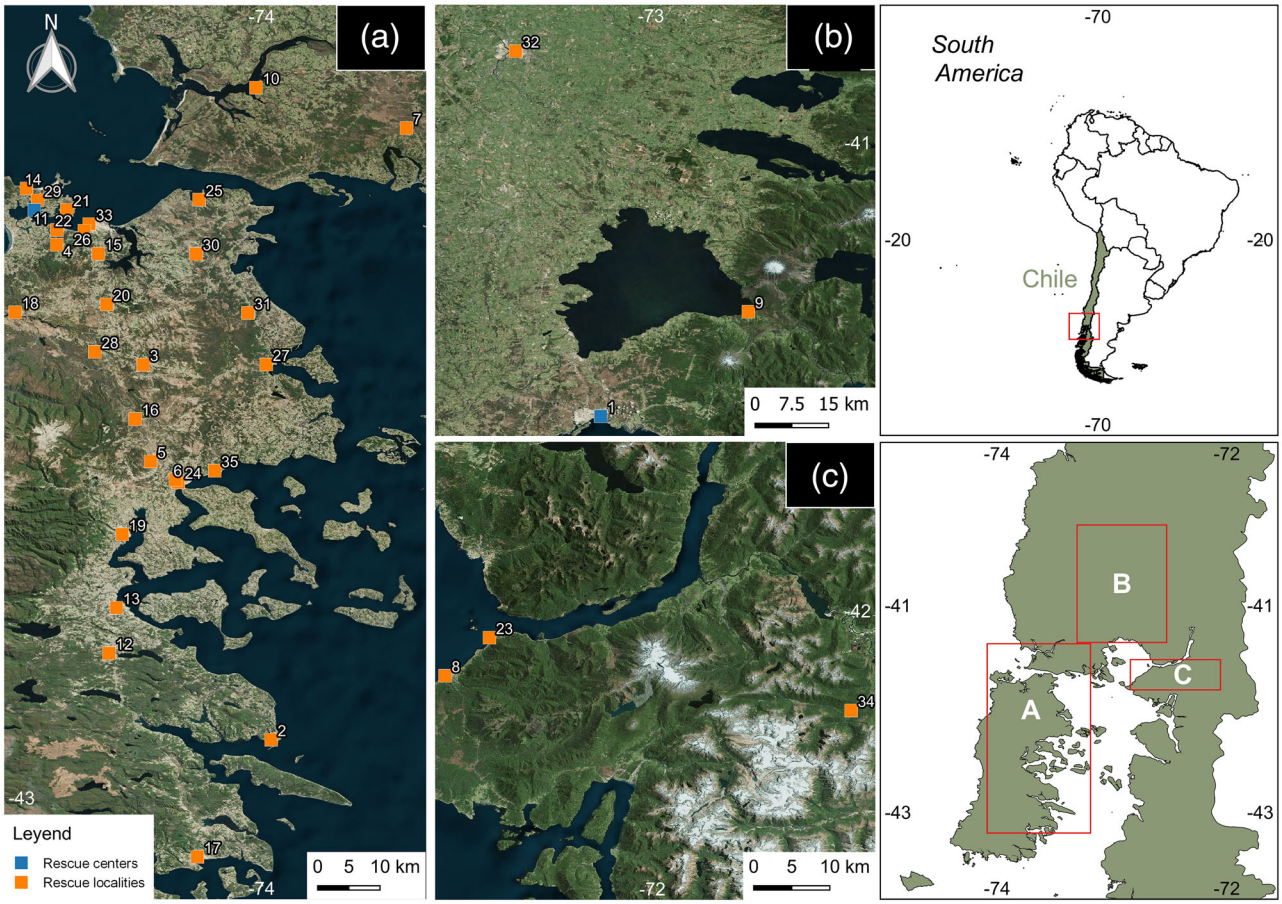

FIGURE 1 Map of southern Chile (Los Lagos region) showing the localities where samples were collected

2.2 | DNA extraction, PCR and sequencing

Total genomic DNA was extracted with the DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer's instructions and eluted in 40 μl of Buffer AE (10 mM Tris–Cl; 0.5 mM EDTA, pH 9.0). Concentration and quality of DNA were measured using an Epoch™ Microplate Spectrophotometer (BioTek Instruments, Inc.); samples with A_{260}/A_{280} DNA absorbance ratio with values ranging from 1.6 to 2.0 were considered pure and suitable for PCR amplification (Khare et al., 2014). Successful DNA extractions from blood and ticks were checked through a conventional PCR (cPCR) targeting the mammalian glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and the tick mitochondrial 16S rRNA genes, respectively.

Babesia detection was performed through cPCR targeting the 18S rRNA, cytochrome oxidase subunit 1 (COI) and cytochrome *b* (*cytb*) genes. *Borrelia* detection was performed by nested PCRs targeting the flagellin (*flaB*) and 16S rRNA (*rrs*) genes. In addition, we sought to amplify *clpA*, *clpX*, *pepX*, *pyrG*, *recG*, *nifS*, *rlpB* and *uvrA* housekeeping genes using an MLST scheme (Margos et al., 2008). DNA of *Borrelia anserina* PL and *Babesia vogeli* was employed as a positive control and ultra-pure water as a negative control.

PCRs were implemented in a thermal cycler ProFlex™ Base 32×3 (Applied biosystems, Inc., Carlsbad, CA, USA) using 2 μl template DNA in 23 μl reaction mixtures containing the following proportions: 12.5 μl DreamTaq Green PCR Master Mix (Thermo Scientific, USA), 1 μl of each primer (0.4 μM) and 8.5 μl of ultra-pure water. PCR products were stained with GelRed® (Biotum, Tehran, Iran) and subjected to electrophoresis in 2% agarose gels. Amplicons were visualized in an ENDURO™ GDS UV (Labnet International, Edison, NJ, USA) transilluminator, and expected-size bands were purified and sequenced in both directions at Macrogen (Seoul, South Korea).

2.3 | Phylogenetic analyses

Sequences recovered in this study were quality-checked and edited to obtain consensus sequences using Geneious Prime® version (v) 2021.2.2 (www.geneious.com). The BLASTn tool (https://blast.ncbi.nlm.nih.gov) was employed to perform pairwise comparisons and identify orthologous sequences. Sequences downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) were used to construct alignments with MAFFT algorithm FFT-NS-i x1000 option (https://mafft.cbrc.jp/alignment/server/). Then, alignments were trimmed to screen out informative regions using the BMGE software with default parameters (Cricuolo & Gribaldo, 2010). BMGE-filtered alignments of *Borrelia* and *Babesia* data sets were used to construct phylogenies with the

maximum likelihood (ML) and Bayesian inference (BI) methods in IQ-TREE v1.6.12 (Nguyen et al., 2015) and MrBayes v3.2.6 (Ronquist et al., 2012), respectively. Protein-encoding sequences present different nucleotide exchange rates (heterogeneity) at the first, second and third positions (Yang et al., 1996, Ronquist et al., 2012). To allow for this heterogeneity, protein-coding gene data sets were split into three partitions (position 1, position 2, and position 3) to select the best-fit evolutionary models and partition schemes (Kainer et al., 2015; Lanfear et al., 2012) using ModelFinder command '-m MFP+MERGE -mrate G' (Kalyaanamoorthy et al., 2017). ML best evolutionary model for non-coding gene were calculated using the ModelFinder command '-m MFP -mrate G' (Kalyaanamoorthy et al., 2017). We used 1000 ultrafast bootstrapping (UFBoot) pseudo-replicates with rapid hill-climbing and stochastic disturbance methods to evaluate the inferred tree robustness. UFBoot values <70% were considered non-significant, between 70% and 94% medium support and ≥95% strong statistical support (Minh et al., 2013).

BI phylogenies were constructed based on the models selected with the MrBayes command 'lset nst = mixed rates = gamma' for non-coding gene (Huelsenbeck, 2004; Ronquist et al., 2012). On the other hand, the best partition schemes as estimated by ModelFinder and the MrBayes command 'lset nst = mixed rates = invgamma' were used to calculate the best models for protein encoding data sets (Huelsenbeck, 2004; Ronquist et al., 2012). Two independent tests of 10^7 generations and four Markov chain Monte Carlo (MCMC) chains were implemented, sampling trees every 1000 generations, and removing the first 25% as burn-in. Tracer v1.7.1 (http://tree.bio.ed.ac.uk/software/tracer/) was used to confirm the correlation and effective sample size of the Markov chains. Bayesian posterior probabilities (BPP) with values ≥0.70 in nodes were considered of strong statistical support (Huelsenbeck & Rannala, 2004). Best-fit models were selected following the Bayesian information criterion (Schwarz, 1978). Trees were visualized and edited with FigTree v1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape 1.1 (https://inkscape.org/es/). When the data set yielded congruent topologies for ML and BI analyses, a strict consensus tree was generated using the Consensus Tree Builder tool in Geneious Prime. A support threshold of 100% was implemented to compare all monophyletic clades in order to merge ML and BI topologies into a sole consensus tree. Independent ML and BI trees are attached as Supporting Information.

3 | RESULTS

3.1 | Tick identification and blood samples

A total of 26 ticks (17 females, five males and four nymphs) were morphologically identified as *I. stilesi*. Expected size amplicons for the tick mitochondrial 16S rRNA gene were obtained for 20 specimens. Four females, one male and one nymph yielded negative results and therefore were excluded from the study. Ticks positive for *Babesia* and *Borrelia* DNA yielded identical 429-bp sequences (ON995413) showing 99.53% (428/430 bp, 100% query cover, 2 gaps, 0 *E*-value) of identity with *I. stilesi* from Chile (DQ061292). A total of 55 blood samples were analysed, and the production of expected size amplicons for *GAPDH* corroborated successful DNA extractions in all cases (Table 1). GenBank accession numbers generated in this study are available in TablesS2–S4.

3.2 | *Babesia* detection

Overall, 20% (4/20) of the ticks (two nymphs and two females) were positive for *Babesia* DNA. Likewise, 10.9% (6/55) of pudus were positive (Table 1). Interestingly, three positive pudus showed clinical signs of ongoing infectious diseases such as lethargy, hepatic haemorrhage and mesenteric lymphadenitis (data not shown). Ten genotypes were obtained for 18S rDNA (1149–1428 bp) and COI (681–1005 bp) genes and five for *cytb* (966–1092 bp) genes. Each of the 18S rDNA, COI and *cytb* genotypes matched *Babesia* s.s. sequences after BLASTn comparisons (data not shown).

With strong Bootstrap/BPP values, 18S rRNA and COI gene phylogenies positioned *Babesia* genotypes detected in *I. stilesi* and pudus in the *Babesia* s.s. group, forming a monophyletic clade within *Babesia* spp. associated with ungulates (Figures 2 and 3). Our 18S rDNA sequences were closely related with *B. odocoilei* isolate 4 (AY661510) recovered from a musk ox (*Ovibos moschatus*) in the United States, and *Babesia* sp. isolate RD61 (AF411337) detected in a reindeer (*Rangifer tarandus*) in that same country (Figure 2). On the other hand, sequences of *Babesia* COI clustered with *B. odocoilei* isolate 138BZAA032 (MG344849) retrieved from an elk (*Cervus elaphus canadensis*) in Canada (Figure 3). In contrast, the *Babesia cytb* sequences formed a discrete lineage within the *Babesia* s.s. group (Figure S3).

The minimal intraspecific genetic identity calculated for 18S rRNA and COI genes of the detected *Babesia* sp. was 99.83% and 98.68%, respectively. After aligning our *Babesia* 18S rDNA and COI sequences with orthologs retrieved from GenBank, the highest pairwise divergences depicted for each *Babesia* sp. or clade were always below the minimum value of interspecific genetic identities of the *Babesia* sp. characterized in this study (Table 2). Few sequences for the *Babesia cytb* gene are available in GenBank; however, pairwise comparisons indicated that *Babesia gibsoni* was the most identical species with the species detected in this study, ranging between 78.60% and 80.12% (Table 2). The genetic evidence unequivocally indicates that the *Babesia* sp. characterized in this study represents a novel species, and the name *Babesia* sp. pudui is provisionally proposed.

3.3 | *Borrelia* detection

DNA of *Borrelia* was not detected in the blood of pudus. However, 5% of ticks (1/20; one female) were positive (Table 1) since we recovered a 665-bp sequence for the *flaB* gene 99.70% (657/659 bp, 99% query cover, 0 gap, 0 *E*-value) identical with *B. chilensis* strain VA1 (CP009910). Furthermore, we obtained a 617-bp *rrs* sequence (ON990079) identical to *B. chilensis* clone 14061 (KY412449) and *B. chilensis* strain VA1 (CP009910). PCR assays targeting MLST genes were implemented to characterize the *Borrelia* strain resulting in

TABLE 1 Sampled and positive animals with the geographical coordinates of collection localities

Species	Locality[a]	Provenance	Geographical coordinates (latitude, longitude)	*Babesia*-positive specimens	*Borrelia*-positive specimens
Pudu puda	Cerefas Universidad San Sebastián, Puerto Montt[1]	Continent	−41.469628, −72.907159	0/3	0/3
	Queilén[2]	Island	−42.885721, −73.468359	0/3	0/3
	Degañ[3]	Island	−42.145274, −73.720717	1/1	0/1
	Pauldeo[4]	Island	−41.908360, −73.891784	0/2	0/2
	Mocopulli[5]	Island	−42.336344, −73.706289	0/2	0/2
	Tehuaco[6]	Island	−42.372438, −73.657162	0/1	0/1
	Calbuco[7]	Continent	−41.677865, −73.201237	1/1	0/1
	Contao[8]	Continent	−41.803322, −72.719169	0/2	0/2
	Ensenada[9]	Continent	−41.213838, −72.545666	0/1	0/1
	Peñol Bajo[10]	Continent	−41.598174, −73.498427	0/1	0/1
	Centro de Conservación Chiloé Silvestre[11]	Island	−41.839786, −73.936015	0/1	0/1
	Lago Tarahuín[12]	Island	−42.714684, −73.788520	0/1	0/1
	Chonchi[13]	Island	−42.625050, −73.774028	0/3	0/3
	Chauman[14]	Island	−41.797195, −73.951494	0/2	0/2
	Mechaico[15]	Island	−41.926147, −73.809907	0/1	0/1
	Butalcura[16]	Island	−42.252443, −73.736915	1/1	0/1
	Quellón[17]	Island	−43.116902, −73.613887	0/4	0/4
	Chepu[18]	Island	−42.041574, −73.973976	1/1	0/1
	Castro[19]	Island	−42.480140, −73.762413	0/3	0/3
	Quichitúe[20]	Island	−42.026219, −73.793279	0/1	0/1
	Guapilacuy[21]	Island	−41.839390, −73.871975	0/1	0/1
	Lechagua[22]	Island	−41.879088, −73.891482	0/1	0/1
	Caleta Puelche[23]	Continent	−41.742766, −72.648612	0/1	0/1
	Dalcahue[24]	Island	−42.377552, −73.651920	1/3	0/3
	Caulin[25]	Island	−41.819313, −73.610747	0/1	0/1
	Hueihue[26]	Island	−41.880609, −73.837354	0/1	0/1
	Quemchi[27]	Island	−42.144713, −73.478056	0/2	0/2
	Puntra[28]	Island	−42.119891, −73.816342	1/1	0/1
	Sector Naval – Faro Corona[29]	Island	−41.820747, −73.929428	0/2	0/2
	El Quilar[30]	Island	−41.926431, −73.616454	0/1	0/1
	Llliuco[31]	Island	−42.042983, −73.514278	0/2	0/2
	Osorno[32]	Continent	−40.576192, −73.114948	0/1	0/1
	Ancud[33]	Island	−41.867489, −73.827690	0/1	0/1
	Los Lagos[34]	Continent	−41.858622, −72.073451	0/1	0/1
	Quiquel[35]	Island	−42.354553, −73.579767	0/1	0/1
				6/55	0/55
Ixodes stilesi	Centro de Conservación Chiloé Silvestre[11]	Island	−41.839786, −73.936015	4/20	1/20

[a]Superscript numbers correlate to the georeferenced localities of Figure 1.

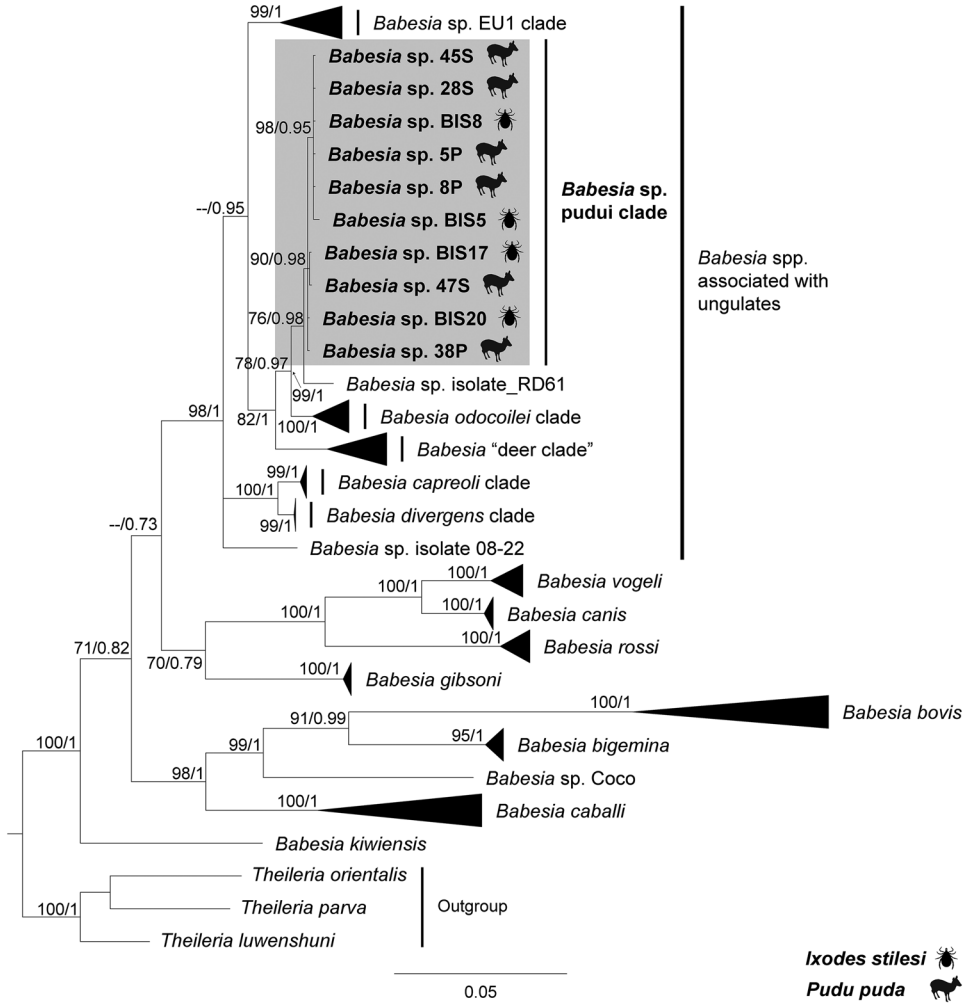

FIGURE 2 Consensus tree of maximum likelihood (ML) and Bayesian inference (BI) phylogenies inferred for 82 sequences of Piroplasmida 18S rRNA gene, using an alignment of 1770 base pairs. Best-fit evolutionary models calculated for ML and BI methods were TIM3+F+G4 and M_{36}, M_{94}, M_{125}, M_{156}, respectively. Bootstrap values and Bayesian posterior probabilities are indicated above or below each branch. The position of the *Babesia* sp. characterized in the present study is highlighted within a grey box. Separated tree topologies are showed in Figure S1.

amplicons of the expected size. Obtained MLST sequences were identical to those of *B. chilensis* strain VA1, except for the *pepX* gene that had one single nucleotide polymorphism. Consequently, the allele 276 was assigned to this locus and is available at http://pubmlst.org/borrelia/. The name 'Chiloé' is given to this genetic variant of *B. chilensis*. Expectedly, phylogenetic analyses of concatenated MLST and *flaB* showed *B. chilensis* strain Chiloé clustering with *B. chilensis* strain VA1 (Figure 4). Moreover, *flaB* phylogeny showed that *B. chilensis* strain Chiloé clusters into a monophyletic clade with several *Borrelia* spp. detected in ticks of the *Ixodes sigelos* group and the rodent *Oligoryzomys longicaudatus* (Figure S8).

4 | DISCUSSION

Babesia spp. are the second most commonly found haemoparasites in mammals after trypanosomes and cause babesiosis in animals and humans (Antunes et al., 2017), a tick-borne disease that has been reported beyond endemic areas (Mathieu et al., 2018; Scott et al., 2021). In this study, we describe *Babesia* sp. pudui as a new genospecies associated with cervids upon calculation of genetic divergence for 18S rRNA, COI and *cytb* genes, and phylogenetic analyses that clearly showed our sequences forming a discrete lineage within *Babesia* s.s. (Figures 2, 3, S3, S4, S5 and S6). In particular, *Babesia* sp. pudui clusters within a clade associated with ungulates (Figures 2 and 3), reinforcing that *Babesia* spp. that parasitize these mammals form a natural group (Hrazdilová et al., 2020). Moreover, the fact that a *Babesia* sp. associated with pudus is phylogenetically closely related with babesias of New World deer (i.e. *Rangifer*, *Odocoileus*) supports the hypothesis that cospeciating would be a common phenomenon in piroplasmids (Schnittger et al., 2022).

Babesia spp. of cervids (i.e. *B. divergens*, *B. capreoli*, *Babesia* sp. EU1, *B. odocoilei* and *Babesia* cf. *odocoilei*) and their associated ticks (i.e. *I. scapularis*, *Ixodes persulcatus* and *I. ricinus*) have been widely reported in Europe and North America (Fanelli, 2021; Hrazdilová et al., 2020; Martínez-García et al., 2021). In contrast, studies of *Babesia* in cervids

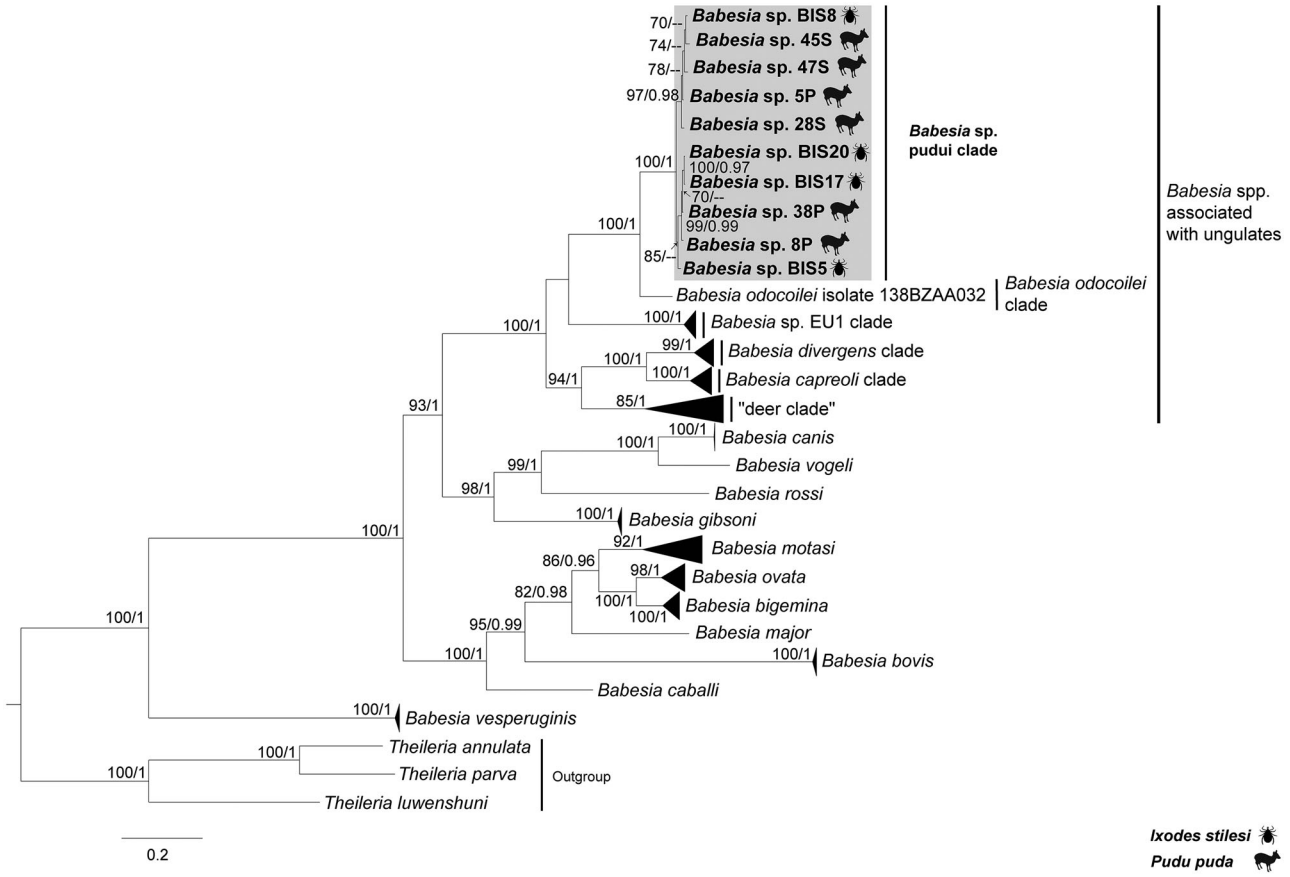

FIGURE 3 Consensus tree of maximum likelihood (ML) and Bayesian inference (BI) phylogenies inferred for 59 sequences of Piroplasmida COI gene, using an alignment of 927 base pairs. Best-fit evolutionary models calculated for ML and BI methods were TN+F+G4 (position 1), TVM+F+G4 (position 2) and TIM2+F+G4 (position 3) and M_{136}, M_{40}, M_{125}, M_{191}, M_{138} (position 1), M_{180}, M_{196}, M_{68}, M_{129}, M_{177} (position 2) and M_{134}, M_{189}, M_{198}, M_{200}, M_{203} (position 3), respectively. Bootstrap values and Bayesian posterior probabilities are indicated above or below each branch. The position of the *Babesia* sp. characterized in the present study is highlighted within a grey box. Separated tree topologies are showed in Figure S2.

and their ectoparasites are few in southern latitudes of the globe. Records of *Babesia* in cervids from South America include *Babesia bovis* and *Babesia bigemina* in *Blastocerus dichotomus* and *Mazama gouazoubira* in Brazil (da Silveira et al., 2011) and *Babesia* spp. in *Mazama rufina* from Ecuador (Diaz et al., 2021). Noteworthy, da Silveira et al. (2011) obtained short sequences for 18S rDNA sequences (i.e. less than 800 bp) that were excluded from our phylogenetic analyses, because small sequences can alter the tree topology for this locus (Barbosa et al., 2019). In Chile, DNA of *Babesia* has been previously detected in domestic dogs (di Cataldo et al., 2020) and wild rodents (*Phyllotis darwini*, *Abrothrix jelskii*) (Santodomingo et al., 2022). Moreover, antibodies against *Babesia* were reported in Andean foxes (*Lycalopex culpaeus*) (di Cataldo et al., 2022). As far as we know, our study addresses the infection of *Babesia* sp. in pudus for the first time.

Interestingly, phylogenies for 18S rRNA and COI genes showed with strong statistical support that *Babesia* sp. pudui relates with *B. odocoilei* (Figures 2 and 3). Although *O. virginianus* is the natural reservoir for *B. odocoilei*, infection in other cervids such as elks and reindeers has been reported as well (Holman et al., 2000; Mathieu et al., 2018). Recently, a molecular survey in humans with symptoms of babesiosis identified *B. odocoilei* as a virulent and persistent pathogen (Scott et al., 2021). Although pathogenic roles of *Babesia* sp. pudui in humans need to be explored, its phylogenetic link with *B. odocoilei* provides an interesting insight into the zoonotic potential of the species.

In wild deer populations, *Babesia* spp. prevail in endemic and stable infections; however, stress imposed by captivity in zoos or wildlife rehabilitation centres might cause a latent *Babesia* infection to flare up into clinical disease (Mathieu et al., 2018; Penzhorn, 2006). In fact, fatal cases of babesiosis have been reported in captive cervids (Mathieu et al., 2018). Importantly, dog attacks, run overs, intensive farming and change in land use that currently affects southern Chile, including Chiloé Island, constitute stressors for pudu populations and could render *Babesia* sp. pudui a matter of concern for the conservation of this deer species. Indeed, in our study half of the *Babesia*-infected pudus showed signs of ongoing infectious disease (data not shown). Therefore, we encourage the veterinary community to consider infections by *Babesia* in their differential diagnoses while attending pudus in rehabilitation centres or zoos.

Pudu deer are commonly parasitized by adults of *I. stilesi* (Nava et al., 2017), and in this study we assessed the presence of *Babesia* DNA in

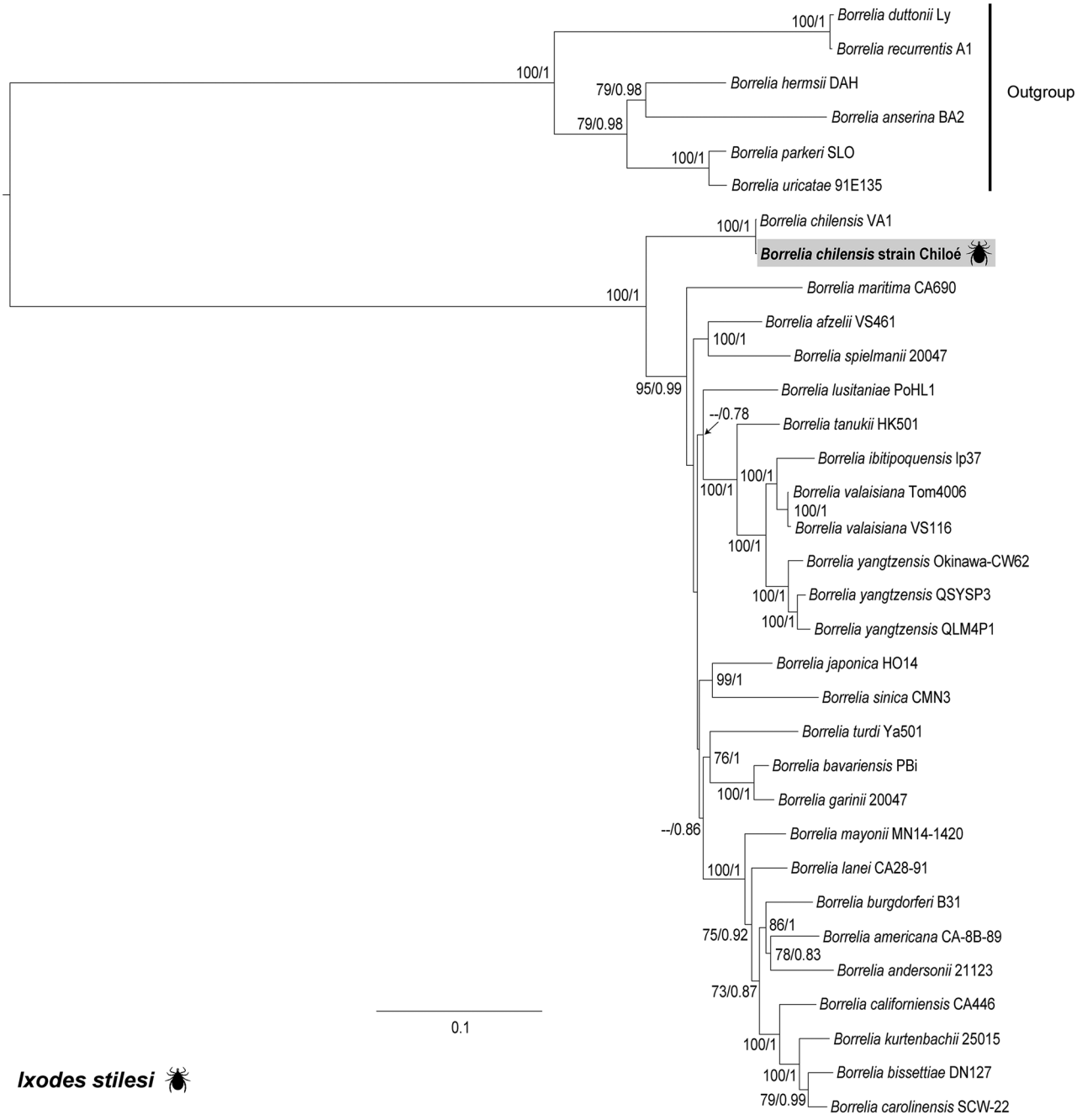

FIGURE 4 Multilocus sequencing typing (MLST) consensus tree of maximum likelihood (ML) and Bayesian (BI) phylogenies inferred for a subset of 33 *Borrelia* spp. with an alignment of 4785 base pairs. Best-fit evolutionary models calculated for ML and B methods were TIM+F+R3 (position 1), GTR+F+I+G4 (position 2) and TVM+F+I+G4 (position 3) and M_{193}, M_{168}, M_{203}, M_{198} (position 1), M_{198}, M_{134}, M_{160}, M_{200}, M_{203}, M_{189} (position 2) and M_{195}, M_{147}, M_{203}, M_{177}, M_{85}, M_{179} (position 3), respectively. Bootstrap values and Bayesian posterior probabilities are indicated above or below each branch. The position of the *Borrelia* strain characterized in the present study is highlighted within a grey box. Separated tree topologies are showed in Figure S7.

attached males and females of this tick species. Although some of the specimens were positive, our evidence is insufficient to acknowledge vector roles (Giannelli et al., 2013). However, given that *Babesia* spp. are transmitted only by ticks, and *I. stilesi* was the sole tick parasitizing pudus in this study, it is reasonable to state that it is the most probable vector for *Babesia* sp. pudui. Future investigations should aim to evaluate the interactions between deer, *I. stilesi* and livestock in the region, because domestic ruminants and cervids inhabiting the same ecosystem can share infections of a given *Babesia* sp. (Cripps et al., 2019).

On the other hand, the fact that blood of pudus was negative for *Borrelia* DNA is not unexpected, since cervids have been reported to be

TABLE 2 Overview table of pairwise sequence identities (%) for *Babesia* 18S rRNA, COI and *cytb* genes based on the alignments used to infer the phylogenetic trees. Extended tables for each gene are available in Figures S4–S6

	Babesia sp. pudui clade	*Babesia* sp. ex *Rangifer tarandus tarandus* RD6	*Babesia odocoilei* clade	*Babesia* sp. EU1 clade	'Deer clade'	*Babesia capreoli* clade	*Babesia* sp. ex *Cervus nippon*	*Babesia divergens* clade	*Babesia gibsoni*	*Babesia canis*	*Babesia rossi*	*Babesia vogeli*
Babesia sp. pudui clade (18S rRNA)	99.83–100	99.22–99.39	98.66–99.30	97.39–98.32	97.30–98	97.67–97.90	97.50–97.70	97.02–97.21	94.82–95.07	92.35–93.53	92.35–93.53	92.03–93.05
Babesia sp. pudui clade (COI)	98.68–100	N/A	92.82–94.13	85.56–87.57	86.65–87.85	85.33–86.56	N/A	85.75–86.92	82.12–82.68	78.76–81.64	80.29–81.64	79.32–80.28
Babesia sp. pudui clade (*cytb*)	99.07–100	N/A	N/A	N/A	N/A	N/A	N/A	N/A	78.60–80.12	78.24–78.99	76.4–76.92	77.96–78.36

incompetent hosts for these spirochetes elsewhere (Kurokawa et al., 2020). Indeed, in a previous *Borrelia* screening performed on pudus the animals were negative, even with positive ticks being collected on them (Verdugo et al., 2017). Therefore, pudus are likely not a reservoir for these spirochetes in nature, but play a fundamental role in the perpetuation and propagation of *I. stilesi*, since they host the adults of this tick species.

Although the ticks analysed in this study were not georeferenced, we aimed to detect and characterize DNA of *B. burgdorferi* s.l. because they came from an insular territory. *Borrelia chilensis* VA1 is to date the sole species of the genus that has been detected in an inland *I. stilesi* population; therefore, we reasoned that the spirochetes in ticks from Chiloé Island would likely represent new genetic variants or species. Only one tick was positive, and the genetic evidence indicated a novel variant of *B. chilensis*, since two loci *flaB* and *pepX* differed 0.93% and 0.18% from the type strain (VA1) for the species, respectively. Chiloé Island became separated from the continent after the coldest Pleistocene glaciation events that occurred near 700,000 years ago, and insular populations of pudu split from the continent <437,000 years ago (Fuentes-Hurtado et al., 2011). It seems then that such a period of isolation would not afford for considerable genetic variability in a clonal population of Bbsl. Indeed, the eight housekeeping genes of the MLST scheme used in our study evolve slowly; therefore, minor genetic differences within a species are likely to represent thousands instead of millions of years of divergence (Margos et al., 2008). This fact reinforces the hypothesis that *B. chilensis* strain Chiloé diverged recently and that its isolation because of the Pleistocene glaciation induced the observed genetic variability. However, our study provides the characterization of *Borrelia* in only one positive tick, so further analyses including *I. stilesi* from a wider range are needed to understand the evolutionary history of *B. chilensis*.

Finally, phylogenies based on the *Borrelia flaB* locus have provided evidence that genotypes closely related with *B. chilensis* also occur in ticks of the *I. sigelos* group, *Ixodes* cf. *neuquenensis* and the rodent *O. longicaudatus* (Muñoz-Leal et al., 2019; Sebastian et al., 2016; Thomas et al., 2020), suggesting an ancestral lineage of Bbsl associated with South American ticks and rodents (Figure S8). Although phylogenetic analyses based on *flaB* exhibit high resolution to separate *Borrelia* genospecies (Fukunaga et al., 1996), they fail to demarcate long-term evolutionary relationships or clonal stability (Margos et al., 2008). To fill the gaps in the evolutionary pathways of *B. chilensis* and related genospecies among ticks of genus *Ixodes* in southern South America, further research should aim at characterizing by MLST other Bbsl genospecies that occur in the region.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Preparation of samples, data curation, data analysis, and the first draft of the manuscript was conceived by Adriana Santodomingo, Sofía Robbiano, Richard Thomas, Catalina Parragué-Migone and Sebastián Muñoz-Leal. All authors commented on posterior versions of the manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data sets generated and/or analysed during the current study are available in this article and Supporting Information.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. Procedures performed in this study were verified and approved by the Bioethics Committee of School of Veterinary sciences, Universidad de Concepción (CBE-07-2022).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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SUPPORTING INFORMATION

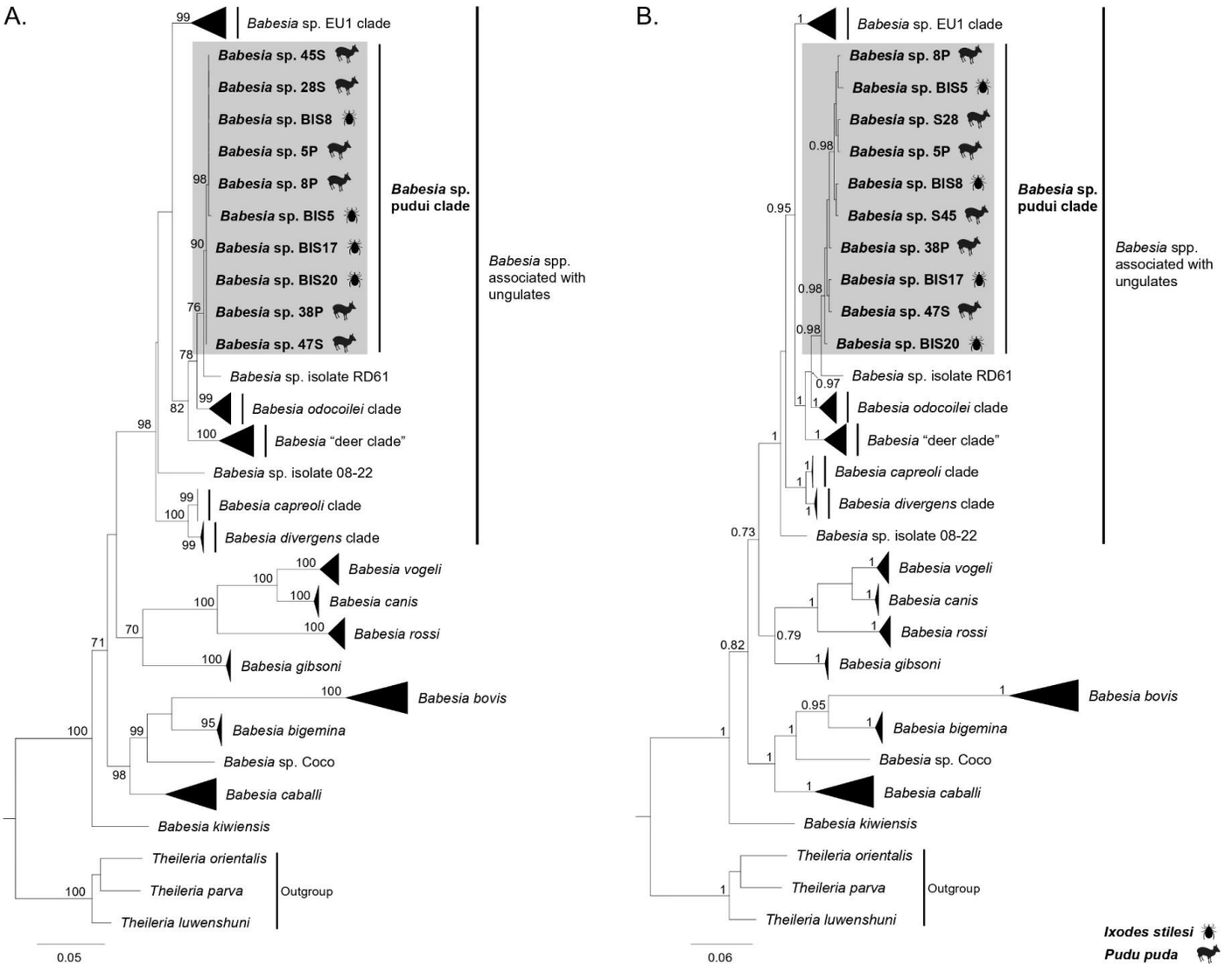

Figure S1. Maximum likelihood (A) and Bayesian inference (B) trees for a subset of Piroplasmida spp. using the 18S rRNA gene.

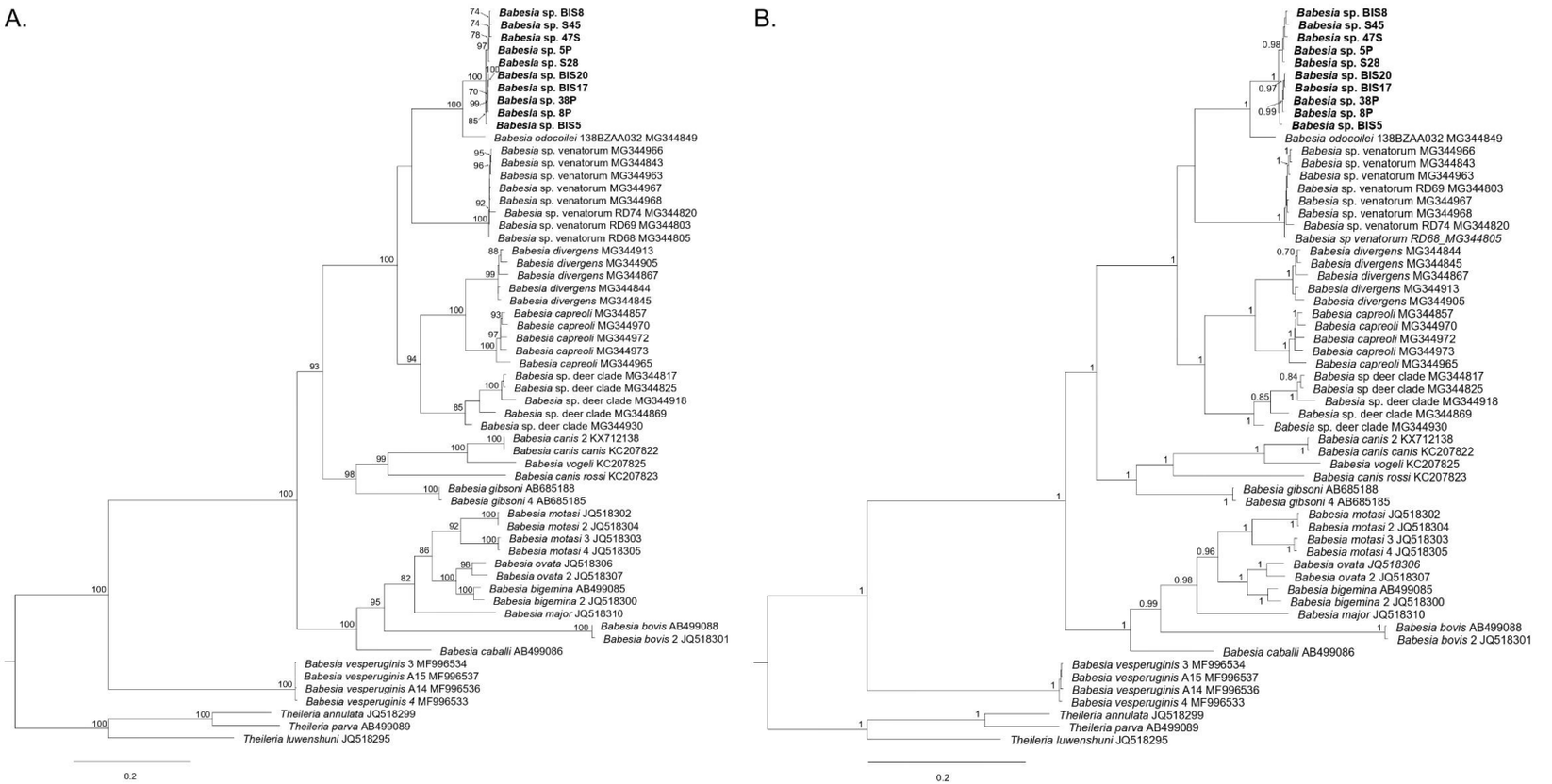

Figure S2. Maximum likelihood (A) and Bayesian inference (B) trees for a subset of Piroplasmida spp. using the COI gene.

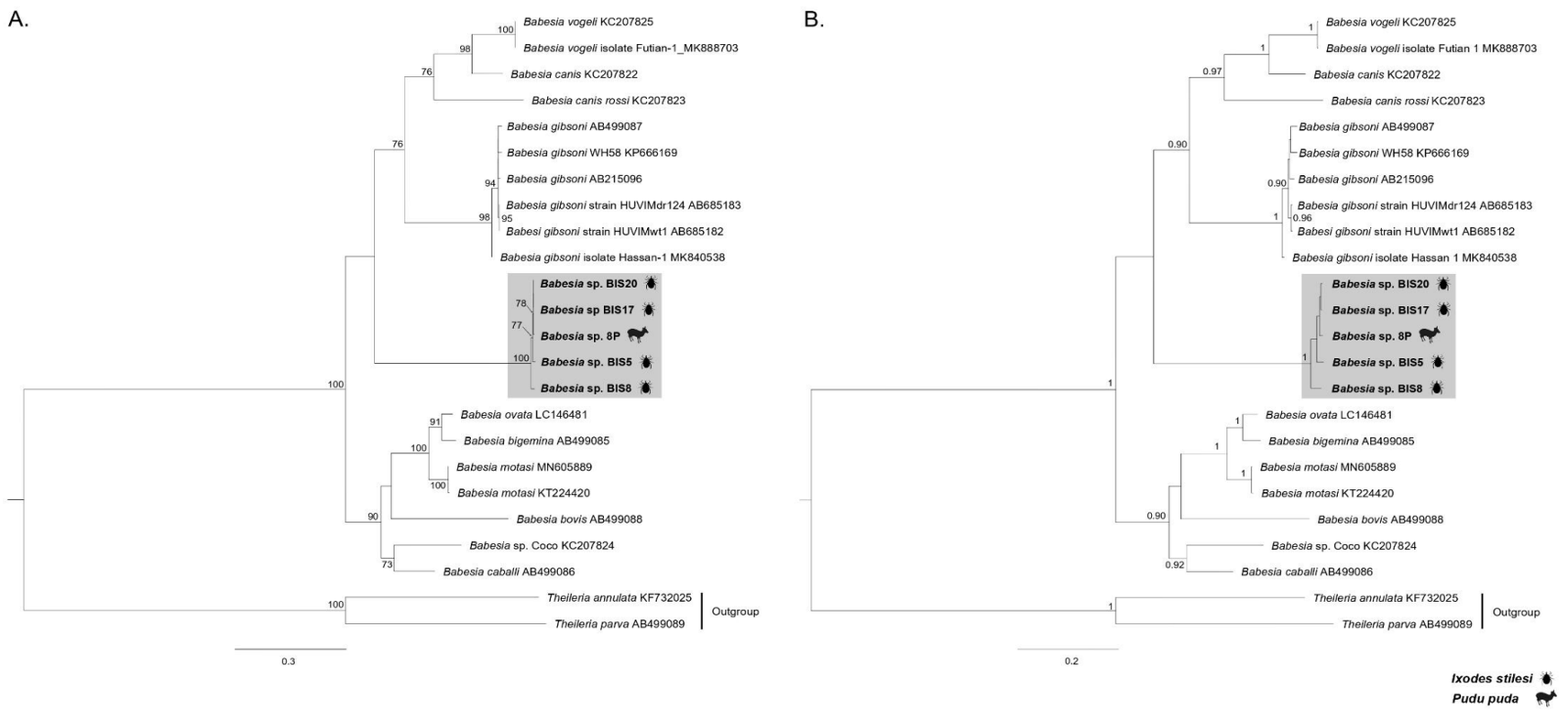

Figure S3. *cytb* maximum likelihood (A) and Bayesian inference (BI) phylogenies inferred for a subset of 24 Piroplasmida sequences with an alignment length of 1,089 base pairs. Best-fit evolutionary models calculated for ML and BI methods were HKY+F+G4 (position 1), TPM3u+F+G4 (position 2), TIM2+F+G4 (position 3); and M_{15}, M_{40}, M_{90}, M_{50}, M_{85} (position1), M_{29}, M_{71}, M_{54}, M_{68}, M_{129}, M_{90} (position2), and M_{134}, M_{198}, M_{189}, M_{200}, M_{203}, M_{200} (position 3), respectively. Bootstrap values and Bayesian posterior probabilities are indicated above or below each branch. The position of *Babesia* spp. characterized in the present study are highlighted within a grey box.

Figure S4. Heatmap matrix of pairwise sequence identities constructed for the 18S rRNA gene of *Babesia* spp. (*Babesia* sp. pudui sequences are highlighted in bold).

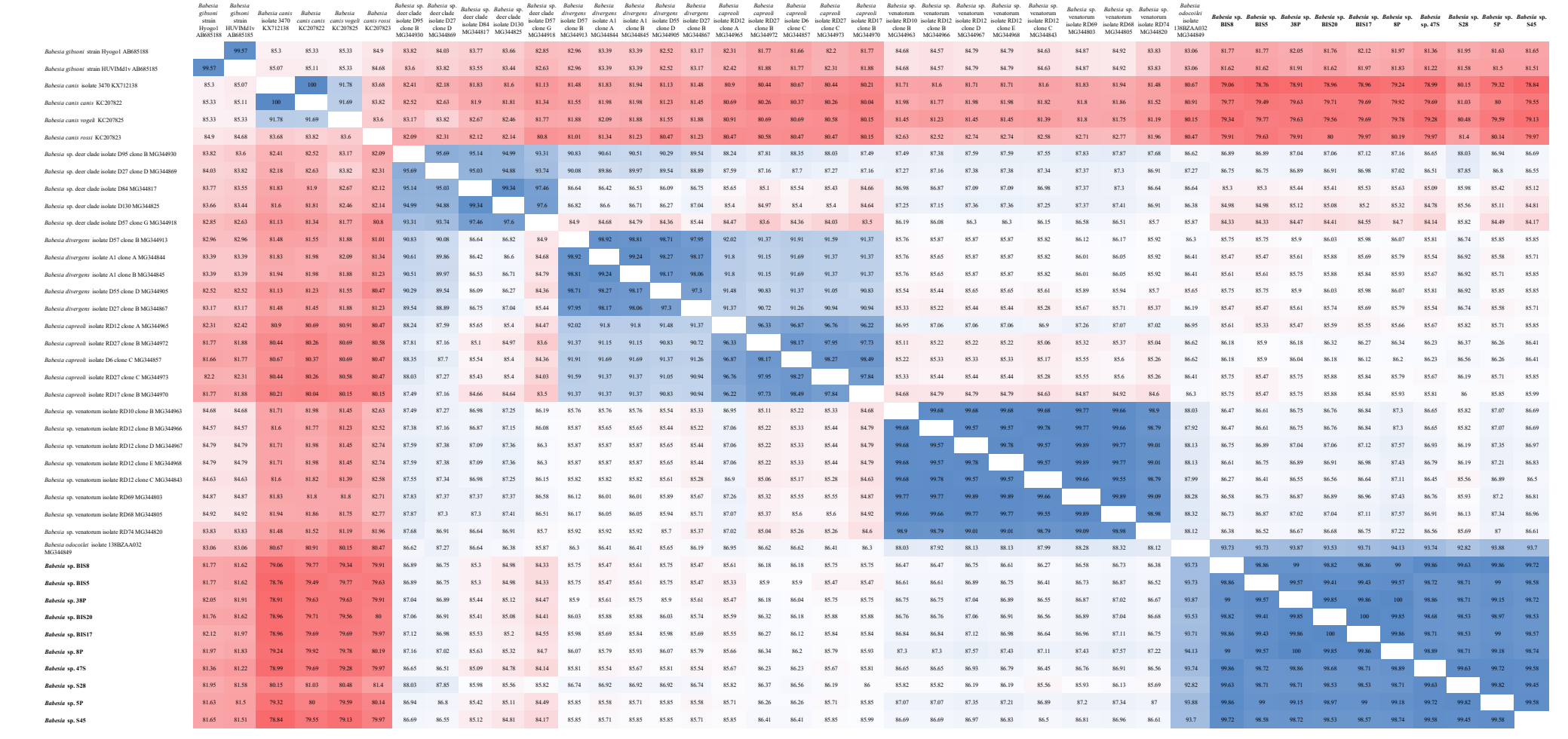

Figure S5. Heatmap matrix of pairwise sequence identities constructed for the COI gene of *Babesia* spp. (*Babesia* sp. pudui sequences are highlighted in bold).

	Babesia gibsoni AB499087	*Babesia gibsoni* isolate WH58 KP666169	*Babesia gibsoni* AB215096	*Babesia gibsoni* strain HUVIMdr124 AB685183	*Babesi gibsoni* strain HUVIMwt1 AB685182	*Babesia gibsoni* isolate Hassan-1 MK840538	*Babesia canis canis* KC207822	*Babesia canis vogeli* KC207825	*Babesia canis vogeli* isolate Futian-1 MK888703	*Babesia canis rossi* KC207823	***Babesia* sp. BIS20**	***Babesia* sp. BIS17**	***Babesia* sp. BIS5**	***Babesia* sp. BIS8**	***Babesia* sp. 8P**
Babesia gibsoni AB499087		99.45	99.53	99.63	99.63	98.5	82	82.46	82.46	81.36	79.81	79.81	79.61	79.3	78.6
Babesia gibsoni isolate WH58 KP666169	99.45		99.53	99.63	99.63	98.5	81.63	82.37	82.37	81.18	79.71	79.71	79.5	79.19	78.42
Babesia gibsoni AB215096	99.53	99.53		99.72	99.72	98.6	81.65	82.49	82.49	81.09	79.81	79.81	79.61	79.3	78.65
Babesia gibsoni strain HUVIMdr124 AB685183	99.63	99.63	99.72		100	98.69	81.74	82.49	82.49	81.27	79.92	79.92	79.71	79.4	78.75
Babesi gibsoni strain HUVIMwt1 AB685182	99.63	99.63	99.72	100		98.69	81.74	82.49	82.49	81.27	79.92	79.92	79.71	79.4	78.75
Babesia gibsoni isolate Hassan-1 MK840538	98.5	98.5	98.6	98.69	98.69		82.21	82.3	82.3	81.09	80.12	80.12	79.81	79.81	79.03
Babesia canis canis KC207822	82	81.63	81.65	81.74	81.74	82.21		89.81	89.81	83.75	78.99	78.99	78.88	78.78	78.24
Babesia canis vogeli KC207825	82.46	82.37	82.49	82.49	82.49	82.3	89.81		100	83.2	78.36	78.36	78.36	78.05	77.96
Babesia canis vogeli isolate Futian-1 MK888703	82.46	82.37	82.49	82.49	82.49	82.3	89.81	100		83.2	78.36	78.36	78.36	78.05	77.96
Babesia canis rossi KC207823	81.36	81.18	81.09	81.27	81.27	81.09	83.75	83.2	83.2		76.92	76.92	76.92	76.81	76.4
***Babesia* sp. BIS20**	79.81	79.71	79.81	79.92	79.92	80.12	78.99	78.36	78.36	76.92		100	99.59	99.28	99.9
***Babesia* sp. BIS17**	79.81	79.71	79.81	79.92	79.92	80.12	78.99	78.36	78.36	76.92	100		99.59	99.28	99.9
***Babesia* sp. BIS5**	79.61	79.5	79.61	79.71	79.71	79.81	78.88	78.36	78.36	76.92	99.59	99.59		99.07	99.48
***Babesia* sp. BIS8**	79.3	79.19	79.3	79.4	79.4	79.81	78.78	78.05	78.05	76.81	99.28	99.28	99.07		99.17
***Babesia* sp. 8P**	78.6	78.42	78.65	78.75	78.75	79.03	78.24	77.96	77.96	76.4	99.9	99.9	99.48	99.17	

Figures S6. Heatmap matrix of pairwise sequence identities constructed for the cytb gene of *Babesia* spp. (*Babesia* sp. pudui sequences are highlighted in bold).

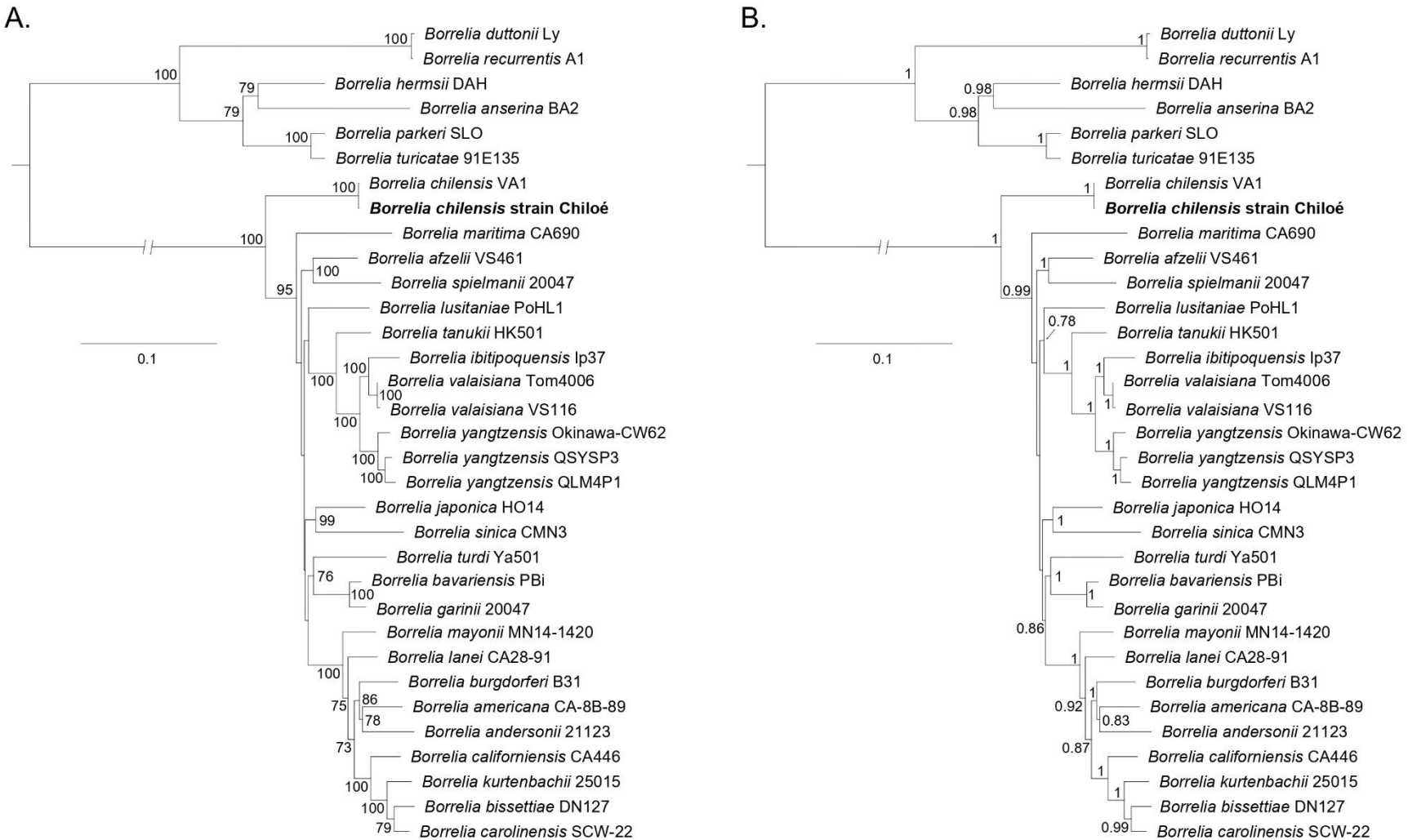

Figure S7. Maximum likelihood (A) and Bayesian inference (B) trees for *Borrelia* MLST.

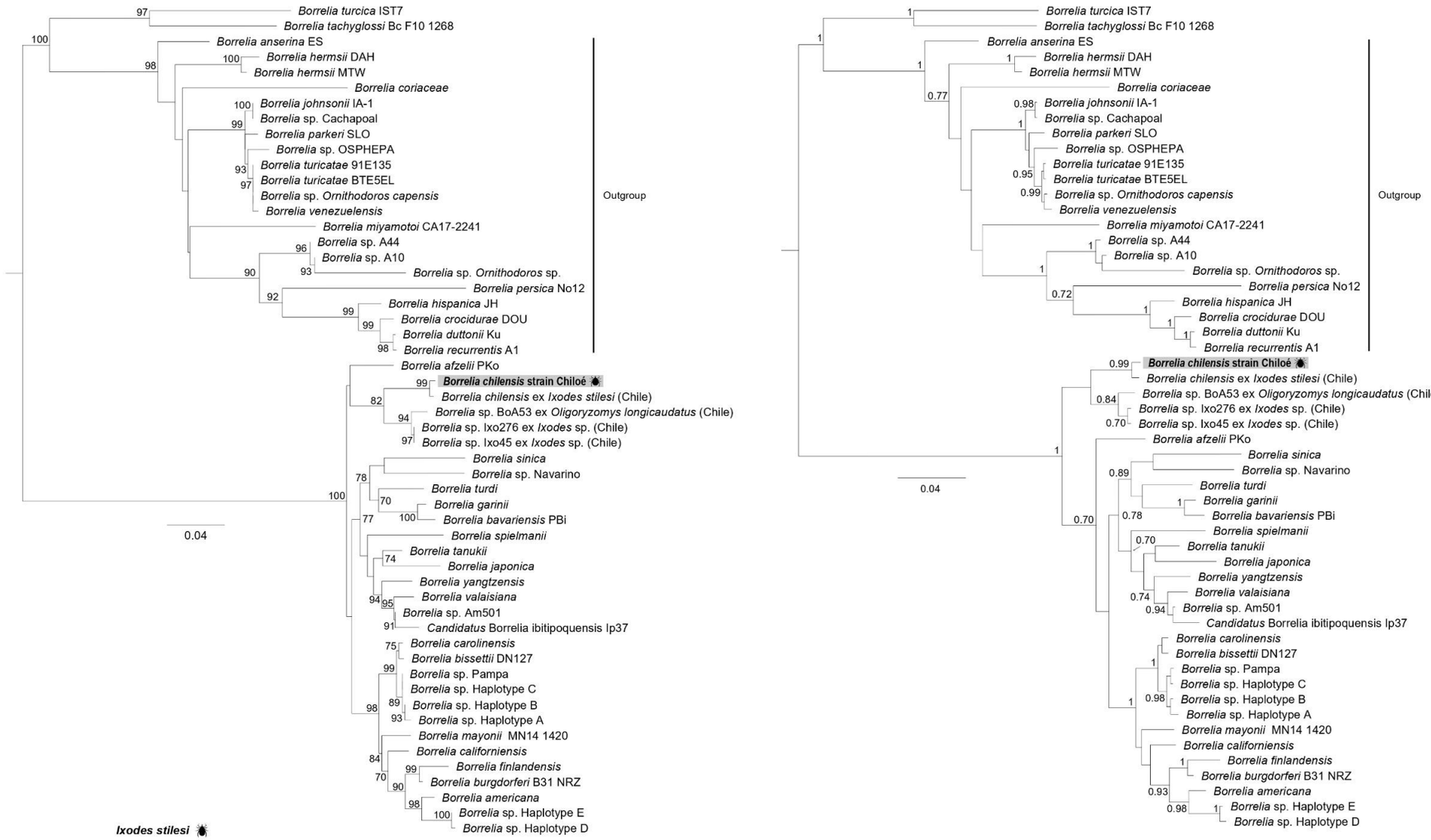

Figure S8. *flaB* Maximum likelihood (A) and Bayesian inference (BI) phylogenies inferred for 54 sequences of *Borrelia* with an alignment of 1,014 base pairs. Best-fit evolutionary models calculated for ML and BI were TVM+F+I+G4 (position 1), HKY+F+I+G4 (position 2) and TIM+F+I+G4 (position 3), and M_{27}, M_{95}, M_{94}, M_{66}, M_{52} (position 1), M_{29}, M_{90} (position 2), M_{85}, M_{134}, M_{189}, M_{177}, M_{147}, M_{125} (position 3), respectively. Bootstrap values and Bayesian posterior probabilities are indicated above or below each branch. The position of the *Borrelia* characterized in the present study, they have highlighted a gray box.

Table S1. Primers and thermal conditions used for PCR detection and genetic characterization of ticks, *Babesia*, and *Borrelia*.

Organism	Gene	Primer	PCR	Sequence	T_o (C°)	Expected length (bp)	Reference
Mammals	*gapdh*	gapdh F gapdh R	Primary	CCTTCATTGACCTCAACTACAT CCAAAGTTGTCATGGATGACC	52	400	Birkenheuer et al. (2003)
Ticks	16S rRNA	16S+1 16S-1	Primary	CCGGTCTCAACTCAGATCAAGT GCTCAATGATTTTTTAAATTGCTGT	*47–48.8 phase 1 50 phase 2	460	Mangold et al. (1998)
Piroplasmida	18S rRNA	Nbab_1F 18SApiR	Primary	AAGCCATGCATGTCTAAGTATAAGCTTTT GGATCACTCGATCGGTAGGAG	60	1500	Oosthuizen et al. (2008) Greay et al. (2018)
	COI	COI-F COI-R	Primary	GGAAGTGGWACWGGWTGGAC TTCGGTATTGCATGCCTTG	59-60	1080	Schreeg et al. (2016)
	cyt*b*	cytb-F cytb-R	Primary	TTAGTGAAGGAACTTGACAGGT CGGTTAATCTTTCCTATTCCTTACG	55	1300	Schreeg et al. (2016)
Borrelia	*flaB*	FLA LL FLA RL FLA LS FLA RS	Primary Nested	ACATATTCAGATGCAGACAGAGGT GCAATCATAGCCATTGCAGATTGT AACAGCTGAAGAGCTTGGAATG CTTTGATCACTTATCATTCTAATAGC	55 55	665 354	Stromdahl et al. (2003)
	16S rRNA	FD3 16S-1	Primary	AGAGTTTGATCCTGGCTTAG TAGAAGTTCGCCTTCGCCTCTG	53	730	Ras et al. (1996)

* The annealing temperature of the first 7 cycles in phase one was increased by 0.3 °C every second cycle from 47 to 48.8 °C, followed by 28 cycles using an annealing temperature of 50 °C in phase 2.

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Table S2. GenBank accession number of *Babesia* phylogenies.

Sequence name	Sequence length (base pairs)	Vertebratae host (or tick host)	Country	GenBank accession number
		18S rRNA		
Babesia sp. deer clade isolate D84	1617	*Cervus elaphus*	Czech Republic	MG344776
Babesia sp. deer clade isolate D93	1555	*Cervus elaphus*	Czech Republic	MG344773
Babesia sp. deer clade isolate D92	1616	*Cervus elaphus*	Czech Republic	MG344775
Babesia sp. MA-2016a isolate v5	1413	Goat	Turkey	KU714605
Babesia sp. MA-2016a isolate v8	1400	goat	Turkey	KU714606
Babesia sp. venatorum isolate RD8	1621	*Capreolus capreolus*	Czech Republic	MG344777
Babesia sp. EU1 strain 182	1504	*Ixodes ricinus*	Italy	GU647159
Babesia sp. EU1 isolate rd2666	1113	Bovine	France	HQ830266
Babesia sp. EU1	1094	*Capreolus capreolus*	Poland	JQ929917
Babesia sp. DD-2004	1727	*Ixodes ricinus*	Slovenia	AY553915
Babesia sp. EU1 isolate EG207	1727	*Ixodes ricinus*	Germany	HM113372
Babesia sp. EU1 clone BAB20	1727	Human	Italy and Austria	AY046575
Babesia sp. EU1 isolate 7627	1727	Sheep	France	FJ215873
Babesia sp. EU1 isolate rd 201	1623	Roe deer	France	EF185818
Babesia sp. EU1 isolate Arnhem	1705	*Rangifer tarandus*	Netherlands	GQ888709
Babesia sp. venatorum	1514	*Ixodes ricinus*	Spain	KM289157
Babesia sp. venatorum	1514	*Ixodes ricinus*	Spain	KM289158
Babesia sp. EU1 isolate Nov-Ip215	1174	*Ixodes persulcatus*	Russia	GU734773
Babesia sp. EU1 isolate hlj48	1571	*Ixodes persulcatus*	China	JQ993425
Babesia sp. YZ-2012 isolate hlj223	1637	*Ixodes persulcatus*	China	JQ993426
Babesia sp. venatorum isolate Irk-Ip14	1172	*Ixodes persulcatus*	Russia	KJ486557
Babesia sp. venatorum	1627	Human	China	KM244044
Babesia sp. venatorum strain HLJ699	1523	*Haemaphysalis concinna*	China	KU204790
Babesia sp. venatorum strain HLJ371	1618	*Ixodes persulcatus*	China	KU204792
Babesia sp. venatorum isolate BvSSR2	1666	*Ixodes persulcatus*	Mongolia	LC005776
Babesia sp. venatorum isolate BvSSR1	1665	*Ixodes persulcatus*	Mongolia	LC005775
Babesia sp. venatorum	1727	Human	China	KF724377
Babesia sp. venatorum isolate BvSSR1	1666	*Ixodes persulcatus*	Mongolia	LC005773
Babesia sp. venatorum isolate BvSSR1	1666	*Ixodes persulcatus*	Mongolia	LC005774
Babesia sp. venatorum isolate BvSSR2	1666	*Ixodes persulcatus*	Mongolia	LC005777
Babesia sp. venatorum isolate Etb5	1588	*Ixodes persulcatus*	Japan	KC493558
Babesia sp. venatorum strain HLJ104	1615	*Ixodes persulcatus*	China	KU204791
Babesia sp. RD1	1671	*Rangifer tarandus*	United States	AF158711
Babesia odocoilei clone BAB104	1727	Data no available	Data no available	AY046577
Babesia odocoilei	1225	Deer	United States	AY144689
Babesia odocoilei 138BZAA032	1607	*Cervus elaphus canadensis*	Canada	KC460321
Babesia odocoilei isolate 3	1658	*Ovibos moschatus*	United States	AY661509
Babesia odocoilei	1605	*Ovis canadensis nelsoni*	United States	AY661502
Babesia odocoilei	1608	Cervus elaphus canadensis	United States	AY661503
Babesia odocoilei isolate 1	1605	*Ovibos moschatus*	United States	AY661507
Babesia odocoilei isolate 4	1658	*Ovibos moschatus*	United States	AY661510
Babesia sp. isolate RD61	1722	*Rangifer tarandus tarandu*	United States	AF411337
***Babesia* sp. pudui BIS5**	**1419**	***Ixodes stilesi***	**Chile**	**ON994400**
***Babesia* sp. pudui BIS8**	**1379**	***Ixodes stilesi***	**Chile**	**ON994401**
***Babesia* sp. pudui BIS17**	**1373**	***Ixodes stilesi***	**Chile**	**ON994402**
***Babesia* sp. pudui BIS20**	**1416**	***Ixodes stilesi***	**Chile**	**ON994403**
***Babesia* sp. pudui 47S**	**1149**	***Pudu puda***	**Chile**	**ON994404**

***Babesia* sp. pudui 38P**	**1428**	***Pudu puda***	**Chile**	**ON994405**
***Babesia* sp. pudui 8P**	**1426**	***Pudu puda***	**Chile**	**ON994406**
***Babesia* sp. pudui 5P**	**1389**	***Pudu puda***	**Chile**	**ON994407**
***Babesia* sp. pudui 45S**	**1331**	***Pudu puda***	**Chile**	**ON994408**
***Babesia* sp. pudui 28S**	**1360**	***Pudu puda***	**Chile**	**ON994409**
Babesia sp. ex Cervus nippon	1041	*Cervus nippon*	Japan	KC465978
Babesia capreoli isolate 2770 clone F6	1724	roe deer	France	FJ944827
Babesia capreoli isolate R1	1670	*Rangifer tarandus*	Germany	KM657248
Babesia divergens	1041	*Cervus nippon*	Japan	AB861507
Babesia divergens isolate 08-41	1432	*Cervus nippon*	Japan	KC465976
Babesia gibsoni isolate: 18sRNA-Kyush	1663	Data no available	Data no available	AB478324
Babesia gibsoni isolate WM-1	1707	Dog	United States	EU084677
Babesia kiwiensis	1509	*Apteryx australis mantelli*	New Zealand	EF551335
Babesia canis rossi strain: N1	1558	Dog	Nigeria	AB935163
Babesia canis rossi strain: N4	1557	Dog	Nigeria	AB935166
Babesia rossi strain NGR clone 82	1637	Dog	Nigeria	JN982353
Babesia canis rossi isolate Dog-44	1590	Dog	Sudan	DQ111760
Babesia canis vogeli	1713	Dog	Italy	AY072925
Babesia canis vogeli from Brazil	1599	Dog	Brazil	AY371196
Babesia canis vogeli isolate Guangxi	1659	*Canis lupus familiaris*	China	KJ939326
Babesia canis vogeli strain TWN2	1675	*Canis familiaris*	Taiwan	HQ148664
Babesia canis canis	1714	Dog	Croatia	AY072926
Babesia canis canis isolate Bd6-2	1258	Dog	Russia	AY962187
Babesia canis canis isolate Dog-1	1585	*Canis familiaris*	Estonia	KT008057
Babesia caballi genotype B2_CABRBEQ	1510	Horse	South Africa	EU642514
Babesia caballi isolate CABEQ51_B1	1468	Horse	South Africa	EU888901
Babesia caballi	1685	Equus caballus	Spain	AY309955
Babesia sp. Coco	1698	*Canis lupus familiaris*	United States	EU109716
Babesia bigemina strain 563	1689	*Bubalus babalis*	China	HQ840960
Babesia bigemina isolate biLushi	1689	Cattle	China	JX495402
Babesia bigemina strain RG	1539	Cattle	Australia	JQ437264
Babesia bovis (BBOV2)	1653	Data no available	Data no available	L19077
Babesia bovis isolate Bareilly	1610	Cattle	India	KF928959
Babesia bovis isolate USDA IA clone 6	1588	*Bos* sp.	United States	HQ264112
Babesia bovis isolate boLushi	1654	Cattle	China	JX495403
Babesia bovis from cow	1649	Cow	Portugal	AY150059
Theileria orientalis from China	1748	Data no available	China	HM538266
Theileria luwenshuni clone Songxian 2	1746	Data no available	China	JX469516
Theileria parva clone Mz13j	1581	*Syncerus caffer*	South Africa	HQ895985
		COI		
Babesia vesperuginis isolate 2_CZ	933	*Nyctalus noctula*	Czech Republic	MF996534
Babesia vesperuginis isolate A.15	933	*Vespertilio murinus*	Austria	MF996537
Babesia vesperuginis isolate A.14	918	*Vespertilio murinus*	Austria	MF996536
Babesia vesperuginis isolate 1_CZ	933	*Nyctalus noctula*	Czech Republic	MF996533
Babesia gibsoni strain Hyogo1	1428	*Canis familiaris*	Japan	AB685188
Babesia gibsoni strain HUVIMd1v	1428	*Canis familiaris*	Japan	AB685185
Babesia canis isolate 3470	864	*Canis aureus*	Romania	KX712138
Babesia canis	1434	Canine	United States	KC207822
Babesia vogeli	1317	Canine	United States	KC207825
Babesia rossi	1434	Canine	United States	KC207823
Babesia sp. deer clade isolate D95 clon	927	*Cervus elaphus*	Czech Republic	MG344930
Babesia sp. deer clade isolate D27 clon	927	*Cervus elaphus*	Czech Republic	MG344869
Babesia sp. deer clade isolate D84	906	*Cervus elaphus*	Czech Republic	MG344817

Babesia sp. deer clade isolate D130	918	*Cervus elaphus*	Czech Republic	MG344825
Babesia sp. deer clade isolate D57 clon	927	*Cervus elaphus*	Czech Republic	MG344918
Babesia divergens isolate D57 clone B	927	*Cervus elaphus*	Czech Republic	MG344913
Babesia divergens isolate A1 clone A	927	Human	Ireland	MG344844
Babesia divergens isolate A1 clone B	927	Human	Ireland	MG344845
Babesia divergens isolate D55 clone D	927	*Cervus elaphus*	Czech Republic	MG344905
Babesia divergens isolate D27 clone B	927	*Cervus elaphus*	Czech Republic	MG344867
Babesia capreoli isolate RD12 clone A	927	*Capreolus capreolus*	Czech Republic	MG344965
Babesia capreoli isolate RD27 clone B	927	*Capreolus capreolus*	Czech Republic	MG344972
Babesia capreoli isolate D6 clone C	927	*Cervus elaphus*	Czech Republic	MG344857
Babesia capreoli isolate RD27 clone C	927	*Capreolus capreolus*	Czech Republic	MG344973
Babesia capreoli isolate RD17 clone B	927	*Capreolus capreolus*	Czech Republic	MG344970
Babesia sp. venatorum isolate RD10 cl	927	*Capreolus capreolus*	Czech Republic	MG344963
Babesia sp. venatorum isolate RD12 cl	927	*Capreolus capreolus*	Czech Republic	MG344966
Babesia sp. venatorum isolate RD12 cl	924	*Capreolus capreolus*	Czech Republic	MG344843
Babesia sp. venatorum isolate RD12 cl	927	*Capreolus capreolus*	Czech Republic	MG344967
Babesia sp. venatorum isolate RD69	879	*Capreolus capreolus*	Czech Republic	MG344803
Babesia sp. venatorum isolate RD12 cl	927	*Capreolus capreolus*	Czech Republic	MG344968
Babesia sp. venatorum isolate RD68	882	*Capreolus capreolus*	Czech Republic	MG344805
Babesia sp. venatorum isolate RD74	909	*Capreolus capreolus*	Czech Republic	MG344820
Babesia odocoilei isolate 138BZAA032	927	*Cervus elaphus canadensis*	Canada	MG344849
Babesia caballi	1141	Equine	United States	AB499086
Babesia major	935	Cattle	China	JQ518310
Babesia motasi	977	Sheep	China	JQ518302
Babesia motasi	976	Sheep	China	JQ518304
Babesia motasi	977	Sheep	China	JQ518303
Babesia motasi	978	Sheep	China	JQ518305
Babesia ovata	977	Cattle	China	JQ518306
Babesia ovata	976	Cattle	China	JQ518307
Babesia bigemina	1434	Bovine	United States	AB499085
Babesia bigemina	977	Cattle	China	JQ518300
Babesia bovis isolate Miyama	1405	Bovine	United States	AB499088
Babesia bovis	964	Cattle	China	JQ518301
***Babesia* sp. pudui BIS5**	974	***Ixodes stilesi***	**Chile**	**ON995389**
***Babesia* sp. pudui BIS8**	974	***Ixodes stilesi***	**Chile**	**ON995390**
***Babesia* sp. pudui BIS17**	907	***Ixodes stilesi***	**Chile**	**ON995391**
***Babesia* sp. pudui BIS20**	944	***Ixodes stilesi***	**Chile**	**ON995392**
***Babesia* sp. pudui 47S**	1002	***Pudu puda***	**Chile**	**ON995393**
***Babesia* sp. pudui 38P**	993	***Pudu puda***	**Chile**	**ON995394**
***Babesia* sp. pudui 8P**	999	***Pudu puda***	**Chile**	**ON995395**
***Babesia* sp. pudui 5P**	1005	***Pudu puda***	**Chile**	**ON995396**
***Babesia* sp. pudui 45S**	978	***Pudu puda***	**Chile**	**ON995397**
***Babesia* sp. pudui 28S**	681	***Pudu puda***	**Chile**	**ON995398**
Theileria annulata	1308	Cattle	China	JQ518299
Theileria parva	1440	Bovine	United States	AB499089
Theileria luwenshuni	1308	Sheep	China	JQ518295
		cytb		
Babesia gibsoni	1092	canine	United States	AB499087
Babesia gibsoni isolate WH58	1092	Dog	China	KP666169
Babesia gibsoni	1105	Dog	Japan	AB215096
Babesia gibsoni strain HUVIMdr124	1071	*Canis familiaris*	Japan	AB685183
Babesia gibsoni strain HUVIMwt1	1071	*Canis familiaris*	Japan	AB685182
Babesia gibsoni isolate Hassan-1	1071	Dog	India	MK840538

Babesia vogeli	1092	canine	United States	KC207825
Babesia vogeli isolate Futian-1	1092	Dog	China	MK888703
Babesia rossi	1092	canine	United States	KC207823
***Babesia* sp. pudui BIS20**	1070	***Ixodes stilesi***	**Chile**	**ON995399**
***Babesia* sp. pudui BIS17**	967	***Ixodes stilesi***	**Chile**	**ON995400**
***Babesia* sp. pudui BIS8**	1051	***Ixodes stilesi***	**Chile**	**ON995401**
***Babesia* sp. pudui BIS5**	1029	***Ixodes stilesi***	**Chile**	**ON995402**
***Babesia* sp. pudui 8P**	1164	***Pudu puda***	**Chile**	**ON995403**
Babesia ovata	1092	Data no available	Japan	LC146481
Babesia bigemina	1092	Bovine	United States	AB499085
Babesia motasi	1092	Sheep	China	MN605889
Babesia motasi strain Tianzhu	1092	Data no available	Data no available	KT224420
Babesia caballi	1092	Horse	Data no available	AB499086
Babesia bovis	1092	Cattle	Data no available	AB499088
Theileria annulata	1092	Bovine	Tunisia	KF732025
Theileria parva	1092	Bovine	United States	AB499089

Table S3. GenBank accession number and PubMLST id of *Borrelia* MLST phylogeny.

Sequence name	Strain ID PubMLST
***Borrelia chilensis* strain Chiloe IS9**	276*
Borrelia chilensis VA1	1424
Borrelia ibitipoquensis lp37	2595
Borrelia valaisiana Tom4006	1870
Borrelia valaisiana VS116	176
Borrelia yangtzensis Okinawa-CW62	1270
Borrelia yangtzensis QSYSP3	239
Borrelia yangtzensis QLM4P1	243
Borrelia tanukii HK501	1457
Borrelia bavariensis Pbi	155
Borrelia garinii 20047	153
Borrelia afzelii VS461	138
Borrelia japonica HO14	1425
Borrelia spielmanii 20047	1001
Borrelia lusitaniae PoHL1	136
Borrelia lanei CA28-91	1949
Borrelia mayonii MN14-1420	1885
Borrelia burgdorferi B31	1
Borrelia carolinensis SCW-22	1450
Borrelia bissettiae DN127	1006
Borrelia californiensis CA446	1453
Borrelia kurtenbachii 25015	1025
Borrelia americana CA-8B-89	1451
Borrelia andersoni i 21123	1135
Borrelia turdi Ya501	1458
Borrelia sinica CMN3	1136
Borrelia maritima CA690	1582
Borrelia duttonii Ly	55
Borrelia recurrentis A1	1876
Borrelia hermsii DAH	56
Borrelia turicatae 91E135	57
Borrelia parkeri SLO	1877
Borrelia anserina BA2	1875
Sequence name	**accession number**
Borrelia chilensis strain Chiloe IS9 *clpA*	ON995405
Borrelia chilensis strain Chiloe IS9 *clpX*	ON995406
Borrelia chilensis strain Chiloe IS9 *nifS*	ON995407
Borrelia chilensis strain Chiloe IS9 *pepX*	ON995408
Borrelia chilensis strain Chiloe IS9 *pyrG*	ON995409
Borrelia chilensis strain Chiloe IS9 *recG*	ON995410
Borrelia chilensis strain Chiloe IS9 *rplB*	ON995411
Borrelia chilensis strain Chiloe IS9 *uvrA*	ON995412

Table S4. GenBank accession number of *Borrelia flaB* phylogeny.

Sequence name	Sequence length (base pairs)	Vertebratae host (or tick host)	Country	*flaB* GenBank accession number
Borrelia turcica IST7	1011	*Hyalomma aegyptium*	Turkey	CP028884
Borrelia tachyglossi Bc-F10-1268	1005	*Bothriocroton concolor*	Australia	CP025785
Borrelia anserina ES	1005	*Argas persicus*	United States	CP013704
Borrelia hermsii DAH	1005	Human	United States	NC_010673
Borrelia hermsii MTW	1005	*Ornithodoros hermsi*	United States	CP005680
Borrelia coriaceae Co53	1002	*Ornithodoros coriaceus*	United States	CP005745
Borrelia johnsonii IA-1	1002	*Carios kelleyi*	United States	EU492387
Borrelia sp. Cachapoal	1002	*Ornithodoros* sp.	Chile	MK112520
Borrelia parkeri SLO	1005	*Ornithodoros parkeri*	United States	CP005851
Borrelia sp. OSPHEPA	634	*Ornithodoros spheniscus*	Chile	MH178396
Borrelia turicatae 91E135	1005	*Ornithodoros turicatae*	United States	CP000049
Borrelia turicatae BTE5EL	1005	human	United States	CP015629
Borrelia sp. *Ornithodoros capensis*	432	*Ornithodoros capensis*	Algeria	MF432464
Borrelia venezuelensis RMA01	1002	*Ornithodoros rudis*	Brazil	MG651650
Borrelia miyamotoi CA17-2241	1008	*Ixodes pacificus*	United States	NZ_CP021872
Borrelia sp. A44	336	*Phyllotis xanthopygus*	Chile	MN596013
Borrelia sp. A10	348	*Phyllotis xanthopygus*	Chile	MN596012
Borrelia sp. *Ornithodoros* sp.	302	*Ornithodoros talaje* species group	Bolivia	HM583797
Borrelia persica No12	587	*Ornithodoros papillipes*	Uzbekistan	NZ_AYOT00000000.1
Borrelia hispanica JH	932	Data no available	Data no availab	GU357615
Borrelia crocidurae DOU	1008	*Ornithodoros sonrai*	Mali	CP004267
Borrelia duttonii Ku	981	Data no available	United Kingdom	DQ346837
Borrelia recurrentis A1	1008	Data no available	France	CP000993
***Borrelia chilensis* strain Chiloe IS9**	665	*Ixodes stilesi*	Chile	**ON995404**
Borrelia spielmanii A14S	456	Human	The Netherlands	DQ111034
Borrelia carolinensis	789	*Ixodes minor*	United States	KF422810
Borrelia chilensis	354	*Ixodes stilesi*	Chile	KY412446
Borrelia sp. Ixo276	307	*Ixodes* sp.	Chile	MH178397
Borrelia sp. BoA53	325	*Oligoryzomys longicaudatus*	Chile	MN596014
Borrelia sp. Ixo45	307	*Ixodes* sp.	Chile	MH187987
Borrelia sp. Am501	987	Data no available	Japan	D82855

Candidatus Borrelia ibitipoquensis Ip37	520	*Ixodes paranaensis*	Brazil	MT130528
Borrelia sp. Pampa	318	*Ixodes longiscutatus*	Brazil	KY657353
Borrelia sp. Haplotype C	499	*Ixodes pararicinus*	Uruguay	JX082313
Borrelia sp. Haplotype B	459	*Ixodes pararicinus*	Uruguay	JX082312
Borrelia sp. Haplotype A	617	*Ixodes pararicinus*	Uruguay	JX082311
Borrelia californiensis CA446	789	*Dipodomys californicus*	United States	KF422809
Borrelia finlandensis SW180-12	789	*Ixodes ricinus*	Poland	MK604301
Borrelia americana SCW-30h	497	*Ixodes minor*	United States	HM802232
Borrelia sp. Haplotype E	482	*Ixodes pararicinus*	Uruguay	JX082315
Borrelia sp. Haplotype D	571	*Ixodes pararicinus*	Uruguay	JX082314
Borrelia sinica CMN3	974	*Niviventer* sp.	China	AB022138
Borrelia sp. Navarino	307	*Ixodes auritulus*	Chile	MH178398
Borrelia turdi	987	*Ixodes turdus*	Japan	D82849
Borrelia garinii	937	Human	Data no availabl	D89899
Borrelia japonica	987	*Ixodes ovatus*	Japan	D82852
Borrelia tanukii	987	*Ixodes tanuki*	Japan	D82847
Borrelia yangtzensis	456	*Crocidura watasei*	Japan	AB526076
Borrelia valaisiana	655	*Ixodes ricinus*	Serbia	MT358278
Borrelia mayonii MN14 1420	1011	Human	United States	NZCP015780
Borrelia afzelii PKo	1011	Data no available	Germany	CP000395
Borrelia bissettii DN127	1011	*Ixodes pacificus*	United States	NC015921
Borrelia burgdorferi B31 NRZ	1011	*Ixodes scapularis*	United States	NZCP019767
Borrelia bavariensis PBi	1011	Human	Germany	CP000013

Parasites & Vectors

RESEARCH Open Access

Wild deer (*Pudu puda*) from Chile harbor a novel ecotype of *Anaplasma phagocytophilum*

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Abstract

Background Deer species play an important role in the enzootic cycles of several *Anaplasma* species. While in the Northern Hemisphere ticks of genus *Ixodes* are well recognized vectors of these intracellular bacteria, less is known regarding the biological cycles of *Anaplasma* spp. in South America.

Methods Using PCR protocols and Sanger sequencing, we assessed the presence of *Anaplasma* spp. in blood and ticks collected on a native deer species (*Pudu puda*) from southern Chile.

Results Based on phylogenetic analyses of the 16S rRNA, *gltA* and *groEL* genes and calculation of average sequence divergence for *groEL*, our results bring to light a novel genovariant of *Anaplasma phagocytophilum* (named strain "Patagonia"). The strain represents a novel ecotype within the *A. phagocytophilum* species complex and was detected in both *P. puda* and their ticks. Using a larger matrix, denser taxon sampling and outgroup, our maximum-likelihood- and Bayesian-inferred phylogenies for *groEL* provide an accurate picture of the topology of *A. phagocytophilum* ecotypes and their evolutionary relationships.

Conclusions This is the first report of an ecotype of *A. phagocytophilum* in South America. Our results provide novel insight into the genetic diversity and ecology of this complex of bacterial lineages. Further studies should elucidate the enzootic cycle of *A. phagocytophilum* strain "Patagonia" and assess its pathogenic potential for pudues, domestic animals and humans in the region.

Keywords Southern Pudu, *Ixodes stilesi*, Wildlife, Molecular detection, Phylogenetics, Tick-borne diseases

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Background

Alphaproteobacteria in the genus *Anaplasma* are intracellular cocobacilli of mammal blood cells transmitted by ticks of genera *Amblyomma*, *Dermacentor*, *Hyalomma*, *Ixodes* and *Rhipicephalus* [1]. *Anaplasma* spp. are infectious agents that cause diseases ranging from harmless to fatal [2, 3]. Among five species and numerous genovariants that have been identified [1], *Anaplasma phagocytophilum* is of animal and public health relevance because of tick-borne fever in ruminants and granulocytic anaplasmosis in equines, canids, felids and humans in the Northern Hemisphere [4, 5].

The genetic diversity of *Anaplasma* spp. has been explored using the conserved 16S rRNA (*rrs*) gene [1]; however, due to its weak intraspecific discriminatory resolution [6], variable loci such as citrate synthase (*gltA*) and the heat-shock operon (*groEL*) have been selected as suitable markers for single-locus genetic analyses [1, 7, 8]. Based on these markers four ecotypes split into seven phylogenetic clusters have been proposed to compose the *A. phagocytophilum* complex in Europe, Asia and North America [1, 7, 8]. A bacterial ecotype is a monophyletic array of strains sharing a similar ecological niche [9, 10], for which the average sequence divergence among groups is significantly higher than the divergence within them for a given gene [9]. *Anaplasma phagocytophilum* ecotypes and clusters have been defined according to their genetics, geographic distribution, enzootic cycles, host preference and pathogenicity [7, 11]. For example, ticks of genus *Ixodes* and cervids constitute the ecological niche for *A. phagocytophilum* ecotypes I and II [1].

Cervids are reservoirs for *Anaplasma* spp. and are often parasitized by ticks of the genus *Ixodes* that transmit these bacteria [12]. For instance, in the Northern Hemisphere, *Ixodes scapularis* and *Ixodes pacificus* (USA), *Ixodes ricinus* (Europe), and *Ixodes persulcatus* (Eurasia) [13] are the known vectors of *A. phagocytophilum*. However, data on the epidemiology of *Anaplasma* spp. is vague in South American cervids [14–20], and restricted to few species from Brazil [14–17], Argentina [19] and Uruguay [18]. In Chile, temperate rainforests (roughly between 35° and 46° S) are the habitat for the pudu (*Pudu puda*), a deer species classified as near threatened [21], which is an important host of adults of the ticks *Ixodes stilesi* and *Ixodes taglei* [22]. Although the eco-epidemiological settings (i.e. *Ixodes* ticks and deer) for an ecotype of *A. phagocytophilum* to occur do exist in Chile, it is currently unknown whether the bacterium occupies this ecological niche in the country. In the present study, we analyzed blood and ticks collected directly from free-ranging pudues from southern Chile. Because only a few *Anaplasma* surveys performed in South American wild cervids have provided short sequences for the 16S rRNA locus (*rrs*) [14–19, 23], we performed genetic screenings with additional molecular markers to detect *Anaplasma* DNA to clarify inter- or intraspecific relationships.

Methods

Sample collection

During a 5-year period (2017–2022), the blood (2–4 ml) of pudues admitted to any one of two wildlife rescue centers, Centro de Conservación Chiloé Silvestre (Nal Bajo, in Chiloé Island; − 41.839786, − 73.936015° W) and Cerefas Universidad San Sebastián (Puerto Montt; − 41.469628, − 72.907159), was collected from the cephalic or saphenous vein using an evacuated tube system (Vacutainer; Beckon, Dickson, and Company, Franklin Lakes, NJ, USA) on the day of admission (Fig. 1).

In addition to blood sampling, ticks were also removed with steel tweezers from various pudues. Blood samples and ectoparasites were kept in sterile tubes containing absolute ethanol and stored at − 80 °C until processing. The morphology of ticks was examined with a Nexius-Zoom (EVO) Stereo Microscope (Euromex Microscopen B.V., Arnhem, The Netherlands) and identified according to Nava et al. [22]. The identity of *Anaplasma*-positive ticks was further validated by sequencing a fragment of

Fig. 1 Map of Chile showing the origin of rescued pudues (black icons) within the Región de Los Lagos, Chile. Brown squares indicate the rehabilitation centers. Maps were constructed with QGIS 3.18.1-Zürich (https://www.gnu.org/licenses). QGIS, Quantum Geographic Information System

the tick mitochondrial (mt) 16S ribosomal RNA (rRNA) gene [22].

DNA isolation

Genomic DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and eluted in 40 µl of buffer AE (10 mM Tris–Cl; 0.5 mM ethylenediaminetetraacetic acid [EDTA], pH 9.0). DNA was quantified with an Epoch™ Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) and assessed for quality at A260/A280 according to Khare et al. [24].

Gene amplification and sequencing

The suitability of the extracted DNA was checked by a conventional PCR (cPCR) assay targeting the mammalian glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and the tick mt 16S rRNA genes, respectively. The primers and thermal conditions used in this study together with their references are shown in Table 1. *Anaplasma* detection was achieved by implementing different nested and hemi-nested PCR protocols targeting the *rrs*, *gltA* and *groEL* genes. DNA of *Anaplasma platys* (OQ155255) was used as the positive control and nuclease-free water was used as the negative control. All PCR reactions were performed in a thermal cycler (ProFlexTM Base 32 × 3; Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) in a final reaction volume of 25 µl (12.5 µl DreamTaq Green PCR Master Mix [Thermo Fisher Scientific], 1 µl of each primer (0.4 µM), 8.5 µl of ultra-pure water and 2 µl template DNA. The PCR products were stained with GelRed® (Biotum, Tehran, Iran), separated by electrophoresis in 2% agarose gels and then visualized using an ENDURO™ GDS UV transilluminator (Labnet International, Edison, NJ, USA). Amplicons with bands of the expected size were purified and Sanger-sequenced at Macrogen (Seoul, South Korea).

Assembly and sequence analyses

Amplicon sequences were quality-checked and edited with Geneious Prime® version (v) 2021.2.2 (www.geneious.com) to generate consensus sequences. Base calls with Phred values ≥ 20 were considered suitable for the analyses [35, 36]. The BLAST® tool (https://blast.ncbi.nlm.nih.gov) was employed to compare obtained nucleotide sequences and identify orthologous sequences.

Phylogenetic analyses

Orthologous sequences downloaded from GenBank (https://www.ncbi.nlm.nih.gov) and consensus sequences were used to build alignments with the MAFFT multiple sequence alignment program using default parameters [37]. The alignments were subsequently trimmed and filtered with Block Mapping and Gathering with Entropy (BMGE) using default parameters to map informative regions for phylogenetics inferences [38].

Table 1 Primers and thermal conditions used for PCR detection and genetic characterization of *Anaplasma* and ticks

Organisms	Gene	PCR	Primer	Sequence	T_o (°C)	Expected length (in bp)	Reference
Mammals	*GAPDH*	Conventional	gapdh F	CCTTCATTGACCTCAACTACAT	52	400	[25]
			gapdh R	CCAAAGTTGTCATGGATGACC			
Ticks	Mitochondrial 16S rRNA (*rrs*)	Conventional	16S + 1	CCGGTCTCAACTCAGATCAAGT		460	[26]
			16S − 1	GCTCAATGATTTTTTAAATTGCTGT			
Anaplasma	16S rRNA (*rrs*)	Conventional	EC9	TACCTTGTTACGACTT	48	1300	[27]
			EC12A	TGATCCTGGCTCAGAACGAACG			[28]
		Nested	A17a	GCGGCAAGCCTCCCACAT	54	1200	[29]
			IS58-1345r	CACCAGCTTCGAGTTAAACC			
	groEl	Conventional	HS1a	AYTGGGCTGGTAYTGAAAT	47	1614	[30]
			GroEl_2R	CGTTCTTACTAGGAACATCAAC			[31]
		Nested	Gro677F	ATTACTCAGAGTGCTTCTCARTG	53	942	[32]
			GroEL_rev2	GCCGACTTTTAGTACAGCAA			[33]
		Heminested	GroEl_2F	TGTAAAGGCGCCTGGTTTCG	55	772	[31]
			GroEl_2R	CGTTCTTACTAGGAACATCAAC			[31]
	gltA	Conventional	F4b	CCAGGCTTTATGTCAACTGC	55	800	[34]
			R1b	CGATGACCAAAACCCAT			
		Nested	EHR-CS136F	TTYATGTCYACTGCTGCKTG	55	650	
			EHR-CS778R	GCNCCMCCATGMGCTGG			

GAPDH Glyceraldehyde-3-phosphate dehydrogenase, *gltA* citrate synthase gene, *groEL* heat-shock operon

Phylogenetic trees were constructed with the Bayesian inference (BI [39, 40]) and maximum-likelihood (ML [41]) methods in MrBayes v 3.2.6 [42] and IQ-TREE v 1.6.12 [43], respectively. As protein-coding genes present different nucleotide exchange rates (heterogeneity) at the first, second and third codon positions [42, 44], datasets were partitioned into the three codon positions (position-1, position-2 and position-3) [42, 44–46]. Then, the Model Finder command "TESTNEWONLYMERGE -mrate G" was implemented to select the best-fit evolutionary models and best-partition scheme for protein-coding gene datasets [47]. The ML best evolutionary models for non-coding genes were calculated using the ModelFinder command "-m TESTNEWONLY -mrate G" [47]. We used rapid hill-climbing and stochastic disturbance methods with 1000 ultrafast bootstrapping pseudo-replicates to evaluate the inferred tree robustness. Bootstrap values < 70%, 70–94% and ≥ 95% were considered non-significant, medium and solid statistical support [48], respectively.

BI phylogenies were constructed based on nucleotide substitution models selected with the MrBayes command "lset nst = mixed rates = gamma" for the non-coding dataset [42, 49]. On the other hand, the best partition schemes computed by ModelFinder and the MrBayes command "lset = mixed rates = invgamma" were used to calculate the best models for protein-encoding datasets [42, 46, 49]. Two independent tests of 20×10^6 generations and four Markov chain Monte Carlo (MCMC) chains were implemented, sampling trees every 1000 generations and removing the first 25% as burn-in. Tracer v1.7.1 [50] was used to confirm the correlation and effective sample size of the MCMC. Bayesian posterior probabilities (BPP) with values > 0.70 in nodes were considered to indicate strong statistical support [51]. All best-fit models and partitions schemes were selected under the Bayesian Information Criterion (BIC) [52]. Trees were visualized and edited with FigTree v 1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape v 1.1 (https://inkscape.org/es/). Congruent topologies between ML and BI analyses were used to produce strict consensus trees in Geneious Prime with the Consensus Tree Builder tool, implementing a support threshold of 100%. The consensus phylogram included all monophyletic clades after comparing ML and BI topologies for each dataset.

Genetic distance analyses

To assess the corrected pairwise distance and determine the average sequence divergence within and among ecotypes, an alignment of 936 bp was constructed with default parameters in MAFFT, including 214 *groEL* sequences of *A. phagocytophilum* with > 70% coverage between them, using *Anaplasma odocoilei* and *A. platys* as outgroups. The corrected pairwise distance was assessed using raxmlGUI [53, 54] for RAxML v 8 [55] with the GTR + GAMMA + I substitution model.

Results

Tick identification and blood samples

A total of 26 hard ticks and 55 blood samples were collected from pudues. All ticks were morphologically identified as *I. stilesi* (17 females, 5 males, 4 nymphs). Amplicons of the expected size were obtained for the mt 16S rRNA gene by PCR in 20 of the 26 tick specimens, with negative results obtained for six ticks (4 females, 1 male, 1 nymph), which were subsequently excluded from the analysis. PCR targeting the *GAPDH* gene in pudu blood resulted in amplicons of the expected size, confirming successful DNA extractions in all cases (Table 2).

Anaplasma detection

Anaplasma DNA was amplified in 8/26 (30.8%) *I. stilesi* (1 nymph, 1 male, 6 females) and in 6/55 (10.9%) pudues (Table 2). Eleven identical sequences were obtained for *rrs* (1,212 bp), 12 for *gltA* (722 bp) and 13 for *groEL* (1,286 bp). Pairwise comparisons between generated sequences indicated one genotype for *rrs*, seven genotypes for *gltA* and 11 genotypes for *groEL*. A mitochondrial genotype of 429 bp retrieved for *Anaplasma*-positive ticks (OP750053) was 99.5% (428/430 bp, 100% query cover, 2 gaps, 0 E-value) identical with a previous sequence of *I. stilesi* from Chile (DQ061292) [56].

After BLASTn comparisons, the *rrs* genotype matched with 94.8% identity *A. phagocytophilum* isolate D2_2 (MK814406), detected in *Canis lupus familiaris* from South Africa [57]; the *gltA* genotypes showed an identity ranging from 82.9% to 83.1% with *A. phagocytophilum* strain Sheep (KP861639) detected in an *Ixodes* sp. collected on a Norwegian White Sheep [58]; and the *groEL* genotypes were 91.4–91.8% identical with *A. phagocytophilum* samc001 (LC496077) detected in *Canis lupus familiaris* from Japan [59].

Phylogenies inferred for the three loci positioned *Anaplasma* genotypes retrieved from *I. stilesi* and pudu blood into the *A. phagocytophilum* clade, forming a monophyletic group (Figs. 2, 3, 4). In particular, the *groEL* phylogeny placed our genotypes in an independent clade related to ecotype III of *A. phagocytophilum* [1] (Fig. 4).

For the *groEL* gene, the average sequence divergence calculated within ecotypes was always less than the average sequence divergence calculated among them, including the ecotype characterized in this study (Table 3). Collectively, the genetic evidence provided by

Table 2 Sampled and *Anaplasma*-positive animals with the geographical coordinates of provenance

Species	Provenance	Locality[a]	Geographical coordinates (latitude, longitude)[b]	*Anaplasma phagocytophilum*[c]
Pudu puda	Continent	Cerefas Universidad San Sebastián, Puerto Montt (1)	− 41.469628, − 72.907159	1/3
	Island	Queilén (2)	− 42.885721, − 73.468359	0/3
	Island	Degañ (3)	− 42.145274, − 73.720717	0/1
	Island	Pauldeo (4)	− 41.908360, − 73.891784	0/2
	Island	Mocopulli (5)	− 42.336344, − 73.706289	0/2
	Island	Tehuaco (6)	− 42.372438, − 73.657162	0/1
	Continent	Calbuco (7)	− 41.677865, − 73.201237	0/1
	Continent	Contao (8)	− 41.803322, − 72.719169	0/2
	Continent	Ensenada (9)	− 41.213838, − 72.545666	0/1
	Continent	Peñol Bajo (10)	− 41.598174, − 73.498427	0/1
	Island	Centro de Conservación Chiloé Silvestre (11)	− 41.839786, − 73.936015	0/1
	Island	Lago Tarahuín (12)	− 42.714684, − 73.788520	0/1
	Island	Chonchi (13)	− 42.625050, − 73.774028	0/3
	Island	Chauman (14)	− 41.797195, − 73.951494	0/2
	Island	Mechaico (15)	− 41.926147, − 73.809907	1/1
	Island	Butalcura (16)	− 42.252443, − 73.736915	1/1
	Island	Quellón (17)	− 43.116902, − 73.613887	0/4
	Island	Chepu (18)	− 42.041574, − 73.973976	1/1
	Island	Castro (19)	− 42.480140, − 73.762413	0/3
	Island	Quichitúe (20)	− 42.026219, − 73.793279	0/1
	Island	Guapilacuy (21)	− 41.839390, − 73.871975	0/1
	Island	Lechagua (22)	− 41.879088, − 73.891482	0/1
	Continent	Caleta Puelche (23)	− 41.742766, − 72.648612	0/1
	Island	Dalcahue (24)	− 42.377552, − 73.651920	1/3
	Island	Caulin (25)	− 41.819313, − 73.610747	0/1
	Island	Hueihue (26)	− 41.880609, − 73.837354	0/1
	Island	Quemchi (27)	− 42.144713, − 73.478056	0/2
	Island	Puntra (28)	− 42.119891, − 73.816342	1/1
	Island	Sector Naval—Faro Corona (29)	− 41.820747, − 73.929428	0/2
	Island	El Quilar (30)	− 41.926431, − 73.616454	0/1
	Island	Lliuco (31)	− 42.042983, − 73.514278	0/2
	Continent	Osorno (32)	− 40.576192, − 73.114948	0/1
	Island	Ancud (33)	− 41.867489, − 73.827690	0/1
	Continent	Los Lagos (34)	− 41.858622, − 72.073451	0/1
	Island	Quiquel (35)	− 42.354553, − 73.579767	0/1
	Total no. positive specimens			*6/55*
Ixodes stilesi	Island	Centro de Conservación Chiloé Silvestre (11)	− 41.839786, − 73.936015	*8/20*

[a] Numbers in parentheses correlate to the sites shown in Fig. 1

[b] Presented in pure numeric format

[c] Number of positive specimens/number of specimens tested

our study points to the finding of a fifth *A. phagocytophilum* ecotype, for which the name *A. phagocytophilum* strain "Patagonia" is proposed. GenBank accession numbers generated in this study are available in Additional file 1: Tables S1, S2).

Discussion

Tick-borne bacteria, including *A. phagocytophilum*, are geographically expanding, probably due to climate change and anthropogenic landscape perturbation, both factors that favor the spread of their vectors synergically [13, 60]. Although *A. phagocytophilum* was previously

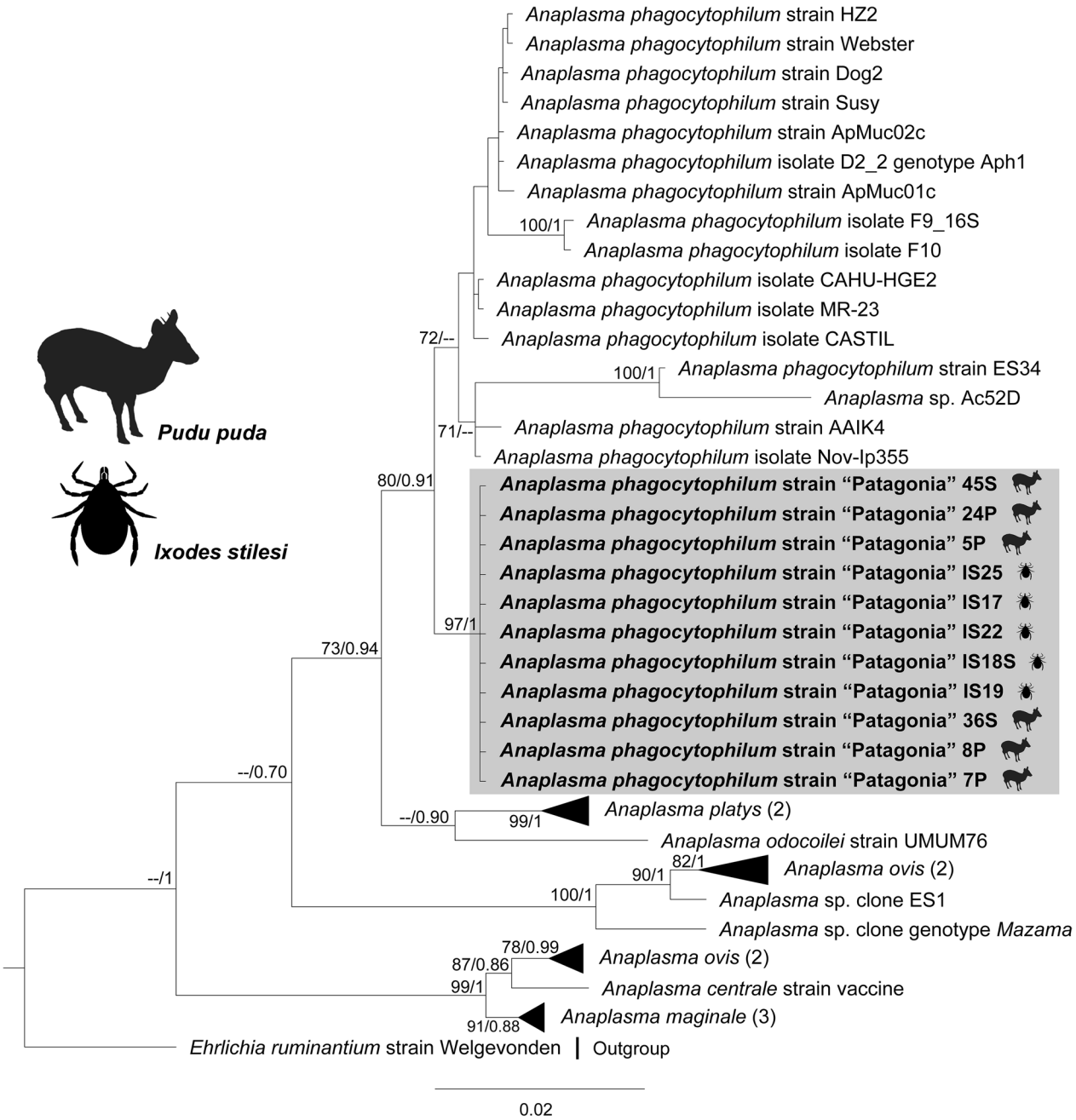

Fig. 2 Maximum likelihood (ML) and Bayesian inference (BI) *rrs* gene consensus tree inferred for a subset of *Anaplasma* spp., using 41 sequences and an alignment of 1,382 bp. Best-fit evolutionary models calculated for the ML and BI methods were TPM3u + F + G4; and M_{90}, M_{177}, M_{85}, M_{152}, M_{179}, M_{117}, M_{195}, respectively. Bootstrap values and Bayesian posterior probabilities (BPP) are indicated above or below each branch. The position of the strain of *Anaplasma phagocytophilum* characterized in the present study is highlighted in a gray box

thought to be a single bacterial species [61], recent phylogenetic reconstructions have revealed a complex of lineages with different pathogeny, geographical distribution, reservoirs and vectors [1]; nevertheless, host range, zoonotic potential and transmission dynamics of this bacterium are still incompletely solved [1, 7, 8, 11].

Based on average divergence of partial *groEL* sequences (Table 3) and strongly supported phylogenies for *rrs*, *gltA*, and *groEL*, in this study we identified a novel genovariant of *A. phagocytophilum* associated with pudues, for which the name "Patagonia" is proposed (Figs. 2, 3, 4). Accordingly, this genovariant has been

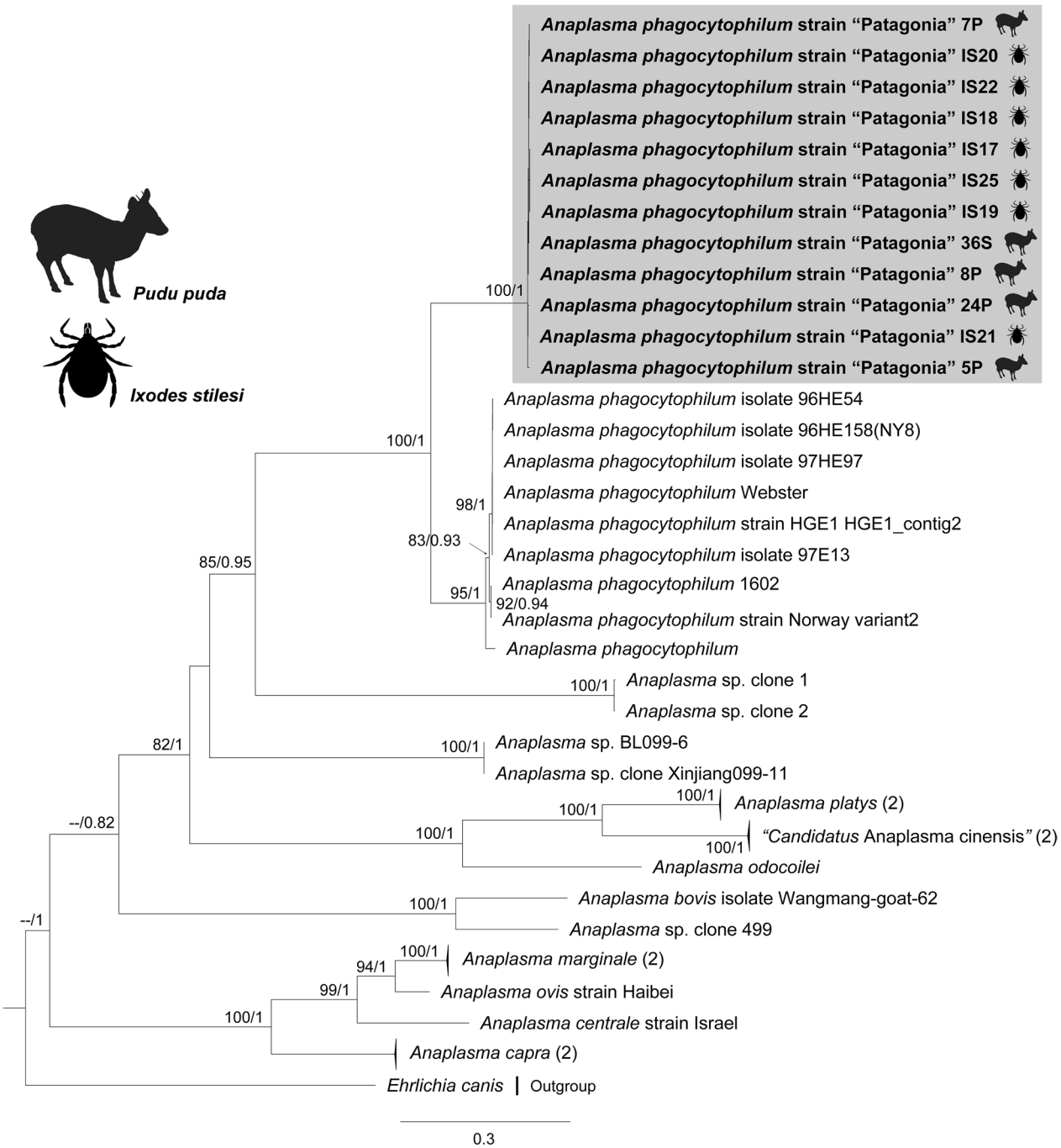

Fig. 3 ML and BI consensus tree inferred for a subset of *Anaplasma* spp., using 40 sequences of the *gltA* gene and an alignment of 1152 bp. Best-fit evolutionary models calculated for the ML and BI methods were GTR + F + I + G4 (position-1), GTR + F + G4 (position-2), HKY + F + I + G4 (position-3); and M_{64}, M_{175}, M_{173}, M_{25}, M_{171}, M_{50}, M_{125} (position-1); M_{80}, M_{135}, M_{164}, M_{166}, M_{145} (position-2); M_{90}, M_{177}, M_{152}, M_{183}, M_{136} (position-3), respectively. Bootstrap values and BPP are indicated above or below each branch. The position of the strain of *A. phagocytophilum* characterized in the present study is highlighted in a gray box

designated as the ecotype V (cluster 8) of *A. phagocytophilum*, which constitutes the first ecotype of this species complex described for South America. Variants of *A. phagocytophilum* are adapted to different hosts and vector species, therefore configuring different enzootic cycles [1, 13]. The fact that *A. phagocytophilum* strain "Patagonia" conforms an additional ecotype suggests that

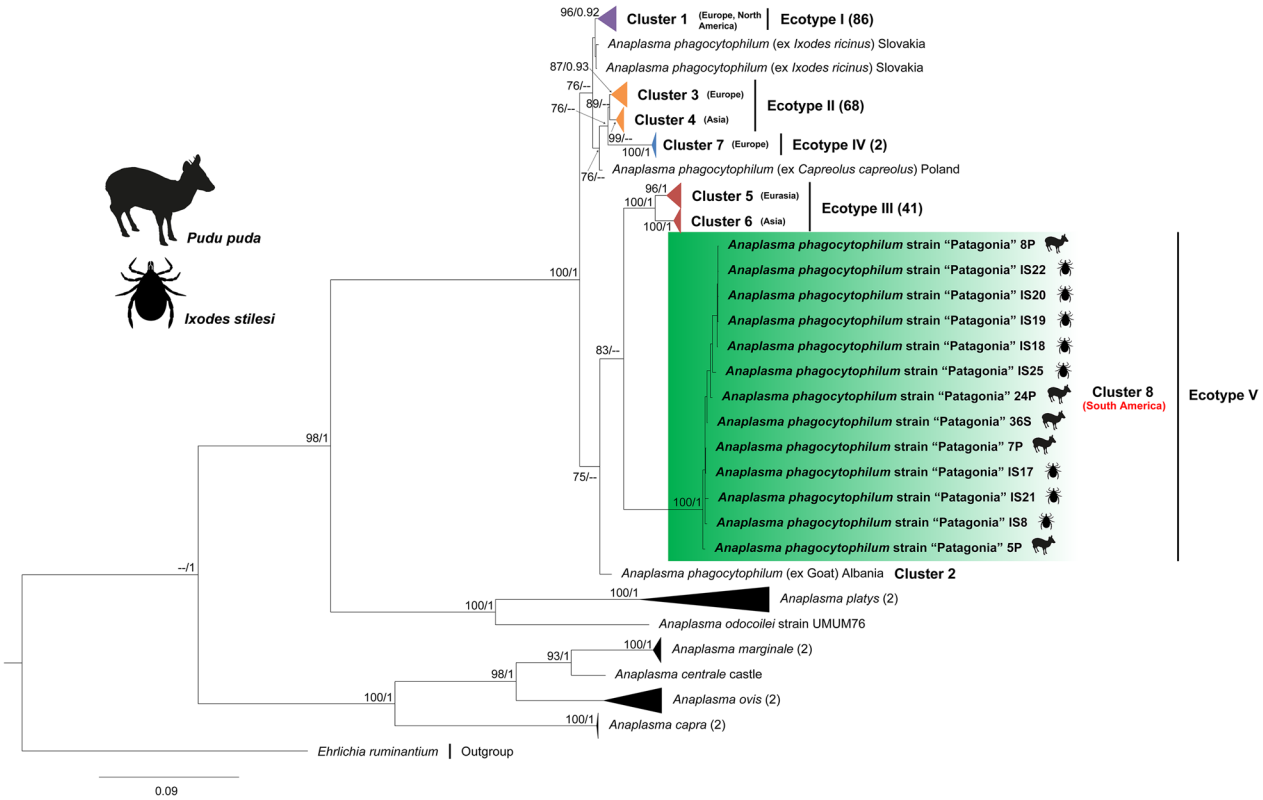

Fig. 4 ML and BI consensus tree inferred for a subset of *Anaplasma* spp., using 226 sequences of the *groEL* gene, and an alignment length of 1224 bp. Best-fit evolutionary models calculated for the ML and BI methods were TIM + F + G4 (position-1); TN + F + G4 (position-2); and K3Pu + F + G4 (position-3); and M_{45}, M_{136}, M_{142}, M_{130}, M_{139}, M_{185} (position-1); M_{81}, M_{40} (position-2); M_{15}, M_{50}, M_{85}, M_{122}, M_{90} (position-3), respectively. Bootstrap values and BPP are indicated above or below each branch. Colors for ecotypes I, II, III and IV were assigned according to Jaarsma et al. [8]. The position of the strain of *A. phagocytophilum* characterized in the present study is highlighted in a green box

Table 3 Average sequence divergence within ecotypes and among ecotypes calculated on the basis of corrected pairwise distances for a subset of *A. phagocytophilum groEl* gene sequences (936 bp)

Ecotype	Ecotype				
	I	II	III	IV	V
I	*0.006007*				
II	0.020384	*0.006410*			
III	0.068461	0.060800	*0.010504*		
IV	0.040475	0.034799	0.065253	*0.000001*	
V	0.090030	0.080672	0.080836	0.079975	*0.003386*

Values highlighted in italics are average sequence divergence within ecotypes

the eco-epidemiology of this novel strain differs from those of the northern latitudes.

Cervids such as roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), white-tailed deer (*Odocoileus virginianus*), fallow deer (*Dama dama*), sika deer (*Cervus nippon*) and their associated ticks (*I. ricinus* and *I. scapularis*) are implicated in the maintenance of endemic cycles of some *A. phagocytophilum* variants (e.g. Ap-V1, B, J, S, W) in northern latitudes [13, 62–65]. In contrast, previous knowledge on *A. phagocytophilum* in South American deer species is vague, limited only to Brazil, and does not support its classification within any ecotype. For example, in their study on the brown brocket deer (*Mazama gouazoubira*), Silveira et al. [15] could not discriminate whether *A. phagocytophilum* or *A. platys* caused the infection using PCR and sequencing protocols. However, a posterior survey revealed that *A. phagocytophilum* would be circulating in brown brocket deer [23]. On the other hand, exposure to *A. phagocytophilum* in Brazilian marsh deer (*Blastocerus dichotomus*) has been reported using indirect immunofluorescence assays [14]. As far as we know, our study is the first multigenic detection of *A. phagocytophilum* DNA in pudu and *I. stilesi*.

Records of *A. phagocytophilum* in South American mammals include rodents (*Cavia* sp. and *Calomys cerqueirai*), peccary (*Tayassu pecari* and *Pecari tajacu*), sloths (*Bradypus tridactylus*) and coati (*Nasua nasua*) [17, 66, 67]. However, due to the use of short fragments

of the *rrs* and *groEL* genes for identification, it is difficult to state whether the *Anaplasma* DNA detected in these mammals corresponded to *A. phagocytophilum* or not. While reports of *A. phagocytophilum* on South American cervids are few, other *Anaplasma* spp. have been recorded in deer in Brazil, such as *Anaplasma bovis* and *Anaplasma* sp. in red brocket deer (*Mazama americana*); *A. bovis*, *Anaplasma marginale* and *A. platys* in marsh deer; and *A. marginale* in brown brocket deer [14–17]. Likewise, the records in South America include *A. platys*, *Anaplasma odocoilei*, *A. marginale* and "*Candidatus* Anaplasma boleense" in marsh deer in Argentina [19], and *Anaplasma* sp. Mazama genotype in brown brocket deer in Uruguay [18].

In Chile, evidence of *A. phagocytophilum* is incipient. Indeed, infection by this bacterium has been reported in horses [68]. However, these results deserve further investigation, since the use of *A. phagocytophilum*-specific primers did not yield positive reactions, and the occurrence of a vector in the area where positive animals were detected is unknown. Further reports of *Anaplasma* spp. in Chile include *A. platys* in dogs, Andean foxes (*Lycalopex culpaeus*), the South American gray fox (*Lycalopex griseus*) [69] and hard ticks (*Rhipicephalus sanguineus* sensu lato). An *Anaplasma*-like agent has also been detected in seabird soft ticks (*Ornithodoros spheniscus*) [70]. Moreover, serological evidence of exposure to *Anaplasma* sp. has been recorded in dogs [71] and humans [71–74]. Our results thus expand current knowledge on vertebrate hosts of *A. phagocytophilum* in the continent.

There is no standardized approach for investigating the genetic diversity and population structure of *Anaplasma* species. Although the *rrs*, *gltA* and *groEL* markers used in this study are currently the most appropriate loci for the genetic characterization of *Anaplasma* spp. [1], *rrs* and *groEL* are conserved and do not have sufficient resolution to segregate some groups when short fragments are analyzed, even in different species of the genus. Therefore, the sequenced fragments must be long enough [1, 6]. Based on the above argument, our phylogenetic analyses did not include sequences shorter than 600 bp.

Previous studies found that the *groEL* gene may delimit lineages (ecotypes, clusters, groups) of *A. phagocytophilum* [1, 7, 8, 11]. Moreover, the discrimination capacity among lineages has improved due to the progressive increase in taxon sampling and the size of the sequences employed in the analyses [1]. Recently, a population study recovered ecotypes I, II, III and IV (mentioned by Jahfari et al. [7] and Jaarsma et al. [8]) as monophyletic but without statistical support for ecotypes I and II [1]. It is worth noting that ecotype IV was designated after including only one sequence in those analyses, and its monophyly was not assessed [1]. In addition, cluster 3 (paraphyletic within ecotype II) lacked statistical support (Electronic Supplementary Material Figure S4. in Rar et al. [1]). Thus, methodological factors, such as the inclusion of an outgroup [10, 75], longer alignments, denser taxon sampling [1, 11] and the application of phylogenetic inferences (BI, ML) [39–41], may circumscribe with higher confidence the monophyly and evolutionary relationships of ecotypes and subclades within *A. phagocytophilum*, as shown in our study.

Applying the above referred methods, ecotypes I, II and IV were depicted as monophyletic lineages with high statistical support (Fig. 4). In particular, ecotype II was only recovered with high support in ML analyses (92% of bootstrap), yet the cluster 3 (Europe) belonging to this ecotype represents a monophyletic group with confident support (0.94/89) (Fig. 4). Our results differ from those of other studies that described these monophyletic groups based on an eco-epidemiological approach without considering systematics [1, 7, 8, 11]. Undoubtedly, ecotype II and cluster 3 represent natural assemblages, but our study shows them now as also phylogenetically supported. Herein described ecotype V was moderately supported in the *groEL*-based ML inference (81% of ultrafast-bootstrap) and closely related to ecotype III (Fig. 4), which is integrated by variants of *A. phagocytophilum* related to small mammals and ticks (Additional file 1: Table S2) [1]. However, the phylogenetic position of the ecotypes should be re-evaluated as new members of the *A. phagocytophilum* complex are discovered.

The presence of *A. phagocytophilum* DNA does not conclusively confirm the role of pudues and *I. stilesi* in the epidemiology of this bacterium or any clinical impact on pudu health. However, the fact that *P. puda* is the sole deer that currently inhabits the areas from which positive animals for this bacterium were recorded [76] strengthens the notion that this cervid could be reservoir of *A. phagocytophilum* strain "Patagonia." In addition, considering the role of *Ixodes* spp. as vectors of *Anaplasma* spp. in the Northern Hemisphere cervids [1], *I. stilesi* and *I. taglei*, two species that commonly parasitize pudues [22], represent potential vectors of *A. phagocytophilum* strain "Patagonia." However, our hypotheses should be tested in experimental studies. Meanwhile, the epidemiological cycle of *A. phagocytophilum* strain "Patagonia" remains unknown.

Conclusions

We report the presence of and ecotype of *A. phagocytophilum* for the first time in South America. The genetic evidence showed conclusively that the *A. phagocytophilum* found in this study is a unique variant, and the name *A. phagocytophilum* strain "Patagonia" is tentatively proposed. The study of the enzootic

cycle of *A. phagocytophilum* strain "Patagonia" is now essential to establish its zoonotic potential and health impact on pudues and further species, such as domestic ruminants. Furthermore, because some variants of *A. phagocytophilum* are infectious agents of public and veterinary health concern, the detection of this bacterium in Chile deserves further attention. Future research should define a standardized approach for genetically characterizing members of *Anaplasma* genus that would afford reliable comparisons, as recommended in Rar et al. [1]. Finally, these findings bring insight into the genetic diversity and ecology of *A. phagocytophilum*.

Abbreviations

BI	Bayesian inference
BLAST	Basic local alignment search tool
gltA	Citrate synthase gene
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase gene
groEL	Heat-shock operon
MAFFT	Multiple alignment using fast Fourier transform
ML	Maximum likelihood
MCMC	Markov chain Monte Carlo
rRNA	Ribosomal ribonucleic acid
rrs	16S rRNA gene

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-023-05657-9.

Additional file 1: Table S1. GenBank accession numbers of the sequences used for *Anaplasma phagocytophilum rrs* and *gltA* phylogenies. Sequences generated in this study are highlighted in bold. **Table S2.** GenBank accession numbers of the sequences used for *Anaplasma phagocytophilum groEL* phylogeny. Sequences generated in this study are highlighted in bold.

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Author contributions
AS, RT, SM-L: material preparation, data collection, analysis, writing of the first draft. AS, RT, SR, JEU, CP-M, JC-S, FV-O, CV-S, DM-A, EH-H, SM-L contributed to the study conception and design and commented on initial versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
GenBank accession numbers generated in this study are available in Additional files 1: Tables S1 and S2.

Declarations

Ethics approval and consent to participate
The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to, and the appropriate ethical review committee approval has been received. Procedures performed in this study were verified and approved by the Bioethics Committee of the School of Veterinary Sciences, Universidad de Concepción (CBE-07-2022).

Consent for publication
Not applicable.

Competing interests
All authors declare that they have no competing interests.

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Publisher's Note

SUPPORTING INFORMATION

Additional file 1: Table S1. GenBank accession numbers of the sequences used for *Anaplasma phagocytophilum* 16S rRNA and *gltA* phylogenies. Sequences generated in this study are highlighted in bold.

Sequence name	**GenBank accession number**	**Host**	**Country**
16S rRNA			
Anaplasma phagocytophilum strain HZ2	CP006616	Human	USA
Anaplasma phagocytophilum strain Webster	NR044762	Human	USA
Anaplasma phagocytophilum strain Dog2	CP006618	Dog	USA
Anaplasma phagocytophilum strain Susy	AY527213	Horse	Sweden
Anaplasma phagocytophilum strain ApMuc02c	JX173652	*Ixodes ricinus*	Australia
Anaplasma phagocytophilum isolate D2_2 genotype Aph1	MK814406	Dog	South Africa
Anaplasma phagocytophilum strain ApMuc01c	JX173651	Dog	Germany

Anaplasma phagocytophilum isolate F9_16S	MW677507	*Ixodes tapirus*	Panama
Anaplasma phagocytophilum isolate F10_16S	MW677508	*Ixodes tapirus*	Panama
Anaplasma phagocytophilum isolate CAHU-HGE2	AF093789	Human	USA
Anaplasma phagocytophilum isolate MR-23	KP276588	*Ixodes pacificus*	USA
Anaplasma phagocytophilum isolate CASTIL	AF172166	Horse	USA
Anaplasma phagocytophilum strain ES34	AB196720	*Cervus nippon yesoensis*	Japan
Anaplasma sp. Ac52D	AB588974	Deer	Japan
Anaplasma phagocytophilum strain AAIK4	KR611719	*Apodemus agrarius*	South Korea
Anaplasma phagocytophilum isolate Nov-Ip355	HM366580	*Ixodes persulcatus*	Russia
***Anaplasma phagocytophilum* 36S**	**OP579238**	***Pudu puda***	**Chile**
***Anaplasma phagocytophilum* 45S**	**OP579239**	***Pudu puda***	**Chile**
***Anaplasma phagocytophilum* 5P**	**OP579240**	***Pudu puda***	**Chile**
***Anaplasma phagocytophilum* 8P**	**OP579242**	***Pudu puda***	**Chile**
***Anaplasma phagocytophilum* 7P**	**OP579241**	***Pudu puda***	**Chile**
***Anaplasma phagocytophilum* IS25**	**OP579248**	***Ixodes stilesi***	**Chile**

***Anaplasma phagocytophilum* IS19**	**OP579246**	***Ixodes stilesi***	**Chile**
***Anaplasma phagocytophilum* IS22**	**OP579247**	***Ixodes stilesi***	**Chile**
***Anaplasma phagocytophilum* 24P**	**OP579243**	***Pudu puda***	**Chile**
***Anaplasma phagocytophilum* IS18**	**OP579245**	***Ixodes stilesi***	**Chile**
***Anaplasma phagocytophilum* IS17**	**OP579244**	***Ixodes stilesi***	**Chile**
Anaplasma platys	LC269820	Dog	Zambia
Anaplasma platys	AY530806	Dog	Spain
Anaplasma odocoilei strain UMUM76	JX876644	*Odocoileus virginianus*	USA
Anaplasma bovis	AB196475	*Haemaphysalis longicornis*	Japan
Anaplasma bovis	U03775	Bovine	Senegal
Anaplasma sp. clone ES1	KC811530	*Elephantulus myurus*	South Africa
Anaplasma sp. clone genotype Mazama	MN817942	*Mazama gouazoubira*	Uruguay
Anaplasma ovis isolate OVI	AF414870	Goat	South Africa
Anaplasma ovis	AJ633049	Goat	China
Anaplasma centrale strain vaccine	AF414868		South Africa

Anaplasma marginale isolate Lushi	AJ633048	Cattle	China
Anaplasma marginale	AF311303	Bovine	USA
Anaplasma marginale from Uruguay	AF414877		Uruguay
Ehrlichia ruminantium strain Welgevonden	NR074155		South Africa
gltA			
***Anaplasma phagocytophilum* IS17**	**OP585594**	***Ixodes stilesi***	**Chile**
***Anaplasma phagocytophilum* IS25**	**OP585602**	***Ixodes stilesi***	**Chile**
***Anaplasma phagocytophilum* IS19**	**OP585593**	***Ixodes stilesi***	**Chile**
***Anaplasma phagocytophilum* 36S**	**OP585597**	***Pudu puda***	**Chile**
***Anaplasma phagocytophilum* 7P**	**OP585599**	***Pudu puda***	**Chile**
***Anaplasma phagocytophilum* IS20**	**OP585600**	***Ixodes stilesi***	**Chile**
***Anaplasma phagocytophilum* IS18**	**OP585598**	***Ixodes stilesi***	**Chile**
***Anaplasma phagocytophilum* IS22**	**OP585601**	***Ixodes stilesi***	**Chile**
***Anaplasma phagocytophilum* 8P**	**OP585596**	***Pudu puda***	**Chile**
***Anaplasma phagocytophilum* 24P**	**OP585595**	***Pudu puda***	**Chile**

***Anaplasma phagocytophilum* IS21**	**OP585592**	***Ixodes stilesi***	**Chile**
***Anaplasma phagocytophilum* 5P**	**OP585591**	***Pudu puda***	**Chile**
Anaplasma phagocytophilum isolate 96HE158(NY8)	AY464138		USA
Anaplasma phagocytophilum Webster	AF304136		USA
Anaplasma phagocytophilum isolate 96HE54	AY464136		Japan
Anaplasma phagocytophilum isolate 97E13	AY464134		USA
Anaplasma phagocytophilum isolate 97HE97	AY464137		USA
Anaplasma phagocytophilum strain HGE1 HGE1_contig2	APHH01000002	Human	USA
Anaplasma phagocytophilum 1602	AF304138	Sheep	Spain
Anaplasma phagocytophilum strain Norway variant2	CP015376	Sheep	Norway
Anaplasma phagocytophilum	AY339602	*Ixodes persulcatus*	Russian
Anaplasma sp. clone 1	JN055361	*Cervus nippon yasoensis*	Japan
Anaplasma sp. clone 2	JN055362	*Cervus nippon yasoensis*	Japan
Anaplasma sp. BL099-6	KJ410280	*Hyalomma asiaticum*	China
Anaplasma sp. clone Xinjiang099-11	JX402608	*Hyalomma asiaticum*	China

Anaplasma platys	AY077620	Dog	Japan
Anaplasma platys	KR011928	*Rhipicephalus* sp.	China
Anaplasma platys	EU516387	Dog	Brazil
"*Candidatus* Anaplasma cinensis" AK-Rm-403	MH716422	*Rhipicephalus microplus*	China
"*Candidatus* Anaplasma cinensis" AK-Rm-228	MH716426	*Rhipicephalus microplus*	China
Anaplasma odocoilei	DQ020101	*Odocoileus virginianus*	USA
Anaplasma bovis isolate Wangmang-goat-62	MH255920	Goat	China
Anaplasma sp. clone 499	JN588561	*Procyon lotor*	Japan
Anaplasma marginale	KX987367	*Rhipicephalus microplus*	China
Anaplasma marginale	AF304140		USA
Anaplasma ovis strain Haibei	CP015994		China
Anaplasma centrale strain Israel	CP001759		Israel
Anaplasma capra Zhengxiaocun-50	MG869310	Goat	China
Anaplasma capra AK-Rm-429	MH716413	*Rhipicephalus microplus*	China
Ehrlichia canis	AY647155		Italy

Additional file 2: Table S2. GenBank accession numbers of the sequences used for *Anaplasma phagocytophilum groEL* phylogeny. Sequences generated in this study are highlighted in bold.

Sequence name	**GenBank accession numbers**	**Host**	**Country**	**Cluster**	**Ecotype**
Anaplasma phagocytophilum isolate Omsk-6_Mglar	MN701631	*Myodes glareolus*	Rusia	5	III
Anaplasma phagocytophilum isolate Omsk-104_Mrut	MN701630	*Myodes rutilus*	Rusia	5	III
Anaplasma phagocytophilum isolate Omsk-65_Itr_m	MN701644	*Ixodes trianguliceps*	Rusia	5	III
Anaplasma phagocytophilum isolate Omsk-Tr7	KF745746	*Ixodes trianguliceps*	Rusia	5	III
Anaplasma phagocytophilum isolate Omsk-Tr3	KF745745	*Ixodes trianguliceps*	Rusia	5	III
Anaplasma phagocytophilum clone Omsk-vole54	KC583431	*Myodes rufocanus*	Rusia	5	III
Anaplasma phagocytophilum isolate Omsk-41_Mrut	MN701636	*Myodes rutilus*	Rusia	5	III
Anaplasma phagocytophilum isolate Omsk-56_Mruf	MN701635	*Myodes rufocanus*	Rusia	5	III
Anaplasma phagocytophilum isolate Omsk-28_Apagr	MN701632	*Apodemus agrarius*	Rusia	5	III
Anaplasma phagocytophilum isolate Omsk-Tr17	KF745747	*Ixodes trianguliceps*	Rusia	5	III
Anaplasma phagocytophilum clone Omsk-vole121	KC583432	*Myodes rutilus*	Rusia	5	III

Anaplasma phagocytophilum isolate 224691FITAFmouse	KF383236	*Ixodes trianguliceps*	Slovakia	5	III
Anaplasma phagocytophilum isolate 228141LITMGvole	KF383232	*Ixodes trianguliceps*	Slovakia	5	III
Anaplasma phagocytophilum isolate 227781LITMGvole	KF383235	*Ixodes trianguliceps*	Slovakia	5	III
Anaplasma phagocytophilum isolate Omsk-51_Mruf	MN701629	*Myodes rufocanus*	Rusia	5	III
Anaplasma phagocytophilum isolate Omsk-23_Mrut	MN701628	*Myodes rutilus*	Rusia	5	III
Anaplasma phagocytophilum isolate Omsk-167_Micagr	MN701633	*Microtus agrestis*	Rusia	5	III
Anaplasma phagocytophilum isolate 220166sMGvole	KF383231	*Myodes glareolus*	Slovakia	5	III
Anaplasma phagocytophilum isolate Sv-shrew70	HQ630617	*Sorex araneus*	Rusia	5	III
Anaplasma phagocytophilum isolate Sv-vole8	HQ630616	*Myodes rutilus*	Rusia	5	III
Anaplasma phagocytophilum strain ItalyHU148	KF031390	*Myodes glareolus*	Italy	5	III
Anaplasma phagocytophilum isolate 227841LITMGvole	KF383233	*Ixodes trianguliceps*	Slovakia	5	III
Anaplasma phagocytophilum isolate Omsk-17_Mrut	MN701639	*Myodes rutilus*	Rusia	6	III
Anaplasma phagocytophilum isolate Omsk-9-13_Mruf	MN609907	*Myodes rufocanus*	Rusia	6	III
Anaplasma phagocytophilum isolate RUS/Alt14-1625-Ipv	KX980041	*Ixodes pavlovskyi*	Rusia	6	III
Anaplasma phagocytophilum isolate Nov-Ip456	HM366570	*Ixodes persulcatus*	Rusia	6	III

Anaplasma phagocytophilum isolate Irk-Ip625	HM366571	*Ixodes persulcatus*	Rusia	6	III
Anaplasma phagocytophilum isolate Nov-vole144	HQ630614	*Myodes rufocanus*	Rusia	6	III
Anaplasma phagocytophilum isolate Kh-vole305	HQ630615	*Myodes rufocanus*	Rusia	6	III
Anaplasma phagocytophilum isolate Tomsk-Ipr1	KF701460	*Ixodes trianguliceps*	Rusia	6	III
Anaplasma phagocytophilum clone Tuva-Ip2947	KC753764	*Ixodes persulcatus*	Rusia	6	III
Anaplasma phagocytophilum isolate Tomsk Dr-1	KY379956	*Dermacentor reticulatus*	Rusia	6	III
Anaplasma phagocytophilum isolate Tomsk-Ipr2	KF701461	*Ixodes persulcatus*	Rusia	6	III
Anaplasma phagocytophilum isolate Tomsk-Ipr3	KF701462	*Ixodes persulcatus*	Rusia	6	III
Anaplasma phagocytophilum isolate Tomsk-Ipr4	KY684729	*Ixodes persulcatus*	Rusia	6	III
Anaplasma phagocytophilum isolate Tomsk-Ipr6	KY684731	*Ixodes persulcatus*	Rusia	6	III
Anaplasma phagocytophilum isolate Tomsk-Ipr8	KY684733	*Ixodes persulcatus*	Rusia	6	III
Anaplasma phagocytophilum isolate Tomsk-Ipr5	KY684730	*Ixodes persulcatus*	Rusia	6	III
Anaplasma phagocytophilum isolate Tomsk-Ipr7	KY684732	*Ixodes persulcatus*	Rusia	6	III
Anaplasma phagocytophilum isolate Omsk-43_Mglar	MN701638	*Myodes glareolus*	Rusia	6	III
Anaplasma phagocytophilum isolate RUS/Alt14-2442-Ipr/Ipv	MG182152	*Ixodes persulcatus*	Rusia	6	III

Anaplasma phagocytophilum isolate G22	AY281818	*Ixodes ricinus*	Germany	1	I
Anaplasma phagocytophilum isolate 472	AF478561	*Capreolus capreolus*	Slovenia	1	I
Ehrlichia phagocytophila #99	AF383227	*Capreolus capreolus*	Switzerland	1	I
Anaplasma phagocytophilum isolate 70	JN005748	*Capreolus capreolus*	Poland	1	I
Anaplasma phagocytophilum isolate 832	AF478553	*Cervus elaphus*	Slovenia	1	I
Ehrlichia phagocytophila	U96730	Sheep	Great Britain	1	I
Ehrlichia phagocytophila	U96729	Goat	Great Britain	1	I
Anaplasma phagocytophilum strain c-D3160	KM215266	*Rupicapra rupicapra*	Slovenia	1	I
Anaplasma phagocytophilum strain tick-EU431	KM215251	*Ixodes ricinus*	Slovenia	1	I
Anaplasma phagocytophilum isolate 2	AF548386	Sheep	Norway	1	I
Anaplasma phagocytophilum strain c-D3155	KM215265	*Rupicapra rupicapra*	Slovenia	1	I
Anaplasma phagocytophilum isolate GC45	HM057228	*Ixodes ricinus*	Spain	1	I
Anaplasma phagocytophilum isolate 0511	HM057225	*Cervus elaphus*	Spain	1	I
Anaplasma phagocytophilum strain c-2829	KM215264	*Rupicapra rupicapra*	Slovenia	1	I
Anaplasma phagocytophilum isolate 09/78	HM057232	*Ixodes ricinus*	Rusia	1	I

Anaplasma phagocytophilum strain bear-9304	KJ622308	*Ursus arctos*	Slovenia	1	I
Anaplasma phagocytophilum isolate 474	AF478563	*Cervus elaphus*	Slovenia	1	I
Anaplasma phagocytophilum strain bear-9503	KJ622307	*Ursus arctos*	Slovenia	1	I
Anaplasma phagocytophilum isolate dog-7425	EU381151	Dog	Slovenia	1	I
Anaplasma phagocytophilum isolate G55	AY281823	*Ixodes ricinus*	Germany	1	I
Anaplasma phagocytophilum GroESL	EF392724	*Ixodes ricinus*	Croatia	1	I
Anaplasma phagocytophilum isolate tick-40	EU381152	*Ixodes ricinus*	Slovenia	1	I
Anaplasma phagocytophilum strain tick-EU343	KM215246	*Ixodes ricinus*	Slovenia	1	I
Anaplasma phagocytophilum isolate 921	AF478558	*Capreolus capreolus*	Slovenia	1	I
Anaplasma phagocytophilum isolate 61g	HM057230	*Ixodes ricinus*	Spain	1	I
Anaplasma phagocytophilum isolate 1	AF548385	Sheep	Norway	1	I
Anaplasma phagocytophilum isolate 09/71	HM057231	*Ixodes ricinus*	Spain	1	I
Anaplasma phagocytophilum strain c-708	KM215263	*Rupicapra rupicapra*	Slovenia	1	I
Anaplasma phagocytophilum isolate W271	AY281844	*Ixodes ricinus*	Germany	1	I
Anaplasma phagocytophilum strain tick-EU108	KM215252	*Ixodes ricinus*	Slovenia	1	I

Anaplasma phagocytophilum isolate I94	AY281828	*Ixodes ricinus*	Germany	1	I
Ehrlichia phagocytophila	AF202895	*Ixodes ricinus*	Switzerland	1	I
Anaplasma phagocytophilum isolate 3C/2310/1	HM057224	*Cervus elaphus*	Spain	1	I
Anaplasma phagocytophilum isolate L6-9	HM057233	*Ixodes ricinus*	Russia	1	I
Anaplasma phagocytophilum isolate tick-43	EU246959	*Ixodes ricinu*	Slovenia	1	I
Anaplasma phagocytophilum isolate GC19	HM057227	*Ixodes ricinus*	Spain	1	I
Anaplasma phagocytophilum strain red-D3217	KM215262	*Cervus elaphus*	Slovenia	1	I
Anaplasma phagocytophilum isolate 473	AF478562	*Cervus elaphus*	Slovenia	1	I
Anaplasma phagocytophilum isolate 707	AF478557	*Cervus elaphus*	Slovenia	1	I
Anaplasma phagocytophilum strain red-D3009	KM215261	*Cervus elaphus*	Slovenia	1	I
Anaplasma phagocytophilum isolate 812 groESL	AF478552	*Cervus elaphus*	Slovenia	1	I
Anaplasma phagocytophilum isolate dog-7414	EU381150	Dog	Slovenia	1	I
Anaplasma phagocytophilum	EU184703	*Sus scrofa*	Slovenia	1	I
Anaplasma phagocytophilum isolate N6	AY281849	*Ixodes ricinus*	Germany	1	I
Ehrlichia sp. 'HGE agent'	AF033101	Human	Slovenia	1	I

Anaplasma phagocytophilum	AF482760	Horse	Germany	1	I
Anaplasma phagocytophilum strain Strong	AY529490	Horse	Sweden	1	I
Anaplasma phagocytophilum isolate 163HFIRQ	KF383241	*Ixodes ricinus*	Slovakia	1	I
Anaplasma phagocytophilum isolate 39FCIRQ	KF383239	*Ixodes ricinus*	Slovakia	1	I
Anaplasma phagocytophilum isolate 187	EU860089	Sheep	France	1	I
Anaplasma phagocytophilum	KF836094	Dog	Brazil	1	I
Anaplasma phagocytophilum strain Susy	AY529489	Horse	Sweden	1	I
Anaplasma phagocytophilum strain Fordyce	EF647585	Horse	USA	1	I
Anaplasma phagocytophilum	DQ680012	Cat	USA	1	I
Anaplasma phagocytophilum	AY219849	Dog	USA	1	I
Ehrlichia equi	AF173989	*Ixodes pacificus*	USA	1	I
Ehrlichia equi	AF173988	*Neotoma fuscipes*	USA	1	I
Anaplasma phagocytophilum clone KC15	AY626252	*Sigmodon hispidus*	USA	1	I
Ehrlichia equi isolate CAMAWI	AF172160	Horse	USA	1	I
Ehrlichia sp. 'HGE agent' isolate CAHU-HGE2	AF172159	Human	USA	1	I

Ehrlichia equi isolate CASOLJ	AF172158	Horse	USA	1	I
Anaplasma phagocytophilum strain GACTR12	DQ088133	*Sylvilagus floridanus*	USA	1	I
Anaplasma phagocytophilum isolate 151	EU157921	*Capreolus capreolus*	Poland	1	I
Anaplasma phagocytophilum strain ST-156	DQ779567	*Cervus elaphus*	Poland	1	I
Anaplasma phagocytophilum isolate 9B13	KC800986	*Alces alces*	Sweden	1	I
Anaplasma phagocytophilum isolate Nf_DU1_HW	JF494841	*Neotoma fuscipes*	USA	1	I
Anaplasma phagocytophilum isolate Nf_1603_HV	JF494836	*Neotoma fuscipes*	USA	1	I
Anaplasma phagocytophilum isolate Nf_1629_HC	JF494835	*Neotoma fuscipes*	USA	1	I
Anaplasma phagocytophilum isolate Nf_1619_HC	JF494834	*Neotoma fuscipes*	USA	1	I
Anaplasma phagocytophilum isolate Dog_CA	JF494833	Dog	USA	1	I
Ehrlichia sp. 'HGE agent'	U72628	Human	USA	1	I
Anaplasma phagocytophilum str. JM	CP006617	Human	USA	1	I
Anaplasma phagocytophilum str. Dog2	CP006618	Dog	USA	1	I
Anaplasma phagocytophilum strain Webster	EU860090	Ruminants	France	1	I
Anaplasma phagocytophilum isolate GV348	MK341070	*Ixodes ricinus*	Slovakia	1	I

Anaplasma phagocytophilum isolate 14DRS	KR092132	*Sus scrofa*	Slovakia	1	I
Anaplasma phagocytophilum isolate J	KF312361	*Ixodes ricinus*	Poland	1	I
Anaplasma phagocytophilum isolate G	KF312358	*Ixodes ricinus*	Poland	1	I
Anaplasma phagocytophilum isolate F	KF312360	*Ixodes ricinus*	Poland	1	I
Anaplasma phagocytophilum isolate E	KF312357	*Ixodes ricinus*	Poland	1	I
Anaplasma phagocytophilum isolate I	KF312359	*Ixodes ricinus*	Poland	1	I
Anaplasma phagocytophilum isolate H	KF312355	*Ixodes ricinus*	Poland	1	I
Anaplasma phagocytophilum isolate Z15	MW762533	*Lepus europaeus*	Czech Republic	1	I
Anaplasma phagocytophilum isolate 16Pl	MG670108	*Procyon lotor*	Poland	1	I
Anaplasma phagocytophilum strain ItalyIRH01241	KF031388	*Ixodes ricinus*	Italy	1	I
Anaplasma phagocytophilum isolate 2916	EU860087	Cow	France	1	I
Anaplasma phagocytophilum isolate 811	AF478551	*Capreolus capreolus*	Slovenia	3	II
Anaplasma phagocytophilum isolate 470	AF478559	*Capreolus capreolus*	Slovenia	3	II
Anaplasma phagocytophilum strain rod-1427	KM215256	*Capreolus capreolus*	Slovenia	3	II
Anaplasma phagocytophilum isolate D21	AY281816	*Ixodes ricinus*	Germany	3	II

Anaplasma phagocytophilum isolate 805	AF478555	*Capreolus capreolus*	Slovenia	3	II
Anaplasma phagocytophilum strain rod-1693	KM215255	*Capreolus capreolus*	Slovenia	3	II
Anaplasma phagocytophilum strain tick-EU136	KM215250	*Ixodes ricinus*	Slovenia	3	II
Anaplasma phagocytophilum isolate G26	AY281820	*Ixodes ricinus*	Germany	3	II
Anaplasma phagocytophilum isolate 794	AF478556	*Capreolus capreolus*	Slovenia	3	II
Anaplasma phagocytophilum strain tick-EU260	KM215249	*Ixodes ricinus*	Slovenia	3	II
Anaplasma phagocytophilum isolate A4 GroEL	AY220469	*Capreolus capreolus*	Austria	3	II
Anaplasma phagocytophilum strain rod-1424	KM215259	*Capreolus capreolus*	Slovenia	3	II
Anaplasma phagocytophilum strain rod-1694	KM215258	*Capreolus capreolus*	Slovenia	3	II
Anaplasma phagocytophilum isolate 806	AF478554	*Capreolus capreolus*	Slovenia	3	II
Anaplasma phagocytophilum isolate A6	AY220470	*Ixodes ricinus*	Austria	3	II
Anaplasma phagocytophilum strain tick-EU322	KM215247	*Ixodes ricinus*	Slovenia	3	II
Ehrlichia phagocytophila #56	AF383225	*Capreolus capreolus*	Switzerland	3	II
Anaplasma phagocytophilum strain rod-1429	KM215257	*Capreolus capreolus*	Slovenia	3	II
Anaplasma phagocytophilum isolate 478	AF478564	*Capreolus capreolus*	Slovenia	3	II

Anaplasma phagocytophilum strain tick-EU329	KM215248	*Ixodes ricinus*	Slovenia	3	II
Anaplasma phagocytophilum strain rod-1691	KM215254	*Capreolus capreolus*	Slovenia	3	II
Anaplasma phagocytophilum isolate I63	AY281825	*Ixodes ricinus*	Germany	3	II
Anaplasma phagocytophilum strain rod-1686	KM215253	*Capreolus capreolus*	Slovenia	3	II
Anaplasma phagocytophilum isolate 47	JN005747	*Capreolus capreolus*	Poland	3	II
Anaplasma phagocytophilum isolate 09	JN005743	*Capreolus capreolus*	Poland	3	II
Anaplasma phagocytophilum strain Italy59_3	KF031400	*Ixodes ricinus*	Italy	3	II
Anaplasma phagocytophilum strain ItalyIRH018611	KF031394	*Ixodes ricinus*	Italy	3	II
Anaplasma phagocytophilum strain ItalyIRH012211	KF031392	*Ixodes ricinus*	Italy	3	II
Anaplasma phagocytophilum strain Italy25	KF031382	*Ixodes ricinus*	Italy	3	II
Anaplasma phagocytophilum strain Italy21b	KF031380	*Ixodes ricinus*	Italy	3	II
Anaplasma phagocytophilum strain ST-128	DQ779568	*Capreolus capreolus*	Poland	3	II
Anaplasma phagocytophilum isolate S40 GroEL	KC800984	*Alces alces*	Sweden	3	II
Anaplasma phagocytophilum isolate Italy737	EU552920	*Ixodes ricinus*	Italy	3	II
Anaplasma phagocytophilum isolate Italy738	EU552918	*Ixodes ricinus*	Italy	3	II

Anaplasma phagocytophilum isolate K	KF312356	*Ixodes ricinus*	Poland	3	II
Anaplasma phagocytophilum isolate fricoe	JX082324	*Ixodes ricinus*	Switzerland	3	II
Anaplasma phagocytophilum isolate erirub	JX082325	*Ixodes ricinus*	Switzerland	3	II
Uncultured *Anaplasma* sp. clone Ip11-2	JQ622144	*Ixodes persulcatus*	Japan	4	II
Anaplasma phagocytophilum isolate Kh-395_Ip	MN989865	*Ixodes persulcatus*	Russia	4	II
Anaplasma phagocytophilum isolate Kh-434_Ip	MN989863	*Ixodes persulcatus*	Russia	4	II
Anaplasma phagocytophilum isolate Irk-Ip776	HM366573	*Ixodes persulcatus*	Russia	4	II
Anaplasma phagocytophilum isolate Irk-Ip820	HM366574	*Ixodes persulcatus*	Russia	4	II
Anaplasma phagocytophilum isolate Kh-chipmunk177	HQ630619	*Tamias sibiricus*	Russia	4	II
Anaplasma phagocytophilum isolate Irk-Ip662	HM366572	*Ixodes persulcatus*	Russia	4	II
Anaplasma phagocytophilum isolate Kh-Ip7	HM366575	*Ixodes persulcatus*	Russia	4	II
Anaplasma phagocytophilum isolate Kh-Ip80	HM366576	*Ixodes persulcatus*	Russia	4	II
Anaplasma phagocytophilum isolate Kh-Ip144	HM366577	*Ixodes persulcatus*	Russia	4	II
Anaplasma phagocytophilum isolate Kh-868_Tsib	MN989862	*Tamias sibiricus*	Russia	4	II
Anaplasma phagocytophilum isolate RUS/Nov14-1682-Ipr/Ipv	MG182154	*Ixodes persulcatus*	Russia	4	II

Anaplasma phagocytophilum isolate RUS/Nov14-1768-Ipv	KX980043	*Ixodes pavlovskyi*	Russia	4	II
Anaplasma phagocytophilum isolate Nov-chipmunk1322	HQ630618	*Tamias sibiricus*	Russia	4	II
Anaplasma phagocytophilum isolate Sv-Ip854	HM366567	*Ixodes persulcatus*	Russia	4	II
Anaplasma phagocytophilum isolate Nov-Ip364	HM366569	*Ixodes persulcatus*	Russia	4	II
Anaplasma phagocytophilum isolate Omsk-373_Ip	MN701641	*Ixodes persulcatus*	Russia	4	II
Anaplasma phagocytophilum clone Omsk-vole83	KC583433	*Myodes glareolus*	Russia	4	II
Anaplasma phagocytophilum isolate Nov-Ip355	HM366568	*Ixodes persulcatus*	Russia	4	II
Anaplasma phagocytophilum isolate Kh-Ip160	HM366578	*Ixodes persulcatus*	Russia	4	II
Anaplasma phagocytophilum strain AAIK2 GroES	KT220191	*Apodemus agrarius*	South Korea	4	II
Anaplasma phagocytophilum strain AAIK3	KT220192	*Apodemus agrarius*	South Korea	4	II
Anaplasma phagocytophilum strain AAIK1	KT192430	*Apodemus agrarius*	South Korea	4	II
Anaplasma phagocytophilum isolate Hongdo-11-1	JX219474	*Ixodes nipponensis*	South Korea	4	II
Anaplasma phagocytophilum clone KWDTAPg	JQ086319	*Haemaphysalis flava*	South Korea	4	II
Anaplasma phagocytophilum isolate KWDAPg	HM752098	*Hydropotes inermis*	South Korea	4	II
Anaplasma phagocytophilum Yeyasu	LC334016	Dog	Japan	4	II

Anaplasma phagocytophilum isolate gw1	KJ677107	Human	South Korea	4	II
Anaplasma phagocytophilum isolate D-SE-63	KU519286	Dog	South Korea	4	II
Anaplasma phagocytophilum isolate S-DD-20	KU519284	Cat	South Korea	4	II
Anaplasma phagocytophilum strain N6Bel	JX133177	*Ixodes ricinus*	Switzerland	3	II
Anaplasma phagocytophilum strain ItalyIRH017411	KF031393	*Ixodes ricinus*	Italy	7	IV
Anaplasma phagocytophilum isolate turmer	JX082323	*Ixodes ricinus*	Switzerland	7	IV
***Anaplasma phagocytophilum* strain Patagonia 5P**	**OP585578**	***Pudu puda***	**Chile**	**8**	**V**
***Anaplasma phagocytophilum* strain Patagonia 7P**	**OP585579**	***Pudu puda***	**Chile**	**8**	**V**
***Anaplasma phagocytophilum* strain Patagonia IS8**	**OP585581**	***Ixodes stilesi***	**Chile**	**8**	**V**
***Anaplasma phagocytophilum* strain Patagonia IS17**	**OP585582**	***Ixodes stilesi***	**Chile**	**8**	**V**
***Anaplasma phagocytophilum* strain Patagonia IS21**	**OP585585**	***Ixodes stilesi***	**Chile**	**8**	**V**
***Anaplasma phagocytophilum* strain Patagonia 36S**	**OP585589**	***Pudu puda***	**Chile**	**8**	**V**
***Anaplasma phagocytophilum* strain Patagonia 24P**	**OP585590**	***Pudu puda***	**Chile**	**8**	**V**
***Anaplasma phagocytophilum* strain Patagonia IS25**	**OP585588**	***Ixodes stilesi***	**Chile**	**8**	**V**
***Anaplasma phagocytophilum* strain Patagonia 8P**	**OP585580**	***Pudu puda***	**Chile**	**8**	**V**

***Anaplasma phagocytophilum* strain Patagonia IS20**	**OP585586**	***Ixodes stilesi***	**Chile**	**8**	**V**
***Anaplasma phagocytophilum* strain Patagonia IS22**	**OP585587**	***Ixodes stilesi***	**Chile**	**8**	**V**
***Anaplasma phagocytophilum* strain Patagonia IS18**	**OP585583**	***Ixodes stilesi***	**Chile**	**8**	**V**
***Anaplasma phagocytophilum* strain Patagonia IS19**	**OP585584**	***Ixodes stilesi***	**Chile**	**8**	**V**
Anaplasma phagocytophilum	AY279085	Goat	Albania	Unassigned	Unassigned
Anaplasma phagocytophilum isolate 77HNIRQ	KF383240	*Ixodes ricinus*	Slovakia	Unassigned	Unassigned
Anaplasma phagocytophilum isolate 5NBZIRQ	KF383238	*Ixodes ricinus*	Slovakia	Unassigned	Unassigned
Anaplasma phagocytophilum isolate 42	EU157920	*Capreolus capreolus*	Poland	Unassigned	Unassigned
Anaplasma platys strain RP	EU516386			Not applicable	Not applicable
Anaplasma platys strain WHBMXZ-126	KX987394	*Boophilus microplus*	China	Not applicable	Not applicable
Anaplasma odocoilei strain UMUM76	JX876642	*Odocoileus virginianus*		Not applicable	Not applicable
Anaplasma marginale isolate CNP_976_2	KY305561	*Syncerus caffer*	South Africa	Not applicable	Not applicable
Anaplasma marginale isolate AEP_1007_3	KY305562	*Syncerus caffer*	South Africa	Not applicable	Not applicable
Anaplasma centrale isolate Ac1_Ug_Ktd_2013_KR5_30h	KY523000	Cattle	Uganda	Not applicable	Not applicable
Anaplasma ovis isolate OVI	AF441131		South Africa	Not applicable	Not applicable

Anaplasma ovis isolate 76	FJ460441		Cyprus	Not applicable	Not applicable
Anaplasma capra strain HLJ-14	KM206275	Human	China	Not applicable	Not applicable
Anaplasma capra isolate Hstaji200	MZ222248	*Equus hemionus onager*	Iran	Not applicable	Not applicable
Ehrlichia ruminantium strain Kumm2	DQ647013		South Africa	Not applicable	Not applicable

CAPÍTULO III: Transmisión experimental de una nueva *Borrelia* relacionada con *Ornithodoros octodontus* (Ixodida: Argasidae) en Chile

Resumen

Las espiroquetas del grupo de borrelias de fiebre recurrente (GFR) del género *Borrelia* son transmitidas por garrapatas y proliferan en ciclos enzoóticos en los que participan *Ornithodoros* spp. (Argasidae) y roedores, principalmente. El aislamiento de estas espiroquetas suele realizarse con un modelo murino en el que se alimentan garrapatas y para luego detectar las espiroquetas transmitidas en la sangre días después. Un experimento de este tipo también prueba que una determinada especie de garrapata es competente en la transmisión de la bacteria en condiciones de laboratorio.

En Sudamérica dos especies de *Ornithodoros* han sido implicadas como vectores de borrelias del GFR. En Chile, existen 11 especies de *Ornithodoros* distribuidas en el norte y centro del país. La evidencia sobre borrelias del GFR en Chile se resume a detecciones moleculares en las garrapatas *Ornithodoros octodontus*, *Ornithodoros spheniscus* y *Ornithodoros* cf. *Atacamensis* y en el roedor *Phyllotis xanthopygus* (Cricetidae). Sin embargo, el reciente hallazgo de una nueva especie de *Borrelia* detectada en *O. octodontus*, una garrapata asociada al roedor *Octodon degus* (Octodontidae), sugiere que la diversidad de *Borrelia* en Chile es aún desconocida.

En este estudio, se recolectó *O. octodontus* en el norte de Chile con el objetivo de determinar experimentalmente su capacidad para transmitir una *Borrelia* sp. a cobayos (*Cavia porcellus*) en condiciones de laboratorio. Se utilizaron dos cobayos para alimentar a ninfas y adultos de *O. octodontus* colectadas en el ambiente y posiblemente infectadas con *Borrelia* sp. Antes y después de la alimentación, se inspeccionaron las espiroquetas en sangre mediante microscopía de campo oscuro y PCR anidada. No se observaron a través de microscopia espiroquetas en sangre, sin embargo, se detectó ADN de *Borrelia* en sangre de un animal 11 días después de alimentar a las garrapatas. Las secuencias genéticas de los genes *flaB*, *clpX*, *pepX*, *recG*, *rplB* y *uvrA* de *Borrelia*, se emplearon para construir análisis filogenéticos de inferencia bayesiana y

máxima verosimilitud. Por un lado, el árbol *flaB* mostró que la *Borrelia* sp. transmitida por *O. octodontus* se agrupaba con la *Borrelia* sp. Alcohuaz, detectada previamente en esa misma especie de garrapata. Por otro lado, las inferencias filogenéticas basadas en la concatenación de los genes *clpX-pepX-recG-rplB-uvrA* indican que la espiroqueta caracterizada se ramifica junto con "*Candidatus* Borrelia caatinga", una especie recientemente descubierta en Brasil. Basándose en el perfil genético presentado en este estudio, se propone el nombre de "*Candidatus* Borrelia octodonta" para la especie transmitida por *O. octodontus*. El hecho de que no se observaran espiroquetas en la sangre de los cobayos, puede reflejar la ocurrencia de una baja espiroquetemia, lo que podría explicarse porque la susceptibilidad de la infección varía en función de la especie de roedor que se utilice en los modelos experimentales. Aunque el reservorio vertebrado de "Ca. *Borrelia octodonta*" sigue siendo desconocido, *O. degus*, debería ser un objetivo futuro para dilucidar esta cuestión.

Experimental transmission of a novel relapsing fever group *Borrelia* harbored by *Ornithodoros octodontus* (Ixodida: Argasidae) in Chile

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Abstract

Tick-borne relapsing fever spirochetes of genus *Borrelia* thrive in enzootic cycles involving *Ornithodoros* spp. (Argasidae) mainly, and rodents. The isolation of these spirochetes usually involves a murine model in which ticks are fed and the spirochetes detected in blood several days later. Such an experiment also demonstrates that a given species of tick is competent in the transmission of the bacteria. Here, soft ticks *Ornithodoros octodontus* were collected in Northern Chile with the objective to experimentally determine its capacity to transmit a *Borrelia* sp. detected in a previous study. Two Guinea pigs (*Cavia porcellus*) were used to feed nymphs and adults of *O. octodontus* and the spirochetes in blood were inspected by dark-field microscopy and nested PCR. Although spirochetes were not seen in blood, DNA was detected in only one animal 11 days after ticks were fed. Genetic sequences of *Borrelia flaB, clpX, pepX, recG, rplB,* and *uvrA* genes retrieved from DNA extraction of positive blood were employed to construct two phylogenetic analyses. On the one hand, the *flaB* tree showed the Borrelia sp. transmitted by *O. octodontus* clustering with *Borrelia* sp. Alcohuaz, which was previously detected in that same tick species. On the other hand, concatenated *clpX-pepX-recG-rplB-uvrA* demonstrated that the characterized spirochete branches together with "*Candidatus* Borrelia caatinga", a recently discovered species from Brazil. Based on the genetic profile presented in this study, the name "*Candidatus* Borrelia octodonta" is proposed for the species transmitted by *O. octodontus*. The fact that spirochete was not observed in blood of guinea pigs, may reflect the occurrence of low spirochetemia, which could be explained because the susceptibility of infection vary depending on the rodent species that is used in experimental models. Although the vertebrate reservoir of "Ca. *Borrelia octodonta*" is still unknown, *Octodon degus*, a rodent species that is commonly parasitized by *O. octodontus*, should be a future target to elucidate this issue.

Keywords: Soft-tick-borne diseases, "*Candidatus* Borrelia octodonta", MLST, phylogeny, vector competence, relapsing fever group.

Introduction

The genus *Borrelia* (*Spirochaetales*) encompasses a group of conical motile bacteria with helical shape (Margos et al. 2020). Spirochetes of this genus thrive in enzootic cycles infecting a wide range of vertebrate hosts and are primarily transmitted by ticks of the families Argasidae (soft ticks) and Ixodidae (hard ticks) (Wang 2015; Barbour 2018; Barbour and Gupta 2021). Three principal groups compose the genus *Borrelia*, but only two include species that pose a risk to human health: (i) the *Borrelia burgdorferi* sensu lato complex, some of which are known to cause Lyme borreliosis; and (ii) the relapsing fever group (RFG), which includes the causative agents of soft-tick-borne relapsing fever (STBRF), a zoonotic disease of global distribution transmitted by ticks of the genus *Ornithodoros* (Margos et al. 2018; Faccini-Martínez et al. 2022).

Ornithodoros spp. naturally transmit *Borrelia* spp. to vertebrate hosts while feeding (Barbour and Gupta 2021). As nidicolous ticks, they parasitize a limited range of vertebrate hosts, rendering the foci of RFG borreliae rather endemic (Barbour and Gupta 2021). In the tick, the spirochetes colonize the midgut and salivary glands and persist through subsequent molts and further bloodmeals (Lopez et al. 2021; Barbour and Gupta 2021). *Ornithodoros* spp. break their fast approximately every three months (Johnson et al. 2016); however, they can starve for years (Francis 1938). Once colonized by spirochetes, ticks remain infected throughout their lifespan (Francis 1938; Johnson et al. 2016).

Soft tick-borne RFG spirochetes seem to have evolved to depend on a specific tick vector for effective transmission, as notably seen in *Ornithodoros* spp., which transmit a specific species of *Borrelia* (Davis 1956; Schwan 1996; Krishnavajhala et al. 2018; Faccini-Martínez et al. 2022). For instance, in South America, some studies implemented experimental models with rodents to demonstrate the competence of *Ornithodoros rudis* and *Ornithodoros* cf. *tabajara* as vectors of RFG spirochetes (Muñoz-Leal et al. 2018; Oliveira et al. 2023), meaning that the ticks can acquire, maintain, and successfully transmit an infectious agent (Sonenshine and Mather 1994). These models have included South American cricetid (Rodentia: Cricetidae) and caviid (Rodentia: Caviidae) rodents as promising animals to recover spirochetes of South American tick species. However, intrinsic (genetic) tick factors that define host predilection, feeding time, tick-host-pathogen interactions, and susceptibility to infection modulate the competence of transmitting a given microorganism

(Beerntsen et al. 2000; Sonenshine 2005; de la Fuente et al. 2017). Therefore, the susceptibility of infection with RFG spirochetes may vary depending on the rodent species that is used in experimental models (Burgdorfer and Mavros 1970; Bermúdez et al. 2021).

In Chile, 11 *Ornithodoros* spp. are distributed across the northern and central regions of the country (González-Acuña and Guglielmone 2005; Muñoz-Leal et al. 2016, 2020, 2023). However, evidence regarding RFG borreliae is still emerging, and currently limited to molecular detections in ticks (Muñoz-Leal et al. 2019a, b; Thompson et al. 2021) and one rodent species (Thomas et al. 2020). Noteworthy the diversity of *Borrelia* in Chile might be underestimated. Based on the report of a novel *Borrelia* sp. detected in *Ornithodoros octodontus*, a tick associated with *Octodon degus* (Rodentia: Octodontidae) (Thompson et al. 2021), the present study aimed to determine whether *O. octodontus* could transmit RFG borreliae under laboratory conditions, and to genetically characterize the detected spirochetes.

Methods

Tick collection and identification

In July of 2021, we visited two localities in the Coquimbo Region, northern Chile: (i) a residential area in El Guindo, Santa Berta, Ovalle Municipality (-30.651693, -71.091803; elevation 346 m) and (ii) a rural area in Illapel Municipality (-31.502081, -71.112455; 604 m) (Fig. 1). One hundred and one ticks were extracted from dens frequented by *O. degus*. Two females, six males, and 82 nymphs were collected at El Guindo, and three females, one male, and seven nymphs at Illapel. In the laboratory, two tubes with living ticks, each one from one locality, were maintained in an incubator at 25ºC and 80% humidity for four months before starting the experiment. All the specimens were identified as *O. octodontus* (Muñoz-Leal et al. 2020).

Feeding of ticks

To determine whether the collected ticks could transmit spirochetes, two guinea pigs (GP1 and GP2, Hartley strain), were infested with each group of ticks. Ninety ticks from El Guindo were fed on GP1, while 11 ticks

from Illapel were fed on GP2. Guinea pigs were anesthetized intramuscularly with a dexmedetomidine and ketamine solution (0.05 mg/kg and 5 mg/kg, respectively) (Hedley 2020), ventrally trichotomized, and ticks introduced into transparent plastic feeding chambers fixed to the abdomen of the animals with a skin-compatible adhesive (Kamar Products, Zionsville, IN, USA). Tick attachment and detachment was visually corroborated. After the meal, all ticks were recovered and placed in an incubator. Atipamezole (1 mg/kg) was inoculated subcutaneously to both guinea pigs to recover of anesthesia (Morrisey and Carpenter 2020). Procedures using laboratory animals were approved by the Ethics Committee of the Faculty of Veterinary Sciences of University of Concepción (CBE-24-2021).

Monitoring of guinea pigs

To detect spirochetes, blood samples were taken within 24 hours after tick exposure and for 20 days thereafter (21 days in total) (Muñoz-Leal et al. 2018; Oliveira et al. 2023). A sample of blood of each Guinea pig was collected before starting the experiment (day zero) and included into the genetic screening to detect *Borrelia* DNA. Two drops of blood were drawn puncturing the animals' ear pinna using a 25-gauge needle (Williams and Kendall 2015). One drop of blood (≈2.5 μL) was expressed onto a slide and 100 fields were observed at 100X using dark-field microscopy. The other drop (≈50 μL) was stored in a microtube at -80°C until processing. Guinea pigs that did not display motile spirochetes within the 21 days of experiment were considered negative and no subsequent bleeding was performed. At the end of the experiment, the animals were sacrificed according to the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (AVMA 2020).

DNA extraction, gene amplification and sequencing

Twenty-one samples of blood per Guinea pig underwent DNA extraction using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and DNA was eluted in 25 μL of buffer AE (10 mM Tris–Cl; 0.5 mM ethylenediaminetetraacetic acid [EDTA], pH 9.0). Subsequently, DNA was quantified and assessed for quality using an Epoch™ Microplate Spectrophotometer (BioTek Instruments,

Inc., Winooski, VT, USA). Samples with an A260/A280 DNA ratio ranging from 1.6-2.0 were deemed suitable for PCR amplification protocols (Khare et al. 2014). A conventional PCR assay targeting the mammalian gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal extraction control (Birkenheuer et al. 2003).

Borrelia screenings were carried out through a nested PCR protocol targeting the flagellin (*flaB*) gene following Stromdahl et al. (2003). Additionally, attempts to amplify the housekeeping genes *clpA*, *clpX*, *pepX*, *pyrG*, *recG*, *nifS*, *rplB*, and *uvrA* using degenerate primers from the *Borrelia* MLST database (https:// pubmlst.org/borrelia) were performed as well. DNA of *Borrelia anserina* PL was employed as a positive control. All PCR reactions were performed in a thermal cycler (ProFlex™ Base 32 × 3; Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Each reaction had a final volume of 25 µL, consisting of 12.5 µL DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), 1 µL of each primer (0.4 µM), 8.5 µL of ultra-pure water and 2 µL of template DNA. Ultra-pure water was used as a negative control in all cases. PCR products were stained with GelRed® (Biotum, Tehran, Iran), separated by electrophoresis in 2% agarose gels, and then visualized using an ENDURO™ GDS UV transilluminator (Labnet International, Edison, NJ, USA). Amplicons displaying bands of the expected size were purified and Sanger-sequenced in both directions at Macrogen (Seoul, South Korea).

Sequence and phylogenetic analyses

To generate consensus sequences, AB1 files were visualized, quality-checked, and edited with Geneious Prime® version (v) 2021.2.2 (www.geneious.com). Base calls with Phred values ≥ 20 were considered suitable for subsequent analyses (Ewing and Green 1998; Ewing et al. 1998). The consensus sequences were then compared with the BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) tool to identify orthologous sequences.

Orthologous sequences recovered from GenBank were aligned with the sequences obtained in this study with MAFFT using default parameters (Katoh and Standley 2013). Subsequently, the alignments were curated with Block Mapping and Gathering with Entropy (BMGE) using default parameters to map informative regions for phylogenetics inferences (Criscuolo and Gribaldo 2010).

Phylogenies were constructed with Maximum likelihood (ML, Felsenstein 1981) and Bayesian inference (BI, Rannala and Yang 1996; Yang and Rannala 1997) methods in IQ-TREE v. 1.6.12 (Nguyen et al. 2015) and MrBayes v. 3.2.6 (Ronquist et al. 2012), respectively. As protein-coding genes exhibit distinct nucleotide exchange rates at the first, second, and third codon positions (heterogeneity) (Yang 1996; Ronquist et al. 2012), datasets were partitioned into three codon positions (position-1, position-2, and position-3) (Yang 1996; Ronquist et al. 2012; Lanfear et al. 2012; Kainer and Lanfear 2015).

For ML analyses, the best-fit evolutionary models and the optimal partition scheme were computed using the ModelFinder command "-m TESTNEWONLYMERGE -mrate G" (Kalyaanamoorthy et al. 2017). To assess the robustness of the inferred tree, we employed rapid hill-climbing and stochastic disturbance methods with 1000 ultrafast bootstrapping pseudo-replicates. Ultrafast-bootstrap values < 70%, 70–94%, and ≥ 95% were interpreted as representing non-significant, moderate, and strong statistical support, respectively (Minh et al. 2013). Best partition schemes for BI were calculated by ModelFinder and MrBayes command "lset = mixed rates = invgamma" (Huelsenbeck et al. 2004; Ronquist et al. 2012; Lanfear et al. 2012). Two independent tests of 20 x 10^6 generations and four Markov Chain Monte Carlo (MCMC) chains were conducted, sampling every cycle until convergence, which was assessed using Tracer v. 1.7.1 (Rambaut et al. 2018). Nodes with Bayesian posterior probabilities (BPP) > 0.70 were considered high statistical support (Huelsenbeck and Rannala 2004). All best-fit models and partitions schemes were selected based on Bayesian Information Criteria (BIC) (Schwarz 1978). Trees were visualized and edited using FigTree v. 1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape v. 1.1 (https://inkscape.org/es/). Consensus trees for both ML and BI were generated for each dataset following the approach of Santodomingo et al. (2022).

Results

Experimental transmission

Not all the ticks introduced into the feeding chamber attached to the hosts' skin. Indeed, only nymphs were engorged and the adults did not fed. The tick feeding time did not exceed 180 minutes. Following tick exposure Spirochetes were not detected during the 21 days of blood examination with dark-field microscopy.

159

160 ***Borrelia* detection**

161 Stored blood samples were analyzed at the end of the transmission experiment. PCRs targeting the *GAPDH*
162 gene yielded amplicons of the expected size, confirming successful DNA extraction for both guinea pigs. All
163 DNA extractions yielded a concentration ranging from ~10 to 15 ng/µL, along with an A260/A280 quality ratio
164 of 1.8 to 2.0. Samples of blood taken at day zero were negative for *Borrelia* DNA in both animals. In GP1, the
165 PCR targeting the *flaB* gene yielded amplicons of the expected size (462 bp) between day 11th to 18th posterior
166 to tick exposure. The animal was negative the day 19th until euthanasia. GP2 was negative for *Borrelia* detection
167 during all the period. One sample yielding marked amplicons for *flaB* PCR in electrophoresis was submitted to
168 the eight-gene MLST characterization. Expected amplicons and good quality sequences were obtained for five
169 of them (*clpX*, *pepX*, *recG*, *rplB*, and *uvrA*). After BLASTn comparisons, the sequence of *flaB* matched with
170 95.44% identity (440/461 bp, 99% query cover, 0 gaps, 0 E-value) the uncultured *Borrelia* sp. clone Omi2MT
171 (MT076262), detected from *Carios mimon* in Brazil (Muñoz-Leal et al. 2021). Subsequent pairwise
172 comparisons of our *Borrelia flaB* genotype with *Borrelia* sp. Alcohuaz (MW981443), detected in *O. octodontus*
173 by Thompson et al. (2021) in Chile, revealed 99.64% similarity (279/280 bp, 99% query cover, 0 gaps, 6e-150
174 E-value).

175 Phylogenies inferred from *flaB* and MLST genes positioned the sequenced *Borrelia* genotype into the
176 RFG borreliae clade. Notably, *Borrelia* sp. Alcohuaz formed a monophyletic group with the *Borrelia* sp.
177 detected in this study. This clade is related to borreliae genotypes characterized from rodents in Chile, including
178 one genotype detected in an undetermined *Ornithodoros* sp. from Bolivia. On the other hand, the concatenated
179 MLST phylogenetic analysis showed the *Borrelia* sp. detected in this study branching independently within a
180 clade including *Borrelia* sp. FMV_PCST_FN from Brazil, *Borrelia recurrentis* and other STBRF borreliae
181 (*Borrelia hispanica*, *Borrelia duttonii*, *Borrelia crocidurae*, *Borrelia persica*), indicating that the characterized
182 species belongs to the relapsing fever group. Given that phylogenetic analyses support the detection of a putative
183 new species, we provisionally propose the name "*Candidatus* Borrelia octodonta" for the spirochete detected
184 in *O. octodontus* (Fig. 2). The name is proposed in allusion to the epithet of the tick species from which the
185 spirochete was transmitted.

Discussion

In the present study, we determined that *O. octodontus* transmitted an RFG *Borrelia* to a guinea pig under laboratory conditions. The fact that only nymphs attached and engorged, confirms the hypothesis that adults of *O. octodontus* this species do not feed, or are very specific on choosing their hosts (Muñoz-Leal et al. 2020). It is well-documented that *Ornithodoros* spp. are proficient at transmitting RFG spirochetes while feeding (Johnson et al. 2016; Jakab et al. 2022). Once ticks are attached, RFG spirochetes in the salivary glands are transmitted and swiftly penetrate the host's skin to reach the bloodstream (Lopez et al. 2021). In this study, *Borrelia* DNA was detected in blood only eleven days after the ticks fed. These findings suggest that the bacterium not only reached the bloodstream but it also was detectable in peripheral blood of the ear pinna, underscoring its dissemination capacity (Liang et al. 2020). Interestingly, our results are in the line with those of Oliveira et al. (2023), who also used guinea pigs as experimental model to recover spirochetes and observed the first peak of spirochetemia between 9 and 11 days after ticks fed on the animals. Collectively, our results suggest that *O. octodontus* is a competent vector of a novel species of *Borrelia* (Sonenshine and Mather 1994; Beerntsen et al. 2000; de la Fuente et al. 2017). However, our inability to detect spirochetes in the blood of GP1 using dark-field microscopy might suggest a low spirochetemia that this novel *Borrelia* sp. could display in *C. porcellus*. Indeed, the propensity of borreliae to infect a given host varies depending on the host species (Burgdorfer and Mavros 1970; Bermúdez et al. 2021).

The use of an experimental murine model to demonstrate tick vectorial competence for borreliae species is a widely used method (Schwan et al. 2012; Muñoz-Leal et al. 2018; Bermúdez et al. 2021; Oliveira et al. 2023). However, research has shown that not all murine models develop infections for a given *Borrelia* sp., even those rodent species sympatric or phylogenetically related with a competent host, a fact that reflects the complexities of the spirochetes' transmission cycles (Burgdorfer and Mavros 1970; Bermúdez et al. 2021). In this context, identifying the competent vertebrate hosts is crucial to gain a more comprehensive understanding of the epidemiological cycle of a given *Borrelia* sp. (Sonenshine 2005).

Once colonized by RFG borreliae, ticks of the genus *Ornithodoros* remain infectious throughout their lifespan (Francıs 1938; Johnson et al. 2016; Jakab et al. 2022). In this sense, the fact that the collected ticks

fasted for four months until the beginning of the experiment, and transmitted a spirochete to one guinea pig, undoubtedly suggests that at least one tick from El Guindo locality was infected (Francıs 1938; Johnson et al. 2016). However, it is possible that more infected ticks were present among the fed specimens, since experimental studies have demonstrated that at least three ticks per mouse are necessary to transmit spirochetes (Boyle et al. 2014; Stewart et al. 2022). Our results support the hypothesis by Thompson et al. (2021) pointing that *O. octodontus* is involved in the transmission of RFG borreliae. Considering that larvae of this tick species feed on the rodent *O. degus* and adult ticks and nymphs are found in their burrows (Muñoz-Leal et al. 2020; Thompson et al. 2021), it is likely that this rodent is involved in the enzootic cycle of this spirochete.

Phylogenies of the *flaB* and MLST genes showed that the detected spirochete forms an independent lineage within the RFG species and is herein named as "Ca. *Borrelia octodonta*". Expectedly, a phylogenetic analysis of *flaB* gene denoted that "Ca. *Borrelia octodonta*" is closely related with *Borrelia* sp. Alcohuaz (MW981443) (Thompson et al. 2021), that was characterized from *O. octodontus* as well, but from a different locality at least 60 km towards the north of El Guindo. This fact aligns with the evidence that *Ornithodoros* species are specific vectors for *Borrelia* species (Davis 1956; Schwan 1996; Krishnavajhala et al. 2018). Consequently, "Ca. *Borrelia octodonta*" and *Borrelia* sp. Alcohuaz are likely the same *Borrelia* species, and the single nucleotide polymorphism in their *flaB* sequences suggests population structuring, which may be influenced by geographical barriers between both locations (Hellgren et al. 2011).

The clade encompassing the borreliae *flaB* sequences retrieved from rodents and ticks in South America exhibited a close relationship with the clade of borreliae associated with *Ornithorodos* ticks of the Old World (*B. hispanica*, *B. duttonii*, *B. crocidurae*, *B. persica*) supporting previous topologies (Fig. 2) (Thompson et al. 2021; Oliveira et al. 2023). In contrast, the MLST phylogeny clearly shows a lack of genetic data for *flaB* genotypes reported in RFG borreliae across South America. It's worth noting that both "*Candidatus* Borrelia caatinga" (Oliveira et al. 2023) and "Ca. *Borrelia octodonta*" stand out as the sole RFG spirochetes from South America in the MLST phylogeny (Fig. 2). To date, our results provide the most comprehensive genetic characterization of a *Borrelia* of the RFG in Chile.

Challenges associated with amplifying the genes in the MLST scheme of RFG borreliae from South America have been previously highlighted (Oliveira et al. 2023). Intriguingly, similar to our findings, Oliveira

et al. (2023) could not amplify the *clpA* and *nifS* genes for "Ca. *Borrelia caatinga*". This suggests that the available primers designed upon RFG borreliae sequences of the Northern Hemisphere may not capture the genetic variability of South American RFG borreliae. Such difficulties underscore the need to generate genomic data to design novel primers and improve the MLST characterization of South American borreliae.

The name "Ca. *Borrelia octodonta*" is herein proposed based on the genomic profiling of the species. However, the isolation, culture, and complete genome sequencing, is needed to formally describe this novel species. Finally, the confirmation that "Ca. *Borrelia octodonta*" is transmitted by *O. octodontus*, poses a probable risk to humans, particularly because *O. octodontus* is associated with *O. degus*, and this rodent establishes their colonies close to human dwellings (Yefi-Quinteros et al. 2018). This fact challenges the boundaries between wild and peridomestic cycles, and while cases of tick-borne relapsing fever do not exist in Chile, attention should be paid as vector ticks are identified.

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Author contributions All authors contributed to the study conception and design, material preparation, and data collection. Analysis was performed by [Adriana Santodomingo], [Richard Thomas], and [Sebastian Muñoz Leal]. The first draft of the manuscript was written by [Adriana Santodomingo] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the supplementary information.

Declarations

Ethics approval Procedures performed in this study were verified and approved by the Bioethics Committee of School of Veterinary Sciences, University of Concepción CBE-24-2021.

Consent to participate Not applicable.

Consent to publish Not applicable.

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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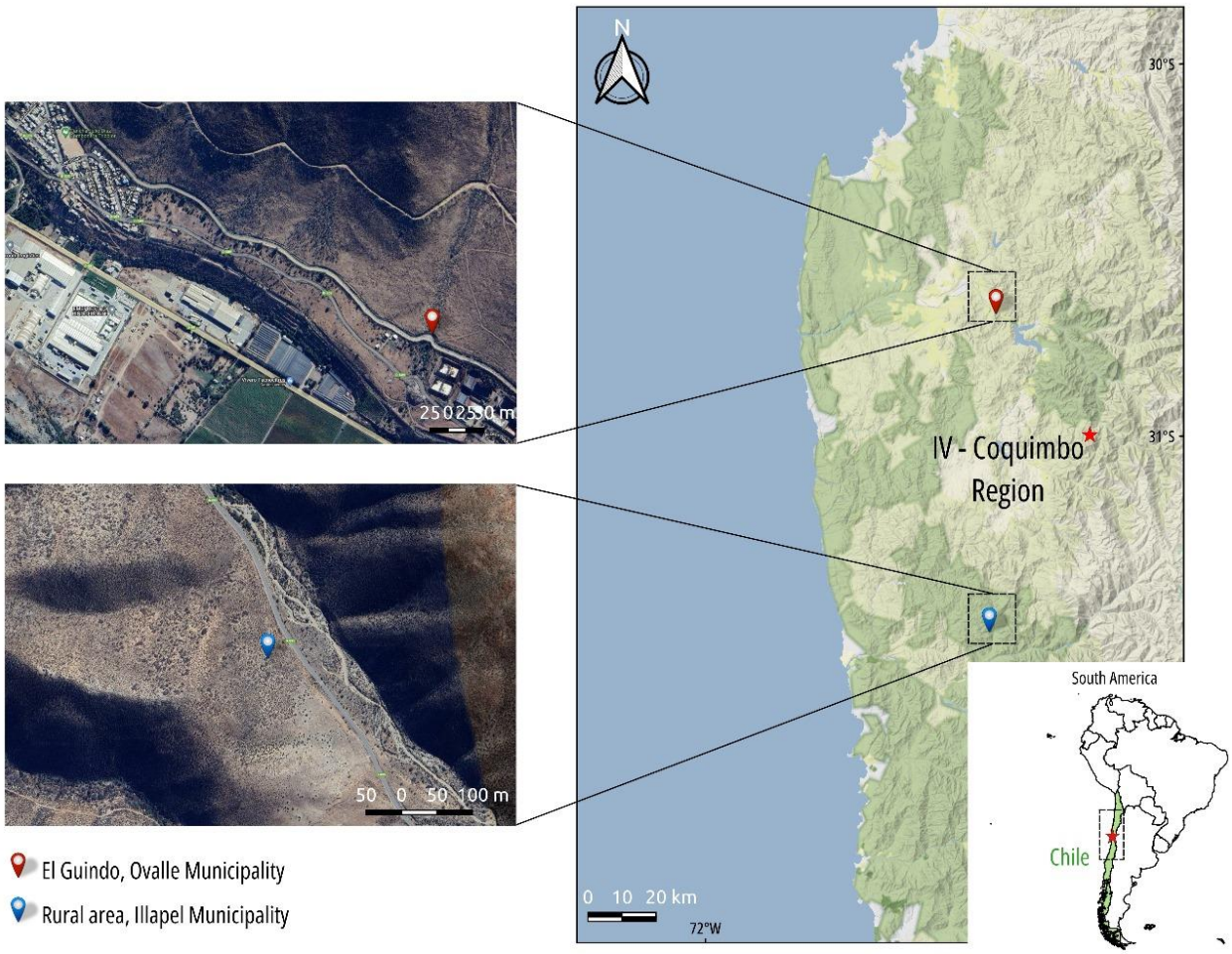

Fig 1. Localities in Coquimbo Region, northern Chile, where soft ticks were collected. The map was created using Quantum Geographic Information System (QGIS) version 3.18.1-Zürich (https://www.gnu.org/licenses).

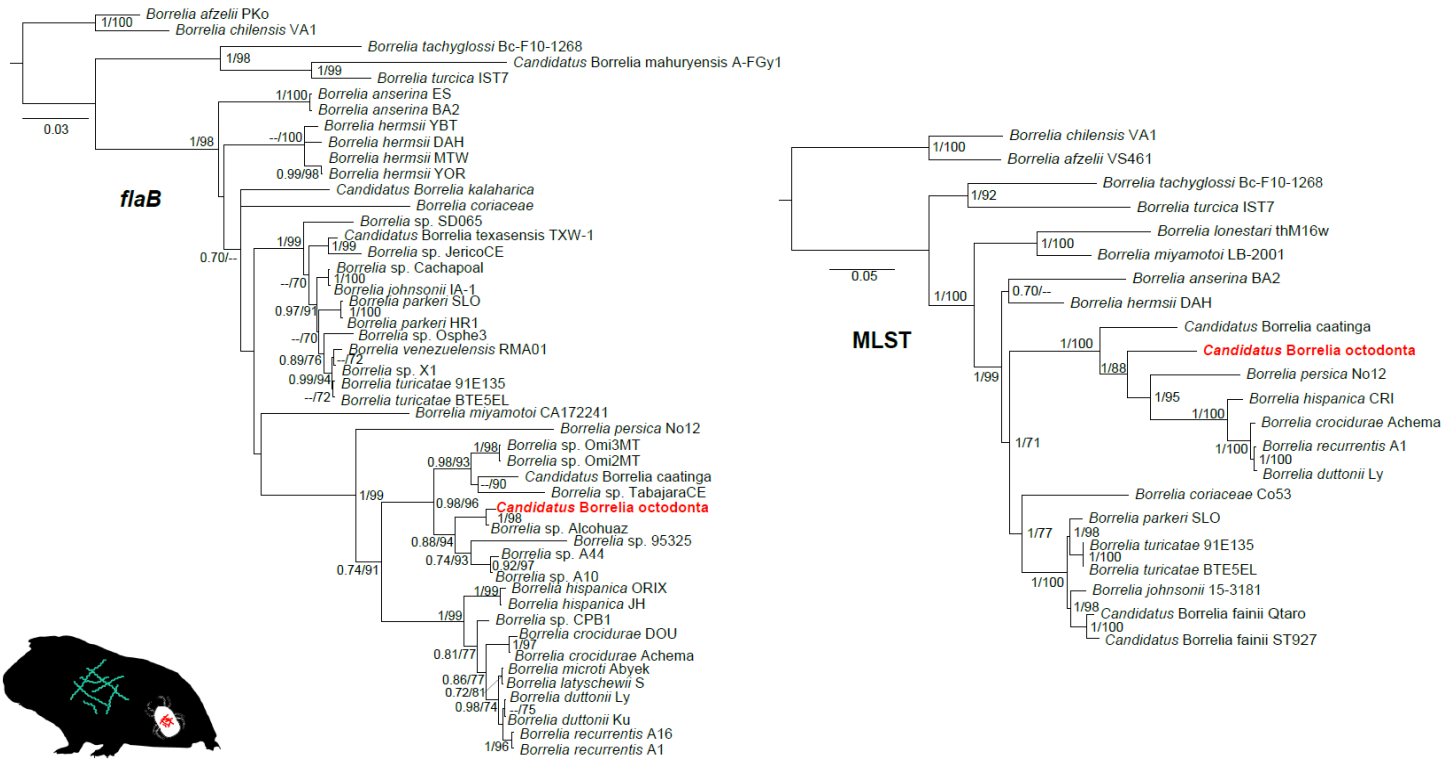

Fig 2. Phylogenetic analyses of relapsing fever group (RFG) *Borrelia* spp. inferred for *flaB* and concatenated *clpA*, *clpX*, *pepX*, *pyrG*, *recG*, *nifS*, *rplB*, and *uvrA* (MLST). The *flaB* tree is based on 47 sequences and an alignment of 612 base pairs; best-fit evolutionary models calculated for ML and BI methods were TVM+F+G4 (position-1), TPM2u+F+G4 (position-2), HKY+F+G4 (position-3); and M_{27}, M_{95} (position-1); M_{34}, M_{123}, M_{129} (position-2); M_{50}, M_{152}, M_{90}, M_{15}, M_{157}, M_{147} (position-3), respectively. The MLST tree is based on 22 sequences and an alignment of 4,776 base pairs; best-fit evolutionary models calculated for ML and BI methods were GTR+F+G4 (position-1), GTR+F+G4 (position-2), TVM+F+G4 (position-3); and M_{202}, M_{175}, M_{203}, M_{193} (position-1); M_{200}, M_{203}, M_{134}, M_{198}, M_{189}, M_{190} (position-2); M_{195}, M_{157}, M_{147} (position-3), respectively. The position of the *Candidatus* Borrelia octodonta characterized in the present study is in red. Trees are drawn to scale.

Numbers above or below tree branches represent Bayesian posterior probabilities/ML bootstrap values. Scale bar indicates nucleotide substitutions per site. GenBank accession numbers of the public sequences used for *Borrelia* phylogenies reconstruction based on *flaB* gen are shown in Table S1. Sequence Type (ST) numbers of the sequences used for *Borrelia* phylogenies reconstruction based on MLST genes are shown in Table S2.

Table S1: GenBank accession numbers of the public sequences used for *Borrelia* phylogenies re-construction based on *flaB* genes

Sequence name	GenBank accession number
flaB	
Borrelia chilensis VA1	KY412446
Borrelia afzelii PKo	CP000395
Borrelia tachyglossi Bc-F10-1268	CP025785
Candidatus Borrelia mahuryensis A-FGy1	CP043682
Borrelia turcica IST7	CP028884
Borrelia anserina ES	CP013704
Borrelia anserina BA2	CP005829
Borrelia hermsii YBT	CP005706
Borrelia hermsii MTW	CP005680
Borrelia hermsii YOR	CP004146
Borrelia hermsii DAH	NC_010673
Candidatus Borrelia kalaharica M15-2747	KT970516
Borrelia coriaceae Co53	CP005745
Borrelia turicatae 91E135	CP000049
Borrelia venezuelensis RMA01	MG651650
Borrelia sp. X1	MF432464
Borrelia turicatae BTE5EL	CP015629
Borrelia sp. Osphe3	MH178396
Borrelia parkeri SLO	CP005851
Borrelia parkeri HR1	CP007022
Borrelia sp. Cachapoal	MK112520
Borrelia johnsonii IA-1	EU492387
Candidatus Borrelia texasensis TXW-1	AF264901
Borrelia sp. JericoCE	MT076261
Borrelia sp. SD065	MG832413
Borrelia miyamotoi CA17-2241	NZ_CP021872
Borrelia persica No12	NZ_AYOT00000000.1
Borrelia sp. Omi3MT	MT076262
Borrelia sp. Omi2MT	MT076262
Borrelia sp. TabajaraCE	MT076263
Borrelia sp. Alcohuaz	MW981443
Borrelia sp. A44	MN596013
Borrelia sp. A10	MN596012
Borrelia sp. 95325	HM583797
Borrelia sp. CPB1	FJ868584
Borrelia recurrentis A16	DQ346829
Borrelia recurrentis A1	CP000993
Borrelia duttonii Ly	CP000976
Borrelia duttonii Ku	DQ346837
Borrelia microti Abyek	JF708951
Borrelia latyschewii S	JF708952
Borrelia crocidurae DOU	CP004267
Borrelia crocidurae Achema	CP003426
Borrelia hispanica ORIX	GU357616
Borrelia hispanica JH	GU357615
Candidatus Borrelia caatinga	OP952107
***Candidatus* Borrelia octodonta**	**OR644722**

Table S2: Sequence Type (ST) numbers of the sequences used for *Borrelia* phylogenies reconstruction based on MLST genes

Sequence name	**ST PubMLST**	**GenBank accession number**
Borrelia chilensis VA1	430	
Borrelia afzelii VS461	70	
Borrelia tachyglossi BcF101268	957	
Borrelia turcica IST7	793	
Borrelia anserina BA2	668	
Borrelia hermsii DAH	104	
Borrelia parkeri SLO	670	
Borrelia turicatae 91E135	116	
Borrelia turicatae BTE5EL	116	
Borrelia johnsonii 15-3181	764	
Candidatus Borrelia fainii Qtaro	958	
Candidatus Borrelia fainii ST927	927	
Borrelia coriaceae Co53	671	
Borrelia recurrentis A1	669	
Borrelia duttonii Ly	101	
Borrelia crocidurae Achema	672	
Borrelia hispanica CRI	805	
Borrelia persica No12	636	
Borrelia lonestari thM16w	735	
Borrelia miyamotoi LB-2001	633	
Candidatus Borrelia caatinga		*clpX* : OP952110; *pepX* : OP952111; *pyrG* : OP952112; *recG* : OP952113; *rplB* : OP952114; *uvrA* : OP952115
***Candidatus* Borrelia octodonta**		***clpX* : OR644727; *pepX* : OR644726; *recG* : OR644725; *rplB* : OR644724; *uvrA* : OR644723**

CONCLUSIONES GENERALES

1 – En los ecosistemas de Chile, los roedores cricétidos examinados parecen no albergar *Anaplasma*. Sin embargo, las especies *Abrothrix jelskii* y *Phyllotis darwini* podrían participar como hospederos en los ciclos epidemiológicos de los nuevos genotipos de *Babesia* sp. LC77 y *Babesia* sp. LC87, detectados en esta investigación. Por su parte, los roedores *Phyllotis xanthopygus* y *Oligoryzomys longicaudatus* podrían tener un rol similar en la epidemiología de los genotipos novedosos de *Borrelia* sp. A10 y *Borrelia* sp. A44. Los resultados sugieren que el Pudú (*Pudu puda*) podría ser un hospedero reservorio natural, tanto para la *Babesia* sp. genoespecie pudui, como para la bacteria *Anaplasma phagocytophilum* genovariante "Patagonia". Así mismo, la detección de estos microorganismos y la nueva genovariante de *Borrelia chilensis*, denominada cepa "Chiloé" en *Ixodes stilesi* sugiere que esta garrapata podría ser un posible vector para estos agentes. En conjunto, estos resultados constituyen el primer informe de infección por *Babesia* spp. y *Borrelia* spp. en roedores cricétidos, y también el primer registro de infección por *Babesia* sp. y *A*. *phagocytophilum* en pudúes en Chile. Es importante aclarar que la detección del ADN de estos microrganismos no confirma de manera concluyente el papel estos animales en la epidemiología de estos agentes infecciosos transmitidos por garrapatas (AITG), ni su posible impacto clínico en la salud de los vertebrados estudiados. Por lo tanto, el rol de estas especies deberá ser confirmado mediante estudios experimentales. Mientras tanto, los ciclos epidemiológicos de *Borrelia* spp., *Babesia* spp. y *Anaplasma phagocytophilum* identificadas en este estudio siguen siendo una incógnita.

2 – En cuanto a la posición filogenética de los microorganismos caracterizados, nuestras inferencias sugieren que corresponderían a nuevos genotipos, genoespecies y/o genovariantes. La genovariante "Patagonia" de *A*. *phagocytophilum*, asociada a pudúes e *I*. *stilesi*, se clasificó como el primer ecotipo sudamericano (ecotipo V; grupo 8) de este complejo de especies. Adicionalmente, mostró una relación moderada con variantes de *A*. *phagocytophilum* vinculadas a roedores. Con relación a los genotipos de *Babesia*

caracterizadas en roedores, destacan *Babesia* sp. LC87 y *Babesia* sp. LC77. El primero se posicionó dentro de un grupo de babesias sudamericanas asociadas con murciélagos y roedores, mientras que el segundo se posicionó en una rama independiente dentro del grupo *microti*. Por su parte, la genoespecie *Babesia* sp. pudi, caracterizada en pudúes e *I*. *stilesi*, se relacionó con la especie zoonótica *Babesia odocoilei*, detectada en cérvidos de Norteamérica. En particular, *Babesia* sp. pudui se agrupó dentro de un clado vinculado a babesias de ungulados, apoyando la hipótesis de que las babesias que parasitan a estos mamíferos forman un grupo natural. En relación con *Borrelia*, los genotipos detectados en roedores, *Borrelia* sp. A10 y *Borrelia* sp. A44, se relacionaron con *Borrelia* sp. 95325 (HM583797) previamente caracterizada en un *Ornithodoros* sp. en Bolivia. Estos a su vez conformaron un grupo monofilético dentro del grupo de borrelias de fiebre recurrente. Por otro lado, el genotipo *Borrelia* sp. A53 se incluyó dentro del grupo de borrelias de Lyme. Asimismo, la genovariante "Chiloé" de *B*. *chilensis* caracterizada en *I*. *stilesi*, se posicionó como hermana de *B*. *chilensis* VA1 dentro del grupo de borrelias de Lyme.

3 – Se determinó que *Ornithodoros octodontus* es capaz de transmitir, bajo condiciones de laboratorio, una *Borrelia* sp. a cobayas. Además, el perfil genético de esta bacteria nos permitió inferir que se trata de una nueva genoespecie denominada "*Candidatus* Borrelia octodonta".

Perspectivas futuras

En resumen, este estudio ha proporcionado información valiosa sobre la presencia y filogenia de AITG en roedores, ciervos y garrapatas en Chile. Aunque se ha detectado ADN de *Anaplasma*, *Borrelia* y *Babesia* en estos mamíferos y garrapatas, la relación exacta de estos animales en la epidemiología de estos agentes infecciosos y su posible impacto clínico aún requiere confirmación a través de estudios experimentales.

En términos de futuras investigaciones, para comprender el papel de roedores, ciervos y garrapatas asociadas en la epidemiología de estos agentes

infecciosos y para investigar los ciclos epidemiológicos de AITG en la región, así como la diversidad genética y las relaciones evolutivas de estos agentes, se pueden considerar varios tipos de estudios y preguntas especificas a resolver:

Estudios epidemiológicos para determinar ¿cuál es la frecuencia de infección de *Anaplasma*, *Borrelia* y *Babesia* en las poblaciones de roedores y ciervos en diferentes regiones de Chile?

Estudios experimentales de transmisión para evaluar ¿cómo se transmiten exactamente estos agentes entre garrapatas y mamíferos? ¿qué factores afectan la transmisión?

Estudios genéticos y filogenéticos para analizar la variabilidad genética de los patógenos para entender su evolución y posible adaptación a diferentes hospedadores y vectores.

Estudios de patogenicidad para evaluar la capacidad de los agentes de causar enfermedad, incluyendo la caracterización de cepas patógenas y su virulencia que permitan determinar ¿qué síntomas y enfermedades clínicas causan estos agentes en los roedores, ciervos y posiblemente en humanos?

Estudios de vigilancia y monitoreo, en donde se implementen programas a largo plazo para observar cambios en la distribución y frecuencias de infección de estos agentes.

Modelización matemática y computacional para crear modelos para predecir la dinámica de transmisión y el impacto de intervenciones y posteriormente resolver ¿qué estrategias de control podrían ser efectivas para gestionar la transmisión de estos agentes en la naturaleza?