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“El caos es un orden sin descifrar”
-José Saramago

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RESUMEN

Las enfermedades zoonóticas representan cerca del 60% de las enfermedades infecciosas. Durante la última década, la prevalencia de las enfermedades zoonóticas ha aumentado, principalmente por efecto del calentamiento climático, así como por el tráfico y caza ilegal de animales silvestres. Dentro de las enfermedades infecciosas, el género *Borrelia* destaca como un grupo de bacterias transmitidas por vector, el cual está dividido en cuatro grupos filogenéticos: (I) borreliosis de Lyme, (II) Fiebre Recurrente, (III) *Borrelia* asociadas con reptiles y (IV) *Borrelia* asociada con monotremas. En México, se ha reportado la presencia de cuatro especies del género *Borrelia*, así como la presencia de garrapatas potencialmente vectores en la zona norte y central de México. Sin embargo, el estudio de la borreliosis en los estados de Guerrero, Estado de México, Puebla y Veracruz, a pesar de existir reportes de vectores, es inexistente. Por esta razón, el objetivo de este trabajo fue analizar la situación actual de la borreliosis en la interface humano-vector-hospedero en la zona central de México, basado en (i) la identificación molecular del género *Borrelia* en vertebrados silvestres y sus garrapatas asociadas, (ii) el establecimiento de la prevalencia de las especies de *Borrelia* detectadas y (iii) la evaluación genética del agente etiológico de *Borrelia burgdorferi* s.s., agente etiológico de la enfermedad de Lyme. Para ello, se llevaron a cabo muestreos dirigidos a vertebrados silvestres en los estados de Guerrero, Estado de México, Puebla y Veracruz. A partir de los ejemplares recolectados, se realizó la búsqueda de garrapatas. Para la detección de *Borrelia* se utilizó un fragmento del 16S ribosomal, uno para el gen de la flagelina y un último fragmento de la proteína de membrana externa A (ospA). Se recuperó un total de 60 garrapatas, identificadas como *Amblyomma dissimile*, en 30 ejemplares de *Rhinella horribilis* en Veracruz. Los análisis filogenéticos revelaron la presencia de un linaje nuevo del grupo de *Borrelia* asociadas con reptiles. En cuanto a mamíferos, se analizaron 11 especies de murciélagos capturados y tres especies de roedores. La reconstrucción filogenética demostró la presencia de dos linajes nuevos de *Borrelia* en murciélagos, uno asociado al grupo de borreliosis de Lyme (Estado de México) y el segundo del grupo de las fiebres recurrentes (Veracruz). En cuanto a los roedores, únicamente en *Habromys schmidlyi* se encontró la presencia de *Borrelia*,

la cual fue identificada como *B. burgdorferi* s.s. El análisis de diversidad genética de *B. burgdorferi* s.s., reveló una estructuración genética débil entre las poblaciones de México, China, Alemania, EUA, Italia, Luxemburgo, identificando un proceso inicial de diferenciación genética en las poblaciones del Oeste de EUA y México, probablemente debido a sus historias evolutivas y patrones ecológicos. Los registros obtenidos representan nuevas localidades para la presencia de *Borrelia* en su totalidad. El reporte del grupo de *Borrelia* asociadas con reptiles representa el primero para Norteamérica, mientras que los registros en murciélagos son los primeros reportes para México. Por otro lado, este es el primer estudio que evalúa la diversidad genética de *B. burgdorferi* s.s. a lo largo de toda su distribución. Finalmente, se reporta el registro más sureño para la distribución de *B. burgdorferi* s.s.

INTRODUCCIÓN

Las enfermedades infecciosas son aquellas que son producidas por un agente patógeno y en su mayoría, son provocadas por bacterias y su transmisión implica múltiples interacciones dinámicas entre poblaciones de fauna silvestre, ganado y humana (Daszak et al. 2000; Jones et al. 2008; Dantas-Torres et al. 2012; Allen et al. 2017; OMS 2019). Cerca del 60% de los agentes patógenos son de origen zoonótico, es decir, son transmitidas de fauna silvestre y/o doméstica hacia el humano, ya sea por contacto directo o por medio de un vector (Woolhouse, 2008; Bueno-Marí et al. 2015; Allen et al. 2017). Por otra parte, la epidemiología es el estudio de la distribución y los determinantes o eventos relacionados con la salud en poblaciones específicas, con el fin de comprender los procesos que influyen en la expansión o transmisión de las enfermedades infecciosas, a través de un enfoque global que incluya los contextos biológicos, físicos, sociales e históricos, presentes en las poblaciones de interés (Susser y Susser 1996; Phillips et al. 2004; Rodríguez-Morales 2005).

Como una forma de abordar de manera integral el concepto de la epidemiología, se propone el enfoque de “Una Salud”, cuyo objetivo es entender y prevenir el brote de enfermedades zoonóticas desde un punto de vista ecosistémico, a partir de la vigilancia epidemiológica tanto en población humana, como en animales (silvestres, domesticados y compañía), y poder utilizar los avances de cada una, para alcanzar un bienestar ecosistémico de salud (Kahn 2006; Conti y Rabinowitz 2011; Zinsstag et al. 2011). Este enfoque, busca integrar los conocimientos de la medicina humana y veterinaria, de la entomología médica y la ecología, con el fin de comprender los mecanismos de mantenimiento de los patógenos en su ciclo selvático, su transmisión a población humana y los cuadros clínicos en pacientes humanos, y así desarrollar enfoques predictivos y poder limitar su posterior propagación tanto en fauna como en población humana (Morse, 1995; Daszak et al. 2000; Jones et al. 2008).

Dentro de las enfermedades infecciosas, destaca el género *Borrelia* como un grupo de bacterias helicoidales Gram negativas que son transmitidas principalmente por garrapatas, así como una especie transmitida por el piojo del cuerpo (Barbour y Hayes 1986; Kurtenbach et al. 2006; Dantas-Torres et al. 2012; Steere et al. 2016).

Este género incluye los agentes etiológicos de la enfermedad de Lyme o borreliosis de Lyme [BL] y Fiebre recurrente [FR] (Dantas-Torres et al. 2012; Steere et al. 2016; Margos et al. 2017; Barbour y Schwan 2018), las cuales son consideradas como enfermedades emergentes (Dantas-Torres et al. 2012).

Los estudios de la borreliosis en México, son escasos, con 38 estudios y únicamente cuatro especies de *Borrelia* reportadas, *B. burgorferi* s.s., *B. duguesii*, *B. mazzottii* y *B. turicatae* (Brumpt et al. 1939; Mazzotti, 1949; Davis, 1956; Guadalupe Gordillo-Pérez et al. 2017; López-Pérez et al. 2019). Hasta el momento, se han reportado 17 especies de animales (14 especies de garrapatas, seis animales silvestres, caballo, perro y dos sinantrópicas: *Rattus rattus* y *Mus musculus*) con exposición y posible infección de *Borrelia*, así como 399 casos humanos entre los años 1939 y 2020 (Colunga-Salas et al. 2020).

Particularmente en México, poco se conoce acerca de las enfermedades emergentes, tanto de origen zoonótico como no zoonótico, sin embargo, esta falta de información puede deberse a la falta de muestreo de los agentes etiológicos, así como a la falta de disposiciones oficiales para la notificación de enfermedades emergentes (Woolhouse, 2008; Colunga-Salas et al. 2020). Lo anterior ha influido a que el estudio de la borreliosis no ha sido sistematizado, por lo que no existe una relación entre los registros en animales y los casos humanos reportados, sin embargo, la mayor cantidad de registros de *Borrelia*, se concentra en la zona norte y centro del país (Colunga-Salas et al. enviado).

ANTECEDENTES

El género *Borrelia* fue formalmente descrito en 1907, después de haber sido considerado como parte del género *Spirochaeta* (Swellengrebel, 1907). Por más de un siglo no se presentaron cambios taxonómicos importantes, sin embargo, en 2014, con base en un análisis filogenómicos utilizando marcadores moleculares conservados (inserciones y deleciones en regiones codificantes conservadas [*Conserved signature inserts and* deletions, CSIs, por sus siglas en inglés]), se demostró que los miembros comprendidos en el grupo de Borreliosis de Lyme (BL) presentaban un número distinto de CSIs en comparación con el grupo de Fiebre recurrente (FR), ante lo cual, se propuso el nuevo género *Borreliella*, en el cual quedaban incluidos únicamente los miembros de BL (Adeolu and Gupta, 2014), con una identidad nucleotídica promedio significativamente distinta del grupo de FR.

A pesar de la evidencia para la descripción de este nuevo género, no toda la comunidad científica lo aceptaba, ante lo cual, un estudio basado en el porcentaje de proteínas conservadas, el cual se ha propuesto como el método más adecuado para la delimitación de géneros bacterianos (Qin et al. 2014), se volvió a proponer al género *Borrelia* como un grupo monofilético (Margos et al. 2018).

Hasta el momento, no existe una clara postura de si el género debe dividirse o no, por lo que los principales defensores de cada hipótesis, siguen realizando estudios sobre esta controversia (Gupta, 2019; Margos et al. 2020). Sin embargo, la postura más aceptada por la comunidad científica, es la unificación en un solo género (Carvalho et al. 2020; Filatov et al. 2020; Morales-Diaz et al. 2020; Răileanu et al. 2020; Sales et al. 2020; Zawada et al. 2020).

Actualmente, el género se subdivide en cuatro grupos principales, el grupo de borrelias asociadas con reptiles (BAR), las borrelias asociadas con monotremas (BAM), el de fiebre recurrente (FR) y el de borreliosis de Lyme (BL) [Figura 1] (Panetta et al. 2017; Gofton et al. 2018).

Todas las especies de este género son transmitidas al hospedero vertebrado por artrópodos hematófagos durante el proceso de alimentación (Burgdorfer et al. 1982; Barbour y Hayes 1986; Kurtenbach et al. 2006; Takano et al. 2011). Hasta el momento, se han reconocido como vectores a las garrapatas duras de la familia Ixodidae para los grupos BAR, BAM y BL, así como para *Borrelia miyamotoi*,

especie del grupo de FR (Fukunaga et al. 1995; A Takano et al. 2011; Loh et al. 2016).

En el caso del grupo de fiebre recurrente, el primer vector que se involucró fue el piojo del cuerpo (*Pediculus humanus*), sin embargo, posteriores trabajos reconocieron la importancia de garrapatas blandas del género *Ornithodoros* como los principales vectores (Scott 1942; Southern y Sanford 1969; Talagrand-Reboul et al. 2018). Hasta el momento, el piojo del cuerpo es el único vector reconocido para *Borrelia recurrentis*, mientras que diversas especies de garrapatas blandas son vectores del resto de las especies de FR (Felsenfeld 1965; Southern y Sanford 1969; Kurtenbach et al. 2006).

Por otra parte, el ciclo de vida de este género bacteriano, inicia con la eclosión del vector en estadio de larva. Una vez que las larvas emergen, éstas pueden infectarse de *Borrelia* al momento de su primera alimentación, siempre y cuando el hospedero esté infectado y la bacteria esté circulando en vía sanguínea. Una vez que el vector adquiere la bacteria, éste será capaz de transmitirla a los próximos hospederos durante cada alimentación [Figura 2] (Felsenfeld, 1965; Piesman, 1993; Kurtenbach et al. 2006; Ai Takano et al. 2012; Krause et al. 2015). Es importante mencionar, que el ser humano es un hospedero accidental, salvo para *B. recurrentis*, donde el ser humano es el único reservorio conocido (Southern y Sanford 1969; Cutler 2009; Cutler et al. 2009).

Únicamente para las especies del grupo de FR, se ha reportado transmisión transovárica, es decir, que las larvas nacen infectadas con borrelia (Varma 1956; Gaber et al. 1984; Rollend 2013; Krause et al. 2015). Sin embargo, se ha reportado que después de entre cinco y nueve generaciones de garrapatas, la patogenicidad de la bacteria disminuye (Barbour y Hayes 1986).

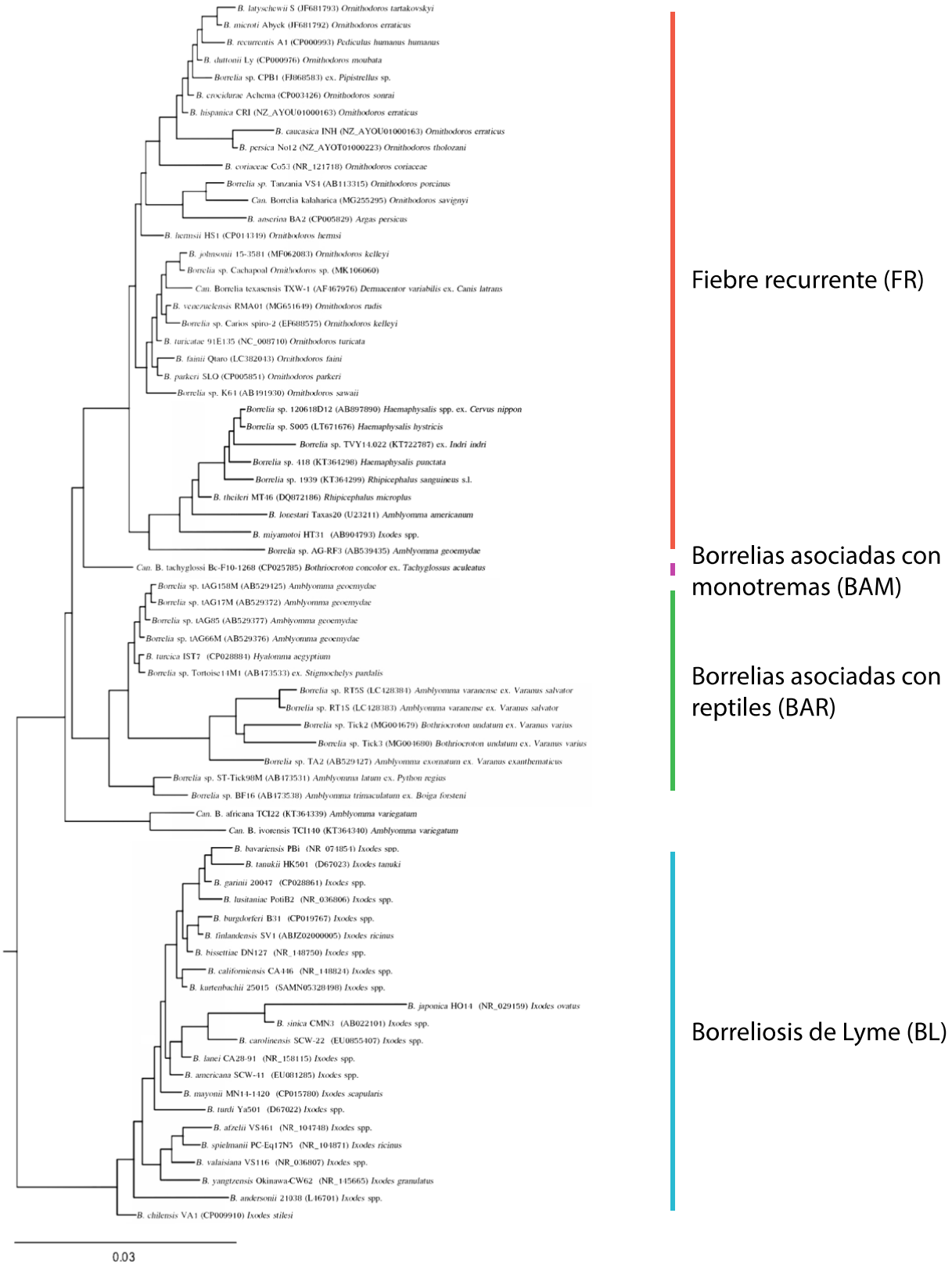

Figura 1. Filogenia del género *Borrelia.* Inferencia Bayesiana a partir del gen 16S rDNA mediante el modelo de sustitución de K2P con distribución gama y sitios invariables. Tomado y modificado de Margos et al. (2020).

Dentro del vector, la bacteria presente en la sangre llega al intestino, donde pasan al hemocele, se replican y posteriormente migran a las glándulas salivales y órganos coxiales (Varma 1956; Barbour y Hayes 1986; Burgdorfer et al. 1989). En el caso del grupo de FR, se ha reportado que las espiroquetas en el ovario, invaden el ovocito en desarrollo. Durante el desarrollo embrionario de la garrapata, las bacterias migran a los ganglios, donde se alojan y perduran hasta que termine el proceso embrionario y puedan invadir posteriormente las glándulas salivales u órganos coxiales (Aeschlimann 1958; Barbour y Hayes 1986).

En la alimentación de las garrapatas, éstas liberan secreciones salivales durante el proceso de corte y penetración a la piel del hospedero vertebrado. Las secreciones salivales evitan la coagulación de la sangre, causando hemorragias, a su vez, la saliva inactiva el sistema del complemento e inhibe la función fagocítica, lo cual facilita que las bacterias ingresen al vertebrado (Ribeiro 1987; Ribeiro et al. 1990; Bowman y Sauer 2004; Anderson y Magnarelli 2008). Por otro lado, los órganos coxiales, donde hay tejidos especializados para excreción del exceso de líquido y solutos que se acumulan en la garrapata durante la alimentación, son especialmente importantes para la transmisión de borrelias en especies de garrapatas donde estos fluidos son liberados cercanos a la cavidad oral del vector [*i.e.* especies del género *Ornithodoros*] (Varma, 1956; Aeschlimann, 1958).

Una vez dentro del hospedero vertebrado, las bacterias se trasladan al torrente sanguíneo, donde circulan y se multiplican por un tiempo entre dos y hasta 10 días, dependiendo de la especie (Barbour y Hayes 1986). A través del torrente sanguíneo, las bacterias migran hacia diversos órganos, como bazo, hígado, riñón y en ciertos casos, cerebro o médula espinal, donde se alojarán por tiempo indefinido (Beck 1937; Barbour y Hayes 1986; Pachner et al. 1995; Pal y Fikrig 2003). Una vez dentro del órgano, la liberación de bacterias al torrente sanguíneo es raro y poco frecuente (Beck 1937; Barbour y Hayes 1986; Pal y Fikrig 2003).

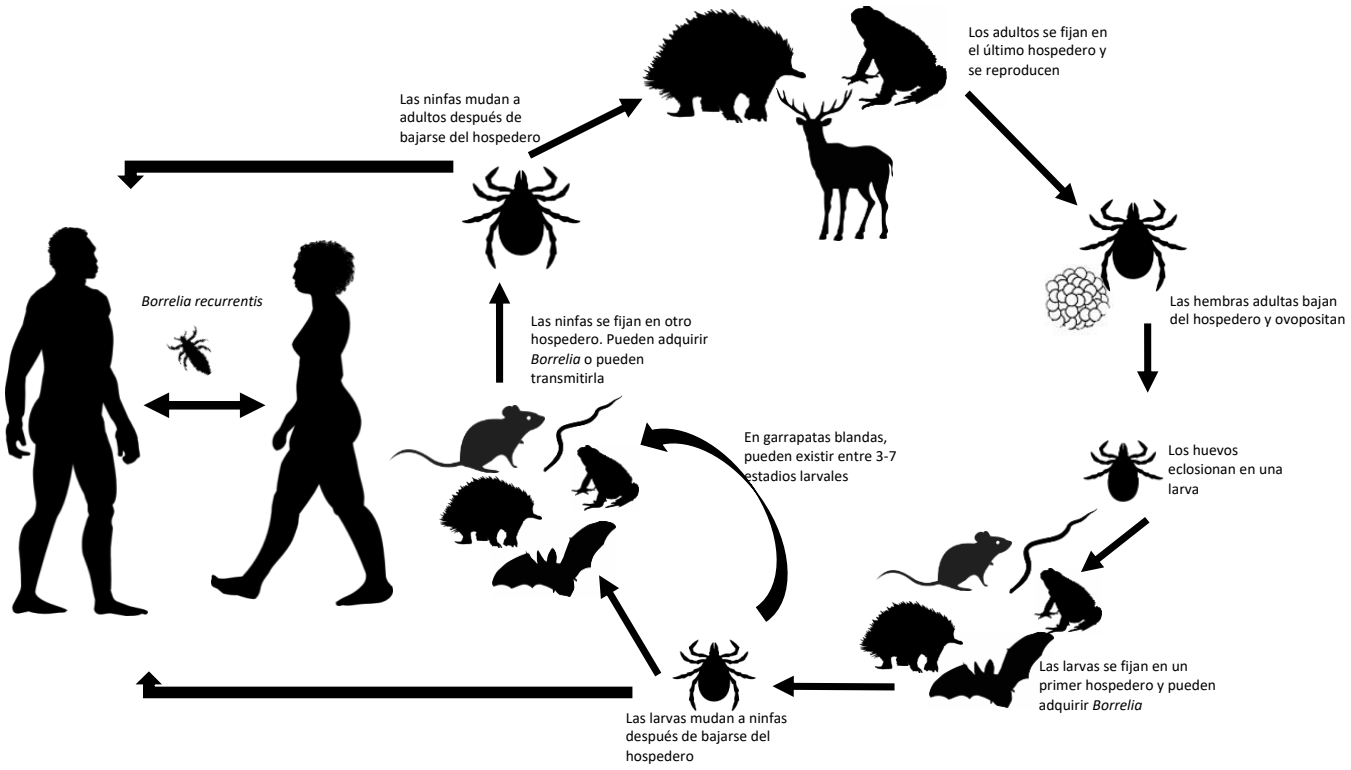

Figura 2. Ciclo de vida del género *Borrelia.* El humano puede fungir como hospedero accidental en cualquier etapa del ciclo de la garrapata, sin embargo, las ninfas y adultos son los que representan un riesgo de infección y transmisión de las especies de *Borrelia* transmitidas por garrapatas.

- *Borrelias asociadas con monotremas*

Este grupo de borrelias, fue reportado por primera vez mediante métodos moleculares por Loh et al. (2016) en ejemplares de *Bothriocroton concolor* e *Ixodes holocyclus* de equidnas (orden Monotrema) en el condado de Queensland y Nuevo Gales, Australia. A partir de análisis de similitud genética, las secuencias recuperadas formaban un clado relacionado con el grupo de FR [Figura 1] (Loh et al. 2016; Panetta et al. 2017; Gofton et al. 2018). Actualmente no existe ninguna especie formalmente descrita, sin embargo, se reconoce la existencia de *Candidatus* Borrelia tachyglossi (Loh et al. 2016, 2017).

Hasta el momento, sólo existen cuatro estudios sobre este grupo y en ellos se

reconoce un linaje filogenético, con la implicación de sólo dos garrapatas como posibles vectores, *Ixodes holocyclus* y *Botriochroton concolor* (Loh et al. 2016, 2017; Gofton et al. 2018).

El orden Monotrema incluye cuatro especies de equidnas y al ornitorrinco (Wilson y Reeder 2005), sin embargo, *Candidatus* Borrelia tachyglossi sólo se ha reportado en equidnas (Loh et al. 2016, 2017; Gofton et al. 2018; Moon et al. 2018), a pesar que *Ixodes ornithorhynchi* ha sido registrada como una garrapata del ornitorrinco (Roberts 1960; Guglielmone y Nava 2010), por lo que estudios en esta especie deben realizarse para evaluar su papel como hospedero de bacterias del género *Borrelia*.

- *Borrelias asociadas con reptiles*

Este grupo de borrelias fue aislada por primera vez de garrapatas duras a partir de muestras de garrapatas de la especie *Hyalomma aegyptium* (Güner et al. 2003). Estos aislados fueron descritos formalmente como *Borrelia turcica*, la cual formaba un clado monofilético de los grupos de FR y BL, los cuales se reconocían hasta ese momento (Güner et al. 2004). Posteriormente, Takano et al. (2010) a partir de muestras de reptiles y de garrapatas asociadas a estos, provenientes de diversos países del este de Europa y Medio Oriente, se reconoce el grupo de BAR. Hasta el momento este grupo es monotípico, siendo *B. turcica* la única especie formalmente descrita, sin embargo existe evidencia de linajes independientes dentro de este grupo que aún no han sido descritos (Güner et al. 2004; Takano et al. 2010, 2011).

Este grupo de borrelias es transmitido por garrapatas duras, hasta el momento se han reconocido seis especies de garrapatas como vectores, *Amblyomma exornatum*, *Amblyomma geoemydae*, *Amblyomma latum*, *Amblyomma trimaculatum*, *Bothriocroton undatum* y *Hyalomma aegyptium*, las cuales tienen afinidad por anfibios y reptiles (Güner et al. 2003; A Takano et al. 2010, 2011; Loh et al. 2016; Panetta et al. 2017). Dada la afinidad y la historia natural de los vectores de este grupo de borrelias, los reptiles son los únicos hospederos vertebrados que se reconocen, entre los cuales resaltan tortugas (*Cuora flavomarginata*, *Mauremys*

mutica y *Testudo greca*), lagartijas (*Varanus exanthematicus*, *Varanus varius* y *Lacerta agilis*) y serpientes [*Python regius* y *Boiga forsteni*] (Takano et al. 2011; Loh et al. 2016; Panetta et al. 2017).

A pesar que este grupo de borrelias está inminentemente ligado con reptiles y sus garrapatas, Panetta et al. (2017) utilizando el gen 16S rRNA, obtiene las relaciones de similitud genética entre las especies válidas del género, utilizando las secuencias disponibles de BAR. Mediante este análisis, se reporta por primera vez a las BAR como un grupo polifilético (Figura 1).

Geográficamente, este grupo se ha reportado en reptiles de Turquía y en Japón, sin embargo, en este último país, los reptiles positivos fueron especies exóticas provenientes de diversos países del este de Europa, Asia y Medio Oriente, específicamente de Sri Lanka, Jordán, Uzbekistán, Zambia, Turquía y Rumania (Takano et al. 2010, 2011).

Hasta el momento, no se han reportado casos humanos cuyo agente etiológico pertenezca a este grupo de borrelias, por lo cual, aparentemente no es un grupo de importancia médica (Takano et al. 2011), lo cual ha repercutido en que el estudio de este grupo de borrelias sea muy escaso a nivel mundial, únicamente con cinco estudios, por lo que más estudios deben realizarse, para corroborar su presencia en otros países (Pablo Colunga-Salas et al. 2018).

- *Fiebre recurrente*

Este grupo fue el primero en reconocerse a partir de muestras de sangre de pacientes con episodios febriles en diversos países de África (Ross y Milne 1904). Las primeras especies descritas se realizaron asumiendo la especificidad vectorial, es decir, cada especie reportada en cada vector, era considerada una especie nueva (Mazzotti 1949; Southern y Sanford 1969; Sakharoff 1981; Barbour y Schwan 2018). Sin embargo, las descripciones más recientes se basan en métodos moleculares (Fukunaga et al. 1995; Margos et al. 2008).

Hasta el momento, este grupo está compuesto por 23 especies válidas y cuatro *Candidatus* (Barbour y Schwan 2018; Talagrand-Reboul et al. 2018). Sin embargo,

a partir de diversos estudios con métodos moleculares, se encontró evidencia que *B. recurrentis* y *B. duttonii* no son dos especies distintas, sino que *B. recurrentis* parece ser una cepa de *B. duttoni* con adaptaciones que le permite mantenerse en y ser transmitida por el piojo del cuerpo (Scott et al. 2005; Lescot et al. 2008; Sally Cutler et al. 2008). Lo anterior, lo han relacionado con la hipótesis propuesta por Nicolle y Anderson, (1927), en la que se menciona que las especies de este grupo se originaron como parásitos de mamíferos pequeños, que se adaptaron al humano a través de la interacción con garrapatas y finalmente adquirieron la capacidad de mantenerse y transmitirse por el piojo del cuerpo.

En cuanto a los vectores, este grupo es el que presenta la mayor diversidad de organismos involucrados. De las 11 especies, nueve de ellas son transmitidas por garrapatas blandas del género *Ornithodoros*, a excepción de *B. miyamotoi* y *B. recurrentis* que son transmitidas por garrapatas duras del género *Ixodes* y *Pediculus humanus* [piojo del cuerpo], respectivamente (Southern y Sanford 1969; Scoles et al. 2001; Wagemakers et al. 2015; Talagrand-Reboul et al. 2018). En el caso específico de *B. recurrentis*, el único reservorio es el humano, por lo cual la infección de fauna silvestre o animales de compañía es nula (Southern y Sanford 1969; Barbour y Hayes 1986; Wagemakers et al. 2015; Talagrand-Reboul et al. 2018).

Los hospederos reconocidos incluyen algunas especies de aves como el pavo (*B. miyamotoi* en *Meleagris gallopavo*), así como diversas especies de mamíferos, entre los que destacan roedores, murciélagos y venados, dónde es común detectar la presencia de la bacteria en muestras de bazo, riñón e hígado (Hindle 1935; Southern y Sanford 1969; Godeluck et al. 1994; Scott et al. 2010; Nieto et al. 2012; Talagrand-Reboul et al. 2018).

El estudio de este grupo fue muy popular durante las epidemias de la Primera y Segunda Guerra Mundial, principalmente entre los mircrobiólogos. Sin embargo, después de la producción en masa de la penicilina y el DDT, que ayudaron a disminuir la tasa de mortalidad a nivel mundial, su estudio disminuyó a partir de la década de 1950 y básicamente los estudios se enfocan a reportes de casos humanos y descripciones de sólo algunas nuevas especies (Barbour y Hayes 1986; Scoles et al. 2001; Nieto et al. 2012; Talagrand-Reboul et al. 2018; Vázquez-

Guerrero et al. 2019).

Este grupo es de importancia médico-veterinaria, ya que *B. crocidurae*, *B. duttonii*, *B. hermsii, B. hispanica, B. miyamotoi*, *B. parkeri*, *B. persica*, *B. recurrentis*, *B. turicatae*, *B. theileri*, *B. baltazardii*, *B. caucasica*, *B. dugesii*, *B. latuschewii, B. mazzotti* y *Candidatus* B. kalaharica, se han comprobado como patógenos a humanos y/o animales (Southern y Sanford 1969; Ciceroni et al. 1994; Fukunaga et al. 1995; Cadavid y Barbour 1998; Scoles et al. 2001; Cutler 2009; Barbour y Schwan 2018). Los cuadros clínicos en población humana se caracterizan por episodios febriles que oscilan entre los 38.7 y 40ºC, con duración de entre 3 y 5 días, a intervalos de una a dos semanas entre sí. Los síntomas iniciales incluyen escalofríos, taquicardia, cefalea y dolor muscular. Los síntomas tardíos pueden incluir ictericia (entre episodios febriles), hepatomegalia y esplenomegalia. El tratamiento incluye el suministro de antibióticos como tetraciclina o doxiciclina por cinco o hasta 10 días (Pilz y Mooser 1936; Southern y Sanford 1969; Arshi et al. 2002; Rebaudet y Parola 2006; Dworkin et al. 2008; Sotelo Cruz y Valencia Mayoral 2012; Wagemakers et al. 2015; Talagrand-Reboul et al. 2018).

En casos donde el tratamiento no es suministrado durante los primeros 10 días puede ocasionar la muerte, la cual, generalmente ocurre posterior al episodio febril, caracterizado por temperaturas corporales bajas, transpiración y bradicardia (Rebaudet y Parola 2006; Dworkin et al. 2008; Talagrand-Reboul et al. 2018). Si se administra el tratamiento en tiempo y forma, la letalidad se reduce a 1 y 3% (Southern y Sanford 1969; Johnson y Golightly 2000; Wagemakers et al. 2015). Pobreza, falta de higiene y hambruna, son factores de riesgo que comúnmente se asocian con altas tasas de infección de fiebre recurrente, con una letalidad entre el 10% y el 15% (Felsenfeld 1965; Southern y Sanford 1969; Johnson y Golightly 2000; Rebaudet y Parola 2006).

En México, el estudio de este grupo es muy antiguo pero escaso y hasta el momento, sólo se han reportado tres trabajos en animales, específicamente en garrapatas, *B. parkeri*, *B. turicatae*, *B. mazzottii* y *B. dugesii* en *Ornithodoros parkeri*, *Ornithodoros talaje* y *Ornithodoros turicata*, respectivamente (Brumpt et al. 1939; Mazzotti, 1949; Davis, 1956). Estos reportes, a su vez, corresponden a las

descripciones formales de dichas especies del género *Borrelia*. La presencia de estas especies de *Borrelia,* se reportaron en los estados de Aguascalientes, Guanajuato y San Luis Potosí (*B. turicatae*) y Coahuila [*B. dugesii*] (Brumpt et al. 1939; Mazzotti, 1949). Hasta el momento, no existen reportes de infección en animales silvestres ni de compañía (Colunga-Salas et al. enviado).

Con respecto a los caso humanos, únicamente se han reportado cinco casos en el periodo comprendido entre el año 1936 y 2019. Cuatro de los casos humanos registrados, se diagnosticaron mediante microscopía y validados por el cuadro clínico (Pilz y Mooser 1936; Sotelo Cruz y Valencia Mayoral 2012), y uno mediante serología a partir de proteínas recombinantes de un cultivo de *B. turicatae* (Vázquez-Guerrero et al. 2019), los cuales fueron reportados en Aguascalientes y Sonora (Pilz y Mooser 1936; Sotelo Cruz y Valencia Mayoral 2012; Vázquez-Guerrero et al. 2019).

- *Borreliosis de Lyme*

Este último grupo está conformado por 21 especies válidas, las cuales conforman un grupo monofilético (Marconi y Garon 1992; Margos et al. 2011, 2017; Panetta et al. 2017). Este grupo es el más estudiado a nivel mundial ya que se incluye el agente causal de la enfermedad de Lyme, *Borrelia burgdorferi* s.l. (Kurtenbach et al. 2006; Schotthoefer y Frost 2015; Margos et al. 2017).

El ciclo enzoótico de las especies de este grupo, es en realidad una red de interacciones complejas que involucran vectores del género *Ixodes*, así como un gran espectro de vertebrados silvestres como reptiles, aves y mamíferos (Barbour y Hayes 1986; Scoles et al. 2001), como hospederos secundarios y definitivos.

Los vectores que se han reconocido para este grupo de borrelias, son especies de garrapatas duras del género *Ixodes*, *I. scapularis, I. pacificus*, *I. ricinus*, *I. minor*, *I. dentatus*, *I. jellisonii*, *I. spinipalpis* e *I. scapularis* (Güner et al. 2004).

Los principales hospederos vertebrados de este grupo de bacterias, son mamíferos pequeños como roedores y musarañas de los géneros *Apodemus*, *Blarina*, *Peromyscus*, *Sciurus*, *Sorex* y *Tamias*, así como el venado cola blanca,

Odocoileus virginianus (Burkot et al. 2000; Huegli et al. 2002; Kurtenbach et al. 2006; Schotthoefer y Frost 2015). Sin embargo, también hay especies de *Borrelia* que se han registrado en aves (*e.g. B. garinii* y *B. valaisiana*) y reptiles (Huegli et al. 2002; Kurtenbach et al. 2006; Pablo Colunga-Salas et al. 2018).

Las especies de importancia médica son *B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, *B. bavariensis*, *B. americana, B. andersonii, B. bissettii, B. spielmanii, B. valaisiana, B. lusitaniae* y *B. mayonii* (Kurtenbach et al. 2006; Schotthoefer y Frost 2015; Steere et al. 2016; Margos et al. 2017). Este grupo de borrelias causan un cuadro clínico similar entre sí, el cual no es letal y suele caracterizarse en una etapa temprana (primeros 15 días posterior a la infección) en el que un eritema migratorio es el signo más característico, acompañado con fiebres, mialgias, artralgias y escalofríos (Burgdorfer et al. 1982; Massarotti et al. 1992; Klempner et al. 2001; Shapiro, 2014; Steere et al. 2016).

Los órganos blanco son bazo, hígado, en los que se ha reportado espleno y hepatomegalias (Cimmino et al. 1989; Duray, 1989), riñón, hígado, músculo esquelético, vejiga, sistema nervioso (cerebro y médula espinal) y corazón, sin embargo, la colonización a estos últimos dos órganos ocurre durante la etapa de diseminación tardía [después de los 30 días de la infección] (Cadavid y Barbour 1998; Shapiro 2014; Steere et al. 2016; Margos et al. 2017).

Específicamente en México, la borreliosis de Lyme es una enfermedad cuya importancia ha aumentado recientemente, sin embargo, su estudio ha sido ocasional y pocos han sido sistemáticos (Colunga-Salas et al. enviado). Desde 1991 hasta el 2020, año en que se reportó el último caso humano, se han registrado 399 casos, los cuales se distribuyen en los estados de Coahuila, Estado de México, Morelos, Nuevo León, Quintana Roo, Sinaloa, Tamaulipas, Veracruz, Yucatán y en la Ciudad de México (Maradiaga-Ceceña et al. 1991; Vargas 1993; Arroyave y Tamez-González 1994; Salinas-Meléndez et al. 1995; Gordillo et al. 1999; Gordillo-Pérez et al. 2003, 2017; Skinner-Taylor et al. 2007, 2016; Vargas et al. 2007; Gordillo-Pérez y Solórzano-Santos 2010; CENAVECE 2013; Macari-Jorge et al. 2017; García-Frade-Ruiz 2018; Garcia-Toribio 2018).

En su mayoría, los casos humanos reportados en México han sido mediante

serología, sin embargo, en algunos de esos casos, se han utilizado kits de Europa y Estados Unidos con antígenos de cepas que circulan en dichos territorios, por lo que no se pueden considerar como casos confirmados. Hasta el momento, sólo se han registrado tres casos confirmados a partir de PCR (Salinas-Meléndez et al. 1995; Vargas et al. 2007), sin embargo, no existen secuencias disponibles.

En cuanto a trabajos con fauna silvestre, desde 1995 hasta 2019 se han registrado 399 reportes de *Borrelia* sp., en 12 especies de garrapatas de los géneros *Amblyomma*, *Dermacentor*, *Ixodes* y *Rhipichepalus*, así como en seis especies de mamíferos silvestres, caballos, perros y animales peridomésticos (*Rattus rattus* y *Mus musculus*, rata y ratón, respectivamente). Estos reportes se distribuyen en Baja California, Coahuila, Jalisco, Estado de México, Michoacán, Nuevo León, Tamaulipas y Yucatán (Salinas-Meléndez et al. 1995; Martinez et al. 1999; Salinas-Meléndez et al. 1999; Tinoco-Gracia et al. 2007; Vargas et al. 2007; Gordillo-Pérez et al. 2009; Tinoco-Gracia et al. 2009; Salinas-Melendez et al. 2011; Guadalupe Gordillo-Pérez et al. 2012; Galaviz-Silva et al. 2013; Feria-Arroyo et al. 2014; Solís-Hernández et al. 2016; Movilla et al. 2016; Solís-Hernández et al. 2018; López-Pérez et al. 2019).

La única especie que se ha confirmado su presencia en México mediante métodos moleculares es *B. burgdorferi* s.s. (López-Pérez et al. 2019), sin embargo, dada la presencia de los vectores y las condiciones climáticas, es probable que estén circulando otras especies pertenecientes a este grupo.

- *"Una Salud"*

Este enfoque tiene como como principal objetivo entender las relaciones entre los patógenos, la fauna silvestre y especies intra y peridomiciliadas, así como de la población humana (Lebel 2002; Conti y Rabinowitz 2011; Zinsstag et al. 2011; Calistri et al. 2013). Este enfoque reconoce que el cuidado de la salud de la población humana, así como la de los animales en la misma comunidad, se beneficia cuando hay colaboración y comunicación entre profesionales de la salud humana y animal (American Medical Veterinary Association 2008; Conti y

Rabinowitz 2011; Dantas-Torres et al. 2012). Así mismo, con este enfoque se busca conocer el riesgo relacionado con las enfermedades infecciosas emergentes, especialmente en zonas donde las civilizaciones humanas han invadido ecosistemas silvestres (Kahn 2006; Dantas-Torres et al. 2012; Cantas y Suer 2014). Conti y Rabinowitz, (2011) explican que los beneficios potenciales de este enfoque incluyen la mejora del diagnóstico y la prevención de enfermedades transmitidas entre animales y personas, la detección temprana de peligros potenciales para la salud ambiental y humana, así como mejorar la satisfacción del paciente.

Dentro de este mismo enfoque, se propone el uso de fauna silvestre como unidades de vigilancia centinela como recurso necesario para proporcionar información crítica sobre la incidencia de la enfermedad, con el fin de poder tomar decisiones para controlar y prevenir enfermedades futuras en población humana, así como evitar o detener la propagación de patógenos (Kahn, 2006; American Medical Veterinary Association, 2008; Woolhouse, 2008; Calistri et al. 2013; Rabinowitz et al. 2013). Finalmente, este tipo de estudios multidisciplinarios pueden ser útiles para comprender patrones de diversidad genética y evolutivos, así como el efecto del cambio climático en la expansión y aparición de enfermedades transmitidas por vector en regiones nuevas, con la finalidad de poder tomar las medidas políticas y de contención necesarias (Conti y Rabinowitz 2011; Rabinowitz et al. 2013; Cantas y Suer 2014).

- *México*

El estudio de la Borreliosis en México se remonta a los primeros casos endémicos de Fiebre recurrente reportados en el estado de Aguascalientes (Pilz and Mooser, 1936), seguidos de la descripción de *Borrelia turicatae* en los estados de Aguascalientes, Guanajuato y San Luis Potosí (Brumpt et al. 1939), *Borrelia duguesi* en el estado de Coahuila (Mazzotti, 1949) y de *Borrelia mazzottii* (Davis, 1956). Después de un periodo de inactividad en el estudio de la borreliosis, a inicios de la década de 1990, se publica el primer estudio serológico de Enfermedad de Lyme en Sinaloa (Maradiaga-Ceceña et al. 1991). A partir de entonces, se han

realizado diversos estudios de *Borrelia* en animales tanto silvestres como domésticos en México, en los estados de Baja California, Chihuahua, Coahuila, Guanajuato, Jalisco, Ciudad de México, Michoacán, Nuevo León, Tamaulipas y Yucatán (Tinoco-Gracia et al. 2007; Vargas et al. 2007; Gordillo-Pérez et al. 2012; Solís-Hernández et al. 2016; López-Pérez et al. 2019).

Por otro lado, los estudios en población humana se han centrado en los estados de Aguascalientes, Coahuila, Estado de México, Ciudad de México, Morelos, Nuevo León, Quintana Roo, Sinaloa, Tamaulipas, Veracruz y Yucatán (Pilz and Mooser, 1936; Maradiaga-Ceceña et al. 1991; Gordillo et al. 1999; Gordillo-Pérez et al. 2007; Macari-Jorge et al. 2017; García-Frade-Ruiz, 2018; Guevara-Valmaña et al. 2019; Vázquez-Guerrero et al. 2019; Rodríguez-García et al. 2020). Sin embargo, la mayoría de los estudios son serológicos, lo cual no permite conocer la especie de *Borrelia* presente, por lo que hacen falta estudios confirmatorios para conocer las especies presentes.

Hasta el momento, sólo se ha reportado la presencia del grupo de BL y de FR en México, de los cuales, el grupo de Borreliosis de Lyme es el más estudiado. De este grupo, se sospecha de la presencia de *B. afzelii* y *B. garinii* en México, sin embargo, las últimas dos especies únicamente se han reportado en Europa y Asia (Baranton et al. 1992; Merle et al. 1993), por lo que si el registro es correcto, probablemente sean casos importados. El único reporte confirmatorio para este género, mediante técnicas de biología molecular, es *B. burgdorferi* s.s. en *Ixodes kingi* de Janos, Chihuahua (López-Pérez et al. 2019), sin embargo, considerando que los vectores de esta especie se distribuyen en zonas altas con climas fríos, es necesario comprobar la transmisión local en dicha zona, por lo que un estudio de diversidad genética podría ayudar a esclarecer la zona de origen probable de dicha infección.

En cuanto a los registros de garrapatas en México, la cuenca del Golfo de México se ha reportado como una zona de alta incidencia de enfermedades infecciosas (Krasnov et al. 2004; Morens y Fauci 2013; Iovine et al. 2015; Allen et al. 2017). Aunado a ello, se han registrado 26 especies del género *Ixodes* en 20 estados del Territorio Nacional, la mayor diversidad se localiza en las regiones de

la Sierra Madre Oriental, Sierra Madre Occidental, Sierra Madre del Sur y en el Cinturón Volcánico Trans-Mexicano (Guzmán-Cornejo y Robbins 2010). De igual forma, la zona del centro y sur del país resulta una zona importante para el estudio del grupo de fiebre recurrente, debido a que es una zona donde se han registrado especies de garrapatas blandas del género *Ornithodoros* (Talagrand-Reboul et al. 2018; Guzmán-Cornejo et al. 2019).

Por otro lado, los géneros de garrapatas *Amblyomma* y *Dermacentor*, son géneros ampliamente distribuidos en México (Guzmán-Cornejo et al. 2011, 2016). En la franja del centro del país, se han registrado 10 especies del género *Amblyomma*, incluyendo los estados de México, Puebla, Guerrero y Veracruz (Guzmán-Cornejo et al. 2011). Mientras que del género *Dermacentor*, se han registrado cinco especies en la región del centro del país (Guzmán-Cornejo et al. 2016).

En lo que respecta con los hospederos, México presenta una alta riqueza de mamíferos, anfibios y reptiles (Ceballos y Oliva 2005; Flores-Villela y García-Vázquez 2014; Parra-Olea et al. 2014). La zona con mayor diversidad de anfibios y reptiles es la zona de Veracruz, mientras que la zona del centro presenta un alto índice de endemismos (Flores-Villela y García-Vázquez 2014; Parra-Olea et al. 2014). En el caso de mamíferos, tanto el orden Rodentia, como el orden Chiroptera son los más diversos y con la distribución más amplia a lo largo del Territorio Nacional, mientras que el venado cola blanca ha incrementado sus poblaciones dentro de su área nativa [desde la península de Yucatán, hasta Coahuila] (Ceballos y Oliva 2005).

Geográficamente, la Faja Volcánica Transmexicana, el cual representa la zona central del país, es la menos estudiada para la presencia del género *Borrelia*, probablemente por lo poco accessible de la zona. Sin embargo, ambientalmente, esta zona se caracteriza por una gran diversidad de ecosistemas, desde bosques mesófilos, hasta zonas cálidas, lo cual permite que exista una amplia diversidad de vertebrados terrestres (Ceballos and Oliva, 2005; Flores-Villela and García-Vázquez, 2014; Marines-Macías et al. 2018). Además, es en la zona central del país, donde se concentra la mayor densidad poblacional (INEGI, 2010).

Dada la presencia de *B. duguesi, B. mazzottii, B. turicatae, B. burgdorferi* s.s. en México, así como la presencia histórica de garrapatas potencialmente vectores para Borrelia reportados en los estados de Guerrero, Estado de México, Puebla y Veracruz, el objetivo general de este trabajo es analizar el panorama epidemiológico del género *Borrelia* en fauna silvestre de estos estados. Y como objetivos particulares:

1. Identificar molecularmente las especies de *Borrelia* presentes en vertebrados silvestres y sus garrapatas de la zona centro de México.
2. Establecer la prevalencia de las especies de *Borrelia* presentes en la zona de estudio por grupo de vertebrados terrestres de la zona central de México.
3. Evaluar la diversidad genética del agente etiólogico de la Enfermedad de Lyme.

CAPÍTULO I. Molecular detection of the reptile-associated *Borrelia* group in *Amblyomma dissimile* from Mexico (Medical and Veterinary Entomology, 2020; doi: 10.1111/mve.12478)

Medical and Veterinary Entomology

Medical and Veterinary Entomology (2020), doi: 10.1111/mve.12478

SHORT COMMUNICATION

Molecular detection of the reptile-associated *Borrelia* group in *Amblyomma dissimile*, Mexico

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Abstract. Recently, thefi rst record of *Borrelia* associated with reptiles in Mexico was published; however, no studies have been done to assess the role of Mexican ticks as potential vectors of this *Borrelia* group. *Amblyomma dissimile* is a hard tick mainly associated with amphibians and reptiles in this country. The aim of this study was to evaluate the presence of *Borrelia* in *A. dissimile* from Mexico. We collected 60 *A. dissimile* individuals attached to 16 *Rhinella horribilis.* DNA was extracted and all specimens were screened individually for *Borrelia* by amplification of a fragment of the 16S rDNA and an additional fragment of the *flagellin* gene. Five ticks were positive for *Borrelia*, DNA sequences corresponded to *Borrelia* sp. and group with sequences of the reptile-associated *Borrelia* group. This is the first report of *Borrelia* in *A. dissimile* and the second report of the reptile-associated *Borrelia* group in Mexico. This study also highlights the importance of this tick species as potential vector of this group.

Key words. *Borrelia turcica*, cane toad, hard tick, pathogen, amphibian.

The genus *Borrelia* comprises several Gram-negative spirochetal species, some of these species have been isolated and recorded in several hard tick species associated with reptiles worldwide. This monophyletic group has been mainly detected in Eastern Europe, Australia, Asia, Middle East and America, in both reptiles and their associated ticks. Despite the lack of studies on the role of ticks as vectors in this group, natural transmission of the reptile-associated *Borrelia* (BAR) group has been suspected in hard ticks of the genera *Ixodes*, *Hyalomma*, *Bothriocroton* and *Amblyomma*, where infections in nymphs have been detected. Thus, these tick species are considered as potential vectors of this *Borrelia* group (Güner *et al.*, 2003; Kalmár *et al.*, 2015).

In Mexico, there is a single study done with *Borrelia* in reptiles, conducted in several species from the coastal state of Veracruz, where the presence of DNA from a member of the reptile-associated *Borrelia* group was recorded. This was closely related with isolates from *Amblyomma trimaculatum* and *Amblyomma varanense* from Japan and Thailand, respectively (Takano *et al.*, 2010; Trinachartvanit *et al.*, 2016; Morales-Diaz *et al.*, 2020). Additionally, other isolates of this *Borrelia* group have been recovered from nine species of the genus *Amblyomma* worldwide, which highlights the importance of this genus as vector of this *Borrelia* group (Güner *et al.*, 2004; Takano *et al.*, 2010; Kalmár *et al.*, 2015; Cicuttin *et al.*, 2019).

From the 26 species of the genus *Amblyomma* recorded in Mexico, *Amblyomma dissimile* Koch, 1844, stands out as an important hard tick, recorded mainly in association with amphibians and reptiles, although it has also been reported incidentally infesting mammals, including humans (Guzmán-Cornejo *et al.*, 2011). This tick species has a Nearctic and Neotropical distribution, and has been recorded in different ecoregions (Guzmán-Cornejo *et al.*, 2011). In Mexico, this tick species is mainly associated with the Mesoamerican cane toad,

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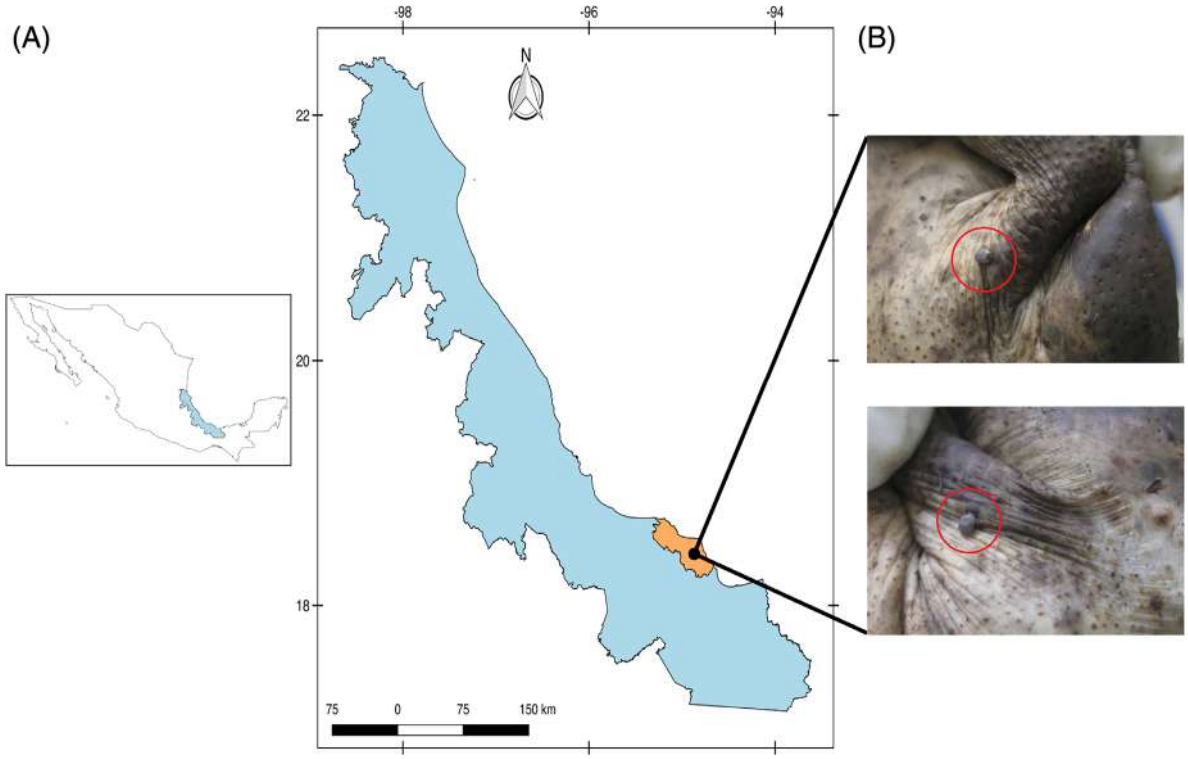

Fig. 1. Sampling done during this study. (A) Sampling site in the state of Veracruz, Mexico; Light blue: the state of Veracruz; Orange: Poza de los Enanos locality. (B) *Amblyomma dissimile* engorged attached to the underarms of *Rhinella horribilis*. [Colourfi gure can be viewed at wileyonlinelibrary.com].

Rhinella horribilis, which is widely distributed in the Pacific and Gulf Coasts near to the margins of water sources and human settlements (Vega-Trejo *et al.*, 2013).

Only few studies have evaluated the diversity of bacterial symbionts and pathogens associated with *A. dissimile*, most of them focused on members of the order Rickettsiales, such as *Rickettsia* and *Anaplasma*, where natural infections with *Anaplasma* sp., *Rickettsia bellii* and *Candidatus* Rickettsia colombianensi (Miranda *et al.*, 2012; Ogrzewalska *et al.*, 2019) have been reported. For this reason, the aim of the current study was to screen *A. dissimile* from Mexico for the presence of *Borrelia* DNA.

Thirty *R. horribilis* toads were manually collected from water sources in Poza de los Enanos (18°30′22″N, 95°02′02″W), close to the Estación de Biología Tropical 'Los Tuxtlas', UNAM, Veracruz, Mexico, during October and November, 2015 (Fig. 1A). After visual inspection of the hosts (Fig. 1B), ticks were removed from the toads, fixed and conserved individually in tubes with 96% ethanol, after this, all hosts were released. Handling of vertebrate hosts was done following all requirements specified in the General Wildlife Federal Law of Mexico (Ley General de Vida Silvestre) under collection permit SGPA/DGVS/09346/16 FAUT-0317 (issued to Leticia M. Ochoa Ochoa), and following the U.K. Animals (Scientific Procedures) Act, 1986.

All collected ticks were identified morphologically as *A. dissimile* using specialized taxonomic keys (Guzmán-Cornejo *et al.*, 2011), and thereafter deposited in the Collection of 'Centro de Medicina Tropical, Facultad de Medicina, UNAM' (CMTFM). After morphological identification, DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen, California, USA) cutting the ticks in the middle and then following manufacturer's guidelines. Larvae and damaged ticks were identified molecularly through amplification of partial DNA sequences (∼440 bp) of the mitochondrial 16S rDNA, using the protocols of Norris *et al.* (Norris *et al.*, 1996). The reaction mixture consisted of 12.5 µL PCR master mix solution (Qiagen), 100 pg. of each primer, 6.5 µL nuclease-free water and 200 ng DNA in a final volume of 25 µL.

To identify the presence of *Borrelia*, a fragment of ∼300 bp of the 16S rDNA gene (Skotarczak *et al.*, 2005) was amplified. Positive samples were further tested for a ∼300 bp fragment of the *flagellin* gene (Assous *et al.*, 2006) following the PCR conditions specified by each author. In all reactions, we included positive (DNA from *Borrelia burgdorferi* s.s. from *Ixodes kingi*, GenBank: MK370994) and negative controls. The PCR products were visualized in 2% agarose gels on the ODYSSEY CLx Imaging System (LI-COR Biosciences), and positive PCR products were sequenced at Macrogen Inc., Korea and at the Laboratorio de Biología Molecular y de la Salud, Instituto de Biología, UNAM. Sequences obtained in this study were deposited in GenBank with the following accession numbers: 16S gene fragment of *A. dissimile*: KY389389-KY389392; 16S gene fragment of *Borrelia*: KY389373, MT192220-MT192223; *flagellin*: MT237183-MT237187.

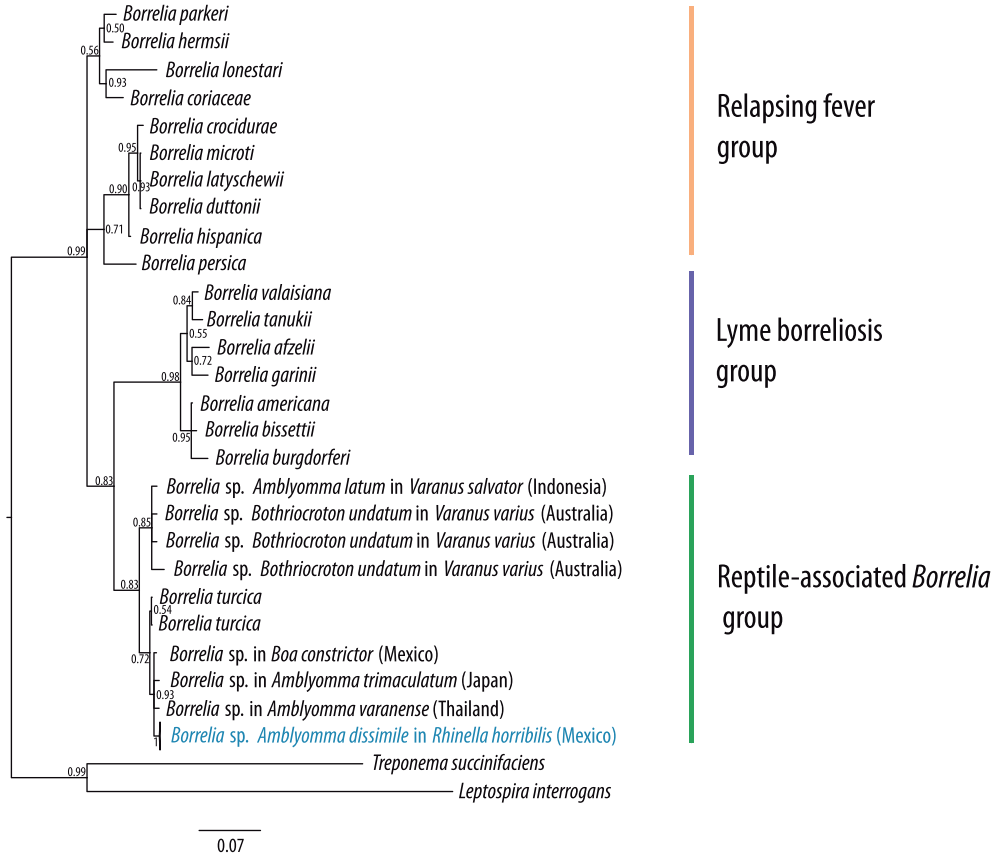

Fig. 2. Phylogenetic reconstruction by codons alignment (16S rDNA + flagellin) for the genus *Borrelia*. The phylogenetic relationships were obtained by Bayesian inference (BI) based on Kimura with gamma distribution (16S complete gene; *flagellin* codon 3), Hasegawa-Kishino-Yano with gamma distribution (*flagellin* codon 1), and General Time Reversible with invariants sites (*flagellin* codon 2) for a total of 885 bp. Posterior probabilities >0.5 are indicated at nodes. Scale bar indicates nucleotide substitutions per site. Sequences obtained in this study (5) are shown in a blue collapsed branch. [Colourfi gure can be viewed at wileyonlinelibrary.com].

Sequences were manually edited using FinchTV 1.5 (Geospiza, Inc., Seattle, WA, U.S.A.), after that, a multiple alignment was performed in Mega X and aligned with MUSCLE, using all available sequences of the reptile-associated *Borrelia* group with other 17 species of the genus *Borrelia* to create a concatenated alignment; *Treponema succinifaciens* and *Leptospira interrogans* were used as outgroups. The best substitution model and partition scheme were determined in PartitionFinder 2. A Bayesian analysis in MrBayes 3.2.3, using the MCMC algorithm and the substitution model previously calculated, was performed considering the specifications used by Morales-Diaz *et al.* (2020).

We found 16 *R. horribilis* parasitized (16/30 = 53%) by 60 *A. dissimile* ticks (10 ♀, 15 ♂, 13 N and 22 L). Sequences obtained of the mitochondrial 16S rRNA gene of a female, male, nymph and larva exhibited a similarity of 99–100% between them, as well as with sequences of *A. dissimile* available in GenBank.

Onlyfi ve ticks tested positive (5/60 = 8.3%) for both *Borrelia* genes, 16S rDNA and *flagellin* [15% N (2/13), 13% ♂(2/15) and 10%♀(1/10)]. All sequences exhibited a 100% similarity between them per gene. Sequences from the 16S rDNA *Borrelia* fragment were 93% similar with sequences of *Borrelia* sp. recovered from *Boa constrictor* from Mexico (MN610439-MN610443) and with sequences of *Borrelia turcica* type strain IST7 from *Hyalomma aegyptium* (CP028884). On the other hand, sequences from the *flagellin* fragment, exhibited a 99% similarity with the respective sequences of *Borrelia* sp. also recovered from *B. constrictor* from Mexico (MT066211-MT066215) and the same *B. turcica* type strain IST7 (CP028884).

With these findings, we provide the first molecular evidence on the presence of *Borrelia* in *A. dissimile* associated with *R. horribilis* for Mexico. Considering the similarity of the sequences retrieved in this study and those from *B. constrictor*, which had been collected in a site of Veracruz (Morales-Diaz *et al.*, 2020) close to where *A. dissimile* of this study were obtained, this makes the tropical region of Veracruz an important area for the study of the reptile-associated *Borrelia* group.

Phylogenetically, the obtained sequences grouped in a monophyletic clade with sequences reported from Mexico, Japan and Thailand with a high branch support, all of them recorded from snakes (Fig. 2). Given the degree of similarity between the other lineages and their phylogenetic position, the obtained sequences represent a putative new lineage of *Borrelia*.

The two phylogenetically close sequences of *Borrelia* of ticks from Japan and Thailand (Fig. 2) recovered from *A. trimaculatum* and *A. varanense*, respectively, were only detected in adult ticks (Takano *et al.*, 2010; Trinachartvanit *et al.*, 2016). Until now, only *B. turcica* has been detected in nymphs of *H. aegyptium* (Takano *et al.*, 2010; Kalmár *et al.*, 2015). Therefore, this study represents the first report of infection of a BAR group lineage other than *B. turcica* in nymphs.

The transstadial transmission of *B. turcica* in *H. aegyptium* (the only known competent vector) is well known. This is one of the most important characteristics for considering a tick species as a vector for this bacterial genus (Kalmár *et al.*, 2015). We now report two *A. dissimile* nymphs and three adults naturally infected with *Borrelia* DNA. For this reason, the potential role of *A. dissimile* as a vector of a putative new *Borrelia* lineage cannot be discarded. Taking together the previous record on the reptile-associated *Borrelia* group of Mexico found in *B. constrictor* (Morales-Diaz *et al.*, 2020) and our current report, it seems feasible that *A. dissimile* could be acting as a vector, since infestations of snakes, including *B. constrictor*, with this hard tick are common in Mexico (Guzmán-Cornejo *et al.*, 2011). However, to confirm this hypothesis, both isolation of these bacteria from *A. dissimile* and transmission studies need to be done to confirm its vector role.

Finally, the role of the cane toad as a host of *Borrelia* remains unclear, since there is a lack of information on the infection of amphibians by this bacterium genus. To dilucidated the role of toads and frogs in the sylvatic cycle of *Borrelia*, further ecological analysis focused on the potential distribution areas of *A. dissimile*, which are warranted to direct the studies and assess the species diversity of the BAR group in Mexico.

In conclusion, *A. dissimile* was found to be naturally infected with a novel putative lineage of the reptile-associated *Borrelia* group. This is a novel association in America and also represents the second record of this *Borrelia* group in Veracruz, Mexico. These results highlight the tropical region of Veracruz as a potential hot-spot for the reptile-associated *Borrelia* group in Mexico. Additionally,fi nding naturally *Borrelia*-infected nymphs opens the possibility to consider *A. dissimile* as a potential vector for *Borrelia* in reptile-lineages of Mexico. However, further studies are needed in order to dilucidated this role in the *Borrelia* sylvatic cycle.

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Author contribution

Pablo Colunga-Salas: Conceptualization, Methodology, Formal analyses, Visualization, Writing – Review & Editing; Sokani Sánchez-Montes: Visualization, Methodology, Review & Editing; Leticia M. Ochoa-Ochoa: Supervision, Review & Editing, Visualization, Resources, Project administration; Estefania Grostieta: Visualization, Review & Editing; Ingeborg Becker: Supervision, Visualization, Resources, Review & Editing, Funding acquisition, Project administration.

Data availability statement

The data that support the results of this study are available in GenBank, under accession numbers for Ticks' 16S: KY389389-KY389392; Borrelia's 16S: KY389373, MT192220- MT192223; flagellin: MT237183-MT237187.

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CAPÍTULO II. *Borrelia* in Neotropical bats: detection of two new phylogenetical lineages (Ticks and Tick-borne Diseases, 2021; doi: 10.1016/j.ttbdis.2020.101642)

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Borrelia in neotropical bats: Detection of two new phylogenetic lineages

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ABSTRACT

The genus *Borrelia* encompasses 50 spirochetal species, several of which are pathogenic and have been detected in a wide range of mammals, especially rodents and cervids. Although the order Chiroptera is the second most diverse mammalian order, and borreliosis represents a human and veterinary health problem in endemic countries, few studies have previously reported infections of *Borrelia* in these flying mammals. For this reason, the aim of the present study was to detect the presence of, and to analyze the diversity of *Borrelia* species in several bat species from Mexico. A total of 69 bats belonging to 11 species were collected and molecular detection of *Borrelia* was performed by amplifying three genes using specific primers. Only five individuals of four bat species (*Saccopteryxbilineata*, *Choeroniscus godmani*, *Sturnira parvidens* and *Lasiurus cinereus*) tested positive for *Borrelia* DNA. We now show the first *Borrelia* record in Mexican bats from two different ecosystems, where previously several potential vector species of the genus *Ixodes* and *Ornithodoros* had been reported. The *Borrelia* sequences obtained from the bats revealed two new putative lineages, one from the relapsing fever group and the second one belonging to the *Borrelia burgdorferi* s.l. complex, both of which are related to zoonotic species. These results highlight the importance of bats as potential hosts of *Borrelia*, and the imperative need of active surveillance in flying mammals in order to understand their potential role in the life cycle of this bacteria genus.

1. Introduction

The genus *Borrelia* comprises several spirochetal Gram-negative bacteria, which are divided into four phylogenetic groups, the relapsing fever group, the reptile-associated *Borrelia*, the monotreme-associated *Borrelia* and the Lyme borreliosis or *Borrelia burgdorferi* s.l. complex (Loh et al., 2017; Margos et al., 2020; Takano et al., 2010; Talagrand-Reboul et al., 2018). They are transmitted by several species of soft ticks (Argasidae: *Ornithodoros* and *Argas*) and hard ticks (Ixodidae: *Amblyomma*, *Bothriocroton*, *Hyalomma*, *Haemaphysalis*, *Ixodes* and *Rhipicephalus*) (Furuno et al., 2017; Margos et al., 2018, 2011; Talagrand-Reboul et al., 2018).

Enzootic transmission cycles of *Borrelia* species include complex networks involving several vertebrate species, mainly mammals. Several members of the orders Rodentia and Artiodactyla are maintenance hosts for both the bacteria and ticks (Kurtenbach et al., 2006; Schotthoefer and Frost, 2015; Steere et al., 2016). Recently, bats have gained importance as hosts for *Borrelia* infections, mainly in South America. However, most of these studies only reported *Borrelia* in ticks attached to these mammals (Table 1). Even though ecological and phylogenetic aspects of bats are well-studied worldwide, little is known on *Borrelia* infections in these mammals. To date, only one fatal case by borreliosis in a *Pipistrellus* sp. bat from the United Kingdom has been recorded, yet more information is needed to determine the susceptibility of bats to *Borrelia* infections (Evans et al., 2009).

There are records of ~1412 bat species worldwide, of which, 9.2 % (130 species) are distributed in Mexico, mainly in the Neotropical region (Burgin et al., 2018; Ceballos and Oliva, 2005; Ramírez-Pulido et al., 2014; Taylor, 2019). This biogeographic region is heterogeneous, with an altitudinal gradient harboring different types of vegetation: tropical rainforest in the lowlands (<1000 m), cloud forest, oak forest and pine-oak forest at intermediate altitudes (1000–2000 m), and pine forest

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at higher altitudes (>2000 m) (Challenger, 1998). In these ecosystems, several species of bats are distributed in caves and shelters, such as old tree trunks, vegetation and old buildings, where these mammals are in close contact with ticks that inhabit these ecological niches (Keirans and Clifford, 1975; Sanchez-Montes et al., 2016).

Since Mexico is rich in bat species, in addition to the fact that *Borrelia* has been associated with these mammals and with their associated ticks in tropical and subtropical areas, the aim of this study was to identify the presence of *Borrelia* in bats from different ecosystems within the Neotropical region of Mexico, using molecular methods.

2. Material and methods

From 2014–2018 several collecting efforts were done (Table 2) in three different Mexican States of the Transmexican Volcanic Belt (Fig. 1). For identification of *Borrelia* sp. in bats, we captured 69 animals of 11 species. Bats were collected using mist nests, under permissions SGPA/DGVS/12142/12[-18] from the Ministry of Environment and Natural Resources [Secretaría del Medio Ambiente y Recursos Naturales]. Males and females without offspring or signs of pregnancy were included in the study. Pregnant females and those carrying offspring were released immediately.

Table 1
Records of the genus *Borrelia* associated with bats and their ticks. Systematic validation of bat species was based on the Mammal Diversity Database (Burgin et al., 2018). Tick validation was based on (Guglielmone et al., 2014). ND = not identified tick species.

Bat species	Tick species	*Borrelia* species	Country	Prevalence (positive/total indv)	Reference
Eptesicus fuscus	ND	Antibodies reactive to *Borrelia hermsii*	Forsyth, Atlanta, Geogia, USA	18 % (2/11)	(Reeves et al., 2006)
			Fulton, Atlanta, Georgia, USA	10 % (1/10)	(Reeves et al., 2006)
Miniopterus schreibersii	*Ixodes vespertilionis*	*Borrelia carolinensis*	Romania	50 % (1/2)	(Michalik et al., 2020)
		Borrelia afzelii	Romania	50 % (1/2)	(Michalik et al., 2020)
		Borrelia spielmanii	Romania	13 % (1/8)	(Michalik et al., 2020)
	Ixodes simplex	*Borrelia carolinensis*	Romania	13 % (1/8)	(Michalik et al., 2020)
		Borrelia afzelii	Romania	25 % (2/8)	(Michalik et al., 2020)
		Borrelia burgdorferi s.s.	Romania	13 % (1/8)	(Michalik et al., 2020)
		Borrelia lanei	Romania	25 % (2/8)	(Michalik et al., 2020)
		Borrelia afzelii/Borrelia valaisiana	Romania	13 % (1/8)	(Michalik et al., 2020)
Myotis brandtii	*Ixodes vespertilionis*	*Borrelia lanei*	Poland	100 % (1/1)	(Michalik et al., 2020)
Myotis bechsteinii	*Ixodes vespertilionis*	*Borrelia garinii/Borrelia lanei*	Poland	100 % (1/1)	(Michalik et al., 2020)
Myotis blythii	*Ixodes vespertilionis*	*Borrelia lanei*	Romania	100 % (1/1)	(Michalik et al., 2020)
Myotis daubentonii	*Ixodes ariadnae*	*Borrelia carolinensis*	Poland	100 % (1/1)	(Michalik et al., 2020)
	Ixodes ricinus	Related to *Borrelia garinii*	Poland	13 % (1/8)	(Michalik et al., 2020)
Myotis emarginatus	*Ixodes ariadnae*	*Borrelia spielmanii*	Poland	100 % (1/1)	(Michalik et al., 2020)
	Ixodes vespertilionis	*Borrelia spielmanii*	Poland	50 % (2/4)	(Michalik et al., 2020)
		Borrelia afzelii	Poland	25 % (1/4)	(Michalik et al., 2020)
		Borrelia lanei	Poland	25 % (1/4)	(Michalik et al., 2020)
Myotis myotis	*Ixodes ariadnae*	*Borrelia spielmanii*	Poland	48 % (12/25)	(Michalik et al., 2020)
		Borrelia afzelii	Poland	16 % (4/25)	(Michalik et al., 2020)
		Borrelia burgdorferi s.s.	Poland	4% (1/25)	(Michalik et al., 2020)
		Borrelia garinii	Poland	4% (1/25)	(Michalik et al., 2020)
		Borrelia lanei	Poland	4% (1/25)	(Michalik et al., 2020)
		Borrelia valaisiana	Poland	4% (1/25)	(Michalik et al., 2020)
		Borrelia carolinensis	Poland	20 % (4/25)	(Michalik et al., 2020)
	Ixodes vespertilionis	*Borrelia carolinensis*	Romania	100 % (1/1)	(Michalik et al., 2020)
	Ixodes ricinus	*Borrelia spielmanii*	Romania	100 % (1/1)	(Michalik et al., 2020)
Myotis mystacinus	*Ixodes ariadnae*	*Borrelia carolinensis*	Poland	100 % (1/1)	(Michalik et al., 2020)
Myotis nattereri	*Ixodes vespertilionis*	*Borrelia lanei*	Poland	100 % (1/1)	(Michalik et al., 2020)
Natalus tumidirostris	—	*Borrelia* sp.	Cueva Macaregua, Santander, Colombia	3% (1/34)	(Marinkelle and Grose, 1968)
Pipistrellus kuhli	ND	*cf. Borrelia spp.*[*]	Tunisia	100 % (1/1)	(Nicolle and Compte, 1905)
		Borrelia cf. recurrentis[*]	Tunisia	100 % (1/1)	(Nicolle and Compte, 1906)
Pipistrellus pipistrellus	*Argas vespertilionis*	Related to *B. burgdorferi* s.l.	United Kingdom	100 % (5/5)	(Hubbard et al., 1998)
Pipistrellus pipistrellus/ Scotophilus sp.	*Argas vespertilionis*	Related to *B. burgdorferi* s.l.	United Kingdom	100 % (8/8)	Hubbard et al., 1998)
Pipistrellus sp.	—	Related to *B. crocidurae, B. recurrentis* and *B. duttonii*	Mevagissy, England, United Kingdom	100 % (1/1)	(Evans et al., 2009)
Rhinolophus hipposideros	*Ixodes vespertilionis*	*Borrelia spielmanii*	Poland	14 % (2/14)	(Michalik et al., 2020)
		Borrelia carolinensis	Poland	7% (1/14)	(Michalik et al., 2020)
		Borrelia afzelii	Poland	21 % (3/14)	(Michalik et al., 2020)
		Borrelia burgdorferi s.s.	Poland	7% (1/14)	(Michalik et al., 2020)
		Borrelia garinii	Poland	29 % (4/14)	(Michalik et al., 2020)
ND	*Carios kelleyi*	Related to *B. parkeri* and *B. turicatae*	Jones Country, Iowa, USA	45 % (14/31)	(Schwan et al., 2009)
		Related to *B. turicatae* and *B. parkeri*	Bellevue and Sabula, Jackson Country, Iowa, USA	2% (3/121)	(Loftis et al., 2005)
		Related to *Borrelia turicatae* and *Borrelia parkeri*	Jones Country, Iowa, USA	NS	(Schwan et al., 2009)

[*] This species were later identified as *Borrelia* (*Spirochaeta*) *vespertilionis* by (Novy and Knapp, 1906).

Table 2
Sampled localities during this study.

State	Locality	Ecosystem	Coordinates	Date
Estado de Mexico	State Park "Picacho de Oro y Plata", Zacualpan (ZN)	Cloud forest	18° 43' N, 99° 46' W	March and April, 2014 May, 2017 April, 2018
Veracruz	Tropical Biology Station "Los Tuxtlas", San Andrés Tuxtla (LT)	Tropical rainforest	18°34′ N, 95°04′ W	October and November, 2015 May, 2016
	"Cueva de los Murciélagos", Sontecomapan (CM)	Tropical rainforest	18° 30' N, 95° 03′ W	May, 2016
	Laguna Escondida, San Andrés Tuxtla (LE)	Tropical rainforest	18° 35' N, 95° 06′ W	May, 2016
Puebla	Necaxa (NX)	Pine-oak forest	20° 12′ N, 98° 01′ W	August, 2018

All captured bats were handled and euthanized based on the guidelines of the American Society of Mammalogy for the Use of Wildlife Mammals in Research (Sikes, 2016). A necropsy was performed on each bat, extracting the spleen, kidney and liver. In order to avoid cross contamination between individuals, the surgical material was disinfected with 10 % chlorine and 96 % ethanol between each individual. Tissues were fixed individually in 96 % ethanol and frozen at -20 °C until processing in the laboratory. All bats were morphologically identified using specialized taxonomic keys (Medellín et al., 2008) and deposited at Colección de Mamíferos, Museo de Zoología "Alfonso L. Herrera", Facultad de Ciencias (MZFC), Universidad Nacional Autónoma de México.

DNA was extracted from all the samples using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). For initial screening of *Borrelia*, a fragment of ~300 bp of the 16S rDNA region (Skotarczak et al., 2005) was amplified. Positive samples were further tested using three other PCR protocols. These included testing for a ~794 bp fragment of the *osp*A gene (Bunikis et al., 2004), a ~280 bp fragment of the *flagellin* gene using the primers proposed by Zore et al. (1999) for the Lyme borreliosis group, and a ~300 bp fragment of the *flagellin* gene for the relapsing fever group (Assous et al., 2006). We performed the PCR thermal conditions specified by each author for each set of primers.

All reaction mixtures consisted of 12.5 µL GoTaq® Green Master Mix, 2X Promega Corporation (Madison, WI, USA), the pair of primers (100 ng each), 6.5 µL nuclease-free water and 50 ng DNA in a final volume of 25 µL. In all reactions, we included a negative (the same reaction mixture with water) and two positive controls (DNA from a *Borrelia burgdorferi* previously isolated from *Ixodes kingi* and from the reptile-associated *Borrelia* from *Amblyomma dissimile*; [Colunga-Salas et al., 2020b; López-Pérez et al., 2019]). PCR products were resolved in 2% agarose gels using SmartGlow™ Pre-Stain of Accuris Instruments (Edison, NJ, USA) and visualized by UV-transillumination. Amplicons of the expected sizes were submitted for their purification and sequencing at Laboratorio de Biodiversidad y la Salud, Instituto de Biología, Universidad Nacional Autónoma de México. The DNA sequences were edited and aligned using 4Peaks V1.8 (Nucleobytes B.V.) and MEGA 6 (Tamura et al., 2013), conducting visual inspections of all sequences. Obtained sequences were submitted to GenBank under the following accession numbers: MT039485-MT039487 and MT039499-MT039500 for 16S rDNA, MT040726-MT040730 for *flagellin* and MN909822-MN909823 for *osp*A.

In order to identify the *Borrelia* species obtained from the bats, sequences of these three genes from all species of the genus *Borrelia* (Margos et al., 2020) and of those previously obtained from bats, available in GenBank, were included in a concatenated alignment with MUSCLE (Edgar, 2004) in command line. The best partition scheme and substitution model of the concatenated data were calculated in PartitionFinder 2 (Lanfear et al., 2017), using *greedy* (Lanfear et al., 2012) and PhyML (Guindon et al., 2010) algorithms.

To analyze the phylogenetic position within the genus *Borrelia* and corroborate the identity of the recovered sequences, a Bayesian analysis in MrBayes 3.2.3 (Ronquist et al., 2012) was performed, using the MCMC algorithm and the substitution model, previously calculated in PartitionFinder 2. Three hot and 1 cold chain were used in two independent runs of 10 million generations and sampling data every 1000 iterations. The final topology was obtained using a majority consensus of 50 % of all trees, considering a burn-in of 20 %. We finally checked the convergence of the results and a good sampling (ESS > 200) in Tracer 1.7.1 (Rambaut et al., 2018). To corroborate whether the obtained sequences were genetic variants or new lineages, we calculated Nei's genetic distances with the *adegenet* package (Jombart et al., 2020) in R, considering the substitution models previously calculated. Nei's distances for the *osp*A gene was not calculated, since no available sequence for the amplified region could be obtained for *B. miyamotoi*.

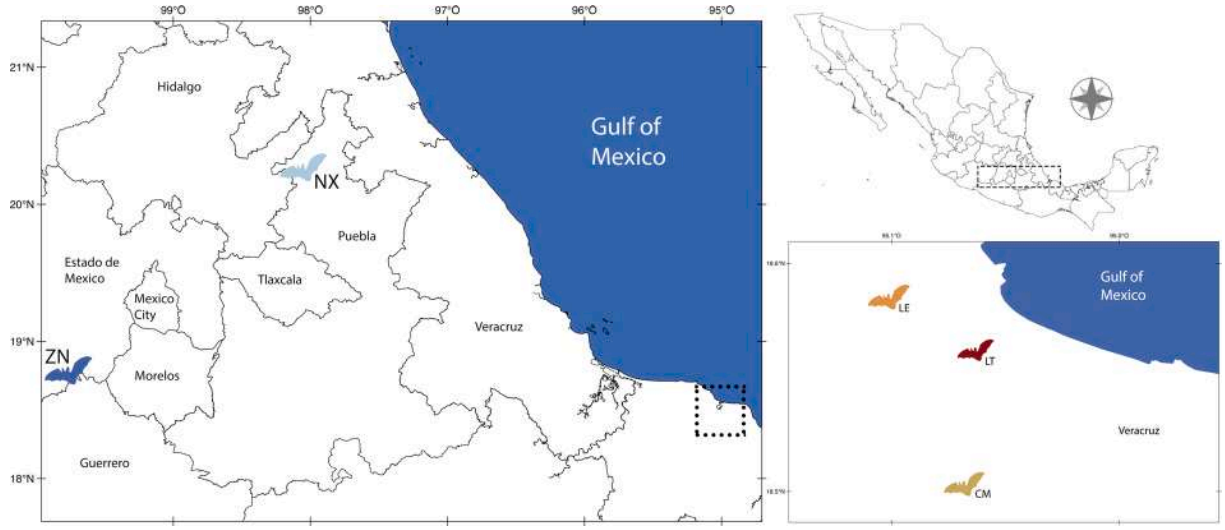

Fig. 1. Sampling sites along the Transmexican Volcanic Belt. Sampling sites: CM = Cueva de los Murciélagos; LE = Laguna Escondida; LT = Los Tuxtlas; NX = Necaxa; ZN = Zacualpan.

3. Results

Eleven species of bats belonging to four families, Emballonuridae (12 individuals), Mormoopidae (16), Phyllostomidae (40) and Vespertilionidae (1) were collected. Phyllostomidae was the most diverse family, with six species, whereas the families Emballonuridae and Vespertilionidae showed the lowest diversity, with a single species each (Table 3). The localities where bats were collected varied: six individuals were recovered from Necaxa, Puebla (NX), while 12 individuals were collected in Laguna Escondida, Veracruz (LE) and in Los Tuxtlas, Veracruz (LT); 19 individuals in Cueva de los Murciélagos, Veracruz (CM) and 20 individuals in Zacualpan, Mexico [ZN] (Table 3).

The initial screening of the 16S rDNA gene showed *Borrelia* DNA in five individuals of four species (2 *S. bilineata*, 1 *C. godmani*, 1 *L. cinereus* and 1 *S. parvidens*) from three families: Emballonuridae, Phyllostomidae and Vespertillionidae. None of the Mormoopidae individuals were positive for *Borrelia*. Of all the samples that tested positive, only the individual of *S. parvidens* and the only specimen of *L. cinereus* were positive for both the *osp*A gene and the ~280 bp fragment of the *flagellin* gene. Samples of *S. bilineata* and *C. godmani* were positive only for the ~300 bp fragment of the *flagellin* gen (Table 3).

The phylogenetic analysis showed that the sequences obtained in this study grouped in two distinct phylogenetic groups: one in the Lyme borreliosis (LB) group and other in the relapsing fever (RF) group (Fig. 2). The LB group contains sequences obtained from the bats *L. cinereus* and *S. parvidens*, whereas the RF group includes sequences obtained from the bats *C. godmani* and *S. bilineata*. It is noteworthy, that the *Borrrelia* sequences obtained from *L. cinereus* and *S. parvidens* bats were 100 % identical with those of the *Borrelia turdi* strain Ya501 (NR_025873) for the 16S rDNA, and 97.85 % identical with *B. turdi* (D82849) for the *flagellin* gene. These sequences were also 98.01 % identical with *B. turdi* retrieved from their vector in Korea, *Ixodes nipponensis* (AB016975) for the *osp*A gen.

The sequences from the RF group obtained from the bats *C. godmani* and *S. bilineata* were 100 % identical with *Borrelia miyamotoi* strain CZ-F1E (CP046389) for the fragment of the 16S rDNA region and 98.72 % identical with *B. miyamotoi* strain Yekat-18 (CP037471) for the *flagellin* gene.

For sequences obtained from *L. cinereus* and *S. parvidens*, the pairwise Nei`s distance with *B. miyamotoi* showed 0% difference in the 16S rDNA region but a 2% difference in the *flagellin* region. On the other hand, sequences retrieved from *S. bilineata* and *C. godmani* and compared with *B. turdi* showed 0% difference with regard to 16S rDNA and 3% for the *flagellin gene*.

Table 3
Detection of *Borrelia* DNA by PCR in Mexican bats. Detection of *Borrelia* DNA was based on 16S rDNA, *flagellin* and *osp*A (only for the Lyme borreliosis group) genes.

Bat species	Percentage of infection per site					
	ZN	LT	CM	LE	NX	Percentage of infection per species
Emballonuridae						
Saccopteryx bilineata	0	0	0	2/12	0	16.6%
Mormoopidae						
Pteronotus davyi	0	0	0/13	0	0	0%
Pteronotus parnelli	0	0	0/2	0	0	0%
Pteronotus personatus	0	0	0/1	0	0	0%
Phyllostomidae						
Artibeus jamaicensis	0/2	0/10	0	0	0	0%
Choeroniscus godmani	0	0	1/3	0	0	33.3%
Dermanura tolteca	0	0/1	0	0	0	0%
Platyrrhinus helleri	0	0/1	0	0	0	0%
Sturnira hondurensis	0/2	0	0	0	0	0%
Sturnira parvidens	1/15	0	0	0	0/6	4.7%
Vespertilionidae						
Lasciurus cinereus	1/1	0	0	0	0	100 %
TOTAL	**10 %**	**0%**	**5%**	**17 %**	**0%**	–

4. Discussion

This study is the first attempt to detect *Borrelia* infections in Mexican bats and now shows two putative novel *Borrelia* species in four bat species from two predominant ecosystems in Mexico: cloud forest and tropical rain forest. To the best of our knowledge, this is also the third report of *Borrelia* infections in bats (Evans et al., 2009; Marinkelle and Grose, 1968), since in the most of the previous studies, the genus *Borrelia* had only been detected in ticks associated with these flying mammals (Table 1). The prevalence of *Borrelia* in Mexican bats per locality (Table 3) was high, as compared to previous ecological studies of *Borrelia* infections in bats [3%] (Marinkelle and Grose, 1968). This suggests that the presence of the genus *Borrelia* could be widely distributed in Mexican bats, however, further studies must be done in order to elucidate the role of bats in the life cycle of the RF and LB groups.

Although the genus *Borrelia* has been studied throughout Mexico, doubts have arisen regarding the species, since most of the diagnostic studies were only done using serological methods, lacking confirmatory evidence (Colunga-Salas et al., 2020a, 2020b; Faccini-Martínez, 2019; Feria-Arroyo et al., 2014; Norris et al., 2015, 2014). Only four formerly named *Borrelia* species have so far been confirmed in Mexico: *Borrelia turicatae* (Brumpt et al., 1939), *Borrelia duguesii* (Mazzotti, 1949), *Borrelia mazzottii* (Davis, 1956) and *Borrelia burgdorferi* s.s. (Colunga-Salas et al., 2020c; López-Pérez et al., 2019). The first three were originally isolated from soft ticks of the genus *Ornithodoros* and then formerly described, whereas *B. burgdorferi* s.s. was confirmed by molecular methods. Taken together, our results are now representing the first confirmatory record of the genus *Borrelia* in flying wild Mexican mammals.

According to the phylogenetic analyses, the sequences from Mexican bats grouped in two different clades, one belonging to the RF group and the second to the LB group. The lineage related to the RF group is phylogenetically related with *B. miyamotoi*, which is widely distributed along the Holarctic region, from Japan to the United States, where it is considered a major public health problem producing relapsing fever-like symptoms (Crowder et al., 2014; Geller et al., 2012; Krause et al., 2015; Sato et al., 2014). Additionally, this monophyletic clade is related with two other pathogenic species: *Borrelia lonestari* that affects humans, causing rashes and fever (Burkot et al., 2001; Stromdahl et al., 2003), and *Borrelia theileri*, that affects cattle, sheep and horses, causing abortions, fever episodes and anemia in several tropical countries (Sharma et al., 2000). Due to the close phylogenetic relationship with *B. miyamotoi*, *B. lonestari* and *B. theileri*, this new lineage from the RF group needs to be more closely studied to clarify its identity and pathogenicity for humans and/or animals.

The new lineage from the LB group forms a monophyletic clade with *B. turdi*, firstly isolated in Japan and now recognized to be widely distributed in Europe (Fukunaga et al., 1996; Norte et al., 2016). So far, this species has been reported infecting birds, mainly migratory, although no pathogenicity has been reported for this vertebrate taxon (Fukunaga et al., 1996; Heylen, 2016). Additionally, this clade is related with two rodent-associated species: *Borrelia yangtzensis*, a species distributed in Asia (Margos et al., 2015), and with *Borrelia valaisiana*, a pathogenic species widely distributed in Asia and Europe that causes a Lyme disease-like illness, and whose principal reservoirs are rodents and

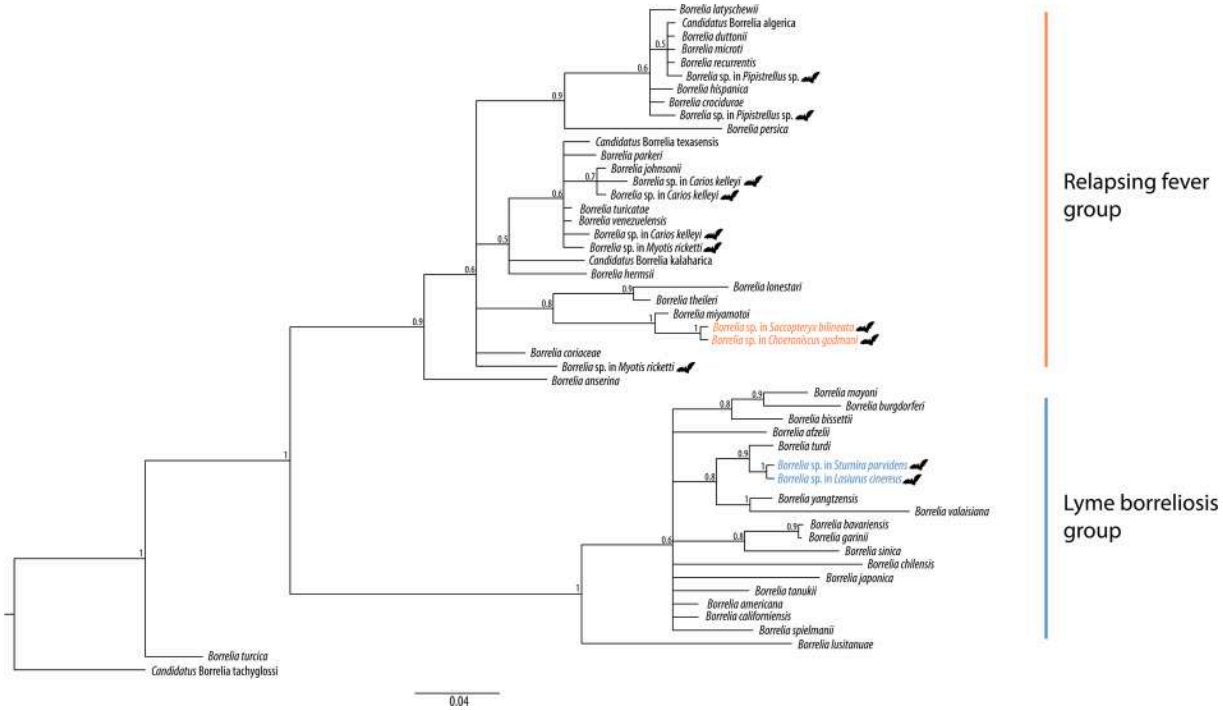

Fig. 2. Bayesian inference (BI) phylogenetic analysis by codons alignment (16S rDNA + *flagellin* + *osp*A genes) for the genus *Borrelia*. The phylogenetic relationships were inferred based on the Hasegawa-Kishino-Yano (Hasegawa et al., 1985) with invariant sites (16S codons 1, 2 and 3; *flagellin* codon 1), Jukes-Cantor (Jukes and Cantor, 1969) with invariant sites (*flagellin* codon 2), Hasegawa-Kishino-Yano with gamma distribution (*flagellin* codon 3, *osp*A codons 1, 3) and General Time Reversible (Tavaré, 1986) with invariants sites (*osp*A codon 2) for a total of 1266 bp. Orange sequences indicate isolates from CM and LE; blue sequences correspond to isolates from ZN. Sequences retrieved from studies done with bats and/or ticks associated with bats are marked with a bat image (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

birds (Hulínská et al., 2009; Socolovschi et al., 2012; Stanek and Reiter, 2011). Given these relationships, it is likely that this putative new lineage related to bats could not represent a public health problem. However, this record represents an enzootic silent focus of *Borrelia* sp. in the region of Zacualpan, Mexico.

The genetic distances between *B. miyamotoi* and the sequences obtained in our study for *S. bilineata* and *C. godmani* support the general findings suggesting that they are a new putative phylogenetic group. Thus, if we compare our data with the genetic distance between two other closely related and valid species, such as *Borrelia duttonii* and *Borrelia microti* (0% for both 16S rDNA and *flagellin*), or with the differences between *B. lonestari* and *B. theileri* (0.7 % for 16S rDNA and 2% for *flagellin*) [Fig. 3], two more distinguishable species, we find that our values lie on the same interval.

Furthermore, our findings are also similar to the comparison of *Borrelia bavariensis* with *Borrelia garinii* (0% for 16S rDNA and 0.7 % for *flagellin*), two closely related species, and *Borrelia yangtzensis* with *B. valaisiana* (0% for 16S rDNA and 1% for *flagellin*) [Fig. 3], two more distinguishable species. It is noteworthy that higher values are obtained when comparing *B. turdi* with sequences of *L. cinereus* and *S. parvidens* for the *flagellin* gene.

The bat species (*S. parvidens* and *L. cinereus*) harboring the novel LB associated-lineage, were captured in one of last patches of Mexican cloud forest in central Mexico (CONABIO, 2010), an ecosystem characterized by low temperatures ranging from 4 to 18 °C and an average altitude of 1700 m (Foster, 2001; Jarvis and Mulligan, 2011). These characteristics are commonly associated with the presence of several species of ticks of the genus *Ixodes*, mainly those from the *Ixodes ricinus* complex, which are the recognized vectors for etiological agents causing Lyme disease and for other *Borrelia* species from the same LB group (Filippova, 1999; Keirans et al., 1999; Movila et al., 2014; Nava et al., 2017).

Despite the molecular evidence of *Borrelia* of the LB group in Mexican bats, their role in the natural transmission of the *B. burgdorferi* s.l. complex remains unclear. Thus, these bats should only be regarded as incidental hosts and not as hosts or reservoirs, since no reports of hard ticks associated to Mexican bats have been published. For this reason, further extensive field trips to enhance studies on ticks must be done. Additionally, more studies on the natural bat-tick transmission and the isolation of *Borrelia* from both hosts are necessary, to clarify the role of bats as hosts and/or reservoirs.

With regard to novel lineage within the RF group, bats were caught in the last Mexican native patch of tropical rainforest with altitudes between 150 and 700 m and an average annual temperature of 26 °C (Dirzo et al., 1997). Historically, in this region only two ticks species (*Ornithodoros talaje* and *Ixodes boliviensis*) have been reported as feeding on mammals and humans (Hoffman, 1930, 1962). Additionally, several hard tick genera have been recorded, such as *Amblyomma, Ixodes* and *Rhipicephalus*. These genera are important since the *Borrelia* lineage obtained from tropical bats clustered together with *B. miyamotoi*, *B. theileri* and *B. lonestari*, which are transmitted by these hard-tick genera. To date, the hard-tick species recorded in the region are only of veterinary importance (Crowder et al., 2014; de Oliveira-Souza-Higa et al., 2020; Guzmán-Cornejo et al., 2011; Guzmán-Cornejo and Robbins, 2010).

From the four positive bat species for *Borrelia*, only *S. bilineata* and *L. cinereus* have insectivorous habits, whereas *C. godmani* is polliniverous and *S. parvidens* is frugivorous, although all of them roost on shelters outside of caves, such as holes in trees, bridges and abandoned buildings (Álvarez and Sanchez-Casas, 1997; Shump and Shump, 1982; Yancey et al., 1998). These ecological habits could suggest that infections possibly occur by ticks found in those habitats. Thus, it is warranted to also focus future search of ticks in these environments.

Although we used several small fragments for molecular detection, it has been shown that 16S and the *flagellin* genes are effective for the delimitation and identification of species of this bacterial genus

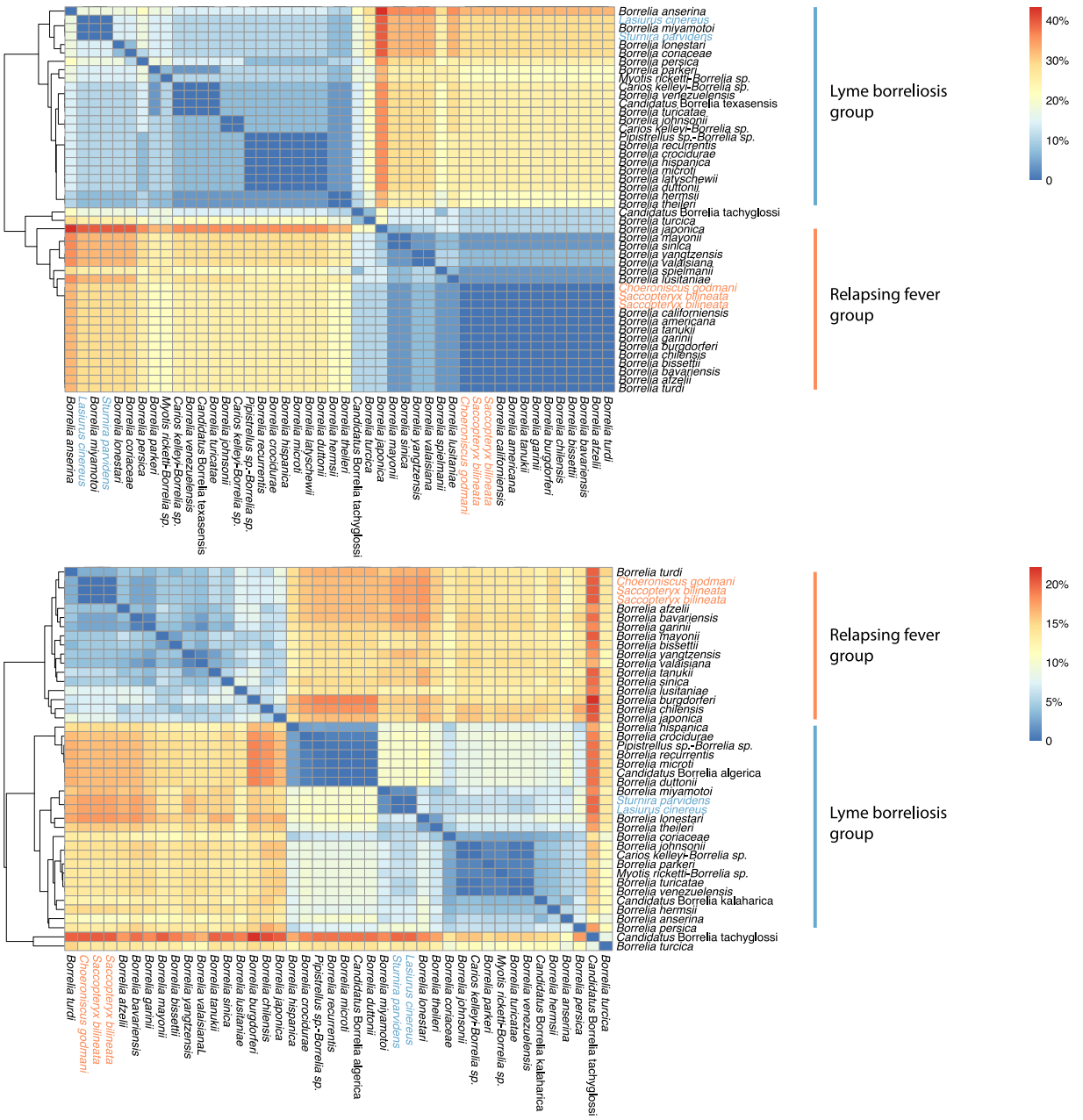

Fig. 3. Heatmap for pairwise Nei's distances for 16S rDNA and *flagellin* genes for several *Borrelia* species. Color gradient represent the percentage of differentiation among species according to the scale bar in the right for the 16S rDNA (A) and the *flagellin* (B) amplified fragments.

(López-Pérez et al., 2019; Morales-Diaz et al., 2020). Our phylogenetic inference suggests the presence of two independent lineages. These need to be further analyzed after successful culture, which would enable a more detailed molecular information with the use of the MLST algorithm for both groups of the genus *Borrelia* (Margos et al., 2008; Morales-Diaz et al., 2020). This would also help elucidate the role of bats in the life cycle of *Borrelia* and whether Mexican bats are reservoirs.

5. Conclusions

We report, for the first time, two putative new lineages of *Borrelia* that were found to infect Neotropical bats of Mexico. One lineage is associated with the *B. burgdorferi* s.l. complex and form a monophyletic clade with *B. turdi*, a species associated with birds in Europe. The other lineage forms a monophyletic clade with *B. miyamotoi,* a pathogenic species of the relapsing fever group. These findings highlight the importance of cloud forest and tropical zones as suitable habitats for the presence of *Borrelia* in Mexico and call for active epidemiological surveillance in those areas.

CRediT authorship contribution statement

Pablo Colunga-Salas: Conceptualization, Methodology, Formal analysis, Visualization, Writing - original draft. **Sokani Sánchez-Montes:** Methodology, Writing - review & editing. **Livia León-Paniagua:** Resources, Writing - review & editing. **Ingeborg Becker:** Supervision, Visualization, Resources, Writing - review & editing, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors report no declarations of interest.

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CAPÍTULO III. Genetic diversity of *Borrelia burgdorferi* sensu stricto: novel strains from Mexican wild rodents

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ORIGINAL ARTICLE

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Genetic diversity of *Borrelia burgdorferi* sensu stricto: Novel strains from Mexican wild rodents

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Abstract

Borrelia burgdorferi s.s. is a Gram-negative spirochaete, the aetiological agent of Lyme disease, the most common vector-borne disease in the Northern hemisphere. Reports on the presence of *B. burgdorferi* in central Mexico have been strongly criticized, since these were based only on unspecific serological methods. Furthermore, the worldwide genetic diversity of *B. burgdorferi* s.s. has not been evaluated. For this reason, the aim of the present study was to confirm the presence of *B. burgdorferi* in the central area of Mexico and to evaluate its relationship with regard to the global genetic diversity of *B. burgdorferi* s.s. To achieve this, fragments of the *flagellin* and the *outer surface protein* A genes were amplified from ear biopsies of the arboreal wild endemic mice *Habromys schmidlyi*. With these sequences, a concatenated Bayesian analysis was performed to confirm the identity of *B. burgdorferi* s.s. Afterwards, the global genetic diversity of this bacterial species was evaluated using our sequences and those available in GenBank. A prevalence of 10.4% (5/48) of *H. schmidlyi* infected with *Borrelia* sp. was detected, and the phylogenetic analyses confirmed the identity of *B. burgdorferi* s.s. Using both genes, the genetic diversity was low. However, genetic structuring analyses revealed that populations of western United States and those from Mexico formed slightly different genetic groups, separated from the populations of the rest of the world. Our study not only confirms the presence of this bacterium in central Mexico, but also shows the most southern record of this bacterium so far. It also highlights the importance of *H. schmidlyi* as a new potential host of this bacterial species. Our study also provides first genetic data on an incipient process of divergence in *B. burgdorferi* s.s. populations of eastern United States and central Mexico.

KEYWORDS

Borreliella, flagellin, *Habromys schmidlyi*, Lyme disease, ospA

1 | INTRODUCTION

Borrelia burgdorferi s.l. is a species complex group of spirochaetes which encompass the aetiological agents of Lyme borreliosis, the most common vector-borne disease in the Northern hemisphere (Clark, Leydet, & Hartman, 2013; Kurtenbach et al., 2006; Saito et al., 2007). This species complex includes 22 validated genospecies, from which *Borrelia burgdorferi* sensu stricto (from now only *B. burgdorferi*) is the main causative agent of the Lyme Disease in North America, Europe and Asia (Margos et al., 2017, 2019; Steere et al., 2016).

The enzootic life cycle of this bacterial species is a complex network that encompass several tick species of the genus *Ixodes*, specifically, from the *Ixodes ricinus* complex (Güner et al., 2004; Kurtenbach et al., 2006). Ixodid ticks from this species complex feed on a broad spectrum of wild vertebrate hosts. Wild rodents of the genus *Peromyscus* have been reported as the main reservoirs of *B. burgdorferi* in North America (Kurtenbach et al., 2006; Schotthoefer & Frost, 2015; Steere et al., 2016).

The distribution and prevalence of *B. burgdorferi* have been widely studied in the Nearctic biogeographical region, especially in wild mammals (Coipan, Van Duijvendijk, Hofmeester, Takumi, & Sprong, 2018; Hovius, van Dam, & Fikrig, 2007; Huegli, Hu, Humair, Wilske, & Gern, 2002; Roome et al., 2017; Schotthoefer & Frost, 2015), including arboreal rodents such as the western gray squirrel *Sciurus griseus* (Lane, Mun, Eisen, & Eisen, 2005) and the eastern chipmunk *Tamias striatus* (Slajchert, Kitron, Jones, & Mannelli, 1997). In the tropical region, there are few records of *Borrelia*-like spirochaetes from arboreal marsupials (*Didelphis albiventris*, *Didelphis marsupialis* and *Phylander opossum*) and arboreal rodents (*Akodon montensis*) (Abel, Marzagão, Yoshinari, & Schumaker, 2000; da Costa, Bonoldi, & Yoshinari, 2002). This opens the possibility of considering arboreal mammals as potential reservoirs of other members of the *B. burgdorferi* s.l. complex in the Nearctic.

In Mexico, the *Borrelia burgdorferi* s.l. complex has been widely studied throughout the country. Yet, most of the studies have been widely criticized since they were carried out using only serological methods with unconfirmed results (Faccini-Martínez, 2019; Feria-Arroyo et al., 2014; Gordillo-Pérez et al., 2018; Norris, Barbour, Fish, & Diuk-Wasser, 2015; Norris, Barbour, Fish, & M Diuk-Wasser, 2014). Based on these serological studies, *Borrelia burgdorferi* has been suspected only in the northern part of the country (Gordillo-Pérez et al., 2009; Vargas et al., 2007). There is only one study made by molecular methods and with available sequences that has demonstrated this complex in *Ixodes kingi* recovered from *Vulpes macrotis* near the Mexican-US border (López-Pérez et al., 2019), but this has not been confirmed in wild mammals.

The central region of Mexico is characterized by a large and intricated mountainous system, the Transmexican Volcanic Belt, whose mountains have an average elevation of 2000 m, where *Quercus* oak forests and patches of primary cloud forest occur alternately in the higher parts throughout the entire region (Marines-Macías, Colunga-Salas, Verde-Arregoitia, Naranjo, & León-Paniagua, 2018). In this mountainous system, various species of ticks of the genus *Ixodes* are distributed (Guzmán-Cornejo, Robbins, & Pérez, 2007). Moreover, in one of the highest areas of this mountainous system, *Habromys schmidlyi*, an arboreal rodent, endemic and phylogenetically closely related to the genus *Peromyscus*, is the most abundant rodent species (León-Paniagua, Navarro-Sigüenza, Hernández-Baños, & Morales, 2007; Marines-Macías et al., 2018). Since the presence of *B. burgdorferi* in this mountainous area of Mexico was doubtful, one of the aims of this study was to analyse by molecular techniques whether this bacterium was present in *Habromys schmidlyi*.

Additionally, since previous attempts to evaluate the genetic diversity of *B. burgdorferi* have focused on several genes including *outer surface protein* and *flagellin* genes, but only at regional levels (Baranton, Seinost, Theodore, Postic, & Dykhuizen, 2001; Brisson, Vandermause, Meece, Reed, & Dykhuizen, 2010; Foretz, Postic, & Baranton, 1997; Mechai, Margos, Feil, Lindsay, & Ogden, 2015; Travinsky, Bunikis, & Barbour, 2010; Wallich et al., 1992), we now calculated the genetic diversity of *B. burgdorferi* at global scale to understand how the genetic diversity is distributed within this species.

2 | MATERIAL AND METHODS

2.1 | Animal sampling

During 2012–2013, an extensive fieldwork was done in the only two known regions harbouring populations of *H. schmidlyi*, in the State Park 'Cerro del Huixteco', within the Sierra de Taxco, Guerrero, Mexico, (18°36′N, 99°36′W), and the State Park 'Picacho de Oro y Plata', Zacualpan, Estado de México, Mexico, and surrounding areas (18°43′N, 99°46′W) (Figure 1). A total of 120 Sherman live-traps were used for rodent trapping in each locality: 60 traps were placed at the ground level, and the remaining 60 traps were placed on the tree trunks and branches, following the transect set on the ground. The arboreal trapping was done using climbing equipment to reach the highest possible point (20 m) [for further sampling information, see Marines-Macías et al., (2018)]. All captured individuals were identified in situ by their external morphology following Romo-Vázquez, León-Paniagua, and Sánchez (2005), and then tagged with an ear notch using a medical punch. Tissues were fixed individually in 96% ethanol. In order to avoid cross-contamination between tissues, the punch was disinfected with 10% chlorine and 96% ethanol between each use. All individuals were screened for the presence of ticks, yet none of the rodents had ticks. After these, all individuals were released in the same place where they had been collected (special permit SGPA/DGVS/12142/12 from the Secretaría del Medio Ambiente y Recursos Naturales).

2.2 | Borrelia molecular detection

For DNA extractions, the entire tissue (~2 cm of diameter) was manually cut in small pieces and placed in 1.5-µl tubes; in this step, also to avoid cross-contamination, the surgical equipment was disinfected (see above). We then used the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) following manufacturer's instructions with a single modification: we added 30 µl of proteinase K in each tube and incubated them at 56°C until the tissues were completely lysed.

For the molecular identification of *Borrelia*, a fragment (~300 bp) of the 16S rDNA region was amplified using the primers and conditions previously reported by Skotarczak et al., (2005). From all positive samples, two additional fragments of ~280 bp of the *flagellin* gene and ~794 bp of the *osp*A gene were amplified, using the primers

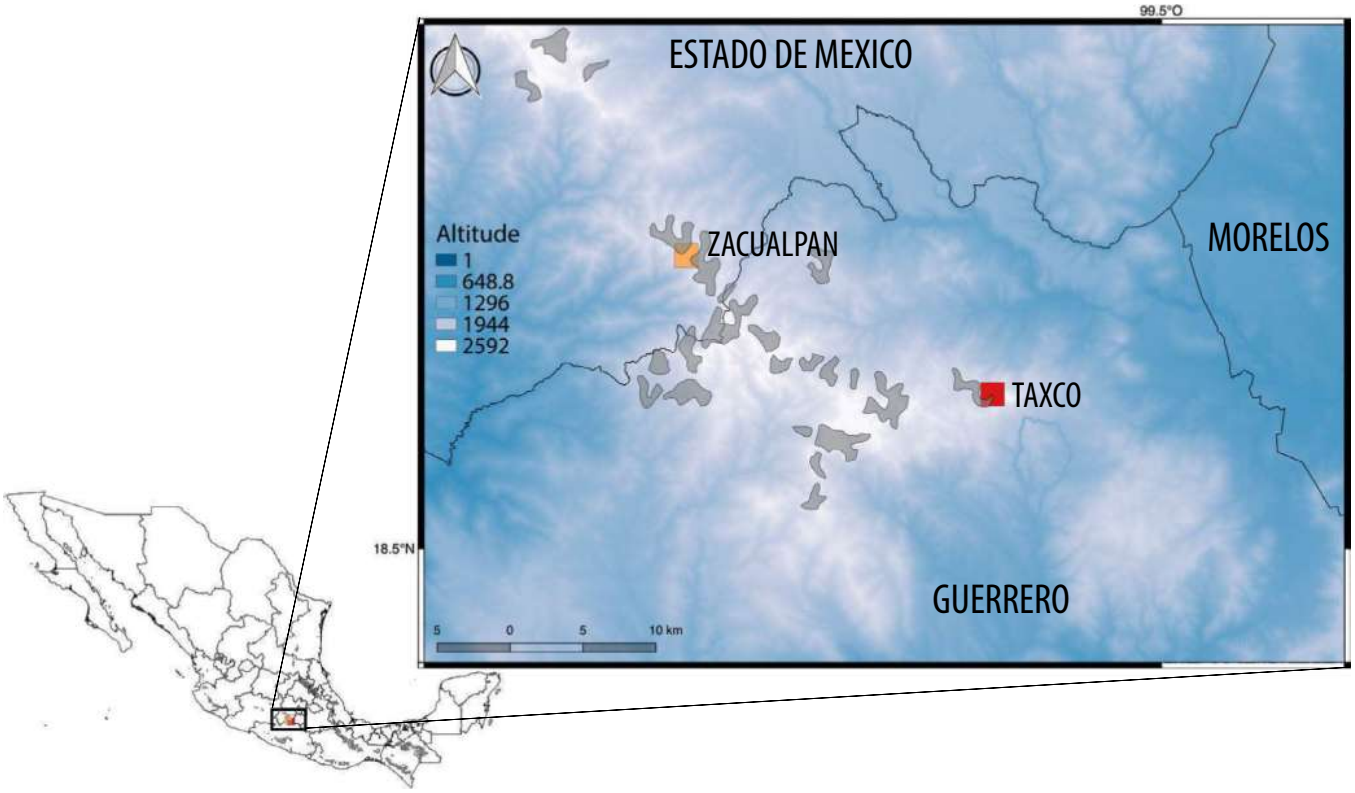

FIGURE 1 Sampling sites for *Habromys schmidlyi*. The gradient of altitude in Mexican mountains is shown in blue scales and distribution of the cloud forest in Mexico according to CONABIO (http://www.conabio.gob.mx/informacion/gis/) in grey shading

and conditions of Zore et al., (1999), and Bunikis et al., (2004), respectively. The reaction mixtures consisted of 12.5 μl of GoTaq® Green Master Mix, 2 × of Promega Corporation (Madison, WI, USA), the pair of primers (100 ng each), 6.5 μl nuclease-free water and 150–250 ng DNA (~2 μl from the total elution of 200 μl) in a final volume of 25 μl. We followed the PCR conditions specified previously for the *flagellin* (Zore et al., 1999) and *osp*A genes (Bunikis et al., 2004). In all reactions, we included a negative (the same reaction mixture with water) and a positive control (DNA from a *Borrelia* sp. previously isolated from *Amblyomma dissimile* ticks attached to *Rhinella horribilis* in Veracruz, Mexico, accession number: KY389373 (as previously reported by Morales-Diaz et al. (2020)).

PCR products were visualized in 2% agarose gels on the ODYSSEY CLx Imaging System (LI-COR Biosciences). All positive PCR products were prepared and sequenced at Laboratorio de Secuenciación Genómica de la Biodiversidad y de la Salud, Instituto de Biología, Universidad Nacional Autónoma de México (UNAM). The DNA sequences were edited and aligned using 4 Peaks V1.8 (Nucleobytes B.V.) and MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018), conducting visual inspections of all sequences.

2.3 | Molecular characterization of Borrelia

To identify the species of *Borrelia* recovered from *H. schmidlyi*, sequences of the 16S rDNA region, the *flagellin* and *osp*A genes from all species of the *B. burgdorferi* s.l. complex available in GenBank were included into a concatenated alignment in MEGA X (Kumar et al., 2018) and aligned with the Muscle algorithm (Edgar, 2004). The best scheme of partition and substitution model of the concatenated data was calculated in Partition Finder 2 (Guindon et al., 2010; Lanfear, Calcott, Ho, & Guindon, 2012; Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2017).

A phylogenetic analysis was performed by the Bayesian inference approach in MrBayes 3.2.7 (Ronquist et al., 2012), using the MCMC algorithm and the substitution model calculated previously. Three hot and one cold chains in two independent runs of 10 million generations, sampling every 1,000 generations, were used. The final topology was obtained using a majority consensus, considering a burn-in of 20%. The convergence of results and good sampling (ESS> 200) was checked in Tracer 1.7.1 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018).

Additionally, all available sequences of *Borrelia burgdorferi* were obtained from GenBank, by manual searching and by a BLAST tool searching only for *flagellin* and *osp*A genes. In order to corroborate the correct identification, a global alignment and a phylogenetic analysis were performed, as previous described, for each gene. Only those sequences clustered in a monophyletic clade were included for further genetic analyses (Figures S1, S2). The 16S rDNA region was not included in the genetic analyses, since this region did not vary within the *B. burgdorferi* s.s. phylogenetic analysis (data not shown).

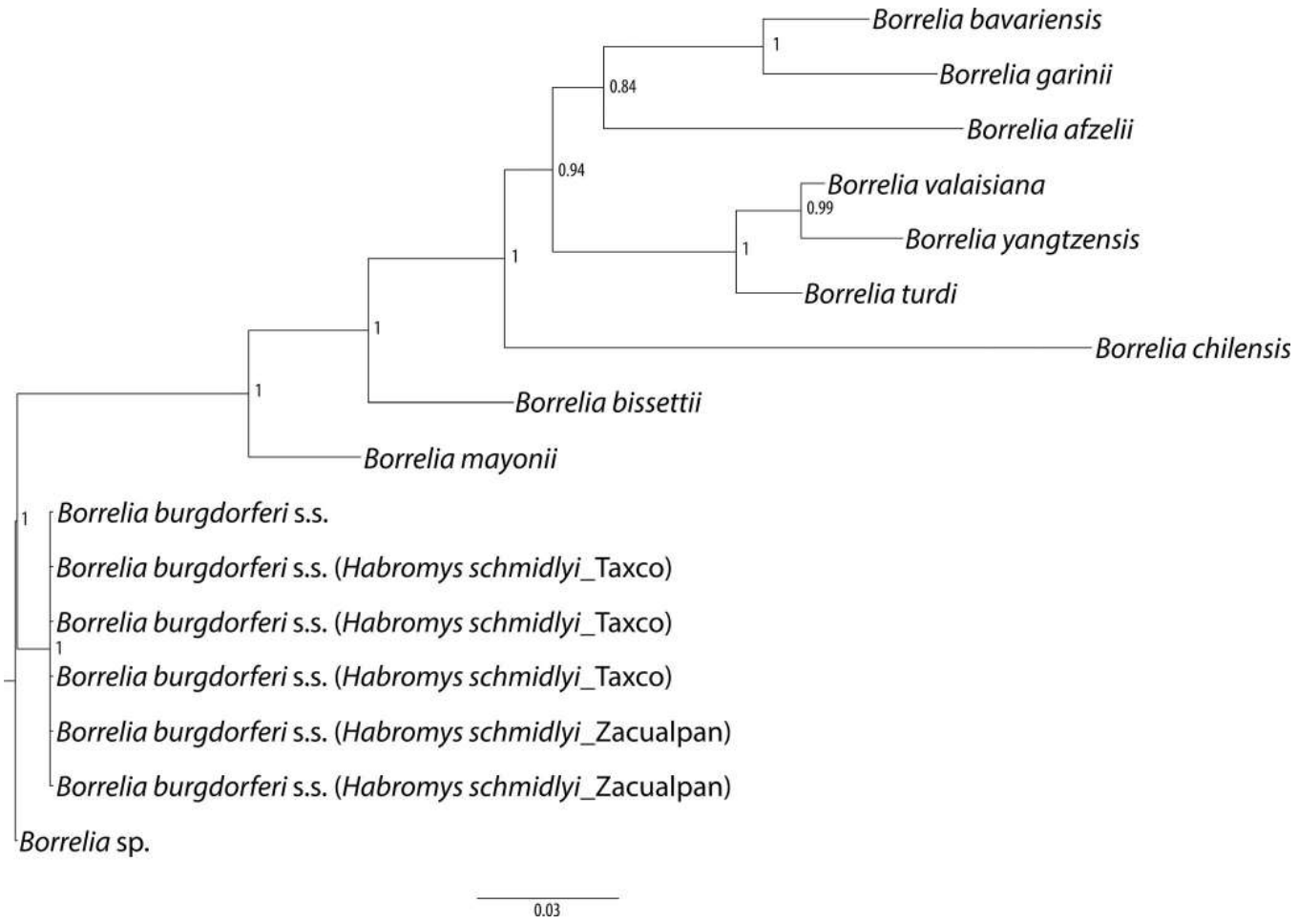

FIGURE 2 Phylogenetic tree inferred from the concatenated alignment (16S rDNA + *flagellin* + *osp*A genes) for the genus *Borrelia*. The phylogenetic relationships were inferred based on the HKY + I substitution model (Hasegawa, Kishino, & Yano, 1985) for a total of 1,309 bp. Posterior probabilities are indicated at each node. Scale bar indicates number of nucleotide substitutions. Sequences generated in this study were submitted to GenBank under accession numbers: MT378393-MT378397 for 16S rDNA region, MG934557-MG934561 for *flagellin* and MN461274-MN461278 for *osp*A

2.4 | Genetic analyses

In order to provide a more detailed account of the genetic diversity and to be able to detect possible populations and subpopulations, a principal coordinates analysis (PCoA) in PAST3 (Hammer, David, & Paul, 2001) was performed for each gene, based on Euclidean distances.

Hierarchical genetic differentiation analysis was tested for both genes by the molecular variance (AMOVA) method in Arlequin 3.5.2.2 (Excoffier & Lischer, 2010). The hierarchical analyses were conducted at three levels: (a) among isolates, equivalent to each sequence; (b) among populations, which were defined by their geographic distribution; and (c) among groups, which were defined by previous analyses. For this, a matrix of pairwise differences, using 10,000 permutations to test the significance of our results, was performed in Arlequin.

Haplotype networks with mutational step estimations were constructed for each gene using the median-joining network in POPART 1.7 (Leigh & Bryant, 2015).

Finally, to detect the geographic and genetic structures for each gene, Geneland analyses were done (Guillot, Renaud, Ledevin, Michaux, & Claude, 2012). From one to six populations were tested, following the *prior* distribution of samples, according to the sampling site referred by each author. In order to do this, one million generations with a thinning of 100, with no coordinate uncertainty, were used.

3 | RESULTS

A total of 48 *H. schmidlyi* individuals were sampled, 29 from Taxco and 19 from Zacualpan. The presence of *Borrelia* DNA was found in biopsies of five individuals (5/48 = 10.4%), three individuals from Taxco and two from Zacualpan. From the five positive individuals, a total of 292 bp were obtained for the 16S rDNA region, 225 bp for the *flagellin* gene, and 792 bp for the *osp*A. According to the phylogenetic analyses, sequences obtained from *H. schmidlyi* grouped together in a monophyletic clade with other sequences of *B. burgdorferi* s.s. from North America and Europe (Figure 2, Figures S1, S2).

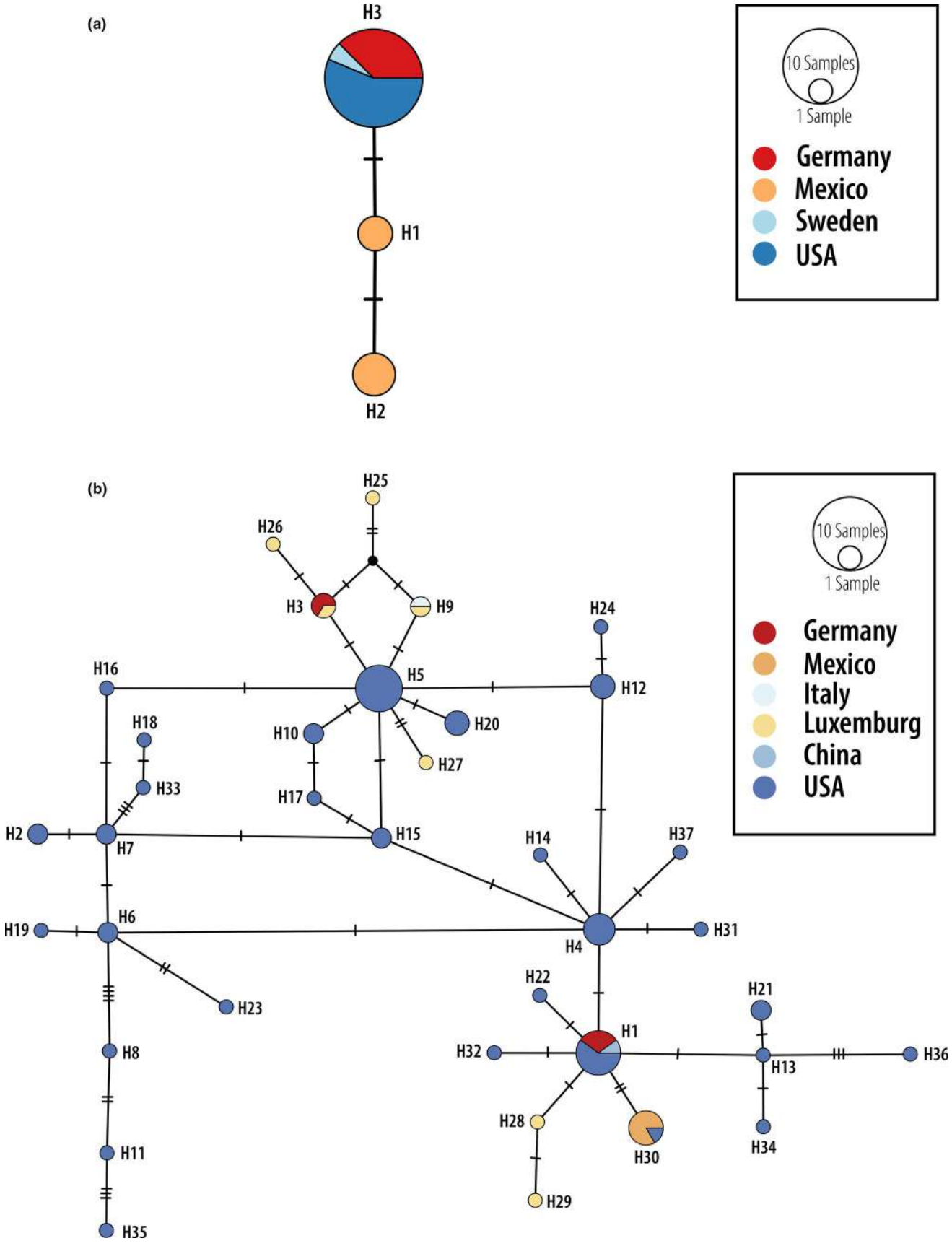

FIGURE 3 Haplotype network analyses for *Borrelia burgdorferi* s.s. (a) Haplotype network inferred with the *flagellin* gene including all available and validated sequences (see Figure S1). (b) Haplotype network inferred with the *osp*A gene including all available and validated sequences (see Figure S2). The colours correspond to the country of origin of each haplotype. Black lines represent the mutational steps between each haplotype

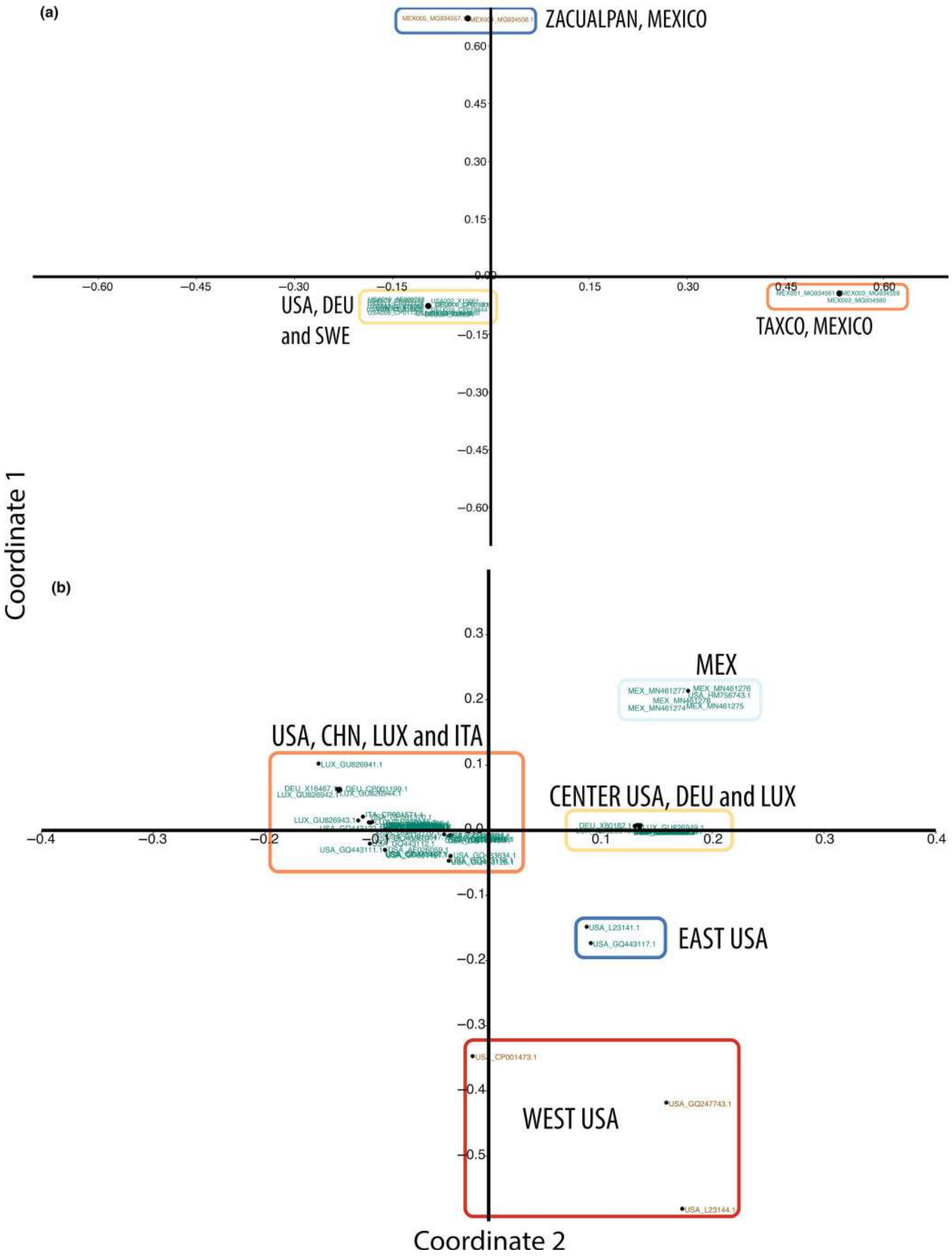
(a)
ZACUALPAN, MEXICO
USA, DEU and SWE
TAXCO, MEXICO
(b)
MEX
USA, CHN, LUX and ITA
CENTER USA, DEU and LUX
EAST USA
WEST USA
Coordinate 1
Coordinate 2

FIGURE 4 PCoA plot of the first and second axes based on Euclidean distances for all available sequences of *B. burgdorferi* s.s. (a) PCoA plot for the partial nucleotide sequence of the *flagellin* gene. The proportion of total variance along the two axes was 93.97% and 6.02%, respectively. (b) PCoA plot for the complete *osp*A gen. The proportion of total variance along the two axes was 37.96 and 10.48%, respectively. CHN, China; DEU, Germany; ITA, Italy; LUX, Luxembourg; Mex, Mexico. Colour squares show the genetic groups obtained from each analysis

TABLE 1 Analysis of molecular variance results for the *flagellin* partial gene

Source of variation	Percentage of variation	Fixation index	*p*-value
Among groups (Mexican populations *vs* the rest of the world)	92.64	$\phi_{CT} = 0.92$	>.001
Among populations within groups	−2.22	$\phi_{SC} = -0.3$	>.001
Within populations	9.58	$\phi_{ST} = 0.9$	**<.001**

Values in bold were statistically significant.

3.1 | Genetic analyses

From the *flagellin* gene, three haplotypes were obtained, with a haplotype diversity of 0.4095 and nucleotide diversity of 0.00224. The haplotype network shows the 'H3' haplotype, both as the most frequent haplotype and the differentiation centre, which includes all samples with the exception of isolates from Mexico, H1 from Taxco and H2 from Zacualpan, both populations were the sites where *H. schmidlyi* was sampled (Figure 3a).

On the other hand, from the haplotype network recovered from the *osp*A gene, 37 haplotypes were recovered, with a haplotype diversity of 0.9530 and nucleotide diversity of 0.00522. This network

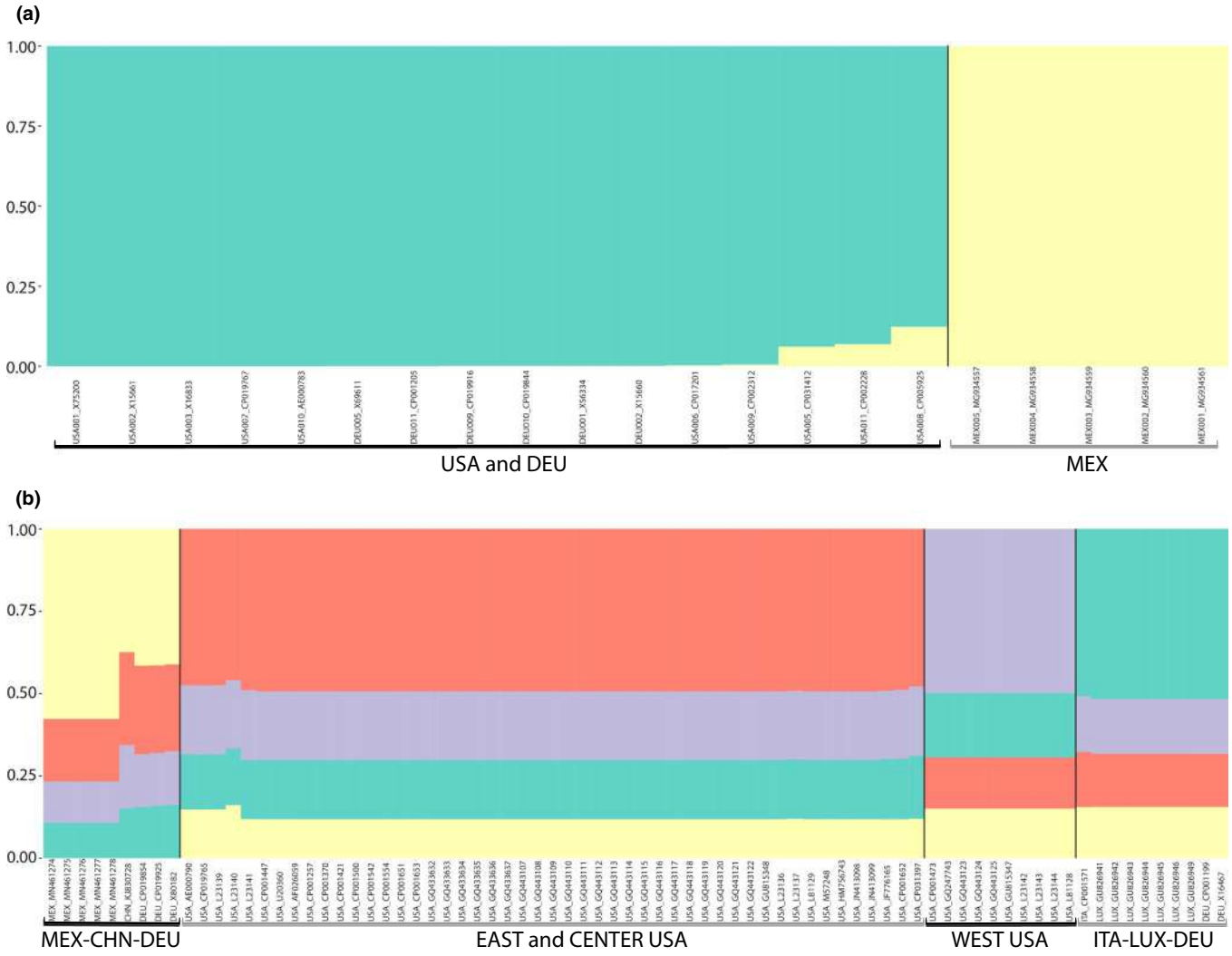

FIGURE 5 Genetic structure of *Borrelia burgdorferi* s.s. samples representing the maximum number of clusters (K). The y-axis represents the identity percentage of each sequence, and black lines separate each population. (a) Geneland analysis results for the partial *flagellin* gen with two populations (K = 2). (b) Geneland analyses for the complete *osp*A gen showing four genetic populations (K = 4). CHN, China; DEU, Germany; ITA, Italy; LUX, Luxemburg; Mex, Mexico

shows intricate relationships among all isolates and two main centres of diversification, H1 and H5, which include isolates from America, Europe and Asia. Meanwhile, haplotypes from North American and Luxemburg isolates are widely distributed along the network and most of them as tip haplotypes (Figure 3b).

The ordination analysis showed three genetic groups for the *flagellin* gene, two of them represent each Mexican population and the last one conformed by the North American-European samples (Figure 4a). The *osp*A sequences formed at least five main genetic groups, where it seems that western United States is the most different group (Figure 4a).

The analysis of molecular variance with the partial *flagellin* gene revealed that 92.64% of variation was among groups (*i.e.* among the Mexican populations and North American-European population), 9.58% was within populations, and − 2.22% was among populations but within groups (Table 1). However, ϕ-statistics obtained with AMOVA, revealed significant variation only within populations [$\phi_{ST} = 0.9$; $p < .0001$], but variation among groups was not statistically significant ($\phi_{CT} = 0.92$; $p > .001$; Table 1). Additionally, the Geneland analysis confirms the presence of two genetic populations with high probability values: Mexican populations in one group, and the rest of the sequences were included in the second group (Figure 5a).

On the other hand, the AMOVA analysis, using the complete *osp*A gene, revealed that 54.31% of variation among groups (*i.e.* western United States and the rest of the world [samples from eastern United States, as well as Mexican, European and Asian samples]), 34.19% was within populations, and 11.44% was among populations but within groups (Table 2). ϕ-statistics revealed a subtle genetic structure among groups, which was not significant ($\phi_{CT} = 0.54$; $p > .0001$; Table 2). With Geneland analysis, four genetic populations were obtained: (a) Mexican-Chinese-eastern German populations, (b) eastern and central USA, (c) western USA, and (d) Italian-Luxemburg-western German populations; however, the individual probability of identity to each group was low (Figure 5b).

4 | DISCUSSION

This is the first confirmatory record of *B. burgdorferi* s.s. in the central region of Mexico and, also, the first record of this bacterium in wild Mexican mammals. Furthermore, this is the most southern record of *B. burgdorferi* s.s. so far, in the transition zone of the Nearctic and Neotropical biogeographical regions. Our report now represents the first association of *B. burgdorferi* with a species of arboreal mouse in Mexico and highlights the importance of arboreal mammals as potential hosts of this bacterium in the wild.

TABLE 2 Analysis of molecular variance results for the *osp*A gene

Source of variation	Percentage of variation	Fixation index	*p*-value
Among groups (Western United States *vs* the rest of the world)	54.37	$\phi_{CT} = 0.54$	>.001
Among populations within groups	11.44	$\phi_{SC} = 0.25$	**<.001**
Within populations	34.19	$\phi_{ST} = 0.66$	**<.001**

Values in bold were statistically significant.

Some studies have tried to relate several species of mammals and various genera of hard ticks from Mexico (*e.g. Amblyomma*, *Dermacentor* and *Ixodes*) as hosts and potential reservoirs of this bacterium (Gordillo-Pérez et al., 2009; López-Pérez et al., 2019; Vargas et al., 2007). However, studies done by Gordillo-Pérez et al. (2009) and Vargas et al. (2007) were based on serological methods to test the infection and, although these were subsequently confirmed by identification of the bacterium by PCR, doubts have arisen. The published sequences have been questioned and presumed to be a probable contamination since they are identical to the sequence of isolate B31, which was used as a positive control (Norris et al., 2015).

Is important to note that transmission of *B. burgdorferi* has been associated with hard ticks of the genus *Ixodes*, specifically of the species from the *I. ricinus* complex (Güner et al., 2004; Kurtenbach et al., 2006). In North America, the main vectors for *B. burgdorferi* are *Ixodes scapularis*, *Ixodes pacificus* and *Ixodes affinis* (Kurtenbach et al., 2006) are generalist ectoparasites feeding from many different vertebrates species (Kurtenbach et al., 2006). Although those hard ticks have not been previously associated with *H. schmidlyi*, it is possible to think that there might be a member of the genus *Ixodes*, not yet recovered, that parasitized this rodent species, thus maintaining the enzootic transmission in the wild, since this tick genus is found mainly inside burrows and nests (Durden, 2006).

The study of the Mexican *Ixodes* is still in its initial period (Guzmán-Cornejo et al., 2007), and many taxonomic and ecological studies are still needed to allow us to understand, which species are involved in the maintenance of enzootic cycles of pathogens, as is the case of the cycle involving *B. burgdorferi* and *H. schmidlyi*. This rodent species has been described as arboreal, living mainly between 1 and 10 m above ground level, and probably makes nests in the understory stratum (Marines-Macías et al., 2018). Despite its arboreal habits, it was shown that this rodent uses holes inside tree trunks to reach the floor and feed; thus, tick search should be focused on these areas.

In the last stage of the life cycle of species from the *Ixodes ricinus* complex, the adults feed mainly on large mammals, which serve as maintenance hosts (Hayes & Piesman, 2003; Kurtenbach et al., 2006). The most important maintenance host in North America for *Borrelia* vectors is *O. virginianus*, since one individual can support large numbers of adult *I. scapularis* (Piesman & Gern, 2004; Spielman, Wilson, Levine, & Piesman, 1985). However, other records show that *I. pacificus* adults also feed on medium-sized mammals, such as foxes of genus *Urocyon*, which can serve as maintenance hosts (Castro & Wright, 2007; Crooks et al., 2001). These two mammals (*O. virginianus* and the grey fox, *Urocyon cinereoargenteus*) are commonly found in cloud

forest of central Mexico and in the Sierra de Taxco and Zacualpan (Ceballos, 2013; León-Paniagua & Romo-Vázquez, 1993), which could be infested by some species of the *I. ricinus* complex, that in turn, keep the populations of *B. burgdorferi* s.s. in circulation.

4.1 | Genetic diversity

To our knowledge, this is the first study using all the available online sequences of the genes *osp*A and *flagellin* to evaluate the worldwide genetic diversity of *B. burgdorferi*. When comparing both genes, the *flagellin* gene seems to be less diverse, with only three haplotypes, compared with the 37 haplotypes of the *osp*A gene. This finding is supported by previous studies where several authors have reported that the outer surface proteins, such as *osp*A, *osp*B and *osp*C, exhibit a higher degree of heterogeneity compared to other genes such as the *flagellin* gene (Baranton et al., 2001; Brisson et al., 2010; Foretz et al., 1997; Travinsky et al., 2010; Wallich et al., 1992).

The genetic diversity analyses of the *flagellin* gene revealed a subtle genetic differentiation between Mexican populations and the rest of the world. This finding is consistent with previous studies obtained from other conserved genes, such as that of the 16S rDNA fragment, from which a restricted gene flow and no evidence of genetic structure were observed among the USA populations of *B. burgdorferi* (Humphrey, Caporale, & Brisson, 2010), as well as with RFLPs analyses from worldwide isolates (Wallich et al., 1992). The Mexican sequences obtained in this study correspond to the first record of *B. burgdorferi* in a cloud forest environment worldwide. These sequences could be associated with unique haplotypes, and additionally, they could also reflect adaptations to the potential vectors and hosts present in the cloud forest ecosystem. The latter makes the search for ticks essential for linking this genetic structuration with ecological and evolutionary drivers.

In contrast, the genetic diversity analysis of the *osp*A gene shows subtle genetic structuration around the world, especially in western USA. An explanation for this subtle genetic structure could be that this protein is necessary for the primary establishment and persistence in the vector, avoiding the immunological system of the ticks (Hovius et al., 2007; Yang, Pal, Alani, Fikrig, & Norgard, 2004). Since *B. burgdorferi* is transmitted by several *Ixodes* species throughout its distribution range, it is likely that this promotes a high variability in the *osp*A gene throughout the different vector species. Additionally, local polymorphism could be maintained by ecological barriers limiting gene flow within unsuitable host species, as has been demonstrated after comparing the genotypes of *B. burgdorferi* in ticks and vertebrates from eastern versus western United States, which showed that these two populations appear to be in a genetic differentiation process (Humphrey et al., 2010).

So far, there are two hypotheses on the origin of *B. burgdorferi* s.s.: some authors have proposed that the centre of origin could be North America (Foretz et al., 1997; Ras, Postic, Foretz, & Baranton, 1997), although the most recent hypothesis proposes that its origin is European (Margos et al., 2008). Our study, using the *flagellin* gene, now supports that the origin of *B. burgdorferi* is possibly in the Holarctic, since Mexican populations represent the youngest populations in more recently colonized regions, based on Bayesian inference and haplotype network. However, with *osp*A gene, the haplotype and phylogenetic relationships among individuals are more complex, likely due to its high variability that could erase historical information. It is important to notice that considering the *osp*A data, the USA and Luxemburg concentrate the largest number of unique haplotypes, with 18 and 5 haplotypes, respectively. The differences in the number of unique haplotypes at each sampling region can be explained by a negative frequency-dependent mechanism, where no area has a single or few unique haplotypes, as has been shown when comparing genetic genotypes of the *osp*C, IGS and *dbp*A genes of *B. afzelii* with the initial infection in its hosts (Barthold, 1999; Coipan et al., 2018). Additional sampling mainly in non-studied areas, genomic data and ancestral reconstruction area analyses could address the interesting question related to the origin of these bacteria.

The *flagellin* gene has been demonstrated to be useful for species delimitation and molecular identification (López-Pérez et al., 2019; Potkonjak et al., 2016), whereas the *osp*A gene has proven to be useful for fine-scale genetic diversity studies, especially to test questions related to local adaptations in different environments.

To conclude, the findings of this study suggest the possibility of an enzootic focus of *B. burgdorferi* s.s. in two populations within the Transmexican Volcanic Belt. The infection of *H. schmidlyi* now reveals a new wild host of this spirochaete and represents the most southern population of *B. burgdorferi* in a region located in the transitional zone between the Nearctic and the Neotropical of high altitude and associated with a cloud forest. Additionally, an incipient genetic differentiation between western United States and Mexico was found, probably due to historical and ecological processes such as an unsuitable host, the adaptation to new vector species, as well as to environmental conditions.

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CONFLICT OF INTEREST

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest, non-financial interest in the subject matter or materials discussed in this manuscript.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. Animals were handled according to National Legislation and with the Guidelines of the American Society of Mammalogists for the Use of Wild Mammals in Research.

DATA AVAILABILITY STATEMENT

The data obtained in this study were submitted in GenBank, under accession numbers: MT378393-MT378397 for 16S rDNA region, MG934557-MG934561 for *flagellin* and MN461274-MN461278 for *osp*A. Furthermore, sequences used in genetic diversity analyses were downloaded from GenBank and accession numbers are shown in all figures.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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CAPÍTULO IV. Lyme disease and Relapsing fever in Mexico: an overview of human and wildlife infections (PLOS ONE, 2020; doi: 10.1371/journal.pone.0238496)

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RESEARCH ARTICLE

Lyme disease and relapsing fever in Mexico: An overview of human and wildlife infections

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Abstract

Lyme borreliosis and Relapsing fever are considered emerging and re-emerging diseases that cause major public health problems in endemic countries. Epidemiology and geographical distribution of these diseases are documented in the US and in Europe, yet in Mexico, studies are scarce and scattered. The aims of this study were (1) to present the first confirmatory evidence of an endemic case of Lyme disease in Mexico and (2) to analyze the epidemiological trend of these both diseases by compiling all the information published on *Borrelia* in Mexico. Two databases were compiled, one of human cases and another of wild and domestic animals in the country. The analysis included the evaluation of risk factors for the human population, the diversity of *Borrelia* species and their geographic distribution. Six *Borrelia* species were reported in a total of 1,347 reports, of which 398 were of humans. Women and children from rural communities were shown to be more susceptible for both Lyme borreliosis and Relapsing fever. The remaining reports were made in diverse mammalian species and ticks. A total of 17 mammalian species and 14 tick species were recorded as hosts for this bacterial genus. It is noteworthy that records of *Borrelia* were only made in 18 of the 32 states, mainly in northern and central Mexico. These results highlight the importance of performing further studies in areas where animal cases have been reported, yet no human studies have been done, in order to complete the epidemiological panorama for Lyme borreliosis and Relapsing fever. Finally, the search for *Borrelia* infections in other vertebrates, such as reptiles and amphibians is recommended to gain a more accurate view of *Borrelia* species and their distribution. The geographical approach presented herein justifies an intense sampling effort to improve epidemiological knowledge of these diseases to aid vector control and prevention programs.

Introduction

Tick-borne pathogens (TBP) have become a public health problem due to the continuous rise in the incidence of human and animal diseases associated with TBPs [1]. Some TBPs that can

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affect both humans and animals include several bacterial members of the genus *Borrelia* [1–3]. This bacterial genus comprises various spirochetal Gram-negative species that are divided into four phylogenetic groups: reptile-associated *Borrelia* (REP), monotreme-associated *Borrelia* (MAB), relapsing fever (RF) and Lyme-borreliosis (LB) [4–6]. The pathogenic species for humans of this bacterial genus are found only in the RF and LB groups [2, 5, 7].

Taxonomically, species of the genus *Borrelia* were initially considered as part of the genus *Spirochaeta*, but in 1907 the genus was divided and the genus *Borrelia* was formally described [8]. For more than a century, there were no important taxonomic changes. However in 2014, phylogenomic and protein studies led to the proposal of creating a new genus for members of the LB group (*Borreliella*) [9]. Further studies on the percentage of conserved proteins were proposed as a suitable method to delimitate the bacterial genera and the genus *Borrelia* was again proposed as a monophyletic genus [10]. To date, controversy remains on whether the genus should be divided [11, 12].

The *Borrelia* species causing RF are mainly transmitted by soft argasid ticks of the genus *Ornithodoros* and the human body louse *Pediculus humanus*, even though hard ticks of the genera *Ixodes*, *Amblyomma* and *Rhipicephalus* have also been implicated as potential vectors [13–15]. To date, 27 *Borrelia* species and two *Candidatus* species have been described as members of this monophyletic group [3, 15]. Of these, 24 and one *Candidatus* species have been recognized as pathogens for humans and/or other mammalian orders (Table 1) [3].

The *Borrelia* species that cause LB are grouped into 22 species [16], which are clustered into a monophyletic group. Of these, 11 species have been found to be the etiological agents of Lyme borreliosis, also known as Lyme disease (Table 1) [2, 17–20]. The competent vectors of these bacterial species are hard ixodid ticks [2, 17].

In Mexico, information on the diversity of *Borrelia* species, of wildlife infections and of human cases remains controversial [55–57]. The current information on this genus is poorly scattered and often published in local bulletins lacking diffusion. Furthermore, no confirmatory evidence of endemic human cases of Lyme disease has been shown in Mexico. Therefore, new insights and more solid evidence of human and animal infections with *Borrelia* are needed to elaborate an actualized map of these bacteria in Mexico.

Thus, the aim of this study was to: A) present to first confirmatory report of an endemic human case of Lyme disease in Mexico, and B) compile all the published records of human and animal infections by *Borrelia* in Mexico, to give an accurate picture of the current epidemiological situation of the genus *Borrelia* in the country.

A) Case report

Clinical summary and test procedures

A 67 years-old, unemployed male patient sought medical attention in August 2017. He was born in the coastal state of Veracruz, Mexico, where his family has a cattle farm. He had a previous history of Brucellosis at age 38. Diabetes had been diagnosed 15 years prior to the current office visit and had been irregularly controlled. He was hypertensive and treated with amlodipine, valsartan and hydrochlorothiazide. He had a history of multiple trips to the State of Veracruz, during which he had seen cattle infested with ticks. After visiting the family farm in the northern part of Veracruz in August 2016, he developed a red skin lesion that lasted several weeks, yet the patient did not seek medical attention. He started having diplopia and fatigue in September 2016. This evolved to stabbing headaches, cramps and pain in his limbs, bilateral hypoesthesia in hands and feet (in gloves and socks) and tachycardia. After having lost 15 Kg, he was hospitalized in March 2017, showing distal strength decrease +/+++ in lower limbs and sensitivity decrease in all modalities (exteroceptive and proprioceptive). A lumbar puncture

Table 1. Pathogenic *Borrelia* species.

Borrelia group	Species	Region/Country	Disease	References
RF	*Borrelia baltazardii*	Iran	TBRF (Tick-borne relapsing fever)	[21]
	Borrelia braziliensis	Brazil	TBRF	[15, 22]
	Borrelia caucasica	Caucasus area	TBRF	[23]
	Borrelia coriaceae	Western North America	Bacteremia of deer	[15, 24]
	Borrelia crocidurae	Western and northern Africa	TBRF, mild symptoms	[3, 15, 25]
	Borrelia dugesii	Mexico	TBRF	[26]
	Borrelia duttonii	Central, eastern and southern Africa	TBRF, Neurological signs, neonatal infections	[3, 15, 27]
	Borrelia graingeri	Kenya	Flu-like syndrome	[28, 29]
	Borrelia harveyi	Kenya	Bacteremia of monkeys	[15, 30]
	Borrelia hermsii	Western North USA, Brirish Columbia (Canada)	TBRF	[15, 31, 32]
	Borrelia hispanica	Iberian Peninsula and northern Africa	TBRF	[15, 33]
	Borrelia latyschewii	Central Asia and Middle East	TBRF, Flu-like syndrome	[15, 34]
	Borrelia lonestari	Southern and eastern United States	Bacteremia of deer	[15, 35]
	Borrelia mazzottii	Mexico, Central America and Western USA	TBRF	[15, 26]
	Borrelia microti	Iran	TBRF	[15, 36]
	Borrelia miyamotoi	Europe, Asia and North America	TBRF, Flu-like syndrome	[15, 37]
	Borrelia parkeri	Western USA	TBRF	[15, 32]
	Borrelia persica	Central Asia, Middle East, Egypt and India	TBRF	[15, 38]
	Borrelia queenslandica	Australia	Bacteremia with relapse in mice	[3]
	Borrelia recurrentis	Africa (Global)*	Louse-borne relapsing fever	[3, 39]
	Borrelia theileri	Africa (Global)**	Bovine borreliosis	[15, 40]
	Borrelia turicatae	British Columbia (Canada), Southwestern and south-central United States and Mexico	TBRF	[15, 41]
	Borrelia venezuelensis	Central America and northern South America	TBRF	[3, 42]
	Candidatus Borrelia kalaharica	Africa	TBRF	[43, 44]
LB	*Borrelia afzelii*	Europe and Asia	Lyme Disease (LD)	[45]
	Borrelia americana	North America	LD	[46]
	Borrelia andersonii	US	LD	[47]
	Borrelia bavariensis	Europe	Lyme borreliosis	[48]
	Borrelia bissettii	North America and Europe	LD	[49]
	Borrelia burgdorferi s.s.	East and West United States and Eastern Europe	LD	[50]
	Borrelia garinii	Europe and Asia	LD	[51]
	Borrelia lusitaniae	Mediterranean basin	LD	[52]
	Borrelia mayonii	Upper midwestern US	Lyme borreliosis	[20]
	Borrelia spielmanii	Europe	LD	[53]
	Borrelia valaisiana	Europe and Japan	LD	[54]

* *B. recurrentis* human cases have been reported in Ethiopia and Sudan, however a worldwide distribution is suspected.

** *B. theileri* cases have been reported in Africa, Australia, North and South America, but due to global bovine trade, it is now considered to be globally distributed.

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was performed, and the spinal fluid showed xanthochromia, glucose 69%, proteins 305 mg/ml, 98 mononuclear and 2 polymorphic nuclear cells (PMN)/μl. He received treatment with ceftriaxone and prednisolone (PDN) for two weeks. The headache remitted, the remaining symptoms improved by 70% and he was able to walk and climb stairs.

In August 2017, he again sought medical attention because symptoms restarted, he reported fatigue, diplopia and bilateral hypoesthesia in hands and feet (in gloves and socks), that made walking clumsy and difficult, fine tremor in his hands and short-term memory loss. On physical exam there was generalized areflexia, no meningeal signs and diminished strength in both lower limbs. He was again hospitalized and blood (5 ml) was taken in 2 vacutainer tubes, one with and one without ethylenediaminetetraacetic acid (EDTA). The sera samples were centrifuged at 2,000 rpm during 10 min and stored at -20˚C until use. DNA of whole blood samples was extracted by columns (DNEasy Blood and Tissue Kit, QIAGEN Inc., DEU), quantified with a spectrophotometer (Nanodrop-1000, Thermo Fisher, USA) and adjusted to a final concentration of 300 ng. Serological analysis was done by Western blotting using anti-*Borrelia burgdorferi*-WESTERNBLOT IgM and IgG kits (EUROIMMUN, Medizinische Labordiagnostik AG, D-23560 Lübeck, DEU), following instructions of the manufacturer. For molecular detection of *Borrelia*, a ~280 bp fragment of the *flagellin* protein gen (*fla*) was amplified by conventional PCR [58]. The reaction mixture consisted of 12.5 μl GoTaq® Green Master Mix, 2X (Promega Corporation, Madison, WI, USA), 2μM of each pair of primers, 6.5 μl nuclease-free water and 50 ng DNA in a final volume of 25 μl [58]. Negative and positive controls were included. As positive control, DNA from *Borrelia* previously isolated from *Amblyomma dissimile* (Colunga-Salas et al. unpublished data, GenBank accession number KY389373) was used. PCR product was visualized in 2% agarose gels with SmartGlow™ Pre-Stain (Accuris Instruments, Edison, NJ, USA) and visualized by UV-transillumination.

A written informed consent was signed by the patient, who was informed of the publication of his case.

Results

The serological analysis by Western blotting was positive for IgG and indeterminate for IgM. The PCR product showed a band of ~280 bp. Sequencing of the PCR product was done at Laboratorio de la Biodiversidad y la Salud, Instituto de Biología, Universidad Nacional Autónoma de México. The sequence was deposited in GenBank with the accession number MN607028. Molecular identification of the patient isolate was done by editing and aligning the sequences manually, including other species of the LB group. Bayesian analysis in MrBayes 3.2.3 [59] was done using the Markov Chain Monte Carlo (MCMC) algorithm with 10,000,000 generations and sampling every 1,000 generations, with a burning of 25% and the substitution model (Hasegawa, Kishino, and Yano model with gamma distribution [HKY+G] [60]) calculated in JModelTest 2 [61] based on the Bayesian Information Criterion. The convergence of the phylogenetic analysis was checked and considered as good when the ESS was higher than 200, in Tracer 1.7.1 [62].

The molecular confirmation by PCR showed that this patient was positive for *B. burgdorferi* s. s. (Fig 1), with a posterior probability of 0.96. The BLAST analysis showed 100% of identity and e value of 4e-133 with sequences of *Borrelia burgdorferi* s.s including those belonging to the strain B31 (Accession numbers: CP019767, AE000783, AB035617, X15661, L29200, AF416433 and Y15088) and other North American sequences. This result represents the first confirmed human autochthonous case of Lyme disease and includes the first available *Borrelia* sequence of Mexico.

B) Compilation of *Borrelia* studies in Mexico from published records

Methodology for databases

In addition to the written informed consent signed by the patient for the Case Report, the current study was approved by the Ethics and Research Committee of the Medical Faculty,

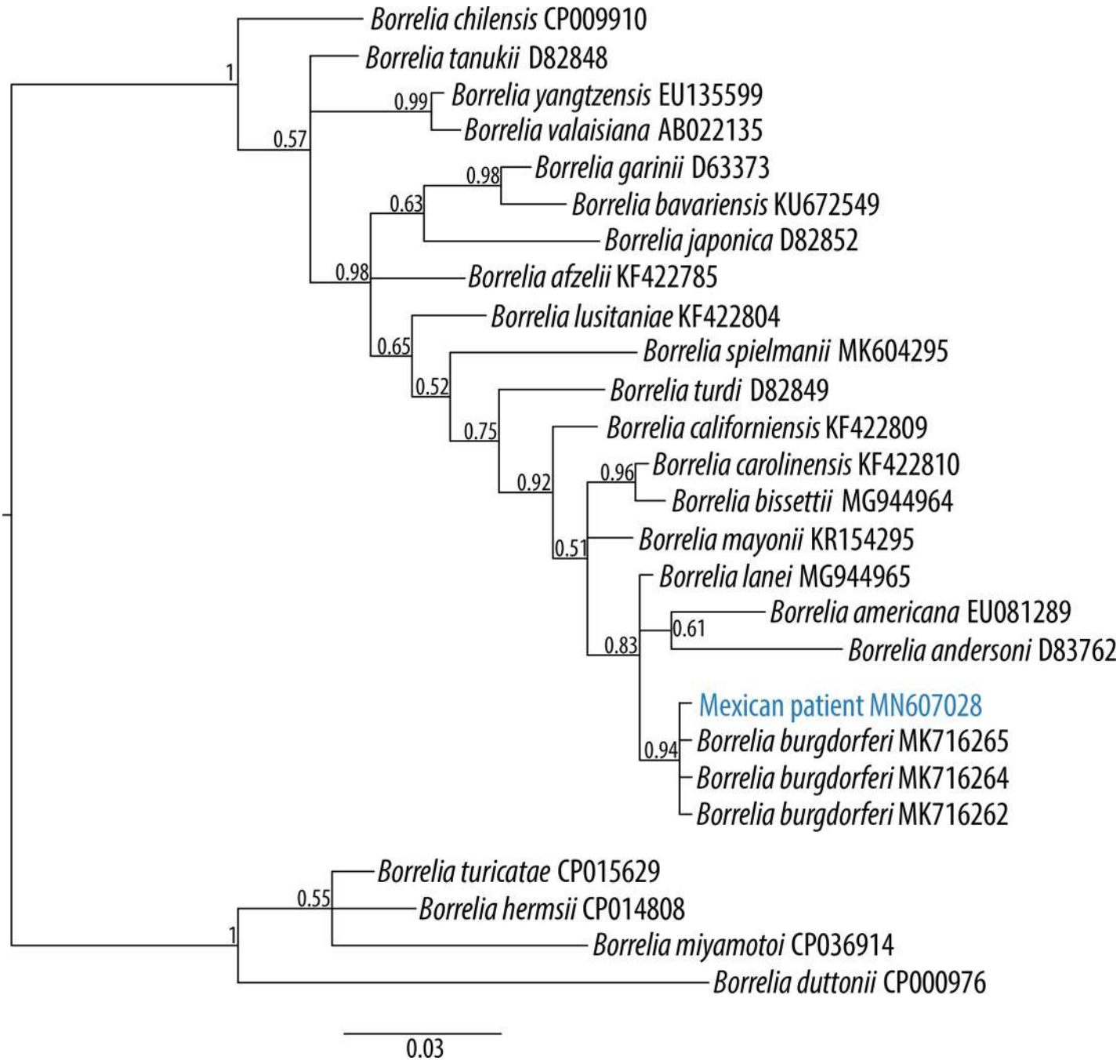

Fig 1. Phylogenetic reconstruction for *flagellin* gene fragment of the Mexican patient with Lyme disease and of several members of the genus *Borrelia*. The phylogenetic relationships were inferred by Bayesian Inference based on the HKY+G substitution model with a total of 255 bp. Posterior probabilities >0.5 are indicated at nodes. Number in parentheses are GenBank accession numbers. Scale bar indicates nucleotide substitutions per site. Blue sequence indicates isolate obtained from the recent Mexican patient.

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UNAM (Comisiones de Investigación y de Ética de la División de Investigación de la Facultad de Medicina, UNAM), with approval number FM/DI/088/2017.

An extensive research of the literature was made to compile a database of published studies of *Borrelia* in human cases, mammals and ticks in Mexico from 1939 to 2020. A combination

of keywords: "Borrelia", "borreliosis", "mammals", "Mexico" and "human cases" were used in specialized databases including BioOne, Elsevier, Highwire, Iris, JSTOR, Pubmed, Scopus, SpringerLink, Wiley Online, Web of Science and Zoological Records, as has been previously proposed [63]. Articles reported in those studies were also analyzed by cross-referencing. Studies published in national and international journals were included.

Human database. For the human database, the information was analyzed according to: (i) *Borrelia* species, (ii) number of human cases, (iii) sex, (iv) age group (newborn [0–2 yo], childhood [3–12 yo], teenager [13–18 yo], adult [19–60 yo] and elderly population [+60 yo]), (v) type of population (rural or urban), (vii) vector species, (viii) year of publication, (ix) detection method and (x) locality.

Additionally, information data from "National Health Information System [Sistema Nacional de Información en Salud] (SINAIS)" published by the Mexican Ministry of Health [Secretaría de Salud] [64] were obtained. This database includes all human borrelia-cases reported between 2000–2013 to the Ministry of Health. All the cases were georeferenced according to the "Catalogue of Keys from the Federal States, Municipalities and Localities" [Catálogo Único de Claves de Áreas Geoestadísticas Estatales, Municipales y Localidades] [65]. The SINAIS database did not include personal information of patients, nor the method of identification of the pathogens, for this reason, these records were only included for the geographical analysis.

Animal database. From each study on mammals and/or ticks, the following information was recorded: (i) *Borrelia* species, (ii) number of positive animals for each species, (iii) order, family, genus and species of the mammalian host (if available), (iv) tick family, genus and species (if available), (v) year of collection, (vi) detection method and (vii) locality. Wild mammalian taxonomy was updated following the most recent taxonomical review for Mexico [66]. For ticks, a review for each genus was used [67–70].

Database analyses. In order to present the first distribution map of *Borrelia* species, we generated a colorimetrical map in R, using the open access layers provided by CONABIO [Comisión Nacional para el Conocimiento y Uso de la Biodiversidad http://www.conabio.gob.mx/informacion/gis/] and functions from the R packages *viridis* [71], *tidyverse* [72], *maps* [73], *ggrepel* [74], *mapproj* [75] and *plotly* [76]. Maps were made for human cases, mammal and tick records, as well as a global map of total reports in Mexico, highlighting the zones with the highest number of cases, using the free and open source geographic information system, QGIS 2.18.9 [https://qgis.org/en/site/].

Results of databases for *Borrelia* studies in Mexico

A total of 1,347 records were obtained from 39 published studies of *Borrelia* in Mexico. Of the total cases, 29.5% (398/1,347) corresponded to humans and 70.5% (949/1,347) were of other animal species (S1 and S2 Tables). Only one study reported both human and animal infections (S1 Table).

Borrelia species were specified in 54% (727) of the 1,347 cases. In the remaining 46% (620 cases), the etiological agent was identified as a member of the *B. burgdorferi* s.l. complex or as a member of the RF group. A total of six *Borrelia* species have been reported in Mexico [*B. afzelii*, *B. burgdorferi* s.s., *B. duguesii*, *B. garinii*, *B. mazzottiii* and *B. turicatae*] (Table 2), of which two are members of the LB group and four of RF.

The most frequent tests used for *Borrelia* detection were serological tests (Enzyme-Linked Immunosorbent Assay [ELISA], Western blotting and immunofluorescence assay [IFA]), used in 25 studies. This was followed by molecular tests (PCR) used in 11 studies. Five non-confirmed human cases were diagnosed by clinical manifestations and light microscopy.

Table 2. *Borrelia* species reported in Mexico.

Borrelia group	*Borrelia* species	No. of records	Type of host
RF	***B. duguesii***	1	Animal
	B. mazzottii	1	Animal
	B. turicatae	549	Animal
LB	***B. afzelii***	2	Human
	B. burgdorferi s.s.	128	Human
		36	Animal
	B. garinii	10	Human

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Human cases. In Mexico, 398 human cases of *Borrelia*-infection were recorded between 1939 and 2020. The most frequent diagnosis was LB in 98.7% (393/398) of the cases. RF was only diagnosed in 1.3% (5/398) of the human cases. Of the 393 cases with LB, in 35.6% (140/393) the etiological agent was identified (*B. afzelii*, *B. garrinii* and *B. burgdorferi* s.s.), using specific diagnosis tests (S1 Table). In contrast, no RF cases reported the infecting *Borrelia* species.

The gender of patient was specified in 231 (58%) of the 398 notified cases (S1 Table). The proportion between sexes was higher in females (134 cases) than males (97 cases). In patients with LB, women were the most frequent group with 58% (131/226), as compared to males showing 42% (95/226) of the cases (S1 Table). The remaining five patients corresponded to RF, which showed an almost equal distribution between sexes, with three cases being females and two cases were males.

The type of community of origin was referred only in 53.5% (213/398) of the cases (S1 Table). Of these 213 cases, 95.7% (204/213) corresponded to urban environments, the remaining 4.3% (9/213) were from rural areas. Human cases of RF showed that 80% (4/5) were reported in rural communities, whereas LB patients were mostly detected in urban communities 97.5% (203/208).

The age of patients was not specified in 94.4% (376/398) of all human cases, yet the remaining 22 cases (17.5%) corresponded mostly to adults 77.2% (17/22).

Animal reports. A total of 949 records of 17 mammalian species (nine wild species, three domesticated species, and two peri-domestic mammals) of six orders were reported as infected by *Borrelia* sp. in 19 studies from Mexico (Table 3; S2 Table). Four species of *Borrelia* were identified, three of which were members of the RF group (*B. duguesii*, *B. mazzottii* and *B. turicatae*) and only one from the LB group (*B. burgdorferi* s.s.) (S2 Table). The RF group was reported in 551 of the 949 records (58%), whereas members of the *B. burgdorferi* s.l. complex were reported only in 398 records [41.9%] (S2 Table).

The order Rodentia showed the highest number of infected species with nine rodent positive species, followed by carnivores with three (Table 3). In both of these mammalian orders, only the *B. burgdorferi* s.l. complex was recorded. In vertebrate hosts, the samples most frequently used for detecting *Borrelia* DNA was blood and/or tissues (305 records). Studies on the 305 mammalian samples only showed infection of an unknown species of the LB group.

Studies on arthropods and potential *Borrelia* vectors revealed 14 species: three soft ticks of the family Argasidae and 11 species of hard ticks of the family Ixodidae (Table 4), of which *I. scapularis* was the most common tick species positive for *Borrelia* DNA (Table 4; S2 Table). Studies on ticks as vectors were done in 570 records. Only 75 records were obtained from ticks retrieved from mammals (S2 Table).

Studies done with free-living ticks (569 records) showed that 96.7% (550/569) contained *Borrelia* causing RF, whereas only 19 records [3.3%] reported the LB group (S2 Table). The specificity of the *Borrelia* groups (only reported in ticks) showed that RF members were

Table 3. Mammalian species associated to *Borrelia* in Mexico.

Mammalian species			Borrelia species
Order	**Family**	**Species [English common name/Spanish common name]**	
Artiodactyla	Cervidae	*Odocoileus virginianus* [White-tailed deer/Venado cola blanca]	*B. burgdorferi* s.l.
	Bovidae	*Bos taurus** [Aurochs /Toro]	*B. burgdorferi* s.l.
Carnivora	Canidae	*Canis lupus familiaris* [Dog/Perro]	*B. burgdorferi* s.l.
		*Vulpes macrotis** [Kit Fox /Zorra del desierto]	*B. burgdorferi* s.s.
	Felidae	*Panthera onca** [Jaguar/Jaguar]	*B. burgdorferi* s.l.
	Procyonidae	*Bassariscus astutus** [Ringtail/Cacomixtle]	*B. burgdorferi* s.l.
			B. burgdorferi s.s.
Lagomorpha	Leporidae	*Sylvilagus floridanus** [Eastern Cottontail/Conejo]	*B. burgdorferi* s.s.
			B. burgdorferi s.l.
Perissodactyla	Equidae	*Equus caballus* [Horse/Caballo]	*B. burgdorferi* s.l.
Primates	Hominidae	*Homo sapiens** [Human/Humano]	*B. burgdorferi* s.s.
Rodentia	Cricetidae	*Microtus mexicanus* [Mexican Vole/Meteorito]	*B. burgdorferi* s.l.
		Neotoma mexicana [Mexican Woodrat/Rata magueyera]	*B. burgdorferi* s.l.
		*Neotoma micropus** [Southern Plains Woodrat/ Rata magueyera]	*B. duguesii*
		Neotomodon alstoni [Volcano Deermouse/Ratón de los volcanes]	*B. burgdorferi* s.l.
		Peromyscus leucopus [White-footed Deermouse/Ratón]	*B. burgdorferi* s.l.
		Peromyscus maniculatus [North American Deermouse/Ratón]	*B. burgdorferi* s.l.
	Heteromyidae	*Heteromys pictus** [Painted Spiny Pocket Mouse/Ratón espinoso]	*B. burgdorferi* s.l.
	Muridae	*Mus musculus* [House Mouse/Ratón de casa]	*B. burgdorferi* s.l.
		Rattus rattus [Roof Rat/Rata negra]	*B. burgdorferi* s.l.

All wild species were updated according to the last taxonomic review of Ramírez-Pulido et al. [66]. Mammalian English common names were updated according to Wilson and Reeder [77] and Spanish common names according to Ceballos and Oliva [78].

* In these studies, the authors did not include host samples when testing for *Borrelia* DNA.

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recovered from argasid tick species, whereas the LB members were only detected in Ixodid tick species.

Geographic analysis. A total of 1,364 cases (both human and animal) were retrieved from published studies (1,347 cases) and from the National Health Information System (17 cases), of which 1,166 (85.4%) were geo-referred (at least at State level). These cases were distributed in 18 of the 32 Mexican states (Fig 2; S1 and S2 Tables). The state with most records was Aguascalientes (409/1,364 = 30%), followed by Nuevo Leon (253/1,364 = 18.5%) and Guanajuato (143/1,364 = 10.4%). The states with no records were Baja California Sur, Campeche, Chiapas, Colima, Durango, Guerrero, Hidalgo, Puebla, Queretaro, Tlaxcala and Zacatecas (Fig 2).

The highest diversity of *Borrelia* species was shown in the central and northeastern regions of the country, being Mexico City, the state with the largest number of species registered (3/6 = 66.7%), followed by Coahuila with two species [2/6 = 33.3%] (Fig 3).

The 1,166 geo-referred cases published both in the literature and by the National Health Information System corresponded mostly to animals 939 (80.5%) and only 19.4% (227) to human cases. Human cases were distributed in northeastern and central Mexico, mostly in the state of Nuevo Leon, where 69 of the 244 geo-referred human cases (28.2%) were reported with regard to their place of origin. This was followed by Tamaulipas with 18.8% (46/244) and Mexico City with 17.2% [42/244] (Fig 4).

Borrelia-infected animals have been reported in northeastern and northwestern regions, central and southeastern Mexico, with the highest density occurring the state of

Table 4. Tick species recorded to be associated with *Borrelia* species in Mexico.

Tick species		Mammalian species	*Borrelia* species
Family	**Species**		
Argasidae	*Ornithodoros turicata*	ND	*B. turicatae*
	Ornithodoros duguesi	*Neotoma micropus*	*B. duguesii*
	Ornithodoros talaje	ND	*B. mazzottii*
Ixodidae	*Amblyomma americanum*	*Homo sapiens sapiens*	*B. burgdorferi* s.s.
	Amblyomma cajennense	*Bos Taurus*	*B. burgdorferi* s.s.
		Canis lupus familiaris	*B. burgdorferi* s.s.
		ND	*B. burgdorferi* s.s.
	Amblyomma mixtum	*Canis lupus familiaris*	*B. burgdorferi* s.l.
	Dermacentor andersoni	ND	*B. burgdorferi* s.s.
	Dermacentor variabilis	ND	*B. burgdorferi* s.l.
	Ixodes affinis	*Canis lupus familiaris*	*B. burgdorferi* s.l.
	Ixodes kingi	*Vulpes macrotis*	*B. burgdorferi* s.s.
	Ixodes scapularis	*Heteromys pictus*	*B. burgdorferi* s.l.
		Panthera onca	*B. burgdorferi* s.l.
		Sylvilagus floridianus	*B. burgdorferi s.s.*
		Sylvilagus floridianus	*B. burgdorferi* s.l.
		ND	*B. burgdorferi* s.l.
	Ixodes spinipalpis	ND	*B. burgdorferi* s.l.
	Ixodes texanus	*Basariscus astutus*	*B. burgdorferi s.s.*
	Ixodes tovari	ND	*B. burgdorferi* s.l.
	Rhipicephalus sanguineus s.l.	*Canis lupus familiaris*	*B. burgdorferi* s.l.

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Aguascalientes, that reported 406 of the 938 animals (43.2% with known place of origin), Nuevo Leon (184/938 = 19.6%) and Guanajuato (142/938 = 15.1%). The lowest density was reported in the central states of Mexico (Michoacan, Coahuila, Jalisco and San Luis Potosi) with less than 1% of the reports (Fig 5).

Discussion

The first record of *Borrelia* in Mexico was reported by Brumpt [79], who recorded *B. turicatae* in specimens of the questing soft tick, *O. turicata*, collected in the states of Aguascalientes, Guanajuato and San Luis Potosi. The first report of *Borrelia* in humans was made by Pilz and Mooser [80], who described three cases of RF in Aguascalientes, based on microscopic evidence of spirochetes in thick-film samples and on symptoms of the patients [80].

It was not until the 1990s when the first record of human exposure to LB was published in Mexico by Maradiaga-Ceceña et al. [81], who reported 32 humans seropositive to strains from the LB group in Sinaloa, yet these studies did not identify the infecting species.

The first report of *Borrelia burgdorferi* s.l. in animals was made by Salinas-Meléndez et al. [82] in DNA obtained from blood of an infected dog. Thus, publications on Lyme disease in Mexico started in the decade between 1990–1999. During this period, the Medical Research Unit of Infectious and Parasitic Diseases of the Mexican Social Security Institute [Unidad de Investigación Médica de Enfermedades Infecciosas y Parasitarias, Centro Médico Nacional SXXI, Instituto Mexicano del Seguro Social] began to study Lyme disease in Mexico. Further studies were done in the states of Nuevo Leon, Sinaloa and Mexico City, that reported human and animal seropositivity throughout the country [81–85].

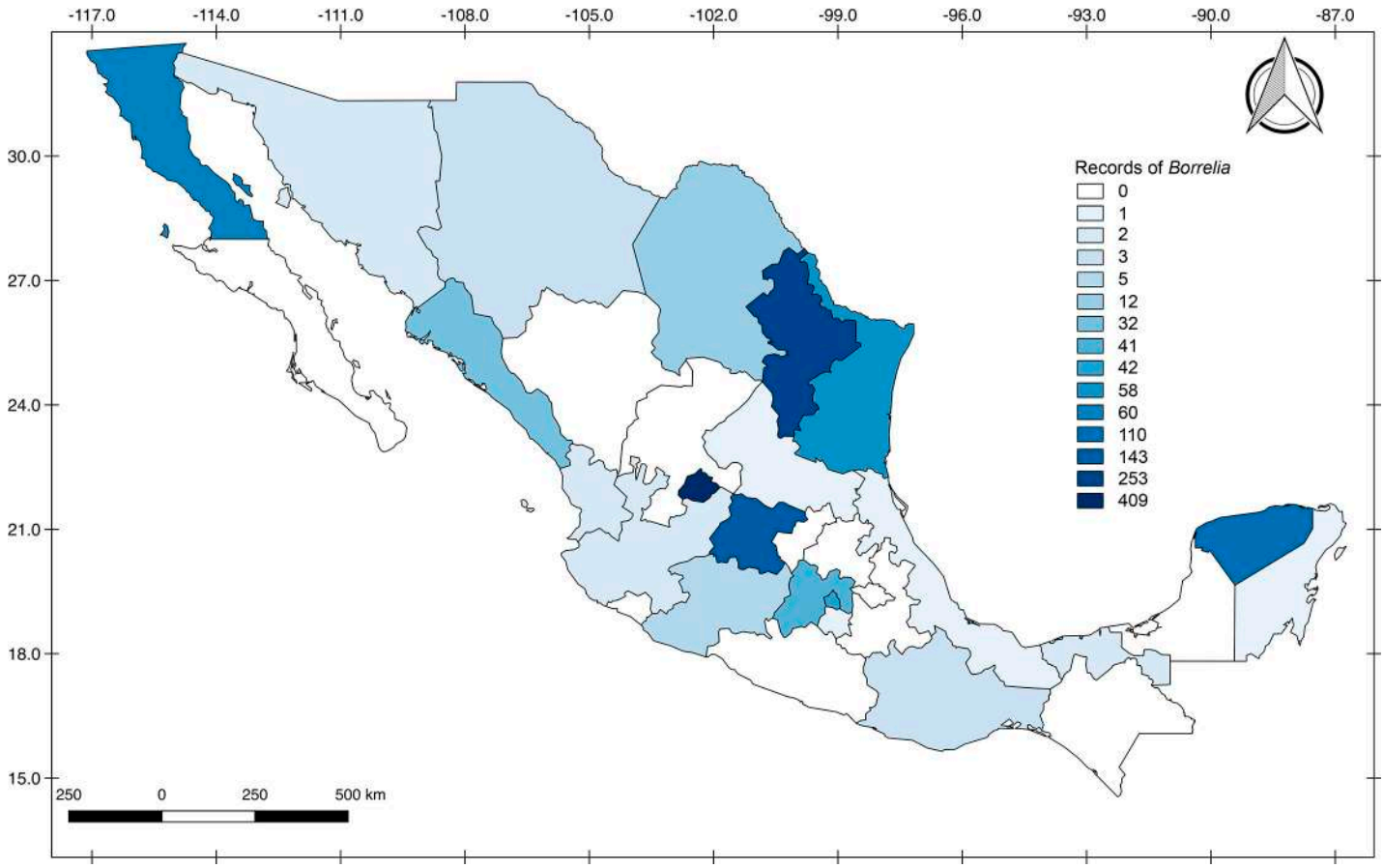

Fig 2. Geographic distribution of the 1,364 geo-referred records of *Borrelia* in Mexico. Records include both human cases and animal infections or expositions to *Borrelia* in Mexico from 1939 to 2020.

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The introduction of confirmatory tests based on PCR and Western blotting allowed a more accurate diagnosis, as compared to traditional tests (thick blood smear and microscopy) [82, 85]. Yet it was not until the early 2010s, that interest in *Borrelia* research intensified and the numbers of publications increased dramatically. Different research centers began to study the distribution of *Borrelia* in animals and human cases [86–88]. More animal species were screened for *Borrelia* DNA and veterinarians started to perform epidemiological surveys in domestic and wild animals [89, 90].

Thus, studies on Lyme disease in Mexico are recent and focus mainly on comparing LB with the other *Borrelia* groups, both in human cases as well as in animals [6, 88, 91]. Historically, LB has been identified in the majority of human cases (almost 97%) in Mexico, as compared to RF. Yet, considering the drastic increase of human cases of borreliosis in countries where the disease is endemic [19, 92, 93], in Mexico an enhanced effort to diagnose and confirm the infection by species of the LB group still needs to be accomplished. Is important to highlight that human cases of LB are mostly reported in urban communities, however, considering the life cycle of ixodid ticks and the transmission cycle of *B. burgdorferi* s.l., it is unlikely that infections originated in these urban areas, since tick larvae and nymphs feed mainly on wildlife [2, 94]. Therefore, it is more likely that humans become infected after incursions into forest areas, where they are exposed to ticks.

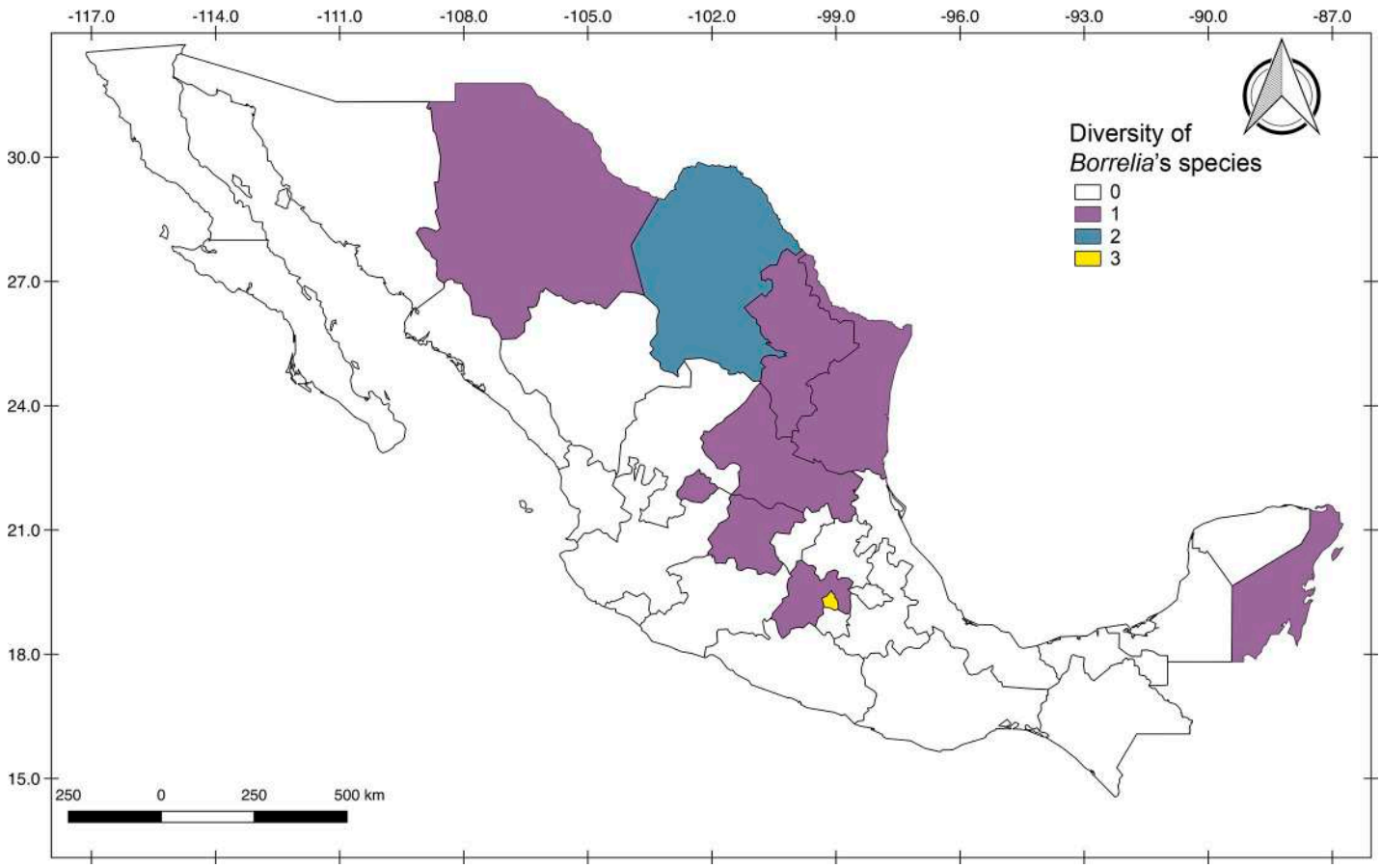

Fig 3. Geographic distribution of the genus *Borrelia* in Mexico according to species richness. Colors indicates the number of species of the bacterial genus per State. Records of bacteria defined as *B. burgdorferi* s.l. were not included, since the species was not specified.

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Currently, it is not possible to establish the origin of RF infections, although the available data seem to indicate that rural communities are the main origin of the infections, yet further studies are required to show a more complete epidemiological panorama. Since RF is regarded a forgotten and neglected tropical disease [15, 95], and given the scarce number of cases with RF reported by the Mexican Ministry of Health [Sistema Nacional de Información en Salud, Secretaría de Salud], it is very likely that many of the patients that are currently reported as having "fever of unknown origin", lacking identification of the etiological agent, are actually patients with RF [64]. It is therefore imperative to consider RF as a differential diagnosis in these types of cases, especially in rural communities, where most of the cases of RF have occurred in Mexico and where a higher risk of contracting the disease exists, as compared to urban communities [15, 95].

Taken together, the current lack of data on both diseases prevent an accurate epidemiological analysis to be made, nor can risk factors for LB or RF be established in Mexico. However, with the information obtained so far, women appear to be more susceptible to *Borrelia* infections than men, both for LB as for RF. This tendency is similar to data reported in Europe and the US [15, 19, 96].

When analyzing the clinical manifestations reported by patients with suspected Lyme disease in Mexico, including the confirmed case report of this study, the general manifestations include fatigue, fever, arthralgia, paresthesia and myalgias [83, 87, 88, 97, 98]. Chronic Lyme

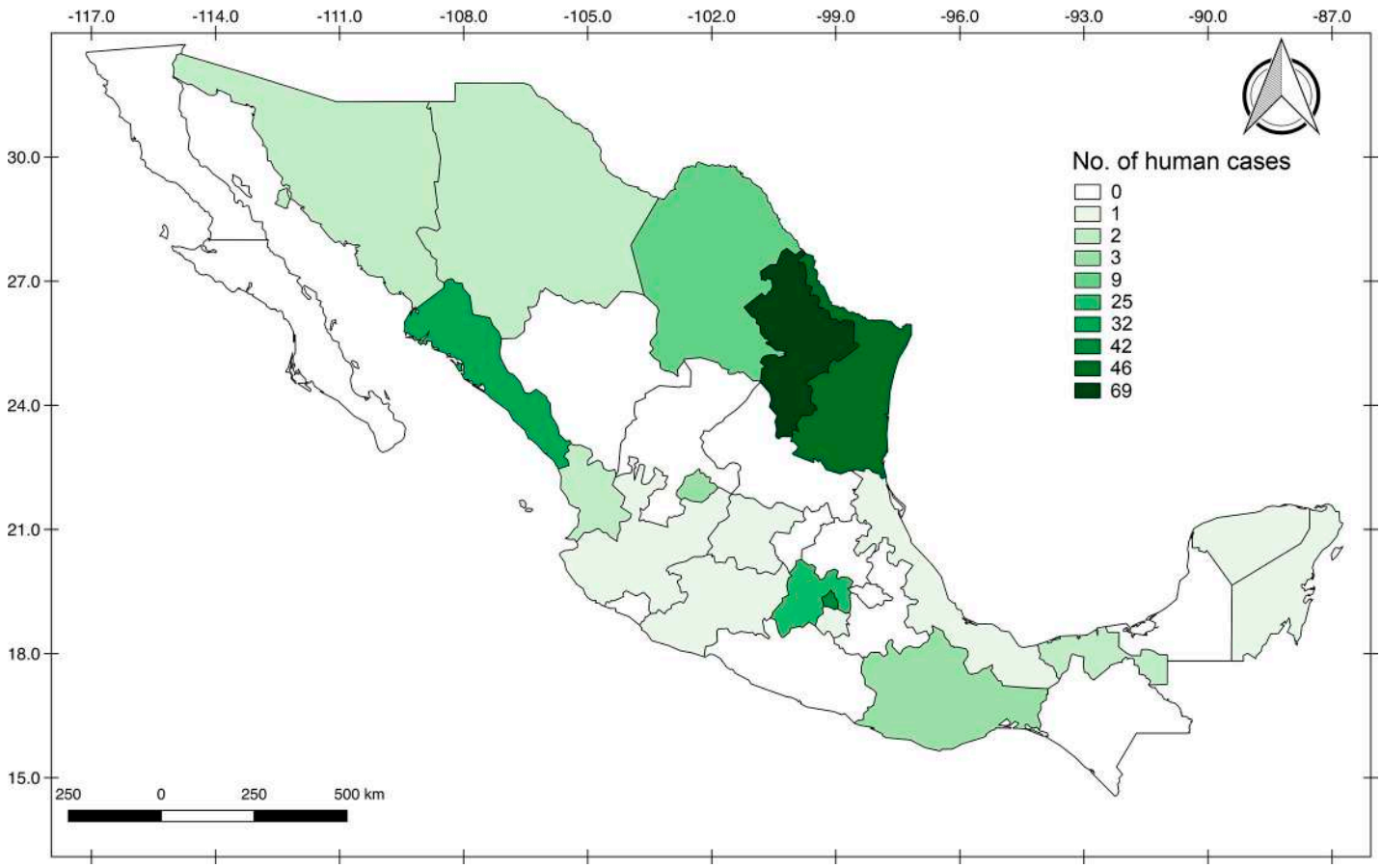

Fig 4. Geographic distribution of *Borrelia*-infected human cases in Mexico. Darker color represents higher density.

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disease often is accompanied by neurological symptoms of neuroborreliosis, including symmetric paresthesia with ascending distribution in all four limbs, meningo-polyradiculoneuropathy (motor and sensory), arthritis [86, 99] and facial palsy, mainly in children [98]. Dermal lesions include irregular and regular *erythema migrans* with reddish edges, pink center, with a clear mononuclear cell infiltrate in the superficial and deep dermis. Additionally, borrelial lymphocytoma lesions with dense nodular lymphocytic infiltrates in the reticular dermis and well-delineated lymphoid follicles can be found. However skin lesions such as acrodermatitis chronica atrophicans are rare [83, 86, 100]. Taking into account the symptoms of the currently reported case, which include diplopia and bilateral hypoesthesia in both upper and lower limbs, tremors in the arms and short-term memory loss, we suggest that these should also be considered as possible symptoms for Lyme disease in Mexican patients.

On the other hand, clinical manifestations reported for RF in Mexican patients differ from those of Lyme disease, since RF patients show fever paroxysms lasting between 2–7 days, which alternate with periods (4–12 days) of apyrexia. The fevers oscillate between 38.5˚C and 40.8˚C, preceded by intense chills. The most common manifestations are headache, exanthems and weakness (found in three of the cases), as well as splenomegaly, hepatomegaly, diaphoresis, epistaxis and photophobia [80, 101]. Blood counts are characterized by eosinophilia, increased platelet counts and moderate leukocytosis, with 80% polynucleated cells [80].

The analysis of *Borrelia* hosts and vectors in Mexico has shown that most of the *Borrelia* infections in animals have been studied in the order Rodentia, which is considered a potential

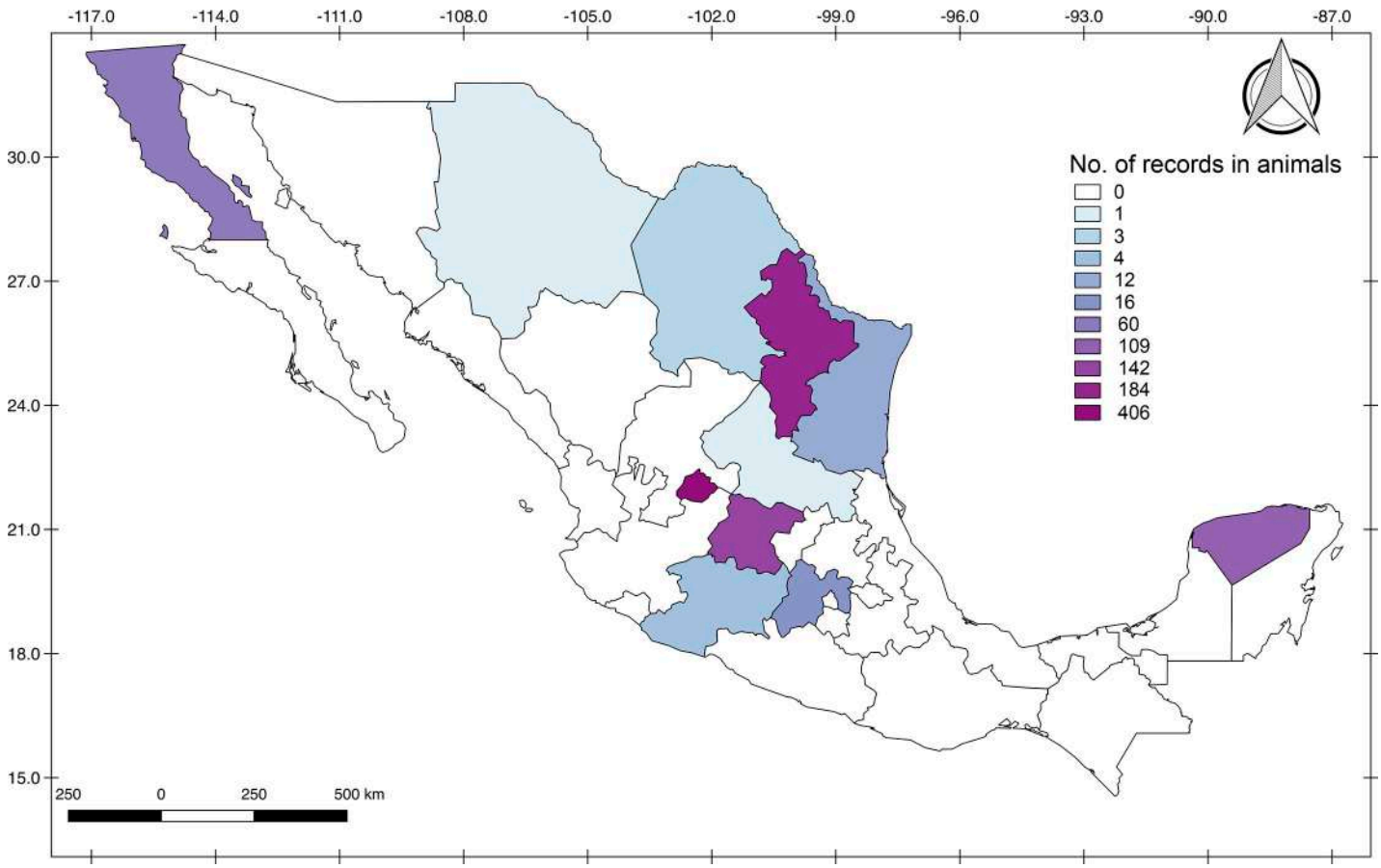

Fig 5. Geographic distribution of *Borrelia* records in wild animals from Mexico. Darker color represents higher density.

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host for *Borrelia* species. This is in accordance with the literature, where this order has been implicated as one of the most important hosts, for both the LB and the RF groups [2, 15, 19]. Thus, several species of genera *Peromyscus* and *Neotoma* have been recognized as reservoirs for many *Borrelia* species in endemic countries [2, 3, 102, 103]. For the LB species, several mammals (mainly rodents and the white-tailed deer) have also been shown to be hosts in Mexico, in which only three *Borrelia* species have been reported. This low number contrasts with the number of species reported in countries endemic for *Borrelia*, such the USA, where nine species of the LB group have been reported [5, 17, 67, 104].

With regard to vector studies done in Mexico, these show that four of the 26 species of the genus *Ixodes* (*I. affinis*, *I. pacificus*, *I. scapularis* and *I. spinipalpis*) are confirmed vectors of species of the LB group. In contrast, only three vectors have been confirmed for *Borrelia* species of the RF group (*Ornithodors coriaceus*, *Ornithodoros turicatae* and *I. scapularis*), which affect several host species (rodents, deer and dogs). The diversity of RF species (three species) reported in Mexico is low, as compared to the eight species reported in the US [3, 15, 67–69]. This highlights the importance of specifying the tick specimens (mainly those recognized as competent vectors) recovered from various hosts, irrespective of whether they have been recognized as reservoirs. Thus, *I. scapularis*, *I. texanus* and *O. duguesi* specimen retrieved from jaguars, cattle, ringtails and rabbits have been shown to test positive for *Borrelia* (S2 Table). The analysis of diverse wild and domestic animal species is relevant, since they could be

playing a role as maintenance hosts for ticks in the ecosystem, as has been evidenced in white-tailed deer [105, 106].

Taken together, the low host and vector diversity reported in Mexico calls for future studies to update the existing historical records [26, 79, 107]. The important difference in the numbers of registered species between Mexico and the US, an endemic and hotspot of the genus *Borrelia*, is probably due to inadequate sampling in Mexico, but mainly because of the use of serological tests without further confirmation of the bacterial presence. It seems warranted to predict higher numbers of both hosts and vectors in Mexico due to important climatic varieties and diversity of ecosystems that provide ideal habitats and opportunities for sustaining diverse vector and host species of this bacterial genus [67, 70, 78].

Previous reports on the diversity of *Borrelia* species in central and northeastern Mexico show that areas with the highest number of records overlap with those showing high probabilities of occurrence of ticks of the genus *Ixodes* [108]. This observation contrasts with the report of Illioldi-Rangel et al., [108] stating that the state of Durango stands out as having a high density of *Ixodes*, yet no records of *Borrelia* have been reported. Clearly, more studies need to be conducted in Durango to validate potential distribution maps.

In the case of the RF, the existing records are related to the distribution of their vectors [79, 81]. The importance of analyzing potential distribution of vectors is based on the fact that it not only permits to direct sampling efforts, but is also crucial for knowledge on vector-borne pathogens in sub-sampled areas and for optimizing epidemiological surveys [108, 109].

To the best of our knowledge, there are no associations between human cases with those of animals or vectors in Mexico. Although human cases have been reported the states Morelos, Oaxaca, Quintana Roo, Tabasco, Veracruz and Mexico City, animal studies are lacking. This situation has also been shown for another groups of pathogens in Mexico such as viruses [63]. The human cases reported in Mexico City most likely refer to patients that were transferred from regional health centers to larger hospitals in the city for better diagnosis and treatment. Natural transmission of *Borrelia* within Mexico City seems highly unlikely. This phenomenon has also been observed for other diseases, in which diagnostic centers are concentrated in larger cities [110]. Sinaloa and Nuevo Leon have become important states, where most of the human *Borrelia* transmission has been reported in Mexico. Regrettably, many cases have only been reported in local epidemiology reports, in pathology departments of hospitals or clinics, or are submitted to local journals with low accessibility and limited distribution, making the information on *Borrelia* difficult to obtain, thus generating inaccurate data.

Since several international organizations and centers have recognized the diseases caused by *Borrelia* as neglected or as a major health threat [1, 93, 95], this now shows the necessity to establish a surveillance program and a specialized reference research center in Mexico, where isolates and *Borrelia* strains can be collected to facilitate more precise information on these pathogens, as well as to identify more specific antigens that could improve the diagnosis.

We consider that our geographic approach with spatial distribution data will now provide valuable information on *Borrelia* for human and animal health authorities and may also be relevant for future control and prevention programs, as well as a guide to direct capture efforts for specific animal studies. Creating a universal and open access database of all published records for scientists, entomologists and public health authorities, can help establish linkages among groups working with the genus *Borrelia* in Mexico. Even though the study of the genus *Borrelia* in Mexico is currently poorly assessed, new work groups are being formed and existing groups are consolidating, which together will increase the knowledge of this bacterial genus in Mexico and complete the epidemiological panorama.

Supporting information

S1 Table. Borreliosis human cases reported in Mexico.
(DOCX)

S2 Table. *Borrelia* detection in Mexican animals.
(DOCX)

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CONCLUSIONES GENERALES

Este estudio generó conocimientos nuevos sobre la presencia de *Borrelia* en vertebrados terrestres y sus garrapatas de la región central de México:

1. Se reporta por primera vez la presencia del grupo de *Borrelia* asociada con reptiles en Norteamérica, específicamente en Veracruz, México.
2. Se registró por primera vez la asociación entre *Borrelia* y *Amblyomma dissimile*.
3. Se reporta un linaje nuevo, el cual probablemente sea una especie nueva del grupo *Borrelia* asociada con reptiles, específicamente del grupo de las serpientes.
4. Se reporta por primera vez la presencia de *Borrelia* en murciélagos de México.
5. Se encontraron dos linajes nuevos, posiblemente dos nuevas especies de *Borrelia* en murciélagos, uno del grupo de *Borrelia burgdorferi* s.l. filogenéticamente relaciona con *Borrelia turdi*; el segundo linaje relacionado con *Borrelia miyamotoi*, del grupo de las fiebres recurrentes.
6. Se registró por primera vez la presencia de *Borrelia burgdorferi* s.s. en ratones arborícolas, específicamente en Taxco de Alarcón, Guerrero y Zacualpan, Estado de México.
7. Se amplía la distribución de *Borrelia burgdorferi* s.s., reportando el registro más sureño, en Taxco de Alarcón, Guerrero.
8. Se registró por primera vez la asociación de *Borrelia* y *Habromys schmidlyi*.
9. Se identifica la posibilidad de un foco enzoótico de *Borrelia burgdorferi* s.s. en dos localidades del Cinturón Volcánico Transmexicano.
10. Se evaluó por primera vez la diversidad genética de *Borrelia burgdorferi* s.s. considerando la distribución completa de la especie.
11. Se recuperó una débil estructuración genética entre las poblaciones de México-China-Alemania, Este y Centro de EUA, Oeste de EUA e Italia-Luxemburgo-Alemania.
12. Se identificó una incipiente diferenciación genética en las poblaciones del Oeste de EUA y las de México, probablemente debido a sus historias

evolutivas, así como a procesos ecológicos y ambientales.

13. Se analizó el panorama epidemiológico de la borreliosis a nivel nacional, generando la información geográfica nacional sobre la presencia de *Borrelia* en población humana y mamíferos silvestres Mexicanos.
14. Se resalta la importancia del bosque mesófilo de montaña del centro del país, como una zona de importancia para el estudio del grupo de *Borrelia burgdorferi* s.l.
15. Se resalta la zona tropical de Veracruz, como una zona importante en la diversidad del grupo de las fiebres recurrentes, así como del grupo de *Borrelia* asociada con reptiles.

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