Universidad Autónoma de Tlaxcala

Posgrado en Ciencias Biológicas

Macroevolución, diversificación y datación de linajes del género de arañas *Loxosceles* Heineken & Lowe, 1832 (Araneae: Sicariidae) de Norteamérica

T E S I S

QUE PARA OBTENER EL GRADO ACADÉMICO DE

DOCTORA EN CIENCIAS BIOLÓGICAS

P r e s e n t a

Claudia Isabel Navarro Rodríguez

Director

Dr. Alejandro Valdez Mondragón

Tlaxcala, Tlax. Febrero, 2025

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Comité tutoral

Dr. Alejandro Valdez Mondragón

Dr. Arturo Estrada Torres

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HOJA DE FINANCIAMIENTO

Este proyecto se realizó en el Laboratorio de Aracnología Tlaxcala (LATLAX) del Instituto de Biología de la UNAM, financiado por el proyecto de Ciencia Básica CONACyT 2016 No.: 282834, titulado: “Arañas de Importancia Medica: Taxonomía integrativa basada en evidencia molecular y morfológica para la delimitación de las especies de arañas mexicanas de arañas violinistas del género *Loxosceles* Heineken & Lowe (Araneae: Sicariidae)-Etapa 1”.

Asimismo, el Programa de Doctorado en Ciencias Biológicas del Centro Tlaxcala de Biología de la Conducta (CTBC) de la Universidad Autónoma de Tlaxcala (UATx) está registrado en el Programa para el Fortalecimiento del Posgrado Nacional y en el Sistema Nacional de Posgrados (SNP)

COORDINACIÓN DEL DOCTORADO EN CIENCIAS BIOLÓGICAS
UNIVERSIDAD AUTÓNOMA DE TLAXCALA
P R E S E N T E

Los abajo firmantes, miembros del jurado evaluador del proyecto de tesis que **Claudia Isabel Navarro Rodríguez** realiza para la obtención del grado de **Doctora en Ciencias Biológicas**, expresamos que, habiendo revisado la versión final del documento de tesis, damos la aprobación para que ésta sea impresa y defendida en el examen correspondiente. El título que llevará es **"Macroevolución, diversificación y datación de linajes del género de arañas *Loxosceles* Heineken & Lowe, 1832 (Araneae Sicariidae) de Norteamérica".**

Sin otro particular, aprovechamos para enviarle un cordial saludo.

A T E N T A M E N T E
TLAXCALA, TLAX., A 09 DE OCTUBRE DEL 2024

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COMITÉ ACADÉMICO
POSGRADO EN CIENCIAS BIOLÓGICAS

Sirva este medio para describir el proceso de revisión de la tesis realizada por el estudiante **Claudia Isabel Navarro Rodríguez** titulada **"Macroevolución, diversificación y datación de linajes del género de arañas *Loxosceles* Heineken & Lowe, 1832 (Araneae Sicariidae) de Norteamérica"** para optar por el grado de **Doctora en Ciencias Biológicas.**

La tesis de **Claudia Isabel Navarro Rodríguez** fue revisada por mí como director de tesis, por los miembros del comité tutoral y por los miembros del comité de examen de grado. En día 27 de enero de 2025, la versión final del documento de tesis se sometió a un análisis de similitud en el programa **Copy-Spider,** marcando poco texto con similitudes **0.23%** (valores admitidos por el programa). Examinando a detalle el resultado, las partes detectadas como similitudes están relacionadas con nombres de instituciones, nombres de los estados de México, de autores y frases de uso común.

Por lo anterior, confirmo que **el estudiante no incurrió en ninguna practica no deseable** en la escritura de la tesis, la cual es un documento original.

Sin mas por el momento reciba un cordial saludo.

CORDIALMENTE
La Paz, Baja California Sur, México; a 27 de enero de 2025

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Investigador Titular "A"
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AGRADECIMIENTOS

Al Posgrado en Ciencias Biológicas del Centro Tlaxcala de Biología de la Conducta (CTBC) de la Universidad Autónoma de Tlaxcala (UATx), por las facilidades otorgadas para desarrollar el proyecto de doctorado.

A la Secretaría de Ciencias, Humanidades, Tecnología e Innovación (SECIHTI) por el apoyo económico a través de una beca (855438), para poder realizar este proyecto de doctorado.

A los miembros del Comité Tutoral, Dr. Alejandro Valdez Mondragón, Dr. Arturo Estrada Torres, Dr. Juan José Morrone Lupi, Dra. Bárbara Cruz Salazar, Dr. Jorge Vázquez Pérez y Dr. Julián Bueno Villegas, por sus revisiones, observaciones y comentarios acertados para mejorar este proyecto. Agradezco enormemente el tiempo invertido en este trabajo.

Al Laboratorio de Aracnología (LATLAX) del Instituto de Biología de la UNAM bajo la dirección del Dr. Alejandro Valdez Mondragón, a quien agradezco especialmente por su apoyo y guía académica, por brindarme las herramientas tanto en campo como el laboratorio para el desarrollo de este proyecto.

Al Instituto de Biología (IBUNAM) y a la Colección Nacional de Arácnidos (CNAN), en especial al Dr. Edmundo González Santillán (curador) y al Dr. Oscar F. Francke (ex curador) Ballvé, por las facilidades para acceder al material biológico, para el desarrollo de este proyecto.

Al Laboratorio de Secuenciación Genómica de la Biodiversidad y de la Salud del IBUNAM, a cargo de la M. en C. Laura Margarita Márquez Valdelamar y de la M. en C. Nelly María López Ortiz, quienes apoyaron en el procesamiento de muestras biológicas.

AGRADECIMIENTOS PERSONALES

A mis padres, cuyo amor y apoyo incondicional han sido el cimiento de mi vida. Gracias por sus enseñanzas, consejos y por estar siempre presentes. Sus palabras de aliento y su ejemplo de perseverancia me inspiraron a alcanzar esta meta. Este logro también es suyo, y les estaré eternamente agradecida.

A mis queridos hermanos, Mari, Ale y Marcos, gracias por celebrar mis logros y por animarme en cada paso del camino. Los quiero mucho, "babys". A mis sobrinos, Mateo y Santiago, su alegría y energía fueron una dosis de felicidad.

A Jared, mi compañero de vida, este logro no sería posible sin tu amor y apoyo incondicional. En cada desafío, en cada desvelo, tu presencia y palabras de aliento me dieron la fuerza para seguir adelante. Gracias por hacerme reír en los momentos difíciles, por creer en mí, por ser mi inspiración y mi mayor fan. Tu aliento constante me impulsó a superar mis límites y alcanzar esta meta que hoy celebramos juntos. Te amo profundamente.

A mis compañeros del LATLAX, con quienes pude coincidir en este camino. En especial a Alma, juntas superamos momentos difíciles y compartimos tantos buenos momentos. Nuestra amistad es un tesoro que me llevo de esta etapa, y estoy segura de que seguirá brillando con el tiempo. A Sam, gracias por las risas, los chismes y los buenos momentos, tu alegría es contagiosa y siempre sabes cómo mejorar el ambiente. Y a Luis, gracias por tu apoyo y por ser un buen compañero.

A “Rebe”, Rebeca Sánchez, quiero agradecer su eficiencia y disposición para ayudarme con los trámites de titulación. Su gestión hizo que todo el proceso fuera mucho más sencillo y llevadero. ¡Gracias por tu apoyo!

Al Dr. Alejandro Valdez Mondragón, mi sincero agradecimiento por su guía académica y apoyo durante la realización de esta tesis. Su dirección enriqueció este trabajo y también brindó herramientas y conocimientos que serán de gran valor en mi futuro profesional. Agradezco su tiempo y la confianza que depositó en mí.

A todas las personas que donaron, enviaron o recolectaron material para este proyecto, les agradezco infinitamente.

Finalmente, y muy importante, a las arañas “violinistas” que hicieron posible esta investigación. Gracias por darme la satisfacción de conocer tantos lugares de México en busca de ustedes.

DEDICATORIA

Esta tesis, donde deje parte de mi vida, está dedicada a:

Jared, gracias por alentarme y acompañarme en todo el proceso, te amo.

Mi familia, por todo su apoyo, los amo.

¡La Ciencia Necesita Mujeres!

"La ciencia del futuro tiene que ser feminista, integradora y humanista"

Raquel Yotti

RESUMEN

El género de arañas de importancia médico-toxicológica *Loxosceles*, comúnmente conocidas como “arañas violinistas”, actualmente está conformado por 147 especies, siendo México el país con la mayor diversidad en el mundo con 40 especies descritas, todas las cuales, pertenecen al grupo más numeroso del género *Loxosceles*, grupo "reclusa". Sin embargo, la amplia variación morfológica en estructuras taxonómicamente importantes como los receptáculos seminales en algunas especies, aunado a su morfología general conservada, dificulta su identificación en algunos casos. El objetivo de este proyecto fue establecer las relaciones filogenéticas entre las especies del grupo “*reclusa”*, además de delimitar a las especies bajo un enfoque de taxonomía integradora. Se utilizaron dos marcadores moleculares para los análisis de delimitación: Citocromo Oxidasa subunidad I (COI) (mitocondrial) e Internal Transcribed Spacer 2 (ITS2) (nuclear). Bajo el criterio del 2% de distancias *p* no corregidas, se implementaron tres métodos de delimitación molecular: 1) ASAP; 2) GMYC, y 3) bPTP. Se realizaron análisis filogenéticos de Inferencia Bayesiana (IB) y Máxima Verosimilitud (MV), datación de linajes y reconstrucción de áreas ancestrales con una matriz concatenada que incluyó los tres genes utilizados (COI+ITS2+28S). Los análisis de delimitación incorporaron 246 terminales correspondientes a 43 especies putativas de *Loxosceles*, de las cuales, 15 se reconocen como especies nuevas, con base en la morfología y la congruencia entre los diferentes métodos moleculares de delimitación. El uso de distintas líneas de evidencia para reconocer linajes confirma que la diversidad de *Loxosceles* en México aún está subestimada, y aunque la morfología del género es conservada sobre todo en pedipalpos de los machos, sigue siendo un criterio robusto para la identificación a nivel de especie. Con base en el análisis de la datación de linajes y la reconstrucción de las áreas ancestrales, planteamos la hipótesis de que la dispersión del género ocurrió de América del Sur a América del Norte antes del cierre definitivo del Istmo de Panamá.

ABSTRACT

The spider genus of medical-toxicological importance *Loxosceles*, commonly known as “violin spiders”, currently is comprised by 147 species, being Mexico the country with the highest diversity worldwide with 40 described species, all of which belong to the largest group of the *Loxosceles* genus, the “recluse” group. However, the wide morphological variation in taxonomically important structures such as seminal receptacles in some species, coupled with their preserved general morphology, makes their identification difficult in some cases. The objective of this project was to establish phylogenetic relationships among the species of the *reclusa* group, as well as to delimit the species under an integrative taxonomy approach. Two molecular markers were used for delimitation analyses: Cytochrome Oxidase subunit I (COI) (mitochondrial) and Internal Transcribed Spacer 2 (ITS2) (nuclear). Under the 2% criterion of uncorrected *p*-distances was implemented, as well as three molecular delimitation methods: 1) ASAP; 2) GMYC, and 3) bPTP. Phylogenetic analyses were performed Bayesian Inference (BI) and Maximum Likelihood (ML), lineage dating and reconstruction of ancestral areas with a concatenated matrix (COI+ITS2+28S). The delimitation analyses incorporated 246 terminals corresponding to 43 putative species of *Loxosceles*, of which 15 are newly recognized in this work as new species, based on morphology and congruence between the different molecular delimitation methods. The use of different sources of evidence to recognize lineages confirms that the diversity of *Loxosceles* in Mexico is still underestimated, and although the morphology of the genus is mainly conserved in pedipalps of males, it remains a robust criterion for identification at the species level. Based on the analysis of lineage dating and reconstruction of ancestral ranges, we hypothesize that the dispersal of the genus occurred from South America to North America before the definitive closure of the Isthmus of Panama.

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1. INTRODUCCIÓN

El género *Loxosceles*, perteneciente a la familia Sicariidae Keyserling, 1880, está conformado actualmente por 147 especies a nivel mundial (WSC, 2025). La mayoría de las especies para el género se encuentran en América del Norte, América Central, América del Sur y África, siendo América del Norte y principalmente México el país con la mayor diversidad en el mundo, registrando un total de 40 especies descritas (Valdez-Mondragón y cols. 2019; Navarro-Rodríguez, 2019; Navarro-Rodríguez y Valdez-Mondragón, 2020; WSC, 2025).

La mayoría de las especies prefiere ambientes y hábitats secos, tales como selvas bajas o matorrales xerófilos y desiertos, no obstante, hay algunas especies que se distribuyen en ambientes tropicales como bosques mesófilos, y selvas medianas y altas, aunque en estos hábitats la diversidad disminuye de forma considerable como se ha observado para México (Valdez-Mondragón y cols. 2018a, 2018b; Navarro-Rodríguez, 2019; Navarro-Rodríguez y Valdez-Mondragón, 2020, 2024). En cuanto a los microhábitats, las especies de este género en México se encuentran frecuentemente a nivel del suelo, debajo o entre montículos de rocas, en laderas secas o cortes de pared en caminos de terracería, y en plantas secas como saguaros, biznagas, opuntias y entre cortezas de árboles como mezquites y acacias. Además, estas arañas son habitantes comunes en cuevas y grutas, principalmente en zona de penumbra y a pocos metros en zona de completa obscuridad, debajo de rocas, en paredes y grietas de roca caliza (Hite y cols. 1966; Gertsch, 1967; Gertsch y Ennik, 1983; Fischer y Vasconcellos-Neto, 2005; Potter, 2005; Binford y cols. 2008; Navarro-Rodríguez, 2019). Estas arañas también tienen hábitos sinantrópicos y pueden encontrarse dentro de casas y habitaciones humanas, en áreas donde el nivel de la luz es bajo, tales como bodegas, grietas en la pared, detrás de muebles o cuadros, bajo mesas o sillas, en estantes o aparadores que almacenen objetos por mucho tiempo (Vetter, 2015). Un ejemplo de esto es *Loxosceles tenochtitlan* Valdez-Mondragón y Navarro-Rodríguez, 2019, especie con localidad tipo en la Alcaldía de Tlalpan en la Ciudad de México, la cual ya ha sido registrada en otras ciudades de Tlaxcala, Puebla e Hidalgo, y la cual hasta el momento solo se ha encontrado en hábitats sinantrópicos (Valdez-Mondragón y cols. 2019; Quijano-Ravell y Castellanos-Sturemark, 2022).

Las arañas presentan distintos métodos de dispersión, uno de los más conocidos y sobresalientes es la técnica llamada "*ballooning*", en las que son transportadas por el viento colgando de un hilo de seda, el cual dejan salir de sus hileras al estar en un punto elevado (Nentwig y cols., 2022). Sin embargo, las especies del género *Loxosceles* perteneciente al clado Synspermiata, no realizan este método de dispersión, ya que se consideran arañas no tejedoras, las cuales se caracterizan por una baja dispersión y hábitos gregarios. De esta manera, se hipotetiza que las expansiones del rango natural de distribución ocurren lentamente y en distancias cortas, aunadas a que algunas especies han sido introducidas por la actividad humana (Binford y cols. 2008).

Respecto a la taxonomía de este grupo de arañas, con base en los caracteres morfológicos de los pedipalpos del género *Loxosceles*, se han propuesto siete grupos de especies en el mundo: *reclusa*, *laeta*, *gaucho*, *spadicea*, *vonwredei*, *rufescens* y *spinulosa* (Gertsch, 1958; Gertsch y Ennik, 1983; Binford y cols. 2008; Duncan y cols. 2010, Fukushima y cols. 2017). El grupo *reclusa* agrupa a las especies con una distribución en Norteamérica desde Estados Unidos, México, parte de Centroamérica y las principales islas de las Antillas (Gertsch y Ennik, 1983; Valdez-Mondragón y cols. 2018b, Valdez-Mondragón y cols. 2019). De acuerdo con Magalhaes y cols. (2017), el género *Loxosceles* hasta ahora, es un grupo natural o monofilético a diferencia del grupo *spinulosa*, el cual se considera el grupo hermano del género *Loxosceles* (Binford y cols, 2008; Magalhaes y cols. 2017). A pesar de esto, la monofilia de los grupos de especies no se ha puesto a prueba hasta el momento.

El grupo de especies *reclusa* es el que presenta mayor diversidad a nivel mundial, principalmente hacia Norteamérica. Existen actualmente dos hipótesis que tratan de explicar cómo fue la colonización del género *Loxosceles* hacia Norteamérica. La primera coincide con la colonización a través del puente de Panamá (Binford y cols. 2008), y la segunda, y más aceptada hasta el momento, propone que la colonización fue a través del puente terrestre que se formó entre Sudamérica y las Islas de las Antillas, llamado GAARlandia (Antillas Mayores y Cresta Aves) (Binford y cols. 2008). Sin embargo, a pesar de que estos trabajos mencionan hipótesis de colonización de sur a norte, no mencionan los procesos que pudieron influir en la gran diversificación de especies en Norteamérica, principalmente en México. De esta manera, el objetivo principal de este trabajo consiste en explicar cómo fue la diversificación de las

especies del género *Loxosceles* en Norteamérica, además de delimitar a las especies (linajes) distribuidas en esta región, pero principalmente en México.

2. ANTECEDENTES

2.1. Clasificación y diversidad del género *Loxosceles*

El género *Loxosceles* se encuentra dentro de la familia Sicariidae Keyserling, 1880, la cual está compuesta además por los géneros *Hexophthalma* Karsch, 1879, con ocho especies y *Sicarius* Walckenaer, 1847, con 21 especies. *Loxosceles* se agrupa dentro del clado Scytodoidea junto con Ochyroceratidae, Scytodidae, Drymusidae y Periegopidae, cuyas características compartidas son los seis ocelos agrupados en tres pares, la presencia de uñas prolaterales bipectinadas en los tarsos I-II y una capucha dorsal distal que cubre las bases de las garras (Labarque y Ramírez, 2012; Wheeler y cols. 2017). La clasificación actual del género *Loxosceles* se presenta a continuación:

Dominio: Eukarya (Chatton, 1925)

Reino: Animalia Linnaeus, 1758

Phylum: Arthropoda Lankester, 1904

Clase: Arachnida Lamarck, 1801

Orden: Araneae Clerck, 1757

Suborden: Opisthothelae Pocock, 1892

Infraorden: Araneomorphae Pocock, 1892

Superfamilia: Scytodoidea Blackwall, 1864

Familia: Sicariidae Keyserling,1880

Género: *Loxosceles* Heineken y Lowe, 1832

Para las especies del género *Loxosceles* se han propuesto diferentes grupos de especies, inicialmente Gertsch (1958) basándose en los caracteres del pedipalpo del macho dividió en dos grupos al género *Loxosceles*: 1) grupo *rufescens* para las especies de Centroamérica, Sudamérica y la especie cosmopolita *L. rufescens*, y 2) grupo *reclusa*: para las especies de Norteamérica, algunos países de Centroamérica y las Antillas. Más tarde para Sudamérica Gertsch (1967) basándose también en los pedipalpos de los machos, y después Gonçalves-de-Andrade y cols.

(2012) incluyeron las características de los receptáculos seminales de las hembras, y distinguieron cuatro grupos de especies: *gaucho*, *spadicea*, *amazonica* y *laeta*. Posteriormente, Binford y cols. (2008) usando evidencia morfológica y molecular, propusieron ocho grupos de especies: *gaucho*, *spadicea*, *amazonica*, *laeta*, *reclusa*, *rufescens*, *vonwredei* y *spinulosa*.

En estudios más detallados con evidencia molecular Duncan y cols. (2010) y morfológica Fukushima y cols. (2017) sinonimizaron a las especies del grupo *amazonica* con las especies del grupo *rufescens*, quedando actualmente siete grupos de especies: cinco distribuidos en América: 1) *reclusa* con 54 especies; 2) *laeta* con 26 especies; 3) *rufescens* con 10 especies; 4) *gaucho* con siete especies; 5) *spadicea* con cuatro especies, y dos para África: 6) *spinulosa* con tres especies y, 7) *vonwredei* con dos especies (Gertsch,1958; Gertsch, 1967; Gertsch y Ennik, 1983; Binford y cols. 2008; Duncan y cols. 2010; Gonçalves-de-Andrade y cols. 2012; Fukushima y cols. 2017; Bertani y cols. 2018). La mayoría de las especies del género *Loxosceles* se encuentra dentro del grupo *reclusa*, grupo al cual pertenecen las especies norteamericanas donde se incluyen todas las especies presentes en México (Figura 1). Sin embargo, actualmente se reconocen especies que no son coincidentes con la morfología descriptiva de los grupos de especies actuales (*L. diaguita* Brescovit, Taucare-Ríos, Magalhaes y Santos, 2017; *L. pallalla*, Brescovit, Taucare-Ríos, Magalhaes y Santos, 2017), por lo cual, se encuentran sin pertenecer o asignarse a ningún grupo de especies hasta el momento.

En América los países con mayor diversidad de especies del género *Loxosceles* son: México con 40 especies, Brasil con 22 especies, Perú con 21 especies, y Estados Unidos con 13 especies (WSC, 2025). De igual manera, aunque con menor número de especies, se tiene registro en Venezuela con ocho especies, Chile con siete especies, Ecuador con seis especies, Argentina con cinco especies, Colombia con cuatro especies, Cuba, Jamaica, Guatemala y Paraguay con tres especies cada uno, Panamá con dos especies y finalmente Belice, Costa Rica y El Salvador con una especie (WSC, 2025).

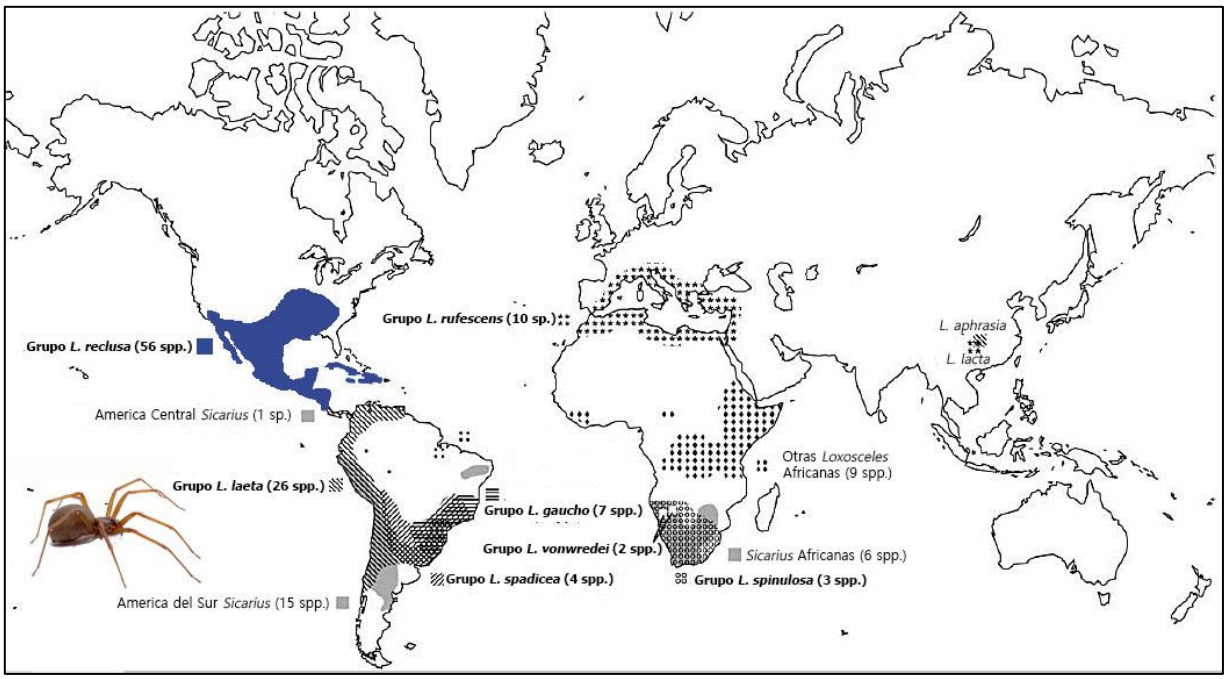

Figura 1. Distribución de los grupos de especies del género *Loxosceles*, en paréntesis se indica el número de especies incluidas por grupo. En azul se representa la distribución del grupo *reclusa*, al que pertenecen las especies norteamericanas incluidas las de México. Tomado y modificado de Binford y cols. (2008).

De las 40 especies que se han descrito para México (Cuadro 1), 38 especies son nativas y dos introducidas: *L. reclusa* (Gertsch y Mulaik, 1940) y *L. rufescens* (Dufour, 1820). Para el caso de Estados Unidos se han descrito actualmente 13 especies, 11 nativas y dos introducidas: *L. laeta* (Nicolet, 1849) nativa de Sudamérica y *L. rufescens* (Dufour, 1820) nativa de la región del Mediterráneo e Islas del Atlántico (Cuadro 1). Las primeras especies descritas para Estados Unidos de América fueron *L. arizonica, L. devia* y *L. reclusa* por Gertsch y Mulaik (1940), posteriormente se describió *L. deserta* por Gertsch (1973) y finalmente se describieron siete especies más por Gertsch y Ennik (1983), siendo este el último trabajo donde se describieron especies para Estados Unidos (Cuadro 1). Para Canadá, hasta el momento no existe un registro oficial de alguna especie de *Loxosceles*, sin embargo, se cuentan con registros dentro de una página web sobre ciencia ciudadana: Recluse or Not (https://spiderbytes.org/recluse-or-not/). Sin embargo, no se tiene certeza de las localidades donde se han encontrado las arañas del género *Loxosceles.* En México, la región con mayor diversidad de especies es el Noroeste, mientras que los estados en México con el mayor número de especies son Baja California Sur

(7), Baja California (5), Guerrero (5), Hidalgo (5) y Sonora (5) (Cuadro 1), y los que presentan el menor número de especies son Aguascalientes, Campeche, Ciudad de México, Jalisco, Michoacán, Quintana Roo, Tabasco, Tlaxcala, Veracruz y Yucatán, con solo una especie registrada (Cuadro 1).

Cuadro 2. Especies del género *Loxosceles* presentes en México (MX) y en Estados Unidos (EU). *Especies introducidas en México. $^{+}$Especies introducidas en EU. **Especies presentes en México y EU.

No.	Especie	Distribución
1	*Loxosceles alamosa* Gertsch y Ennik, 1983	MX: Sonora
2	*Loxosceles apachea*** Gertsch y Ennik, 1983	MX: Aguascalientes, Chihuahua, Zacatecas y Durango, EU: Arizona, Nuevo México y Texas
3	*Loxosceles aranea* Gertsch, 1973	MX: Querétaro
4	*Loxosceles arizonica* Gertsch y Mulaik, 1940	EU: Arizona y California
5	*Loxosceles aurea* Gertsch, 1973	MX: Durango y Coahuila
6	*Loxosceles baja* Gertsch y Ennik, 1983	MX: Baja California Sur
7	*Loxosceles barbara* Gertsch y Ennik, 1983	MX: Baja California Sur
8	*Loxosceles belli* Gertsch, 1973	MX: Coahuila
9	*Loxosceles blanda* Gertsch y Ennik, 1983	EU: Texas y Nuevo México
10	*Loxosceles boneti* Gertsch, 1958	MX: Guerrero
11	*Loxosceles candela* Gertsch y Ennik, 1983	MX: Nuevo León
12	*Loxosceles carmena* Gertsch y Ennik ,1983	MX: Baja California Sur
13	*Loxosceles chinateca* Gertsch y Ennik, 1983	MX: Oaxaca y Veracruz
14	*Loxosceles colima* Gertsch, 1958	MX: Colima, Jalisco y Nayarit
15	*Loxosceles coyote* Gertsch y Ennik, 1983	MX: Sonora
16	*Loxosceles deserta*** Gertsch,1973	MX: Baja California, EU: California
17	*Loxosceles devia*** Gertsch y Mulaik, 1940	MX: Tamaulipas y Nuevo León, EU: Texas
18	*Loxosceles francisca* Gertsch y Ennik, 1983	MX: Baja California
19	*Loxosceles huasteca* Gertsch y Ennik, 1983	MX: San Luis Potosí
20	*Loxosceles insula* Gertsch y Ennik, 1983	MX: Colima

21	*Loxosceles jaca* Gertsch y Ennik,1983	MX: Hidalgo
22	*Loxosceles kaiba* Gertsch y Ennik,1983	EU: Arizona
23	*Loxosceles laeta*[+] (Nicolet, 1849)	EU: Massachusetts, Kansas y California
24	*Loxosceles luteola* Gertsch, 1973	MX: Nuevo León
25	*Loxosceles malintzi* Valdez-Mondragón, Cortez-Roldán, Juárez-Sánchez y Solís-Catalán, 2018	MX: Guerrero, Morelos y Puebla
26	*Loxosceles manuela* Gertsch y Ennik, 1983	MX: Baja California
27	*Loxosceles martha* Gertsch y Ennik, 1983	EU: California
28	*Loxosceles misteca* Gertsch, 1958	MX: Guerrero
29	*Loxosceles mulege* Gertsch y Ennik,1983	MX: Baja California Sur
30	*Loxosceles nahuana* Gertsch, 1958	MX: Hidalgo
31	*Loxosceles palma******* Gertsch y Ennik ,1983	MX: Baja California, EU: California
32	*Loxosceles reclusa**** **** Gertsch y Mulaik, 1940	MX: Tamaulipas EU: Centro de Estados Unidos Americanos.
33	*Loxosceles rothi* Gertsch y Ennik, 1983	MX: Baja California Sur
34	*Loxosceles rufescens******[+] (Dufour, 1820)	MX: Chihuahua, EU: amplia distribución
35	*Loxosceles russelli* Gertsch y Ennik, 1983	EU: California
36	*Loxosceles sabina* Gertsch y Ennik, 1983	EU: Arizona
37	*Loxosceles seri* Gertsch y Ennik, 1983	MX: Sonora y Sinaloa
38	*Loxosceles sonora* Gertsch y Ennik, 1983	MX: Sonora y Sinaloa
39	*Loxosceles tehuana* Gertsch, 1958	MX: Oaxaca y Chiapas
40	*Loxosceles tenango* Gertsch, 1973	MX: Hidalgo y San Luis Potosí
41	*Loxosceles tenochtitlan* Valdez-Mondragón y Navarro-Rodríguez, 2019	MX: Tlaxcala, Estado de México y Ciudad de México, Hidalgo
42	*Loxosceles teresa* Gertsch y Ennik, 1983	MX: Tamaulipas
43	*Loxosceles tlacolula* Gertsch y Ennik, 1983	MX: Oaxaca
44	*Loxosceles tolantongo* Navarro-Rodríguez y Valdez-Mondragón, 2020	MX: Hidalgo
45	*Loxosceles valdosa* Gertsch,1973	MX: San Luis Potosí y Tamaulipas
46	*Loxosceles yucatana* Chamberlin y Ivie, 1938	MX: Campeche, Tabasco, Yucatán y Quintana Roo.
47	*Loxosceles zapoteca* Gertsch,1958	MX: Guerrero y Puebla

2.2 Sistemática filogenética, diversificación y datación de la familia Sicariidae

En cuanto a estudios filogenéticos con la familia Sicariidae, Magalhaes y cols. (2017) realizan la primera filogenia basada en datos fenotípicos, morfológicos y características del veneno. Aunque se incluyeron especies de los tres géneros que componen la familia, el estudio estuvo conformado en su mayoría por representantes del género *Sicarius*. Los resultados arrojan dos grupos monofiléticos que corresponden a dos géneros: 1) *Hexophthalma* Karsch, 1879 para las especies de África, y 2) *Sicarius* Walckenaer, 1847 para las especies de América. En cuanto al género *Loxosceles* se recupera como un grupo monofilético y como grupo hermano a *L. simillima* (Magalhaes y cols. 2017; Magalhaes y cols. 2019; Magalhaes y cols. 2022).

Respecto a estudios evolutivos basados en caracteres tanto morfológicos como moleculares con *Loxosceles*, aún son escasos. Binford y cols. (2008) realizaron el primer análisis filogenético con datos moleculares para establecer los patrones de divergencia y de vicarianza entre *Loxosceles* y *Sicarius* de África (ahora género *Hexophthalma*) causados por la deriva continental. En dicho estudio incluyeron datos de representantes de todas las regiones geográficas nativas de *Loxosceles* y *Hexophthalma*, exceptuando *Loxosceles* de África del Norte y Central. Para el análisis utilizaron los genes 28S, COI, 16S y NADHI. Sin embargo, en este trabajo se incluyeron solamente secuencias correspondientes a cuatro especies de México: *L. baja, L. boneti, L. colima* y *L. chinateca*. Los resultados apoyan la hipótesis de que el ancestro común más reciente entre *Loxosceles* y *Sicarius* provino de Gondwana Occidental, antes de la separación de África y Sudamérica. Asimismo, se mencionan dos hipótesis sobre la colonización de las especies de *Loxosceles* hacia Norteamérica. La primera menciona que la colonización fue a través del puente de Panamá, mientras que la segunda y más aceptada es a través del puente que se formó entre Sudamérica y las Islas de las Antillas, llamado GAARlandia (Greater Antilles + Aves Ridge). Sin embargo, no aclaran qué factores pudieron influir en la diversificación del género en Norteamérica, sobre todo en la parte continental (Binford y cols. 2008).

Planas y Ribera (2014) describieron la diversificación, colonización y delimitación de las especies de *Loxosceles* en las Islas Canarias en el Océano Atlántico, probaron la monofilia del grupo y delimitación de linajes evolutivos de *Loxosceles* de África occidental y la cuenca mediterránea, ya que son las especies que están estrechamente relacionadas con las áreas

vecinas. Utilizando el registro fósil, calibraron y explicaron los periodos de diversificación del género. Para los análisis moleculares, utilizaron los genes COI, 16S, NADH, H3 e ITS2. Los resultados de los análisis filogenéticos mostraron un clado bien soportado para las ocho especies de las Islas Canarias, revelando la presencia de especies de *Loxosceles* endémicas del archipiélago. Los principales eventos de colonización entre las islas ocurrieron durante el Mioceno tardío y la dispersión entre islas fue la principal fuerza impulsora para la diversificación en el grupo, además de algunos eventos de especiación posteriores.

Después, Planas y cols. (2014) realizaron un análisis de datación molecular para ubicar los principales eventos de diversificación de *L. rufescens* en la cuenca del Mediterráneo, donde además discuten el centro de origen de esta especie. Como resultado, encontraron 11 linajes evolutivos dentro de *L. rufescens* y fecharon los principales eventos de diversificación en el período de las glaciaciones del Pleistoceno, la ubicación de estos refugios pleistocénicos a la par de su compleja orografía y, además, la baja capacidad de dispersión de esta especie fueron clave para la alta diversidad genética actual de *Loxosceles rufescens.* Asimismo, encontraron que los principales refugios se concentran en Magreb, una región al norte del continente africano, y que coinciden con la ubicación de cuatro linajes evolutivos. Esto apoya la hipótesis de que el norte de África es el centro de origen de *L. rufescens* como lo había mencionado previamente Gertsch (1967), Duncan y cols. (2010), Nentwig y cols. (2017), y Taucare-Ríos y cols. (2018). Como conclusión, mencionan que la diversidad genética fue resultado de especiación por alopatría promovida por sucesivas glaciaciones durante el pleistoceno.

2.3 Delimitación molecular de especies en el género *Loxosceles*

En los últimos años, el uso de secuencias de ADN se ha implementado como una evidencia más dentro de la taxonomía, utilizándolo principalmente para la delimitación de especies morfológicamente indistinguibles o cuya morfología es poco variable entre especies. No obstante, actualmente también se utiliza para acelerar el descubrimiento e identificación de especies, sobre todo en grupos cuya diversidad esta subestimada (Dayrat, 2005; Grabowski y cols. 2017). La evidencia molecular es usada a la par de utilizar otro tipo de evidencias, tales como morfológicas o biogeográficas, utilizándolos simultáneamente para generar hipótesis de

linajes o especies más sustentadas (Dayrat, 2005; DeSalle, 2005; Navarro-Rodríguez y Valdez-Mondragón, 2020).

En algunos grupos de arañas donde la morfología es muy conservada o con poca variación como el caso del género *Loxosceles*, el uso de evidencia molecular es muy útil para el descubrimiento de la diversidad no descrita y la delimitación de especies (Valdez-Mondragón y cols. 2019; Navarro-Rodríguez y Valdez-Mondragón, 2020). Aunado a esto, es imprescindible el uso de un enfoque integrador para el reconocimiento de las especies, con la finalidad de poder corroborar las hipótesis generadas desde múltiples enfoques y bajo distintas líneas de evidencia (Dayrat, 2005: DeSalle y cols. 2005; Padial y cols. 2010).

El primer estudio taxonómico integrador sobre el género *Loxosceles* fue el de Ribera y Planas (2009) donde utilizaron el marcador mitocondrial Citocromo Oxidasa subunidad I (COI) describiendo una especie nueva para el género, con distribución en Túnez: *Loxosceles mrazig*. Esta especie presentó un alto grado de similitud morfológica con *L. gaucho* Gertsch, 1967 una especie que se distribuye en Brasil. Sin embargo, se encontraron diferencias morfológicas en los pedipalpos de los machos y la coloración del cuerpo. Además, los datos moleculares con COI revelaron una distancia (*p*) genética mayor al 20%, corroborando que se trataba de linajes o especies distintas.

Posteriormente, Planas y Ribera (2015) utilizaron el gen mitocondrial COI y el nuclear ITS2 para delimitar a las especies de las Islas Canarias y describieron seis especies nuevas: *Loxosceles mahan*, *L. bentejui*, *L. tazarte*, *L. guayota*, *L. tibicena* y *L. hupalupa*, todas endémicas de las Islas Canarias. Las distancias (*p*) genéticas no corregidas entre las especies descritas para COI fueron superiores al 12%, y los datos de haplotipos con ITS2 también corroboraron las diferentes especies. Morfológicamente, encontraron dificultad para distinguir a las especies debido a la variación sutil en los caracteres sexuales. Las principales diferencias en los machos se concentraron en la forma del émbolo, mientras que, en las hembras las diferencias se encontraron en la forma general de los receptáculos seminales.

Para el caso de México, Valdez-Mondragón y cols. (2019) describieron *L. tenochtitlan* del Valle de México, a partir de datos morfológicos y moleculares, utilizando genes mitocondriales y nucleares (COI e ITS2). Aunque esta especie es morfológicamente similar a *L. misteca*, las distancias genéticas calculadas con COI fueron del 13.8% y 4.2% para los datos

de ITS2. Los métodos de delimitación de especies recuperaron un grupo monofilético bien soportado para *L. tenochtitlan* del Valle de México y también para la especie *L. misteca* de Guerrero, especies anteriormente consideradas como la misma (Navarro-Rodríguez, 2019). Valdez-Mondragón y cols. (2019) no solamente utilizaron evidencia morfológica tradicional y molecular, sino además, incluyeron morfología lineal, geométrica y modelaje de distribución de especies, siendo el primer trabajo de taxonomía integradora para el género en Norteamérica.

El trabajo más reciente es el de Navarro-Rodríguez y Valdez-Mondragón (2020), donde describieron, con datos morfológicos y moleculares (COI e ITS2), a *Loxosceles tolantongo* del estado de Hidalgo. Esta especie resultó ser morfológicamente similar con *L. jaca* también del estado de Hidalgo, sin embargo, la principal diferencia se encuentra en la tibia del pedipalpo del macho, además, las distancias genéticas entre especies fueron mayores al 10% para COI. A pesar de la extensa revisión taxonómica realizada Gertsch y Ennik (1983), estos dos últimos trabajos de taxonomía integradora permitieron conocer que la diversidad de especies del género *Loxosceles* para México es subestimada.

2.4 Factores biogeográficos, diversificación y datación molecular

Los cambios a gran escala en el entorno físico también pueden influir en los patrones de diversificación al cambiar la distribución y la conectividad de las masas de tierra o el clima global y/o regional (Dimitrov y Hormiga, 2020). Mastretta-Yanes y cols. (2015) realizaron un estudio sobre la historia climática, geológica y filogeográfica del altiplano mexicano, en donde explican cómo la actividad volcánica transformó parte del territorio mexicano durante el Pleistoceno, principalmente en el Cinturón Volcánico Transmexicano (CVT), ya que la mayoría de sus volcanes emergieron durante esta época. Además, mencionaron cómo las fluctuaciones climáticas, junto con el vulcanismo fueron impulsores de la diversificación en el altiplano mexicano. Ellos mencionan dos mecanismos que pudieron haber promovido la diversificación: 1) la generación de barreras geográficas que promovieron la especiación alopátrica, 2) el surgimiento de nuevas montañas para la colonización y posterior divergencia. También, mencionan que, al diferenciar a las poblaciones o especies, la mayoría de los patrones genéticos

se asocian con el pleistoceno como consecuencia de las fluctuaciones climáticas y la actividad volcánica durante esa época.

Halffter (2017) y Halffter y Morrone (2017), explican cómo la convergencia de las regiones biogeográficas Neártica y Neotropical (Zona de Transición Mexicana ZTM) han originado la composición y riqueza de especies, donde el clima y las cordilleras que alberga han favorecido el desplazamiento y establecimiento de distintos taxa. En ambos trabajos mencionan que el uso de técnicas moleculares como el uso del ADN mitocondrial y nuclear permite comprobar o refutar las hipótesis formuladas de linajes monofiléticos, así como los tiempos y eventos de distribución propuestos. Además, señalan distintos patrones de distribución según la procedencia de las especies, que pueden ser de origen septentrional-holártico, o de origen sudamericano-neotropical.

Para el grupo de los arácnidos, Schramm y cols. (2021) realizaron un estudio de datación y divergencia molecular del género monotípico de amblipígidos o arañas látigo *Acanthophrynus*, en específico de la especie *A. coronatus*, con distribución en las regiones tropicales, siendo un claro ejemplo de artrópodos estenotópicos y de dispersión reducida, donde analizan los factores que han impulsado su diversificación. Hipotetizan que el bosque caducifolio funcionó en este grupo como un conducto de dispersión y a su vez una barrera en los momentos de cambios paisajísticos o paleoclimáticos, promoviendo la diversificación principalmente por medio de la vicarianza, pero también por una posterior dispersión a través de las selvas bajas. Asimismo, tomando en cuenta la compleja historia del paisaje mexicano, ellos sugieren que el surgimiento del CVT condujo a la diversificación de *Acanthophrynus*, recalcando que los ciclos glaciales/interglaciares son impulsores de la diversificación en el Neotrópico Mexicano. Como resultado, obtuvieron que la primera divergencia entre *Acanthophrynus* ocurrió en el Mioceno tardío o principios del Plioceno, 6.95 o 5.21 millones de años (Ma), lo cual coincide con la orogénesis del CVT, que comenzó en el Mioceno y entre los ciclos glacial/interglaciar del Plioceno. Finalmente, discuten que gran parte de la diversidad tropical puede ser el producto de repetidos procesos de especiación impulsada por vicarianza y dispersión, en asociación a un paisaje heterogéneo y un clima fluctuante (Simões y cols. 2016).

Para el caso de las arañas, y en específico para arañas del clado Synspermiata, Valdez-Mondragón y Francke (2015) realizaron una estimación de los tiempos de divergencia del

género *Ixchela* (familia Pholcidae), los autores discuten el tiempo de divergencia dentro del contexto de la complejidad histórica biogeográfica y geológica de México. Como resultado, encontraron que el género *Ixchela* divergió en el Mioceno tardío, y la divergencia entre los clados internos del género ocurrió en el Plioceno tardío, siendo la mayoría de los eventos de especiación durante el Pleistoceno, pues los cambios climáticos inducidos por las glaciaciones repetidas influyeron significativamente en la diversificación del grupo. Estos patrones de diversificación y especiación ocurrieron primordialmente en los Bosques de Montaña templados, con el surgimiento de la Sierra Madre del Sur de Oaxaca, el Cinturón Volcánico Transmexicano, la Sierra Madre Oriental y en el componente biótico Mesoamericano, en la Sierra de Chiapas.

Para el género *Loxosceles*, Magalhaes y cols. (2022) realizaron la revisión de tres especies fósiles de las arañas reclusas (*L. aculicaput* Wunderlich, 2004, *L. defecta* Wunderlich, 1988 y *L. deformis* Wunderlich, 1988) preservadas en ámbar dominicano del Mioceno. Para corroborar la identidad y confiabilidad de los fósiles, estimaron la filogenia de la familia Scytodoidea incluyendo datos de especies extintas y fósiles, además de datos morfológicos y moleculares. Como resultados encontraron que *L. defecta* Wunderlich, 1988 y *L. deformis* Wunderlich, 1988 pertenecen al género *Loxosceles*, por lo cual estos ejemplares se podrán usar de manera confiable como puntos de calibración para la datación de las filogenias del género y otros grupos de arañas. Además, asignan a *L. defecta* dentro del grupo de especies *reclusa* y la cual podría estar relacionada con las especies existentes de las Antillas. Para el caso de *L. aculicaput* Wunderlich, 2004 el holotipo fue asignado al género *Drymusa* (*Drymusa aculicaput*).

La alta riqueza de especies y la amplia distribución del género *Loxosceles* en México, lo convierte en un excelente modelo y grupo interesante en el aspecto biogeográfico. Esto permite investigar y entender cuáles fueron los posibles procesos macroevolutivos de diversificación dentro de un contexto biogeográfico histórico, explicando también su gran diversidad de Norteamérica.

3. JUSTIFICACIÓN

El género de arañas *Loxosceles* es altamente diverso en Norteamérica, principalmente en el biogeográfico y geológicamente complejo territorio mexicano. Sin embargo, no hay un estudio sobre los procesos biogeográficos que influyeron en su diversificación, lo cual es de importancia para el caso de México al ser el país con la mayor diversidad de especies a nivel mundial. De esta manera, las herramientas filogenéticas, la datación de linajes y la reconstrucción de áreas ancestrales explicarán dicha diversificación.

4. HIPÓTESIS

1. La diversificación del grupo *reclusa* se dio antes del Mioceno-Plioceno debido a los cambios orográficos y de hábitat importantes durante ese periodo en Norteamérica.

2. El grupo *reclusa* es un grupo monofilético, debido a que las especies de Norteamérica y el Caribe se originaron a partir de un ancestro en común.

4.1PREDICCIONES

1. La datación del grupo *reclusa* será previa al Mioceno-Plioceno y la reconstrucción de las áreas ancestrales recuperará a la Sierra Madre Oriental (SMOr), la Sierra Madre del Sur (SMS) y el Cinturón Volcánico Transmexicano (CVT) como principales impulsores de la diversificación del género *Loxosceles* en Norteamérica, durante el Mioceno medio y Plioceno.
2. Se espera encontrar un clado bien definido conformado por las especies del género *Loxosceles* que habitan Norteamérica y las Islas del Caribe, con un alto soporte estadístico.

5. OBJETIVOS

5.1. General

Determinar la diversidad de especies y establecer las relaciones filogenéticas del género *Loxosceles* en Norteamérica mediante un enfoque integrador de datos moleculares, morfológicos y biogeográficos, para inferir los procesos que impulsaron su diversificación.

5.2. Específicos

1. Delimitar especies mediante el uso de los marcadores moleculares mitocondriales (COI) y nucleares (ITS2) corroborándolos con evidencia morfológica (pedipalpos en machos y receptáculos seminales en hembras).

2. Probar la monofilia del grupo de especies *reclusa* con datos moleculares.

3. Estudiar la diversificación del género *Loxosceles* en un contexto biogeográfico norteamericano, mediante el uso de los marcadores mitocondriales COI y nucleares 28S e ITS2.

4. Describir las especies nuevas descubiertas bajo un contexto de taxonomía integradora (evidencia molecular y morfológica).

6. MATERIALES Y MÉTODOS

El proyecto consistió en cuatro partes esenciales: 1) revisión morfológica de los ejemplares de *Loxosceles*, 2) obtención de las secuencias genéticas para los análisis moleculares, 3) delimitación de especies con datos moleculares y morfológicos (taxonomía integradora), realización de análisis filogenéticos, datación de linajes, reconstrucción de áreas ancestrales, y 4) descripción de especies nuevas (taxonomía alfa).

A continuación, se describen de manera sintetizada los materiales y métodos para los estudios morfológicos y moleculares del trabajo en general. Además, en cada capítulo, los materiales y métodos correspondientes se describen en detalle.

6.1 Material biológico

6.1.1 Colecciones científicas. Se revisaron en total 4,167 ejemplares tanto machos, como hembras y juveniles, pertenecientes a 36 especies del género *Loxosceles* depositados en las siguientes colecciones nacionales y extranjeras: LATLAX: Laboratorio de Aracnología Tlaxcala, Laboratorio Regional de Biodiversidad y Cultivo de Tejidos Vegetales (LBCTV), Instituto de Biología UNAM, unidad Tlaxcala (responsable: Dr. Alejandro Valdez Mondragón); CNAN: Colección Nacional de Arácnidos, Instituto de Biología, Universidad Nacional Autónoma de México (IBUNAM), México D. F. (responsable: Dr. Edmundo González Santillán); CIBNOR: Centro de Investigaciones Biológicas del Noroeste, La Paz, Baja California Sur, México (responsable: Dra. María L. Jiménez Jiménez); AMNH: American Museum of Natural History, Nueva York, Estados Unidos (responsable: Dr. Lorenzo Prendini). (SDSU) Department of Biology, San Diego State University, San Diego, California, Estados Unidos (responsable: Richard Cripps).

6.1.2 Trabajo de campo

Se llevaron a cabo expediciones a campo a distintas localidades y estados de la República Mexicana para recolectar material del género *Loxosceles.* Entre 2017-2022 se efectuaron 19 salidas a 28 estados de México (Cuadro 2). Se realizaron recolectas en campo de las localidades previamente mencionadas en trabajos publicados con el género, haciendo énfasis en las localidades tipo de las especies y localidades nuevas (Figura 2). Se recolectaron machos, hembras y juveniles para los estudios morfológicos y moleculares, recabando además información sobre el hábitat y microhábitat donde fueron recolectados. Los ejemplares recolectados en campo se fijaron en alcohol etílico al 80% para estudios morfológicos y al 96% para estudios moleculares. Los especímenes para estudios moleculares se almacenaron en el laboratorio a -20°C.

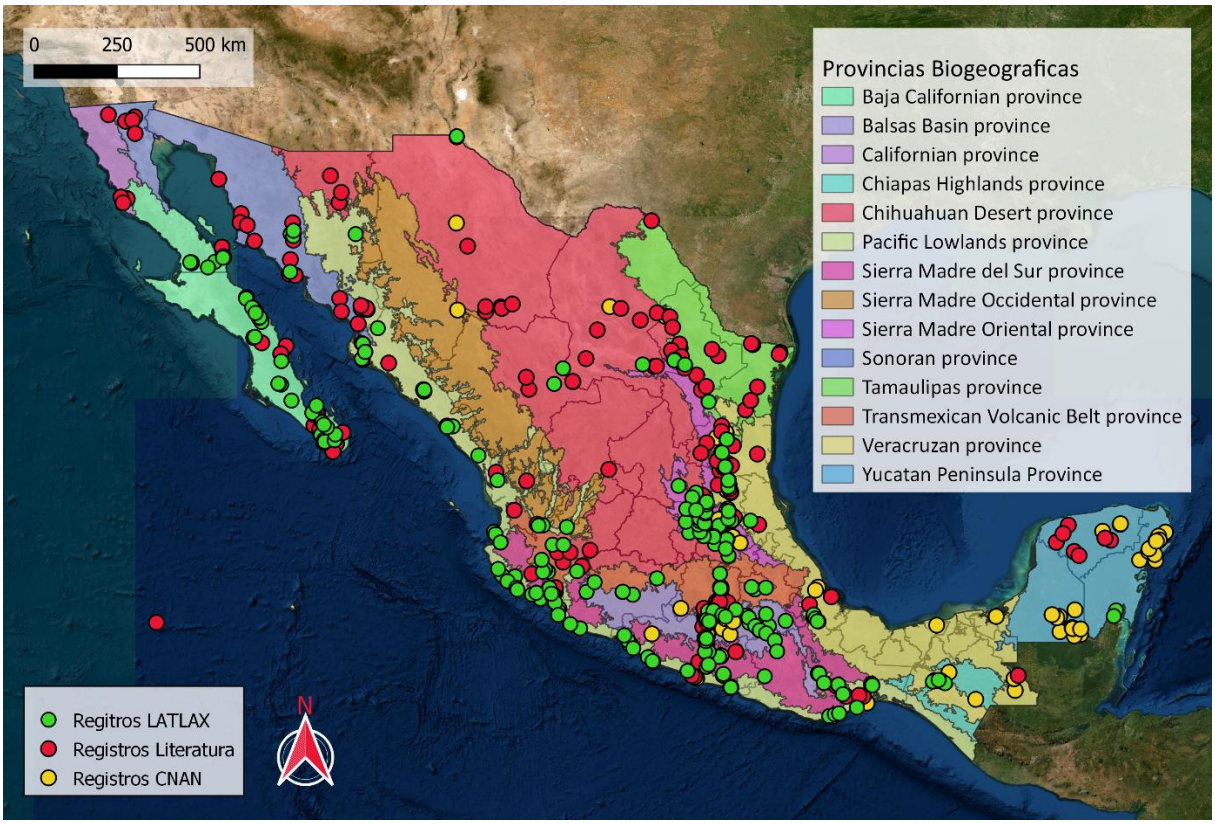

Figura 2. Registros totales del género *Loxosceles* para la República Mexicana actualizados hasta el año 2024. Las provincias biogeográficas propuestas por Morrone y cols. (2017) se presentan en colores.

Cuadro 2. Recolectas en campo, material de donación y préstamos de las especies de *Loxosceles* de México realizados entre 2017-2022. M= machos, H= hembras, J= juveniles.

Colecta No.	Mes de colecta	Estado/País	Localidades muestreadas	No. ejemplares identificados			Especies
				M	H	J	
Año 2017							
1	Marzo	Puebla	6	4	6	49	*L. malintzi*
2	Marzo	Hidalgo	9	7	14	90	*L. jaca* *L. nahuana* *L. tenango*
3	Abril	Tlaxcala	1	0	0	1	*L. tenochtitlan*
4	Abril	Tlaxcala	1	1	4	17	*L. tenochtitlan*
5	Mayo	Puebla	3	6	10	91	*L. malintzi* *L. malintzi*
6	Julio	Puebla-Oaxaca	23	6	7	59	*L. tehuana* *L. tlacolula*

7	Septiembre	Guerrero	8	8	30	112	*L. misteca* *L. malintzi*. *L. zapoteca*
			Año 2018				
8	Marzo	Oaxaca	10	3	7	99	*L. chinateca*
9	Mayo	Tlaxcala	1	0	24	2	*L. tenochtitlan*
10	Mayo	Hidalgo	11	27	68	153	*L. jaca* *L. nahuana* *L. tenango* *L. tolantongo*
11	Junio	Tlaxcala	1	2	0	50	*L. tenochtitlan*
12	Octubre	Guerrero	16	39	107	207	*L. misteca* *L. zapoteca* *L. malintzi*
			Año 2019				
13	Enero	Hidalgo	1	1	6	4	*L. tolantongo*
14	Junio	Jalisco/ Colima	15	1	21	137	*L. colima*
15	Agosto	Sinaloa Baja California Baja California Sur Coahuila Colima Durango Guerrero Hidalgo Michoacán Nayarit Nuevo León Oaxaca Puebla San Luis Potosí Tamaulipas	60	37	56	221	*L. sonora* *L. baja* *L. francisca* *L. mulege* *L. rothi*
16	Octubre		70	24	78	148	*L. apachea* *L. aurea* *L. belli* *L. colima* *L. devia* *L.* aff. *devia* *L. jaca* *L. luteola* *L. malintzi* *L. nahuana* *L. reclusa* *L. tehuana* *L. tenango* *Loxosceles* sp *Loxosceles.* sp *L. valdosa* *L. zapoteca*
			Año 2020				
17	Noviembre	Nayarit Jalisco Colima	65	129	369	205	*L. colima* *L.* aff. *colima* *Loxosceles.* sp *Loxosceles.* sp

		Michoacán Guerrero Hidalgo Morelos					*Loxosceles.* sp *L. totolapa* *L. zapoteca* *L. nahuana*
Año 2021							
18	Octubre 2021	Querétaro San Luis Potosí Guanajuato	42	21	84	196	*L. nahuana* *L.* aff. *aranea* *L. huasteca*
Año 2022							
19	Septiembre 2022	Oaxaca Chiapas	11	43	58	110	*Loxosceles.* sp *Loxosceles.* sp *Loxosceles.* sp *L.* aff. *tlacolua* *L. tehuana*
Material de donación							
20	2005-2006	Chihuahua	1	4	4	1	*L. apachea* *L. rufescens*
21	2017	San Luis Potosí	1	3	13	23	*Loxosceles.* sp *Loxosceles.* sp *L. devia*
22	2017-2018	Ciudad de México	5	8	9	15	*L. tenochtitlan*
23	Marzo 2019	Estado de México	1	0	1	0	*L. tenochtitlan*
24	Julio 2020	Sonora	1	4	8	4	*L. sonora*
25	Junio 2021	Chiapas	2	0	1	4	*L. tehuana*
26	Septiembre 2021	Quintana Roo	1	3	4	5	*L. yucatana*
27	Septiembre 2021	Guanajuato	1	4	4	0	*L. huasteca*
28	Noviembre / diciembre 2021	Sinaloa Sonora	6	7	15	25	*Loxosceles.* sp *Loxosceles.* sp *L. sonora*
29	Enero 2022	Sinaloa	2	7	16	6	*Loxosceles.* sp *L. sonora*
30	Marzo 2022	Guanajuato	1	1	2	0	*Loxosceles.* sp
31	Revisión CNAC abril 2022	Sinaloa Zacatecas	2	147	138	4	*Loxosceles.* sp *L. colima*
32	Abril/ Mayo/ Julio 2022	Sinaloa	4	3	9	30	*Loxosceles.* sp *Loxosceles.* sp
33	Agosto 2022	Sonora	1	1	3	1	*L. sonora*
34	Julio 2022	Campeche	1	0	1	2	*L. yucatana*
35	Abril 2022	EU: California	5	6	3	0	*L.* aff. *martha* *L. arizonica* *L. blanda* *L. deserta*

Material revisado - Colección Nacional de Arácnidos (CNAN)							
36	Revisión abril/2022	Baja California Chihuahua Coahuila Durango Guanajuato Guerrero Morelos Nuevo León Oaxaca Puebla Sinaloa Sonora Tamaulipas Guatemala	39	206	156	21	*L.* aff. *apachea* *L.* aff. *baja* *L.* aff. *belli* *L.* aff. *boneti* *L.* aff. *devia* *L. apachea* *L. aranea* *L.belli* *L. candela* *L. coyote* *L. devia* *L. francisca* *L. guatemala* *L. manuela* *L. malintzi* *Loxosceles.* sp *L. mulege* *L. seri* *L. sonora* *Loxosceles.* sp *Loxosceles.* sp *L. valdosa*
Total:		**28 estados**	**428 localidades**	**764**	**1336**	**2067**	**36 especies**

6.2 Revisión morfológica e identificación de material

La identificación de las especies se llevó a cabo en el Laboratorio de Aracnología (LATLAX) del Laboratorio Regional de Biodiversidad y Cultivo de Tejidos Vegetales (LBCTV), Instituto de Biología UNAM, unidad Tlaxcala. Se revisó material de donación de Campeche, Chiapas, Guanajuato, Quintana Roo, Sinaloa, Sonora y California, Estados Unidos, colectado en el 2021 y 2022 (Cuadro 2).

Se realizaron disecciones de pedipalpos en ejemplares machos y receptáculos seminales en ejemplares hembras adultos, esto para identificarlos a nivel de especie. Para la identificación, se utilizó la clave de identificación para *Loxosceles* de América del Norte de Gertsch y Ennik (1983) aunado a material bibliográfico adicional. Se revisaron 764 machos, 1336 hembras y 2067 juveniles del género *Loxosceles* (Cuadro 2).

De las 47 especies descritas para Norteamérica, se incluyeron en el estudio 36 (lo que representa el 76% de las especies), esto debido a que las 11 especies restantes son especies

introducidas (*L. laeta*) o que a pesar del esfuerzo de muestreo en campo no se pudieron obtener (*L. alamosa, L. barbara, L. boneti, L. carmena, L. insula, L. kaiba, L. palma, L. russelli, L. sabina* y *L. teresa*) (Cuadro 3).

Cuadro 3. Especies del género *Loxosceles* de Norteamérica consideradas para el estudio.

No.	Especie	Material utilizado		Distribución
		Morfología	Molecular	
1	*L.apachea*	x	x	Chihuahua, Zacatecas y Durango, EU: Arizona, Nuevo México y Texas
2	*L.aranea*	x	x	Querétaro
3	*L.arizonica*	x	x	EU: Arizona y California
4	*L.aurea*	x	x	Durango y Coahuila
5	*L.baja*	x	x	Baja California Sur
6	*L.belli*	x	x	Coahuila
7	*L.blanda*	x	x	EU: Texas y Nuevo México
8	*L.candela*	x	-	Nuevo León
9	*L.chinateca*	x	x	Oaxaca y Veracruz
10	*L.colima*	x	x	Colima, Jalisco y Nayarit
11	*L.coyote*	x	-	Sonora
12	*L.deserta*	x	x	Sonora, Baja California, EU: Nevada, Utha, Arizona y California
13	*L.devia*	x	x	Tamaulipas y Nuevo León, EU: Texas
14	*L.francisca*	x	x	Baja California Norte
15	*L.huasteca*	x	x	San Luis Potosí
16	*L.jaca*	x	x	Hidalgo
17	*L.luteola*	x	x	Nuevo León
18	*L.malintzi*	x	x	Guerrero, Morelos y Puebla
19	*L. manuela*	x	-	Baja California Norte
20	*L.martha*	x	x	EU: California
21	*L.misteca*	x	x	Guerrero
22	*L.mulege*	x	x	Baja California Sur
23	*L.nahuana*	x	x	Hidalgo
24	*L.reclusa*	x	x	Tamaulipas EU: Zona centro
25	*L.rothi*	x	x	Baja California Sur
26	*L.rufescens*	x	-	Chihuahua, EU: amplia distribución
27	*L.seri*	x	-	Sonora y Sinaloa
28	*L.sonora*	x	x	Sonora y Sinaloa
29	*L.tehuana*	x	x	Oaxaca y Chiapas
30	*L.tenango*	x	x	Hidalgo y San Luis Potosí
31	*L.tenochtitlan*	x	x	Tlaxcala, Estado de México y Ciudad de México
32	*L.tlacolula*	x	x	Oaxaca
33	*L.tolantongo*	x	x	Hidalgo
34	*L.valdosa*	x	x	San Luis Potosí y Tamaulipas
35	*L.yucatana*	x	x	Campeche, Tabasco, Yucatán y Quintana Roo.
36	*L.zapoteca*	x	x	Guerrero y Puebla
	Total:	**36**	**31**	

6.3 Análisis moleculares

La separación de tejido, extracción de ADN, amplificación por PCR y purificación se realizaron en el Laboratorio de Biología Molecular del LBCTV, Instituto de Biología UNAM-Tlaxcala, Ciudad de Tlaxcala.

6.3.1 Separación de tejido

Se separó tejido de 307 ejemplares de 31 especies del género *Loxosceles* (Cuadro 3); según la localidad, se tomaron muestras de tres y hasta seis ejemplares cuando fue posible. Para *L. manuela*, *L. rufescens*, *L. candela*, *L. coyote* y *L. seri* no se realizó extracción de ADN debido a que la fecha de colecta del material biológico era muy antigua, lo que puede afectar la calidad del ADN.

6.3.2 Extracción de ADN, cuantificación, amplificación y secuenciación

La extracción del ADN dependió del material disponible y del tamaño de los ejemplares obtenidos. El tejido utilizado para extracción de ADN fue aislado principalmente de patas completas (adultos), y del prosoma y opistosoma (juveniles). Se utilizaron kits de extracción Qiagen DNeasy, siguiendo los protocolos de Navarro-Rodríguez y Valdez-Mondragón (2020). Posterior a la extracción de ADN, se realizó la cuantificación de este, utilizando un espectrofotómetro Colibri Microvolume Spectrometer-Titertek Berthold, donde se coloca 1 µL de extracción ADN. Para estimar la pureza del ADN se considera la proporción de la absorbancia a 260 nm y 280 nm. El rango aceptado como ADN puro es a partir de 1.8 unidades, proporciones menores indican la presencia de proteínas. Se utilizaron tres genes para los estudios moleculares: COI (gen mitocondrial), ITS2 y 28S (genes nucleares). La amplificación se llevó a cabo mediante la técnica de PCR (Polymerase Chain Reaction). Posteriormente se realizó la purificación de los productos de PCR para eliminar componentes no deseados como enzimas, nucleótidos, cebadores y componentes del tampón. Esta se llevó a cabo con un kit de purificación QIAquick QIAGEN, siguiendo los protocolos del fabricante. La secuenciación de ambas cadenas de los genes amplificados por PCR fue mediante Sanger. Según la disponibilidad de ejemplares, se envió a secuenciar el ADN de por lo menos cinco ejemplares por especie y se realizó en el Laboratorio Temático de Secuenciación Genómica de la Biodiversidad y la Salud,

a cargo de la M. en C. Laura Márquez Valdelamar, del Instituto de Biología, UNAM, Ciudad Universitaria, Ciudad de México, México.

6.4 Edición de secuencias

6.4.1 Identidad y ensamble de secuencias

Después de la secuenciación de Sanger, se recibieron las secuencias en ambas direcciones: Forward (5´-3´) and Reverse (3´-5´). Posteriormente, se corroboró la identidad de las secuencias en ambas direcciones y posibles contaminaciones en BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), en la plataforma GenBank del NCBI. Se descartaron las secuencias con baja calidad y/o poca cantidad de nucleótidos. Se utilizaron los programas Geneious v.8.1.9 (Rozen y Skaletsky, 2000) para visualizar la calidad de las secuencias, el cromatograma y el número de nucleótidos por secuencia (Forward/Reverse). Se utilizó el programa Bioedit v. 7.0.5.3 (Hall, 1999) para la edición y el ensamble de las secuencias (Forward/ Reverse) para generar la secuencia consenso de cada uno de los especímenes enviados a secuenciar. Una vez editadas las secuencias, se depositaron en la plataforma de acceso público en línea GenBank del NCBI (https://www.ncbi.nlm.nih.gov/genbank/).

6.4.2 Alineamiento múltiple de secuencias

Después de obtener la matriz con todas las secuencias editadas, se subió en formato fasta (.fas) para el alineamiento múltiple de secuencias a la plataforma en línea MAFFT (Multiple sequences Alignment based on Fast Fourier Transform). Esto se realizó con la finalidad de obtener una matriz de secuencias alineadas por cada marcador (COI, ITS2, 28S). Finalmente, con el programa Mesquite v. 3.81 (Maddison y Maddison, 2023) se obtuvo una matriz concatenada de los marcadores de COI+ ITS2 +28S.

7. RESULTADOS

Los resultados presentados a continuación, están estructurados por capítulos; el capítulo I, fue el artículo requisito y ya fue publicado, mientras que los capítulos II y III se encuentran en proceso de finalización y posterior publicación.

7.1 Capítulo 1. Artículo sobre delimitación de especies.

Violins we see, species we don´t… Species delimitation of the spider genus *Loxosceles* Heineken & Lowe (Araneae: Sicariidae) from North America using morphological and molecular evidence.

Artículo publicado en la revista *Zootaxa*. En este capítulo se presenta un enfoque integrador con el uso de marcadores nucleares y mitocondriales (ITS2 y COI) aunado a evidencia morfológica para la delimitación de especies. Para ello se implementaron el criterio del 2% y distancias *p* no corregidas con Neighbor Joining (NJ), aunado al uso de tres métodos de delimitación molecular para el descubrimiento y validación de las especies del género *Loxosceles*, esto bajo el criterio de congruencia: 1) Automated Simultaeous Analysis Phylogenetics (ASAP); 2) General Mixed Yule Coalescent (GMYC) y 3) Bayesian Poisson Tree Proceses (bPTP). Estatus: **Publicado.**

Cita: Navarro-Rodríguez, C. I., & Valdez-Mondragón, A. (2024). Violins we see, species we don't… Species delimitation of the spider genus *Loxosceles* Heineken & Lowe (Araneae: Sicariidae) from North America using morphological and molecular evidence. *Zootaxa*, 5428(4), 527-548. https://doi.org/10.11646/zootaxa.5428.4.4

7.2 Capítulo 2. Artículo sobre la filogenia y diversificación del grupo de especies *reclusa* en América del Norte.

Phylogenetics systematics and diversification of the spider genus *Loxosceles* Heineken & Lowe 1832 (Araneae: Sicariidae) from North America. Este artículo se enfoca en la reconstrucción filogenética del grupo *reclusa*, así como en el conocimiento de la diversificación del género *Loxosceles* en un contexto biogeográfico norteamericano bajo la datación de linajes con BEAUTI y BEAST y la reconstrucción de áreas ancestrales con el programa RASP. Para ello, se implementaron análisis filogenéticos de Inferencia Bayesiana (IB) y Máxima Verosimilitud (ML). Estatus: **En preparación.**

7.3 Capítulo 3. Artículo sobre taxonomía y descripción de especies nuevas del género *Loxosceles* en México.

Nine new species of the spider genus *Loxosceles* Heineken & Lowe (Araneae, Sicariidae) from Mexico. En este artículo se presentan avances en la taxonomía del género, donde se incluye la descripción de nueve especies nuevas del género *Loxosceles* en México. Las descripciones están basadas en machos y hembras adultas, colectados en seis estados diferentes del país. Estatus: **En preparación.**

CAPÍTULO 1:
ARTÍCULO SOBRE DELIMITACIÓN DE ESPECIES

Violins we see, species we don´t… Species delimitation of the spider genus *Loxosceles* Heineken & Lowe (Araneae: Sicariidae) from North America using morphological and molecular evidence.

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https://doi.org/10.11646/zootaxa.5428.4.4

Zootaxa 5428 (4): 527–548
https://www.mapress.com/zt/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.5428.4.4
http://zoobank.org/urn:lsid:zoobank.org:pub:85B0D966-AB65-44B6-9CB4-92F9DD0CB010

Violins we see, species we don't… Species delimitation of the spider genus *Loxosceles* Heineken & Lowe (Araneae: Sicariidae) from North America using morphological and molecular evidence

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Abstract

In modern systematics, different sources of evidence are commonly used for the discovery, identification, and delimitation of species, especially when morphology fails to delineate between species or in underestimated species complexes or cryptic species. In this study, morphological data and two DNA barcoding markers—cytochrome *c* oxidase subunit I (COI) and internal transcribed spacer 2 (ITS2)—were used to delimit species in the spider genus *Loxosceles* from North America. The molecular species delimitation analyses were carried out using three different methods under the corrected *p*-distance Neighbor-Joining (NJ) criteria: 1) Assemble Species by Automatic Partitioning (ASAP), 2) General Mixed Yule Coalescent model (GMYC), and 3) Bayesian Poisson Tree Processes (bPTP). The analyses incorporated 192 terminals corresponding to 43 putative species of *Loxosceles*, of which 15 are newly recognized herein, as putative new species, based on morphology and congruence between molecular methods with COI. The average intraspecific genetic distance (*p*-distance) was <2%, whereas the average interspecific genetic distance was 15.6%. The GMYC and bPTP molecular methods recovered 65-79 and 69 species respectively, overestimating the diversity in comparison with morphology, whereas the ASAP method delimited 60 species. The morphology of primary sexual structures (males palps and female seminal receptacles) was congruent with most of the molecular methods mainly with COI, showing that they are robust characters for identification at the species level. For species delimitation COI was more informative than ITS2. The diversity of *Loxosceles* species is still underestimated for North America, particularly in Mexico which holds the highest diversity of this genus worldwide.

Key words: DNA barcoding, integrative taxonomy, Synspermiata, violinist spiders, molecular methods

Resumen

En la sistemática moderna, las diferentes fuentes de evidencia se utilizan comúnmente para el descubrimiento, identificación y delimitación de especies, principalmente cuando la morfología no logra delimitar especies en complejos de especies o especies crípticas. En este estudio, se utilizaron datos morfológicos y dos marcadores de código de barras de ADN: 1) Citocromo *c* Oxidasa subunidad I (COI) y 2) Espaciador Transcrito Interno 2 (ITS2) para la delimitación de especies del género de arañas *Loxosceles* de Norteamérica. Los análisis moleculares de delimitación de especies se llevaron a cabo utilizando tres métodos diferentes bajo el criterio de Neighbor-Joining (NJ) con distancias-*p* corregidas: 1) Assemble Species by Automatic Partitioning (ASAP), 2) General Mixed Yule Coalescent model (GMYC), y 3) Bayesian Poisson Tree Processes (bPTP). Los análisis incorporaron 192 terminales correspondientes a 43 especies putativas de *Loxosceles* de las cuales 15 se reconocen recientemente en este trabajo como especies nuevas putativas, con base en la morfología y la congruencia entre los diferentes métodos moleculares con COI. La distancia genética (distancias *p*) promedio intraespecífica fue <2%, mientras que la distancia promedio interespecífica fue del 15.6%. Los métodos moleculares GMYC y bPTP recuperaron 65-79 y 69 especies respectivamente, sobreestimando la diversidad en

Accepted by I.L.F. Magalhaes: 29 Feb. 2024; published: 25 Mar. 2024

comparación con la morfología, mientras que el método ASAP delimitó 60 especies. La morfología de las estructuras sexuales primarias (palpos de los machos y receptáculos seminales de las hembras) fue congruente con la mayoría de los métodos moleculares, principalmente con COI, lo que demuestra que son caracteres robustos para la identificación de las especies. Para la delimitación de las especies COI fue más informativo que ITS2. La diversidad de especies de *Loxosceles* aún está subestimada en Norteamérica, principalmente en México donde se encuentra la mayor diversidad de especies a nivel mundial.

Palabras clave: Código de Barras de ADN, taxonomía integradora, Synspermiata, arañas violinistas, métodos moleculares

Introduction

The spider genus *Loxosceles* Heineken & Lowe, 1832 belongs to the family Sicariidae Keyserling, 1880 and is currently composed of 149 species worldwide (WSC, 2024). Mexico has the highest diversity in the world, with 40 currently described species including 38 native species and two introduced: *L. reclusa* (Gertsch & Mulaik, 1940) from southeastern USA and *L. rufescens* (Dufour, 1820) from the Mediterranean region (Valdez-Mondragón *et al.* 2018a; Valdez-Mondragón *et al.* 2019; Navarro-Rodríguez 2019; Navarro-Rodríguez & Valdez-Mondragón 2020; WSC 2024). In North America, *Loxosceles* spiders are mainly found in dry habitats, such as xerophilous scrub, deciduous forest, and deserts (Valdez-Mondragón *et al.* 2019; Navarro-Rodríguez & Valdez-Mondragón 2020). The northern region of Mexico is the most diverse in terms of species numbers, whereas the diversity decreases to the south of the country where the habitat is more tropical, such as the tropical rainforests of the Yucatan Peninsula (Valdez-Mondragón *et al.* 2019; Navarro-Rodríguez & Valdez-Mondragón 2020).

Historically, traditional spider taxonomy has been based on the morphology of primary sexual structures that are useful features for species-level identification, such as epigyna, seminal receptacles, or spermathecae in females and palps in males. Species identification by morphology is a complicated task in spiders mainly because only adult specimens can be used, which are sometimes difficult to collect because often only males, only females, or even only immatures specimens are found (Barrett & Hebert 2005). Also, some groups tend to have conservative morphology or lack morphological variation among species, which complicates their identification. Furthermore, polymorphisms have been detected in the genitalia of some spider species (Huber & González 2001; Valdez-Mondragón 2010; Huber & Carvalho 2019; Huber & Villareal 2020). Several spider taxonomic groups have traditionally been recognized as being difficult to identify with morphology given their homogeneous and conservative traits, including some mygalomorphs spiders such as tarantulas (Hamilton *et al.* 2014; Ortiz *et al.* 2018; Candia-Ramírez & Francke 2021), trapdoor spiders (Satler *et al.* 2013; Wilson *et al.* 2018; Xu *et al.* 2020), and the "lampshade" spiders (genus *Hypochilus* Marx) (Ciaccio *et al.* 2022). Araneomorph spiders of the Synspermiata clade, including *Loxosceles*, also fit this situation (Gertsch 1958; Brignoli 1969; Planas & Ribera 2015; Valdez-Mondragón *et al.* 2019; Navarro-Rodríguez 2019; Navarro-Rodríguez & Valdez-Mondragón 2020), given the simplicity of their sexual structures that render identification at the species level difficult.

On the other hand, some spider groups show a wide variation in primary sexual structures. Sometimes, this is considered wide intraspecific variation, but in most cases it is the result of underestimated or cryptic biodiversity. Species of the genus *Loxosceles* from North and South America show wide morphological variation mainly in the seminal receptacles on females, which has been widely reported previously by Brignoli (1969), Gertsch (1958), Buckup (1980), Gertsch & Ennik (1983), and recently for Mexican species by Valdez-Mondragón *et al.* (2019), Navarro-Rodríguez (2019) and Navarro-Rodríguez & Valdez-Mondragón (2020). This morphological variation complicates the identification to species level for some cases. For this reason, Gertsch & Ennik (1983), Valdez-Mondragón *et al.* (2019), and Navarro-Rodríguez and Valdez-Mondragón (2020) suggest caution when using these sexual characters for taxonomic purposes at the species level in *Loxosceles*, or to use these in conjunction with other evidence such as somatic features, molecular data or even ecological and biogeographical data.

Genomes accumulate nucleotide substitutions at a rate that is influenced by gene flow, changes in population size, natural selection, and recombination (Smith & Carstens 2022). The patterns of nucleotide variation across individuals sampled within a species complex will retain information about the complex's recent history, and molecular methods used to delimit species attempt to access this information (Smith & Carstens 2022). Integrative taxonomy is a framework for delimiting and describing taxa by integrating various types of data, such as morphology

and molecular data, as well as different methodologies or approximations (Dayrat 2005; Will *et al*. 2005; Pante *et al*. 2015).

In biological groups where the traditional taxonomy is ambiguous or where speciation has occurred without changes in morphological features, molecular data is an important tool for delimiting species (Hebert *et al*. 2003 a,b; Hebert *et al*. 2004; Barrett & Hebert 2005). Using an integrative approach that corroborates hypotheses generated from multiple approaches and under different lines of evidence is essential, with species limits expected to show congruency across all results (DeSalle *et al*. 2005; Carstens *et al*. 2013).

Nowadays, molecular methods are becoming popular as a methodological tool to support species delimitation, identification, and recognition of underestimated biodiversity. There are phenotypic approaches based on genetic distances (e.g., NJ, ASAP) (Hebert *et al*. 2003a, b; 2004) and those that incorporate coalescent or tree models (e.g., GMYC, PTP, BPP) (Pons *et al*. 2006; Zhang *et al*. 2013; Yang 2015; Luo *et al*. 2018), with the latter being more robust for species delimitation. Although such methods are useful approximations for the delimitation of lineages or species, they are often implemented in addition to other types of data or approximations such as geometric morphometrics (Rohlf & Marcus 1993; Wilson *et al*. 2021; Seiter *et al*. 2022; Solís-Catalán 2020) or distributional analyses (e.g., niche models and species distribution) (Raxworthy *et al*. 2007; Rissler 2007; Valdez-Mondragón *et al*. 2019; Zhang *et al*. 2020; Hazzi & Hormiga 2021; Cortez-Roldán 2018, 2022). These methods and approximations can differ amongst themselves and often show results that depend on the study group (Carstens *et al*. 2013; Valdez-Mondragón 2020; Nolasco & Valdez-Mondragón 2022). Therefore, it is recommended to use more than one molecular method to define more precise species hypotheses, in combination with morphological evidence as part of an integrative approach (Dayrat 2005; DeSalle *et al*. 2005; Padial *et al*. 2010; Carstens *et al*. 2013).

In the past few years, the use of combined morphological and molecular evidence for the study of species diversity in the genus *Loxosceles* has increased. Ribera & Planas (2009) and Planas & Ribera (2015) used mitochondrial and nuclear markers to identify and discover new species of Caribbean *Loxosceles*. Using COI, 16S, and H3, Tahami *et al*. (2017) discovered a new species from three Iranian caves. For North America, Valdez-Mondragón *et al*. (2019) and Navarro-Rodríguez & Valdez-Mondragón (2020) published integrative taxonomy and species delimitation studies with molecular and morphological evidence for the first time for North American species, concluding that the diversity of species in the genus is underestimated in North America but mainly in Mexico, where more sampling is required.

As such, we presented the most complete sampling of North American species of *Loxosceles* so far with the objective of implement different species delimitation methods for North American species of *Loxosceles* using two molecular markers, mitochondrial COI and nuclear ITS2, and to corroborate these with morphological evidence of sexual structures in an integrative taxonomic context to obtain robust species hypotheses.

Material and methods

Biological material

The specimens were collected by hand and deposited in 80% ethanol for morphological studies and in 96% ethanol for molecular studies, labeled with their collection data. The specimens and additional material examined are deposited with their codes in the following collections: CARCIB = Arachnological Collection, Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, Baja California Sur, Mexico. CNAN = National Collection of Arachnids, Instituto de Biología, Universidad Nacional Autónoma de Mexico (IB-UNAM), Mexico City, Mexico (additional material). Observations of the specimens were done using a Zeiss model Stemi 508 stereoscopic microscope. The dissection of seminal receptacles in females and palps in males was performed for species identification. The female genitalia were immersed in potassium hydroxide (KOH-10%) for 1–5 minutes to digest and clean the soft tissue around the seminal receptacles. Left male palps and female genitalia were dissected for identification to the species level and for photography, being immersed in ethanol 70% gel and covered with a thin layer of distilled water to minimize diffraction during photography following Valdez-Mondragón & Francke (2015) and Navarro-Rodríguez & Valdez-Mondragón (2020). Photographs were taken using a Zeiss Axiocam 506 color camera attached to a Zeiss AXIO Zoom V16 stereo microscope. Images of palps and seminal receptacles were edited in Adobe Photoshop CS6. Species-level identification was carried out following Gertsch & Ennik (1983), Valdez-Mondragón *et al*. (2018, 2019), and Navarro-Rodríguez & Valdez-Mondragón (2020) for *Loxosceles* from North America.

Taxon sampling

The molecular analyses were based on a total of 192 sequences from 43 putative species based previously on morphology (S1). Sixty-two COI sequences and 40 ITS2 sequences published by Binford *et al*. (2005), Petersen *et al.* (2017), Valdez-Mondragón *et al.* (2019) and Navarro-Rodríguez & Valdez-Mondragón (2020) were downloaded from the GenBank database (www.ncbi.nih.gov) (S1). In addition, 130 new sequences of COI and 113 of ITS2 (Figs 2, 3, red circles) from 43 species across Mexico and the USA were added (S1). Since this study is focused only on species delimitation within *Loxosceles* and not on the phylogenetic relationships, the outgroup taxa *Loxosceles mrazig* Ribera & Planas, 2009 was used only for rooting purposes (S1).

DNA extraction, amplification, and sequencing

Tissue selection, DNA extraction, PCR amplification, and purification were performed at the Laboratorio de Biología Molecular del Laboratorio Regional de Biodiversidad y Cultivo de Tejidos Vegetales (LBCTV), IB-UNAM, Tlaxcala. For DNA extraction, a Qiagen DNeasy Tissue Kit was used, following the protocols of Valdez-Mondragón & Francke (2015) and Navarro-Rodríguez & Valdez-Mondragón (2020). For DNA extraction, two legs from adult specimens, three or four legs from juveniles, and in a few cases of immature specimens the entire specimen, were dissected. The tissue was stored in 96% ethanol in a -20°C freezer for posterior DNA extraction. Two genes were used for the molecular analyses: cytochrome *c* oxidase subunit I (COI) (mitochondrial gene) and internal transcribed spacer 2 (ITS2) (nuclear gene). For the amplification of COI, we used two primer sets: LCO1490/HCO2198 and LCO1490-JJ/HCO2198-JJ (Folmer *et al.* 1994; Astrin & Stüben 2008), while for ITS2, the primer set 5.8SF and CAS28sB1d were used (Ji *et al.* 2003; Planas & Ribera 2014) (Table 1). The PCR (Polymerase Chain Reaction) parameters for COI and ITS2 were performed following the protocols by Valdez-Mondragón *et al.* (2019) and Navarro-Rodríguez & Valdez-Mondragón (2020). The sequencing of both chains (5′–3′ and 3′–5′) of the PCR products was performed in a Sequencer Genetic Analyzer RUO Applied Biosystems Hitachi model 3750xL at the Laboratory of Molecular Biology and Health, IB-UNAM, Mexico City.

TABLe 1: Primer sets used in this work for PCR amplification of the COI and ITS2 gene regions.

Gene	Primer name	Primer sequence (5′–3′)	Reference
COI	LCOI490	5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'	Folmer *et al.* (1994), Astrin & Stüben (2008)
	HCO2198	5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'	
	LCOI490-JJ	5'-CHA CWA AYC ATA AAG ATA TYG G-3'	
	HCO2198-JJ	5'-AWA CTT CVG GRT GCV CAA ARA ATC A-3'	
ITS2	5.8SF	5'-CAC GGG TCG ATG AAG AAC GC-3'	Ji *et al.* (2003), Planas & Ribera (2014)
	CAS28sB1d	5'-TTC TTT TCC TCC SCT TAY TRA TAT GCT TAA-3'	

DNA sequence alignment and editing

BioEdit v. 7.0.9.0 (Hall 1999) and Geneious v. 8.1.9 (Rozen & Skaletsky 2000) programs were used for molecular sequence editing. A multiple sequences alignment for each marker separately (COI and ITS2) was performed in the online version of MAFFT (Multiple sequences Alignment based on Fast Fourier Transform) with default parameters: Auto (FFT-NS-2, FFTNS-i or L-INS-i; depending on data size). In some cases, sequence alignment was done manually and edited using BioEdit v. 7.0.5.3 (Hall 1999). Geneious v. 8.1.9 was used to assess sequence quality.

Molecular analyses and species delimitation methods

For species delimitation, the uncorrected *p*-distance under Neighbor Joining (NJ) phenetic analysis was implemented

as an initial criterion of species separation. Posteriorly, three molecular methods were carried out and shown over a Maximum Likelihood (ML) tree: 1) Assemble Species by Automatic Partitioning (ASAP) online version (Puillandre *et al.* 2021) based on pairwise genetic distances, 2) General Mixed Yule Coalescent (GMYC) (method based on coalescence) (Pons *et al.* 2006) using GMYC online version (https://species.h-its.org/gmyc/), and 3) Bayesian Poisson Tree Process (PTP) (method based on trees) (Zhang *et al.* 2013; Kapli *et al.* 2017) using the online version (https://species.h-its.org/ptp/). The ML tree was also estimated using the web server IQ-Tree (Trifinopoulos *et al.* 2016).

Uncorrected p-distances under Neighbor Joining (NJ)

First, sequence evolution models were selected using ModelFinder v. 1.6.12 (Kalyaanamoorthy *et al.* 2017) in the web server IQ-Tree (Trifinopoulos *et al.* 2016). The Neighbor Joining (NJ) analysis of uncorrected genetic distances (*p*) was performed in MEGA v. 10.1.7 (Kumar *et al.* 2016). The selected parameters were as follows: Number of replicates = 1000, bootstrap support values = 1000 (significant values ≥50%), Substitution type = nucleotide, Model = *p* distance, Substitution to Include = d: Transitions+Transversions, Rates among Sites = Gamma distributed (G), Missing Data Treatment = Pairwise deletion, Select Codon Position = 1st+2nd+3rd+Noncoding Sites.

Assemble Species by Automatic Partitioning (ASAP)

This method is based on pairwise genetic distances of single locus sequences and scores the most probable groups into a tree (Puillandre *et al.*, 2021) using an aligned FASTA format input matrix. Analyses were conducted within the online version (https://bioinfo.mnhn.fr/abi/public/asap) using K2P distances and only considering partitions showing the lowest asap-score. This is an ascending hierarchical clustering method, which merges sequences into groups that are successively merged further until all sequences form a single group, then the partitions are equivalent to each sequence merge step (Nolasco & Valdez-Mondragón 2022).

General Mixed Yule Coalescent (GMYC)

For the General Mixed Yule Coalescent Model (GMYC)-based delimitation method (Pons *et al.* 2006), first, ultrametric trees were constructed. Phylogenetic analyses were conducted under BEAUTi and BEAST 2.6.0 (Drummond *et al.* 2012) using the Coalescent model (constant population). An independent lognormal relaxed clock was applied to each partition, and each analysis was run for 40×10^6 generations. Convergence was assessed with TRACER v. 1.6 (Rambaut & Drummond 2014). The TREEANNOTATOR program v. 2.6.0 (a BEAST package) was used to build maximum clade credibility trees, after discarding the first 25% of generations as burn-in. Following gene tree inference, GMYC was implemented in the web interface for single and multiple threshold GMYC (https://species.h-its.org/gmyc/). Time thresholds were used to delimit species in a Maximum Likelihood context using ultrametric trees (Ortiz & Francke 2016).

Bayesian Poisson tree processes (bPTP)

For the Bayesian Poisson Tree Process (bPTP) bounding method (Zhang *et al.* 2013), an input likelihood tree using the Bayesian variant of the Poisson Tree Process model (Yao *et al.* 2016) was used. Analyses were conducted using the online version (https://species.h-its.org/) with default options: rooted tree, MCMC generations = 100000, Thinning = 100, Burn-in = 0.1, Seed = 123. The congruence integration criterion was selected for species delimitation, which is based on the congruence of the different evidence used (morphology, criteria, and methods) to reach a consensus and generate robust species hypotheses (Fonseca *et al.* 2008; Padial *et al.* 2010; Navarro-Rodríguez 2019). In addition, the integration by accumulation criterion was applied, which assumes that any source of evidence, even one, can be the basis for species discovery of species. In this approach, consistency is desirable but not considered necessary (Padial *et al.* 2010).

Results

Intra and inter specific genetic distances

The corrected *p*-distances based only in COI data group 53 putative species of *Loxosceles* from North America; 43 of these 53 putative species were corroborated with morphology, because in some species the morphology of adult specimens (males and females) was not available for identification (Fig. 1, question marks) or it was not possible to review them, as was the case with the Caribbean species. The average interspecific genetic distances among analyzed *Loxosceles* species from North American were 15.6% for COI (S2) and 8.8% for ITS2 (S3). The average intraspecific genetic distance for COI was 1.2%. While this value was <2% for most species (46 of 53), seven species (*L.* aff. *apachea*, *L. baja*, *L. cubana L.* aff. *martha*, *L. mulege*, *L. rothi and L. taino*) showed average intraspecific genetic distances between 2.1% and 10.1% with high bootstrap support values (>97%) (stars in Fig. 1, S2). For ITS2, the average intraspecific genetic distance was 0.3% (S3). For COI the bootstrap support values were high (99%) for most species (Fig. 1).

Of the 55 described species that comprise the *reclusa* group, we worked with 36 species, which represent 67% of the species sequenced, therefore only 18 species (33%) of the group remain to be sequenced (Table 2).

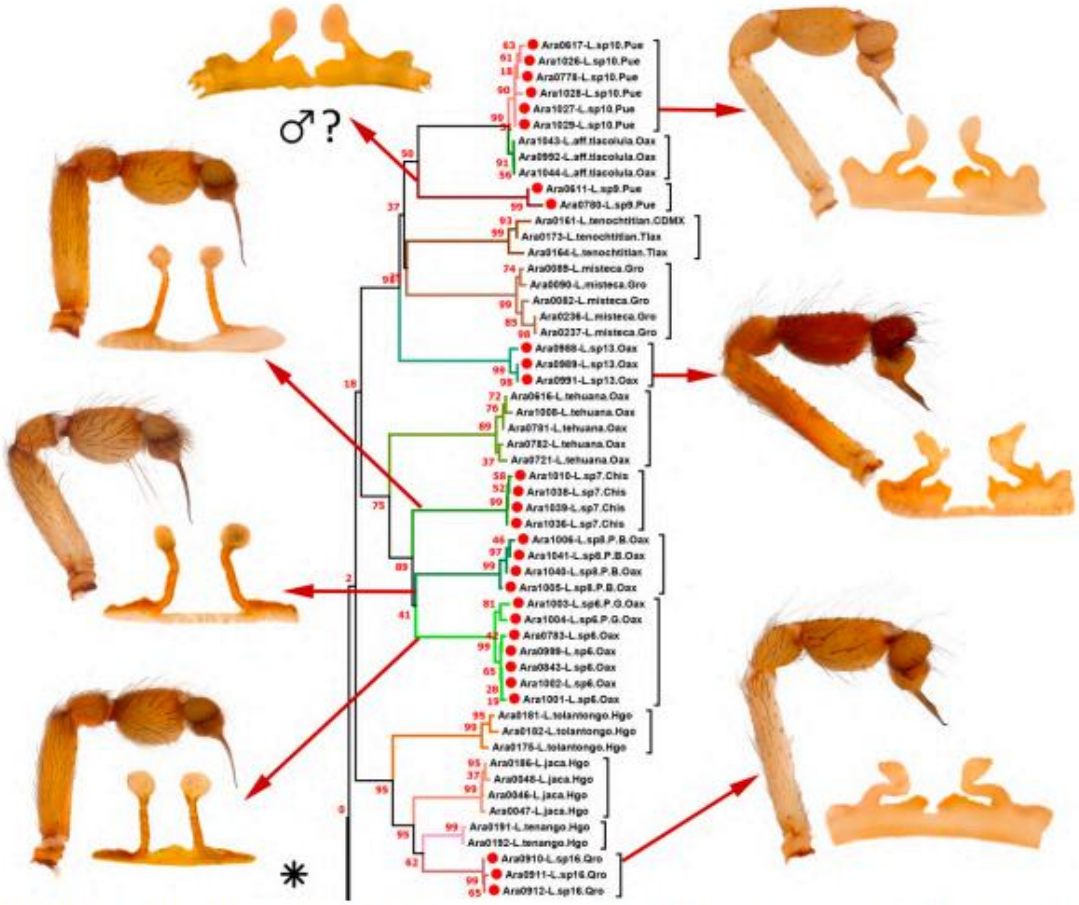

FIGURe 1. Neighbor-Joining (NJ) tree with corrected *p*-distances using COI barcode sequences from different species of *Loxosceles* (*reclusa* group) from North America. Branch colors indicate putative species. Male palps and female seminal receptacles are shown for each putative new species. Numbers above branches indicate significant bootstrap support values (> 50%). Red circles represent samples for each putative new species. Question marks represent specimens where morphology was not available. Stars indicate possible species complexes (see text for discussion).

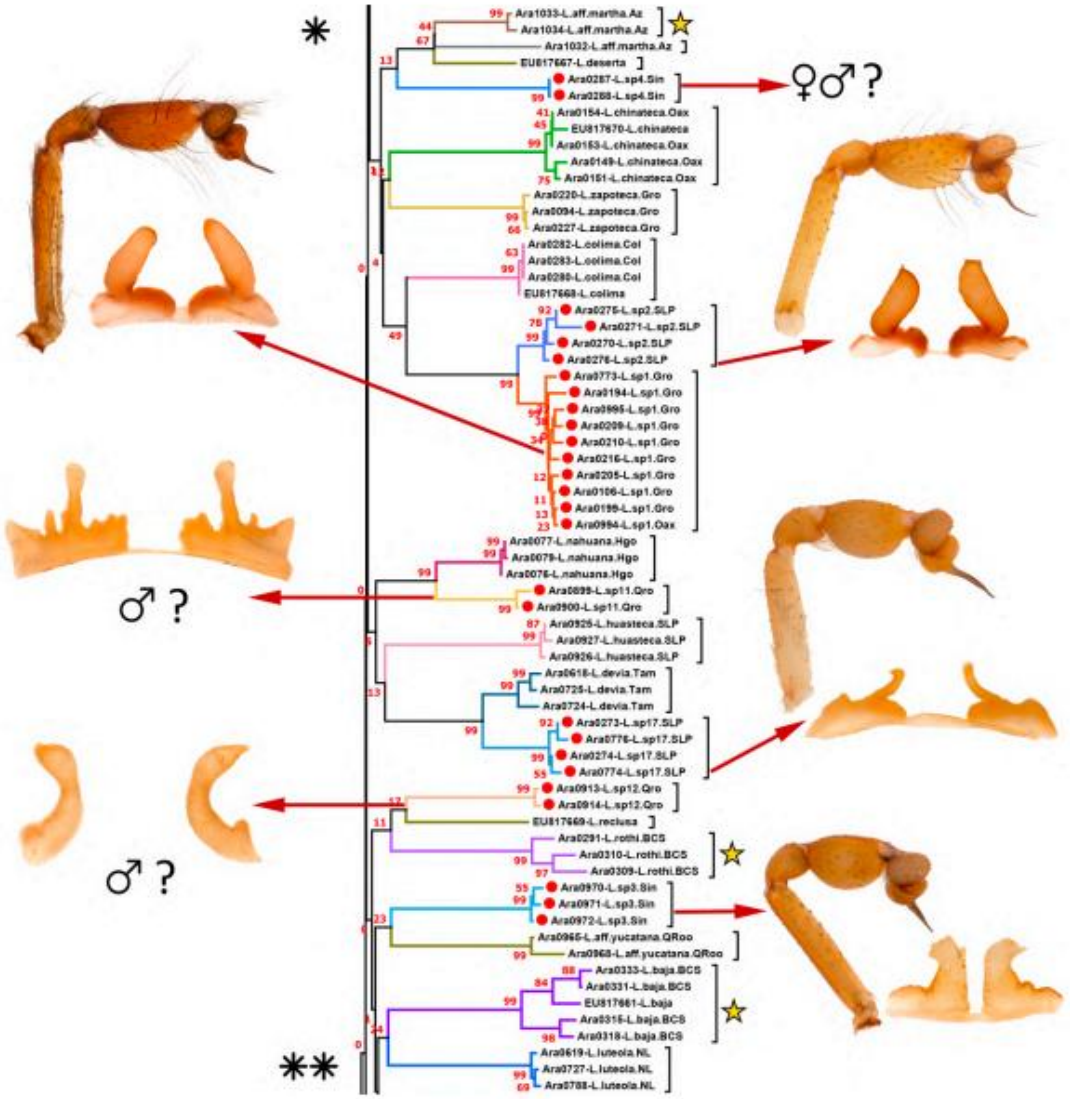

FIGURe 1. (Continued). Neighbor-Joining (NJ) tree with corrected *p*-distances using COI barcode sequences from different species of *Loxosceles* (*reclusa* group) from North America. Branch colors indicate putative species. Male palps and female seminal receptacles are shown for each putative new species. Numbers above branches indicate significant bootstrap support values (> 50%). Red circles represent samples for each putative new species. Question marks represent specimens where morphology was not available. Stars indicate possible species complexes (see text for discussion).

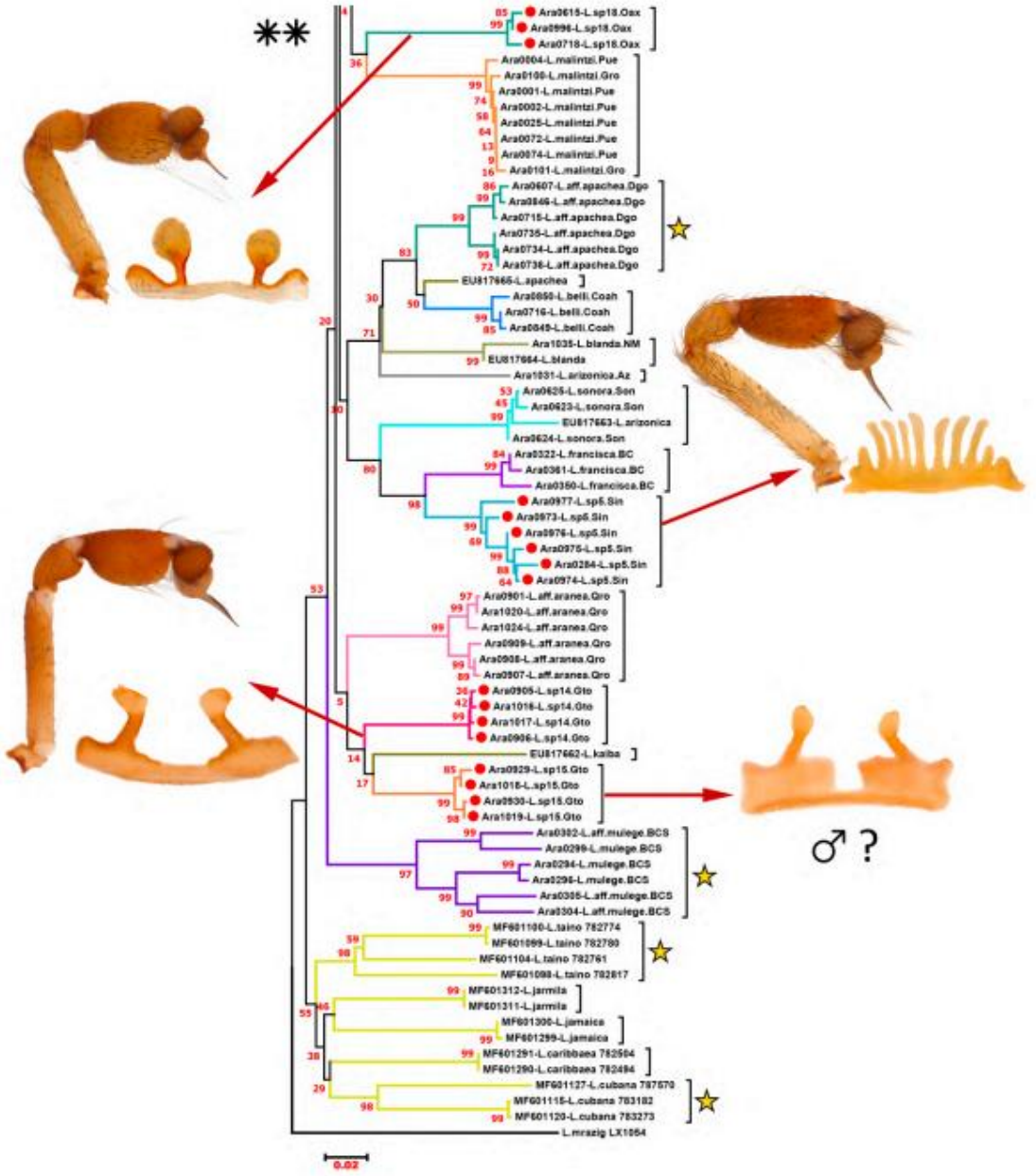

FIGURe 1. (Continued). Neighbor-Joining (NJ) tree with corrected *p*-distances using COI barcode sequences from different species of *Loxosceles* (*reclusa* group) from North America. Branch colors indicate putative species. Male palps and female seminal receptacles are shown for each putative new species. Numbers above branches indicate significant bootstrap support values (> 50%). Red circles represent samples for each putative new species. Question marks represent specimens where morphology was not available. Stars indicate possible species complexes (see text for discussion).

TABLe 2. Species of *Loxosceles* (*reclusa* group) that have not been sequenced so far. MX: Mexico, USA: the United States of America, CA: Central America, AN: Antilles.

No.	Species of *Loxosceles*	Distribution
1	*L. alamosa* Gertsch & Ennik, 1983	MX: Sonora
2	*L. aurea* Gertsch, 1973	MX: Durango, Coahuila
3	*L. barbara* Gertsch & Ennik, 1983	MX: Baja California Sur
4	*L. boneti* Gertsch, 1958	MX: Guerrero, CA: El Salvador
5	*L. candela* Gertsch & Ennik, 1983	MX: Nuevo León
6	*L. carmena* Gertsch & Ennik, 1983	MX: Baja California Sur
7	*L. coyote* Gertsch & Ennik, 1983	MX: Sonora
8	*L. guatemala* Gertsch, 1973	CA: Guatemala
9	*L. insula* Gertsch & Ennik, 1983	MX: Colima
10	*L. maisi* Sánchez-Ruiz & Brescovit, 2013	AN: Cuba
11	*L. manuela* Gertsch & Ennik, 1983	MX: Baja California
12	*L. mogote* Sánchez-Ruiz & Brescovit, 2013	AN: Cuba
13	*L. palma* Gertsch & Ennik, 1983	USA: California, MX: Baja California
14	*L. rica* Gertsch & Ennik, 1983	CA: Costa Rica
15	*L. russelli* Gertsch & Ennik, 1983	USA: California
16	*L. seri* Gertsch & Ennik, 1983	MX: Sonora, Sinaloa
17	*L. teresa* Gertsch & Ennik, 1983	MX: Tamaulipas
18	*L. virgo* Gertsch & Ennik, 1983	AN: Virgin Is.

Molecular methods and species delimitation

COI. For the ASAP delimitation analysis under COI, 10 partitions with the best asap-scores were obtained, from which the one with the lowest rank was selected, finding 60 species. The ASAP partition with the lowest rank underestimates the number of species (*Loxosceles* sp1-Gro with *Loxosceles* sp2-SLP, and *Loxosceles* aff. *tlacolula* with *Loxosceles* sp10-Pue) (Fig. 2). The tree-based methods (GMYC and bPTP) had some discrepancies with the morphology since in some cases the number of species was overestimated in comparison with ASAP. In the GMYC delimitation analysis, 65 and 79 species were recovered under single threshold (SN) and multi threshold (MT) implementation respectively (Fig. 2). In comparison with the other methods, MT implementation of GMYC was the approximation that recovered the higher number of species with 79 (Fig. 2). In the bPTP delimitation analyses, 69 species were recovered under ML and BI approximations (Fig. 2). In particular cases, such as *L. taino* and *L. mulege* each sequence as recovered as a single species (Fig. 2). Some species recovered under morphology were not recovered in some of the delimitation methods, such as *Loxosceles* sp1-Gro, *Loxosceles* sp2 SLP or *Loxosceles* sp10 Pue and *Loxosceles* aff. *tlacolula* Oax which are recovered as a single species in all the delimitation methods (Fig. 2). In the case of *Loxosceles* sp8 P B Oax, *Loxosceles* sp7 Chis, and *Loxosceles* sp6 (Oax and P G Oax), there was not morphological variation, however in most of the molecular methods they are separated as three different species, except GMYC (MT) where the species *Loxosceles* sp6 Oax is overestimated (Fig. 2). For COI, of the 43 morphologically recognized species, 23 (*L. colima* Col, *L. tehuana* Oax, *L. arizonica* Az, *L. belli* Coah, *Loxosceles* sp3 Sin, *L. jaca* Hgo, *L. tenango* Hgo, *Loxosceles* sp16 Qro, *Loxosceles* sp15 Gto, *Loxosceles* sp14 Gto, *L. zapoteca* Gro, *L. luteola* NL, *L. huasteca* SLP, *Loxosceles* sp12 Qro, *Loxosceles* sp18 Oax, *L. malintzi* Pue, *L. devia* Tam, *Loxosceles* sp17 SLP, *Loxosceles* sp11 Qro, *L. nahuana* Hgo, *Loxosceles* sp13 Oax, *L. misteca* Gro and *Loxosceles* sp9 Pue) were recovered with both morphological and molecular data as well as all the delimitation methods (Fig. 2).

ITS2. The ITS2 gen was less informative than COI for species delimitation (Fig. 3). Although fewer species were recovered, the sampling for this marker had fewer sequences in comparison with the analyses implemented under COI. With the sequences used for ITS2, ASAP delimitation analysis recovered only 31 species, being the molecular method with the least recovered species. In comparison, GMYC recovered 57 species under SN and MT implementations (Fig. 3). The bPTP molecular method under ML and BI recovered 45 species (Fig. 3). In accordance with the morphological evidence, species such as *Loxosceles* sp3 Sin, *L. sonora* Son, *Loxosceles* sp5

Sin, *L.* aff. *yucatana* QRoo, *L. rothi*, *L. luteola*, *L. valdosa*, *Loxosceles* sp9 Pue, *L. huasteca* SLP, *Loxosceles* sp12 Qro, and *Loxosceles* sp16 Qro were recovered and congruent also in all the molecular methods (Fig. 3).

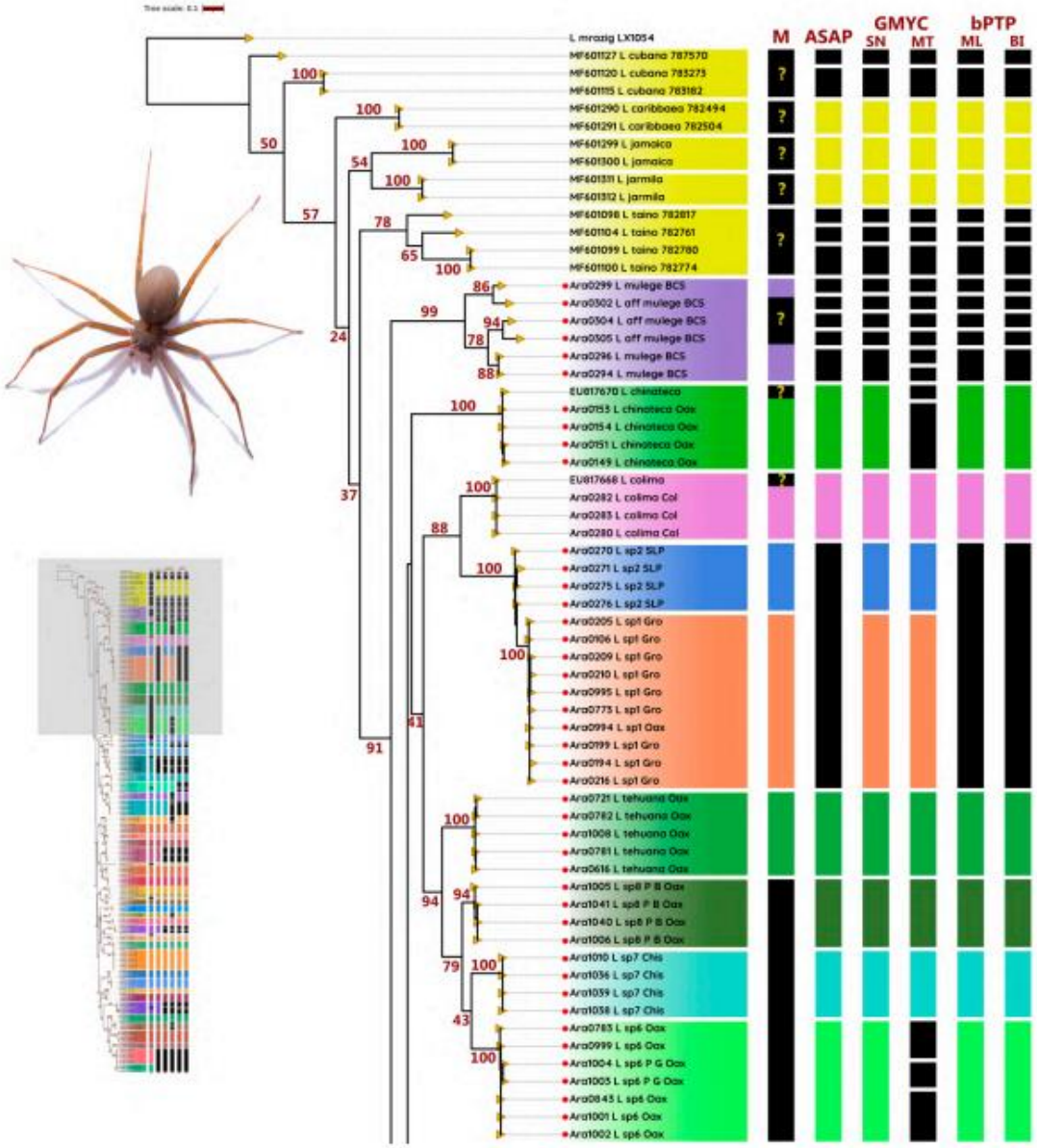

FIGURe 2. Maximum Likelihood (ML) phylogenetic tree constructed with COI of species of *Loxosceles* (*reclusa* group) from North America. Colors of bars indicate different species. Bars represent molecular delimitation methods. Bar abbreviations: Morphology (M); ASAP; GMYC with single (SN) and multi (MT) thresholds; bPTP with ML and Bayesian Inference (BI). Numbers below bars represent species recovered for each delimitation method. Red numbers on branches represent bootstrap support values (>90%). Question marks represent specimens where morphology was not available. Red circles represent new sequences used in this study.

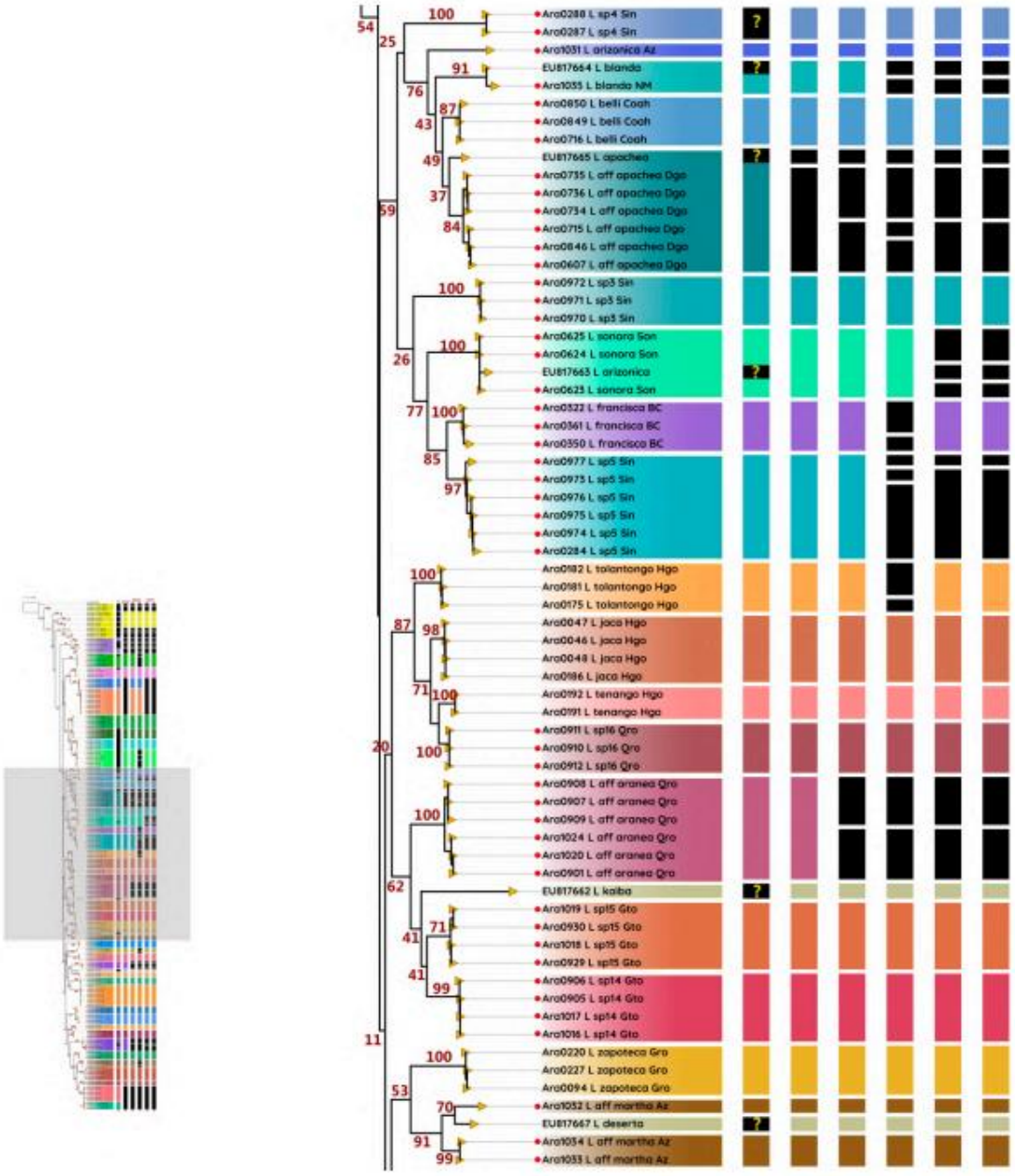

FIGURe 2. (Continued). Maximum Likelihood (ML) phylogenetic tree constructed with COI of species of *Loxosceles* (*reclusa* group) from North America. Colors of bars indicate different species. Bars represent molecular delimitation methods. Bar abbreviations: Morphology (M); ASAP; GMYC with single (SN) and multi (MT) thresholds; bPTP with ML and Bayesian Inference (BI). Numbers below bars represent species recovered for each delimitation method. Red numbers on branches represent bootstrap support values (>90%). Question marks represent specimens where morphology was not available. Red circles represent new sequences used in this study.

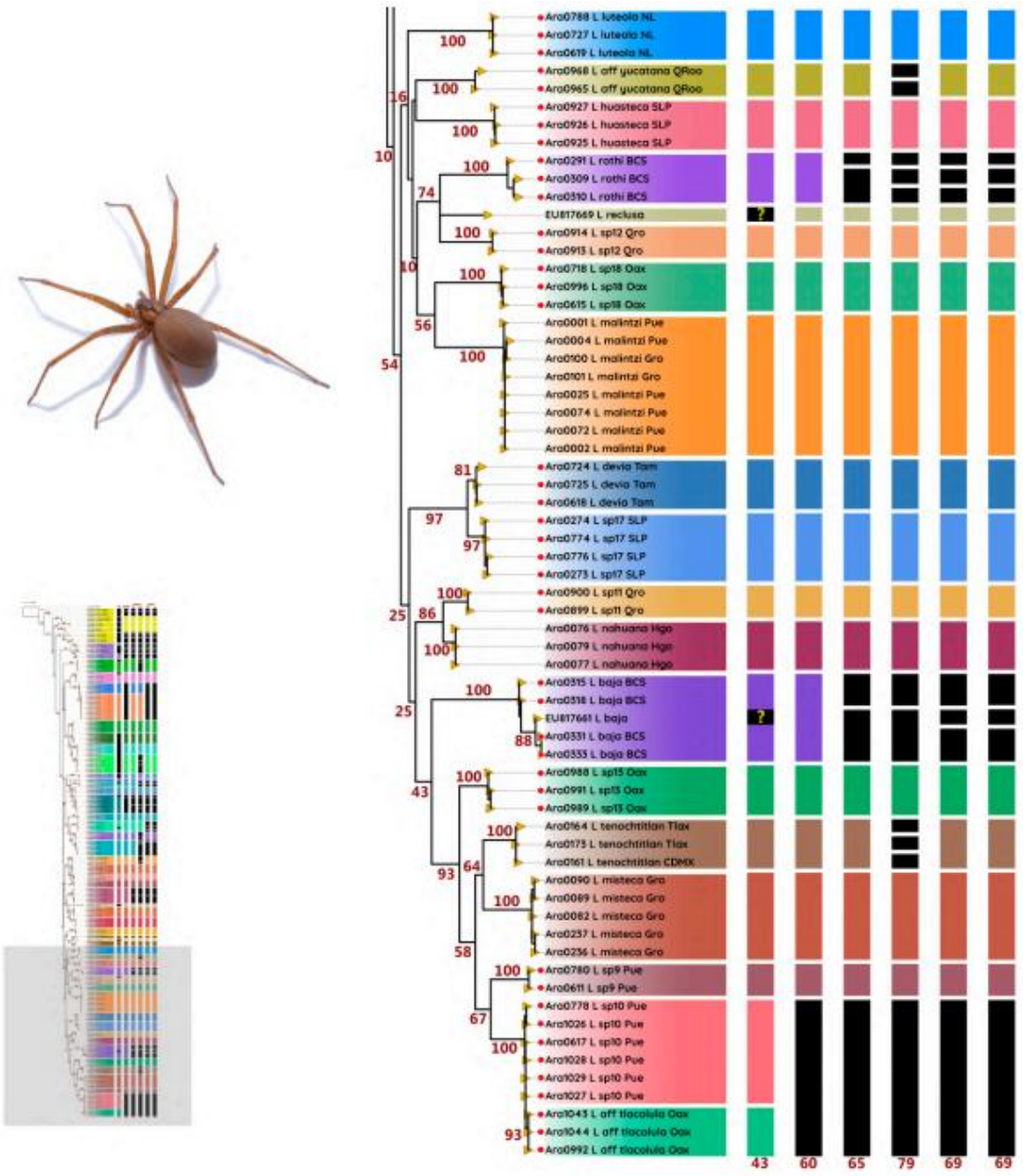

FIGURe 2. (Continued). Maximum Likelihood (ML) phylogenetic tree constructed with COI of species of *Loxosceles* (*reclusa* group) from North America. Colors of bars indicate different species. Bars represent molecular delimitation methods. Bar abbreviations: Morphology (M); ASAP; GMYC with single (SN) and multi (MT) thresholds; bPTP with ML and Bayesian Inference (BI). Numbers below bars represent species recovered for each delimitation method. Red numbers on branches represent bootstrap support values (>90%). Question marks represent specimens where morphology was not available. Red circles represent new sequences used in this study.

Discussion

The work of a taxonomist is based on delimiting, describing, and identifying species (Cook *et al.* 2010), for which different types of data are used that provide further support to the proposed species hypotheses. The use of molecular data (DNA sequences) is helpful in groups that show conserved morphology with low variation (Hendrixson *et al*. 2013; Hamilton *et al.* 2014; Ortiz *et al.* 2018; Xu *et al.* 2020; Hazzi & Hormiga 2021; Ciaccio *et al.* 2022), making their identification complicated. This is the case for the spider genus *Loxosceles* (Gertsch 1958; Brignoli 1969; Gertsch & Ennik 1983; Planas & Ribera 2015; Valdez-Mondragón *et al.* 2019; Navarro-Rodríguez & Valdez-Mondragón 2020; Juárez-Sánchez 2022).

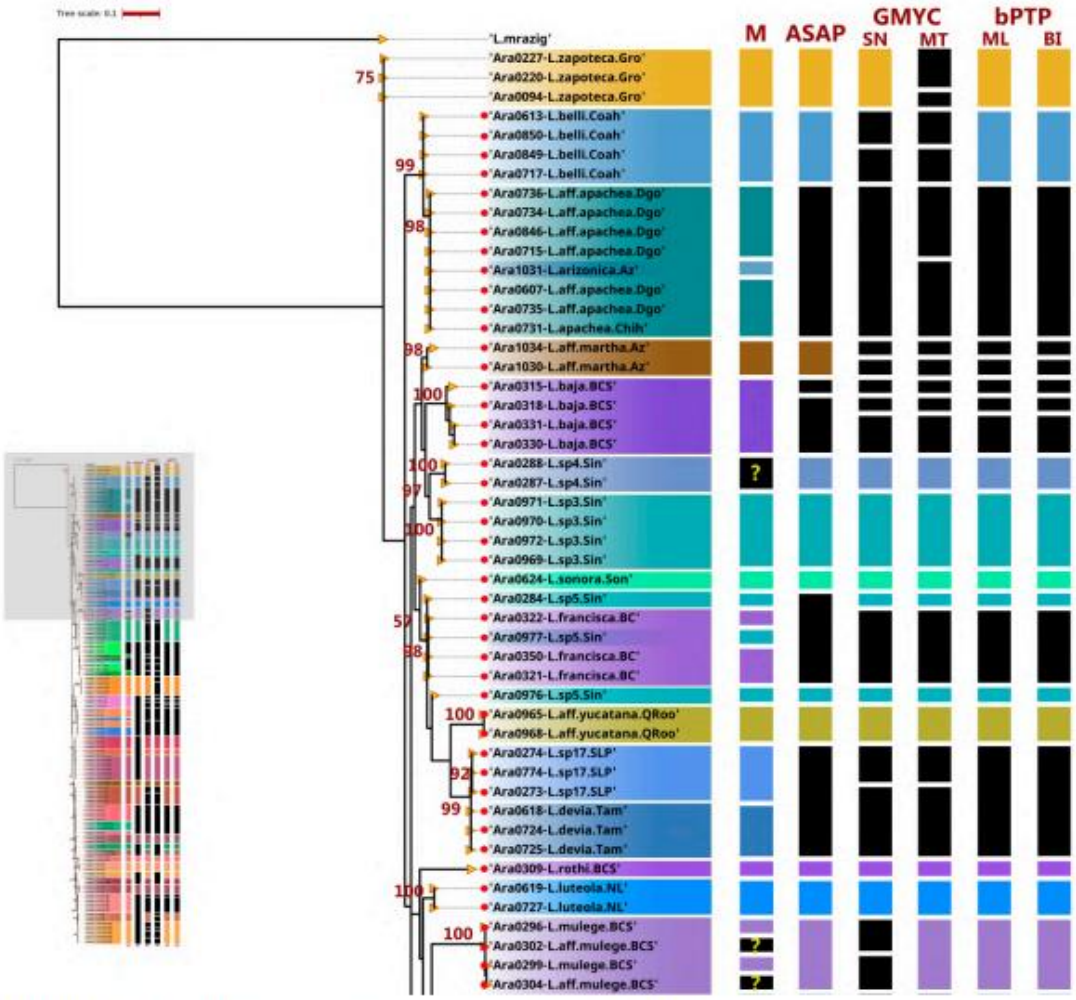

FIGURE 3. Maximum Likelihood (ML) phylogenetic tree constructed with ITS2 of species of *Loxosceles* (*reclusa* group) from North America. Colors of bars indicate different species. Bars represent molecular delimitation methods. Bar abbreviations: Morphology (M); ASAP; GMYC with single (SN) and multi (MT) thresholds; bPTP with ML and Bayesian Inference (BI). Numbers below bars represent species recovered for each delimitation method. Red numbers on branches represent bootstrap support values (>90%). Question marks represent specimens where morphology was not available. Red circles represent new sequences used in this study.

The first integrative species delimitation works using both morphology and molecular data with *Loxosceles* were carried out by Ribera & Planas (2009) and Planas & Ribera (2015) for Canary Islands species. They incorporated mitochondrial and nuclear markers into species delimitation methods to identify and discover new species, showing congruence with morphology. For North American species, Valdez-Mondragón *et al*. (2019) and Navarro-Rodríguez & Valdez-Mondragón (2020) published integrative taxonomy and species delimitation studies with molecular and morphological evidence, focusing on Mexican species. They used different molecular methods, as well as geometric morphometry and niche modeling tools, to corroborate the delimitated species and to uncover cryptic diversity.

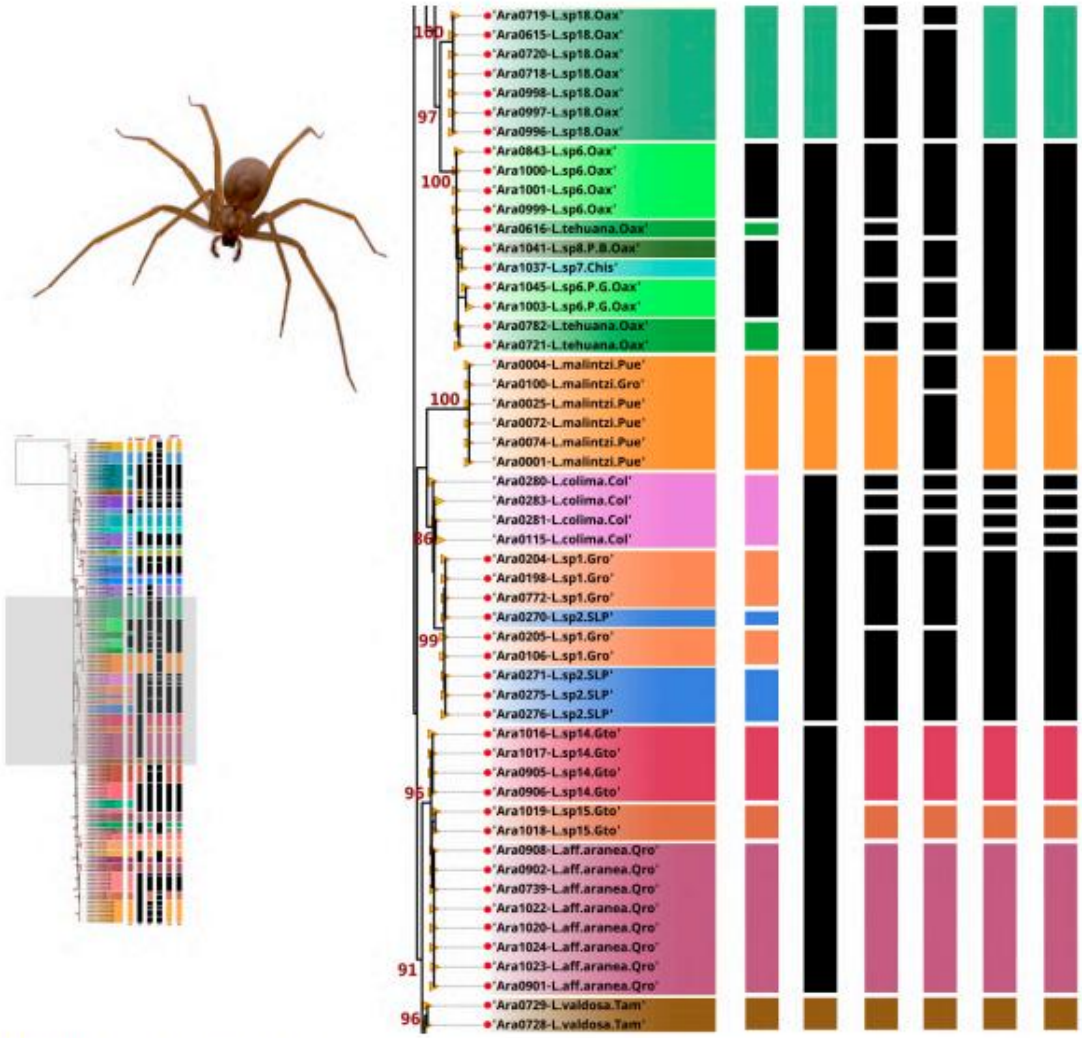

FIGURE 3. (Continued). Maximum Likelihood (ML) phylogenetic tree constructed with ITS2 of species of *Loxosceles* (*reclusa* group) from North America. Colors of bars indicate different species. Bars represent molecular delimitation methods. Bar abbreviations: Morphology (M); ASAP; GMYC with single (SN) and multi (MT) thresholds; bPTP with ML and Bayesian Inference (BI). Numbers below bars represent species recovered for each delimitation method. Red numbers on branches represent bootstrap support values (>90%). Question marks represent specimens where morphology was not available. Red circles represent new sequences used in this study.

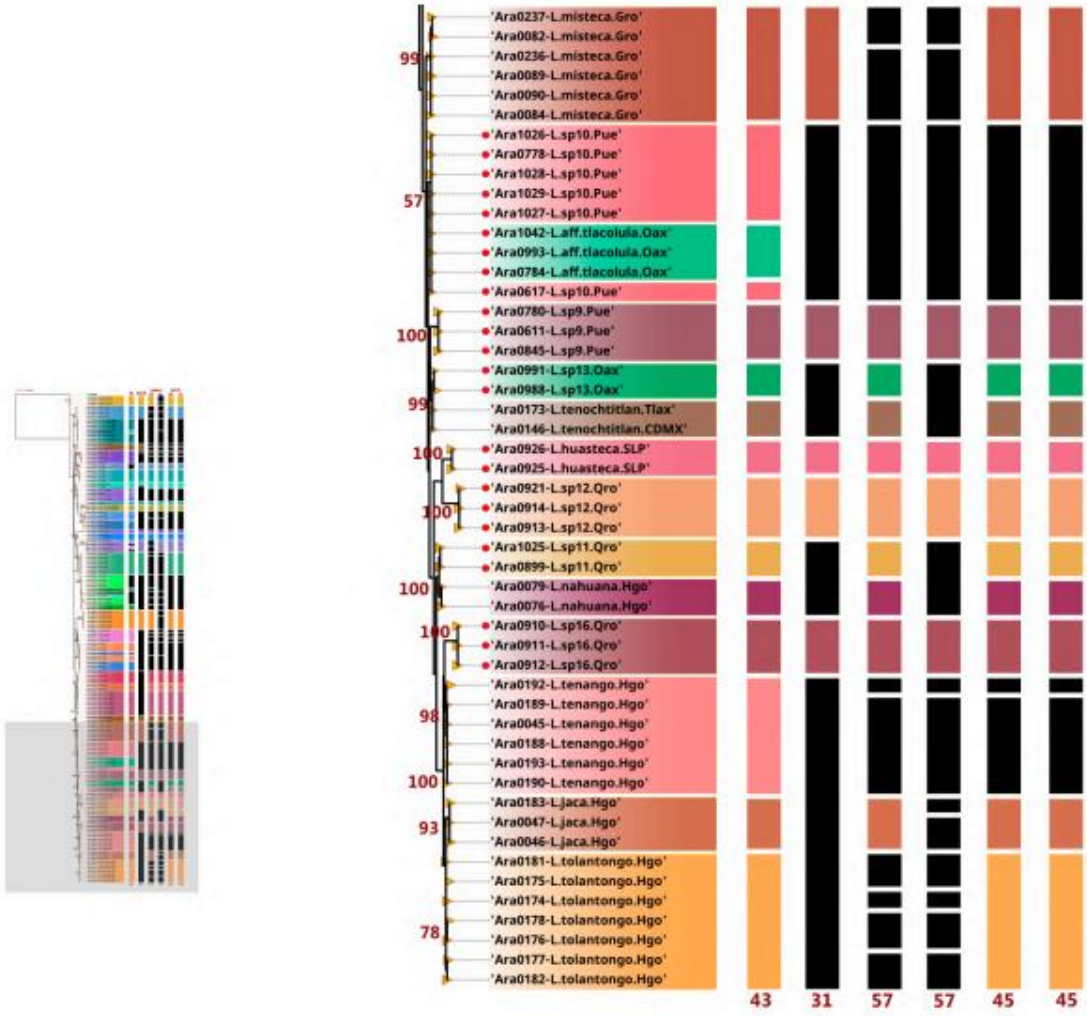

FIGURE 3. (Continued). Maximum Likelihood (ML) phylogenetic tree constructed with ITS2 of species of *Loxosceles* (*reclusa* group) from North America. Colors of bars indicate different species. Bars represent molecular delimitation methods. Bar abbreviations: Morphology (M); ASAP; GMYC with single (SN) and multi (MT) thresholds; bPTP with ML and Bayesian Inference (BI). Numbers below bars represent species recovered for each delimitation method. Red numbers on branches represent bootstrap support values (>90%). Question marks represent specimens where morphology was not available. Red circles represent new sequences used in this study.

The present work represents the most complete sampling of North American *Loxosceles* to date and, as previous studies have shown, corroborates that the diversity of the genus *Loxosceles* in North America remains underestimated and undescribed yet, and that some North American species/populations may have wide morphological and even genetic variation (Duncan *et al.* 2010; Planas & Ribera 2015; Tahami *et al.* 2017; Valdez-Mondragón *et al.* 2019; Navarro-Rodríguez 2019; Navarro-Rodríguez & Valdez-Mondragón 2020; Juárez-Sánchez 2022). Thanks to the use of integrative taxonomy in recent years, new putative species have been able to be identified that were previously considered as single species with wide distributions, and even wide morphological variation in primary sexual structures (Valdez-Mondragón *et al.* 2018b; Valdez-Mondragón *et al.* 2019; Navarro-Rodríguez & Valdez-Mondragón 2020; Juárez-Sánchez 2022).

Although the NJ analyses with genetic *p*-distances represent single phenetic analyses without phylogenetic

propose, they provide a good initial approximation to understand the genetic variation within and among *Loxosceles* species. Herein, we were able to corroborate previously described species, as well as uncover putative new species. Interspecific genetic distances with COI support their delimitation, ranging from 7–17% for most species, with an average of 15.6%. These results are similar to previous works that used molecular evidence for species delimitation of Mediterranean *Loxosceles* species with COI and reported average genetic distances of 17.7% (Ribera & Planas 2009), 13.7% (Planas & Ribera 2014), and 16.9% (Massa & Ribera 2021). Another study on *Loxosceles* species from Iran reported an average interspecific genetic distance of 16.2% (Tahami *et al.* 2017). For North American species, the average genetic distances previously reported have been 13.8% for COI and 4.2% for ITS2 (Valdez-Mondragón *et al.* 2019) and 15.9% for COI and 8.4% for ITS2 (Navarro Rodríguez & Valdez-Mondragón 2020). Recently, Juárez-Sánchez (2022) reported average interspecific genetic distances for the *Loxosceles* "*colima*" species complex of 10.88% for COI and 2.48% for ITS2, recovering five putative species within the complex (*in prep.*).

In comparison with GMYC and bPTP, the ASAP method recovered the lowest number of species. These results are similar to those found by Nolasco & Valdez-Mondragón (2022) working with the spider genus *Physocyclus* (Pholcidae). According to Luo *et al.* (2018), gene flow has a detrimental impact on GMYC and bPTP methods, which are sensitive to the ratio between population size and divergence time. The GMYC-MT method recovered the highest number of species for COI and ITS2.

Some species have not been confirmed with morphological evidence (e.g., *Loxosceles* sp4-Sin) due to no adult specimens being available (Fig. 1, question marks); for Caribbean species, we only accessed the molecular data but not morphology, in order to review and confirm the delimitation of COI (Fig. 2, question marks). In other cases, only the morphological evidence of females (seminal receptacles) was available (*Loxosceles* sp9-Pue, *Loxosceles* sp11-Qro, *Loxosceles* sp12-Qro, and *Loxosceles* sp15-Gto). In these cases, the molecular evidence was congruent with female morphology, and they can thus be proposed as initial hypotheses of putative new species (Padial *et al.* 2009) until complete morphological evidence is obtained for their validation. As mentioned in previous works with *Loxosceles*, only subtle variations were observed in the morphology of sexual structures (Planas & Ribera 2015), which complicates identification. Furthermore, some Mexican species show wide variation in their morphology of seminal receptacles, but are more robust taxonomically in their pedipalp morphology, which can be used to corroborate species-level divisions (Valdez-Mondragón *et al.* 2019; Navarro-Rodríguez & Valdez-Mondragón 2020). Therefore, it is advisable to have a complete series of both males and females to morphologically validate a putative species and/or understand its morphological intra- or interspecific variation (Hillis *et al.* 2021).

This study identified possible species complexes in the cases of *Loxosceles baja*, *L. mulege*, and *L. rothi*, which show inconsistencies among molecular delimitation methods and morphological evidence. All three of these species are distributed in the Baja California Peninsula (BCP), characterized by high levels of species endemism in spiders and a complex biogeographic history (Crews & Hedin 2006; Ceccarelli *et al.* 2023). In *L. baja*, the NJ analysis found an average intraspecific genetic distance of 3.8%, however no morphological differences were found among the different specimens of this species. Similarly, all methods except ASAP found inconsistencies with this species, delimiting more than two different species. In the case of *L. mulege*, the NJ analysis found an average intraspecific genetic distance of 8.6%, which is considered high for intraspecific genetic distance. Unfortunately, in this case, only males and females from one locality were included in this study (Fig. 2: Ara0299, Ara0296 and Ara0294), and no adult specimens were collected from the remaining localities. Therefore, it was not possible to assess any morphological differences between populations. Furthermore, all delimitation methods delimited more than four species within *L. mulege*. In *L. rothi*, the NJ analysis found an average intraspecific genetic distance of 2.9%; however, similar to the case of *L. baja*, no morphological differences were found among the different specimens of this species, and all methods except ASAP found inconsistencies by delimiting more than two different species. In these three cases, more morphological evidence is necessary to corroborate their statuses as putative new species, as well as congruence across different delimitation methods to obtain robust species hypotheses. The BCP represents the most diverse region for species diversity in *Loxosceles*, with nine described species to date; however, more sampling and deeper studies are necessary to understand the diversity of this region and the complex biogeographic history of these species.

Another similar case was seen with *L.* aff. *apachea*-Dgo; the NJ analysis found an average intraspecific genetic distance of 3.0%, recovering it as two different putative species. Similarly, the GMYC and bPTP methods delimited the taxon as two or more species. In terms of morphology, adult specimens of both males and females were assessed, yet the morphology was found to be very similar among all specimens, especially in the pedipalps. It would not be

surprising for further cryptic diversity to be uncovered, as was the case for the species from the Sonora and Pacific Lowlands (northern part) provinces. This taxon was believed to represent only one species (*L. sonora*) until this study, where three putative new species (*Loxosceles* sp3-Sin, *Loxosceles* sp4-Sin, and *Loxosceles* sp5-Sin) were uncovered based on genetic distances, different methods of molecular species delimitation, and morphology.

There are other cases of conflict with some sequenced species such as Ara1032 identified as *Loxosceles* aff. *martha* due to morphological characteristics, however, there is a lack of material to corroborate this identification since there is only one male specimen. Therefore, it would be necessary to collect females specimens to confirm the identification or corroborate whether it is really *L. deserta* with close distribution. Also, as mentioned by Gertsch & Ennik (1983) about the distribution of *L. deserta*, *L. martha*, *L. russelli*, *L. arizonica*, and *L. palma*, these species can be found in sympatry. Another similar case is EU817663 identified as *L. arizona*, which is grouped with the sequences of *L. sonora*; this might be a misidentification, as in the work of Gertsch (1958) where some specimens from Sonora were erroneously assigned to *L. arizonica*, later corrected in Gertsch & Ennik (1983). In this way, a revision of the sequenced specimen (EU817663) would be necessary to corroborate its identity.

As mentioned by Hamilton *et al.* (2011), delineating species with solely molecular evidence is rarely possible, and using additional information such as biogeographic data is necessary. In addition to molecular and morphological evidence, microhabitat information can reveal important information that might have driven the diversification of species within the genus *Loxosceles*. This has been suggested for other spider families within Synspermiata such as Pholcidae by Eberle *et al.* (2018), whose diversification rate was found to depend on microhabitat occupation (ground, leaves, or spaces among big rocks on the ground). In the case of *Loxosceles* spiders, Souza & Ferreira (2018) showed that the morphology of *L. troglobia* is associated with cave environments, finding the following troglomorphic characteristics: eyes with reduced diameters, the absence of pigment around the eyes, appendage elongation, and pale coloration. In the Mexican species of *Loxosceles*, Valdez-Mondragón *et al.* (2019) found variation in leg I lengths between males of *L. misteca* and *L. tenochtitlan*. The longer legs of *L. misteca* might be associated with the microhabitat where the species commonly inhabits; karstic caves from Guerrero and Morelos. Another example of how microhabitat and ecology can drive the diversification of *Loxosceles* species is *L. tlacolula* from Oaxaca. This species has one of the smallest body sizes and is characterized by having short legs, inhabiting the spaces among small rocks on the ground. Phenotypic and ecological data hold further power for illuminating the process of speciation and allowing researchers to distinguish among population- and species-level variation (Cadena & Zapata 2021). The availability of molecular data does not eliminate the need for phenotypic and ecological data; rather, by combining molecular, phenotypic, and ecological data, researchers can better understand how genetic divergence, phenotypic divergence, and ecological divergence differ across putative species, which in turn should inform taxonomic efforts and shed light on speciation and diversification processes in a way that either data type on its own could not (Winker 2009; Cadena & Zapata 2021).

In conclusion, the COI data showed better resolution than the ITS2 region as was also seen with previously studied species of *Loxosceles* from North America (Valdez-Mondragón *et al.* 2019; Navarro-Rodríguez and Valdez-Mondragón, 2020). In addition to molecular evidence, sexual characters should also be considered for species delimitation of North American spiders, while somatic structures could also provide strong data. As proposed by Carstens *et al.* (2013), probing different methods or lines of evidence (e.g., phenotypic, molecular, ecological, and even biogeographical data) in necessary to properly implement species delimitation and for understanding the diversity and diversification of biological groups. As modern taxonomists, we must keep in mind that our first perception of what constitutes a species is based mainly on the morphological features that we can see on the specimens in the field. However, speciation without morphological changes can occur, and the use of an integrative taxonomic approach is necessary to understand the diversity of megadiverse groups such as spiders.

Acknowledgements

The first author thanks the PhD (Doctorado) Program of the Centro Tlaxcala de Biología de la Conducta (CTBC), Universidad Autónoma de Tlaxcala, Tlaxcala City, and the Consejo Nacional de Humanidades, Ciencias y Tecnologías (CONAHCyT) for scholarship support during their PhD in Biological Science. The second author thanks the program "Jóvenes investigadores por México (ex Cátedras CONAHCyT)" and CONAHCyT for scientific support for the project No. 59: "Laboratorio Regional de Biodiversidad y Cultivo de Tejidos Vegetales (LBCTV),

Instituto de Biología, Universidad Nacional Autónoma de México (UNAM)-Tlaxcala". The second author also thanks SEP-CONAHCyT for financial support of the project of Basic Science (Ciencia Básica) 2016, No. 282834. We thank Dr. Edmundo González Santillán and Dr. Oscar F. Francke (ex-curator) of the Colección Nacional de Arácnidos (CNAN), Instituto de Biología, UNAM, for providing specimen loans, MSc. Laura Márquez Valdelamar for the molecular sequencing of the samples, Brett O. Butler for the English language review of the manuscript, and the reviewers for their comments and suggestions that improved the quality of the manuscript. We also thank the students of the Laboratory of Arachnology (LATLAX), IBUNAM, Tlaxcala, for their help in the field and processing the material in the laboratory. We thank to M.Sc. Carlos Palacios-Cardiel (Technician of the Centro de Investigaciones Biológicas del Noroeste CIBNOR) by provide support in the storage of the material at the Laboratory of Arachnology (CARCIB). Thank you to everyone who donated specimens, especially to Biol. Jair Alcántar and Dr. Rodrigo Monjaraz-Ruedas for their field collection efforts. Specimens were collected under Scientific Collector Permit FAUT-0309 from the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT), Mexico, provided to Alejandro Valdez-Mondragón (corresponding author).

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Supplementary material

Supplementary material 1. species sequences

Supplementary material 2. genetic distances COI

Supplementary material 3. genetic distances ITS2

CAPÍTULO 2
ANÁLISIS FILOGENÉTICOS

Phylogenetics and diversification of the spider genus *Loxosceles* Heineken & Lowe (Araneae: Sicariidae) from North America

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Abstract

The spider genus *Loxosceles* better known as “brown recluse spiders” or “violin spider”, are known by their venomous bites worldwide, being the most diverse genus into the family Sicariidae. In this study we corroborated the monophyly of the *recluse* species group distributed in North America using molecular evidence under mitochondrial and nuclear markers (COI+ITS2+28S). Bayesian Inference (BI) and Maximum Likelihood (ML) phylogenetic analyses were implemented. The analysis recovered the *recluse* group as monophyletic. We hypothesized that the *reclusa* group appeared approximately 16.33 million years ago (Mya) (middle Miocene), and its diversification events occurred during the Late Miocene and part of and part of the early Pliocene. Potentially the main ancestral areas that influenced species diversification in North America were the Sierra Madre Oriental (SMOr), Sierra Madre del Sur (SMS) and Balsas Basin Province (BP), during the Miocene. Based on analysis of lineage dating and reconstruction of ancestral areas, we hypothesize that previously to the diversification in North America the dispersal of the genus occurred from South America to North America before the final closure of the Isthmus of Panama, through dispersal over water.

Key words. Systematics, Synspermiata, Biogeography, Neartic, Neotropical.

Introduction

Currently, the spider genus *Loxosceles* Heineken & Lowe, 1832 is composed by 147 species, and belongs to the family Sicariidae Keyserling, 1880, which includes also the genera *Hexophthalma* Karsch, 1879 with eight species and *Sicarius* Walckenaer, 1847 with 21 species (WSC, 2025). Sicariidae is grouped within the clade Scytodoidea along with Ochyroceratidae Fage, 1912, Psilodercidae Machado, 1951, Scytodidae Blackwall, 1864, Drymusidae Simon, 1893, and Periegopidae Simon, 1893, whose shared characteristics are the six-ocellus grouped in three pairs, bipectinate prolateral claws on tarsi I-II and a distal dorsal hood covering the claw bases (Labarque & Ramírez, 2012; Wheeler *et al*., 2017; Kulkarni, *et al.,* 2023).

Based on the morphological characters of the palps, the genus *Loxosceles* has been classified into seven species groups worldwide: *reclusa*, *laeta*, *gaucho*, *spadicea*, *vonwredei*, *rufescens*, and *spinulosa* (Gertsch,1958; Gertsch & Ennik, 1983; Binford *et al.,* 2008; Duncan *et al.,* 2010, Fukushima *et al.,* 2017). The *reclusa* group includes those species with distribution in North America from the United States, Mexico, part of Central America and the main Antillean Islands. Mexico is the country with the highest of *Loxosceles* diversity of species in the world, with 38 native and two introduced: *Loxosceles reclusa* (Gertsch & Mulaik, 1940) from southeastern USA and *Loxosceles rufescens* (Dufour, 1820) from the Mediterranean region (Gertsch & Ennik, 1983; Valdez-Mondragón *et al.*, 2019). Also, under integrative taxonomy, several new species from Mexico remains to be described (Navarro-Rodríguez & Valdez-Mondragón, *in prep.*).

According to Magalhaes *et al.* (2017) based on phenotypic, morphological data and venom characteristics, the genus *Loxosceles* is a monophyletic genus, recovering *Loxosceles simillima* as a sister group. Regarding the monophyly of the species groups, based on molecular evidence using mitochondrial and nuclear data Binford *et al.* (2008) recover as monophyletic the clade with the North American species and as sister group the Caribbean species, which are represented by nine species, supporting as well the *reclusa* group as monophyletic. Furthermore, Binford *et al.* (2008) proposed the presence of a common ancestor for Sicariidae in western Gondwana, which diversified before the separation of the African and South American continents.

About the dispersion of the genus *Loxosceles* along North America, this is previously hypothesized to have occurred via the land bridge called GAARlandia (Greater Antilles and Aves Ridge) around 33–35 My, which connected South America with the Antillean Islands and subsequently dispersed to North America (Binford *et al*., 2008). However, so far, any study has mentioned the processes that may have influenced the diversification of the genus in North America under a biogeographic approach, being the region with the highest species diversity worldwide. Also, Binford *et al*. (2008) only included into their analyses four species from Mexico, without any hypothesis of diversification.

Mexico has a complex biogeographic and geological history, which is composed of different mountain ranges and chains: the Sierra Madre Oriental, the Sierra Madre Occidental, and the Sierra Madre del Sur which emerged mainly in the Inferior and Middle Cenozoic (Mastretta-Yanes *et al*., 2015) and the Trans-Mexican Volcanic Belt emerging in the Miocene-Middle extending its orogeny until the late Pleistocene (Ferrari *et al*., 2012; Mastretta-Yanes *et al*., 2015). Furthermore, Mexico is located between two large biogeographic regions (Nearctic and Neotropical) (Morrone, 2005), where both converge in the Mexican Transition Zone (Morrone, 2020). Halffter and Morrone (2017), conducted a study where explain how the convergence of the Nearctic and Neotropical biogeographic regions (Mexican Transition Zone) has been originated the composition and richness of species, where the climate and the mountain range it hosts have favored the displacement and establishment of different taxa. For the case of arachnids, Schramm *et al.* (2021) conducted a molecular dating and divergence study of the whip spiders *Acanthophrynus coronatus* Butler, 1873, considering the complex history of

Mexican landscape, suggesting that the emergence of the Transmexican Volcanic Belt led to the diversification of *Acanthophrynus* emphasizing that glacial/interglacial cycles are drivers of diversification in the Mexican Neotropics. For spiders, Valdez-Mondragón and Francke (2015) estimate the divergence times of the genus *Ixchela* (Pholcidae), finding that most speciation events may have occurred mainly during the Pleistocene, where climatic changes induced by repeated glaciations significantly influenced the diversification of the genus. These diversification and speciation patterns occurred mainly in the Temperate Montane Forests, with the emergence of the Sierra Madre del Sur of Oaxaca, the Trans-Mexican Volcanic Belt, the

Sierra Madre Oriental and in the Sierra de Chiapas. It is likely that these geological events have also driven the diversification of the genus in North America.

For study the biogeographical factors might have been influencing *Loxosceles* species diversification in North America, it is advisable to integrate phylogenetic and biogeographic analyses, molecular clocks and, when possible, the fossil record (Morrone, 2005). This to have a better estimation of the formulated hypotheses of monophyletic groups, as well as distribution times and events (Halffter & Morrone, 2017).

In this way, the aim of this study is to understand the diversification of *Loxosceles* species of the *reclusa* group in North America under molecular evidence into a biogeographic context. It also incorporates a hypothesis on the divergence times and ancestral areas reconstruction that influenced the diversification of *Loxosceles* from North America, based on the molecular clock and the fossil record.

Material and methods

Biological material

The specimens were collected by hand and deposited in 80% ethanol for morphological identification and in 96% ethanol for molecular studies, labeled with their collection data. The specimens and additional material examined were deposited with their codes in the following collections: CARCIB = Collection of Arachnology, Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, Baja California Sur, Mexico. CNAN = National Collection of Arachnids, Instituto de Biología, Universidad Nacional Autónoma de Mexico (IB-UNAM), Mexico City, Mexico (additional material). Observations and identification of the specimens were done using a Zeiss model Stemi 508 stereoscopic microscope. The dissection of seminal receptacles in females and palps in males was implemented for species identification. The female genitalia were immersed in potassium hydroxide (KOH-10%) for 1–5 minutes to digest and clean the soft tissue around the seminal receptacles. Left male palps and female genitalia were dissected for identification to the species level. Species-level identification was carried out following Gertsch & Ennik (1983), Valdez-Mondragón *et al*. (2018, 2019), and Navarro-Rodríguez & Valdez-Mondragón (2020).

Taxon sampling

The molecular phylogenetic analyses were based on a total of 246 sequences from 36 species (S1) of a concatenated matrix of COI, ITS2 and 28S genes. 204 COI sequences and 154 ITS2 sequences published by Binford *et al.* (2005), Petersen *et al*. (2017), Valdez-Mondragón *et al.* (2019) and Navarro-Rodríguez & Valdez-Mondragón (2020, 2024) were downloaded from the GenBank database (www.ncbi.nih.gov) (S1). In addition, 85 new sequences of 28S from species across Mexico and the USA were added for this study (S1).

Outgroup selection

The criterion of outgroup comparison was used to test the monophyly of the *reclusa* group (Table 1). The outgroup selection was based on previous phylogenetic hypotheses of the genera within the Synspermiata clade (Binford *et al*., 2008; Ubick *et al*., 2017; Wheeler *et al*., 2017; Magalhaes *et al*., 2019; Taucare-Ríos & Piel, 2021). To test the monophyly of the genus *Loxosceles*, the following outgroups were selected: *Drymusa serrana* Goloboff & Ramírez, 1992, *Dysdera silvativa* Schmidt, 1981, *Hexophthalma spatulata* (Pocock, 1900), *Kukulcania mexicana* Magalhaes & Ramírez, 2019, *Scytodes fusca* Walckenaer, 1837, *Scytodes thoracica* (Latreille, 1802) and *Sicarius thomisoides* Walckenaer, 1847. In addition, species from four *Loxosceles* species groups were selected as outgroups: 1) *spadicea* group*: Loxosceles spadicea* Simon, 1907, 2) *rufescens* group*: Loxosceles rufescens* (Dufour, 1820), *Loxosceles amazonica* Gertsch, 1967, *Loxosceles mrazig* Ribera & Planas, 2009, 3) *spinulosa* group: *Loxosceles spinulosa* Purcell, 1904, and 4) *vonwredei* group: *Loxosceles vonwredei* Newlands, 1980. Sequences of *Loxosceles cubana* Gertsch, 1958 and *Scytodes thoracica* (Latreille, 1802) were used to calibrate the trees. Outgroup sequences were obtained also from the GenBank database (S1). For divergence dating and calibration see sections below.

DNA extraction, amplification and sequencing

Tissue selection, DNA extraction, PCR amplification, and purification were performed at the Laboratorio de Biología Molecular del Laboratorio Regional de Biodiversidad y Cultivo de Tejidos Vegetales (LBCTV), IB-UNAM, Tlaxcala. For DNA extraction, a Qiagen DNeasy Tissue Kit was used, following the modified protocols of Valdez-Mondragón & Francke (2015)

and Navarro-Rodríguez & Valdez-Mondragón (2020). For DNA extraction, two legs in adult specimens, three or four legs in juveniles, and in a few cases of immature specimens the entire specimen were dissected. The tissue was stored in 96% ethanol in a -20°C freezer for posterior DNA extraction. The final DNA extractions were stored in a -70°C freezer. Three genes were used for the molecular analyses: cytochrome *c* oxidase subunit I (COI) (mitochondrial gene), internal transcribed spacer 2 (ITS2) and 28S ribosomal RNA (nuclear genes). For the amplification of COI, we used two primer sets: LCO1490/HCO2198 and LCO1490-JJ/HCO2198-JJ (Folmer *et al.*, 1994; Astrin & Stüben, 2008), for ITS2, the primer set 5.8SF and CAS28sB1d were used (Ji *et al.*, 2003; Planas & Ribera, 2014), and for 28S the primer set was 28S-B1 and 28S-B2 (Table 1). The PCR (Polymerase Chain Reaction) parameters for COI and ITS2 were performed following the protocols by Navarro-Rodríguez & Valdez-Mondragón (2020, 2024). The PCR (Polymerase Chain Reaction) parameters for COI and ITS2 were performed following the protocols by Valdez-Mondragón *et al*. (2019) and Navarro-Rodríguez & Valdez-Mondragón (2020, 2024). The protocol for 28S was as follows: initial heating phase of 15 min at 95 °C, 35 amplification cycles of 35 s at 95 °C (denaturing), 1 min at 62 °C (alignment), and 1 min at 72 °C (elongation), with a final elongation of 10 min at 72 °C. Gel electrophoresis was carried out with 0.5% agarose using the molecular weight marker Perfect DNA 100 bp Ladder Novage to calculate fragment size of amplifications. Gels were visualized in a photodoc BioDoc-It2 Imager 315 Imaging System LMS-20 Transilluminator. PCR products were purified using a QIAquick Qiagen purification kit. A Veriti Applied-Biosystems 96 Well Thermal Cycler was used for amplifications, in total volume of 20 microliters: 2.3 µl of injectable H_2O, 2 µl of Q-Solution, 10 µl of Multiplex-Mix a QIAGEN Kit, 3.2 µl of oligonucleotide (forward and reverse) and 2.5 µl of DNA. The sequencing of both chains (5′–3′ and 3′–5′) of the PCR products was performed in a Sequencer Genetic Analyzer RUO Applied Biosystems Hitachi model 3750xL, at the Laboratory of Molecular Biology and Health, IB-UNAM, Mexico City.

DNA sequence alignment and editing

The molecular sequences in the raw. ab1 format were edited using the programs BioEdit version 7.0.9.0 (Hall, 1999) and Geneious version 8.1.9 (Rozen & Skaletsky, 2000). The edited

sequences were saved in .fasta format and used in a multiple sequences alignment, performed in the MAFFT (Multiple sequences Alignment based on Fast Fourier Transform) online version (https://mafft.cbrc.jp/alignment/server/) using its alignment strategy: Auto (FFT-NS-2, FFTNS-i or L-INS-i; depending on data size). In some cases, sequence alignment was done manually and edited using BioEdit software. Geneious software was used to assess sequence qualities. A concatenated matrix (COI+ITS2+28S) was constructed using Mesquite version 3.81 software by Maddison & Maddison (2023).

Table 1: Primers sets used for PCR amplification for COI, ITS2 and 28S.

Gene	Primer name	Primer sequence (5′–3′)	Reference
COI	LCOI490	5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'	Folmer *et al*. (1994)
	HCO2198	5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'	
	LCOI490-JJ	5'-CHA CWA AYC ATA AAG ATA TYG G-3'	Astrin y Stüben, (2008)
	HCO2198-JJ	5'-AWA CTT CVG GRT GCV CAA ARA ATC A-3'	
ITS2	5.8SF	5'-CAC GGG TCG ATG AAG AAC GC-3'	Ji *et al*. 2003; Planas y Ribera, (2014)
	CAS28sB1d	5'-TTC TTT TCC TCC SCT TAY TRA TAT GCT TAA-3'	
28S	28S-B1	5'-GACCGATAGCAAACAAGTACCG-3'	Bruvo-Madarić *et al*. (2005)
	28S-B2	5'-GATTAGTCTTTCGCCCCTATA-3'	

Phylogenetic analysis

Maximum Likelihood Analysis (ML)

Phylogenetic analyses were performed in IQ-Tree web server (http://iqtree.cibiv.univie.ac.at/) (Trifinopoulos *et al.*, 2016), using the concatenated matrix (COI+ITS2+28S). Models of sequence evolution were selected using IQ-Tree web server (http://iqtree.cibiv.univie.ac.at/) under the Akaike information criteria (AIC). The selected model for each gene were the next: COI = GTR+F+I+G4 (1st, 2nd and 3rd codon positions) and GTR+F+G4 for ITS2 and 28S. The ML analysis was calculated with 1000 replicates of Bootstrap (significant values ≥ 80%) (Hillis & Bull, 1993). The phylogenetic tree was edited using the program ITOL version 6.7.3 (Letunic & Bork, 2021) and Adobe Photoshop CS6.

Bayesian Inferences Analysis (BI)

The phylogenetic analyses were carried out in the CIPRES Science Gateway website (https://www.phylo.org) (Miller *et al*., 2010) and in MrBayes v.3.2 (Ronquist & Huelsenbeck, 2003) using the concatenated matrix (COI+ITS2+28S). Models of sequence evolution were selected using IQ-Tree web server (http://iqtree.cibiv.univie.ac.at/) under the Bayesian Information Criteria (BIC). The selected model for each gene were the next: COI = GTR+F+I+G4 (1st, 2nd and 3rd codon positions), ITS = TVM+F+G4, and 28S = TIM3+F+G4. The BI analyses were run with four parallel Markov chains with the following parameters: MCMC (Markov Chain Monte Carlo) generations = 50 000 000, sampling frequency = 1000, print frequency = 1000, number of runs = 2, number of chains = 8, MCMC burnin = 2500, sumt burnin = 2500, sump burnin = 2500. TRACER version 1.6 (Rambaut and Drummond 2003–2009) was used to analyze the parameters and the Effective Sample Size (ESS) of the MCMC to ensure the runs converged. FigTree version 1.4.3 was used to visualize the tree with the posterior probability values (PP) at nodes. The generated trees were edited using iTOL version 6.7.3 and Adobe Photoshop CS6.

Divergence dating

To estimate the divergence times of the lineages of *Loxosceles* from North America, the relaxed molecular clock model was used, where molecular substitution rates are not constant and can vary (Magallón, 2018). To carry out the divergence dating we used the programs BEAUti (Bayesian Evolutionary Analysis Utility) version 1.7.5 (Drummond *et al*., 2012) and BEAST (Bayesian Evolutionary Analysis SamplingTrees) version 1.7.5 (Drummond *et al.*, 2012). For the calibration of the molecular clock, two alternatives were used: 1) the fossil record, and 2) the substitution rate of COI. First, the fossil record of the Synspermiata clade, mainly of the subfamily Scytodoidea, was used as minimum ages, using *Loxosceles defecta* Wunderlich, 1988 from the Dominican amber (15-20 Mya) (Neogene) described by Wunderlich, (1988) (Iturralde-Vinent and MacPhee (1996) represented by *Loxosceles cubana* Gertsch, 1958 sequences. Also, *Scytodes weitschati* Wunderlich, 1988 described by Wunderlich, (1988) from Baltic amber (43 to 47-8 Mya) (Paleogene) and represented by *Scytodes thoracica* (Latreille, 1802) sequences was used. The estimated substitution rate of COI is 0.0178, which was included in the analysis

as an additional calibration point (Papadopoulou *et al*., 2010). The concatenated COI+ITS2+28S matrix was used to calculate divergence times. The divergence time interval of the lineages and their internal relationships was established by calculating 95% of the highest posterior divergence interval (HPD) for each *tmrca* (time of the most recent common ancestor) at each node. The results under the molecular clock with the lognormal relaxed clock model were subsequently analyzed using TRACER to evaluate the parameters and ESS of the MCMC. TRACER and TreeAnnotator version 1.7.5. (Rambaut & Drummond, 2002–2013) were further used to select the number of generations with low posterior probabilities discarded as burn-in (25%). The parameters used in BEAUti for the analysis with BEAST were as follows: Model = Lognormal relaxed clock (Uncorrelated), estimate; Tree Prior = A birth-death was applied; Substitution and Site Heterogeneity model = GTR+I+G; Base frequencies = Empirical; Number of Gamma Categories = 4; MCMC length of chain = 40 000 000. FigTree was used to visualize the topology of the tree with the posterior probability values (PP) at nodes. The generated trees were edited using iTOL version 6.7.3 and Adobe Photoshop CS6.

Ancestral Areas Reconstruction

The software RASP version 4.2 (Reconstruction Ancestral States in Phylogenies) (Yu *et al*., 2015, 2020) was used to perform the reconstruction of dispersal, vicariance and ancestral area events by Statistical-Dispersal Vicariance Analysis (S-DIVA) and Bayesian Binary MCMC (BBM). An input consensus tree generated from a Bayesian Inference analysis was used. For a first analysis, a statistical dispersion-variance analysis (S-DIVA) was performed to infer the biogeographic history through multiple phylogenetic trees. The analyses were done including 14 biogeographical provinces proposed by Morrone *et al.* (2017) and Morrone (2019) for North America. The R package 'BioGeoBEARS' (Matzke 2014) was used to select the historical biogeography model (Nepokroeff *et al*., 2003). The R package 'adephylo' (Jombart *et al*., 2010) and 'geiger' version 2.0.6.2 (Pennell, *et al*., 2014) were also used to estimate the phylogenetic signal.

Results

Phylogenetic analyses

According to IB and ML phylogenetic analyses of the concatenated matrix (COI+ITS2+28S), the *reclusa* species group are recovered as monophyletic with 98% Bootstrap support values under ML and 98% Posterior Probabilities (PP) values under BI (Fig. 1).

Under the ML phylogenetic analysis for Mexican species, seven main clades were recovered. The first, clade A (Fig. 1) includes five species: *Loxosceles* sp. 18, *L. tehuana*, *Loxosceles* sp. 8, *Loxosceles* sp. 7 and *Loxosceles* sp. 6, with a Bootstrap value of 98% and 99% PP. All these species are distributed mainly in southeastern Mexico, in the states of Oaxaca and Chiapas state. The second, clade B, includes four species: *L. kaiba, L. devia, L. huasteca,* and *Loxosceles* sp. 17, with a low Bootstrap support value of 84% and 49% PP. These species are distributed mainly in San Luís Potosí and Tamaulipas. The third one, clade C, contains five species: *Loxosceles* sp. 3, *Loxosceles* sp. 4, *L. baja, L.* aff. *martha* and *L. deserta,* with Bootstrap values of 69% and 75% PP.. The fourth, clade D, consists of seven species: *Loxosceles* sp. 5, *L. francisca, L. apachea, L. arizonica, L. blanda, L. belli* and *L. sonora*, with 100% Bootstrap and PP support values. For clades C and D, the species are distributed mainly in northwestern Mexico and the southwestern United States, in the states of California, Arizona, Nuevo Mexico, Baja California, Baja California Sur, Sonora, Sinaloa, Coahuila y Durango. The fifth, clade E, comprises four species: *Loxosceles* sp. 1, *Loxosceles* sp. 2, *L.* colima and *L. malintzi*, with Bootstrap and PP support values of 94%. These species are distributed in San Luís Potosí, Colima, Guerrero, Puebla and Oaxaca. The sixth, clade F, comprises seven species: *L. misteca, L. tenochtitlan, Loxosceles* sp. 9, *L.* aff. *tlacolula* and *Loxosceles* sp. 10, *Loxosceles* sp. 13 and *L. valdosa,* with Bootstrap support values of 73% and 91% PP. These species are distributed in Guerrero, Puebla, Oaxaca, Tlaxcala, CDMX and Tamaulipas. Finally, the seven clade, clade G, comprises 10 species: *L. jaca, Loxosceles* sp. 16, *L. tenango, L. tolantongo, L. rothi, L. nahuana, Loxosceles* sp. 11, *L.* aff. *aranea, Loxosceles* sp. 14 and *Loxosceles* sp. 15, with a low Bootstrap support value of 51% and 20% PP (Fig. 1). Most species are distributed in central Mexico in the states of Guanajuato, Hidalgo and Queretaro.

For clades A, D and G there is some biogeographical correspondence, clade A is composed of species distributed towards the southeastern region of the country, between the

Sierra Madre del Sur and Pacific Lowlands provinces. For clade D, all species are distributed in the northwestern region, mainly in the provinces of Baja California, Sonora, Pacific Lowlands and Chihuahuan Desert. Finally, clade G, the species that comprise it, are found in the central region distributed in the provinces of the Sierra Madre Oriental and Chihuahuan Desert.

Lineage dating and reconstruction of ancestral areas

The combination of the substitution rate of COI (0.0178) and the calibration points: *Loxosceles defecta* Wunderlich, 1988 from the Dominican amber (15-20 Mya) and *Scytodes weitschati* Wunderlich, 1988, from Baltic amber (43 to 47-8 Mya), result in the divergence event of "*reclusa"* group was 16.33 Mya (15.15-21.01, 95% of the highest posterior density interval; HPD) (Fig. 2) during the Middle Miocene. The first divergence event within the Caribbean and North American species occurring 15.91 Mya ago (15.26-17.28, 95% HPD) during the Middle Miocene. For the Caribbean species, the first divergence event between them occurred approximately 14.19 Mya ago (10.82 -17.11 95% HPD) during the Middle Miocene (Fig. 2).

This estimate also indicates that the probably first diversification events of the *reclusa* group in Mexico occurred approximately 11.65 Mya ago (8.4-15.06, 95% HPD) during the Middle Miocene. Subsequent speciation events occurred within the different clades of the *reclusa* group in Mexico approximately 5 to 7.5 Mya ago (Fig. 2) during the Late Miocene and part of de Pliocene.

The topology of the divergence time tree was similar to the ML and BI inference (Fig. 1). The first divergence event within clade A, occurred at 7.32 Mya and subsequently speciation events within this clade occurred between 0.25 and 2.56 Mya. For the clade B, the first divergence event was 7.6 Mya and speciation events occurred between 1.44 and 6.16 Mya. For clades C and D, the divergence ages were similar at 6.5 and 6.1 Mya respectively, after speciation events occurred between 1.89 and 5.17 Mya. For clade E, the first divergence event was 7.73 Mya and speciation events occurred around 0.91 and 5.83 Mya. For clade F, divergence event occurred at 7.3 Mya, and speciation events between 2.16 and 5.4 Mya. For clade G, first divergence event was 6.21 Mya and speciation events occurred between 1.32 and 4.35 Mya (Fig. 2).

Most of the diversification events for the genus *Loxosceles* occurred during the late Miocene, whereas the speciation events occurred during the Pliocene and some in part of the late Miocene.

The reconstruction of ancestral areas (AA) shows six main biogeographical provinces that influenced the diversification of *Loxosceles:* 1) Sierra Madre Oriental (SMOr), 2) Balsas Basin Province (BP), 3) Veracruzan Province (VP), 4) Chihuahuan Desert Province (CD), 5) Sierra Madre del Sur (SMS) and 6) Baja Californian Province (BC).

The divergence of the *reclusa* group seems to have occurred by dispersal (Fig. 3, green circle), and the dispersal towards Central America may have been the first event that influenced its diversification (Fig. 3). As for the species from the Caribbean and North America, dispersal and vicariance influenced their diversification (Fig. 3, yellow circle), and the dispersal towards the Caribbean Islands was the driving force behind the diversification. The Sierra Madre Oriental and Sierra Madre del Sur were recovered as the main ancestral areas that drove the diversification of the *Loxosceles* genus in Mexico.

The diversification of clade A, which includes five species: *Loxosceles* sp. 18, *L. tehuana, Loxosceles* sp. 8, *Loxosceles* sp. 7 and *Loxosceles* sp. 6, was driven mostly by the Sierra Madre del Sur and the Veracruzan Province. For clade B, which is made up of four species: *L. huasteca, L. kaiba, L. devia* and *Loxosceles* sp. 17 were driven by the ancestral areas of Sierra Madre Oriental and Tamaulipeca Province. For clades C and D, composed of five and seven species respectly: *L. baja, L. martha, L. deserta, Loxosceles* sp. 4 and *Loxosceles* sp. 3; *Loxosceles* sp. 5, *L. francisca, L. apachea, L. arizonica, L. blanda, L. belli* and *L. sonora*, were driven by the same ancestral areas of Baja Californian Province, Sonora Province, Pacific Lowlands Province and Chihuahuan Desert Province. For clade E, composed of only four species: *Loxosceles* sp. 1, *Loxosceles* sp. 2, *L. colima* and *L. malintzi*, was determined by the Balsas Province, Sierra Madre del Sur, Pacific Lowlands Province, Sierra Madre Oriental and Trans-Mexican Volcanic Belt. For clade F, which includes seven species: *L. misteca, L. tenochtitlan, L. tlacolula, L. valdosa, Loxosceles* sp9, *Loxosceles* sp10 and *Loxosceles* sp13, was driven mainly by the ancestral areas of the Balsas Province, Sierra Madre del Sur, the Trans-Mexican Volcanic Belt and Veracruzan provinces. Finally, Clade G consisting of 10 species,

the ancestral areas determining its diversification were Sierra Madre Oriental, Veracruzan Province and Chihuahuan Desert Province.

Diversification among species of the genus *Loxosceles* has been driven by both vicariance and dispersal events, the latter mainly between the Late Miocene and Pliocene (2.6-8 Mya) (Fig. 3).

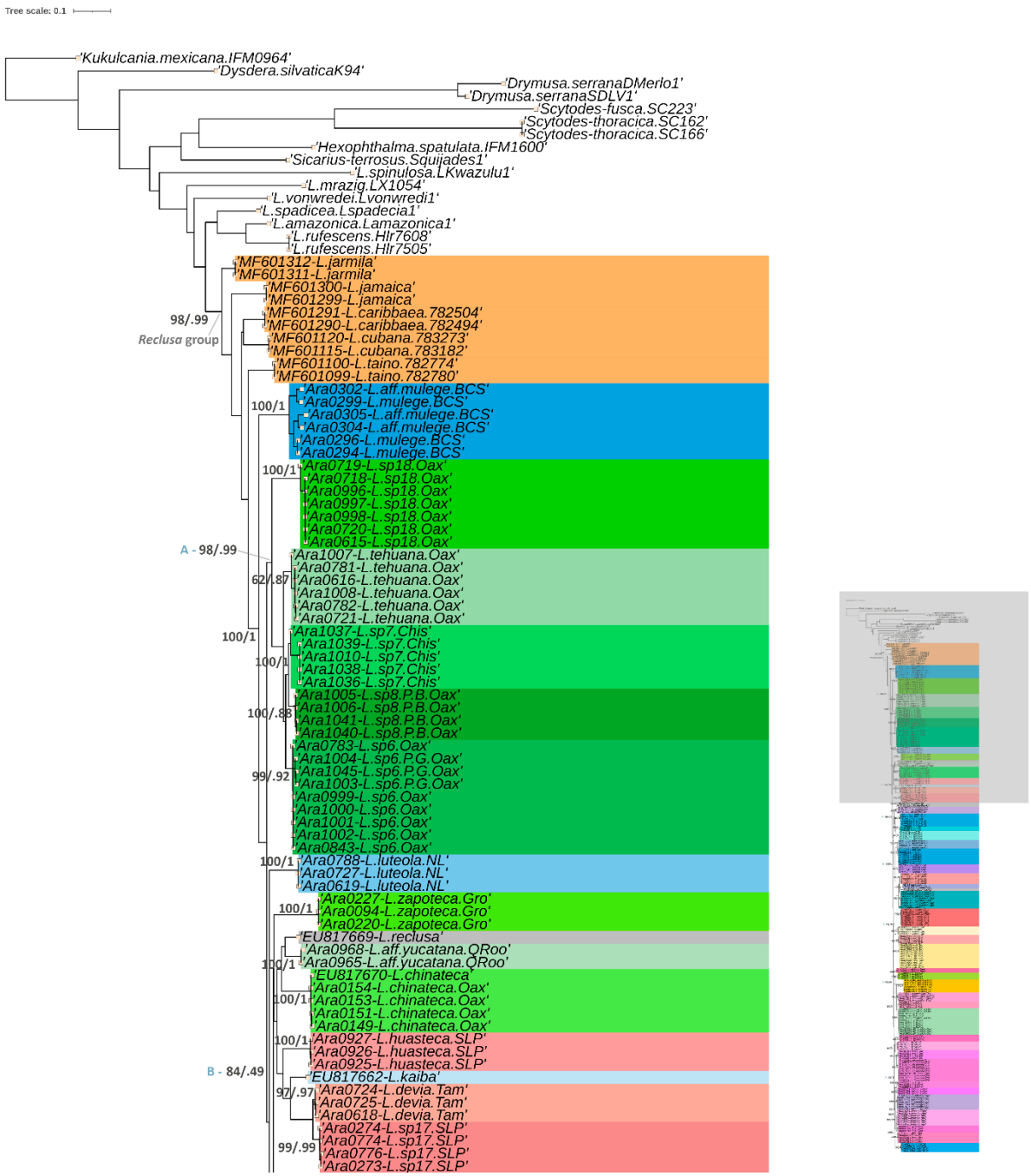

Figure 1. Maximum Likelihood Tree (highest log: -17982.91) inferred from a concatenated matrix (COI+ITS2+28S) of *Loxosceles* species belonging to the North American *reclusa* group. Numbers on the branches represent Bootstrap values under ML and Posterior Probabilities under BI.

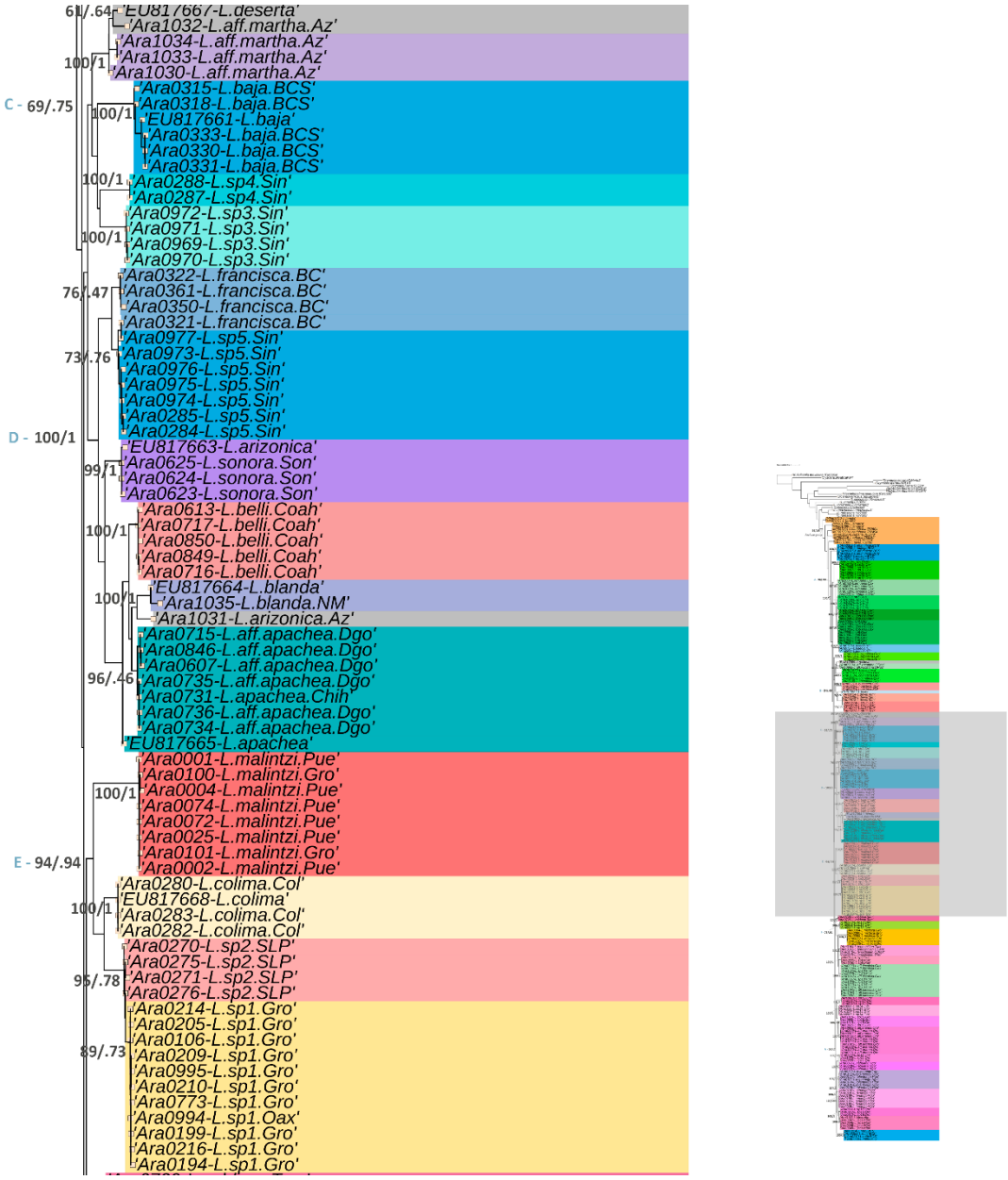

Figure 1. Continuation.

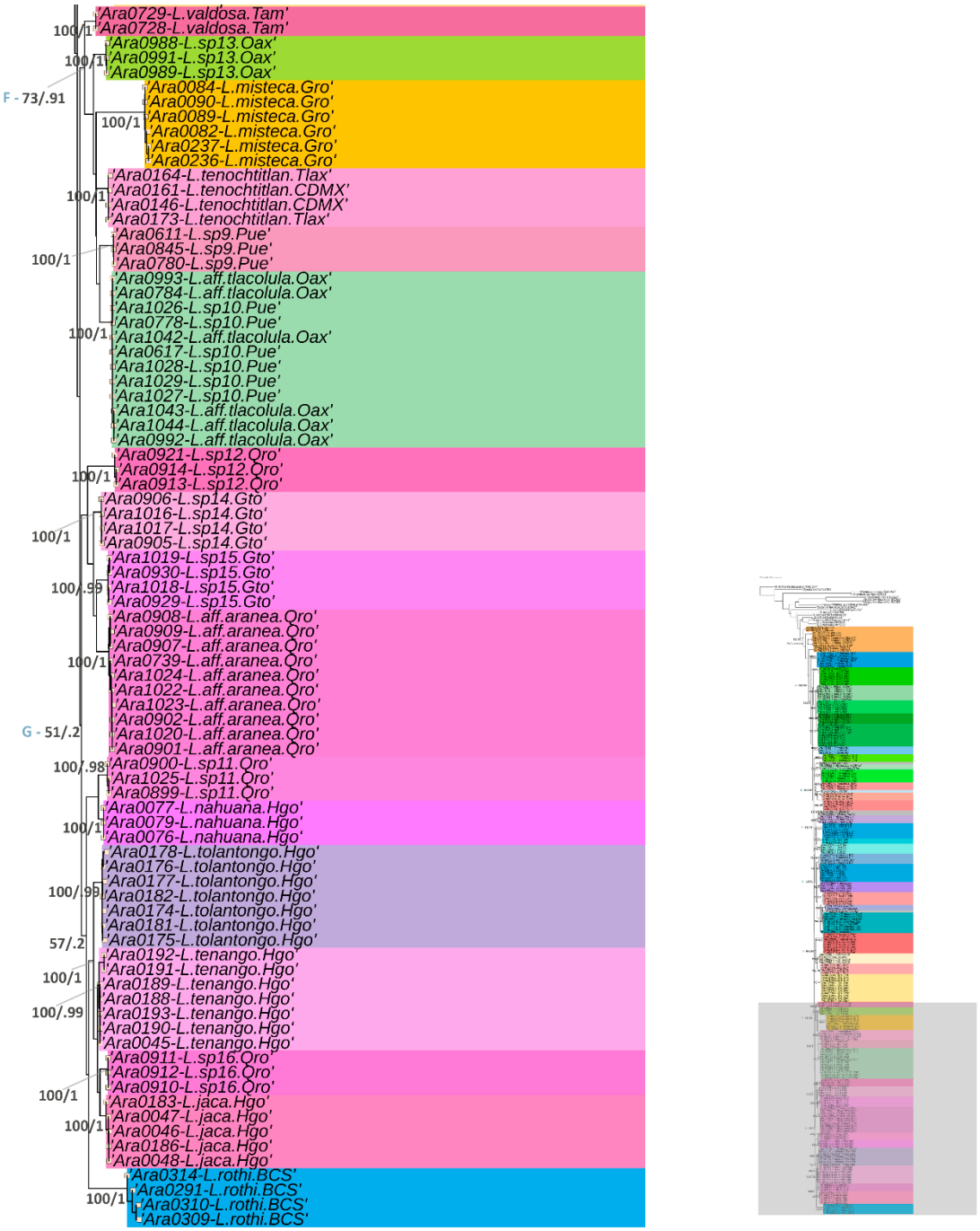

Figure 1. Continuation.

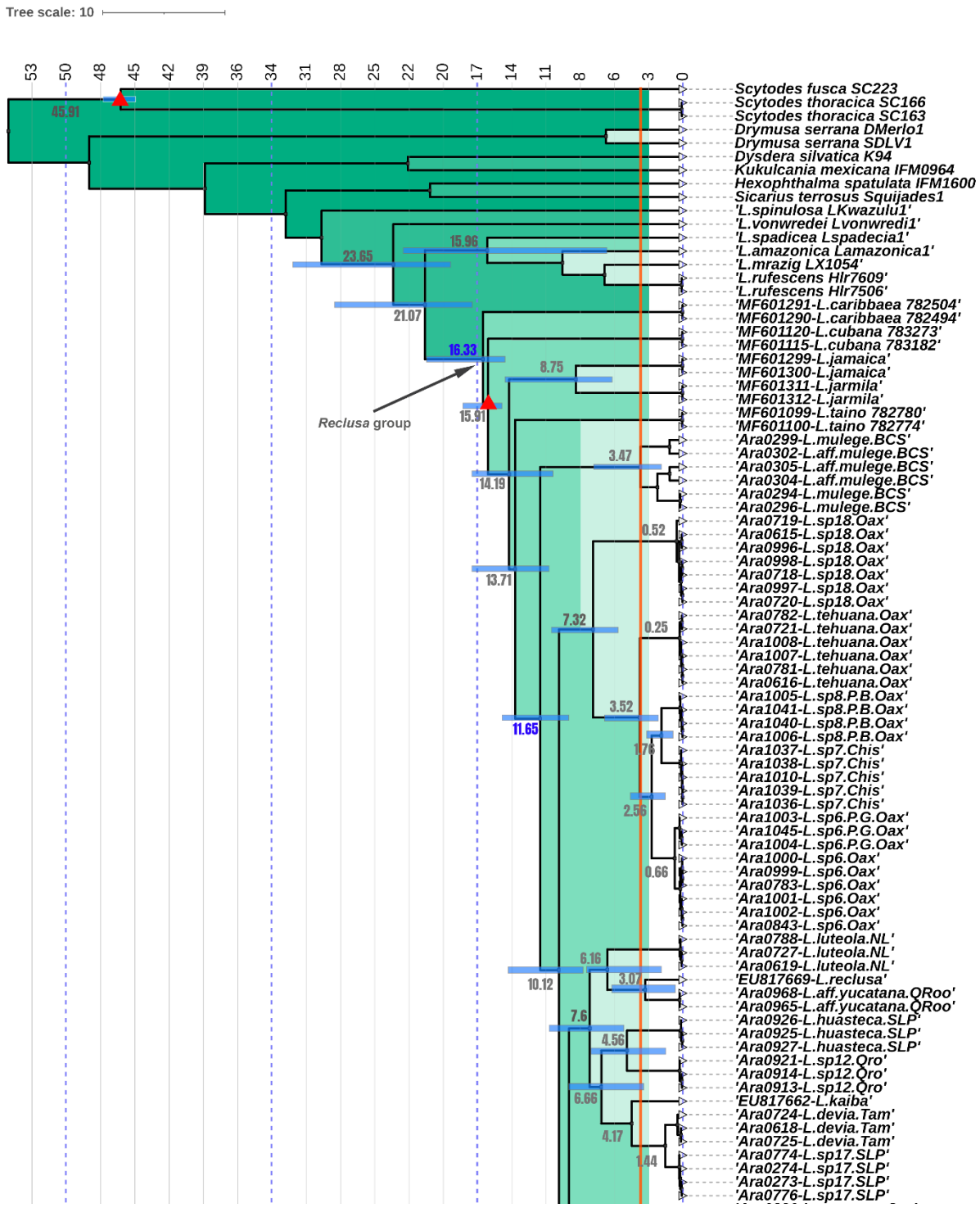

Figure 2. Chronogram dated with the COI substitution rate and fossil record of a concatenated matrix (COI+ITS2+28S) lineages of the genus *Loxosceles* of the *reclusa* group, under a relaxed clock. Numbers on the bars indicate the average age of each node in millions of years (Mya). Red triangles indicate calibration points. Orange vertical line represents speciation events.

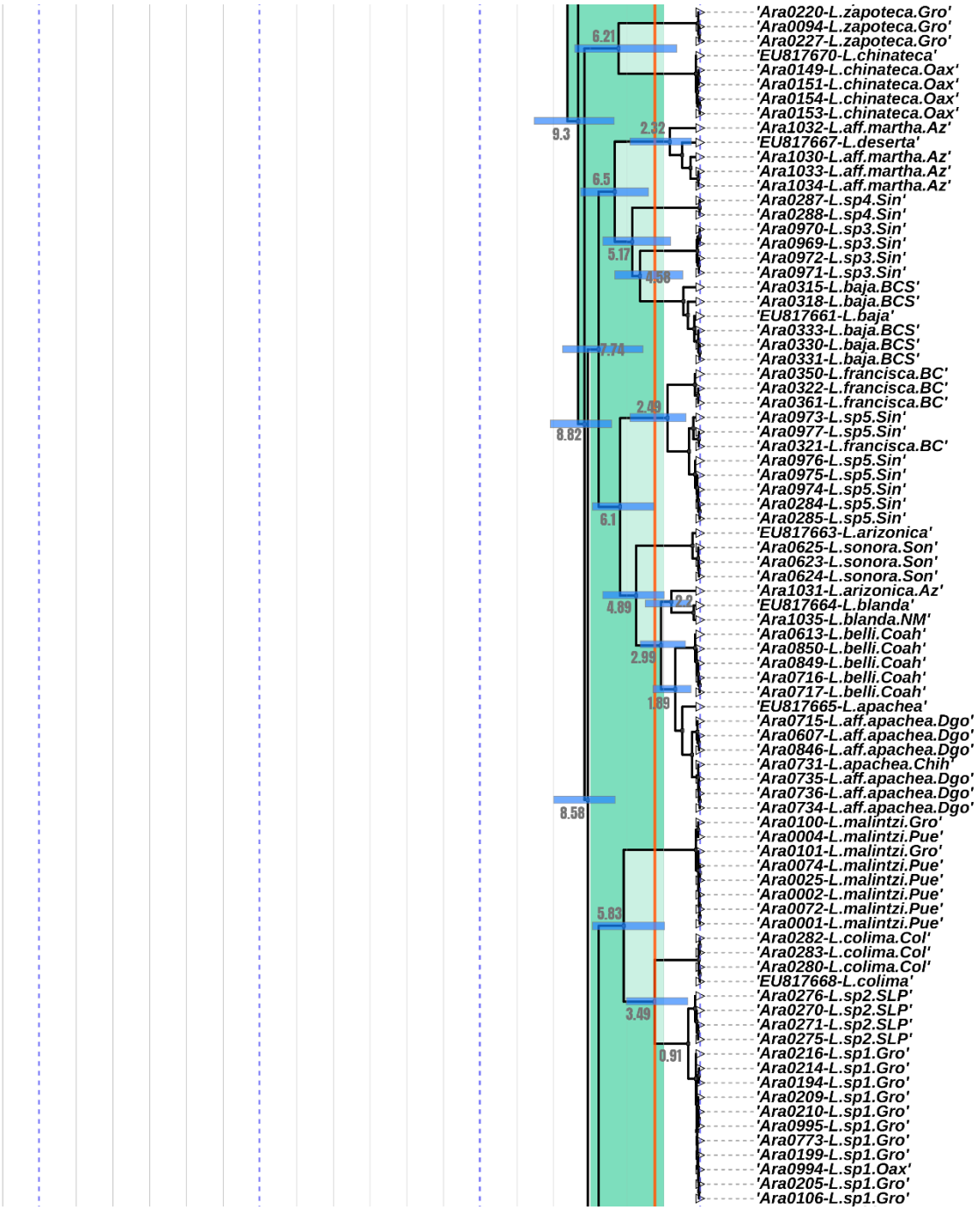

Figure 2. Continuation

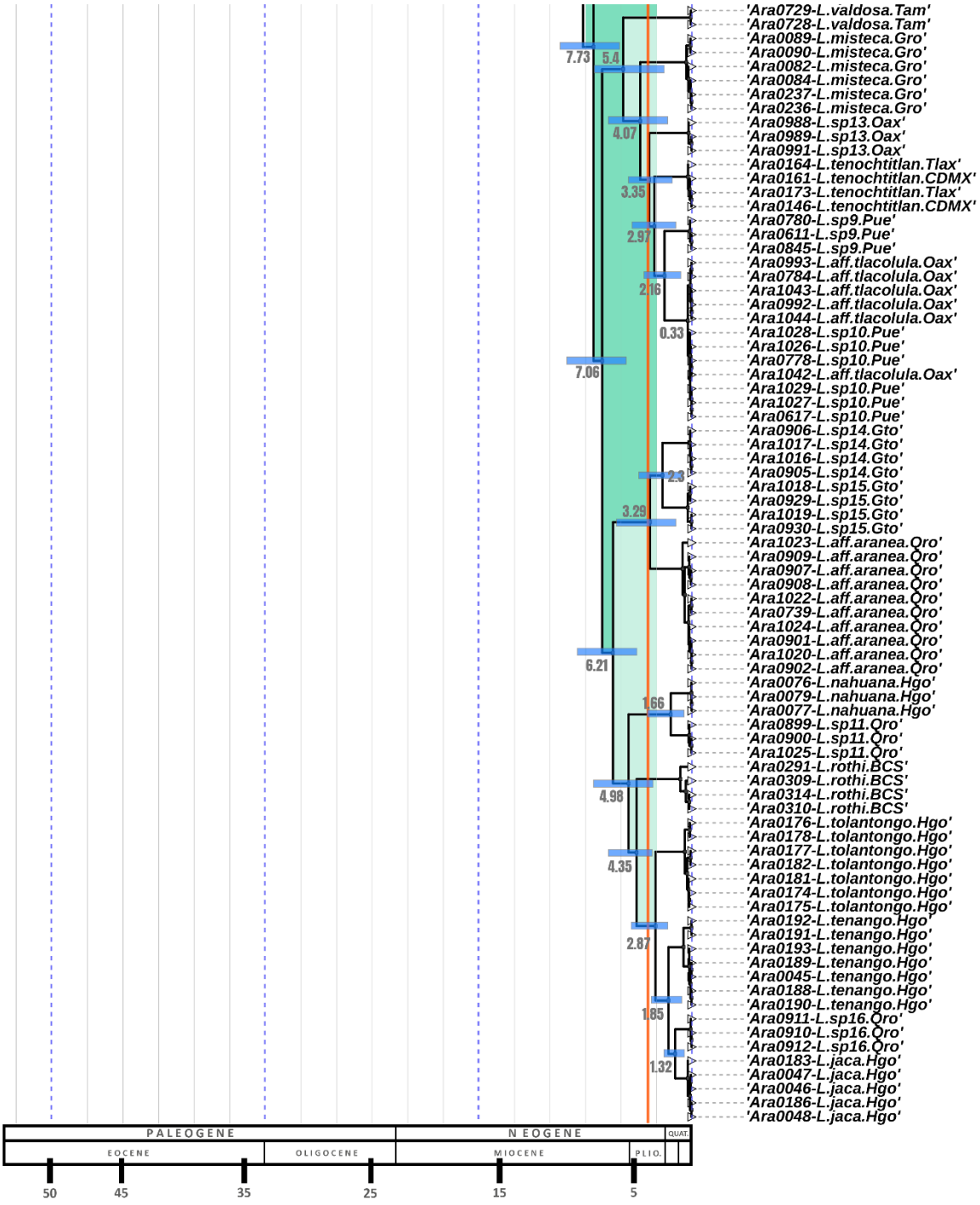

Figure 2. Continuation

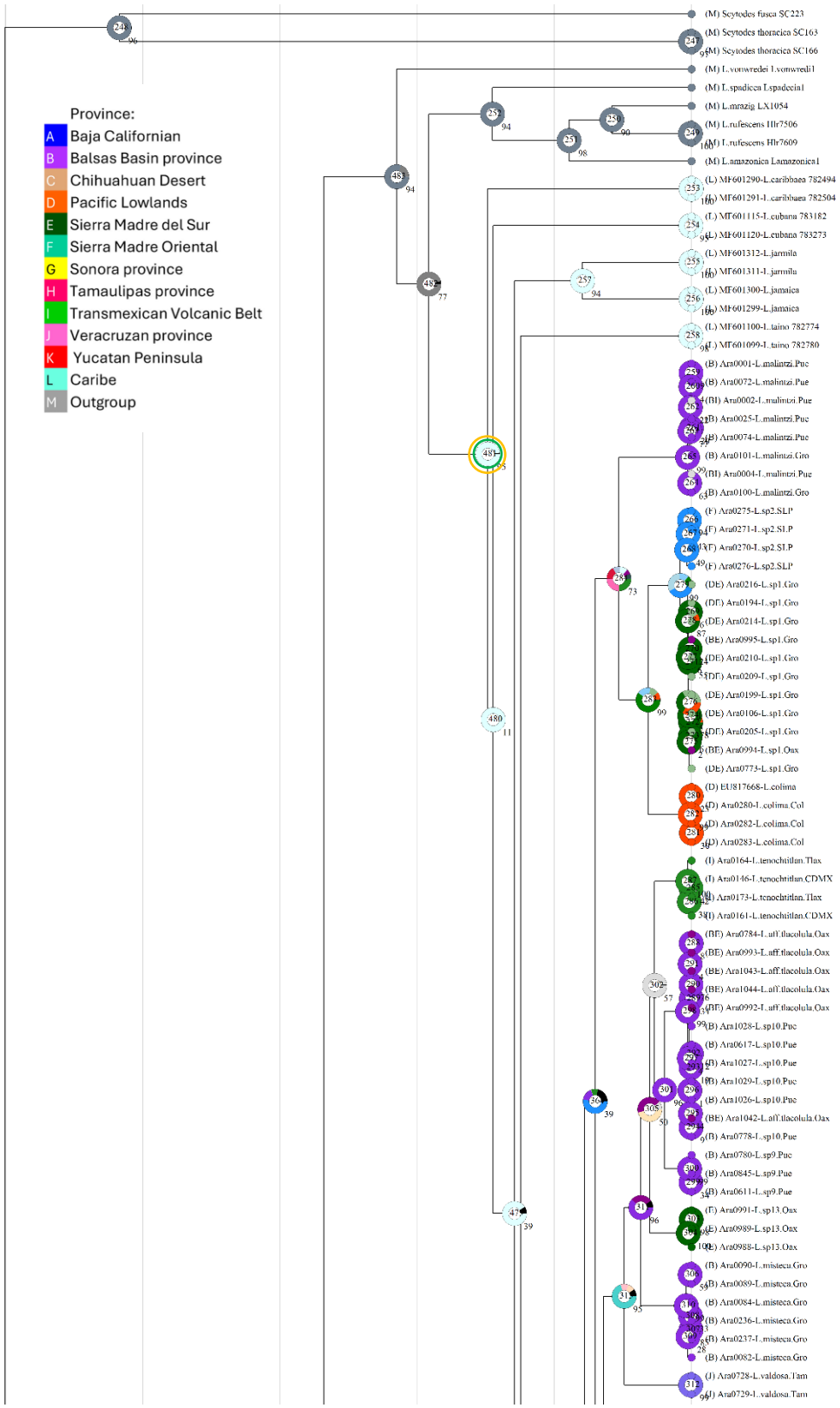

Figure 3. Ancestral areas reconstruction of the diversification pattern of *Loxosceles* species from the North American *reclusa* species group. Circles in color on the nodes indicate the ancestral areas corresponding to the biogeographical provinces. Green circle outline = dispersion. Yellow circle outline = vicariance.

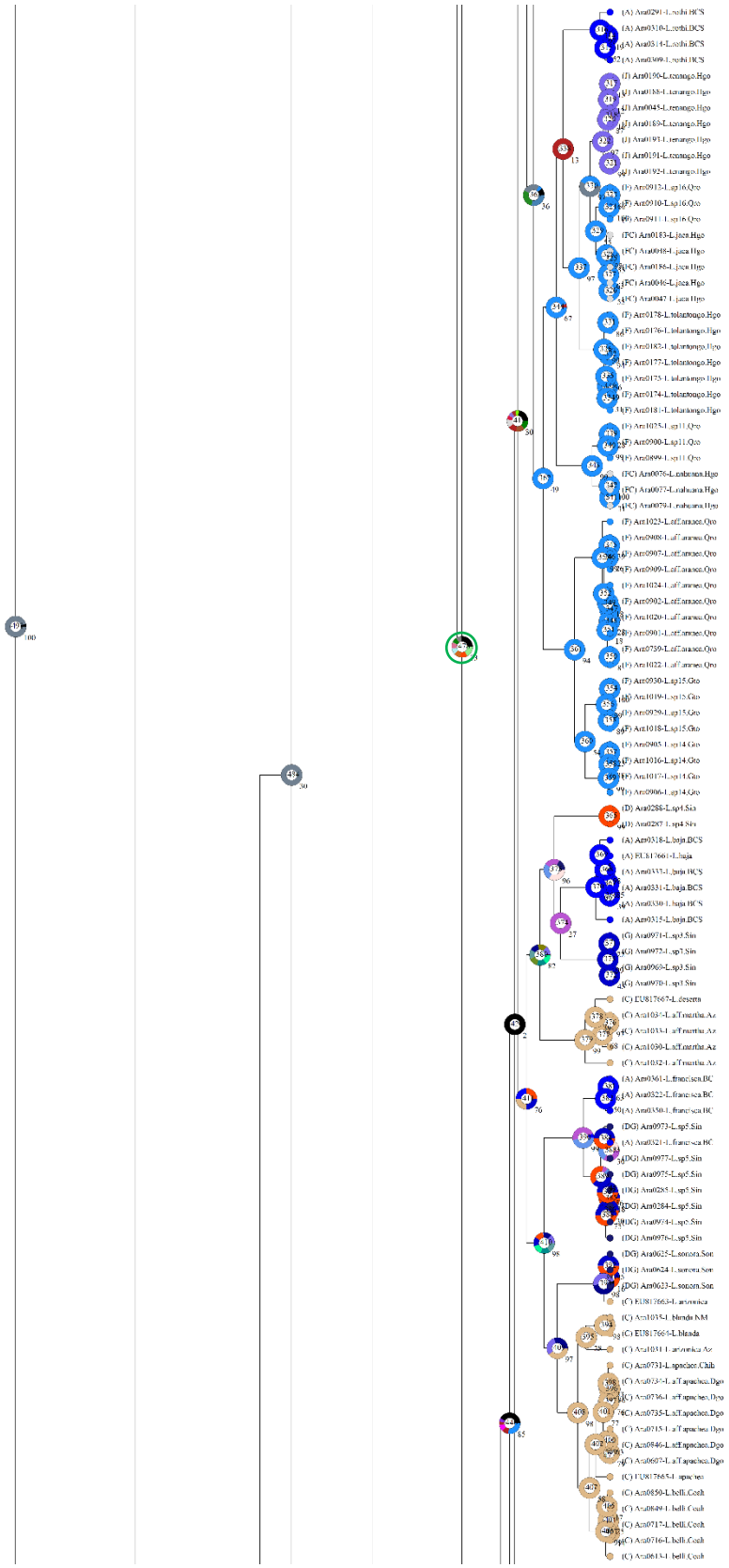

Figure 3. Continuation.

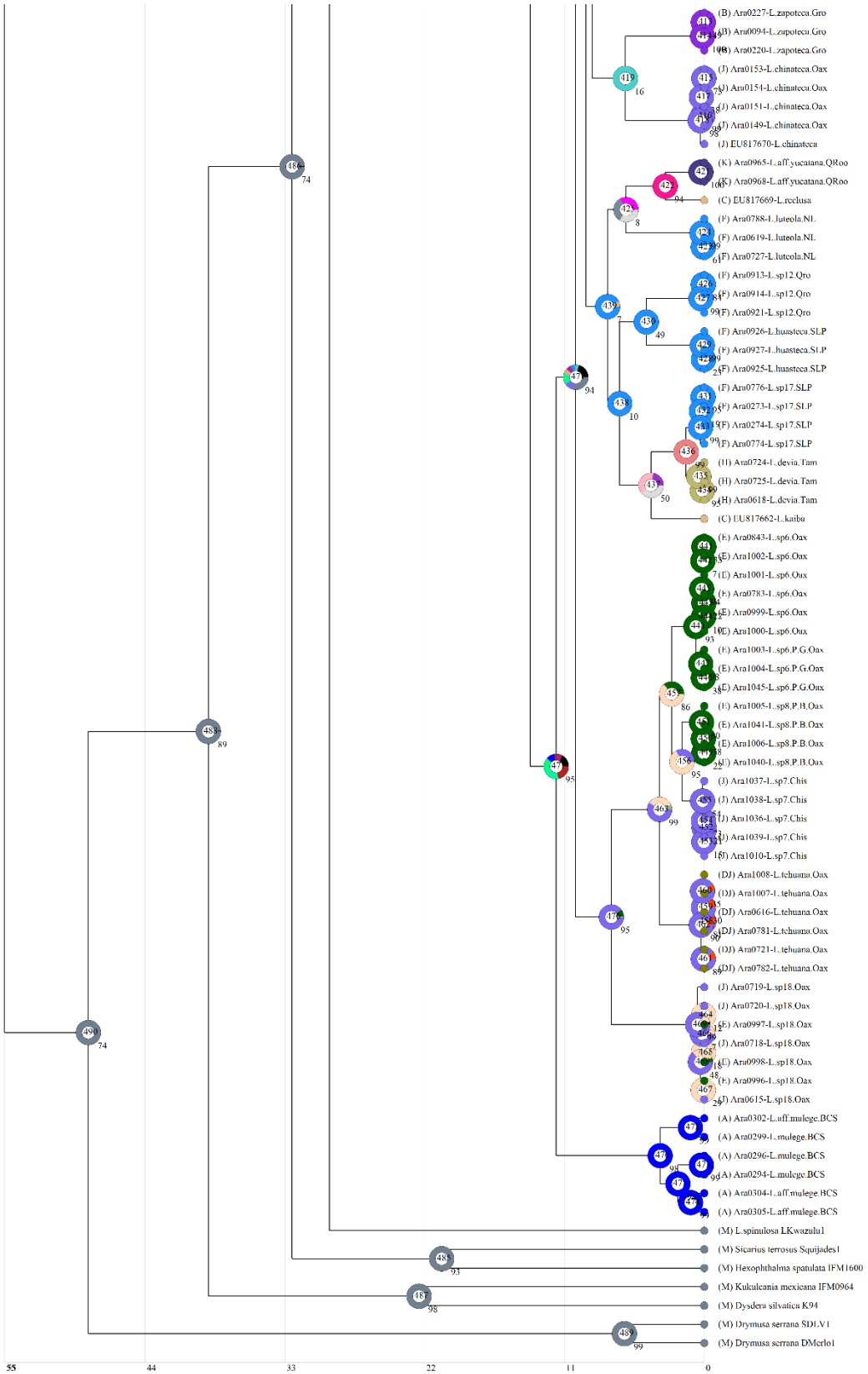

Figure 3. Continuation.

Discussion

Phylogenetic analyses

In previous analyses based on molecular data, the monophyly of the genus *Loxosceles* has already been tested by Binford *et al*. (2008), however, the sampling of taxa used was low. From North American and Caribbean region, only 14 sequences belonging to 13 species of the *reclusa* group were included, representing only 26% of the North American species. In this work, 219 sequences of 43 species of the genus *Loxosceles* were included, which represents more than 60% of the species of the *reclusa* group. Although 18 described species of the genus *Loxosceles* of the *reclusa* group have yet to be included, we consider that the current taxa sampling is sufficient to test the monophyly of the *reclusa* group.

Based on phylogeny, the *reclusa* group is recovered as monophyletic, with 99% support based on the concatenated matrix (COI+ITS2+28S) (Fig. 1), also using only COI and COI+ITS2. In contrast to Binford *et al.* (2008) where they recover monophyly only with the 28S and 16S+ND1 genes, but not under only COI. But when they included COI in a concatenated matrix, they obtained a better resolution in the nodes. The same step in this work was followed by concatenating all the genes, and better support for the nodes was obtained.

Duncan *et al.* (2010) also mention that the COI gene alone does not recover monophyletic groups in the case of *Loxosceles*, but when concatenating or using other genes they do support monophyly. As is the case in this work, where the *reclusa* group is recovered as monophyletic by including the concatenated matrix (COI+ITS2+28S). As also reported by Taucare-Ríos and Piel (2021), they recover as monophyletic the species of the *reclusa* group with a PP support under IB of 100% with a concatenated COI + 28S matrix.

Molecular dating and reconstruction of ancestral areas

According with the dating hypothesis, Binford *et al*. (2008) mention that the *reclusa* species group has a minimum age of 20 Mya and further mention that it could potentially be much older, where this group is expected to be at least 33 Mya old. However, with our estimation in this work, we found that the divergence times of the *reclusa species* group are more recent (16.33 million years).

Based on the distribution of the genus *Loxosceles* in North America, it was observed that the *reclusa* group has a distribution coincident with the Mexican Plateau distribution pattern (Halffter, 2017), which is characterized by lines of South American origin and ancient penetration in the Mexican Transition Zone, with diversification in the Mexican Plateau that may expand into the southwestern of the United States (Halffter, 2017).

Mexico has a complex biogeography due to the convergence of two major biogeographic regions (Nearctic and Neotropical) and the convergence of both in the Mexican Transition Zone (Valdez-Mondragón & Francke, 2015), which, with the emergence of the biogeographic provinces that compose it, may have influenced the diversification of the genus *Loxosceles*, very similar to what Mastretta-Yanes *et al.* (2015) for some species, as the emergence of the Transmexican Volcanic Belt was the main driver of diversification in the Mexican Plateau.

Our results based on lineage dating and ancestral range estimation (Fig. 3) indicate that the *reclusa* group was in North America after 16 Ma and has a diversification after the formation of the GAARlandia land bridge, therefore not consistent with the hypothesis proposed by Binford et al. (2008) of the dispersal of the genus through this land bridge.

Based on this result, we hypothesize that the dispersal of the genus occurred from South America to North America before the final closure of the Isthmus of Panama. This has been previously reported for other groups of spiders with little or poor dispersal capacity (Esposito, & Prendini, 2019; Shapiro *et al.*, 2022; Magalhaes *et al*., 2024) so we do not rule out this hypothesis.

We also hypothesize that dispersal to the Caribbean Islands (Greater Antilles) was possibly from Central America or even from North America, as has occurred with other groups of arachnids where dispersal was from the continent to the islands (Esposito, & Prendini, 2019; Crews & Esposito 2020; Magalhaes *et al.*, 2024). Although our sampling includes most of the North American species, it is still necessary to include Central American species to corroborate these hypotheses. The genus *Loxosceles* may have undergone rapid diversification, since the ages of diversification are similar for most clades. This may have been driven by mountain range orogeny.

Acknowledgements

The first author thanks the PhD (Doctorado) Program of the Centro Tlaxcala de Biología de la Conducta (CTBC), Universidad Autónoma de Tlaxcala, Tlaxcala City, and the Secretaria de Ciencias, Humanidades, Tecnología e Innovación (SECIHTI) for scholarship support during their PhD in Biological Science. The second author thanks the program "Jóvenes investigadores por México (ex Cátedras CONAHCyT)" and SECIHTI for scientific support for the project No. 59: "Laboratorio Regional de Biodiversidad y Cultivo de Tejidos Vegetales (LBCTV), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM)-Tlaxcala". The second author also thanks SEP-SECIHTI for financial support of the project of Basic Science (Ciencia Básica) 2016, No. 282834. We thank Dr. Edmundo González Santillán and Dr. Oscar F. Francke (ex-curator) of the Colección Nacional de Arácnidos (CNAN), Instituto de Biología, UNAM, for providing specimen loans, MSc. Laura Márquez Valdelamar for the molecular sequencing of the samples, Brett O. Butler for the English language review of the manuscript, and the reviewers for their comments and suggestions that improved the quality of the manuscript. We also thank the students of the Laboratory of Arachnology (LATLAX), IBUNAM, Tlaxcala, for their help in the field and processing the material in the laboratory. Specimens were collected under Scientific Collector Permit FAUT-0309 from the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT), Mexico, provided to Alejandro Valdez-Mondragón (corresponding author).

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CAPÍTULO 3.

ESTUDIOS TAXONÓMICOS

Nine new species of the spider genus *Loxosceles* Heineken & Lowe (Araneae, Sicariidae) from Mexico

Nueve especies nuevas del género de arañas *Loxosceles* Heineken & Lowe, 1832 (Araneae, Sicariidae) para México

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Abstract

Nine new species of the spider genus *Loxosceles* from Mexico are described. The descriptions of the species are based on male and female adult specimens: *Loxosceles* sp1 sp. nov. from Guerrero, *Loxosceles* sp2 sp. nov. from San Luis Potosí, *Loxosceles* sp3 sp. nov. from Sinaloa, *Loxosceles* sp5 sp. nov. from Sinaloa, *Loxosceles* sp13 sp. nov. from Oaxaca, *Loxosceles* sp14 sp. nov. from Guanajuato, *Loxosceles* sp16 sp. nov. from Queretaro, *Loxosceles* sp17 sp. nov. from San Luis Potosí, and *Loxosceles* sp18 sp. nov. from Oaxaca. With the description of the new species herein, the "recluse" group increases from 55 to 64 described species, Mexico is the country holding the greatest diversity of species worldwide with 49.

Key words. Taxonomy, reclusa group, North America, violin spiders

Nota sobre las descripciones de especies:

En la sección de descripción de especies, se presenta solamente una descripción general de cada una de las especies nuevas (macho y hembras), ya que serán nombradas y publicadas posteriormente en una revista indexada.

Introduction

Commonly known because their venomous bites which generate symptoms called loxoscelism (Vetter, 2015; Lopes et al. 2021), the spiders genus *Loxosceles,* belonging to the family Sicariidae Keyserling, 1880 is currently comprised of 147 species worldwide (WSC, 2025). Mexico holds the highest diversity in the world, with a total of 40 described species, 38 native and two introduced: *Loxosceles reclusa* (Gertsch & Mulaik, 1940) and *Loxosceles rufescens* (Dufour, 1820), not including the new species described herein (Gertsch & Ennik, 1983; Valdez-Mondragón et al. 2019; Navarro-Rodríguez, 2019; Navarro-Rodríguez and Valdez-Mondragón, 2020; WSC, 2025).

For species of the genus *Loxosceles* different species groups have been proposed based on pedipalp characters (Gertsch, 1958; Gertsch, 1967; Binford et al. 2008; Duncan et al. 2010; Fukushima et al, 2017). So far, seven species groups are currently recognized: 1) *reclusa* group with 55 species; 2) *laeta* group with 26 species; 3) *rufescens* group with 10 species; 4) *gaucho* group with seven species; 5) *spadicea* group with four species; 6) *vonwredei* group, and 7) *spinulosa* group with three species (Gertsch,1958; Gertsch, 1967; Gertsch and Ennik, 1983; Binford et al. 2008; Duncan et al. 2010; Gonçalves-de-Andrade et al. 2012; Fukushima et al. 2017; Bertani et al. 2018; WSC, 2025). Most species (55) are included in the *reclusa* group, which is characterized by having the tarsus of the male palpus wider than long and widely lobed on the prolateral side (Gertsch, 1958; Gertsch, 1967; Gertsch and Ennik, 1983). The North American species belong to this group, which includes all the species present in Mexico.

About the taxonomic history of *Loxosceles* species from Mexico, the first described species was *Loxosceles yucatana* by Chamberlin and Ivie (1938), subsequently Gertsch (1958 and 1973) described six and seven species respectively. The largest revision and more complete for species from North America was done by Gertsch and Ennik (1983), describing for the first

time 20 species from Mexico, which also includes the unique identification key so far which is used to species identification of the *reclusa* group. Subsequently, the male of *Loxosceles mulege* Gertsch and Ennik, 1983 was described by Jiménez and Llinas (2005). In the last decade, the taxonomic description of Mexican species of *Loxosceles* has been based in morphological but also molecular data, describing: *Loxosceles malintzi* Valdez-Mondragón, Cortez-Roldán, Juárez-Sánchez and Solís-Catalán, 2018 based only in morphology; and *Loxosceles tenochtitlan* Valdez-Mondragón and Navarro-Rodríguez, 2019 and *Loxosceles tolantongo* Navarro-Rodríguez and Valdez-Mondragón, 2020 based on morphological and molecular evidence (integrative approaching).

North American species, mainly in Mexico, are mainly found in dry or arid habitats, rarely being found in tropical environments, where the diversity decrease. The microhabitats of these spiders include under big rocks or, among small rocks, leaf litter, and dry or rotten agaves and cacti plants, as well as commonly on walls inside caves in karstic regions (Valdez-Mondragón *et al.* 2018a; 2018b; 2019; Navarro-Rodríguez, 2019; Navarro-Rodríguez & Valdez-Mondragón, 2020). These spiders are also highly tolerant to disturbed places and high temperatures and can be found in anthropized or urban environments such as warehouses or inside houses behind pictures frames, furniture, or under/inside boxes (Sandidge & Hopwood, 2005; Cortez-Roldán, 2018; Valdez-Mondragón *et al.* 2019; Romiti *et al.* 2021).

In this taxonomic contribution we describe based on adults male and female specimens nine new species from the states of Guanajuato, Guerrero, Querétaro, Oaxaca, San Luis Potosí, and Sinaloa, in Mexico. With this contribution the number of species described for the genus *Loxosceles* increases to 158, and the *reclusa* group to 64, Mexico as the country with the highest number of described species at 49.

Material and methods

Biological material

The specimens were collected by hand and deposited in ethanol 80% for morphological studies, labeled with their collection data. The specimens and additional material examined were deposited with their codes in the following collections: CARCIB = Collection of Arachnology, Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, Baja California Sur, Mexico. CNAN = National Collection of Arachnids, Instituto de Biología, Universidad Nacional Autónoma de Mexico (IB-UNAM), Mexico City, Mexico (additional material). Observations and identification of the specimens were done using a Zeiss model Stemi 508 stereoscopic microscope. The dissection of seminal receptacles in females and palps in males was implemented in ethanol 80%. The female genitalia were immersed in potassium hydroxide (KOH-10%) for 1–5 minutes to digest and clean the soft tissue around the seminal receptacles. Left male palps and female genitalia were dissected for species identification and for photography, being immersed in ethanol 70% gel and covered with a thin layer of distilled water to minimize diffraction during photography following Navarro-Rodríguez & Valdez-Mondragón (2020). Photographs were taken using a Zeiss Axiocam 506 color camera attached to a Zeiss AXIO Zoom V16 stereo microscope. Images of palps and seminal receptacles were edited in Adobe Photoshop CS6. Species-level identification was carried out following Gertsch & Ennik (1983), Valdez-Mondragón *et al*. (2018, 2019), and Navarro-Rodríguez & Valdez-Mondragón (2020). The descriptions were done following Valdez-Mondragón *et al.* (2018, 2019) and Navarro-Rodríguez & Valdez-Mondragón (2020). All measurements in the descriptions are in millimeters (mm). The distribution map was made using QGIS v. 3.16.6 (QGIS.org). Four fieldtrips were made to different states in Mexico to collect additional material of different species: Guerrero (2018), Guanajuato (2021), Querétaro (2021) and Oaxaca (2022).

Abbreviations

AME anterior median eye

PLE posterior lateral eye

PME posterior median eye

Taxonomy

Family **SICARIIDAE** Keyserling,1880

Genus ***Loxosceles*** Heineken y Lowe, 1832

Type species: *Loxosceles citigrada* Heineken and Lowe, 1832: 322, pl. 48, figs. 1-14 (= *Scytodes rufescens* Dufour, 1820: 203, pl. 76: Figs. 5 a-d), at present *Loxosceles rufescens* (Dufour, 1820).

The *reclusa* species group (Gertsch and Ennik, 1983).

Diagnosis. Tarsus of male palpus broader than long and broadly lobed on the prolateral side (Figs. 2, 5, 8, 11, 14, 17, 20, 23, 26). The eyes of anterior row larger, in less recurved row, with anterior median eyes separated from anterior laterals by one diameter or less (Figs. 1, 4, 7, 10, 13, 16, 19, 22, 25).

Distribution. Species from North America, adjacent areas of Central America, and Antilles. (Gertsch and Ennik, 1983).

Species included. 55 described species, see WSC (2025) for a complete list of species.

Loxosceles sp1-Gro (sp. nov.)

(Figures 1-3)

Type material: MEXICO: Guerrero: Male holotype from Juan R. Escudero (CARCIB Ara0519) of 325km, Autopista del Sol at the height of the río Papagayo, 2018, A. Valdez, I. Navarro, P. Solís, A. Juárez, A. Cabrera Cols. Paratypes: 2 males (CARCIB Ara0519), 4 females (CARCIB Ara0519), same data as holotype.

Other material examined. MEXICO: Guerrero: 2 males, 14 females (CARCIB Ara0516, Ara0517), Copala, 2018, A. Valdez, I. Navarro, P. Solís, A. Juárez, A. Cabrera Cols. 5 males, 13 females (CARCIB Ara0518), Ayutla de los Libres, 2018, A. Valdez, I. Navarro, P. Solís, A. Juárez, A. Cabrera Cols. 8 males, 13 females (CARCIB Ara0519) same data as holotype. 5 males, 3 females (CARCIB Ara0520), Quechultenango, 2018, A. Valdez, I. Navarro, P. Solís, A. Juárez, A. Cabrera Cols. 2 males, 12 females (CARCIB Ara0521), Mochitlan, 2018, A. Valdez, I. Navarro, P. Solís, A. Juárez, A. Cabrera Cols.

General diagnosis. *Palp*: Femora reddish orange, long and thin, with stridulatory pick basally (Fig. 2A); patellae reddish orange; tibiae dark reddish orange, almost straight dorsally, ventrally curved, wider in distal half than proximal (Figs. 2A, 2C). Tarsus oval, dark reddish brown; bulb oval, embolus nearly straight with slight curvature distally, slightly curved at tip (Figs. 2A, 2C, 2D, 2E). *Genital area*: Seminal receptacles elongate, rounded apically, curved inwards. Base of the spermathecae broad, with the inner side strongly sclerotized

Distribution. MEXICO: Guerrero (Figs. 1-3)

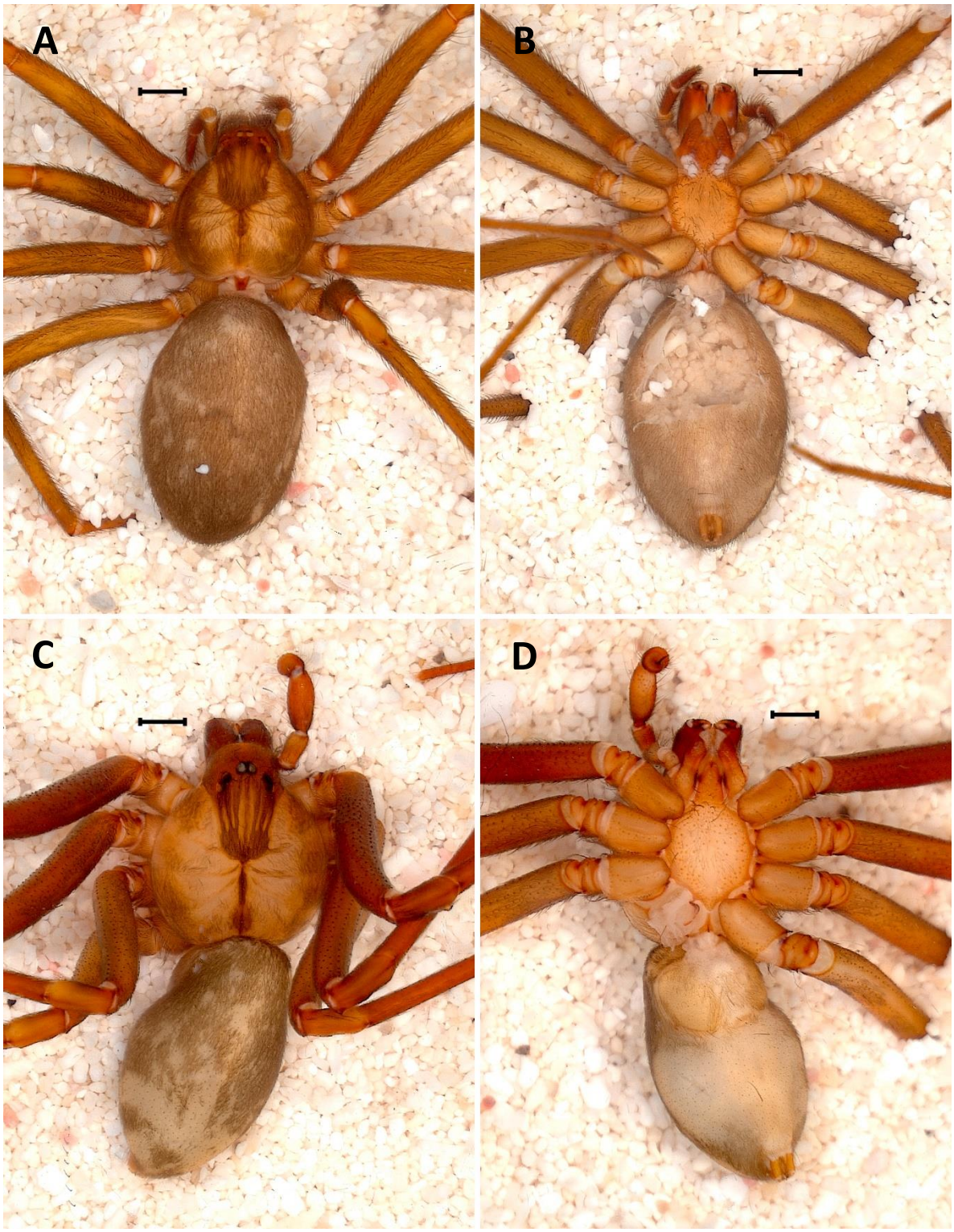

Figure 1. Female and male specimens of *Loxosceles* sp1 sp. nov. Guerrero. **A)** Habitus, dorsal view female; **B)** Habitus, ventral view female; **C)** Habitus, dorsal view male; **D)** Habitus, ventral view male. Scale bars: 1 mm.

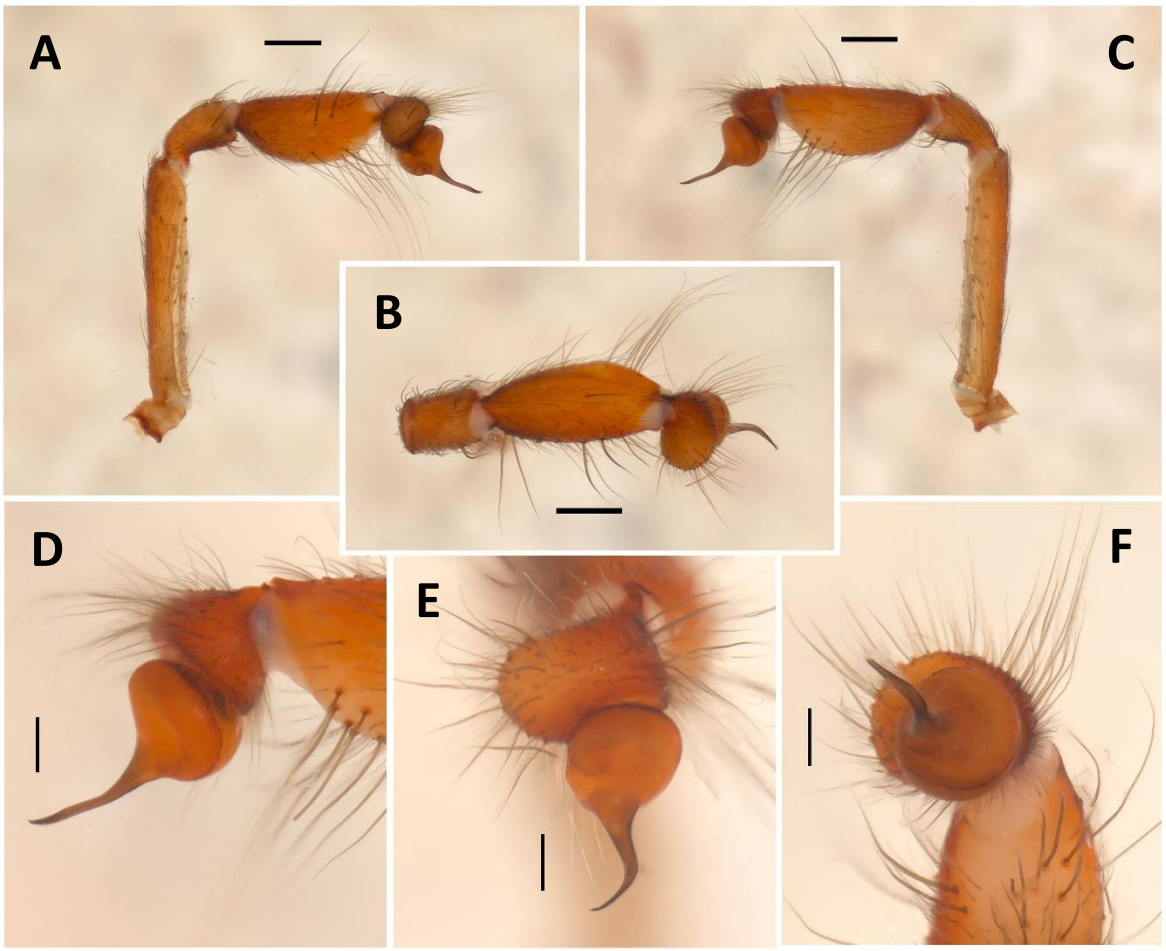

Figure 2. *Loxosceles* sp1 sp. nov. Guerrero. Male Holotype left palp: **A-C)** prolateral, dorsal and retrolateral views respectively; **D-F)** Detail of the bulb and embolus, retrolateral, dorsal and apical views, respectively. Scale bars: 0.5 mm (**A-C**), 0.2 mm (**D-F**).

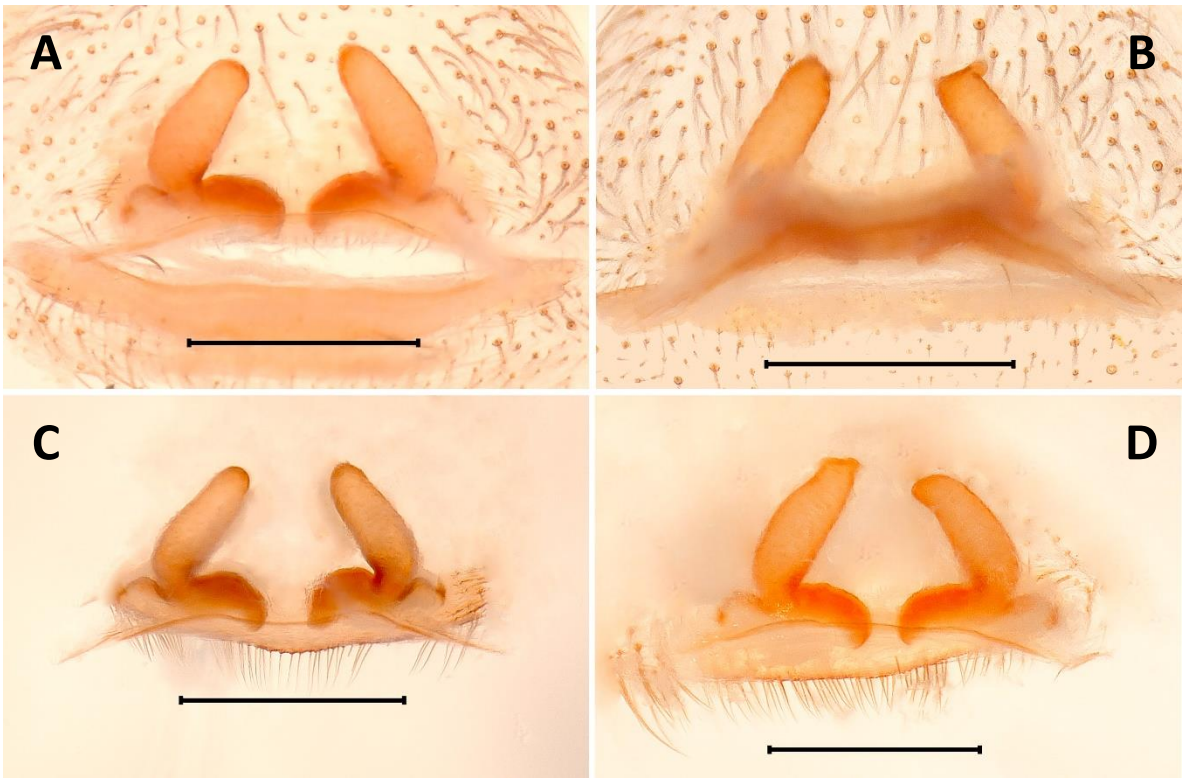

Figure 3. *Loxosceles* sp1 sp. nov. Guerrero. Female internal genitalia. Variation of the seminal receptacles: **A, B)** 325km, Autopista del Sol a la altura del río Papagayo, Juan R. Escudero. **C, D)** Balnerario "Los Manantiales", carretera Quechultenango-Colotlipa, Quechultenango, Dorsal view. Scale bars: 0.5 mm.

***Loxosceles* sp2-SLP (sp. nov).**

(Figures 4-6)

Type material: MEXICO: San Luis Potosí: Male holotype from Ciudad Valles (CARCIB Ara0603) 2.5 Km east of Ejido Laguna del Mante, Reserva de la Biosfera El Abra-Tanchipa, Rincón de Pames, 2017, E. González-Santillán Col. Paratypes: 1 male, 2 females (CARCIB Ara0603) same data as holotype.

Other material examined. MEXICO: San Luis Potosí: 2 females (CARCIB Ara0603), same data as holotype.

General diagnosis. *Palp:* Femora pale brown, long and thin, with stridulatory pick basally (Fig. 5A); patellae light brown; tibiae light brown, almost straight dorsally, ventrally curved, thicker distally than proximally (Figs. 5A, 5C). Tarsus oval, light brown; bulb oval, with embolus slightly curved from medially, tip twisted medially (Figs. 5A, 5C, 5D, 5E). *Genital area:* seminal receptacles elongate, almost rounded apically, except for a few small projections on the apical side. Base of spermathecae broad, with the inner side strongly sclerotized.

Distribution. MEXICO: San Luis Potosí (Figs. 4-6)

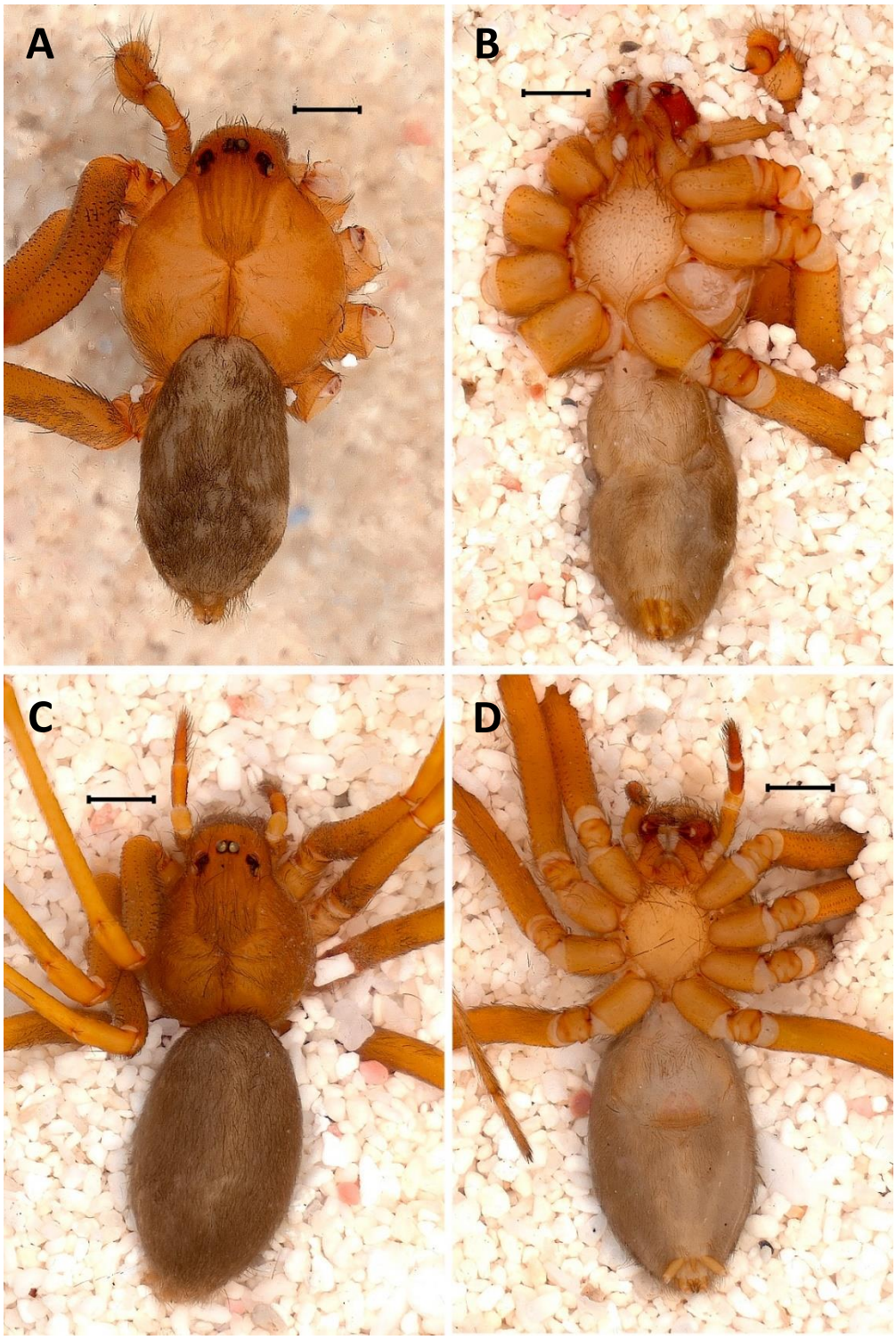

Figure 4. Female and male specimens of *Loxosceles* sp2 sp. nov. San Luis Potosí. **A)** Habitus, dorsal view male; **B)** Habitus, ventral view male; **C)** Habitus, dorsal view female; **D)** Habitus, ventral view female. Scale bars: 1 mm.

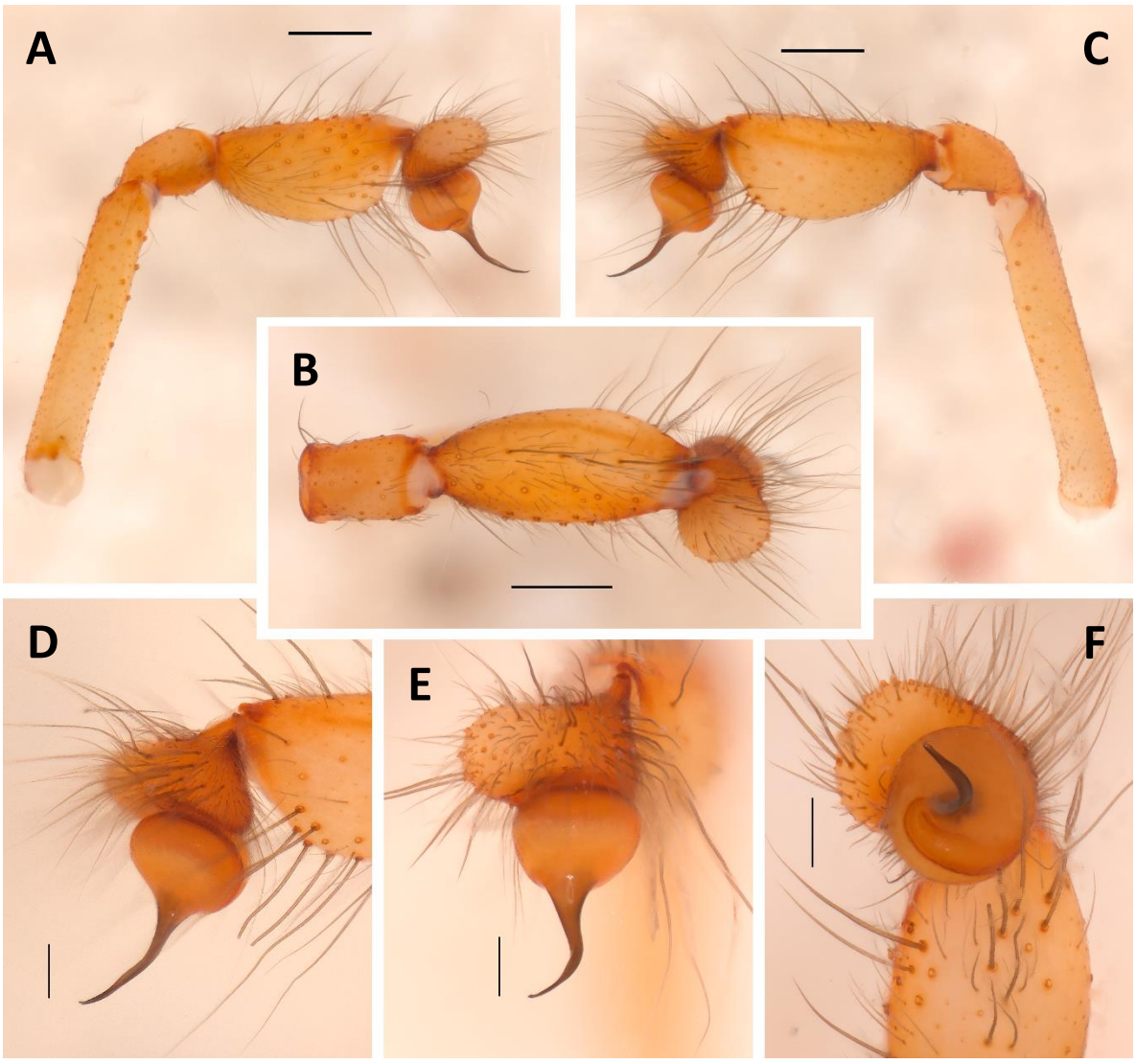

Figure 5. *Loxosceles* sp2 sp. nov. San Luis Potosí. Male Holotype left palp: **A-C)** prolateral, dorsal and retrolateral views respectively; **D-F)** Detail of the bulb and embolus, retrolateral, dorsal and apical views, respectively. Scale bars: 0.5 mm (**A-C**), 0.2 mm (**D-F**).

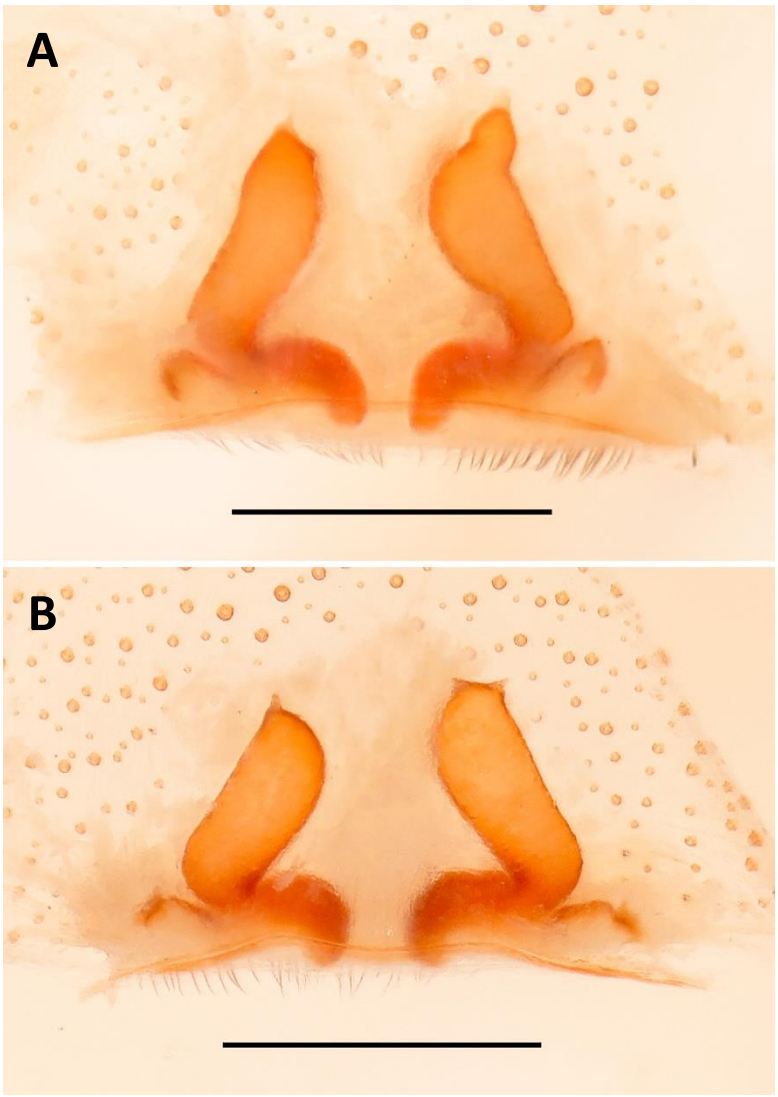

Figure 6. *Loxosceles* sp2 sp. nov. San Luis Potosí. Female internal genitalia. Variation of the seminal receptacles: **A, B)** 2.5 Km al E de Ejido Laguna del Mante, Reserva de la Biosfera El Abra-Tanchipa, Rincón de Pames, Ciudad Valles. Dorsal view. Scale bars: 0.5 mm.

***Loxosceles* sp3-Sin (sp. nov).**

(Figures 7-9)

Type material: MEXICO: Sinaloa: Male holotype from Ahome (CARCIB Ara0991) Sierra de Barobampo “Cañón del Diablo", 2022, Jair Alcántar Cols. Paratypes: 4 females (CARCIB Ara0991), same data as holotype. 1 male (CARCIB Ara0993) Ahome, 2022, Jair Alcántar Cols.

Other material examined. MEXICO: Sinaloa: 1 male, 11 females (CARCIB Ara0961), Ahome, 2022, Jair Alcántar Cols. 2 females (CARCIB Ara0992), Ahome, 2022, Jair Alcántar Cols. 1 female (CARCIB Ara0993), Ahome, 2022, Jair Alcántar Cols.

General diagnosis. *Palp:* Femora pale orange, long and thin, with stridulatory pick basally (Fig.8A); patellae light brown; tibiae light brown, curved dorsally and ventrally, almost circular, thicker medially (Figs. 8A, 8C). Tarsus oval, light brown; bulb oval, with straight embolus, widest at base, with ventral canal (Figs. 8A, 8C, 8D, 8E). *Genital area:* Seminal receptacles apically broad, boot-shaped. Internally straight and slightly inclined towards the external part. Base of spermathecae broad, with sclerotized external face.

Distribution. MEXICO: Sinaloa (Figs. 7-9)

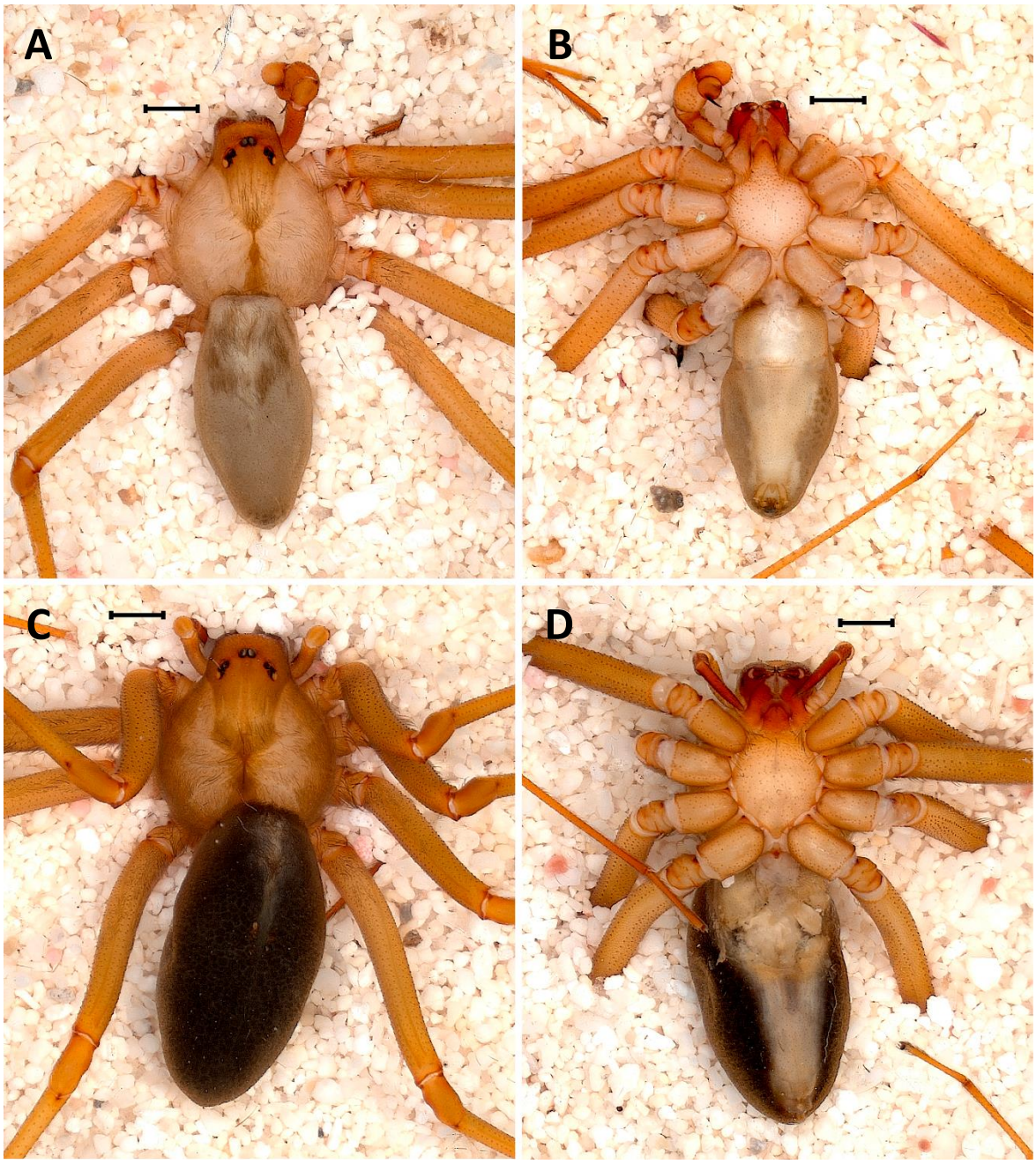

Figure 7. Female and male specimens of *Loxosceles* sp3 sp. nov. Sinaloa. **A)** Habitus, dorsal view male; **B)** Habitus, ventral view male; **C)** Habitus, dorsal view female; **D)** Habitus, ventral view female. Scale bars: 1 mm.

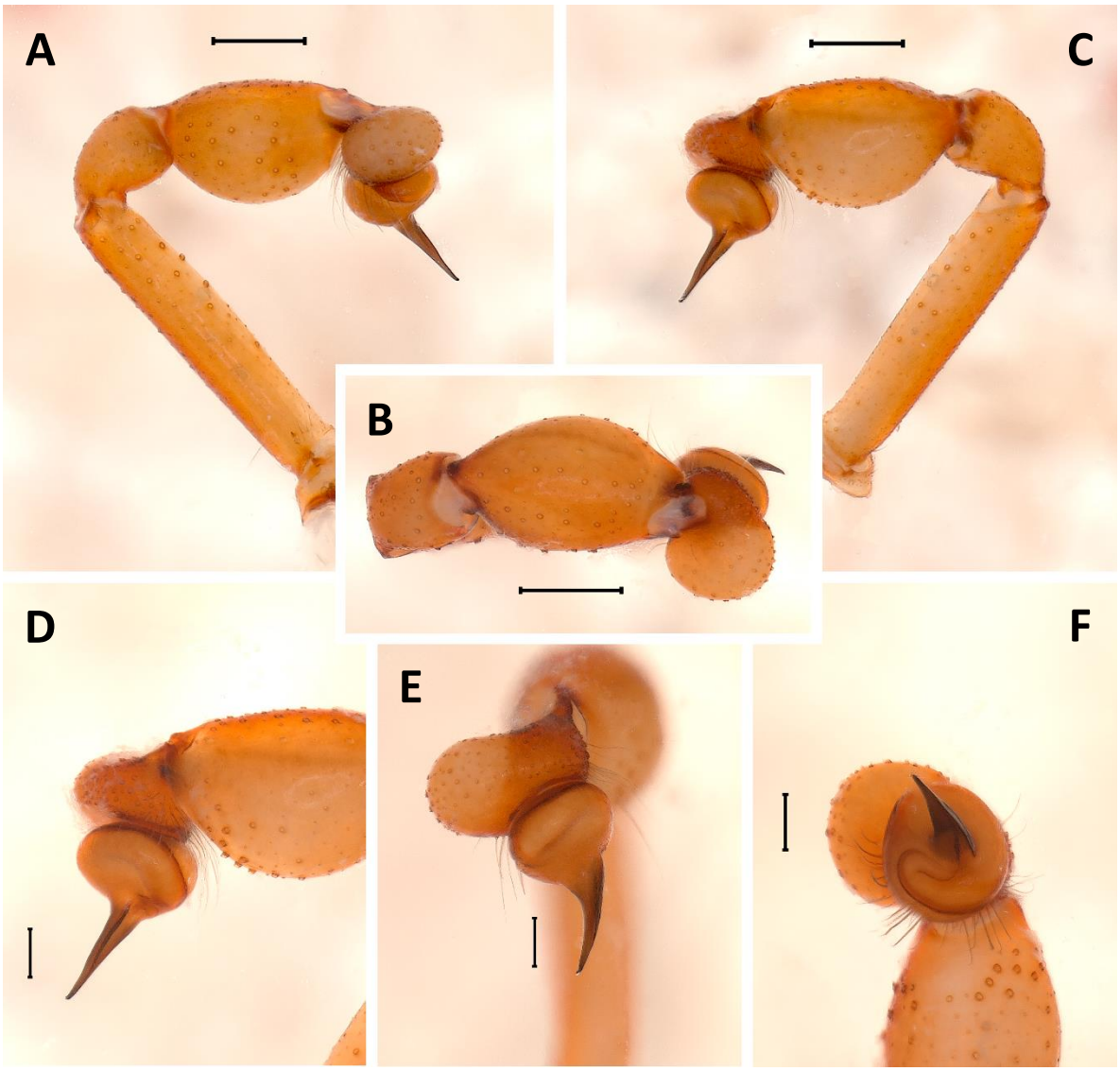

Figure 8. *Loxosceles* sp3 sp. nov. Sinaloa. Male Holotype left palp: **A-C)** prolateral, dorsal and retrolateral views respectively; **D-F)** Detail of the bulb and embolus, retrolateral, dorsal and apical views, respectively. Scale bars: 0.5 mm (**A-C**), 0.2 mm (**D-F**).

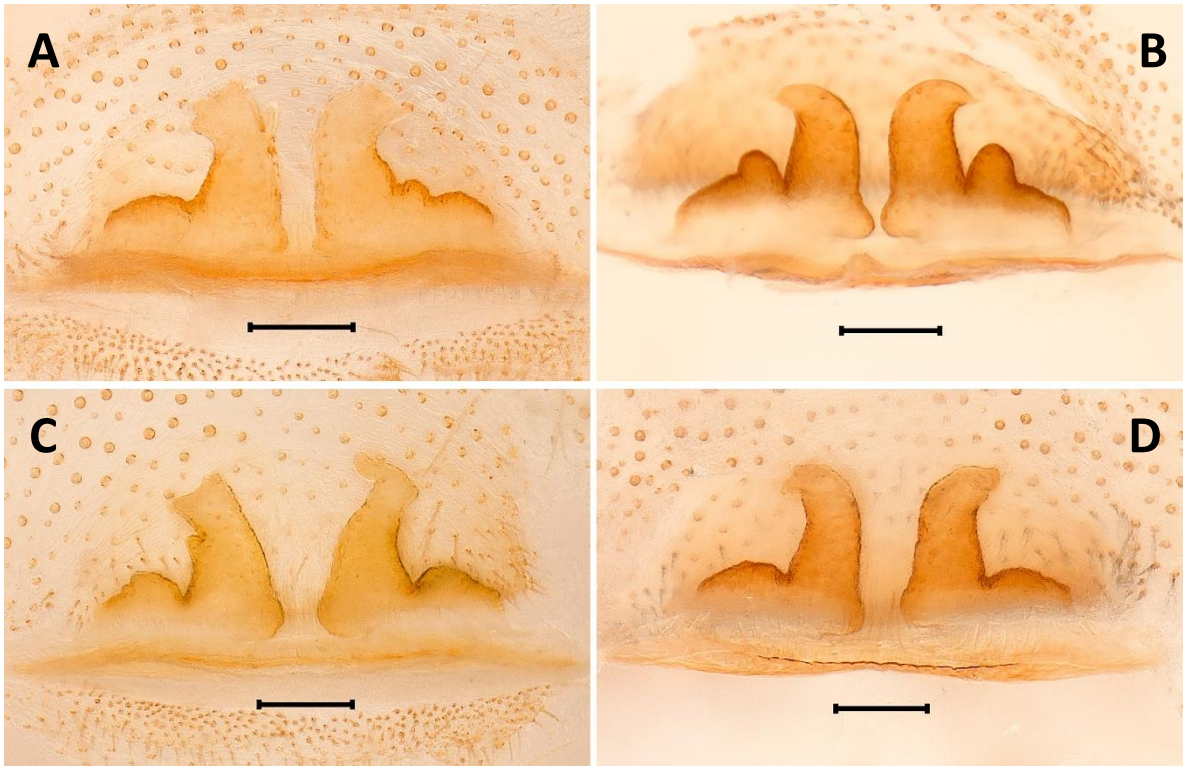

Figure 9. *Loxosceles* sp3 sp. nov. Sinaloa. Female internal genitalia. Variation of seminal receptacles: **A, B, C)** Sierra de Barobampo a 600m del Cañón del Diablo, Ahome. **D)** Sierra de Barobampo "Cañón del Diablo", Ahome. Dorsal view. Scale bars: 0.2 mm.

***Loxosceles* sp5-Sin (sp. nov).**

(Figures 10-12)

Type material: MEXICO: Sinaloa: Male holotype from Ahome (CARCIB Ara0965) Cerro de Iturbide, to 900m from ejido Rosendo G. Castro, 2022, Jair Alcántar Cols. Paratypes: 6 males (CARCIB Ara0965), 10 females (CARCIB Ara0965), same data as holotype.

Other material examined. MEXICO: Sinaloa: 1 male, 4 females (CARCIB Ara0963), Ahome, 2021, Jair Alcántar Cols. 6 females (CARCIB Ara0964), Ahome, 2022, Jair Alcántar Cols. 2 females (CARCIB Ara0995), Ahome, 2022, Jair Alcántar Cols. 1 male (CARCIB Ara0996), Ahome, 2022, Jair Alcántar Cols.

General diagnosis. *Palp:* Femora pale brown, long and thin, with stridulatory pick basally (Fig.11A); patellae light brown; tibiae reddish brown, curved and ventrally thicker distally, (Figs. 11A, 11C). Tarsus oval, light brown; bulb oval, with straight, slender, long embolus (Figs. 11A, 11C, 11D, 11E). *Genital area:* Seminal receptacles elongate, with nine slender projections, mostly rounded and wider apically. Base of spermathecae broad.

Distribution. MEXICO: Sinaloa (Figs. 10-12)

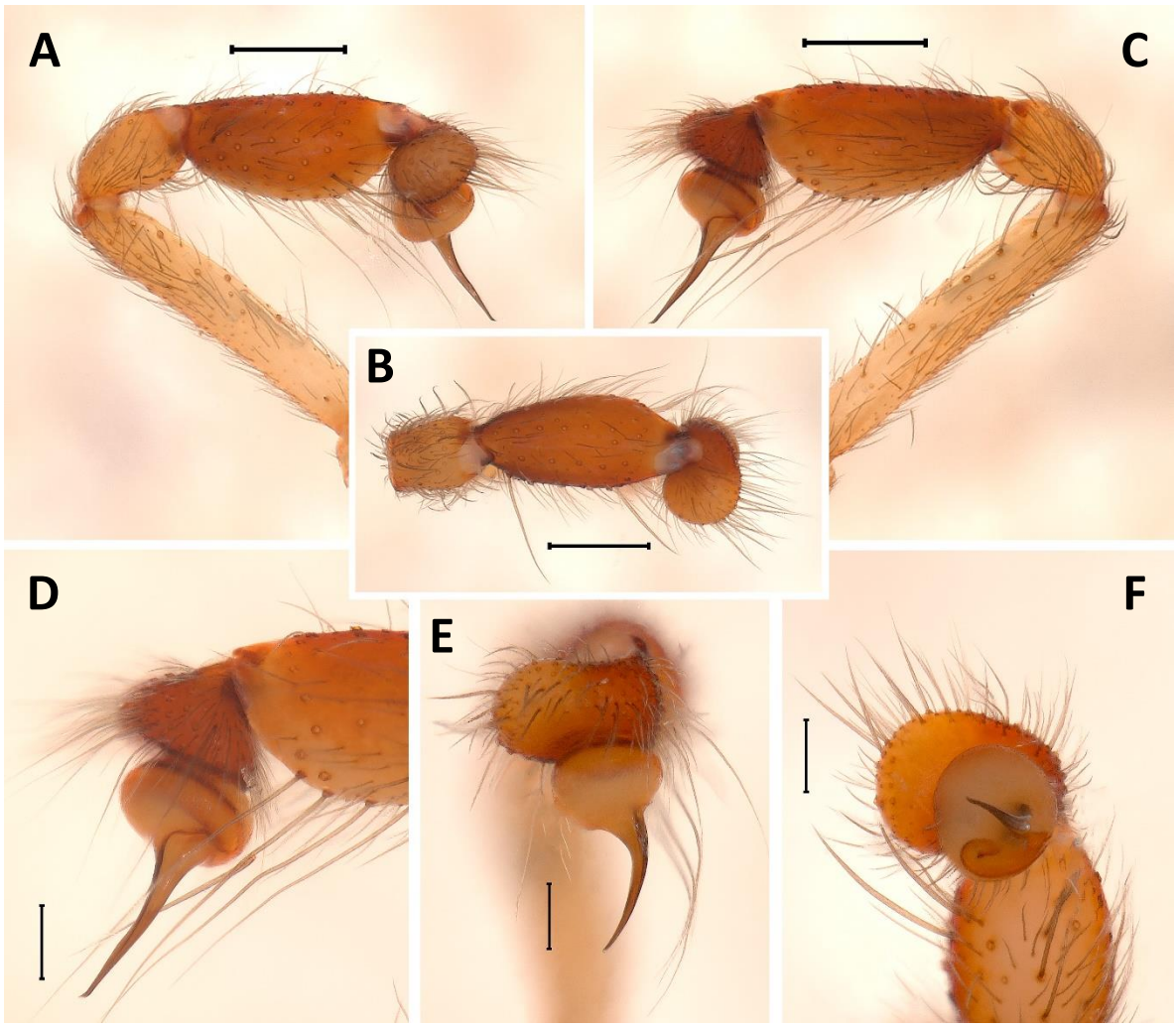

Figure 11. *Loxosceles* sp5 sp. nov. Sinaloa. Male Holotype left palp: **A-C)** prolateral, dorsal and retrolateral views respectively; **D-F)** Detail of the bulb and embolus, retrolateral, dorsal and apical views, respectively. Scale bars: 0.5 mm (**A-C**), 0.2 mm (**D-F**).

***Loxosceles* sp13-Oax (sp. nov).**

(Figures 13-15)

Type material: MEXICO: Oaxaca: Male holotype from Villa de Chilapa de Díaz (CARCIB Ara1007) 3.5 km to Southwest of Chilapa de Díaz, 2022, A. Valdez, S. Nolasco, A. Juárez Cols. Paratypes: 3 males, 5 females (CARCIB Ara1007), same data as holotype.

Other material examined. MEXICO: Oaxaca: 2 males, 3 females (CARCIB Ara1008) Villa de Chilapa de Díaz, 2022, A. Valdez, S. Nolasco, A. Juárez Cols.

General diagnosis. *Palp:* Femora reddish orange, long and thin, with stridulatory pick basally (Fig.14A); patellae reddish orange; tibiae dark reddish brown, ventrally curved and thicker medially, (Figs. 14A, 14C). Tarsus oval, dark reddish; bulb oval, with straight embolus, thick to one-third, with thin, slightly raised tip (Figs. 14A, 14C, 14D, 14E). *Genital area:* Seminal receptacles asymmetrical, slightly curved and inclined towards the inner part. Base of spermathecae with small projections. Base broad, with the inner side strongly sclerotized.

Distribution. MEXICO: Oaxaca (Figs. 13-15)

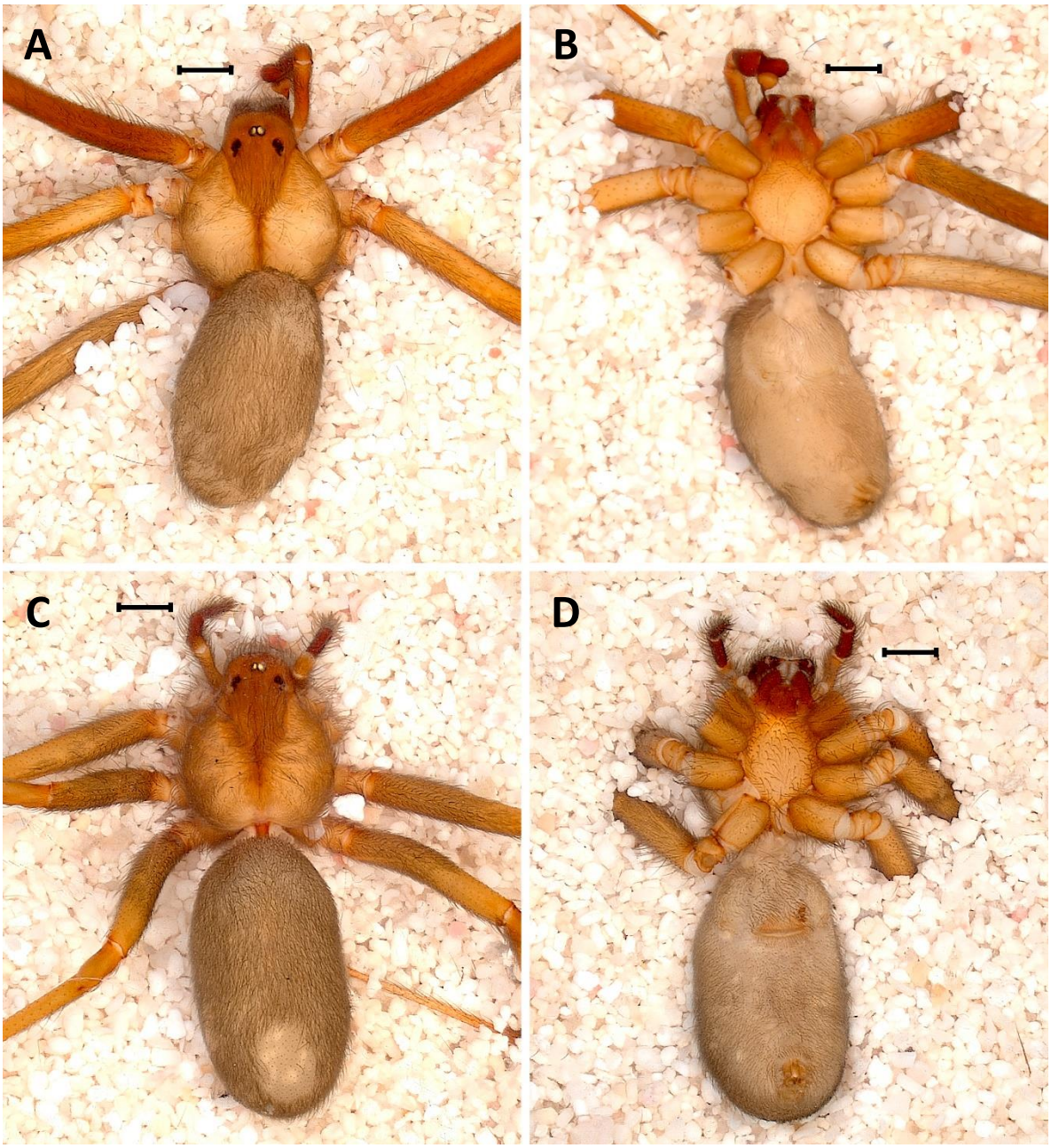

Figure 13. Female and male specimens of *Loxosceles* sp13 sp. nov. Oaxaca. **A)** Habitus, dorsal view male; **B)** Habitus, ventral view male; **C)** Habitus, dorsal view female; **D)** Habitus, ventral view female. Scale bars: 1 mm.

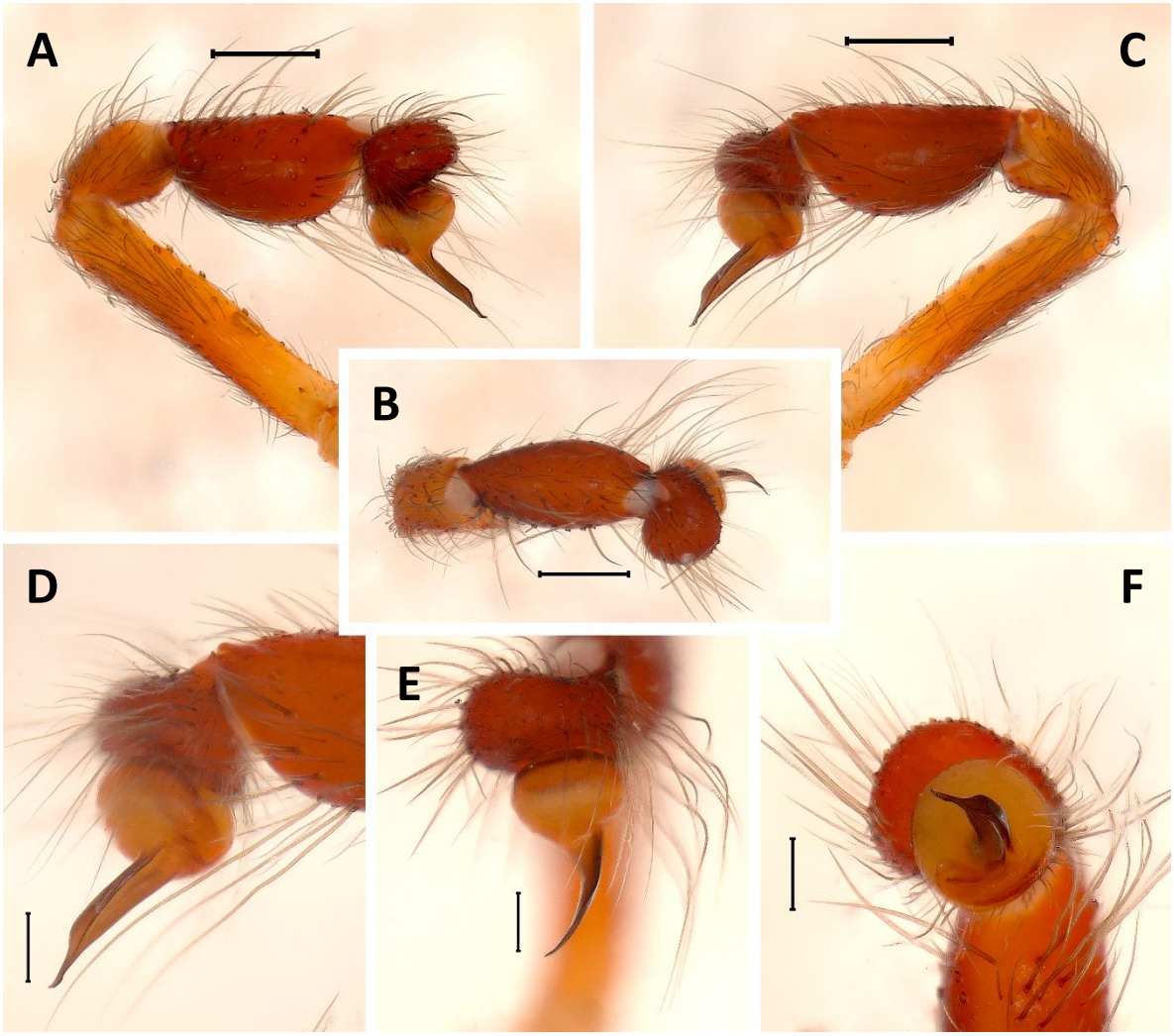

Figure 14. *Loxosceles* sp13 sp. nov. Oaxaca. Male Holotype left palp: **A-C)** prolateral, dorsal and retrolateral views respectively; **D-F)** Detail of the bulb and embolus, retrolateral, dorsal and apical views, respectively. Scale bars: 0.5 mm (**A-C**), 0.2 mm (**D-F**).

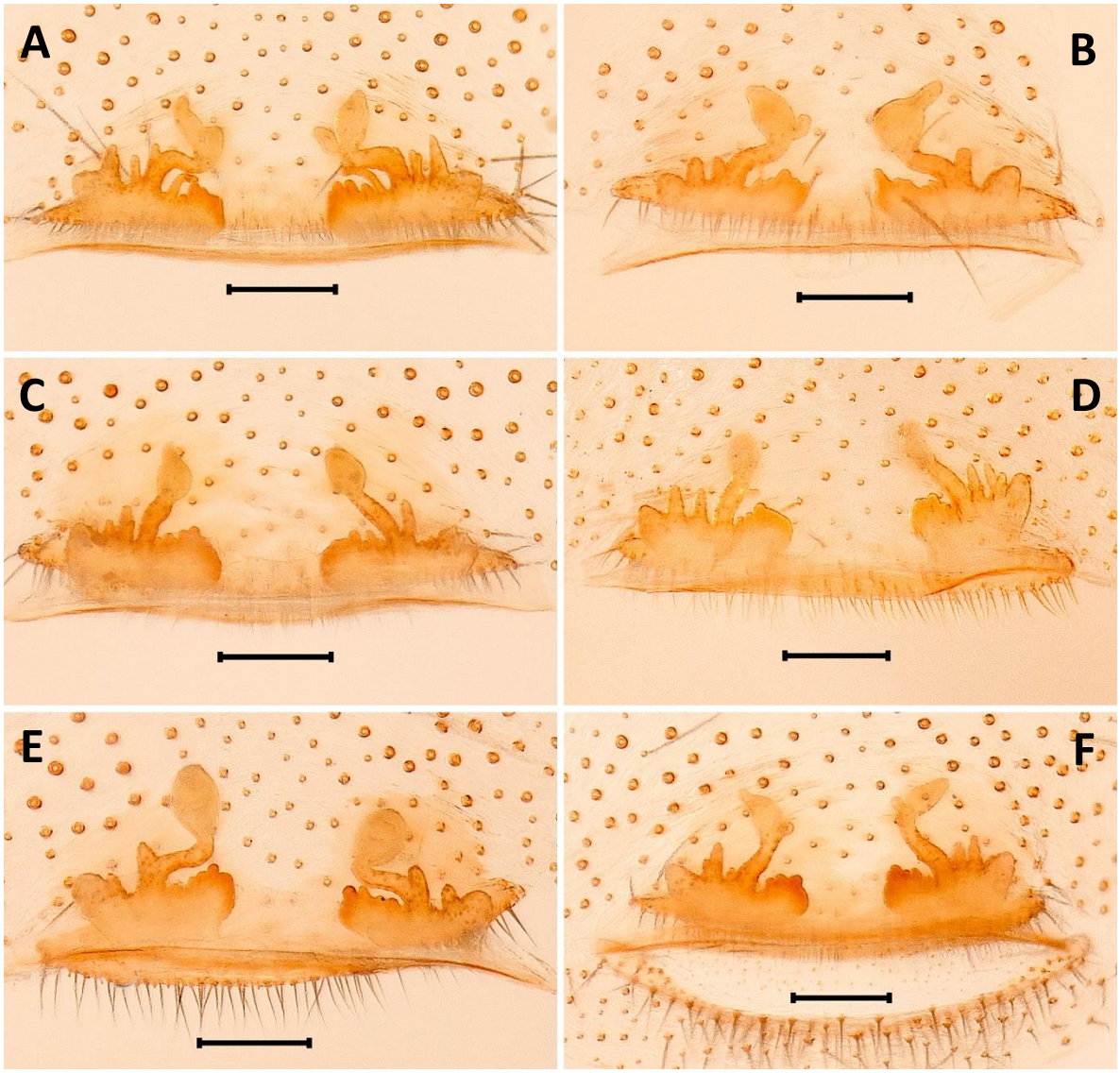

Figure 15. *Loxosceles* sp13 sp. nov. Oaxaca. Female internal genitalia. Variation of the seminal receptacles: **A, B, C, D, E, F)**. 3.5 km al SE de Chilapa de Díaz, Villa de Chilapa de Díaz. Dorsal view. Scale bars: 0.2 mm.

***Loxosceles* sp14-Gto (sp. nov).**

(Figures 16-18)

Type material: MEXICO: Guanajuato: Male holotype from Santa Catarina (CARCIB Ara0914) 6 Km NO of Santa Catarina, 2021, A. Valdez, A. Juárez, L. Cabrera, S. Nolasco Cols. Paratypes: 1 males, 2 females (CARCIB Ara0914), same data as holotype.

Other material examined. MEXICO: Guanajuato: 2 males, 2 females (CARCIB Ara0914), same data as holotype.

General diagnosis. *Palp:* Femora reddish orange, long and thin, with stridulatory pick basally (Fig.17A); patellae reddish orange; tibiae reddish brown, ventrally curved and thick from proximal to distal, (Figs. 17A, 17C). Tarsus oval, reddish brown; bulb oval, with straight embolus, with thin, slightly curved tip (Figs. 17A, 17C, 17D, 17E). *Genital area:* Seminal receptacles slender, wider apically. Base of the spermathecae inclined towards the inner side, sclerosed in the basal part and part of the lobe.

Distribution. MEXICO: Guanajuato (Figs. 16-18)

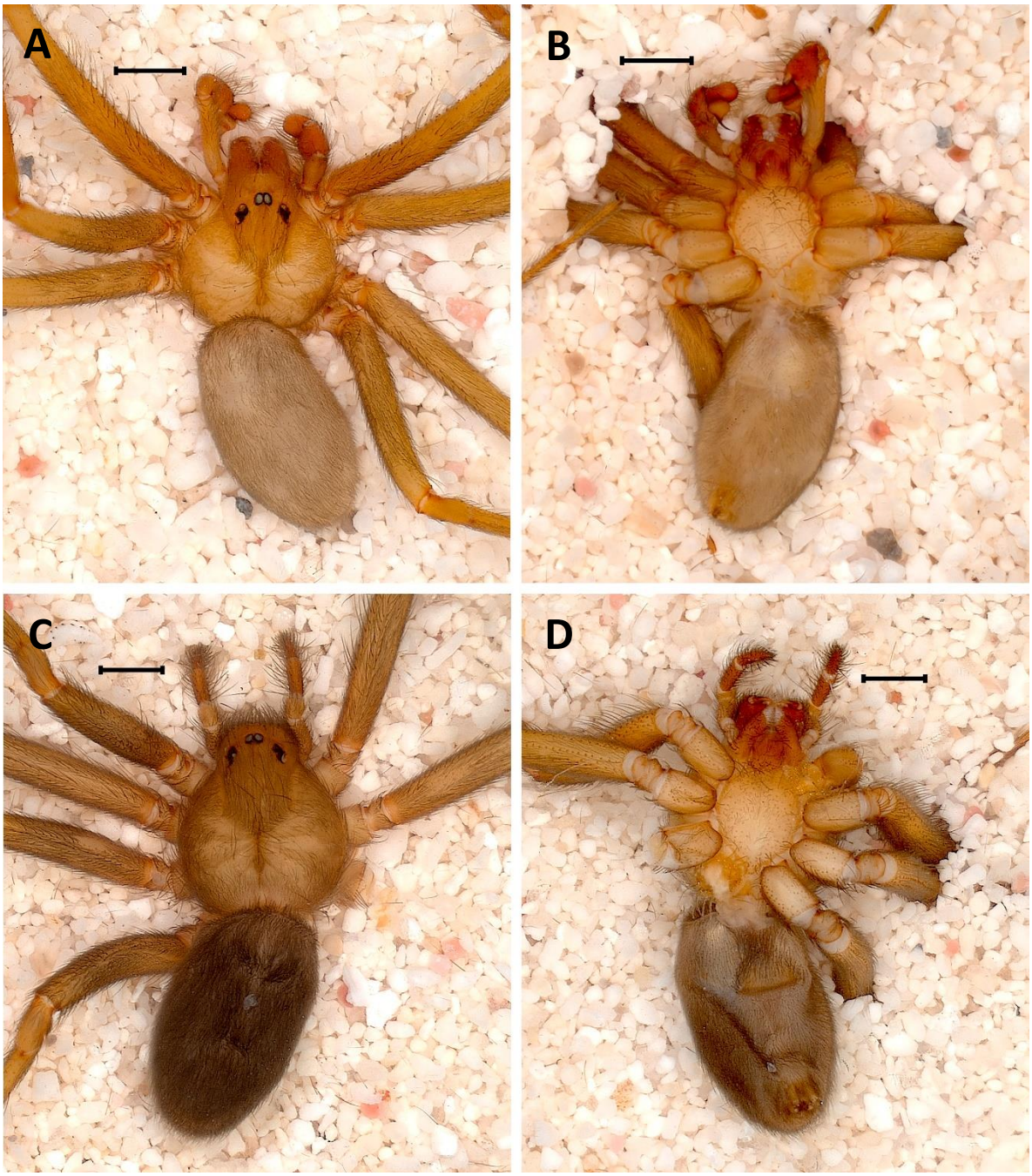

Figure 16. Female and male specimens of *Loxosceles* sp14 sp. nov. Guanajuato. **A)** Habitus, dorsal view male; **B)** Habitus, ventral view male; **C)** Habitus, dorsal view female; **D)** Habitus, ventral view female. Scale bars: 1 mm.

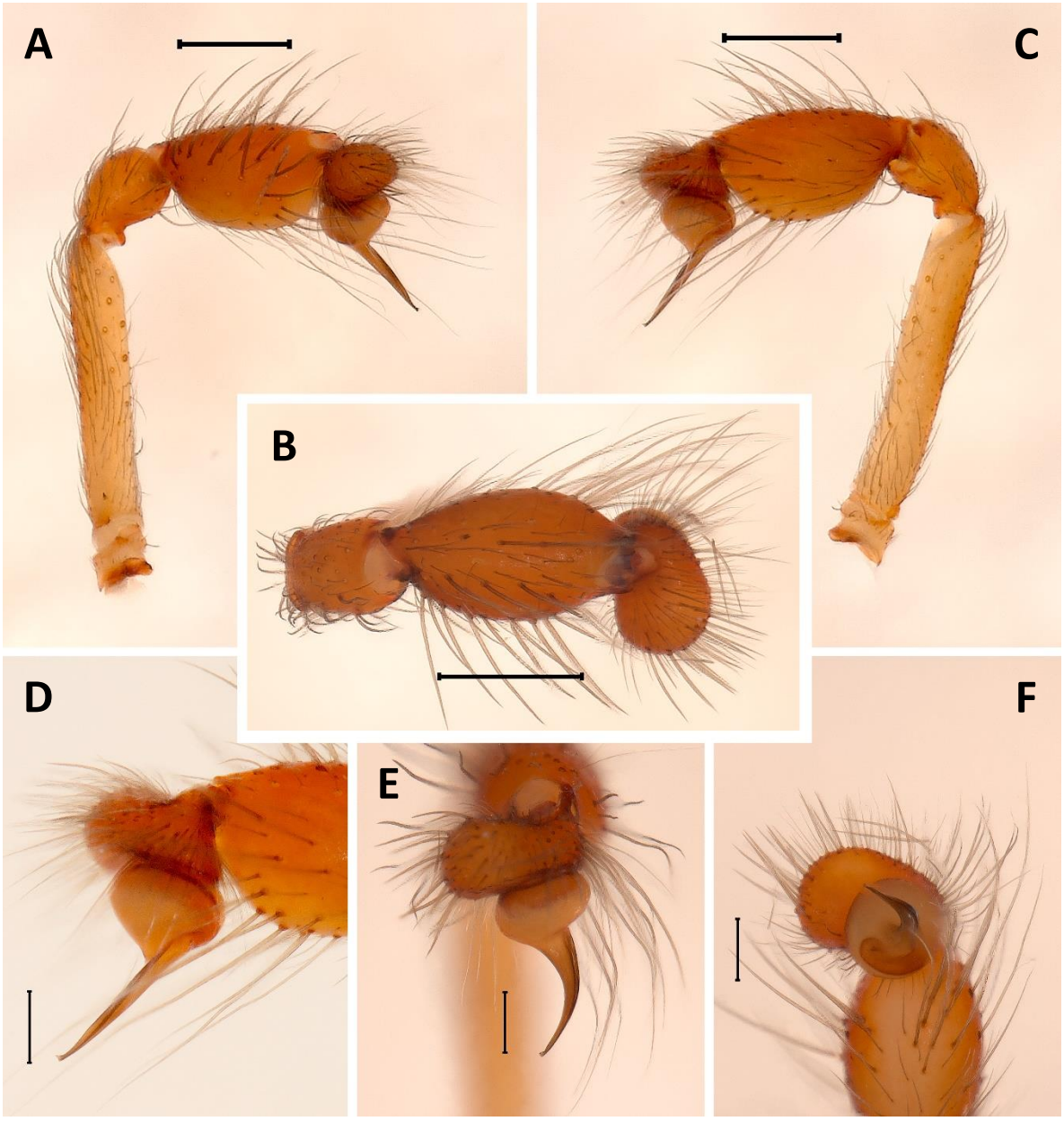

Figure 17. *Loxosceles* sp14 sp. nov. Guanajuato. Male Holotype left palp: **A-C)** prolateral, dorsal and retrolateral views respectively; **D-F)** Detail of the bulb and embolus, retrolateral, dorsal and apical views, respectively. Scale bars: 0.5 mm (**A-C**), 0.2 mm (**D-F**).

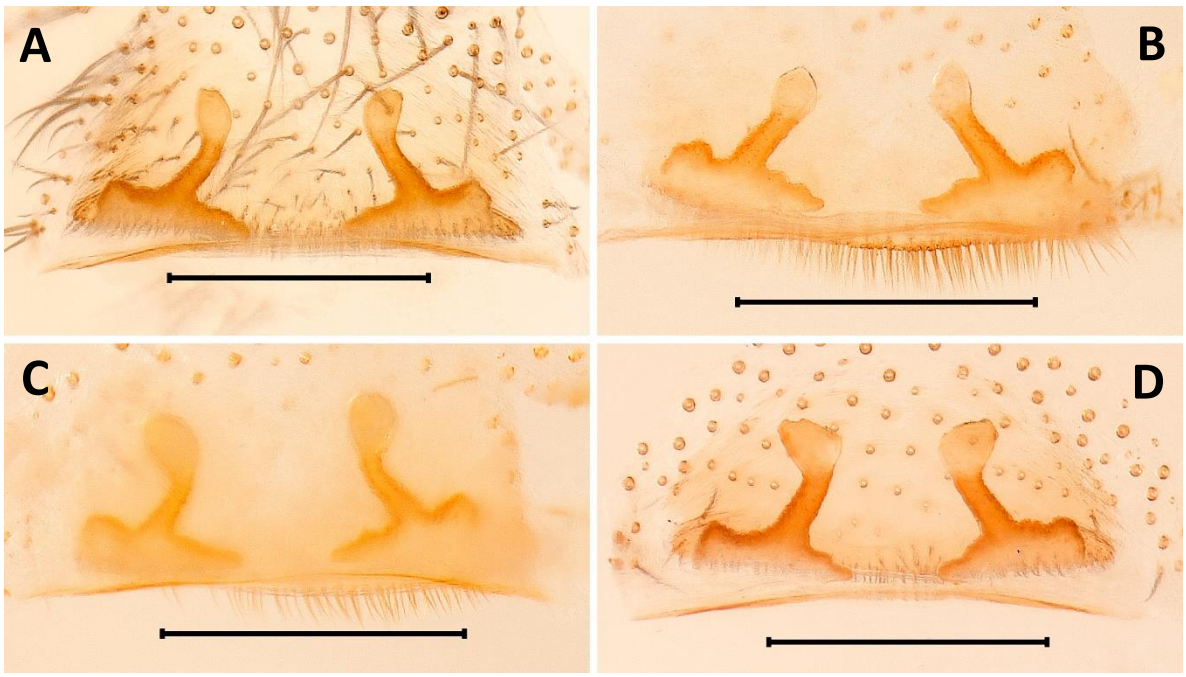

Figure 18. *Loxosceles* sp14 sp. nov. Guanajuato. Female internal genitalia. Variation of seminal receptacles: **A, B, C, D)** 6 Km al NO de Santa Catarina, Santa Catarina. Dorsal view. Scale bars: 0.5 mm.

***Loxosceles* sp16-Qro (sp. nov).**

(Figures 19-21)

Type material: MEXICO: Querétaro: Male holotype from Pinal de Amoles (CARCIB Ara0917) Campamento Las Trancas, 2021, A. Valdez, A. Juárez, L. Cabrera, S. Nolasco Cols. Paratypes: 1 males, 4 females (CARCIB Ara0917), same data as holotype.

Other material examined. MEXICO: Querétaro: 2 males, 3 females (CARCIB Ara0917), same data as holotype.

General diagnosis. *Palp:* Femora pale brown, long and thin, with stridulatory pick basally (Fig.20A); patellae pale brown; tibiae brown, ventrally curved and thick (Figs. 20A, 20C). Tarsus oval, brown; bulb oval, with straight embolus, with thin, slightly curved tip (Figs. 20A, 20C, 20D, 20E). *Genital area:* Seminal receptacles S-shaped, inclined towards the inner side. Base of the spermathecae broad, and slightly sclerotized.

Distribution. MEXICO: Querétaro (Figs. 19-21)

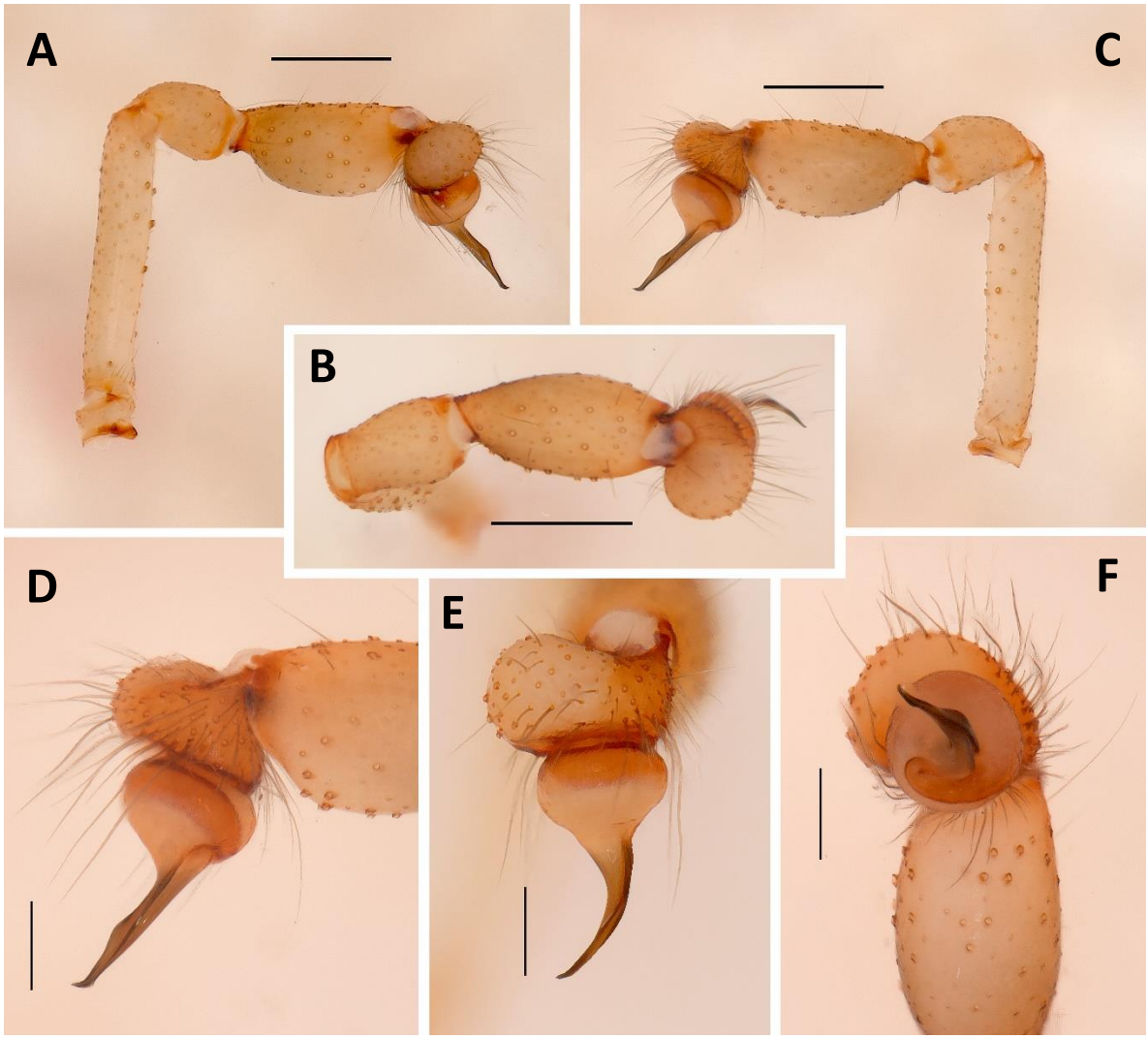

Figure 20. *Loxosceles* sp16 sp. nov. Querétaro. Male Holotype left palp: **A-C)** prolateral, dorsal and retrolateral views respectively; **D-F)** Detail of the bulb and embolus, retrolateral, dorsal and apical views, respectively. Scale bars: 0.5 mm (**A-C**), 0.2 mm (**D-F**).

Loxosceles sp17-SLP (sp. nov).

(Figures 22-24)

Type material: MEXICO: San Luis Potosí: Male holotype from Ciudad Valles (CARCIB Ara0604) 2.5 Km al E de Ejido Laguna del Mante, Reserva de la Biosfera El Abra-Tanchipa, Rincón de Pames, 2017, E. González-Santillán Col. Paratypes: 2 females (CARCIB Ara0604), same data as holotype.

Other material examined. MEXICO: San Luis Potosí: 2 females (CARCIB Ara0607), same data as holotype.

General diagnosis. *Palp:* Femora pale brown, short and thin, with stridulatory pick basally (Fig. 23A); patellae pale brown; tibiae pale brown, ventrally curved and thick, almost circular (Figs. 23A, 23C). Tarsus oval, pale brown; bulb oval, with curved embolus, thin-tipped (Figs. 23A, 23C, 23D, 23E). *Genital area:* Seminal receptacles slender, inclined towards the inner side. Base of spermathecae broad, with the inner side strongly sclerotized.

Distribution. MEXICO: San Luis Potosí (Figs. 22-24)

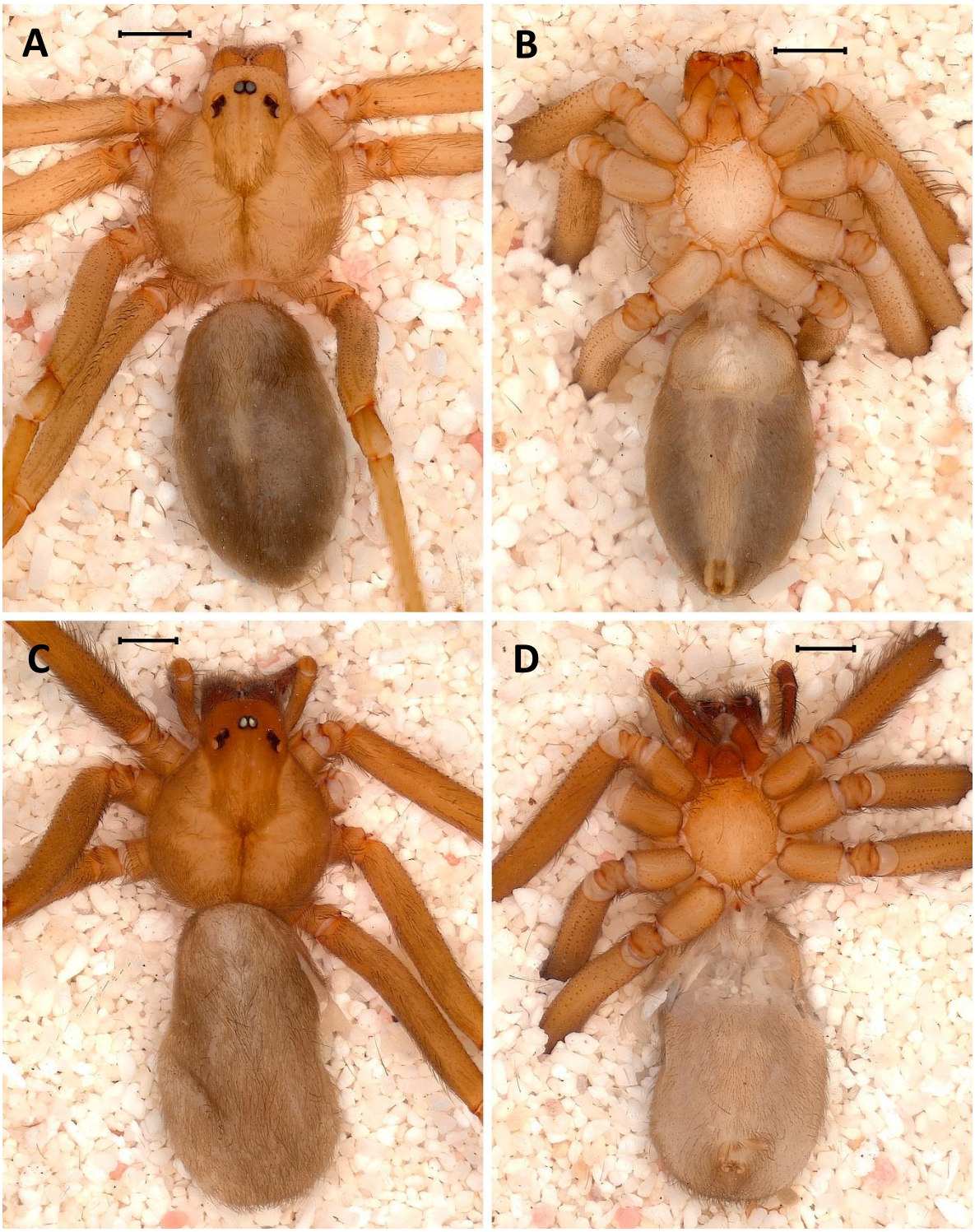

Figure 22. Female and male specimens of *Loxosceles* sp17 sp. nov. San Luis Potosí. **A)** Habitus, dorsal view male; **B)** Habitus, ventral view male; **C)** Habitus, dorsal view female; **D)** Habitus, ventral view female. Scale bars: 1 mm.

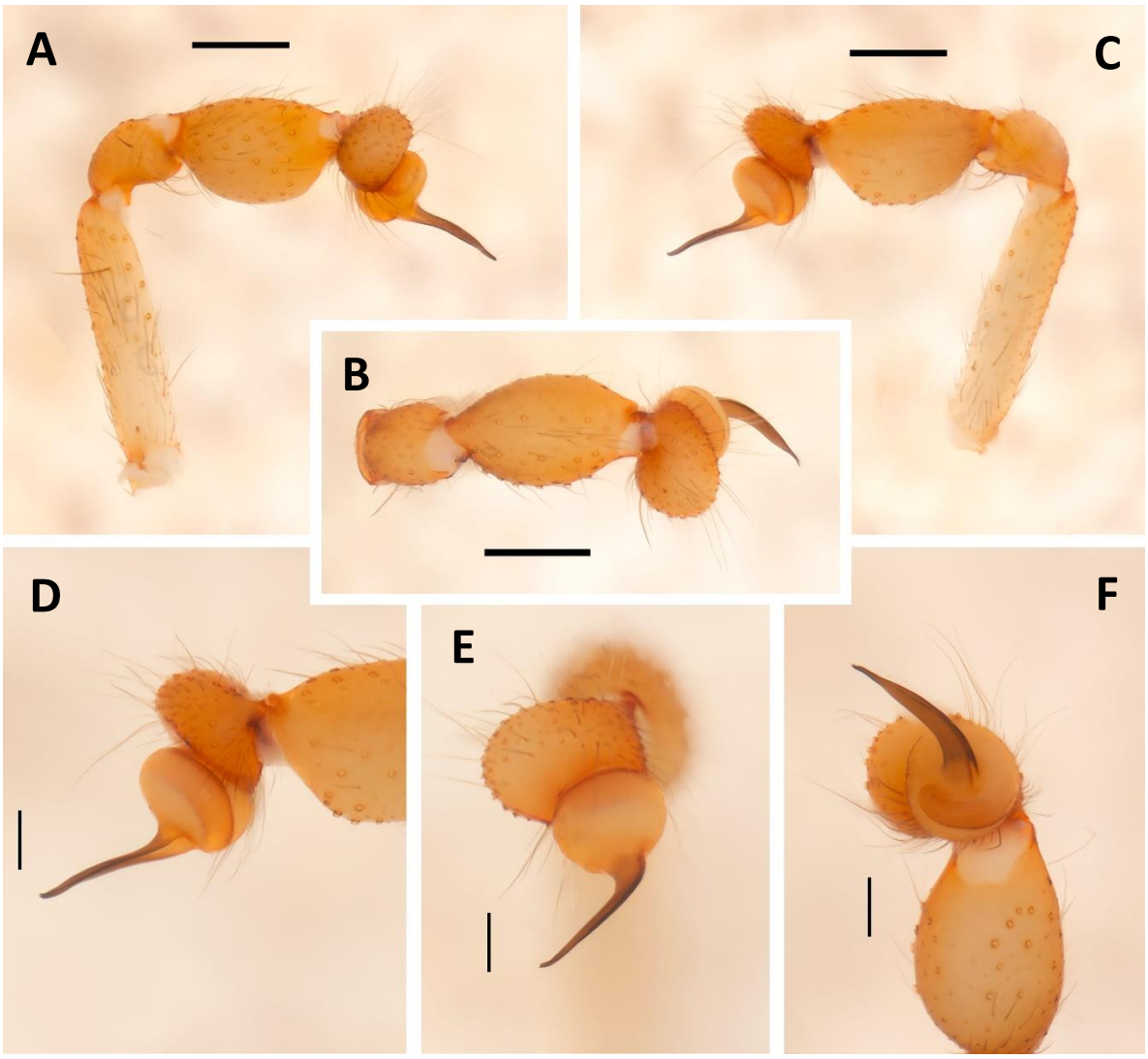

Figure 23. *Loxosceles* sp17 sp. nov. San Luis Potosí. Male Holotype left palp: **A-C)** prolateral, dorsal and retrolateral views respectively; **D-F)** Detail of the bulb and embolus, retrolateral, dorsal and apical views, respectively. Scale bars: 0.5 mm (**A-C**), 0.2 mm (**D-F**).

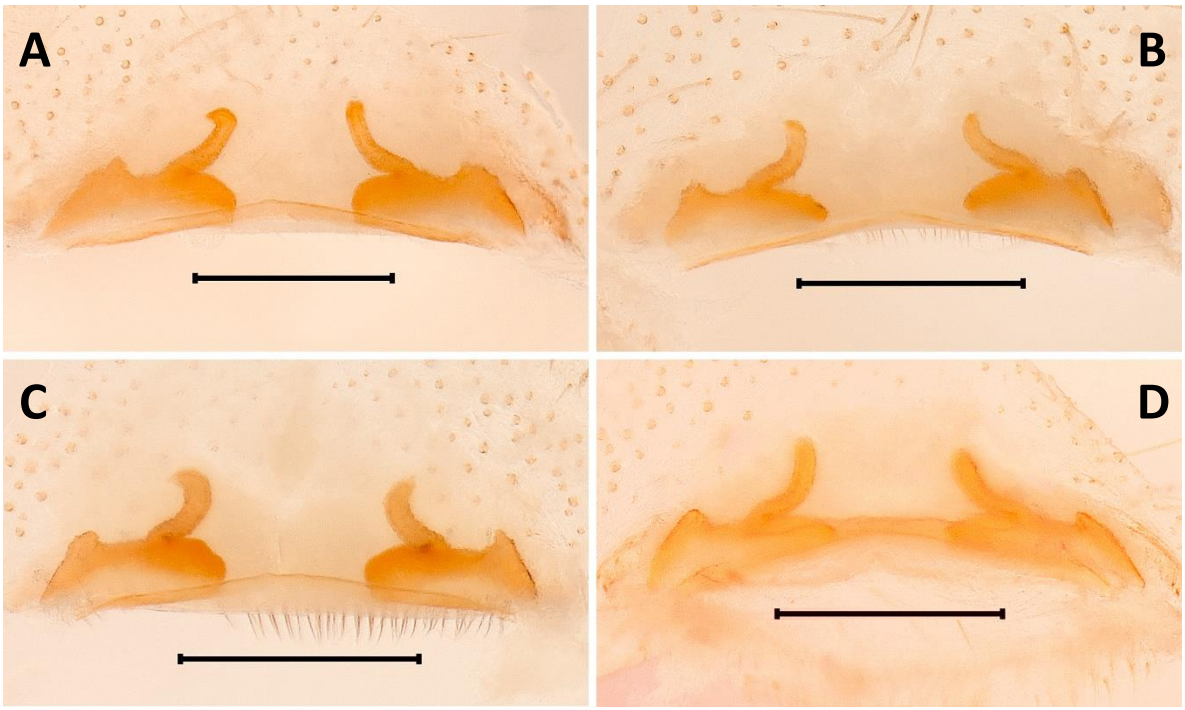

Figure 24. *Loxosceles* sp17 sp. nov. San Luis Potosí. Female internal genitalia. Variation of seminal receptacles: **A, B, C, D)** 2.5 Km al E de Ejido Laguna del Mante, Reserva de la Biosfera El Abra-Tanchipa, Rincón de Pames, Ciudad Valles. Dorsal view. Scale bars: 0.5 mm.

***Loxosceles* sp18-Oax (sp. nov).**

(Figures 25-27)

Type material: MEXICO: Oaxaca: Male holotype from San Pedro Totolapa (CARCIB Ara1011) 4 km North of San Pedro Totolapa, 2022, A. Valdez, S. Nolasco, A. Juárez Col. Paratypes: 4 male, 4 females (CARCIB Ara1011) same data as holotype.

Other material examined. MEXICO: Oaxaca: 3 males (CARCIB Ara1011), same data as holotype. 3 females (CARCIB Ara0152) San Pedro Totolapa, 2017, A. Valdez, M. Cortez, A. Juárez, J. Valerdi Cols. 5 females (CARCIB Ara0569), San Pedro Totolapa, 2019, A. Valdez, B. Hubber, A. Cabrera Cols.

General diagnosis. *Palp:* Femora pale brown, short and thick, with stridulatory pick basally (Fig. 26A); patellae pale brown; tibiae brown, dorsally and ventrally curved and thick, almost circular, projecting distally (Figs. 26A, 26C). Tarsus oval, Brown; bulb oval, with straight, slender embolus, tip slightly elevated (Figs. 26A, 26C, 26D, 26E). *Genital area:* Seminal receptacles short, wider apically, circular in shape. Base of spermathecae small, sclerosed at basal part of lobe.

Distribution. MEXICO: Oaxaca (Figs. 25-27)

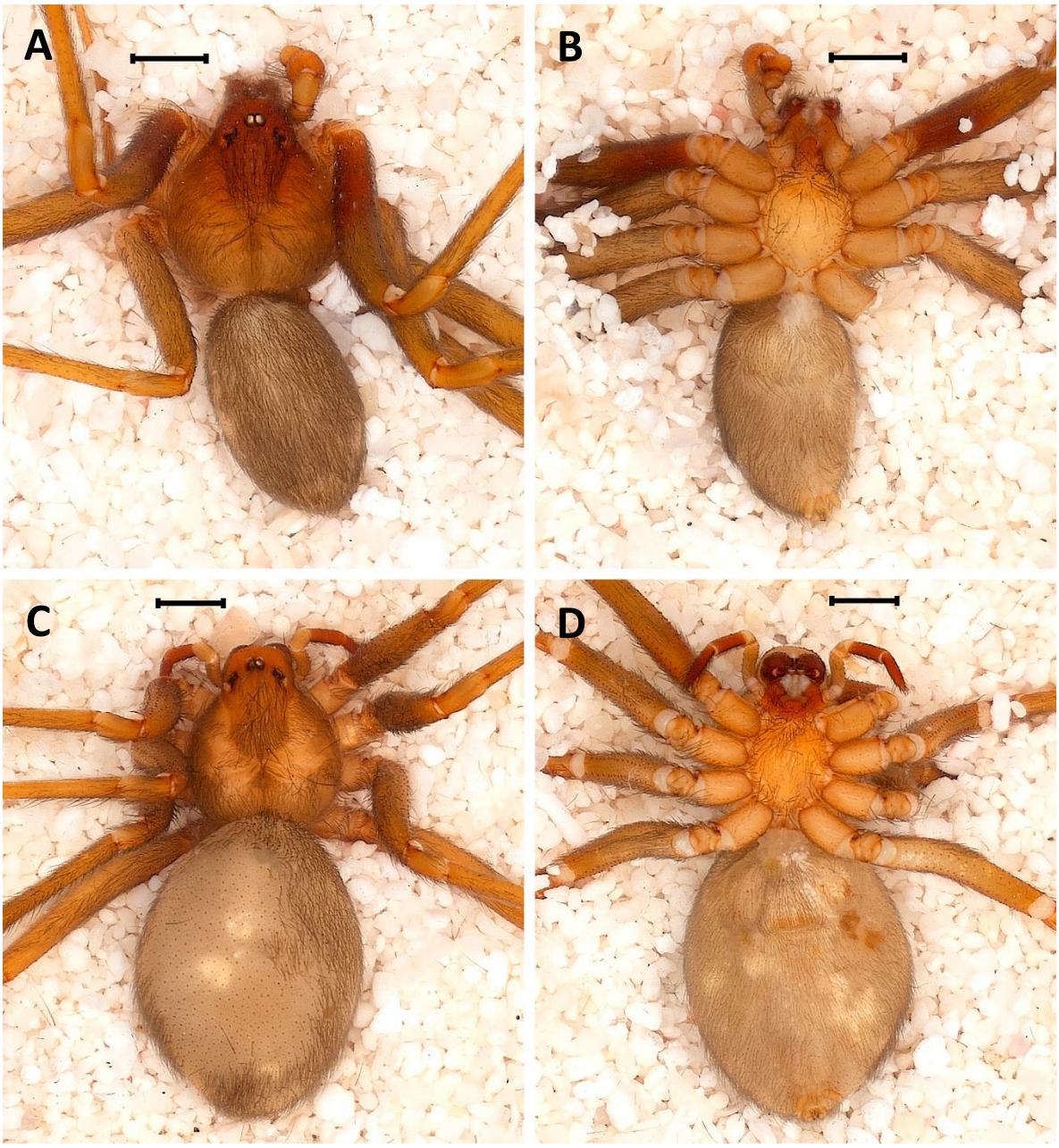

Figure 25. Female and male specimens of *Loxosceles* sp18 sp. nov. Oaxaca. **A)** Habitus, dorsal view male; **B)** Habitus, ventral view male; **C)** Habitus, dorsal view female; **D)** Habitus, ventral view female. Scale bars: 1 mm.

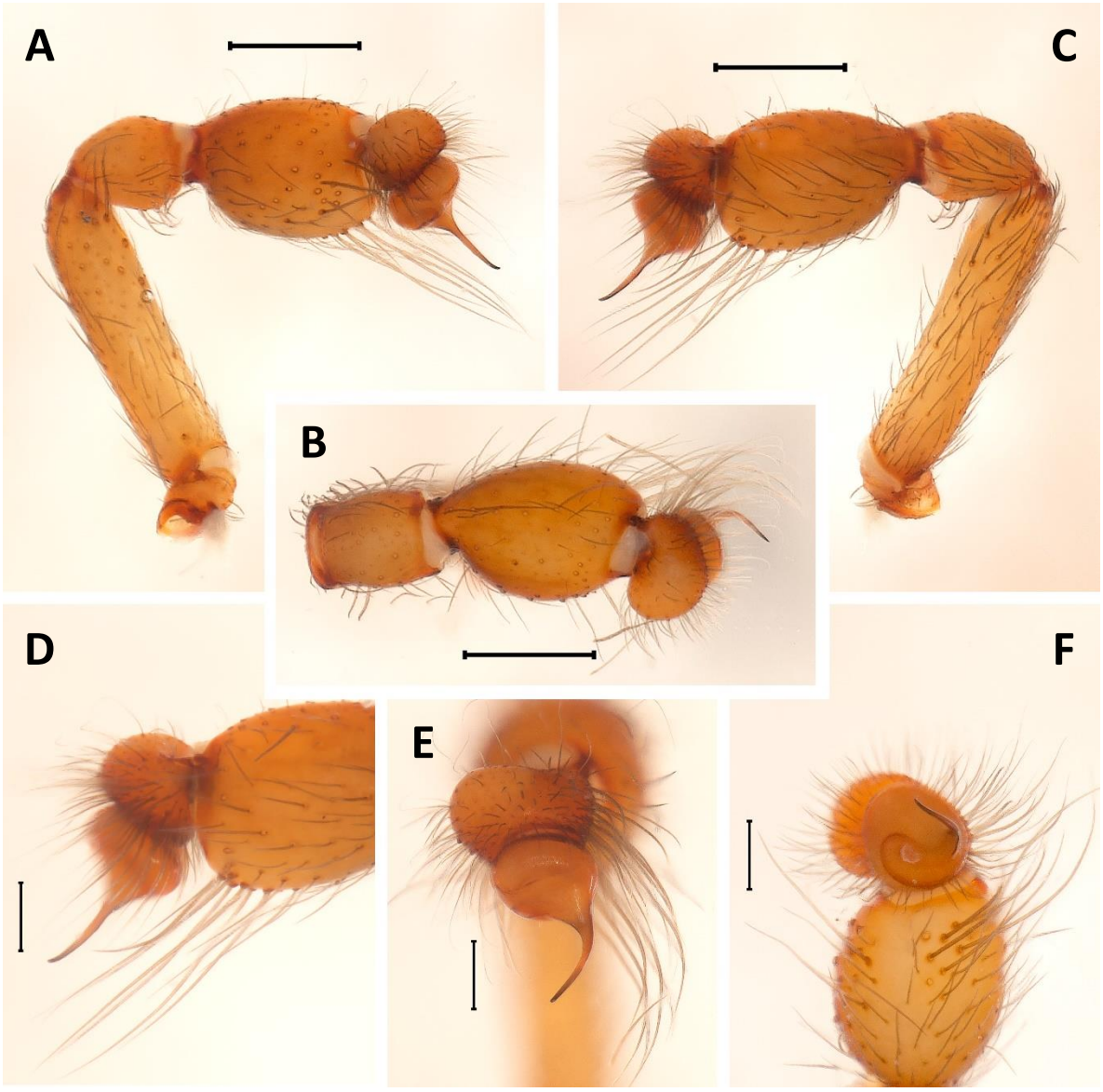

Figure 26. *Loxosceles* sp18 sp. nov. Oaxaca. Male Holotype left palp: **A-C)** prolateral, dorsal and retrolateral views respectively; **D-F)** Detail of the bulb and embolus, retrolateral, dorsal and apical views, respectively. Scale bars: 0.5 mm (**A-C**), 0.2 mm (**D-F**).

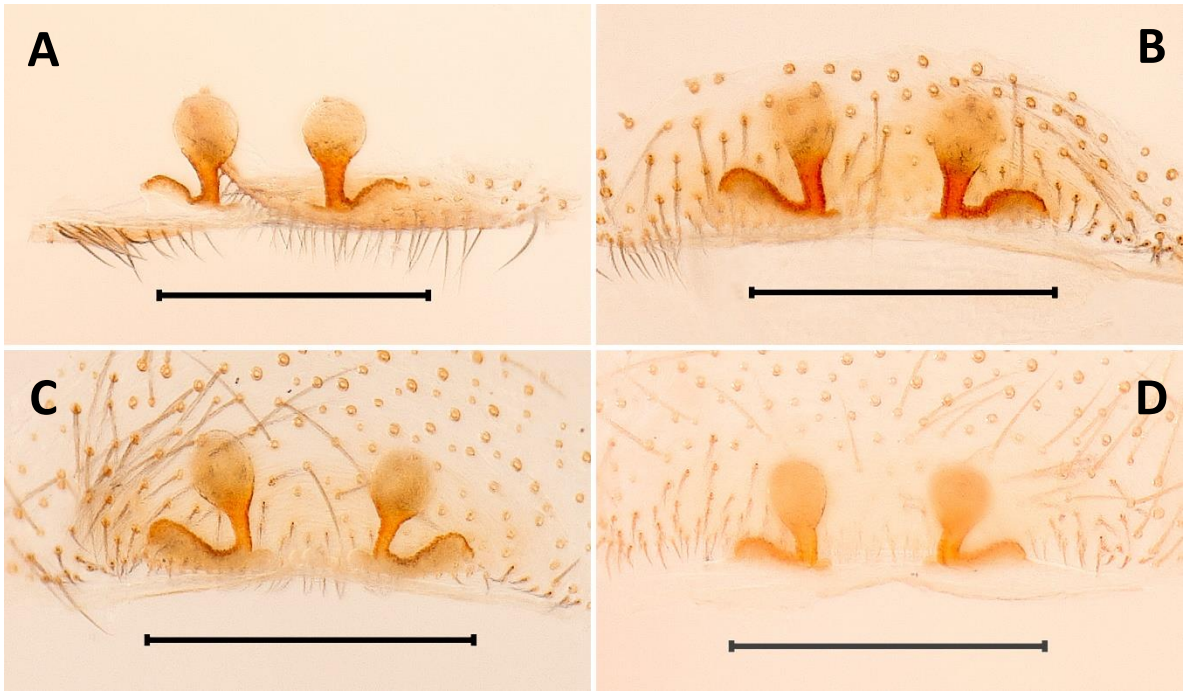

Figure 27. *Loxosceles* sp18 sp. nov. Oaxaca. Female internal genitalia. Variation of seminal receptacles: **A, B, C, D)** 4 km al N de San Pedro Totolapa. San Pedro Totolapa. Dorsal view. Scale bars: 0.5 mm.

Discussion

The most extensive taxonomic work for species of the genus *Loxosceles* in Mexico was done 42 years ago by Gertsch and Ennik (1983) where they described 20 species for the country, based on morphological characters of males and females, represented with drawings and sometimes only for one of the sexes.

Despite recent work with the genus *Loxosceles* in Mexico on the description of new species (Jiménez and Llinas, 2005; Valdez-Mondragón et al. 2018b; Valdez-Mondragón et al. 2019; Navarro-Rodríguez and Valdez-Mondragón, 2020), an updated taxonomic study or revision of the genus had not been carried out, reviewing most of the species present in Mexico. Therefore, this work includes the description of the largest number of species of *Loxosceles* in Mexico, previously corroborated with integrative evidence (morphology and molecular) (Navarro-Rodríguez and Valdez-Mondragón, 2025; *in prep.*).

For the species described here based on previous work by Navarro-Rodríguez and Valdez-Mondragón, (2024) they found an intraspecific p-distance of 15.6% with COI. While six (*Loxosceles* sp3 sp. nov, *Loxosceles* sp13 sp. nov., *Loxosceles* sp14 sp. nov., *Loxosceles* sp16 sp. nov., *Loxosceles* sp17 sp. nov., and *Loxosceles* sp18 sp. nov.) of the nine species corroborated by all methods, on par with morphology, for the remaining three species (*Loxosceles* sp1 sp. nov., *Loxosceles* sp2 sp. nov., *Loxosceles* sp3 sp. nov., and *Loxosceles* sp5 sp. nov.) only two of the three methods corroborate these species, likewise on par with morphology.

Although Gertsch (1958) mentioned that the identification of some species of the *Loxosceles* genus could be complicated, mainly due to the wide variation present in the seminal receptacles of the females, for which reason he suggested reviewing a good series to corroborate the species. Also, Gertsch (1973 and 1983) mentioned that, despite this wide variation reported for females, the seminal receptacles turned out to be more useful and informative than the pedipalps of males. However, in the case of most Mexican species, the shape of the pedipalp turns out to be more useful and informative when corroborating the species, since a wide variation has been found in the seminal receptacles of most Mexican species, which complicates their identification (Valdez-Mondragón et al. 2018b; Navarro-Rodríguez, 2019).

In the case of the pedipalps, although Gertsch (1983) did not describe the variation of the pedipalps, later studies with Mexican species do report it (Valdez-Mondragón et al. 2018b; Navarro-Rodríguez, 2019; Valdez-Mondragón et al. 2019; Navarro-Rodríguez and Valdez-Mondragón, 2020) and corroborate that most of the time the shape of the pedipalp is more useful to corroborate the species, since it presents more intraspecifically conserved diagnostic characters and does not vary even belonging to different localities.

In terms of geographic distribution, the biogeographic provinces with the highest number of *Loxosceles* spiders were: 1) the Balsas Basin, 2) the Sierra Madre Oriental, 3) the Pacific Lowlands and 4) the province of Baja California. In these provinces, dry and tropical deciduous forests predominate, as well as scrublands, characteristic habitats of the genus *Loxosceles*, although there are species that are distributed in tropical humid forests, as is the case of *L. chinateca* and *L. yucatana.* In the case of Sierra Madre Oriental province, the records are mainly found in the east of the province where elevations are lower, and the climate is more tropical (Valdez-Mondragón et al. 2018). With this contribution the number of species described for the genus *Loxosceles* increases to 158, and the *reclusa* group to 64, Mexico as the country with the highest number of described species at 49.

Acknowledgements

The first author thanks the PhD (Doctorado) Program of the Centro Tlaxcala de Biología de la Conducta (CTBC), Universidad Autónoma de Tlaxcala, Tlaxcala City, and the Secretaria de Ciencias, Humanidades, Tecnología e Innovación (SECIHTI) for scholarship support during their PhD in Biological Science. The second author thanks the program "Jóvenes investigadores por México" and SECIHTI for scientific support for the project No. 59: "Laboratorio Regional de Biodiversidad y Cultivo de Tejidos Vegetales (LBCTV), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM)-Tlaxcala". The second author also thanks SEP-SECIHTI for financial support of the project of Basic Science (Ciencia Básica) 2016, No. 282834. We thank Dr. Edmundo González Santillán and Dr. Oscar F. Francke (ex-curator) of the Colección Nacional de Arácnidos (CNAN), Instituto de Biología, UNAM, for providing specimen loans, Brett O. Butler for the English language review of the manuscript, and the reviewers for their comments and suggestions that improved the quality of the manuscript. We also thank the students of the Laboratory of Arachnology (LATLAX), IBUNAM, Tlaxcala, for their help in the field and processing the material in the laboratory. Specimens were collected under Scientific Collector Permit FAUT-0309 from the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT), Mexico, provided to Alejandro Valdez-Mondragón (corresponding author).

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8. CONCLUSIONES GENERALES

1. Al integrar múltiples líneas de evidencia, este estudio ha permitido identificar 15 nuevas especies putativas del género *Loxosceles* en Norteamérica. Estos resultados resaltan la importancia del enfoque taxonómico integrador para conocer la diversidad de especies y sugieren que la diversidad del género ha sido subestimada.

2. Los resultados obtenidos indican que el gen COI es un marcador útil y de referencia para la delimitación de especies del género *Loxosceles*, ya que brinda mayor resolución y es más informativo en comparación con ITS2 y 28S.

3. La morfología conservada del género *Loxosceles* complica la identificación a nivel de especie. Sin embargo, la morfología de los pedipalpos de los machos sigue siendo un rasgo útil para la identificación y diagnosis de las especies, destacando la importancia de su uso en la taxonomía del género.

4. Los análisis filogenéticos basados en la matriz concatenada (COI+ITS2+28S), respaldados por altos valores de Bootstrap y Probabilidad Posterior, confirman la monofilia del grupo de especies "*reclusa*" bajo reconstrucción de ML e IB.

5. La aparición de distintas barreras biogeográficas, además de la poca capacidad de dispersión del grupo han propiciado la diversificación de este género en México, estando mayormente influida según la datación por la orogenia de la Sierra Madre del Sur durante el Oligoceno y la orogenia de la Sierra Madre Oriental durante el Eoceno.

6. Con base en los análisis de datación de linajes y reconstrucción de áreas ancestrales, descartamos la hipótesis de que el género *Loxosceles* se dispersó a través del puente terrestre formado entre las islas del Caribe y Sudamérica, llamado GAARLandia, en cambio sugerimos que su dispersión fue a través de Centroamérica previo a la formación del Istmo de Panamá.

9. REFERENCIAS GENERALES

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10. PUBLICACIONES

10.1. Artículos adicionales publicados o en proceso de publicación durante el Doctorado

Artículo de Divulgación. Link de acceso: https://www.revistacyn.com/actual/1127

Articulo Científico: Aceptado y en prensa para su publicación en la revista Acta Zoológica Mexicana (nueva serie).

Presence of bacteria *Wolbachia* (Rickettsiales, Ehrlichiaceae) in Synspermiata spiders, including the first record for the family Sicariidae (Araneae, Araneomorphae)

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Abstract

Infection by the endosymbiotic bacteria *Wolbachia* (Ehrlichiaceae) is recorded in a wide variety of arthropods, including spiders. Within the order Araneae, there are previous reports of the infection of *Wolbachia* in Synspermiata spiders. Under molecular studies using the mitochondrial molecular marker Cytochrome *c* oxidase 1 (CO1) with the genera *Physocyclus* (Pholcidae) and *Loxosceles* (Sicariidae), we detected the presence of *Wolbachia* in one species of *Physocyclus* and seven species of *Loxosceles* from Mexico, which represent the fifth record for the family Pholcidae and the first records for Sicariidae. According with previous works and our results, the infection of *Wolbachia* in spiders has been recorded in 19 families, 70 genera, and 122 species. Linyphidae is the the family with the highest records of infection by *Wolbachia* with 27 genera and 43 species. With these records, four families of spiders from the Synspermiata clade have been documented so far with the presence of *Wolbachia*: Dysderidae, Pholcidae, Telemidae, and Sicariidae, being Sicariidae the family with the highest records with seven species, which are documented in this work.