

## Research article

<urn:lsid:zoobank.org:pub:8C465C60-7228-4051-AFFD-96E1EBE1A67A>**Description of a new species of *Loxosceles* Heineken & Lowe (Araneae, Sicariidae) recluse spiders from Hidalgo, Mexico, under integrative taxonomy: morphological and DNA barcoding data (*COI+ITS2*)**Claudia Isabel NAVARRO-RODRÍGUEZ<sup>1</sup> & Alejandro VALDEZ-MONDRAÓN<sup>2,\*</sup><sup>1</sup>Laboratory of Arachnology (LATLAX), Laboratorio Regional de Biodiversidad y Cultivo de Tejidos Vegetales (LBCTV), Instituto de Biología, Universidad Nacional Autónoma de Mexico (UNAM), sede Tlaxcala, Ex-Fábrica San Manuel, San Miguel Contla, 90640 Santa Cruz Tlaxcala, Tlaxcala, Mexico.<sup>1</sup>Posgrado en Ciencias Biológicas, Centro Tlaxcala de Biología de la Conducta (CTBC), Universidad Autónoma de Tlaxcala (UATx), Carretera Federal Tlaxcala-Puebla, Km. 1.5, C. P. 90062, Tlaxcala, Mexico.<sup>2</sup>CONACYT Research Fellow. Laboratory of Arachnology (LATLAX), Laboratorio Regional de Biodiversidad y Cultivo de Tejidos Vegetales (LBCTV), Instituto de Biología, Universidad Nacional Autónoma de Mexico (UNAM), sede Tlaxcala, Ex-Fábrica San Manuel, San Miguel Contla, 90640 Santa Cruz Tlaxcala, Tlaxcala, Mexico.<sup>2</sup>Colección Nacional de Arácnidos (CNAN), Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de Mexico (UNAM), Ciudad Universitaria, Apartado Postal 04510, Coyoacán, Mexico City, Mexico.<sup>\*</sup>Corresponding author: [lat\\_mactans@yahoo.com.mx](mailto:lat_mactans@yahoo.com.mx)<sup>1</sup>Email: [biobella66@hotmail.com](mailto:biobella66@hotmail.com)<sup>1</sup><urn:lsid:zoobank.org:author:B3AD9D20-D15E-4363-8A04-88C1C2F9374E><sup>2</sup><urn:lsid:zoobank.org:author:F043A1C7-2B83-40C9-A74E-82C92F00725A>

**Abstract.** Based on an integrative taxonomic approach, a new species of the genus *Loxosceles* Heineken & Lowe, 1832, is described from the state of Hidalgo, Mexico. *Loxosceles tolantongo* sp. nov. is described based on DNA barcoding using cytochrome *c* oxidase subunit 1 (*COI*) and internal transcribed spacer 2 (*ITS2*), and morphology. For species delimitation, four molecular methods were implemented: 1) corrected *p*-distances under neighbor joining (NJ); 2) automatic barcode gap discovery (ABGD); 3) general mixed yule coalescent model (GMYC) and 4) Bayesian Poisson tree processes (bPTP). The new species morphologically resembles *L. jaca*, another species from Hidalgo, but there are morphological differences mainly in the tibiae of the male palp, the seminal receptacles of the females and also the high genetic *p*-distances. *COI* was more informative than *ITS2* for the genetic separation; however, both concatenated genes (*COI+ITS2*) present robust evidence for species delimitation. *Loxosceles tolantongo* sp. nov. is considered a unique species for four reasons: 1) it can be diagnosed and distinguished by morphological characters (of the male palps mainly, but also of the seminal receptacles of the females); 2) the genetic *p*-distances with *COI* were high (>10%); 3) the molecular species delimitation methods were congruent under *COI* and *COI+ITS2*; and 4) under *COI* and *COI+ITS2*, the new species is a putative sister group of *L. jaca*+*L. tenango*.

**Keywords.** Integrative taxonomy, molecular markers, Synspermiata, species delimitation, Hidalgo.

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## Introduction

Spiders of the genus *Loxosceles* Heineken & Lowe, 1832 are better known as ‘violin spiders’, ‘recluse spiders’ or ‘brown recluse spiders’, and there are currently 139 species worldwide (World Spider Catalog 2020). Gertsch (1958, 1967) and Gertsch & Ennik (1983) proposed that the species of *Loxosceles* belong to eight species groups: *reclusa*, *laeta*, *amazonica*, *gaucho*, *spadicea*, *rufescens*, *vonwredei* and *spinulosa*. However, Duncan *et al.* (2010) and Fukushima *et al.* (2017) synonymized the species group *amazonica* with the species group *rufescens* based on molecular data. Therefore, the genus is currently composed of seven species groups (Valdez-Mondragón *et al.* 2019). North America is the region that has the highest diversity with more than 50 species, all of them belonging to the *reclusa* species group and distributed mainly in Mexico (Gertsch & Ennik 1983; Valdez-Mondragón *et al.* 2018b, 2019). Mexico is the country with the highest diversity of species worldwide, with a total of 39 species described, 37 native species and two introduced: *Loxosceles reclusa* Gertsch & Mulaik, 1940 and *Loxosceles rufescens* (Dufour, 1820) (Gertsch 1958, 1973; Gertsch & Ennik 1983; Valdez-Mondragón *et al.* 2018a, 2018b). Baja California Sur, Baja California and Sonora are the states with the greatest diversity in the country, with five species each (Valdez-Mondragón *et al.* 2018a, 2018b). The preferred habitats of the species of *Loxosceles* from Mexico are mainly dry and tropical forests, including tropical deciduous forests, and deserts; however, some species, such as *L. chinateca* Gertsch & Ennik, 1983 and *L. yucatana* Chamberlin & Ivie, 1938, are distributed exclusively in tropical rain forests (Valdez-Mondragón *et al.* 2019). Also, some species have been recorded from caves, a preferred microhabitat of some species, e.g., *L. misteca* Gertsch, 1958, *L. boneti* Gertsch, 1958, *L. chinateca*, *L. tehuana* Gertsch, 1958, *L. tenango* Gertsch, 1973 and *L. yucatana* (Valdez-Mondragón *et al.* 2018a, 2018b, 2019). In the last two years, two species have been described from Mexico, *Loxosceles malintzi* Valdez-Mondragón, Cortez-Roldán, Juárez-Sánchez & Solís-Catalán, 2018 from the states of Puebla, Morelos and Guerrero; and *Loxosceles tenochtitlan* Valdez-Mondragón & Navarro-Rodríguez, 2019 from Mexico City, and the states of Mexico and Tlaxcala (Valdez-Mondragón *et al.* 2018a, 2018b, 2019).

Historically, the taxonomy of spiders has been based mainly on traditional morphology, using genitalic characters, such as male palps and seminal receptacles in females. Sexual characters in spiders are robust and important morphological characters that are still used to separate species and to provide a diagnosis. This means that genitalia evolves, on average, more rapidly than non-genital morphological traits (Huber 2003; Huber & Dimitrov 2014). Furthermore, somatic characters are useful as additional evidence to separate species in some groups of spiders. Thus coloration, color pattern, body proportions and even extreme size differences are useful traits for species separation (Huber *et al.* 2005; Huber & Dimitrov 2014; Valdez-Mondragón *et al.* 2019). Traditional morphology alone cannot determine species boundaries in some cases due to the intraspecific variation in sexual structures, and the genus *Loxosceles* is a good example (Brignoli 1968; Gertsch & Ennik 1983; Valdez-Mondragón *et al.* 2019). However, the male palps of *Loxosceles* remain a good character for species identification because of the little morphological variation in comparison with the seminal receptacles of the females, as was demonstrated recently in the description of *L. malintzi* and *L. tenochtitlan* by Valdez-Mondragón *et al.* (2018b, 2019).

In spider groups with complicated morphology, the use of molecular markers helps in the discovery of undescribed diversity and species delimitation, as was demonstrated, for example, by Planas & Ribera (2015) with the genus *Loxosceles*. Although DNA barcodes are being applied in systematics as a

useful tool to resolve species delimitation problems, modern taxonomy includes many different sources of evidence, such as traditional morphology, ecology, reproduction and biogeography (integrative taxonomy). Recently described *L. tenochtitlan* by Valdez-Mondragón *et al.* (2019) represents the first species of the genus described using an integrative taxonomic approach with multiple lines of evidence, including traditional morphology, geometric and lineal morphology, molecular markers (*COI* and *ITS2*), and even the biogeographic methods of ecological niche modeling.

In this study, a new species of *Loxosceles* from the state of Hidalgo is described based on morphological and molecular evidence using an integrative taxonomic approach.

## Material and methods

### Biological material

Specimens were hand-collected and preserved in ethanol (80%) for morphology and ethanol (96%) for molecular studies. The type specimens and the additional examined material are deposited in the following repositories:

- CNAN = National Collection of Arachnids, Institute of Biology, Universidad Nacional Autónoma de Mexico (IBUNAM), Mexico City, Mexico (type specimens).  
LATLAX = Laboratory of Arachnology, Laboratorio Regional de Biodiversidad y Cultivo de Tejidos Vegetales (LBCTV), IBUNAM, Tlaxcala City, Mexico (additional material).

Descriptions and observations of the specimens were done using a Zeiss Discovery V8 stereo microscope. The digital photographs of the specimens were taken with a Zeiss Axiocam 506 color camera attached to a Zeiss AXIO Zoom V16 stereo microscope. Photographs were edited using Photoshop CS6. The male palps and female genitalia were dissected in ethanol (80%). The female genitalia were cleaned in potassium hydroxide (KOH – 10%) for 5 to 10 minutes, following Valdez-Mondragón *et al.* (2018b) protocol. The habitus, male palps and female genitalia were submerged in 96% alcohol gel (ethanol) and covered with a thin layer of distilled water to minimize diffraction during photography (Valdez-Mondragón & Francke 2015; Valdez-Mondragón *et al.* 2019). The descriptions were done following Valdez-Mondragón *et al.* (2018b, 2019). All morphological measurements are given in millimeters (mm). The distribution map was made using Q-QGIS ver. 2.18.

### Abbreviations

- AME = anterior median eyes  
PLE = posterior lateral eyes  
PME = posterior median eyes

### Taxon sampling

The molecular analyses presented here are based on a total of 50 individuals from eleven species, including the new species described here and two outgroups to root the trees: *Loxosceles rufescens* (Dufour, 1820) and *Scytodes thoracica* (Latreille, 1802) (Table 1). Three different partitions were used (*COI*: 674 bp, *ITS2*: 435 bp and *COI* + *ITS2*: 1109 bp).

### DNA extraction, amplification and sequencing

Specimens for DNA extraction were preserved in ethanol (96%) and kept at -20°C. DNA was isolated from legs, prosoma, or complete specimens in the case of immatures. DNA extractions were done using a Qiagen DNeasy Tissue Kit following the protocol by Valdez-Mondragón *et al.* (2019). DNA fragments included approximately 650 bp of the cytochrome *c* oxidase subunit 1 (*COI*) mitochondrial gene and 435 bp of the Internal Transcribed Spacer 2 (*ITS2*) nuclear gene. The fragments were amplified

**Table 1** (continued on the next page). Specimens sequenced for each species of *Loxosceles* Heineken & Lowe, 1832 and *Scytodes* Latreille, 1804, DNA voucher numbers, localities and GenBank accession numbers.

Species	DNA voucher LATLAX	Locality	GenBank accession number	
			COI	ITS2
<i>L. colima</i>	Ara0115	Mexico: Colima	MK936303	MK957224
<i>L. colima</i>	Ara0280	Mexico: Colima	MN512430	MN525282
<i>L. colima</i>	Ara0281	Mexico: Colima	—	MN525283
<i>L. colima</i>	Ara0282	Mexico: Colima	MN512431	—
<i>L. colima</i>	Ara0283	Mexico: Colima	MN512432	MN525284
<i>L. jaca</i>	Ara0186	Mexico: Hidalgo	MK936292	MK957194
<i>L. jaca</i>	Ara0048	Mexico: Hidalgo	MK936293	—
<i>L. jaca</i>	Ara0046	Mexico: Hidalgo	MN512427	MK957192
<i>L. jaca</i>	Ara0047	Mexico: Hidalgo	MN512428	MK957193
<i>L. jaca</i>	Ara0183	Mexico: Hidalgo	MN512429	MK957199
<i>L. malintzi</i>	Ara0100	Mexico: Guerrero	MK936282	MK957220
<i>L. malintzi</i>	Ara0001	Mexico: Puebla	MK936283	MK957218
<i>L. malintzi</i>	Ara0002	Mexico: Puebla	MK936284	—
<i>L. malintzi</i>	Ara0025	Mexico: Puebla	MK936285	MK957219
<i>L. malintzi</i>	Ara0072	Mexico: Puebla	MK936286	MK957222
<i>L. malintzi</i>	Ara0074	Mexico: Puebla	MK936287	MK957223
<i>L. malintzi</i>	Ara0101	Mexico: Guerrero	MK936288	—
<i>L. malintzi</i>	Ara0004	Mexico: Puebla	MK936289	MK957221
<i>L. misteca</i>	Ara0082	Mexico: Guerrero	MK936272	MK957212
<i>L. misteca</i>	Ara0089	Mexico: Guerrero	MK936273	MK957215
<i>L. misteca</i>	Ara0090	Mexico: Guerrero	MK936274	MK957214
<i>L. misteca</i>	Ara0084	Mexico: Guerrero	MK936275	MK957213
<i>L. misteca</i>	Ara0236	Mexico: Guerrero	MK936276	MN525280
<i>L. misteca</i>	Ara0237	Mexico: Guerrero	MK936277	MN525281
<i>L. nahuana</i>	Ara0076	Mexico: Hidalgo	MK936297	MK957216
<i>L. nahuana</i>	Ara0077	Mexico: Hidalgo	MK936298	—
<i>L. nahuana</i>	Ara0079	Mexico: Hidalgo	MK936299	MK957217
<i>L. tenango</i>	Ara0191	Mexico: Hidalgo	MK936290	—
<i>L. tenango</i>	Ara0192	Mexico: Hidalgo	MK936291	MK957201
<i>L. tenango</i>	Ara0045	Mexico: Hidalgo	—	MK957195
<i>L. tenango</i>	Ara0189	Mexico: Hidalgo	—	MK957196
<i>L. tenango</i>	Ara0190	Mexico: Hidalgo	—	MK957197
<i>L. tenango</i>	Ara0193	Mexico: Hidalgo	—	MK957198
<i>L. tenango</i>	Ara0188	Mexico: Hidalgo	—	MK957200
<i>L. tenochtitlan</i>	Ara0146	Mexico: Mexico City	MK936278	MK957209
<i>L. tenochtitlan</i>	Ara0161	Mexico: Mexico City	MK936279	—
<i>L. tenochtitlan</i>	Ara0173	Mexico: Tlaxcala	MK936280	MK957210
<i>L. tenochtitlan</i>	Ara0164	Mexico: Tlaxcala	MK936281	MK957211

**Table 1** (continued). Specimens sequenced for each species of *Loxosceles* Heineken & Lowe, 1832 and *Scytodes* Latreille, 1804, DNA voucher numbers, localities and GenBank accession numbers.

Species	DNA voucher LATLAX	Locality	GenBank accession number	
			CO1	ITS2
<i>L. tolantongo</i> sp. nov.	Ara0175	Mexico: Hidalgo	MK936294	MK957208
<i>L. tolantongo</i> sp. nov.	Ara0181	Mexico: Hidalgo	MK936295	MK957206
<i>L. tolantongo</i> sp. nov.	Ara0182	Mexico: Hidalgo	MK936296	MK957207
<i>L. tolantongo</i> sp. nov.	Ara0174	Mexico: Hidalgo	–	MK957202
<i>L. tolantongo</i> sp. nov.	Ara0176	Mexico: Hidalgo	–	MK957203
<i>L. tolantongo</i> sp. nov.	Ara0177	Mexico: Hidalgo	–	MK957204
<i>L. tolantongo</i> sp. nov.	Ara0178	Mexico: Hidalgo	–	MK957205
<i>L. zapoteca</i>	Ara0094	Mexico: Guerrero	MK936300	MK957225
<i>L. zapoteca</i>	Ara0220	Mexico: Guerrero	MK936301	MN525285
<i>L. zapoteca</i>	Ara0227	Mexico: Guerrero	MK936302	MN525286
<i>L. rufescens</i>	–	Grecia: Peloponeso	–	KR864735
<i>Scytodes thoracica</i>	–	Turquía: Antalya	KR864739	–

using the primers in Table 2. Amplifications were carried out in a Veriti Applied-Biosystems 96 Well Thermal Cycler, in a total volume of 25 µl: 3 µl DNA, 8.7 µl H<sub>2</sub>O, 12.5 µl Multiplex PCR Kit of QIAGEN and 0.4 µl of each molecular marker (forward and reverse). The PCR program for *CO1* was as follows: initial step 1 min at 95°C; amplification 35 cycles of 30 s at 95°C (denaturation), 30 s at 48°C (annealing), 1 min at 72°C (elongation); and final elongation 5 min at 72°C. PCR program for *ITS2* was as follows: initial step 3 min at 94°C; amplification 40 cycles of 30 s at 94°C (denaturation), 1 min at 53°C (annealing), 1 min at 72°C (elongation); and final elongation 5 min at 72°C. PCR products were checked to analyze length and purity on 1% agarose gels with a marker of 100 bp and purified directly using the QIAquick PCR Purification kit of QIAGEN. DNA extraction and amplification were performed at the Molecular Laboratory at the Laboratorio Regional de Biodiversidad y Cultivo de Tejidos Vegetales (LBCTV), Institute of Biology, Universidad Nacional Autónoma de Mexico (UNAM), Tlaxcala City. Sequencing was performed at the Molecular Laboratory in the Institute of Biology, UNAM, Mexico City. Sequencing of both strands (5'-3' and 3'-5') of PCR products were performed in a Sequencer Genetic Analyzer RUO Applied Biosystems Hitachi model 3750xL. Sequence data of *CO1* and *ITS2* are deposited in GenBank with accession numbers: MK936272–MK936303 and MN512427–MN512432 for *CO1* and MK957192–MK957225 and MN525280–MN525286 for *ITS2* (Table 1).

### DNA sequence alignment and editing

Sequences were edited with the programs BioEdit ver. 7.0.5.3 (Hall 1999) and Geneious ver. 10.2.3 (Kearse *et al.* 2012). Sequences were aligned online using the default gap opening penalty of 1.53 in MAFFT (Multiple Sequence Alignment based on Fast Fourier Transform) ver. 7 (Katoh & Toh 2008) using the following alignment strategy: Auto (FFT-NS-2, FFTNS-i or L-INS-i, depending on data size). These aligned matrices were subsequently used in analyses.

### Molecular analyses, species delimitation and haplotypes networks

For molecular species delimitation, the following methods were used for analyzing the concatenated *CO1* + *ITS2* matrix (1109 characters): 1) corrected *p*-distances under neighbor joining (NJ) using MEGA ver. 7.0; 2) automatic barcode gap discovery (ABGD) online version (Puillandre *et al.* 2012) using both uncorrected and K2P distance matrices; 3) general mixed yule coalescent model (GMYC) (Pons *et al.*

**Table 2.** Primers used for each molecular marker for PCR.

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

2006) using GMYC web server (<https://species.h-its.org/gmyc/>); and 4) Bayesian Poisson tree processes (bPTP) (Zhang *et al.* 2013; Kapli *et al.* 2017) using web server (<https://species.h-its.org/ptp/>).

#### P-distances under neighbor joining (NJ)

The bootstrap values in the NJ analysis were calculated with the following commands: Number of replicates = 1000, bootstrap support values = 1000 (significant values  $\geq 50\%$ ), Substitution type = nucleotide, Model = Kimura 2-parameter, Substitution to Include = d: Transitions + Transversions, Rates among Sites = Gamma distributed (G), Missing Data Treatment = Pairwise deletion, Select Codon Position = 1<sup>st</sup> + 2<sup>nd</sup> + 3<sup>rd</sup> + Noncoding Sites.

#### Starting trees under Maximum Likelihood (ML) and Bayesian Inference (BI)

The approaches for DNA barcoding tree-based delimitation explicitly use the phylogenetic species concept. An input starting tree generated with ML using MEGA ver. 7.0 and BI performed by MrBayes ver. 3.1.2 (Ronquist & Huelsenbeck 2003) were implemented, and the analysis recognized monophyletic clusters by searching differential intra- and interspecific branching patterns (Ortiz & Francke 2016). The ML analysis was calculated with the parameters for *COI* and *ITS2*: Number of replicates = 1000, bootstrap support values = 1000 (significant values  $\geq 50\%$ ), Models of sequence evolution selected using jModelTest = GTR, Rates among sites = G+I, No. of discrete Gamma Categories = 6, Gaps Data Treatment = Complete deletion, Select Codon Position = 1<sup>st</sup> + 2<sup>nd</sup> + 3<sup>rd</sup> + Noncoding Sites, ML Heuristic Method = Subtree-Pruning-Regrafting – Extensive (SPR level 5), Initial Tree for ML = Make initial tree automatically (Default – NJ/BioNJ). The BI analyses were run with four parallel Markov chains with the following parameters: MCMC (Markov Chain Monte Carlo) generations = 20 000 000, sampling frequency = 1000, print frequency = 1000, number of runs = 2, number of chains = 4, MCMC burn-in = 2500, sumt burn-in = 2500, sump burn-in = 2500, Models of sequence evolution selected using jModelTest = GTR, Rates among sites = G+I, Select Codon Position = 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup>. TRACER ver. 1.6 (Rambaut & Drummond 2014) was used to analyze the parameters and the effective sample size (ESS) of the MCMC to ensure the runs converged. FigTree ver. 1.4.3 was used to visualize the topology of the tree with the posterior probability values (PP) at nodes. The models of sequence evolution were selected using the Akaike information criterion (AIC) in jModelTest ver. 2.1.10 (Posada & Buckley 2004). The models selected for *COI* and *ITS2* for each partition block were: GTR+G+I (1<sup>st</sup> and 2<sup>nd</sup> codon positions) and GTR+G (3<sup>rd</sup> position). The model selected for *ITS2* was GTR+G.

#### Automatic barcode gap discovery (ABGD)

The ABGD species delimitation method uses recursive partitioning with a range of prior intraspecific divergence and relative gap widths, estimating the threshold between intra- and interspecific genetic variation, generating species-level groupings (Ortiz & Francke 2016). ABGD analyses were conducted using both uncorrected and K2P distance matrices with default options: Pmin = 0.001, Pmax = 0.1, Steps = 10, Relative gap width (X) = 1, Nb bins = 20.

### General mixed yule coalescent model (GMYC)

The GMYC species delimitation method applies single (Pons *et al.* 2006) or multiple (Monaghan *et al.* 2009) time thresholds to delimit species in a Maximum Likelihood context, using ultrametric trees (Ortiz & Francke 2016). Phylogenetic analyses were run in BEAST ver. 2.6.0 (Drummond *et al.* 2012) using a coalescent (constant population) tree prior. Independent lognormal relaxed clock was applied to each partition, for analyses  $20 \times 10^6$  generations were run. Convergence was assessed with TRACER ver. 1.6 (Rambaut & Drummond 2014). TREEANNOTATOR ver. 2.6.0 (BEAST package) was used to build maximum clade credibility trees, after discarding the first 25% of generations by 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/>). The backend of this web server runs the original R implementation of the GMYC model authored by Fujisawa & Barraclough (2013). A single threshold was used for the concatenated matrix.

### Bayesian Poisson tree processes (bPTP)

The PTP species delimitation method (Zhang *et al.* 2013) is similar to GMYC, but uses substitution calibrated (not ultrametric) trees to avoid the potential flaws in constructing time calibrated phylogenies (Zhang *et al.* 2013; Ortiz & Francke 2016). We employed the Bayesian variant of the method (bPTP) on the online version (<https://species.h-its.org/ptp/>). It was run on the Bayesian gene trees with default options: rooted tree, MCMC generations = 100 000, Thinning = 100, Burn-in = 0.1, Seed = 123. Haplotypes network for *COI* was constructed to visualize the mutations among haplotypes of species using the TCS algorithm (Clement *et al.* 2000) in PopArt ver. 1.7 (Leigh & Bryant 2015). The trees generated were edited using Adobe Photoshop CS6.

## Results

Phylum Arthropoda von Siebold, 1848  
Class Arachnida Cuvier, 1812  
Order Araneae Clerck, 1757  
Family Sicariidae Keyserling, 1880

Genus *Loxosceles* Heineken & Lowe, 1832

Type species: *Loxosceles citigrada* Heineken & Lowe, 1832 (= *Scytodes rufescens* Dufour, 1820), currently *Loxosceles rufescens* (Dufour, 1820).

*Loxosceles tolantongo* sp. nov.

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Figs 1–9, 16–33, 36–39

### Differential diagnosis

*Loxosceles tolantongo* sp. nov. morphologically resembles *L. jaca* Gertsch & Ennik, 1983, also from Hidalgo. However, in lateral view, the palp tibia of the male *L. tolantongo* sp. nov. is slightly longer than in *L. jaca* and the curvature of the basal-ventral part of the tibia is less pronounced than in *L. jaca* (Figs 20, 22, 30, 33), where it is prominent and totally curved (Figs 34–35, 44, 46). In dorsal view, the palp tibia of *L. tolantongo* sp. nov. is thinner and longer (Fig. 21), whereas in *L. jaca* it is wider and shorter (Fig. 45). In the new species, in lateral view, the embolus is slightly wider basally than that of *L. jaca* (Figs 20, 22 vs Figs 44, 46). In lateral and dorsal views, the tip of the embolus in *L. tolantongo* sp. nov. is slightly curved (Figs 20, 22, 24), whereas in *L. jaca* it is sigmoid (Figs 44, 46, 48). The seminal receptacles of the females of both species are similarly S-shaped (Figs 36–39, 40–43). However, the shape of the base of the receptacles is different: in *L. tolantongo* sp. nov. it is oval-shaped (Figs 28, 36–39), whereas in *L. jaca* it is square-shaped (Figs 40–43). Also, the base of seminal receptacles is more sclerotized in *L. tolantongo* sp. nov. than in *L. jaca* (Figs 28, 36–39, 40–43).

## Etymology

The species name is a noun in apposition and refers to the type locality of the new species: the Tourist Center Grutas de Tolantongo, located in the Mezquital Valley, Hidalgo, Mexico.

## Material examined

### Holotype

MEXICO – Hidalgo • ♂; Municipality of Cardonal, Tourist Center Grutas de Tolantongo; 20.6503° N, 99.0047° W; 1315 m a.s.l.; 17 Mar. 2017; A. Valdez, E. Briones, J. Valerdi, A. Juárez and M. Sánchez leg.; night collecting; CNAN-T01317.

### Paratypes

MEXICO – Hidalgo • 1 ♂; same collection data as for holotype; CNAN-T01319 • 1 ♂; same collection data as for holotype; 22 May 2018; A. Valdez, J. Valerdi, A. Cabrera, P. Solís and I. Navarro leg.; diurnal collecting; CNAN-T01320 • 2 ♀♀; same collection data as for preceding; CNAN-T01321, CNAN-T01324 • 1 ♂; Municipality of Cardonal, Tourist Center Grutas de Tolantongo, 500 m west of entrance No. 5; 20.6446° N, 98.9973° W; 1481 m a.s.l.; 22 May 2018; A. Valdez, J. Valerdi, A. Cabrera,



**Figs 1–5.** Live male holotype (CNAN-T01317) of *Loxosceles tolantongo* sp. nov. from the type locality: Tourist Center Grutas de Tolantongo, Municipality of Cardonal, Hidalgo, Mexico. Photos 1–4 by Alejandro Valdez-Mondragón (2019); photo 5 by Claudia Isabel Navarro-Rodríguez (2019).

P. Solís and I. Navarro leg.; diurnal collecting; CNAN-T01318 • 2 ♀♀; same collection data as for preceding; CNAN-T01322, CNAN-T01323.

#### Other material

MEXICO – Hidalgo • 1 ♂, 5 immatures; Municipality of Cardonal, Tourist Center Grutas de Tolantongo, 500 m west of entrance No. 5; 20.6446° N, 98.9973° W; 1481 m a.s.l.; 22 May 2018; A. Valdez, J. Valerdi, A. Cabrera, P. Solís and I. Navarro leg.; diurnal collecting; LATLAX Ara-0488 • 1 ♂, 1 immature; same collection data as for holotype; 23 May 2018; LATLAX Ara-0490 • 2 immatures; same collection data as for holotype; 22 May 2018; A. Valdez, J. Valerdi, A. Cabrera, P. Solís and I. Navarro leg.; diurnal collecting; LATLAX Ara-0489 • 1 ♂, 2 immatures; same collection data as for holotype; LATLAX Ara-0137 • 6 ♀♀, 4 immatures; Municipality of Cardonal, Tourist Center Grutas de Tolantongo, ‘El Paraíso’ trail; 20.6502° N, 99.0002° W; 1312 m a.s.l.; 24 Jan. 2019; A. Valdez, P. Solís, M. Cortez, J. Sánchez and D. Montiel leg.; LATLAX Ara-0527, Ara-0528.

#### Description

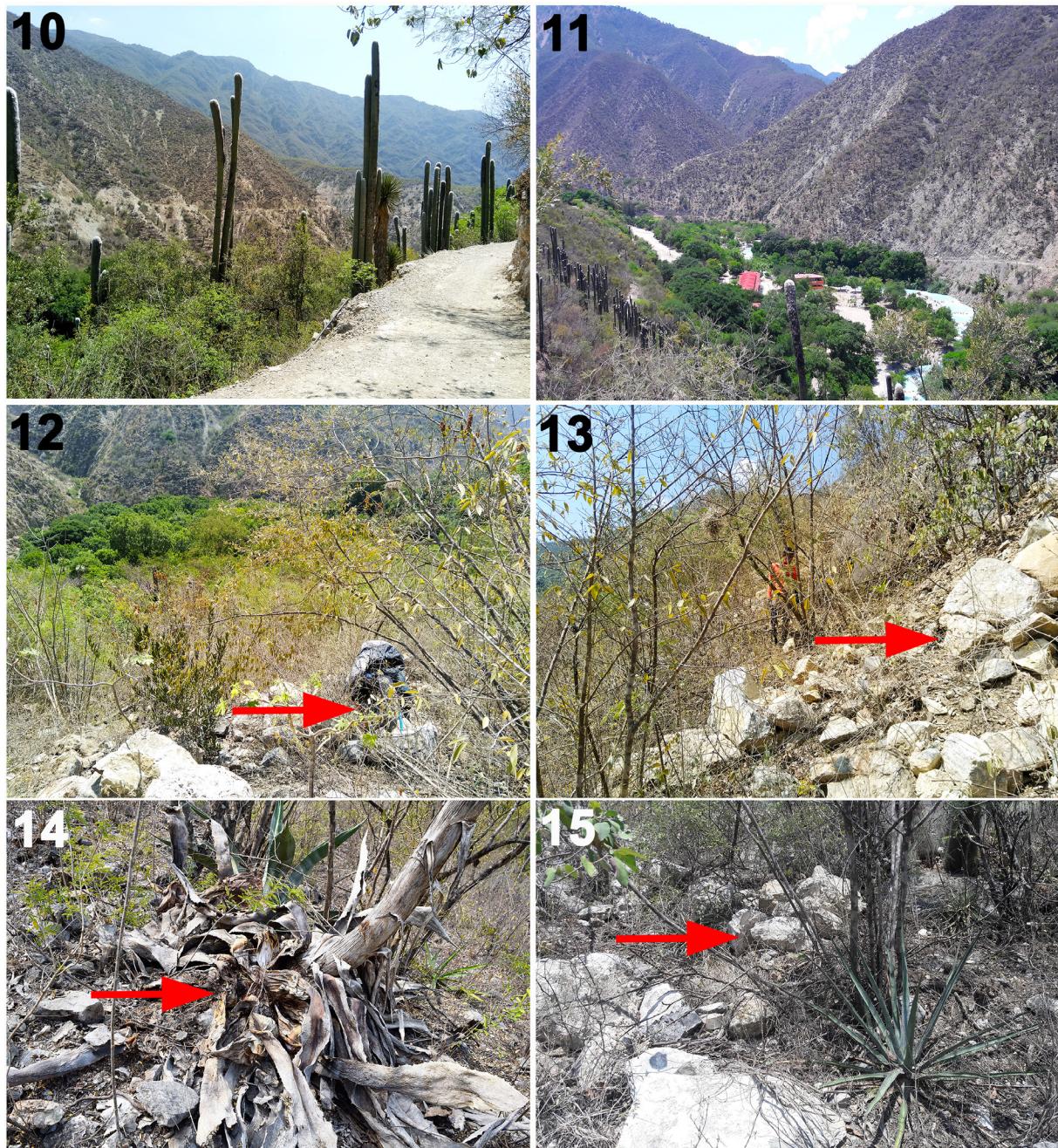
##### Male (holotype CNAN-T01317)

Specimen collected manually, preserved and observed in 80% ethanol.

MEASUREMENTS. Total length 5.60. Carapace: 2.60 long, 2.30 wide. Clypeus length 0.25. Diameter of AME 0.20, PME 0.17, PLE 0.13; AME-PME 0.11. Labium: length 0.44, width 0.50. Sternum: length 1.40, width 1.25. Leg lengths: I (total 14.80): femur 4.0/patella 0.9/tibia 4.6/metatarsus 4.1/tarsus 1.2; II (15.20): 4.4/0.7/4.6/4.4/1.1; III (12): 3.6/0.6/3.2/3.6/1; IV (14.60): 4/0.7/3.7/4.9/1.3. Leg formula: 2-1-4-3.



**Figs 6–9.** Live female paratype (CNAN-T01321) of *Loxosceles tolantongo* sp. nov. from the type locality: Tourist Center Grutas de Tolantongo Municipality of Cardonal, Hidalgo, Mexico. Photos 6–7 by Alejandro Valdez-Mondragón (2019); photos 8–9 by Claudia Isabel Navarro-Rodríguez (2019).



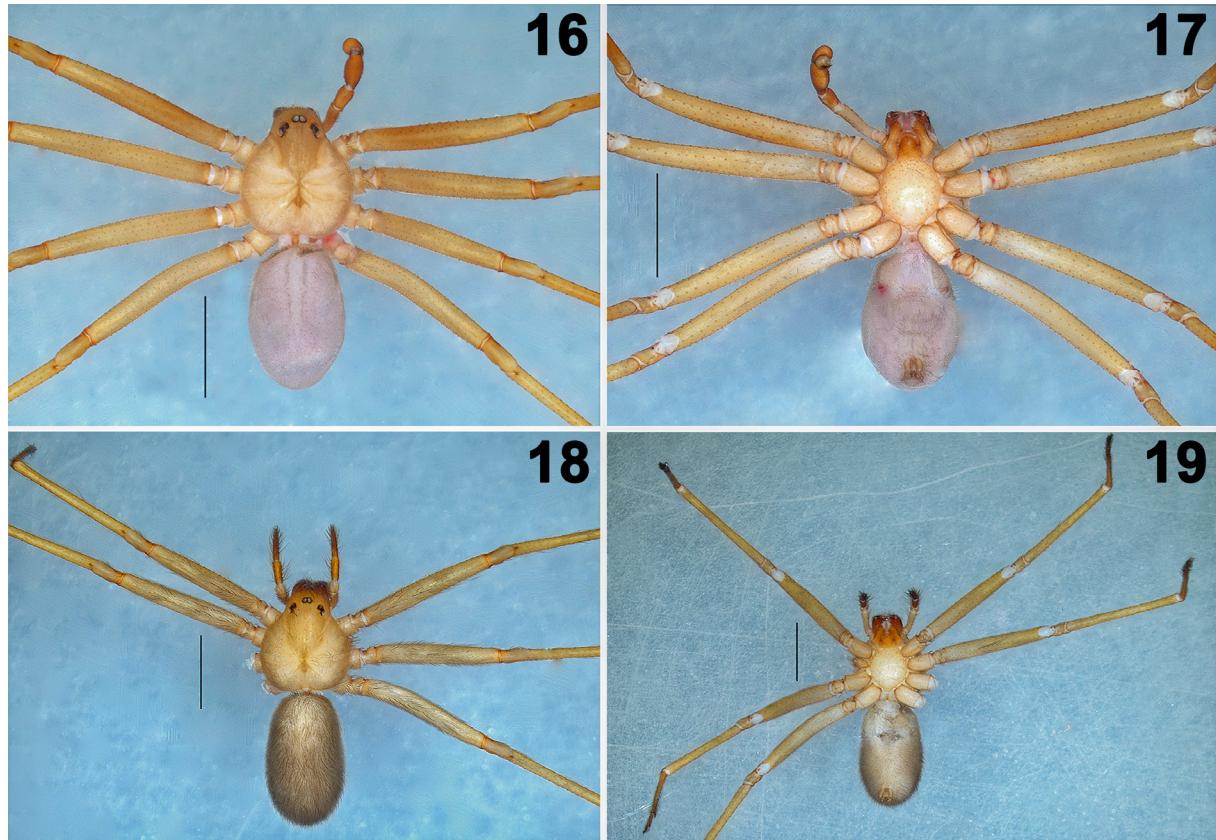
**Figs 10–15.** Habitat and microhabitat of *Loxosceles tolantongo* sp. nov. **10–11.** Xerophytic forest from the type locality: Tourist Center Grutas de Tolantongo, Municipality of Cardonal, Hidalgo, Mexico. **12–15.** Microhabitat situated 500 m west of entrance No. 5 to the Tourist Center Grutas de Tolantongo, Municipality of Cardonal, Hidalgo, Mexico (arrows indicate the microhabitat where the specimens can be found: under big rocks and inside of rotten and dry agave plants). Photos 10, 12–14 by Claudia Isabel Navarro-Rodríguez (2018); photos 11, 15 by Alejandro Valdez-Mondragón (2018).

**PROSOMA.** Carapace reddish, longer than wide, pyriform, dorsally with slightly dark brown ‘violin’ pattern (Figs 1–5, 16, 26), which is darker brown than rest of body (Figs 1–5, 16, 26). Carapace without lateral spots like other species. Six eyes in three groups, clypeus reddish brown (Figs 1–5, 16, 26). Sternum pale reddish, longer than wide (Fig. 17). Labium reddish brown, wider than long, fused to the sternum (Fig. 17). Endites pale brown basally, reddish brown distally and white apically. Endites longer than wide, rounded basally (Fig. 17).

**LEGS.** Coxae, trochanters, femora and patella reddish brown, paler on femora III and IV (Figs 1–5, 16–17), and the rest of the leg browner (Figs 1–5, 16).

**CHELICERAE.** Fused basally, reddish brown, stridulatory lines laterally. Fangs reddish brown, with thin setae around them (Fig. 17).

**OPISTHOSOMA.** Dark gray, paler posteriorly (Fig 1–5, 16), oval, longer than wide, and high (Figs 1–5, 16–17). Region of gonopore pale gray, with small setae. Colulus reddish brown, conical (Fig. 17). Spinnerets reddish brown, cylindrical; anterior lateral spinnerets longest, posterior median spinnerets smallest, with long setae. Tracheae opening near posterior margin of opisthosoma.



**Figs 16–19.** *Loxosceles tolantongo* sp. nov. **16–17.** Habitus of ♂ holotype (CNAN-T01317), dorsal and ventral views, respectively. **18–19.** Habitus of ♀ paratype (CNAN-T01321), dorsal and ventral views, respectively. Scale bars = 2 mm.

PALPS. Trochanters and femora pale reddish, paler ventrally in femora; patellae, tibiae reddish brown, longer than wide, wider in distal half than basal half (Figs 20, 22, 30–33). Tarsus oval, reddish brown, bulb spherical, embolus short, thick at base, narrowed to fine point (Figs 20–25).

**Female** (paratype CNAN-T01264)

Specimen collected manually, preserved and observed in 80% ethanol.

MEASUREMENTS. Total length 6.64. Carapace: 2.71 long, 2.56 wide. Clypeus length 0.34. Diameter of AME 2, PME 1.8, PLE 1.8; AME-PME 0.2. Labium: length 0.42, width 0.57. Sternum: length 1.56, width 1.36. Leg lengths: I (total 12.42): femur 3.6/patella 0.88/tibia 3.36/metatarsus 3.40/tarsus 1.18; II (16.65): 3.8/1.0/3.8/3.45/4.6; III (11.72): 3.60/0.96/3.04/3.06/1.06; IV (13.23): 3.44/0.96/3.52/4.0/1.31. Leg formula: 2-4-1-3.



**Figs 20–25.** *Loxosceles tolantongo* sp. nov., ♂ holotype (CNAN-T01317). 20–22. Left palp, prolateral, dorsal and retrolateral views, respectively. 23–25. Detail of the bulb and embolus, retrolateral, dorsal and apical views, respectively. Scale bars: 20–22 = 0.5 mm; 23–25 = 0.2 mm.

Differs from male as follows:

**PROSOMA.** Carapace brown and pale brown, with less defined dark brown ‘violin’ pattern (Figs 6–9, 18, 27). Larger carapace. Sternum paler brown (Fig. 19). Labium more reddish brown. Endites more reddish brown.

**LEGS.** Coxae, trochanters, femora, patella and tibiae brown (Figs 6–9, 18–19). Metatarsi and tarsi dark brown (Figs 6–9, 18–19).

**CHELICERAE.** Darker reddish brown.

**OPISTHOSOMA.** Brown and pale brown (Figs 6–9, 18–19). Spinnerets paler brown.

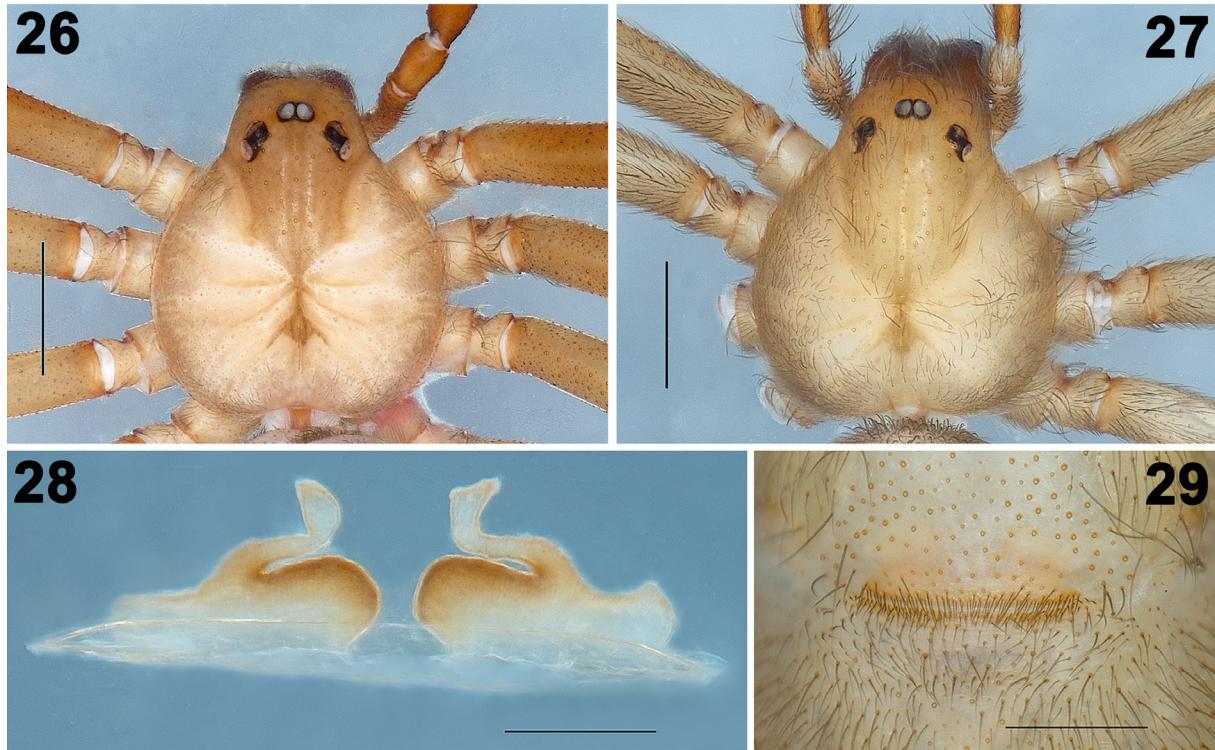
**PALPS.** Trochanters, femora and patellae pale brown, tibiae and tarsi reddish brown. Tibiae cylindrical, tarsi conical (Figs 18–19).

**GENITAL AREA.** Seminal receptacles asymmetric, S-shaped (Fig. 28). Base of seminal receptacles oval and wide, sclerotized (Fig. 28). See variation section for more details (Figs 36–39).

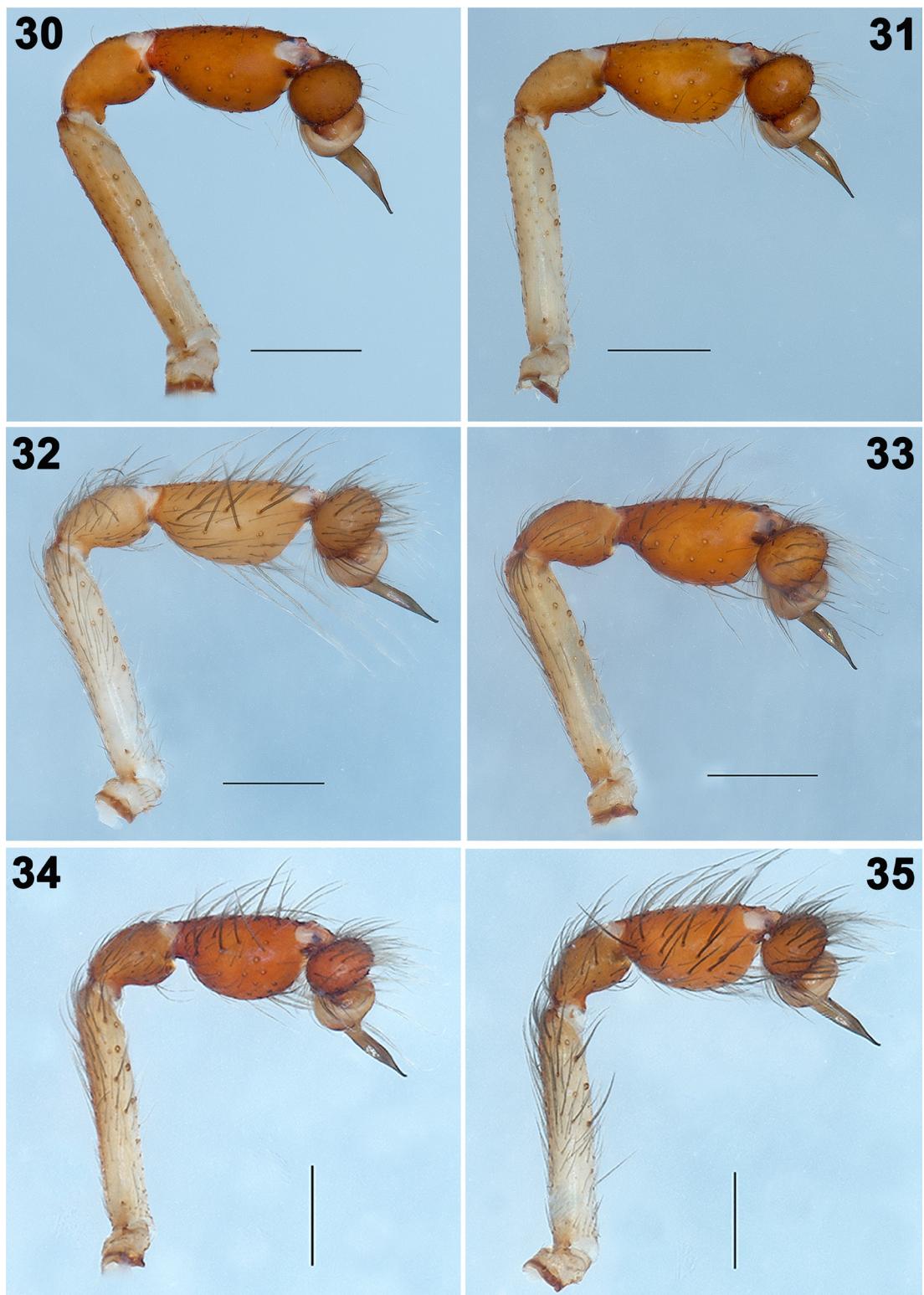
### Variation

#### Males

Males collected from the Tourist Center Grutas de Tolantongo and from 500 m west of entrance No. 5 are pale brown, with legs the same color as carapace; in some cases the legs are darker than the body. The male holotype is darker brown than all other specimens. The male collected from ‘El Paraíso’ trail is dark



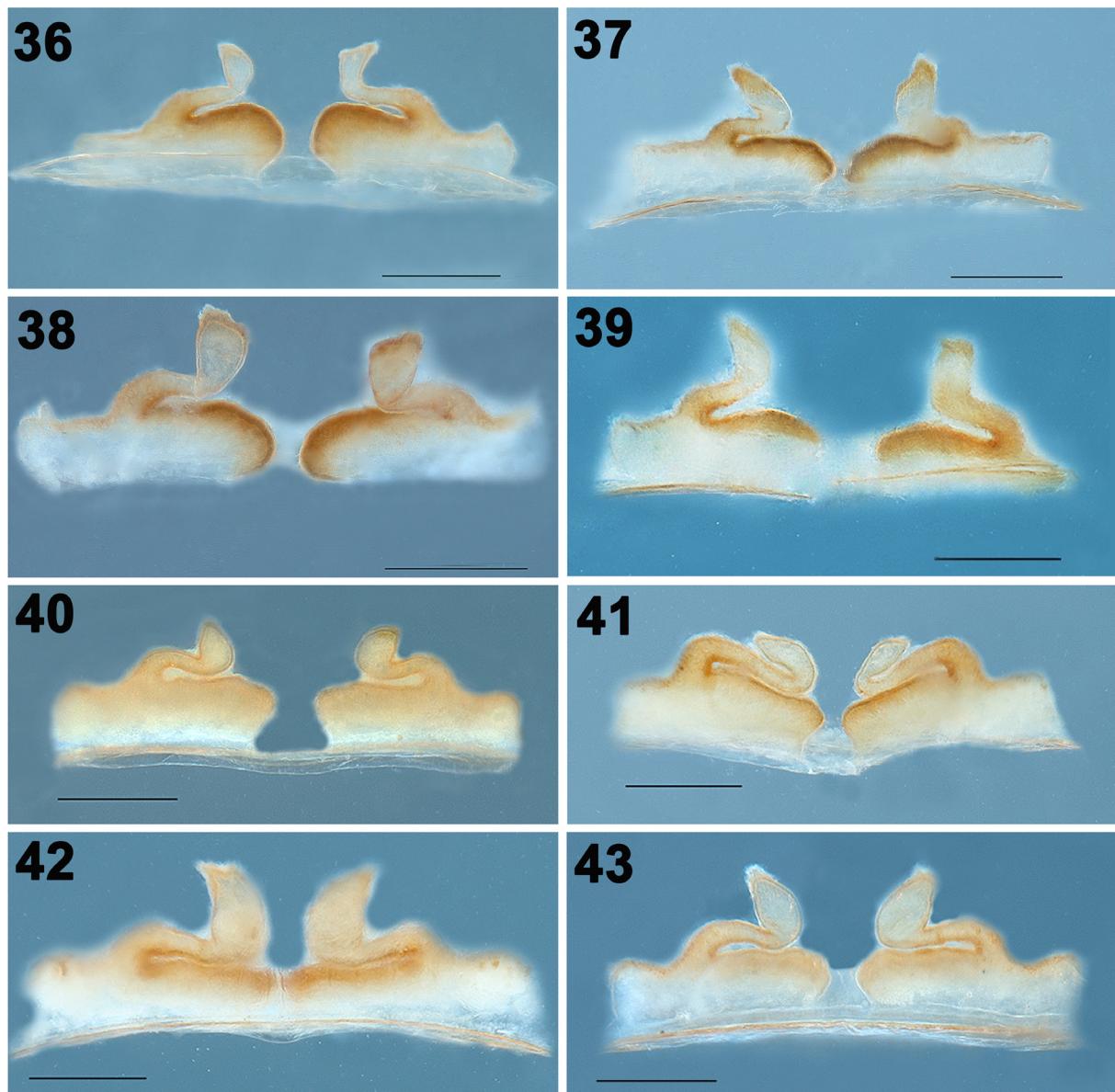
**Figs 26–29.** *Loxosceles tolantongo* sp. nov. 26–27. Caparace of ♂ holotype (CNAN-T01317) and ♀ paratype (CNAN-T01321), respectively. 28–29. ♀ paratype (CNAN-T01321). 28. Seminal receptacles. 29. Genital area, ventral view. Scale bars: 26–27 = 1 mm; 28 = 0.2 mm; 29 = 0.5 mm.



**Figs 30–35.** Variation of the male palps, left palps, prolateral views. **30–33.** *Loxosceles tolantongo* sp. nov. **30–32.** Tourist Center Grutas de Tolantongo, Municipality of Cardonal, Hidalgo (type locality). **33.** 500 m west of the entrance No. 5 to the Tourist Center Grutas de Tolantongo, Municipality of Cardonal, Hidalgo. **34–35.** *Loxosceles jaca* Gertsch & Ennik, 1983. 2.5 km north of Jacala de Ledezma, Municipality of Jacala de Ledezma, Hidalgo. Scale bars = 0.5 mm.

reddish brown, with legs the same color as body. Tourist Center Grutas de Tolantongo, Municipality of Cardonal ( $N = 5$ ): Tibia I 4.1–5.4 ( $x = 5$ ); carapace length (CL) 2.2–2.6 ( $x = 2.5$ ), carapace width (CW) 2.1–2.3 ( $x = 2$ ). 500 m west of entrance No. 5, Municipality of Cardonal ( $N = 2$ ): Tibia I 4.1–4.3 ( $x = 4$ ), CL 2.2–2.4 ( $x = 2$ ), CW 2.0–2.2 ( $x = 2$ ). ‘El Paraíso’ trail, Municipality of Cardonal ( $N = 1$ ): Tibia I 5.2, CL 2.7, CW 2.4.

The male palps present a little variation. In the case of the holotype the palp has more marked coloration and the embolus is wider at the base (Figs 20–25, 30–33).

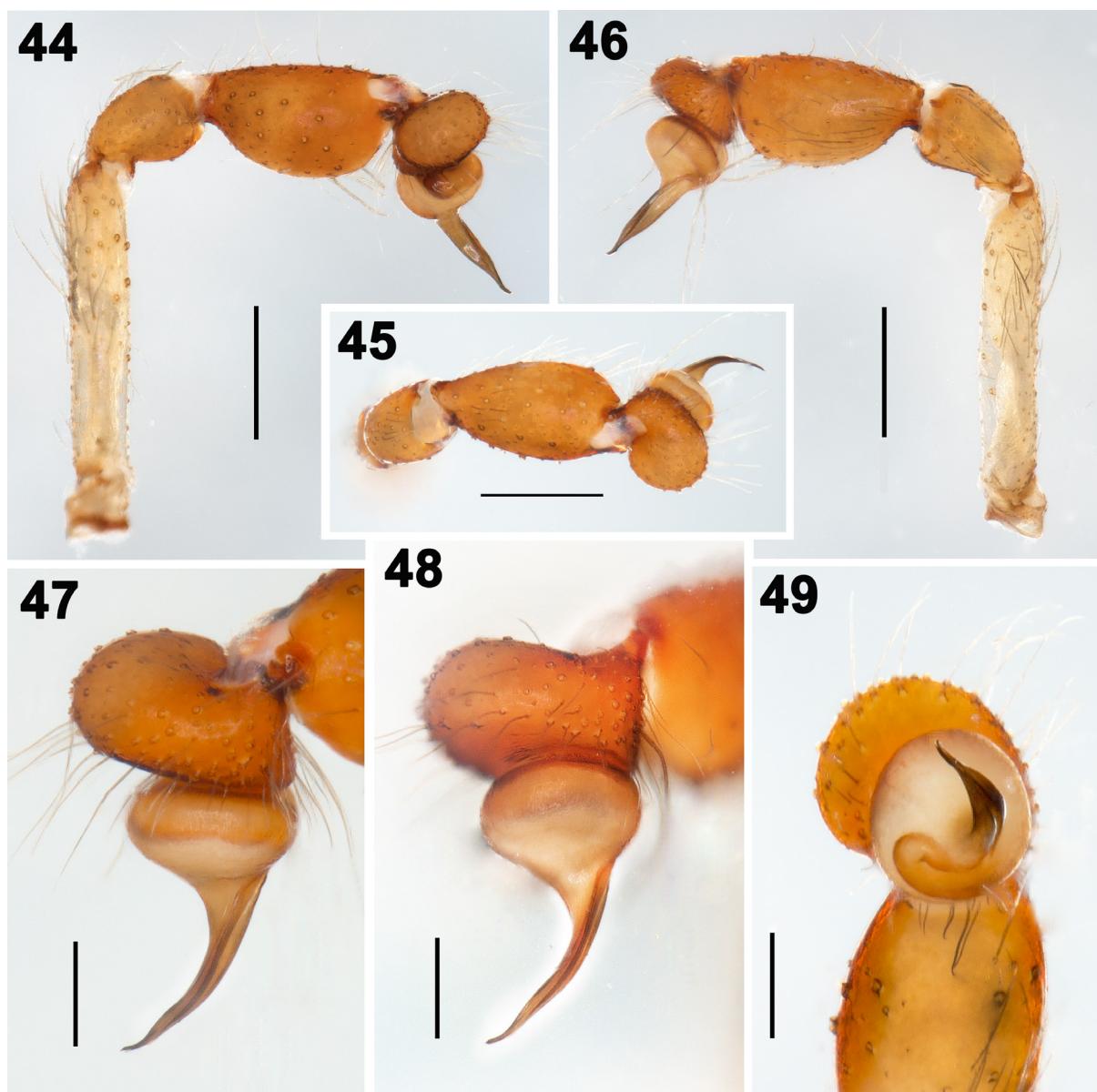


**Figs 36–43.** Variation of the seminal receptacles of females, dorsal view. **36–39.** *Loxosceles tolantongo* sp. nov. **36, 39.** Tourist Center Grutas de Tolantongo, Municipality of Cardonal. **37–38.** 500 m west of the entrance No. 5 to the Tourist Center Grutas de Tolantongo, Municipality of Cardonal. **40–43.** *Loxosceles jaca* Gertsch & Ennik, 1983. 2.5 km north of Jacala de Ledezma, Municipality of Jacala de Ledezma, Hidalgo. Scale bars = 0.2 mm.

### Females

The female collected from the Tourist Center Grutas de Tolantongo is pale brown on carapace and legs. Females from 500 m west of entrance No. 5 are dark brown on carapace and legs. Females from ‘El Paraiso’ trail are dark brown on carapace, with light brown legs. Tourist Center Grutas de Tolantongo, Municipality of Cardonal ( $N = 2$ ): Tibia I 3.3–3.3 ( $x = 3$ ), CL 2.2–2.7 ( $x = 3$ ), CW 2.0–2.5 ( $x = 2$ ). 500 m west of entrance No. 5, Municipality of Cardonal ( $N = 2$ ): Tibia I missing–3.2, CL 2.6–2.6 ( $x = 3$ ), CW 2.2–2.3 ( $x = 2$ ). ‘El Paraiso’ trail, Municipality of Cardonal ( $N = 6$ ): Tibia I 3.1–3.9 ( $x = 3$ ), CL 2.4–2.7 ( $x = 3$ ), CW 2.2–2.4 ( $x = 2$ ).

The seminal receptacles of females are S-shaped, asymmetrical and broadly variable in shape, even in specimens from the same locality (Figs 36–39). The apical lobes are rounded in some specimens, oval in



**Figs 44–49.** *Loxosceles jaca* Gertsch & Ennik, 1983. **44–46.** Left palp, prolateral, dorsal and retrolateral views respectively. **47–49.** Detail of the bulb and the embolus: retrolateral, dorsal and apical views, respectively. Scale bars: 44–46 = 0.5 mm; 47–49 = 0.2 mm.

others, or even with a sharp tip (Figs 28, 36–39). The base of the seminal receptacles is variable, in some specimens it is wider, more rounded and more sclerotized than in others (Figs 28, 36–39).

### Natural history

The specimens of *L. tolantongo* sp. nov. were collected in a xerophytic forest, the native vegetation type around the Touristic Center Grutas de Tolantongo (Figs 10–15). The microhabitat where the specimens were collected was under big rocks, and from inside rotten and dry agave plants (Figs 12–15). Some specimens were collected close to each other.

### Distribution

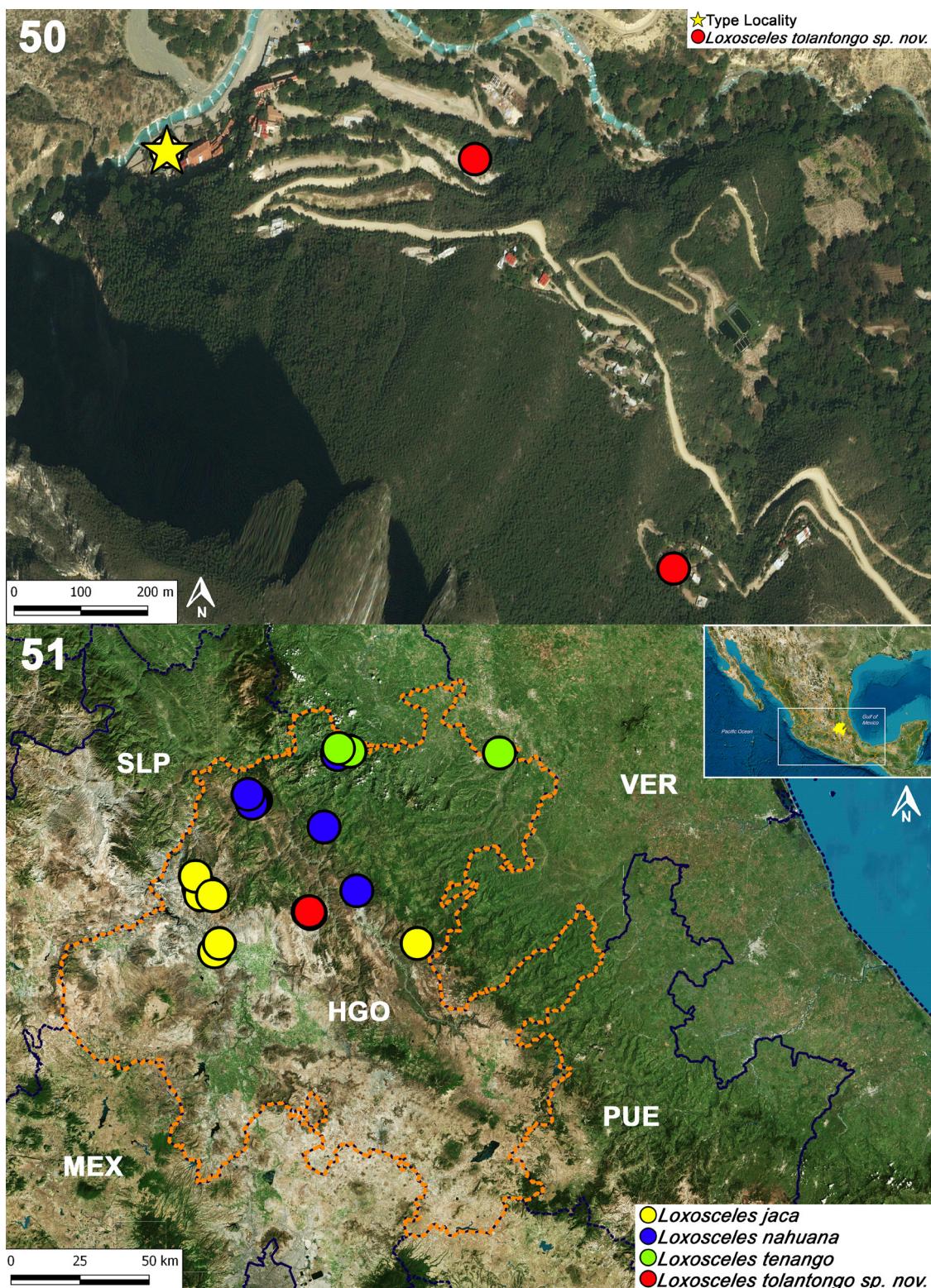
MEXICO: Hidalgo (Figs 50–51).

### Molecular analyses and species delimitation

The analyzed matrices include 49 individuals of ten species of *Loxosceles*, 38 individuals for the *COI* data set and 42 individuals for *ITS2* (Table 1, Figs 52–56). Specimens used in this study, GenBank accession numbers and localities of the specimens are listed in Table 1. The average genetic *p*-distance among analyzed species was 15.9% for *COI* and 8.4% for *ITS2* (Figs 52–53). Corrected *p*-distances from the *COI* and *ITS2* data recovered nine species of *Loxosceles*, both with high bootstrap support values (Figs 52–53). Based on the genetic analyses, *L. tolantongo* sp. nov. is closely related to *L. jaca*+*L. tenango* with *COI* (Fig. 52), and with *L. jaca* with *ITS2* (Fig. 53); with an average *p*-distance between *L. tenango* and *L. tolantongo* sp. nov. of 9.6%, and between *L. jaca* and *L. tolantongo* sp. nov. of 10.5% for *COI* and 1.8% for *ITS2* (Tables 3–4). Molecular analyses with *COI* and the concatenated matrix (*COI*+*ITS2*) (Figs 54, 56) indicate that four different species delimitation methods, including the morphology, were congruent to delimit *L. tolantongo* sp. nov. as a valid and different species, and recovered a total of nine species. However, using the ABGD species delimitation method under recursive partitions (RP), 14, 11 and 10 species were recovered (Fig. 54). The molecular analyses are consistent with the morphology; the nine species of *Loxosceles* used in this study were previously described only with morphological characters, except for *L. tenochtitlan* which was described with different lines of evidence (morphological and molecular) by Valdez-Mondragón *et al.* (2019). Molecular analyses with *ITS2* indicate that of the four different molecular species delimitation methods, only NJ and GMYC (Yule) were consistent with the morphology for delimitation of *L. tolantongo* sp. nov. (Figs 53, 55). ABGD and bPTP species delimitation methods do not recover *L. tolantongo* as a different species, but they recover other species, such as *L. zapoteca* Gertsch, 1958, *L. colima* Gertsch, 1958 (except with initial partitions (IP) (ABGD)) and *L. malintzi* (Fig. 55). The haplotype network analysis with *COI* data is concordant with the results of the different species delimitation analyses (Fig. 57). There were >10 mutations between haplotypes under *COI* for all the species (Fig. 57). Regarding *L. tolantongo* sp. nov. and *L. jaca*+*L. tenango*, the haplotype network was concordant with the species delimitation, and showed a total of 39 and 36 mutations respectively between haplotypes under *COI* (Fig. 57).

### Discussion

There are currently two important tasks to which DNA barcodes markers are being applied in modern systematics. The first is distinguishing between species (equivalent to species identification or species diagnosis) and the second is the use of DNA data to discover new species (equivalent to species delimitation and species description) (DeSalle *et al.* 2005). For some groups of organisms, such as spiders, traditional morphology in some cases fails to determine species boundaries, and identifying morphologically inseparable cryptic or sibling species requires a new set of taxonomic tools, including the analysis of molecular data (Jarman & Elliott 2000; Witt & Hebert 2000; Proudlove & Wood 2003; Hebert *et al.* 2003, 2004; Bickford *et al.* 2007; Hamilton *et al.* 2011, 2014, 2016; Ortiz & Francke 2016; Valdez-Mondragón *et al.* 2019).



**Figs. 50–51.** Distribution records of *Loxosceles tolantongo* sp. nov. from Hidalgo. **50.** Known records of *Loxosceles tolantongo* sp. nov. from the Tourist Center Grutas de Tolantongo, Municipality of Cardonal, Hidalgo, including the type locality. **51.** Distribution records of the four species of *Loxosceles* from Hidalgo, Mexico. Abbreviations: HGO = Hidalgo; MEX = Estado de México; PUE = Puebla; SLP = San Luis Potosí; VER = Veracruz.

**Table 3.** Genetic *p*-distance matrix from the *COI* data among *Loxosceles tolantongo* sp. nov., *L. tenango* Gertsch, 1973 and *L. jaca* Gertsch & Ennik, 1983. Average *p*-distance = 6.7%.

Species	1	2	3	4	5	6	7	8	9
1. Ara0191 – <i>L. tenango</i>									
2. Ara0192 – <i>L. tenango</i>	0.000								
3. Ara0186 – <i>L. jaca</i>	0.071	0.068							
4. Ara0048 – <i>L. jaca</i>	0.073	0.070	0.002						
5. Ara0046 – <i>L. jaca</i>	0.070	0.070	0.002	0.004					
6. Ara0047 – <i>L. jaca</i>	0.067	0.068	0.002	0.004	0.005				
7. Ara0183 – <i>L. jaca</i>	0.085	0.085	0.017	0.018	0.018	0.020			
8. Ara0175 – <i>L. tolantongo</i> sp. nov.	0.095	0.094	0.102	0.105	0.104	0.102	0.118		
9. Ara0181 – <i>L. tolantongo</i> sp. nov.	0.096	0.098	0.098	0.101	0.104	0.100	0.123	0.008	
10. Ara0182 – <i>L. tolantongo</i> sp. nov.	0.094	0.097	0.097	0.100	0.103	0.099	0.123	0.010	0.005

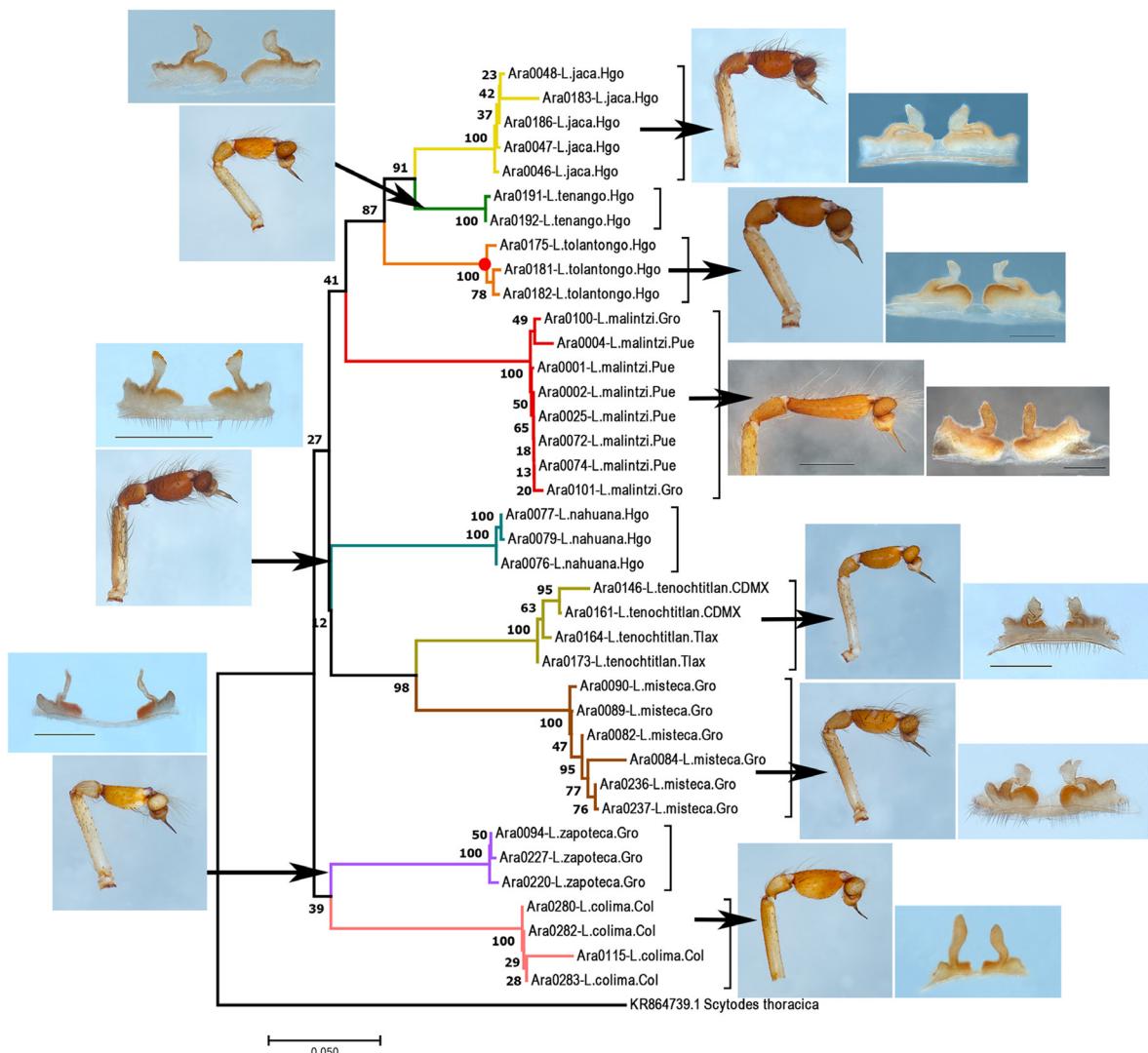
**Table 4.** Genetic *p*-distance matrix from the *ITS2* data between *Loxosceles tolantongo* sp. nov. and *L. jaca* Gertsch & Ennik, 1983. Average *p*-distance = 1.8%.

Species	1	2	3	4	5	6	7	8	9	10
1. Ara0046 – <i>L. jaca</i>										
2. Ara0047 – <i>L. jaca</i>	0.003									
3. Ara0186 – <i>L. jaca</i>	0.021	0.018								
4. Ara0183 – <i>L. jaca</i>	0.006	0.003	0.015							
5. Ara0174 – <i>L. tolantongo</i> sp. nov.	0.016	0.013	0.020	0.015						
6. Ara0176 – <i>L. tolantongo</i> sp. nov.	0.015	0.012	0.019	0.015	0.006					
7. Ara0177 – <i>L. tolantongo</i> sp. nov.	0.019	0.015	0.023	0.018	0.009	0.009				
8. Ara0178 – <i>L. tolantongo</i> sp. nov.	0.021	0.018	0.025	0.020	0.012	0.006	0.015			
9. Ara0181 – <i>L. tolantongo</i> sp. nov.	0.012	0.009	0.015	0.012	0.003	0.003	0.006	0.009		
10. Ara0182 – <i>L. tolantongo</i> sp. nov.	0.017	0.015	0.021	0.017	0.009	0.009	0.006	0.014	0.006	
11. Ara0175 – <i>L. tolantongo</i> sp. nov.	0.025	0.021	0.029	0.024	0.015	0.015	0.018	0.021	0.012	0.015

In recent studies, molecular evidence has suggested that the known diversity within the genus *Loxosceles* could be highly underestimated (Binford *et al.* 2008; Duncan *et al.* 2010; Planas & Ribera 2014, 2015; Tahami *et al.* 2017; Valdez-Mondragón *et al.* 2019). One important factor leading to the underestimation is widespread intraspecific variation in sexual structures, mainly in the seminal receptacles of the females (Figs 36–43), as was noted in this work and previously by Brignoli (1968), Gertsch & Ennik (1983), and recently by Valdez-Mondragón *et al.* (2018b, 2019) in the case of the species from Mexico. However, sexual structures such as the male palps remain a good character for species identification because of their little morphological variation in comparison with the seminal receptacles of the females (Valdez-Mondragón *et al.* 2018a, 2018b, 2019). It is important to consider that male palps may be remarkably similar, as indicated for *L. tolantongo* sp. nov and *L. jaca* (which showed slight differences), which can also make species identifications difficult, thus contributing to such underestimations. Although *L. tolantongo* sp. nov. morphologically resembles *L. jaca*, the new species could be diagnosed based on the sexual characters of the male palps (Figs 20–25) and the seminal receptacles of the females (Figs 28, 36–39). Furthermore, molecular markers provided additional evidence to delimit the new species within an integrative taxonomic context. Identifying morphologically inseparable cryptic or sibling species

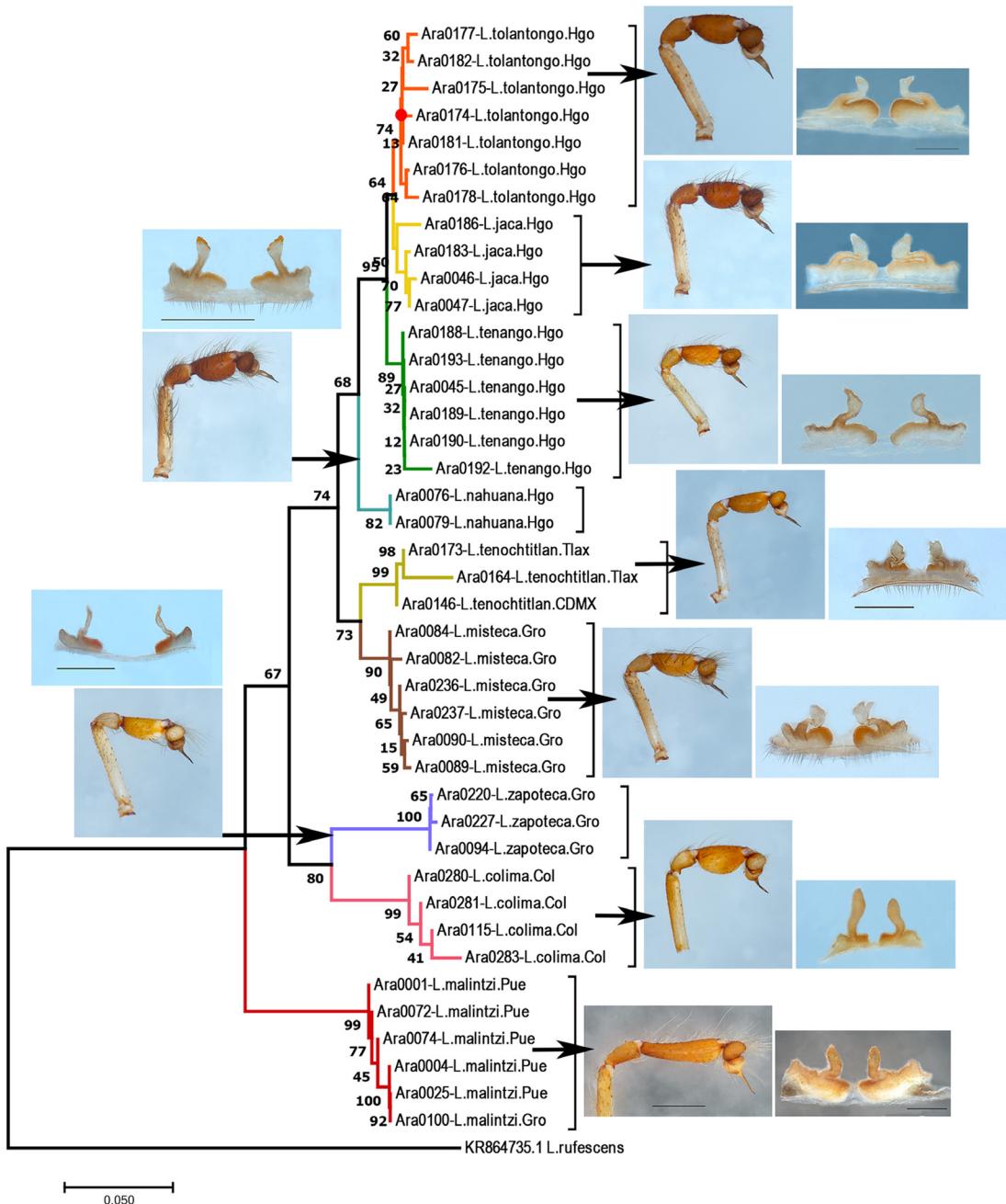
requires a new set of taxonomic tools, including DNA and additional sources of evidence (integrative taxonomy) (Jarman & Elliott 2000; Witt & Hebert 2000; DeSalle *et al.* 2005; Hebert *et al.* 2003, 2004; Bickford *et al.* 2007; Hamilton *et al.* 2011, 2014, 2016; Ortiz & Francke 2016, Valdez-Mondragón *et al.* 2018a, 2018b, 2019).

As was mentioned by Hamilton *et al.* (2011), we can rarely delimit species based only on molecular data, and that is why, in addition to the molecular evidence, the geographical data provides information to delimit these species. *Loxosceles jaca* and *L. tolantongo* sp. nov. were found in different localities, Cardonal and Jacala, Hidalgo, respectively. The type locality of the new species is located in the Tolantongo Canyon, 500 m deep, where the Tolantongo river is located, which can serve as a geographic barrier for *Loxosceles* spiders that usually have limited dispersions (Sandidge & Hopwood 2005; Binford *et al.* 2008; Vetter 2008, 2015; Foelix 2011). Also, both species were found in different vegetation types: *L. tolantongo* sp. nov. was found in a xerophytic forest between 1315 and 1481 m a.s.l. , under



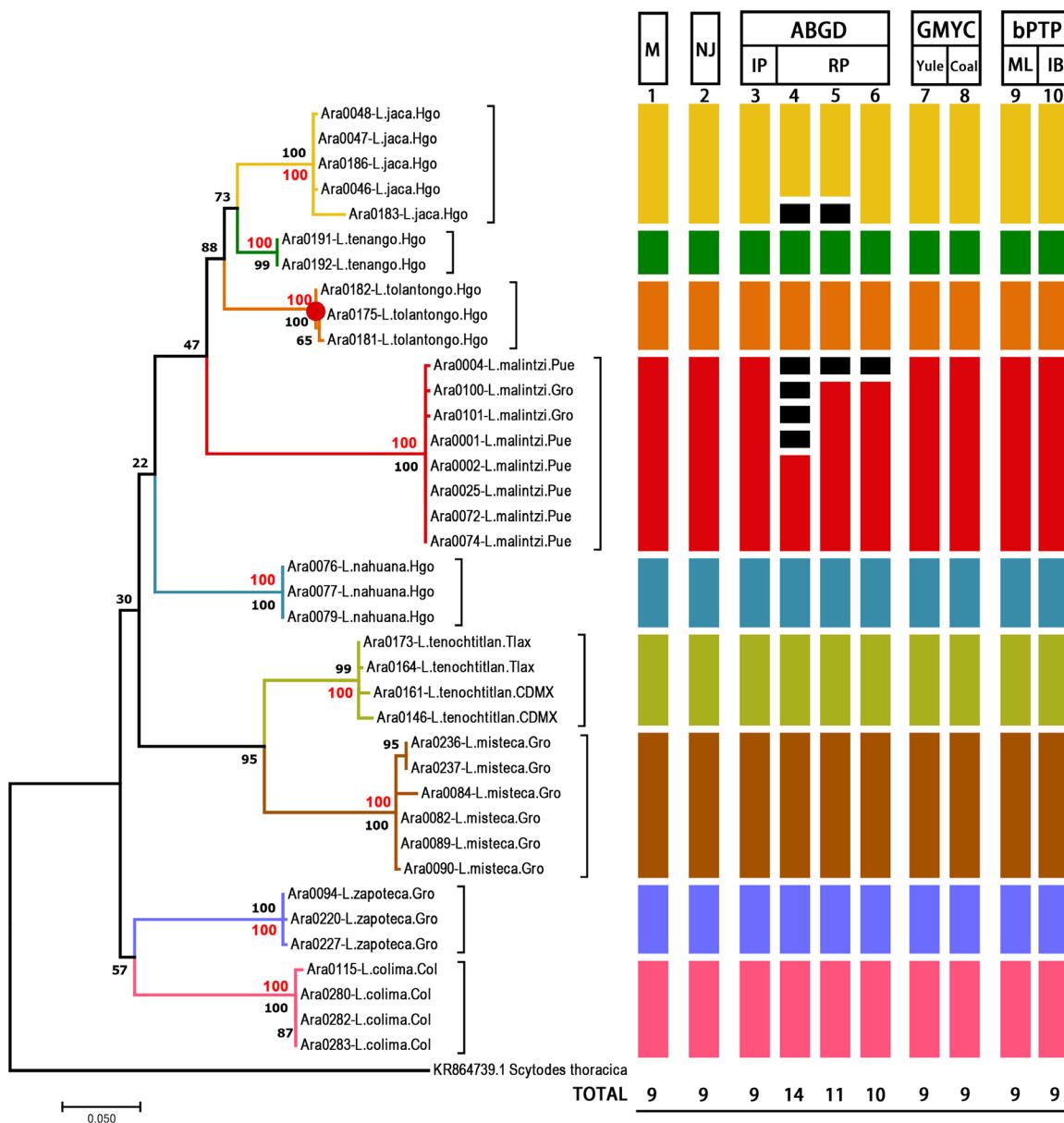
**Fig. 52.** Neighbor joining tree constructed from *COI* data of nine species of *Loxosceles* Heineken & Lowe, 1832 from Mexico. Colors of branches indicate different species. Numbers on nodes are bootstrap support values. Red circle at node represents *Loxosceles tolantongo* sp. nov.

rocks and dry agave plants, whereas *L. jaca* was found in shrub vegetation at 1290 m a.s.l. , mainly within dry agave leaves. The vegetation type seems to play an important role in the ecological niche and delimitation of distribution for some species of *Loxosceles* from Mexico, as was demonstrated by Valdez-Mondragón *et al.* (2019) with the ecological niche modeling of *L. tenochtitlan*, a widespread species from the central region of Mexico.



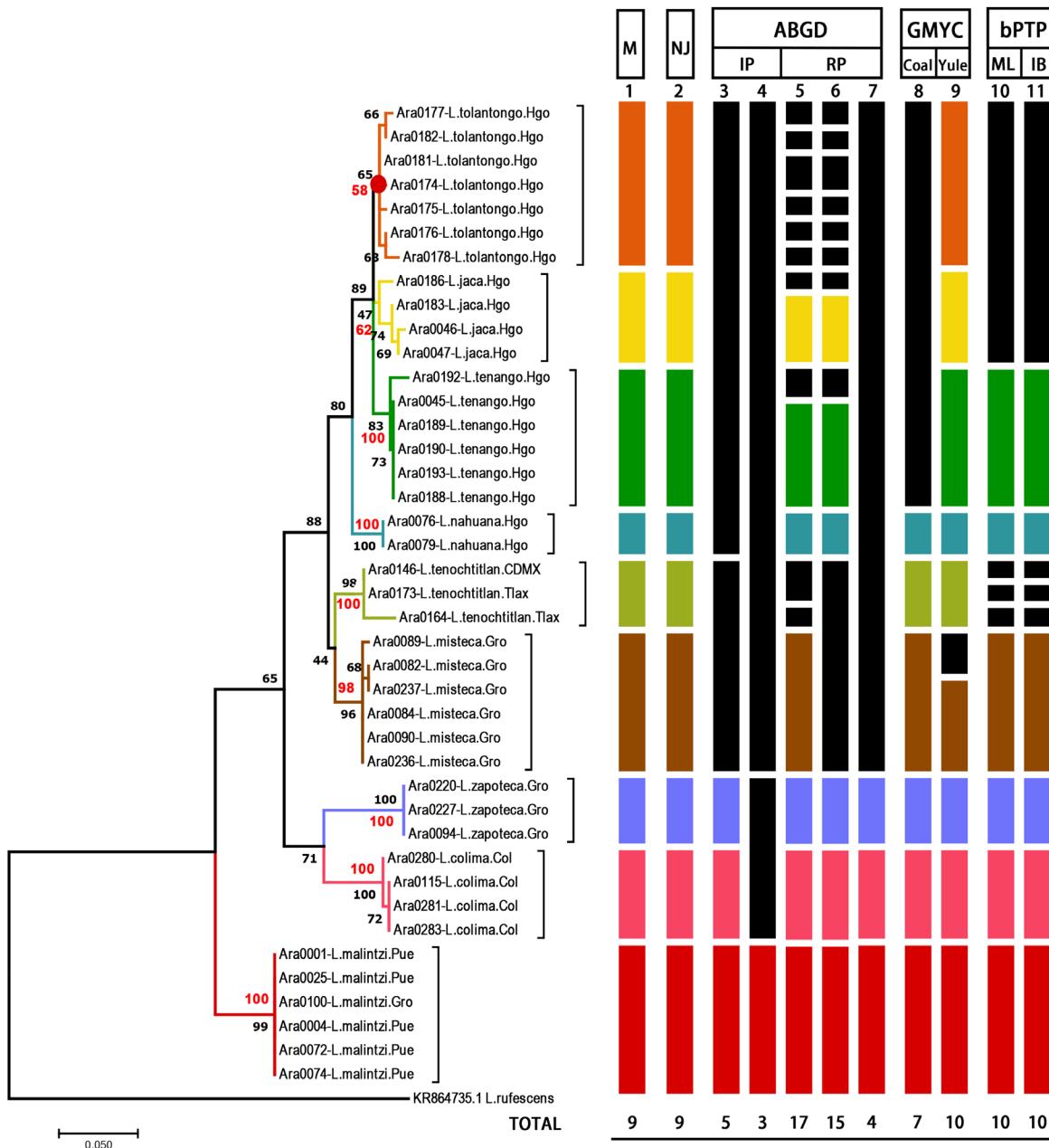
**Fig. 53.** Neighbor joining tree of ITS2 data of nine species of *Loxosceles* Heineken & Lowe, 1832 from Mexico. Colors of branches indicate different species. Numbers at nodes represent bootstrap support values. Red circle at node represent *Loxosceles tolantongo* sp. nov.

Planas & Ribera (2014, 2015) found genetic distances between species of *Loxosceles* from the Canary Islands to be >12% using *COI*, whereas Tahami *et al.* (2017) found genetic distances between species from the Middle East ranging from 17.5 to 20.6% for *COI*. In this work, the genetic distances were over 15% between the different species. They also reported average low genetical distances inside the same species (0.26% and less than 1%) and in this work the intraspecific genetic distances are low



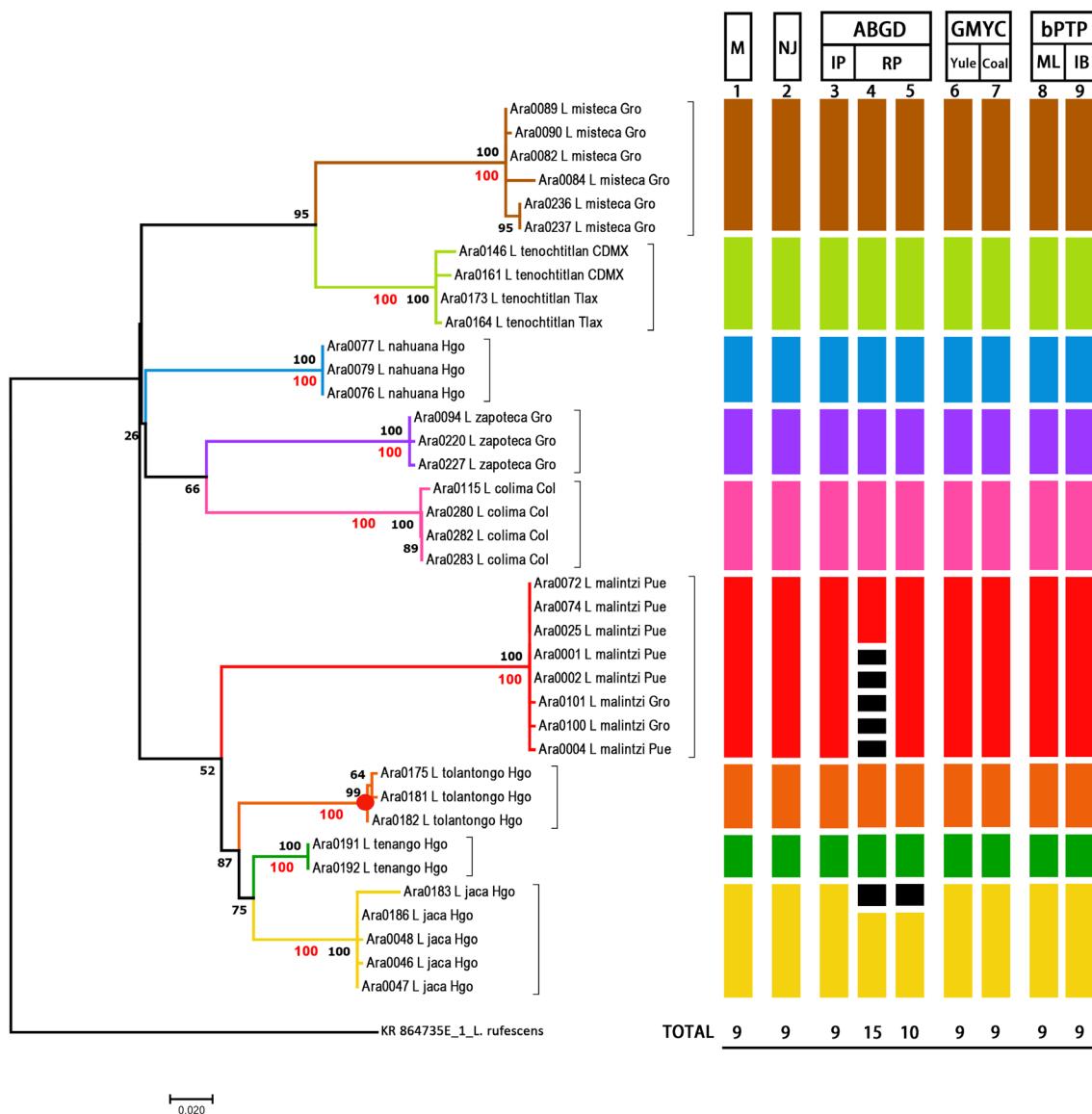
**Fig. 54.** Maximum likelihood tree inferred from *COI* gene of species of *Loxosceles* Heineken & Lowe, 1832 from Mexico. Colors of branches and bars indicate different species. Numbers above bars represent the delimitation methods: 1 = morphology (M); 2 = neighbor joining (NJ); 3 = ABGD with initial partitions (IP); 4–6 = ABGD with recursive partitions (RP); 7 = GMYC yule analysis; 8 = GMYC coalescent analysis; 9 = bPTP with ML; 10 = bPTP with IB. Numbers below bars represent species recovered for each delimitation method. Red numbers on branches correspond to Bayesian posterior probabilities, black numbers are bootstrap support values from the ML analysis.

(<1%) (Tables 3–4; Fig. 52). The corrected genetic *p*-distances showed that *COI* performed better for species delimitation than *ITS2* (Figs. 52–55). Agnarsson (2010) concluded that *ITS2* was insufficient to solve the relationships between closely related species, but also indicated that *ITS2* is an easily amplified and sequenced marker for use in spider phylogeny despite its limits at lower taxonomic levels.



**Fig. 55.** Maximum likelihood tree inferred from *ITS2* gene of species of *Loxosceles* Heineken & Lowe, 1832 from Mexico. Colors of branches and bars indicate different species. Numbers above bars represent the delimitation methods: 1 = morphology (M); 2 = neighbor joining (NJ); 3–4 = ABGD with initial partitions (IP); 5–7 = ABGD with recursive partitions (RP); 8 = GMYC yule analysis; 9 = GMYC coalescent analysis; 10 = bPTP with ML; 11 = bPTP with IB. Numbers below bars represent species recovered for each delimitation method. Red numbers on branches correspond to Bayesian posterior probabilities, black numbers are bootstrap support values from the ML analysis.

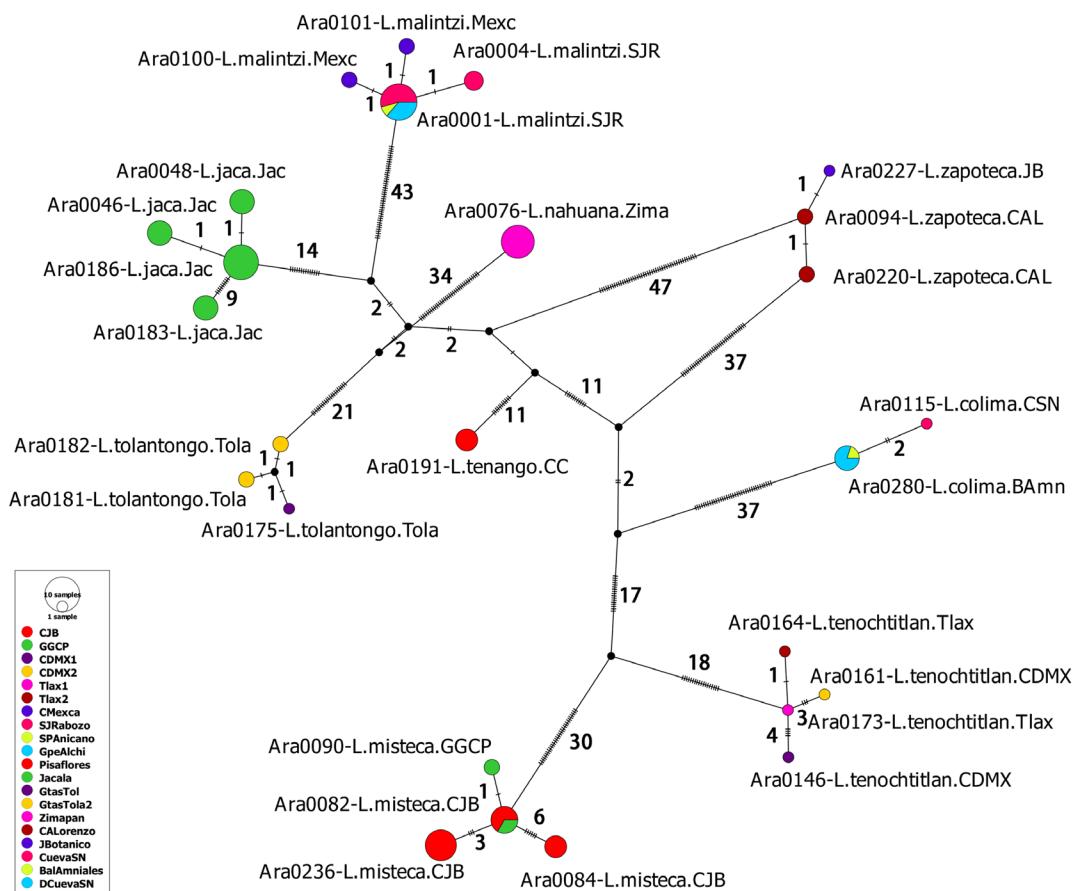
The uses of the ABGD species delimitation method are recursive partitioning, with a range of prior intraspecific divergence and relative gap width to estimate the threshold between intra- and interspecific variation which intends to produce species-level groupings (Ortiz & Francke 2016). However, this species delimitation method is sensitive to sampling effect, as was demonstrated in the spider genera *Aphonopelma* Pocock, 1901 and *Bonnetina* Vol, 2000 from North America by Hamilton *et al.* (2014) and Ortiz & Francke (2016), respectively, tending to give moderately over-splitting solutions as was the case of our work (Fig. 55). PTP is similar to GMYC, but uses substitution calibrated (not ultrametric)



**Fig. 56.** Maximum likelihood tree inferred from the concatenated matrix (*COI* + *ITS2*) of species of *Loxosceles* Heineken & Lowe, 1832 from Mexico. Colors of branches and bars indicate different species. Numbers above bars represent the delimitation methods: 1 = morphology (M); 2 = neighbor joining (NJ); 3 = ABGD with initial partitions (IP); 4–5 = ABGD with recursive partitions (RP); 6 = GMYC yule analysis; 7 = GMYC coalescent analysis; 8 = bPTP with ML; 9 = bPTP with IB. Numbers below bars represent species recovered for each delimitation method. Red numbers correspond to Bayesian posterior probabilities, black numbers are bootstrap support values from the ML analysis.

trees to avoid the potential flaws in constructing time-calibrated phylogenies, as was proposed by Zhang *et al.* (2013).

The work of Cao *et al.* (2016) with the spider genus *Pseudopoda* Jäger, 2000 (Sparassidae Bertkau, 1872) suggested using concatenated fragments *COI*+*ITS2*, which identifies and diagnoses species in a more appropriate way than the separated mitochondrial barcodes. In this work, the concatenated matrix showed that all species delimitation methods were congruent with the morphology, recovering *L. tolantongo* sp. nov. as a separated species (Figs 54–56). Carstens *et al.* (2013) suggested that researchers should apply a wide range of species delimitation analyses to their data and place their trust in delimitations that are congruent across methods. Using several species delimitation methods, incongruence across the different results is evidence of a difference in the power to detect cryptic lineages across one or more of the approaches used to delimit species and could indicate that assumptions of one or more of the methods have been violated. In these cases the assumptions for species delimitations should be conservative (Carstens *et al.* 2013). In this work, the different molecular species delimitation methods and their variants were congruent and consistent to separate *L. tolantongo* sp. nov. of *L. jaca*+*L. tenango*. *Loxosceles tolantongo* sp. nov. is considered a unique species for four reasons: 1) it can be diagnosed and distinguished by morphological characters (male palps mainly); 2) the high genetic *p*-distances with *COI* (>10%); 3) the four different molecular species delimitation methods were congruent under



**Fig. 57.** Haplotype network from the *COI* data obtained with TCS using PopArt. Each circle represents the haplotypes found in nine species of *Loxosceles* Heineken & Lowe, 1832 from Mexico. Numbers on branches indicate the number of mutations between haplotypes.

*COI* and *COI+ITS2* (concatenated matrix); 4) under *COI* and *COI+ITS2*, *L. tolantongo* sp. nov. is a putative sister group of *L. jaca*+*L. tenango*.

Although the new species described herein has a small range of occurrence, it is considered as not threatened. The Tourist Center Grutas de Tolantongo, which belongs to the Sociedad Cooperativa Ejidal Grutas Tolantongo (Cooperative Ejido Society of the Tolantongo Grottos), has managed to preserve much of the resort and the ejido land around it in its natural state, preserving many native species distributed in the region.

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