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Taxonomy and systematics

Ixchela azteca (Araneae: Pholcidae), a widespread spider species from Central Mexico: Underestimated diversity or morphological and genetic variation?

Ixchela azteca *(Araneae: Pholcidae), una especie de araña ampliamente distribuida en el centro de México: ¿diversidad subestimada o variación morfológica y genética?*

Alejandro Valdez-Mondragón *, Samuel Nolasco-Garduño

Centro de Investigaciones Biológicas del Noroeste, S.C., Programa Académico de Planeación Ambiental y Conservación, Laboratorio de Aracnología, Km. 1 Carretera a San Juan de La Costa "El Comitán", 23205 La Paz, Baja California Sur, Mexico

*Corresponding author: lat_mactans@yahoo.com.mx (A. Valdez-Mondragón)

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Abstract

We describe the morphological and genetic variation in *Ixchela azteca* Valdez-Mondragón & Francke (Pholcidae) from central Mexico, based on morphological and DNA barcoding evidence. Species delimitation employing the barcoding locus included 3 methods under the corrected genetic *p*-distances 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 genetic analyses found a genetic *p*-distance of 3.5% between 2 populations of *I. azteca*. The molecular methods and morphology were not congruent in delimiting and recognizing 2 possible different species. Instead, 2 allopatric populations of *I. azteca* are recognized. Also, an updated taxonomic revision of *I. azteca* is included. In conclusion, the incongruence between the molecular delimitation methods for species delimitation and the morphological, genetic, ecological, and biogeographic evidence maintain *I. azteca* as a widespread species across central Mexico, with allopatric populations in temperate and semiarid regions.

Keywords: Species delimitation; Integrative taxonomy; CO1; Ecology; Biogeographic provinces

Resumen

Describimos la variación morfológica y genética en *Ixchela azteca* Valdez-Mondragón y Francke (Pholcidae) del centro de México, basado en evidencia morfológica y código de barras de DNA. La delimitación de especies con el locus de código de barras incluyó 3 métodos bajo el criterio de distancias genéticas *p* corregidas "neighbor-joining" (NJ): *1)* "Assemble Species by Automatic Partitioning" (ASAP), *2)* "General Mixed Yule Coalescent model"

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(GMYC), and *3)* "Bayesian Poisson Tree Processes" (bPTP). Los análisis genéticos entre 2 poblaciones de *I. azteca* encontraron una distancia genética *p* de 3.5%. Los métodos moleculares y morfología no fueron congruentes para delimitar y reconocer 2 posibles especies, pero sí se reconocen 2 poblaciones alopátricas de *I. azteca*. Adicionalmente, se incluye una revisión taxonómica actualizada de *I. azteca*. En conclusión, la incongruencia de los métodos de delimitación molecular para la delimitación de especies en combinación con la evidencia morfológica, genética, ecológica y biogeográfica, corrobora a *I. azteca* como una especie ampliamente extendida en la región central de México, con poblaciones alopátricas en zonas templadas, pero también en regiones semiáridas.

Palabras clave: Delimitación de especies; Taxonomía integrativa; CO1; Ecología; Provincias biogeográficas

Introduction

The spider family Pholcidae is the ninth-largest spider family in the world and the most diverse within the Synspermiata clade (WSC, 2025). Commonly known as cellar spiders or daddy long-legs spiders, Pholcidae is currently composed of 97 genera and 1,901 species (WSC, 2025). This family is among the most diverse and abundant web-building spiders in the world, mainly occurring in tropical and subtropical forests and with a high number of synanthropic and troglobite species (Huber, 2000, 2011, 2018; Valdez-Mondragón, 2010).

The genus *Ixchela* Huber, 2000 (subfamily Modisiminae) currently contains 22 described species (Valdez-Mondragón, 2013, 2020; Valdez-Mondragón & Francke, 2015; WSC, 2025). The genus is composed of relatively large pholcid spiders mainly distributed in Mexico and Central America. These spiders inhabit temperate climate zones in pine, oak, or pine-oak forests at moderately high elevations (1,000-2,950 m) (Valdez-Mondragón, 2013, 2020; Valdez-Mondragón & Francke, 2015). The monophyly of the genus is supported based on morphological and molecular evidence (CO1+16S), with the genus' divergence time estimated to have occurred in the late Miocene (Valdez-Mondragón & Francke, 2015).

Although pholcid spiders generally have a relatively uniform and conservative somatic morphology, their genitalia (palps and epigyna) and secondary sexual characters (male chelicerae) are highly informative for morphological identification at species level. These morphological features are a source of important and robust evidence and are commonly used for species delimitation in most groups of spiders (Huber, 2003). Spider genitalia usually show little intraspecific, but conspicuous interspecific variation (Eberhard, 1985; Eberhard et al., 1998). However, in certain groups with highly conservative morphology, or species with a wide geographic distribution, species delimitation and identification require additional diagnostic features to differentiate underestimated species diversity. In contrast, other species of spiders have wide geographic variation and even show intrasexual dimorphism within males or females. In the last 4 decades, research has focused on understanding the variation among individuals, leading sexual variation to be understood as the differences exhibited not only between males and females but also among individuals of the same sex within a species (Andersson, 1994; Barraclough, 1995; Gross, 1996; Huber, 2021).

The mitochondrial cytochrome c oxidase subunit 1 (CO1) DNA barcode gene has emerged as the standard animal barcoding marker for many taxa, including spiders (Astrin et al., 2006; Correa-Ramírez et al., 2010; Graham et al., 2015; Hamilton et al., 2011, 2014, 2016; Hebert, Cywinska et al., 2003; Hebert, Ratnasingham et al., 2003; Hebert et al., 2004; Ortiz & Francke, 2016; Planas & Ribera, 2015; Nolasco & Valdez-Mondragón, 2022a; Tahami et al., 2017). The integrative taxonomy approach is generally accepted as being more effective in the diagnosis, delimitation, and description of species or even to understand the variation in different populations within species.

Ixchela azteca Valdez-Mondragón & Francke, 2015 from central Mexico and *Ixchela abernathyi* (Gertsch, 1971) from northeast Mexico represent the species with the widest distributions in comparison with other species of the genus, mainly in temperate regions. However, Valdez-Mondragón and Francke (2015) reported body size variation for populations of *I. azteca* from temperate regions, bringing into question if this is merely geographic variation in the same species or is evidence of the presence of different species not yet described. Because of that, morphological and molecular evidence are needed to test if *I. azteca* has a widespread distribution in temperate and semiarid habitats or represents different species.

In this study, we described based on morphological evidence and DNA barcoding (CO1) the variation of different populations of *I. azteca* including 3 populations from temperate regions of central Mexico and a population from a semiarid habitat in Guanajuato state, Mexico. In addition, an updated taxonomic revision of *I. azteca* is included herein, including new records for the species.

Materials and methods

The specimens used in this study are deposited in the Colección Nacional de Arácnidos (CNAN), Instituto de Biología, Universidad Nacional Autónoma de México (IBUNAM), Mexico City; and the Colección Aracnológica (CARCIB), Centro de Investigaciones Biológicas del Noroeste, CIB, La Paz, Baja California Sur, Mexico. The specimens from Guanajuato, Mexico are deposited at the CARCIB. Morphological structures, such as female epigyna and male palps, were dissected in ethanol (80%) and cleared in potassium hydroxide (KOH-10%). Habitus, chelicerae, palps, and epigyna were placed on 96% gel alcohol to facilitate positioning and covered with a thin layer of liquid ethanol (80%) to minimize diffraction during photography. The analyzed morphological structures were female epigyna and male palps, dorsal and ventral habitus, and male chelicerae.

A Zeiss Axiocam 506 color camera attached to a Zeiss AXIO Zoom.V16 stereoscope was used to photograph specimen structures. For measurements, the specimens were observed using a Zeiss Discovery V8 stereoscope. All measurements are in millimeters (mm). The distribution map was generated using QGIS v. 2.18.17 “Las Palmas”. Photographs and a map were edited using Adobe Photoshop CS6. Morphological abbreviations follow Valdez-Mondragón (2013, 2020) and Valdez-Mondragón and Francke (2015). Abbreviations: ALE, anterior lateral eyes; AME, anterior median eyes; E, embolus; FAC, frontal apophysis of chelicerae; MSE, median septum of epigynum; PAB, prolateroventral apophysis of bulb; PLE, posterior lateral eyes; PME, posterior median eyes; PP, pore plates; PR, procursus; SAC, sclerotized apophysis of chelicerae; VPP, ventrobasal protuberance of procursus.

The DNA barcoding locus was obtained from a total of 59 specimens belonging to 19 species of *Ixchela* previously described by Valdez-Mondragón (2013, 2020) and Valdez-Mondragón and Francke (2015), and combined with available CO1 sequences, including *I. azteca* and a single outgroup (*Physocyclus dugesi* Simon) for the genetic *p*-distances analysis. Species used in the molecular analyses are listed in Supplementary material (S1). *Physocyclus dugesi* (subfamily Arteminae) was used only to root the tree. The comparison among sequences of *I. azteca* and the rest of the species’ sequences was to analyze the intra and interspecific genetic variation. For samples of *I. azteca*, 1 sequence (CNAN Ara-0160) from Km 46 highway Toluca-Valle de Bravo, Estado de México (type locality of the species); 2 sequences (CNAN Ara-0158 and Ara-0159) from Cueva del Fraile, Gustavo A. Madero, Mexico City; and 2 sequences (CNAN Ara-0169 and Ara-0170) from Cueva del Diablo, Tepoztlán, Morelos were used for the analysis. Specimens used in this study, including GenBank accession numbers and locality information are listed in S1.

DNA was isolated from legs (1 complete leg per sample), and extractions were performed with a Qiagen DNeasy Tissue Kit following a modified protocol described in Valdez-Mondragón and Francke (2015) and Valdez-Mondragón (2020). DNA fragments corresponding to 658 bp of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene were amplified using the same primers (LCO1490 and HCO2198) as in Valdez-Mondragón and Francke (2015) and Valdez-Mondragón (2020). Amplifications were carried out using a Veriti 96 Well Thermal Cycler (Applied Biosystems) following the modified protocol of Valdez-Mondragón and Francke (2015) and Valdez-Mondragón (2020). PCR products were checked to analyze length and purity on 1% agarose gels with a marker of 100 bp and purified directly from the QIAquick PCR Purification kit of Qiagen. DNA extraction and PCR amplification were performed at Laboratorio de Biología Molecular, Laboratorio Regional de Biodiversidad y Cultivo de Tejidos Vegetales (LBCTV), Instituto de Biología, Universidad Nacional Autónoma de México (IB-UNAM), Tlaxcala. Bi-directional Sanger sequencing was performed at the Laboratorio de Secuenciación Genómica de la Biodiversidad y la Salud (LANABIO), IB-UNAM, Mexico City. The generated sequences were saved in FASTA format. All newly generated sequences were deposited in GenBank with the accession numbers OR106002-OR106006 (see S1).

Sequences were aligned using the default gap opening penalty of 1.53 in the online MAFFT platform (multiple alignment using fast fourier transform), version 7 (Katoh & Toh, 2008) using the following alignment strategy: auto (FFT-NS-2, FFTNS-I, or L-INS-i; depending on data size). Inspection and editing of sequences and alignments was done using GENEIOUS version 10.2.6 (Kearse et al., 2012) and BioEdit version 7.0.5.3 (Hall, 1999). The matrix obtained from the multiple sequence alignments was then used for subsequent analyses.

To assess species delimitation with the barcoding locus, we first conducted a phenetic analysis with the neighbor joining (NJ) method to visualize the uncorrected *p*-distances using the program MEGA v. 7.0 (Tamura et al., 2007). We subsequently used the reconstructed NJ tree to carry out analyses with the following 3 methods: *1)* Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al., 2021): we ran this analysis on the online platform (https://bioinfo.mnhn.fr/abi/public/asap/) with the parameters suggested by Nolasco and Valdez-Mondragón (2022a); *2)* General Mixed Yule Coalescent model (GMYC) (Pons et al., 2006): this analysis was performed using the GMYC web server (https://species.h-its.org/gmyc/); and *3)* Bayesian Poisson Tree Processes (bPTP) (Kapli et al., 2017; Zhang et al., 2013): this method was conducted using the web server (https://species.h-its.org/ptp/).

p-*distances under neighbor joining (NJ)*

The genetic distances tree was reconstructed with MEGA v. 7.0 (Tamura et al., 2007), using the following commands: No. replicates = 1,000, bootstrap support values = 1,000 (significant values ≥ 50%), substitution type = nucleotide, model = *p*-distance, substitution to include = d: transitions + transversions, rates among sites = gamma distributed with invariant sites (G+I), missing data treatment = pairwise deletion.

Automatic Barcode Gap Discovery (ABGD): this method is implemented to find gaps in genetic divergence, considering that the intraspecific genetic variation is smaller than the interspecific divergences. First, the method generates a prior data partition of the data into putative species (initial partitions, IP). Then, these initial partitions are recursively partitioned until there is no further partitioning of the data (recursive partitions, RP). ABGD analyses were carried out in the online platform (https://bioinfo.mnhn.fr/abi/public/abgd/) using the following options: K2P distances non-corrected, pmin = 0.001, pmax = 0.1, steps = 10, relative gap width (X) = 1, nb bins = 20. Assemble Species by Automatic Partitioning (ASAP): this is an ascending hierarchical clustering method, merging sequences into groups that are successively further merged until all sequences form a single group. A partition is the equivalent to each sequence merge step, then, the software analyzes all partitions and scores the most probable groups on the tree (Puillandre et al., 2021). ASAP analyses were run in the online platform (https://bioinfo.mnhn.fr/abi/public/asap/) using Kimura (K80) distance matrices and configurated with following parameters: substitution model = *p*-distances, probability = 0.01, best scores = 10, fixed seed value = -. General Mixed Yule Coalescent (GMYC): this 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). To generate the ultrametric tree, phylogenetic analyses were carried out in BEAUti and BEAST v.1.10.4 software (Drummond et al., 2012) using a coalescent (constant population) tree prior. Independent log normal clock uncorrelated was applied with its respective evolutionary model and substitution rates. The models of sequence evolution were selected using jModelTest version 2.1.10 using the Akaike information criterion (AIC) (Posada & Buckley, 2004). The models selected for CO1 for each partition block were GTR+G+I (1st and 2nd codon positions) and GTR+G (3rd codon position). For the analyses, 40 million iterations were run 5 times, independently. The program Tracer 1.6 (Rambaut & Drummond, 2003) was used to evaluate the convergence values. Tree annotator v. 2.6.0 (BEAST package) was used to construct maximum clade credibility trees, after discarding the first 25% of generations ("burnin") of the 5 independent runs. Finally, the GMYC method was implemented in the web service (https://species.h-its.org/gmyc/), which uses the original R implementation of the GMYC model (Fujiwasa & Barraclough, 2013). Bayesian Poisson Tree Processes (bPTP): this species delimitation method is similar to GMYC, but it does not use an ultrametric tree as input. This is because the models of speciation rate are implemented directly using the numbers of substitutions calculated from the branch lengths. The Bayesian and maximum likelihood variants were carried out on the online version (https://species.h-its.org/ptp/), with the following options: rooted tree, MCMC = 1,000,000, thinning = 100, burn-in = 0.1, seed = 123. The trees obtained in all analyses were edited with iTOL online version (https://itol.embl.de/) (Letunic & Bork 2021) and Photoshop CS6. We used the congruence integration criteria to delimit species, which is based on the correspondence among the different molecular methods to generate high support for a species hypothesis (DeSalle et al., 2005, Hamilton et al. 2011, Navarro-Rodríguez & Valdez-Mondragón, 2020, 2024; Nolasco & Valdez-Mondragón, 2022a; Valdez-Mondragón, 2020).

The approaches for DNA barcoding tree-based delimitation explicitly use the phylogenetic species concept, where the analysis recognizes a monophyletic cluster by searching differential intra and inter-specific branching patterns (Ortiz & Francke, 2016).

Results

According to the morphology of primary (male palps and female epigyna) and secondary (male chelicerae) sexual structures, the populations of *Ixchela azteca* showed geographical variation in morphological traits (Fig. 1). The males of the 3 populations have the same shape of the palp (Figs. 1, 8, 9; Valdez-Mondragón & Francke, 2015: Figs. 38, 39); however, there are differences in the shape of the male chelicerae (Figs 1, 4, 6, 7; Valdez-Mondragón & Francke, 2015: Figs. 36, 37, 40) and female epigyna (Figs. 10-13; Valdez-Mondragón & Francke, 2015: Fig. 36). The populations from the temperate region of Cueva del Fraile, Mexico City (CNAN Ara-0158 and Ara-0159), the population from the type locality (Estado de

México, CNAN Ara-160), and the population from Cueva de Diablo, Morelos (CNAN Ara-0169 and Ara-0170) have the frontal apophysis of chelicerae (FAC) shorter (Valdez-Mondragón & Francke, 2015: Fig. 40) than the males from the population of the dry region of Guanajuato, which have a longer and conical FAC (Fig. 7). The female epigynum from Guanajuato is square-shaped in ventral view (Fig. 10), whereas in the other 3 populations from temperate region of central Mexico (Mexico City, Estado de México and Morelos) it is more trapezoidal (Valdez-Mondragón & Francke, 2015: Fig. 42). In frontal view, the conical projection in the population from Guanajuato is smaller (Fig. 12) than the one observed in females from central Mexico (Valdez-Mondragón & Francke, 2015: Fig. 44). Finally, females from the 3 populations of the temperate region of central Mexico have a longer and straighter epigynum in lateral view (Valdez-Mondragón & Francke, 2015: Fig. 41) than the population from the dry region of Guanajuato, which have a shorter and curved epigynum (Fig. 13). The dorsal pattern of the carapace is similar between populations of *I. azteca*, with lateral spots on the carapace and a dark region along the fovea (Fig. 5; Valdez-Mondragón & Francke, 2015: Fig. 35). In comparison with the rest of the species of the genus, *I. azteca* so far is the only species with morphological variation in the primary (male palps and female epigyna) and secondary (male chelicerae) sexual structures.

The CO1 matrix included 59 terminals (including 1 outgroup) and 601 aligned positions. The uncorrected CO1 average distances between the populations of *I. azteca* from temperate regions and the dry region of Guanajuato was 3.5% (Table 1), whereas the average genetic distances among the remaining species of *Ixchela* was 12.1% (Table 1). Bootstrap support for the populations from Guanajuato was 100% (Fig. 1, red branch), whereas the group with the populations from temperate regions in central Mexico had a bootstrap support of 59% (Fig. 1, green branch).

Molecular methods for species delimitation

In the maximum likelihood (ML) phylogram obtained with CO1 (Fig. 2), most of the species assignments based on the current taxonomy were congruent with all species delimitation methods, except for the specimens assigned to *I. azteca* (Fig. 2). The GMYC model recovered a total of 21 species, and the 2 clades of *I. azteca* (Fig. 2, green and red branches) were recovered as a single species exclusive of *I. azteca* CNAN-Ara0169 and Ara0170, which were recognized as a different species (Fig. 2). The ASAP method recognized a total of 18 species, recovering both clades of *I. azteca* as a single species (Fig. 2). Finally, the bPTP method recovered a total of 25 species of *Ixchela* under both variants (BI and ML); however, similar to the GMYC analysis, the specimens CNAN-Ara0169 and Ara0170 were recognized as a different species from *I. azteca* (Fig. 2). The first clade of *I. azteca* (Fig. 2, green branch) did not receive significant bootstrap support (58%), a result also observed in the NJ analysis, whereas the clade from Guanajuato (Fig. 2, red branch) was supported by a high bootstrap value (100%). The *I. azteca* samples CNAN-Ara0169 and Ara0170 had a high bootstrap support of 99% for their sister relationship (Fig. 2).

Family Pholcidae C. L. Koch, 1850
Genus *Ixchela* Huber, 2000

Diagnosis and description. For an updated diagnosis and description of the genus, see Valdez-Mondragón (2013, 2020) and Valdez-Mondragón and Francke (2015).

Taxonomic summary

Type species. Coryssocnemis furcula F. O. Pickard-Cambridge, 1902, by original designation of Huber (2000). Type locality: female holotype from Tecpam, región de los Altos (cerro Tecpam, Departmento Chimaltenango), Guatemala, around 2,300 m, Otto Stoll. Godman & Salvin Coll., in BMNH (F. O. Pickard-Cambridge, 1902; Huber, 1998).

Composition. Twenty-two species. See the World Spider Catalog (2025) for the specific list of species.

Distribution. Ixchela is widely distributed from northeastern Mexico to Nicaragua. Although specimens from Nicaragua were not examined in this work, Huber (2000) examined 1 male of an undescribed species from this country deposited in the Museo Entomológico Nicaraguense (León, Nicaragua).

Ixchela azteca Valdez-Mondragón & Francke, 2015
I. azteca Valdez-Mondragón & Francke, 2015: 29, f. 1-6, 18-44 (Description ♂, ♀).
Figs. 3-13, 16-19

Diagnosis and description. See Valdez-Mondragón and Francke (2015).

Taxonomic summary

Type material. Mexico: Estado de México: ♂ holotype (CNAN T0763) [26 August 2011; A. Valdez, J. Mendoza, D. Barrales, R. Monjaraz, E. Miranda Cols.] from Km 46 highway Toluca-Valle de Bravo (19°15’21.6” N, 100°04’00.8” W; 2,315 m asl) (examined). Paratypes: 1 f# (CNAN T0764); 1 ♀, 3 juv. (CNAN T0765), same data as holotype (examined). See Valdez-Mondragón and Francke (2015) for the complete list of material examined from Estado de México, Guerrero, Mexico City, Michoacán, and Morelos.

Table 1

Average genetic *p*-distance under NJ among the different species of *Ixchela* analyzed with CO1. The value in bold indicates the average *p*-distance between the populations from temperate regions (A) and the population from the dry region in Guanajuato (B) of *I. azteca* (3.5%) (bold). The average genetic *p*-distance under NJ between the species of *Ixchela* was 12.1%.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. *Ixchela juarezi*																		
2. *Ixchela jalisco*	0.099																	
3. *Ixchela grix*	0.107	0.099																
4. *Ixchela abernathyi*	0.110	0.109	0.118															
5. *Ixchela tzotzil*	0.105	0.118	0.107	0.110														
6. *Ixchela pecki*	0.123	0.108	0.112	0.125	0.120													
7. *Ixchela azteca* A	0.116	0.091	0.102	0.120	0.131	0.136												
8. *Ixchela tlayuda*	0.119	0.104	0.120	0.096	0.127	0.127	0.115											
9. *Ixchela azteca* B	0.123	0.098	0.113	0.128	0.135	0.143	0.035	0.112										
10. *Ixchela zapatai*	0.094	0.099	0.111	0.112	0.115	0.117	0.117	0.116	0.112									
11. *Ixchela panchovillai*	0.113	0.118	0.127	0.122	0.124	0.149	0.120	0.115	0.116	0.116								
12. *Ixchela franckei*	0.129	0.100	0.129	0.131	0.131	0.106	0.129	0.138	0.137	0.120	0.135							
13. *Ixchela taxco*	0.124	0.108	0.120	0.139	0.133	0.129	0.118	0.131	0.121	0.084	0.138	0.137						
14. *Ixchela mixe*	0.141	0.133	0.121	0.153	0.153	0.139	0.141	0.145	0.154	0.140	0.155	0.133	0.144					
15. *Ixchela huberi*	0.130	0.122	0.129	0.129	0.131	0.144	0.124	0.117	0.121	0.119	0.122	0.142	0.143	0.127				
16. *Ixchela huasteca*	0.112	0.124	0.135	0.138	0.142	0.140	0.123	0.112	0.119	0.109	0.137	0.136	0.130	0.153	0.137			
17. *Ixchela placida*	0.113	0.129	0.134	0.117	0.139	0.148	0.122	0.126	0.126	0.126	0.079	0.122	0.145	0.156	0.134	0.134		
18. *Ixchela mendozai*	0.111	0.082	0.105	0.076	0.116	0.116	0.106	0.084	0.107	0.099	0.120	0.122	0.123	0.131	0.120	0.115	0.127	
19. *Ixchels purepecha*	0.121	0.127	0.128	0.136	0.147	0.144	0.123	0.127	0.128	0.118	0.123	0.151	0.119	0.156	0.145	0.134	0.134	0.126

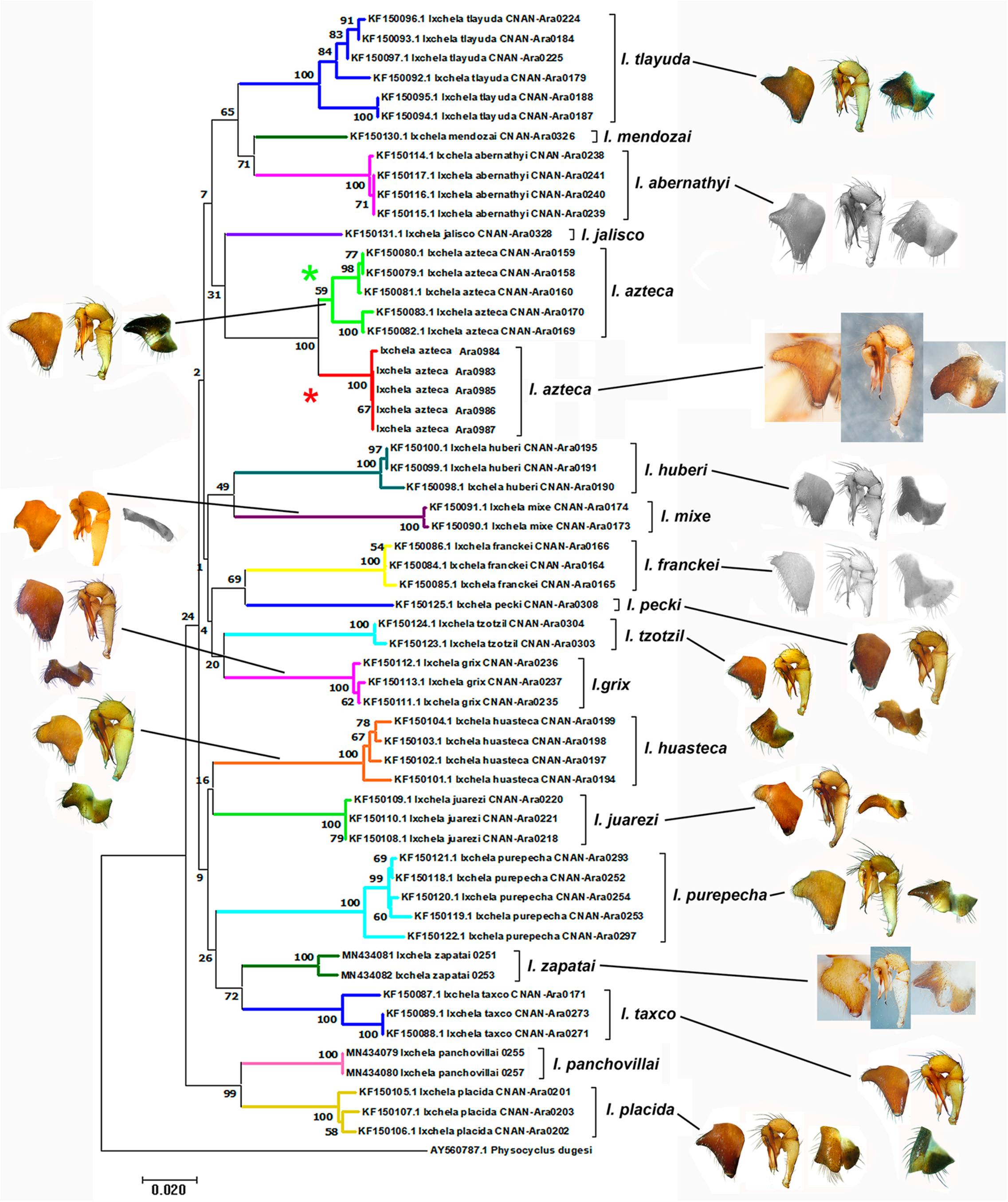


Figure 1. Neighbor-joining tree constructed with *p*-distances of the CO1 barcode sequences from different specimens and species of *Ixchela*. Branch colors indicate species, red and green branches and asterisks represent populations of *Ixchela azteca*. Male chelicera, male palp, and female epigyna are shown to illustrate the variation at species level and within *I. azteca*. Numbers on branches indicate bootstrap support values (> 50%).

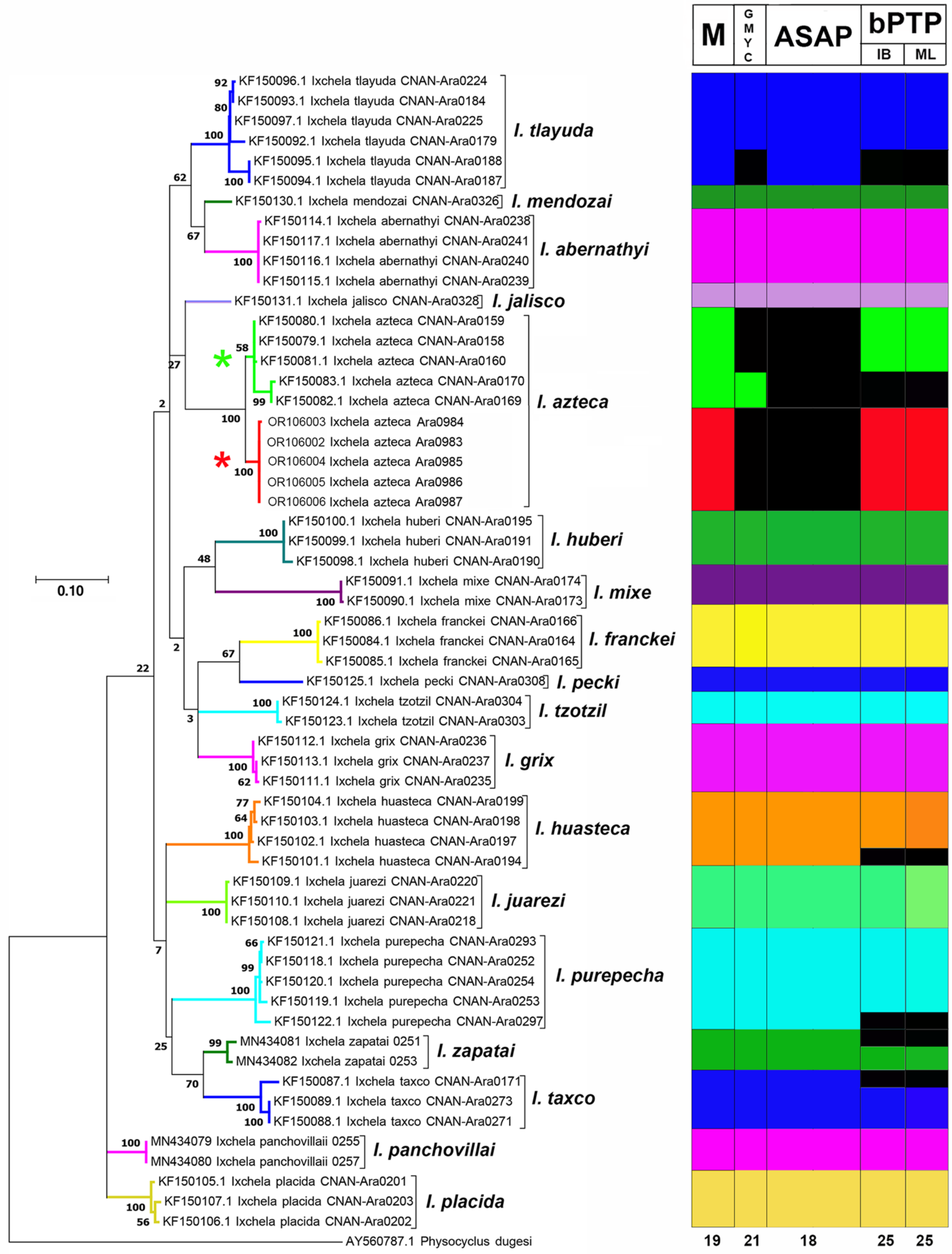
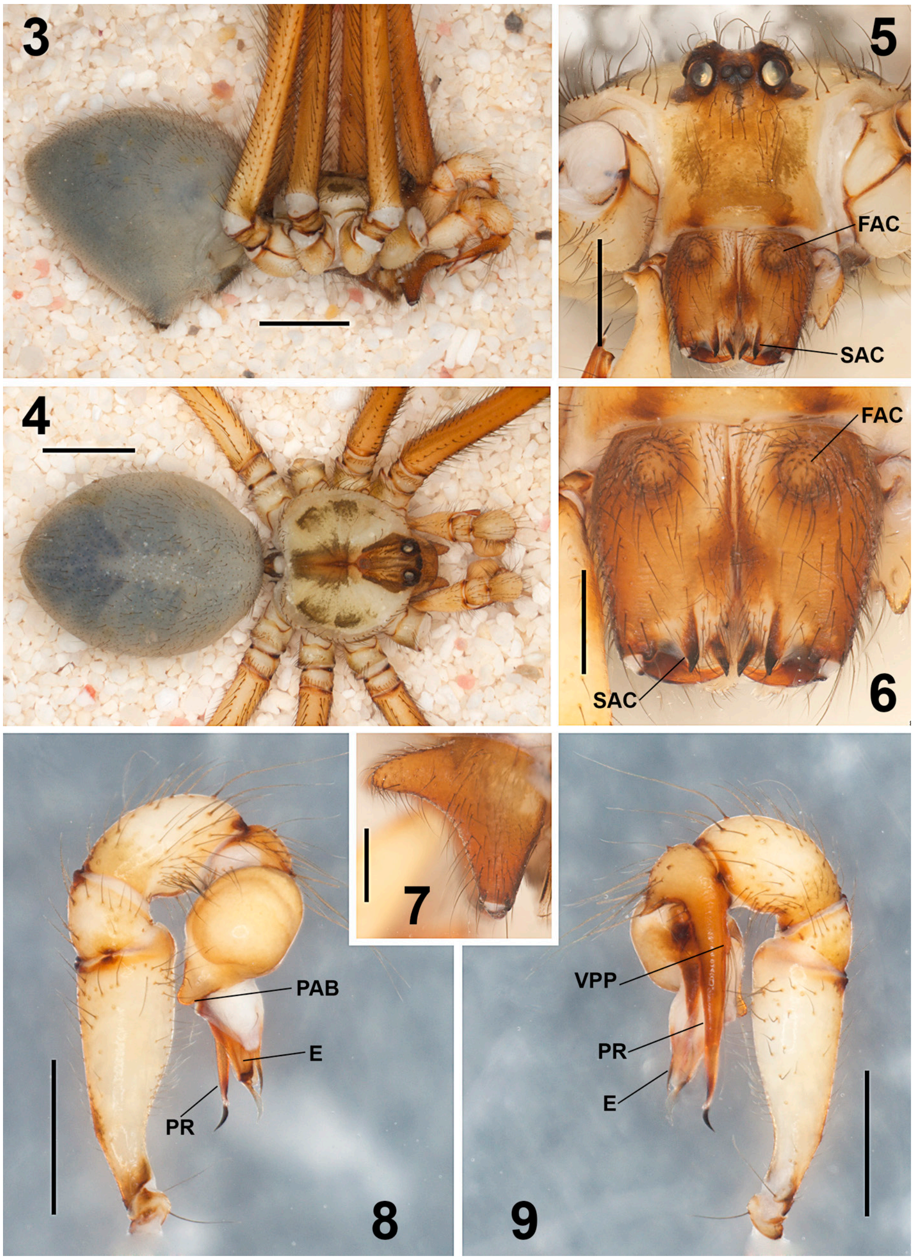


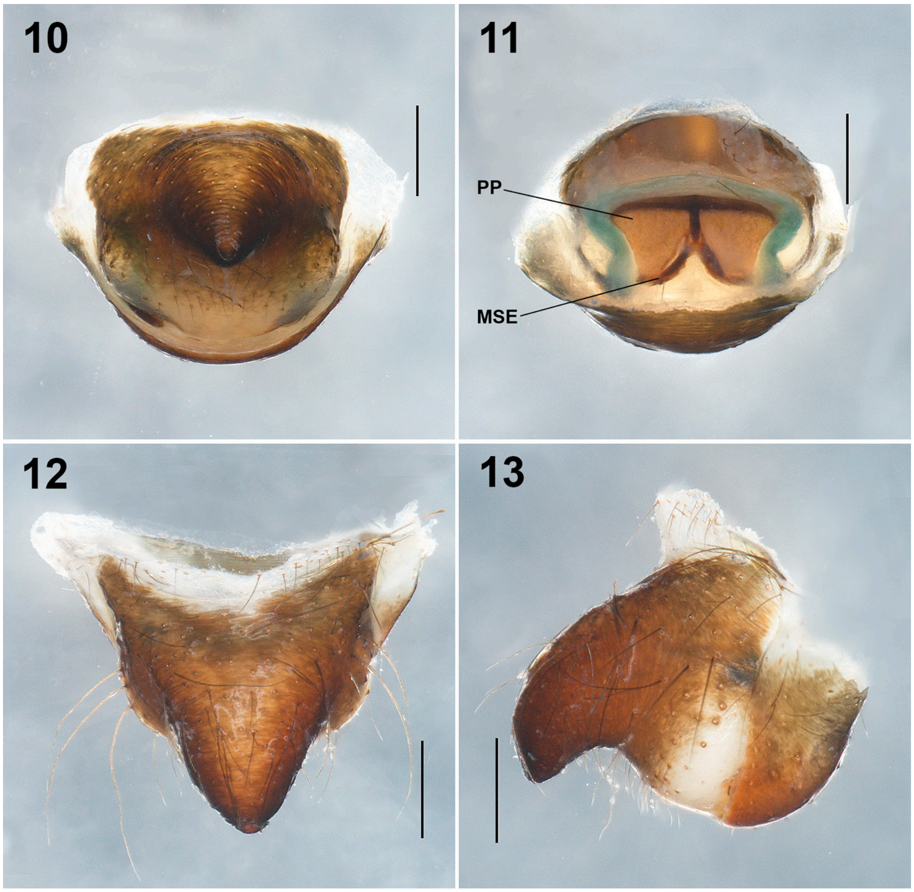
Figure 2. Maximum likelihood tree of *Ixchela* constructed with CO1. Bar colors represent putative species in the tree and in the columns, which represent the different species delimitation methods. Red and green branches and asterisks represent populations of *Ixchela azteca*. Numbers below the columns represent the species recovered in each species delimitation method (not considering *Physocyclus dugesi*). Numbers above branches represent bootstrap support values for ML (> 50%). Column abbreviations: morphology (M); GMYC with single (SN) and multi (MT) thresholds; ASAP; bPTP with maximum likelihood (ML) and Bayesian inference (BI) variants.



Figures 3-9. *Ixchela azteca* Valdez-Mondragón & Francke, 2015 from Cortazar, Guanajuato, Mexico. Male: 3-4, habitus, lateral and dorsal views, respectively; 5, carapace and chelicerae, frontal view; 6-7, chelicerae, frontal and left lateral views, respectively; 8-9, left palp, prolateral and retrolateral views, respectively. E: Embolus, PAB: prolateroventral apophysis of bulb, PR: procursus, SAC: sclerotized apophysis of chelicerae, VPP: ventrobasal protuberance of procursus. Scale bars: Figs. 6, 7 = 0.5 mm, Figs. 5, 8, 9 = 1 mm, Figs 3, 4 = 2 mm.

New material examined. Mexico: Guanajuato: 1 ♂, 2 ♀, 2 juv. (CARCIB) [14 October 2021; A. Valdez, A. Juárez, L. Cabrera, S. Nolasco Cols.] from 7.5 km southeast of Cortazar (20°25’31.1” N, 100°54’51.8” W; 2,160 m asl), Municipality Cortazar (night collecting).

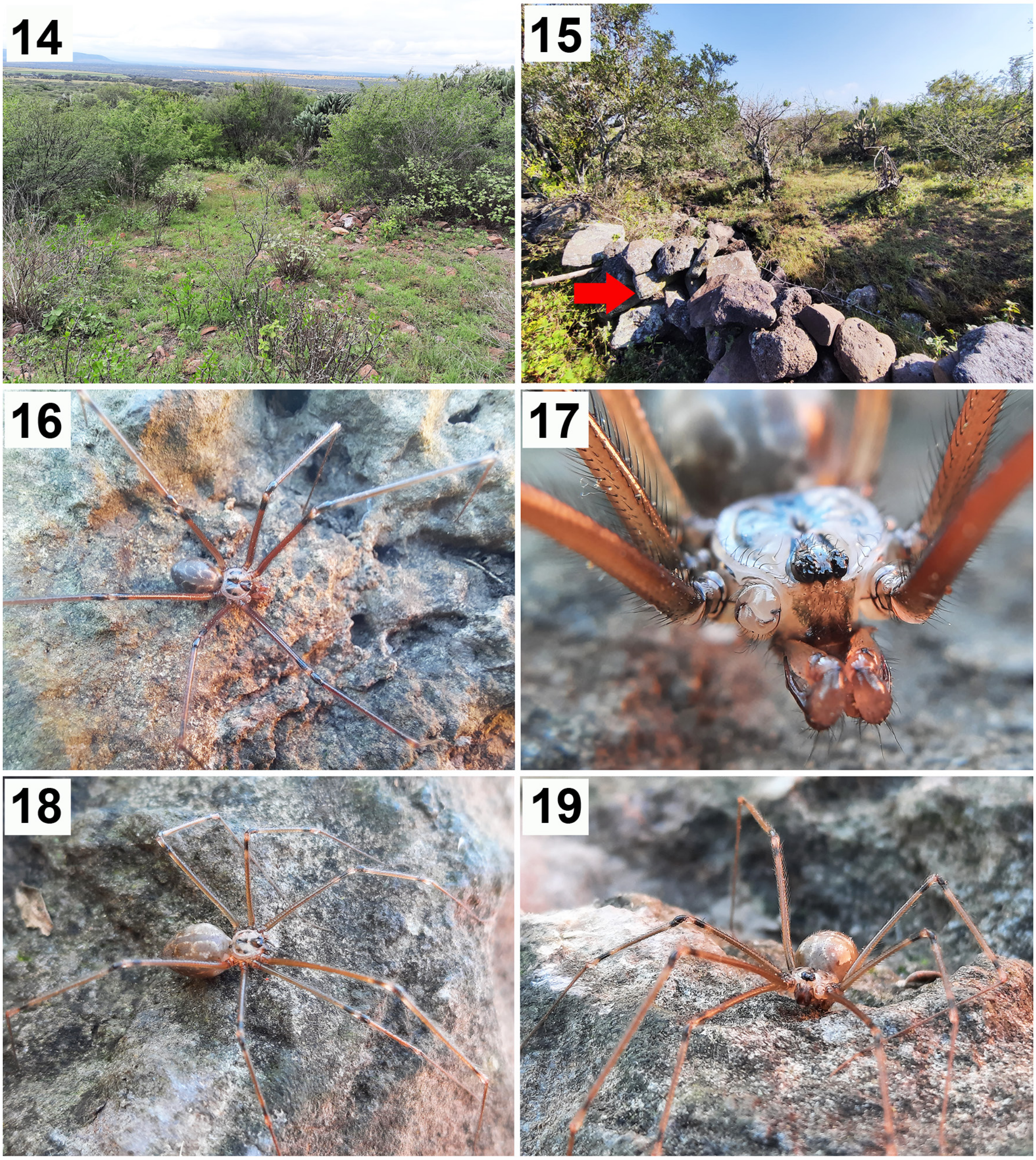
Morphological variation. Male specimens from Cueva del Diablo (Morelos, Mexico) and from Gruta de Tziranda were notably smaller than specimens from the type locality and the other localities, including the new specimens from Guanajuato. Female specimens from Grutas de



Figures 10-13. *Ixchela azteca* Valdez-Mondragón & Francke, 2015 from Cortazar, Guanajuato, Mexico. Female epigynum: 10, ventral view; 11, dorsal view; 12, frontal view; 13, left lateral view. MSE: Median septum of epigynum, PP: pore plates. Scale bars: 0.5 mm.

Tziranda (Michoacán, Mexico) and road to Cueva del Fraile (Mexico City, Mexico) were notably smaller than specimens from the other localities, including the new specimens from Guanajuato. Specimens from Cueva del Diablo have paler coloration on carapace and legs than specimens from other localities. The opisthosomal coloration can vary from grey, pale grey, blue, or pale blue, potentially related to the prey consumed. Males: Cueva del Diablo (Morelos, Mexico) (n = 4), tibia I: 10.87-12.50 ($\bar{x}$ = 11.74). Cueva del Diablo, La Peña (Estado de México, Mexico) (n = 1): tibia I: 18.12. Cueva de Peña Blanca (Estado de México, Mexico) (n = 2), tibia I: 13.75, 14.00. Gruta de Tziranda (Michoacán, Mexico) (n = 1): tibia I: 11.00. Road to Cueva del Fraile and Cueva del Fraile, respectively (Mexico City, Mexico) (n = 2): tibia I: 9.50, 12.25. 7.5 km southeast of Cortazar (Guanajuato, Mexico) (n = 1): tibia I: 13.63. Females: Cueva del Diablo (Morelos, Mexico) (n = 7): tibia I: 7.50-16.00 ($\bar{x}$ = 12.77). Km 34 highway Toluca-Zitácuaro (Estado de México, Mexico) (n = 3): tibia I: 6.60-12.5 (x = 9.50). 5 km W of Casahuates (Guerrero, Mexico) (n = 3): tibia I: 9.37-15.25 ($\bar{x}$ = 11.95). El Naranjo (Michoacán, Mexico) (n = 2): tibia I: 9.50, 11.75. 7 km SE of Ciudad Hidalgo (Michoacán, Mexico) (n = 2): tibia I: 9.7, 11.37. Gruta de Tziranda (Michoacán, Mexico) (n = 4): tibia I: 7.10-10.2 ($\bar{x}$ = 8.70). Road to Cueva del Fraile (Mexico City, Mexico) (n = 2): tibia I: 10.12, 11.87. 7.5 km southeast of Cortazar (Guanajuato, Mexico) (n = 2): tibia I: 9.87, 11.87 ($\bar{x}$ = 10.87).

Natural history. Valdez-Mondragón and Francke (2015) reported that the specimens from Estado de México and Guerrero were collected on their sheet webs in oak-pine and pine forests, inside cavities on walls along road-cuts in wet and shaded areas covered with roots. The male collected in the Instituto de Biología, UNAM was walking on a wall inside a classroom. The specimens from Cueva del Fraile, Mexico City, and Gruta de Tziranda,



Figures 14-19. Habitat and live specimens of *I. azteca*. 14-15, Thorny scrub forest at 2,160 m asl. from 7.5 km southeast of Cortazar, Municipality Cortazar, Guanajuato, Mexico (red arrow indicates the microhabitat where the specimens were collected); 16-17, live male specimens; 18-19, live female specimens.

Michoacán were collected on their sheet webs inside the caves, close to the walls. Specimens collected outside the Cueva del Fraile were among boulders in shady moist areas, whereas specimens from Cueva del Diablo, Morelos were collected in the cave entrance and inside the cave, where humidity was c. 70% and temperatures cold. These specimens were collected on their sheet webs, and it was very common to find prey remains in their webs, mainly large leafcutter ants of the genus *Atta* (subfamily Myrmicinae). The specimens from Cortazar, Guanajuato, were collected in a disturbed thorny scrub forest (Figs. 14, 15) with columnar cacti and nopales (*Opuntia* sp.). These

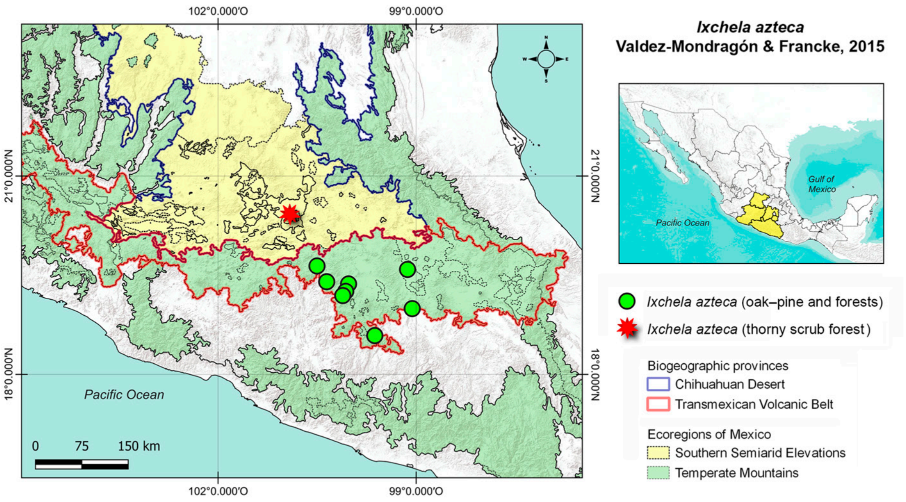


Figure 20. Distribution records of *I. azteca* in the Chihuahuan Desert biogeographic province (Southern Semiarid Elevations ecoregion) and in the Transmexican Volcanic Belt biogeographic province (Temperate Mountains ecoregion). Map by Mayra Cortez Roldán.

specimens were observed on their sheet webs built among walls of big rocks (Figs. 14, 15 red arrow, 16-19).

Distribution. Mexico: Estado de México, Guanajuato, Guerrero, Mexico City, Michoacán, Morelos (Fig. 20).

Discussion

The evolutionary aspects of morphological variation, including dimorphism and polymorphism within and among sexes, has been widely studied in several animal groups. Also, assessing species boundaries is a central debate in modern systematics, particularly in cases where morphology presents wide variations or lacks information at deep levels. The use of DNA barcoding approaches has been applied in modern systematics in 2 different ways: first, to distinguish among described species (equivalent to species identification or species diagnosis), and second, to discover new species (incorporating species delimitation methods and species descriptions) (DeSalle et al., 2005). In this way, another taxonomic task is to establish the limits between species or identify those species with wide morphological and genetic variation among populations.

Regarding the genus *Ixchela*, molecular methods for species delimitation have been used in parallel with morphological features to obtain the most robust evidence for species delimitation and diagnoses. Valdez-Mondragón (2020) described *I. panchovillai* and *I. zapatai* using 4 molecular species delimitation methods and morphology. All molecular methods were congruent with the morphology of primary and secondary sexual features (male palps, male chelicerae, and female epigyna). However, in the case of *I. azteca*, despite the population from Guanajuato (Fig. 2, green branch) being genetically divergent from the populations from central Mexico (Fig. 2, red branch) by a *p*-distance value of 3.5% (Table 1), the molecular species delimitation methods were not congruent with morphology, recovering the 2 clades as a single species. Previous studies on species delimitation in pholcid spiders have usually found congruence between species limits hypothesized a priori based on morphology and those recovered using mitochondrial markers (Nolasco & Valdez-Mondragón, 2022a; Valdez-Mondragón, 2020). However, similar to *I. azteca*, morphological variation of sexual structures within the same species has been previously reported in other pholcid spiders. Huber and Pérez-González (2001) reported a case of morphological variation in the female genitalia of 3 species in the genus *Ciboneya* Huber & Pérez-González, 2001, which even have different female morphs in the same population. Valdez-Mondragón (2010) described discontinuous interspecific variation in the epigyne shape in females of *Physocyclus enaulus* Crosby, 1926 with 3 distinct morphotypes. Such variation has been observed in insular pholcids as Huber and Acurio (2021) reported for *Aymaria conica* (Banks, 1902), with wide morphological variation in the female´s genitalia. In the case of *I. azteca*, the epigyna of the population from Guanajuato is wider and more curved than in the populations from central Mexico, which are longer and less curved. Males of the different *I. azteca* populations also show morphological variation, mainly in the chelicerae, a robust and very important feature at species level in the family Pholcidae. Males from Guanajuato have longer apophyses than males from central Mexico.

Pholcid spiders show a great variety of morphological features that allow for delimitation and diagnosis to the species level in most cases. Primary sexual structures such as male palps and female genitalia, as well as secondary sexual structures such as male chelicerae, are important and useful characters for identifying and diagnosing pholcids species (Huber et al., 2018; Nolasco & Valdez-Mondragón, 2022b; Valdez-Mondragón, 2020). However, as Huber and Pérez-González (2001) suggest, it is possible that taxonomists are strongly biased against discovering genitalic variation such as dimorphisms or even polymorphisms, simply because the genitalia have traditionally been used with an overwhelming priority for species delimitation and identification. This is why taxonomic descriptions should (ideally) be based on a large series of specimens to analyze both intra- and inter-specific morphological and genetic variation. This practice also allows us to identify cases of morpho-clines, dimorphisms, or even polymorphisms.

Although the genetic *p*-distance under CO1 between both populations of *I. azteca* was 3.5% (Table 1), this mitochondrial marker is susceptible to over- and underestimating diversity in some cases; therefore, the limits interpretation must be considered with caution (Astrin et al., 2006; Ortiz & Francke, 2016). Additional informative mitochondrial (e.g., 16S) or even nuclear (e.g., ITS1, ITS2, or 28S) markers, as well as further morphological evidence, would help to obtain better resolution of species hypotheses (Astrin et al., 2006; Agnarsson, 2010; Navarro-Rodríguez & Valdez-Mondragón, 2020; Nolasco & Valdez-Mondragón, 2022a; Ortiz & Francke, 2016; Planas & Ribera, 2015; Valdez-Mondragón et al., 2019).

For the most part, the natural distribution of the genus *Ixchela* is in temperate climate zones, principally in pine, oak, or pine-oak forests, between 1,000-2,950 m of elevation (Valdez-Mondragón, 2013). However, species such as *Ixchela juarezi* Valdez-Mondragón, 2013 have been recorded in thorny scrub forest. The population of *I. azteca* from Cortazar, Guanajuato, was similarly collected in a disturbed thorny scrub forest in the Chihuahuan Desert biogeographic province. Although intensive collecting efforts have targeted low elevations, *Ixchela* seems to occur only above 1,000 m asl mainly in temperate climate regions (Valdez-Mondragón, 2013, 2020; Valdez-Mondragón & Francke, 2015). Species such as *I. juarez* and *I. azteca* that are found in arid and semiarid elevations might represent relict species or populations resulting from vicariant events, and the temperate mountains likely served as a conduit for dispersal. Allopatric populations of *I. azteca* may be a product of the repeated appearance and disappearance of orographic barriers and habitat fragmentation across a complex landscape formed in response to past orogenic events and climate oscillations, mainly in the Transmexican Volcanic Belt (TVB) biogeographic province (transition zone between the Nearctic and Neotropical regions), as was demonstrated by Valdez-Mondragón and Francke (2015) in the genus *Ixchela*.

Based on the available evidence, we propose that the allopatric populations of *I. azteca* assessed herein belong to the same species considering the following: *1)* although morphological variation is evident, this variation could be influenced by environmental effects, genetic mutations, developmental perturbations, or even geographic variation as was suggested in other groups by Debat and David (2001); *2)* although 2 clades of *I. azteca* were recovered under ML with a genetic *p*-distance of 3.5%, the populations from central Mexico present a low bootstrap support value (59%) and 2 subclades were found; *3)* incongruence among molecular species delimitation methods was observed with regards to *I. azteca*, with some methods recovering both clades as a single species; and *4)* the population from Guanajuato was collected in a thorny scrub forest (Chihuahuan Desert province-Southern Semiarid Elevations ecoregion), while the rest of the specimens from central Mexico where collected in temperate pine-oak forests (TVB province-Temperate Mountains ecoregion) (Fig. 20). We suggest that the observed morphological and genetic variation might be due to the geographic variation of the species.

In conclusion and considering the suggestions of Carstens et al. (2013), while probing different methods or lines of evidence is necessary for properly implementing species delimitation, it is better to be conservative about species delimitation when the information and results are incongruent to avoid over-splitting species.

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