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Molecular Phylogenetics and Morphometrics Reveal a New Endemic Jumping Pitviper (Serpentes: Viperidae: *Metlapilcoatlus*) from the Sierra Madre Oriental of Mexico

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ABSTRACT.—The jumping pitvipers of the genus *Metlapilcoatlus* are generally associated with montane environments and are widely distributed from central and southern Mexico to Panama. In this study, we analyzed the phylogenetic position and compared the morphology of a population from the Sierra Madre Oriental, within the Sierra Gorda Biosphere Reserve, to other species of jumping pitvipers. We implemented two mtDNA gene fragments (cyt *b* and ND4) of 50 specimens of the five *Metlapilcoatlus* species for phylogenetic reconstruction using Bayesian inference and maximum likelihood. We used a linear discriminant analysis and comparative statistics of external and hemipenial characters to assess the morphological differences among *Metlapilcoatlus* lineages. Our analyses support a strong genetic and morphological distinction of *Metlapilcoatlus nummifer* populations established to the north of the Trans-Mexican Volcanic Belt (Hidalgo, Northern Veracruz, and Querétaro) from other *Metlapilcoatlus* taxa. Based on this evidence we describe the populations from the north as a new species.

RESUMEN.—Las víboras saltadoras del género *Metlapilcoatlus* generalmente están asociadas con ambientes de montaña y se encuentran ampliamente distribuidas desde el centro y sur de México hasta Panamá. En este estudio, analizamos la posición filogenética y comparamos la morfología de una población de la Sierra Madre Oriental, dentro de la Reserva de la Biosfera Sierra Gorda, con otras especies de víboras saltadoras. Utilizamos dos fragmentos de genes de ADNmt (cyt *b* y ND4) de 50 especímenes de las cinco especies de *Metlapilcoatlus* para reconstrucciones filogenéticas utilizando inferencia bayesiana y máxima verosimilitud. Utilizamos un análisis discriminante lineal y estadísticas comparativas de caracteres externos y hemipeniales para evaluar las diferencias morfológicas entre los linajes de *Metlapilcoatlus*. Nuestros análisis respaldan una distinción genética y morfológica de las poblaciones de *M. nummifer* establecidas al norte de la Faja Volcánica Transmexicana (Hidalgo, Norte de Veracruz y Querétaro) de otros taxones de *Metlapilcoatlus*. Con base en esta evidencia, describimos las poblaciones del norte como una nueva especie.

Over the last 20 yr, considerable efforts have been made to elucidate the taxonomic complexity and evolutionary history of various groups of New World snakes (Pyron et al., 2013; Jadin et al., 2014, 2020; Figueroa et al., 2016). One group that has received particular attention during this time are the viperids (Gutberlet and Harvey, 2004; Castoe and Parkinson, 2006; Fenwick et al., 2009; Quijada-Mascareñas and Wüster, 2009; Jadin et al., 2011; Alencar et al., 2016). Because of the representativeness of the viperid group in the Americas and the relatively well-known phylogenetic relationships for certain genera, they are of great importance as ecological and phylogeographical models (Castoe et al., 2009; Alencar et al., 2018; Jadin et al., 2019). And, although New World pitvipers have been described since the beginning of modern taxonomy (e.g., *Crotalus horridus* Linnaeus 1758), many new taxa continue to be uncovered (Jadin et al., 2012; Townsend et al., 2013; Grünwald et al., 2015; Doan et al., 2016; Carrasco et al., 2019; Timms et al., 2019) suggesting greater species diversity than is currently recognized.

The jumping pitvipers were first separated as a distinct genus, *Atropoides*, by Werman (1992) and originally included three species—*Atropoides nummifer*, *Atropoides olmec*, *Atropoides picadoi*—removed from the genus *Porthidium* (sensu Campbell and Lamar, 1989). The genus *Atropoides* was later expanded to six species with the elevation of *Atropoides mexicanus* and *Atropoides*

occiduus from subspecies status within *A. nummifer* (Campbell and Lamar, 2004) and the description of *Atropoides indomitus* (Smith and Ferrari-Castro, 2008). Collectively, the jumping pitvipers are widely distributed from central Mexico through the mountain ranges of Guatemala and Honduras and into Panama (Campbell and Lamar, 2004; Fig. 1). Concurrent with the changes in taxonomy, the evolutionary history of all six taxa has been inferred using both molecular and morphological data sets (Castoe et al., 2003, 2005; Smith and Ferrari-Castro, 2008; Jadin et al., 2010) and further studies have linked the species diversification of the genus to geologic events in the late Miocene and Pliocene epochs (Castoe et al., 2009; Daza et al., 2010). Although some studies have found *Atropoides* to be monophyletic (Castoe et al., 2005, 2009; Jadin et al., 2010), others have recovered relationships suggesting paraphyly of the genus (Castoe et al., 2003, 2006; Jadin et al., 2011; Pyron et al., 2013; Alencar et al., 2016). Attempting to rectify the issue of paraphyly, Campbell et al. (2019) restricted the generic name *Atropoides* to *Atropoides picadoi*, the type species for the genus, and allocated the remaining five species to a new genus, *Metlapilcoatlus*. Given the literature we accept this conclusion and add morphological support in that *A. picadoi* attains a significantly larger size, lacks nasorostrals, and averages fewer gulars, interrials, middorsal scale rows, prefoveals, subfoveal rows, and supralabials and higher numbers of posterior intercanthals, subcaudals, and ventrals from species of *Metlapilcoatlus* (Jadin et al., 2010).

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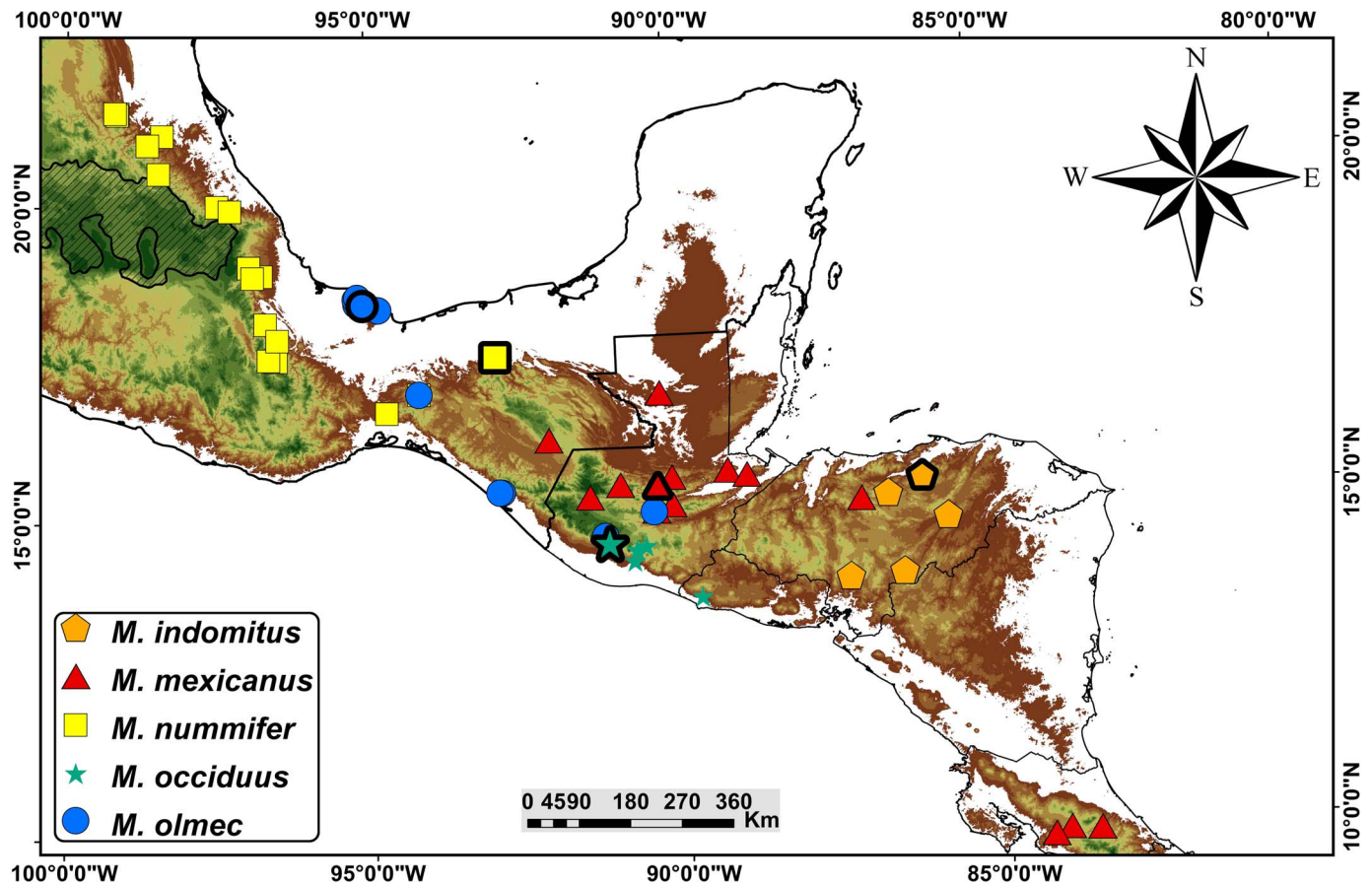


FIG. 1. Distribution map of the specimens used in this study for morphological and molecular analysis of *Metlapilcoatlus* spp. in Mexico and Central America. Bolded icons represent type localities for each species. Shaded regions represent the Transmexican Volcano Belt (TVB).

The Mexican highlands harbor a significant amount of the North American biodiversity and an endemism level that is barely comparable to that of other regions (Mastretta-Yanes et al., 2015). Driving these faunal characteristics are considerable topographic and climatic variations in the area (Flores-Villela, 1993; Ochoa-Ochoa and Flores-Villela, 2006; Bryson et al., 2011). Within these highlands lies the state of Querétaro that is located in the biogeographic confluence zone of the Nearctic and Neotropical regions where three important physiographic provinces in the country converge: the Trans-Mexican Volcanic Belt (TMVB), the Mesa Central, and the Sierra Madre Oriental (Dixon et al., 1972; Padilla and Pineda-López, 1997; Gámez et al., 2012; Morrone, 2014). Given this region's complex biogeographic history, a high biodiversity would be expected. However, studies documenting the herpetofaunistic diversity in the state of Querétaro are scarce (Nieto-Montes de Oca and Pérez-Ramos, 1999; Dixon and Lemos-Espinal, 2010).

In our study, we explore populations of *Metlapilcoatlus* from an important region of the Mexican highlands, near the northern limit of the distribution for this genus, that previous studies have left out. We used two mtDNA gene fragments to assess the phylogenetic position of a population of *Metlapilcoatlus* within the Sierra Gorda Biosphere Reserve in the Sierra Madre Oriental, revealing that the population represents a novel taxon. Furthermore, characters of external and hemipenial anatomy were compared across taxa, and we found diagnosable traits distinguishing this new taxon from its congeners. Therefore, we describe a new species of jumping pitviper that is endemic to this region of Mexico.

MATERIALS AND METHODS

Species Delimitation.—In this study, we used the same proxy to define species concept as in Meik et al. (2018) and Padial et al. (2010). Hypotheses for initial species delimitation were based on morphological and molecular data from Jadin et al. (2010) and Castoe et al. (2009). Then, to evaluate hypotheses for species boundaries, we provide evidence from multiple data classes taking into account three main criteria according to Carbajal-Marquez et al. (2020): 1) evolutionary history of widely recognized lineages obtained by molecular and morphological data, 2) cohesion of morphological traits in multivariate space, and 3) statistical comparison with congeneric taxa.

Morphological Analyses.—The morphological data of 22 characters of lepidosis (Supplemental Material 1) follows Werman (1992), Wüster et al. (1996), Gutberlet (1998), Gutberlet and Harvey (2002), Fenwick et al. (2009), and Jadin et al. (2010). We include seven novel specimens of *Metlapilcoatlus* from Querétaro, México belonging to the National Collection of Amphibians and Reptiles (Colección Nacional de Anfibios y Reptiles: CNAR), the Zoology Museum “Alfonso L. Herrera” (MZFC) of the Universidad Nacional Autónoma de México and compared with 58 specimens from other species and populations (Supplemental Material 2). In the present study we did not account for sexual dimorphism as a source of variation because including specimens from both sexes can provide a more conservative assessment of divergence (Meik et al., 2018).

We examined hemipenes from 16 specimens across all species of *Metlapilcoatlus* in order to compare hemipenial features

TABLE 1. GenBank numbers for DNA sequences generated in this study. Sequences are derived from specimens deposited at the Museo de Zoología de la Facultad de Ciencias, Universidad Nacional Autónoma de México (MZFC).

Taxon	ID	Voucher	Locality	cyt <i>b</i>	ND4
<i>Metlapilcoatlus</i> sp.	<i>Metlapilcoatlus</i> sp.	MZFC 35381	México: Jalpan: Valle Verde	MW729752	MW729737
		MZFC 35382	México: Jalpan: Valle Verde	MW729753	MW729738
		MZFC 35382	México: Jalpan: Valle Verde	MW729754	–
		MZFC 35384	México: Jalpan: San Juan de los Durán	MW729755	MW729739
		MZFC 35385	México: Jalpan: San Juan de los Durán	MW729756	MW729740
		MZFC 35386	México: Jalpan: Valle Verde	MW729757	–

among the species (Supplemental Material 3). However, we did not include hemipenial structures in the morphometric analyses because we were only able to examine a few hemipenes of the different species and we wanted to avoid the increase of the distances between groups due to sampling bias. Terminology used in the description of the hemipenes follows that proposed by Dowling and Savage (1960) and Jadin et al. (2010).

For morphological analysis we performed a principal component analysis (PCA), using species hypotheses from Castoe et al. (2009) and Jadin et al. (2010). Subsequently, we use a linear discriminant analysis (LDA) to determine morphological differences among *Metlapilcoatlus* species. All analyses were performed using the software R (R Core Team, 2018). Our analysis aims to predict which group a new individual will most likely belong to, knowing only the individual's variable profile (Klecka, 1980). Subsequently, a morphometric comparison was carried out with nonparametric Mann-Whitney test for individual samples. The Mann-Whitney tests were performed only between the species with highest morphological similarity (*M. nummifer*) and the target populations of our study.

Laboratory Methods.—DNA was extracted using tissue samples (muscle and liver), through a standard proteinase K digestion to be purified later (*sensu* Girish et al., 2005). Genetic data included two coding regions of the mitochondrial genes cytochrome *b* (cyt *b*) and NADH dehydrogenase subunit 4 (ND4), which adds up to 68 taxa and 1,388 characters used in this study. All regions were amplified via PCR (*sensu* Arévalo et al., 1994; Parkinson et al., 2002; Castoe et al., 2005; Castoe and Parkinson, 2006). The PCR procedures used primers described by Arévalo et al. (1994), Kocher et al. (1989), and Tsai et al. (2007). Subsequently, obtained sequences were concatenated and aligned through the software Geneious Basic (Kearse et al., 2012). Additionally, 10 new sequences of specimens from the Sierra Gorda de Querétaro were generated and deposited into GenBank (Table 1).

Phylogenetic Analyses.—Mitochondrial DNA sequences were obtained from six *Metlapilcoatlus* species and outgroup taxa acquired from previous studies (Parkinson et al., 2002; Castoe et al., 2003, 2005; Wüster et al., 2005; Castoe and Parkinson, 2006) deposited in GenBank (Supplemental Material 4) and combined

with our novel sequences. Bayesian inference (BI) analyses were conducted using MrBayes v3.0b4 software (Huelsenbeck and Ronquist, 2003). Each analysis performed 5×10^6 generations and a 25% fraction of generations from each run was removed as burn-in, in accord with other studies. Gene fragments were analyzed under the GTR + G model without partitions within the matrix as selected by JModeltest software (Posada, 2008). All estimations were recovered each 1,000 generations. To carry out maximum likelihood (ML) analyses, RAxML-HPC software was used (Stamatakis, 2006). A total of 1×10^6 iterations were made to obtain the final reconstruction, and the support of the nodes using the model GTRGAMMA with default parameters. The external group—composed of the sister genera *Cerrophidion* and *Porthidium*—was selected a priori, according to what was described by Castoe et al. (2005) and Jadin et al. (2010, 2012).

Finally, we utilized 32 nucleotide sequences of the ND4 gene fragment to compute pairwise comparisons as a measure of estimated genetic distances between species of *Metlapilcoatlus* and *Atropoides* (Table 2). Analyses were conducted in MEGA6 (Tamura et al., 2013) using the Kimura two-parameter model (Kimura, 1980) and nucleotides from all three codon positions. All positions containing gaps and missing data were eliminated, providing a total of 384 positions in the final data set.

RESULTS

Morphological Analyses.—Our principal component analysis showed high morphological overlap among all species of *Metlapilcoatlus*, even between highly differentiated mtDNA clades such as *M. mexicanus* and *M. nummifer*. The first two axes accounted for 36.2% of the morphological variation. PC1 explained 20.6%, with number of foveals, posterior intercanthals, and subfoveal rows loading strongly. PC2 explained 15.6%, with number of nasorostrals, number of body blotches, and number of intersupraoculars loading strongly. Further analysis of focus group and closely related *M. nummifer* revealed high levels of morphological overlapping (Supplemental Material 5).

Discriminant analysis showed that there are highly significant differences (Wilks's $\lambda = 0.0014$; $F_{132,310} = 4.849$; $P = 0.00001$)

TABLE 2. Pairwise sequence divergences of the ND4 gene fragment between *Atropoides picadoi* and *Metlapilcoatlus* species as defined in this study. The number of base substitutions per site from averaging over all sequence pairs between groups are shown below the diagonal while standard error estimate(s) are shown above. Values for *Metlapilcoatlus* sp. are in bold.

	1	2	3	4	5	6	7
1. <i>A. picadoi</i>		0.014	0.014	0.015	0.014	0.014	0.013
2. <i>M. indomitus</i>	0.112		0.011	0.011	0.010	0.011	0.011
3. <i>M. mexicanus</i>	0.124	0.084		0.008	0.010	0.009	0.007
4. <i>M. nummifer</i>	0.124	0.075	0.045		0.011	0.008	0.006
5. <i>M. occiduus</i>	0.122	0.071	0.083	0.084		0.011	0.010
6. <i>M. olmec</i>	0.118	0.081	0.070	0.051	0.093		0.009
7. <i>Metlapilcoatlus</i> sp.	0.118	0.082	0.047	0.028	0.080	0.057	

TABLE 3. Loadings of the variables used in the discriminant analysis (LDA) performed to analyze the different species of the genus *Metlapilcoatlus*.

Variable	Axis 1	Axis 2
Nasorostral scales	0.067	-0.002
Number of subfoveals rows	0.012	0.047
Anterior internasals	0.009	-0.006
Canthals	0.007	-0.018
Gular scales	0.005	0.020
Subcaudal scales	0.003	-0.010
Interoculabials scales	0.002	0.025
Subnasal scales	0.002	0.021
Mid dorsal Scale Rows (MDSR)	0.001	0.003
Scales contacting third supralabial	0.000	-0.008
Supralabial scales	-0.001	0.006
Postocular scales	-0.001	0.027
Scales between the <i>rictus</i> and dorsals	-0.001	0.009
Ventrals	-0.005	-0.007
Interrictal scales	-0.009	0.005
Prefoveal scales	-0.011	0.036
Scales contacting supraocular	-0.013	-0.003
Posterior intercantals	-0.013	-0.005
Postorbital stripe height	-0.014	0.005
Intersupraocular scales	-0.015	0.004
Body blotches	-0.016	-0.006
Subocular scales	-0.037	0.033
% explained variance	47	19

among *Metlapilcoatlus* species. Overall, 92.5% of specimens were correctly classified in the groups defined a priori based on our phylogenetic analysis. Lepidosis characters that had significant values to discriminate among species were number of nasorostral scales, number of subfoveal rows, number of subocular scales, and number of dorsal blotches (Table 3). Discriminant analysis separated species with 66% of variance explained in the first two axes. The first axis explained 47% of the variance, whereas the second axis explained 19% (Fig. 2; Table 3). On axis 1, number of blotches at the sides of the body, number of ventral scales, intersupraoculars, posterior intercantals, and suboculars were the variables that loaded negatively on the axis and that separate *M. indomitus* and *M. occidius* from the rest of the species. The number of nasorostral scales, anterior internasals, and subcaudals, weighed positively on axis 1, and they are features shared, mainly, between *M. mexicanus* and *M. nummifer*. Axis 2 allows to set *Metlapilcoatlus* sp. apart from the rest; these species are the only ones that weighed negatively on this axis. The number of interoculabial scales, subfoveal rows, and intersupraoculars scales weighed positively, grouping most of the species. Traits that weighed negatively on this axis were number of canthal scales, number of scales in contact with third supralabial, and number of ventral scales.

Eight variables obtained significant values to be able to discriminate among species of the genus (Table 4). However, these variables do not wholly represent the differences of *Metlapilcoatlus* sp. with the highest morphological similarity to *M. nummifer*. The comparison of these two taxa suggest that they differ significantly from each other in the number of scales in the mid part of the body ($W = 140.5$, $P = 0.001$), number of posterior intercantals ($W = 37$, $P = 0.01$), number of foveal scales ($W = 32.5$, $P = 0.008$), number of interoculabial scales ($W = 150.5$, $P = 0.0007$), number of scales in contact with third supralabial ($W = 32$, $P = 0.004$), number of nasorostral scales ($W = 43$, $P = 0.02$), number of intersupraocular scales ($W = 30$, $P = 0.004$), and postorbital stripe height ($W = 147$, $P = 0.002$; Fig. 3).

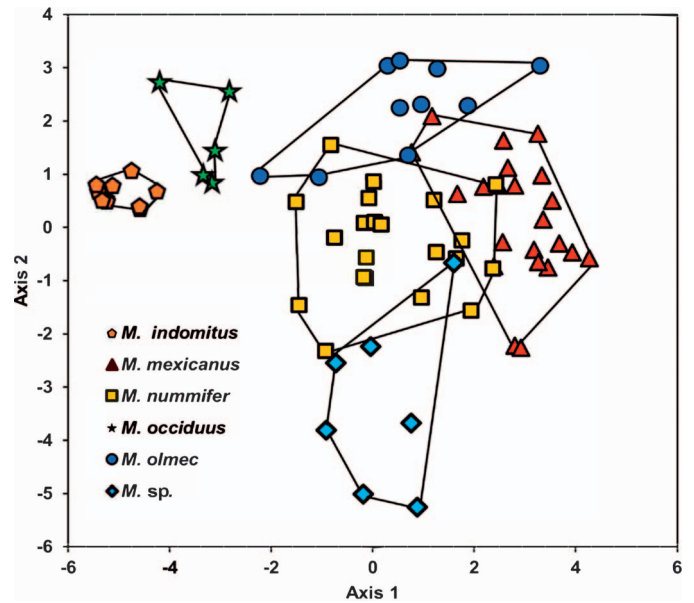


FIG. 2. Discriminant analysis performed to determine morphological variation between the six species of *Metlapilcoatlus*.

Hemipenis Description.—*Metlapilcoatlus* sp. hemipenes (hemipenis length [HL] = 13.21 mm; hemipenis width [HW] = 2.24 mm), are significantly smaller compared to sizes reported for similar sized males of *M. mexicanus* (HL > 21 mm; HW = 11 mm), *M. nummifer* (HL > 16 mm; HW = 9 mm), and *M. olmec* (HL > 20 mm; HW = 10 mm). In *Metlapilcoatlus* sp. the *sulcus spermaticus* insertion continues longitudinally in centrolinear position over the sulcate side of the hemipenis, in comparison to *M. mexicanus*, *M. nummifer*, and *M. olmec*, whose *sulcus spermaticus* curves ventro-diestrally and terminates inserted at the tip of the lobes. Hemipenes are strongly bilobed, as in the majority of viperids, subcylindrical and capitated, extending from the caudal base to the seventh or eighth subcaudal scale; the *sulcus spermaticus* is bifurcated and runs centrolinearly in each of the lobes until their apex. The walls of the *sulcus spermaticus* are robust and well defined with borders barely ornamented by spines; the bifurcation point is located towards the basal part of the body of the hemipenis. Each of the *sulcus spermaticus* divisions runs longitudinally to the hemipenis body on its sulcate side to the lobes' apex, where it is inserted apically in each of them. Basally, in the sulcate side, the hemipenis is devoid of ornaments. However, towards the apical part, in the zone near the lobes, there are hooks and spines of great size, which decrease as they approach the basal part. Lobes are narrower than the hemipenis body. Each lobe is homogeneously ornamented with rows of

TABLE 4. Wilks's lambda and significance values of the variables used in the discriminant analysis (LDA).

Variable	Wilks's lambda	F	P
Intersupraocular scales	0.002	4.549	0.000
Number of body blotches	0.002	4.461	0.001
Number of subcaudal scales	0.002	3.161	0.01
Number of interoculabial scales	0.001	2.931	0.015
Scales between <i>rictus</i> and dorsals	0.001	2.573	0.029
Number of nasorostral scales	0.001	2.505	0.033
Postorbital stripe height	0.001	2.332	0.045
Number of anterior internasal scales	0.001	2.301	0.048

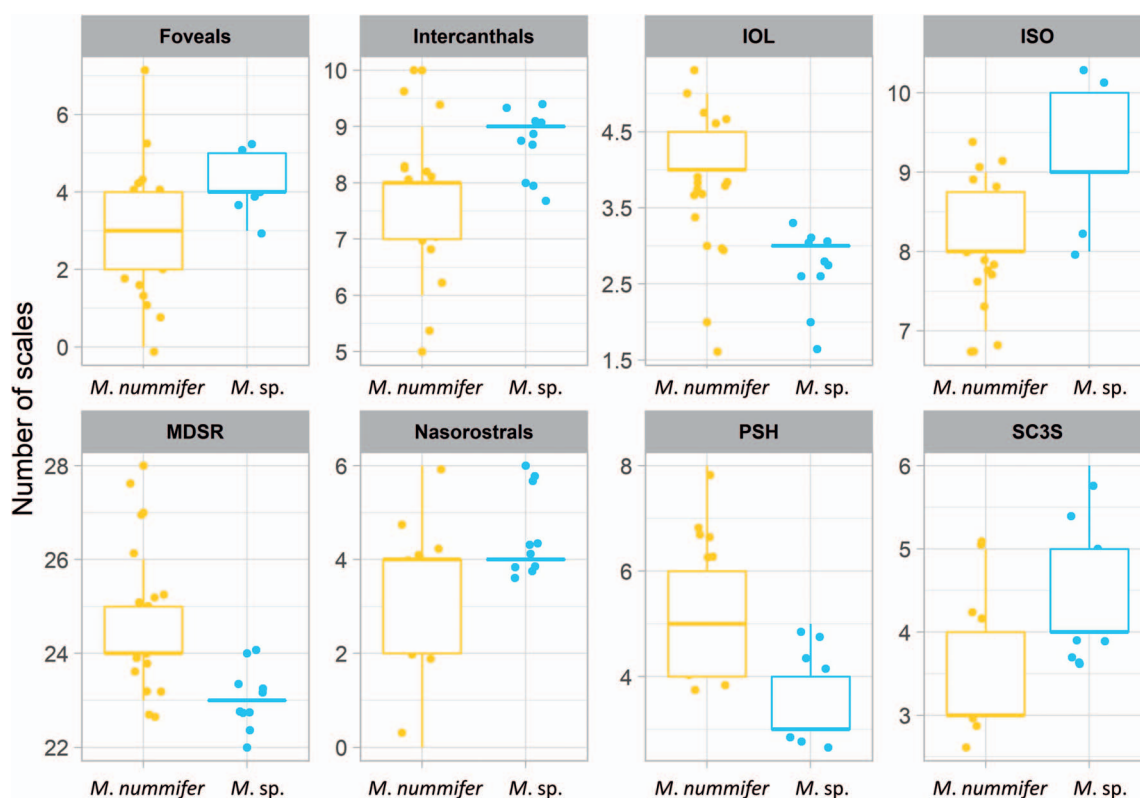


FIG. 3. Comparison of the main lepidotic characters used to differentiate between *Metlapilcoatlus nummifer* and *Metlapilcoatlus* sp. (A) Foveal scales ($W = 32.5$, $P = 0.008$), (B) intercahthals scales ($W = 37$, $P = 0.01$), (C) interoculabial scales ($W = 150.5$, $P = 0.0007$), (D) postorbital stripe height ($W = 147$, $P = 0.002$), (E) intersupraocular scales ($W = 30$, $P = 0.004$), (F) middorsal scale rows ($W = 140.5$, $P = 0.001$), (G) nasorostral scales ($W = 43$, $P = 0.02$), (H) scales contacting third supralabial ($W = 32$, $P = 0.004$).

calyces in the basal region and a zig-zag pattern in the apical region (of the lobes; Fig. 4).

Regarding hemipenial morphology, the group formed closest to *Metlapilcoatlus* sp. is *M. mexicanus*, *M. nummifer*, and *M. olmec*. However, *Metlapilcoatlus* sp. differs from this group and the rest of the species in the genus because their hemipenes are proportionally smaller, position of *sulcus spermaticus* is centro-linear with regard to the sulcate side of the hemipenis, and the walls of the *sulcus spermaticus* are much thicker with regard to the other species in the genus. The hemipenes of *Metlapilcoatlus* sp. also have a greater number of spines at the base of the lobes, a smaller calyculate area, and are proportionally narrower towards the tip of each lobe. Hemipenes lobes are distally ornamented by calyces, medially by spines, and basally by little spinules separated by naked areas (asulcate side), or totally naked (sulcate side).

Phylogenetic Relationships.—The phylogenetic relationships and branch support were congruent in both analyses. The genus *Metlapilcoatlus* was recovered as monophyletic, but without support, in both phylogenies with *A. picadoi* being sister to the rest of the *Metlapilcoatlus* taxa (Fig. 5). The *Metlapilcoatlus* taxa were divided into two strongly supported main clades; the first one formed by *M. indomitus* as sister to *M. occiduus*, which are sister to the other four. The second clade is, in turn, divided into two monophyletic groups. The first contains a weak sister relationship between different populations of *M. mexicanus* and *M. olmec*, and the second clade is strongly supported and comprised of three subclades with different populations of *M. nummifer*: the first one is formed by populations from Central Veracruz, the second one by populations from Northern

Veracruz, and the last one by populations from Northern Hidalgo and the adjacent part of Querétaro.

DISCUSSION

Morphometrics.—Despite the fact that multivariate analyses are widely used to examine variation of shape and size, and species delimitation in other reptile groups such as lizards and snakes (Velasco and Herrel, 2007; Angarita-Sierra, 2014; Passos et al., 2018), there are few viperid studies that have used this approach (Fernandes et al., 2004). We found significant differences among *Metlapilcoatlus* species. Our findings are quite important considering that previous studies have had difficulties clarifying the species diversity of *Metlapilcoatlus* because of the great morphological variation within species in the genus and the lack of information regarding type specimens (Campbell and Lamar, 2004; Smith and Ferrari-Castro, 2008; Jadin et al., 2010). It can be observed in the present study that *M. indomitus*, *M. occiduus*, and *Metlapilcoatlus* sp. are separated from the rest of the species (Fig. 2). This is probably because they share few synapomorphies with the *M. nummifer* complex, such as poorly or undeveloped nasorostral scales, lower number of ventral scales, higher number of subfoveal rows, higher number of oculabial scales, number of subcaudals, and the number of blotches at the sides of the body. In the case of the species belonging to the *M. nummifer* complex, there is a higher variation of traits (e.g., infralabials, intersupraoculars, subcaudals, supralabials, etc.) in the observed specimens and, as a result, there is overlap in the morpho space (Fig. 2). However, our analysis detected significant differences among species of this complex. Levels of variation pointed out in

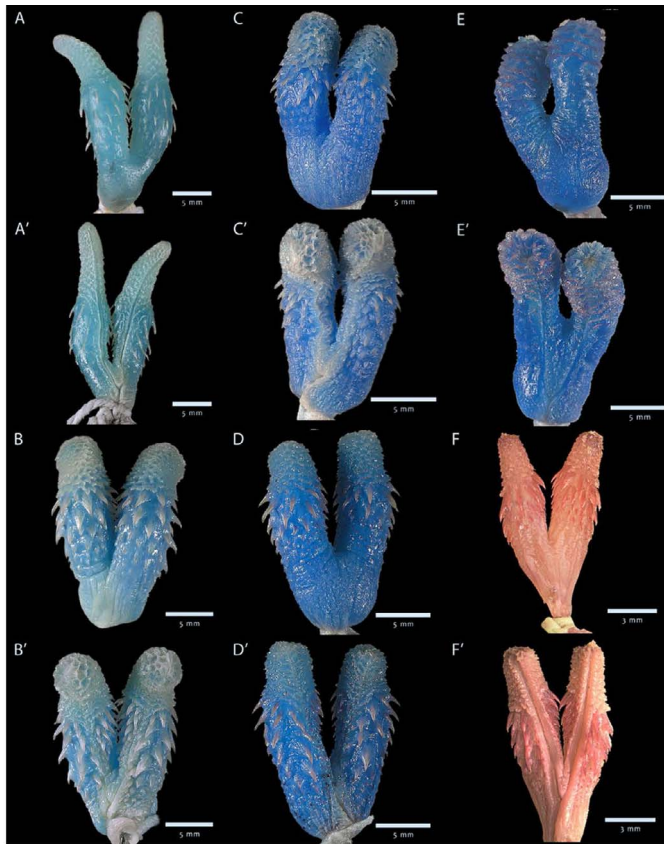


FIG. 4. Hemipenes of the species of the genus *Metlapilcoatlus*. First, the asulcate view is shown and the sulcate face of the hemipenes of each species is printed with a premium letter. (A) *Metlapilcoatlus indomitus* (UTA R-52952), (B) *M. mexicanus* (UTA R-45500), (C) *M. nummifer* (UTA R-24842), (D) *M. olmec* (UTA R-25113), (E) *M. occiduus* (UTA R-26415), and (F) *Metlapilcoatlus* sp. (MZFC 35382).

the present study show the latent difficulty in establishing boundaries among species using morphological characters only. For instance, the nasorostral scales are considered to be a diagnostic trait in this genus although we discovered here that it is a highly variable trait. Such is the case of *M. indomitus* in which a variation ranging from zero to two nasorostral scales has been recorded (Jadin et al., 2010) compared to specimens of *Metlapilcoatlus* sp. that have as many as six nasorostral scales.

Hemipenial Morphology.—Hemipenial characters function as good indicators of relationship among species (Dowling, 2002; Jadin et al., 2010). The *sulcus spermaticus* in particular is a structure of hemipenial morphology that has been widely used as a diagnostic trait to establish boundaries among serpent and other reptile species (Angarita-Sierra, 2014; Sturaro et al., 2018). Despite that there are certain intraspecific levels of variation among hemipenial characters, it is uncommon to find variation in the *sulcus spermaticus*, because most of the variation in snakes is found in structures like the number of spines and calyces (Dowling and Savage, 1960). In the case of *Metlapilcoatlus* sp., the *sulcus spermaticus* is much thicker and voluminous to that of its congeners.

Phylogenetic Relationships.—The relationships recovered in our study are consistent with those obtained previously (e.g., Castoe et al., 2003, 2005, 2009; Daza et al., 2010; Jadin et al., 2010). The genus *Metlapilcoatlus* was recovered as monophyletic, though with low support, with regard to *Atropoides*, *Cerrophidion* and *Porthidium*. Additionally, our analyses found that *M. indomitus*

and *M. occiduus* form a clade that is a sister group to a clade of *M. mexicanus*, *M. nummifer*, and *M. olmec*, with *M. mexicanus* and *M. olmec* being sister to each other. Within *M. nummifer*, we found a split from among the populations to the South of the TMVB (Teziutlán, Puebla; Córdoba, Veracruz; Ixhuatlán del Café, Veracruz; Northern Veracruz) and those that inhabit locations north of the TMVB (Huejutla, Hidalgo; Hueyacocotla, Veracruz; Jalpan, Querétaro; Fig. 6). Our results suggest that the TMVB played an important role in the genetic and morphological structuring of the clade comprised by *Metlapilcoatlus* sp. and *M. nummifer*, specifically at the end of its formation in its oriental part 2.5 Ma (Becerra, 2005; Castoe et al., 2009). This information suggests that interaction among populations to the north and south of the TMVB may have been constant until 2.5 Ma (Marshall and Liebherr, 2000; Becerra, 2005). If we consider that this genus of viperids is strongly associated with tropical montane cloud forest, it is possible that residual distribution of this type of vegetation throughout the Sierra Madre Oriental, its wide vertical distribution (600–3,200 m above sea level [a.s.l.]), and the effect of vegetal associations adjacent to this elevation gradient (González-Espinoza et al., 2012) have promoted distinct evolutionary histories.

TAXONOMIC CONCLUSIONS

An integrative taxonomic approach that includes biogeographical, ecological, molecular, and morphological evidence is crucial to infer the origin, boundary, and evolution of species properly (Padiál et al., 2010). Our study shows significant genetic and morphological (external and hemipenial) differences between *Metlapilcoatlus* populations from the south of the Sierra Madre Oriental and the eastern limit of the TMVB. Additionally, we found strong phylogenetic support of each *Metlapilcoatlus* lineage obtained in this study corresponding with important biogeographic boundaries, enabling us to elucidate the factors that influence the isolation of populations across different spatial scales. For these reasons, we describe a new species for this genus of viperid.

SYSTEMATIC ACCOUNTS

Metlapilcoatlus borealis sp. nov.

Fig. 7

Metlapilcoatlus nummifer (Taylor, 1949); *Metlapilcoatlus nummifer* (Castoe et al., 2009); *Metlapilcoatlus nummifer* (Jadin et al., 2010)

Holotype.—Universidad Nacional Autónoma de México, Museo de Zoología “Alfonso L. Herrera” MZFC 35381, field number OFV 1331, female of El Pílon, Jalpan de Serra Municipality, Querétaro, México, 1,134 m (21.499°, –99.173°) collected on 30 June 2015 by Jacinto Chávez.

Paratype.—Universidad Nacional Autónoma de México, Museo de Zoología “Alfonso L. Herrera” MZFC 35382, field number OFV 1336, male of Valle Verde, Jalpan de Serra Municipality, Querétaro, México, 1,132 m (21.497°, –99.170°) collected on 18 July 2015 by Jacinto Chávez.

Diagnosis.—*Metlapilcoatlus borealis* is a medium-sized, moderately robust viper (TL: males 657 mm, females 594 mm), has 22–25 rows of scales over the mid part of the body, nasorostral scales 4–6, ventrals 130–132, subcaudals 26–35, supraoculars 8–10, interoculars 2–3, scales from 21–26 shields at the sides of the body before the cloaca. Additionally, *Metlapilcoatlus borealis* possess three unique nucleotides for cyt *b* at positions 21 (C),

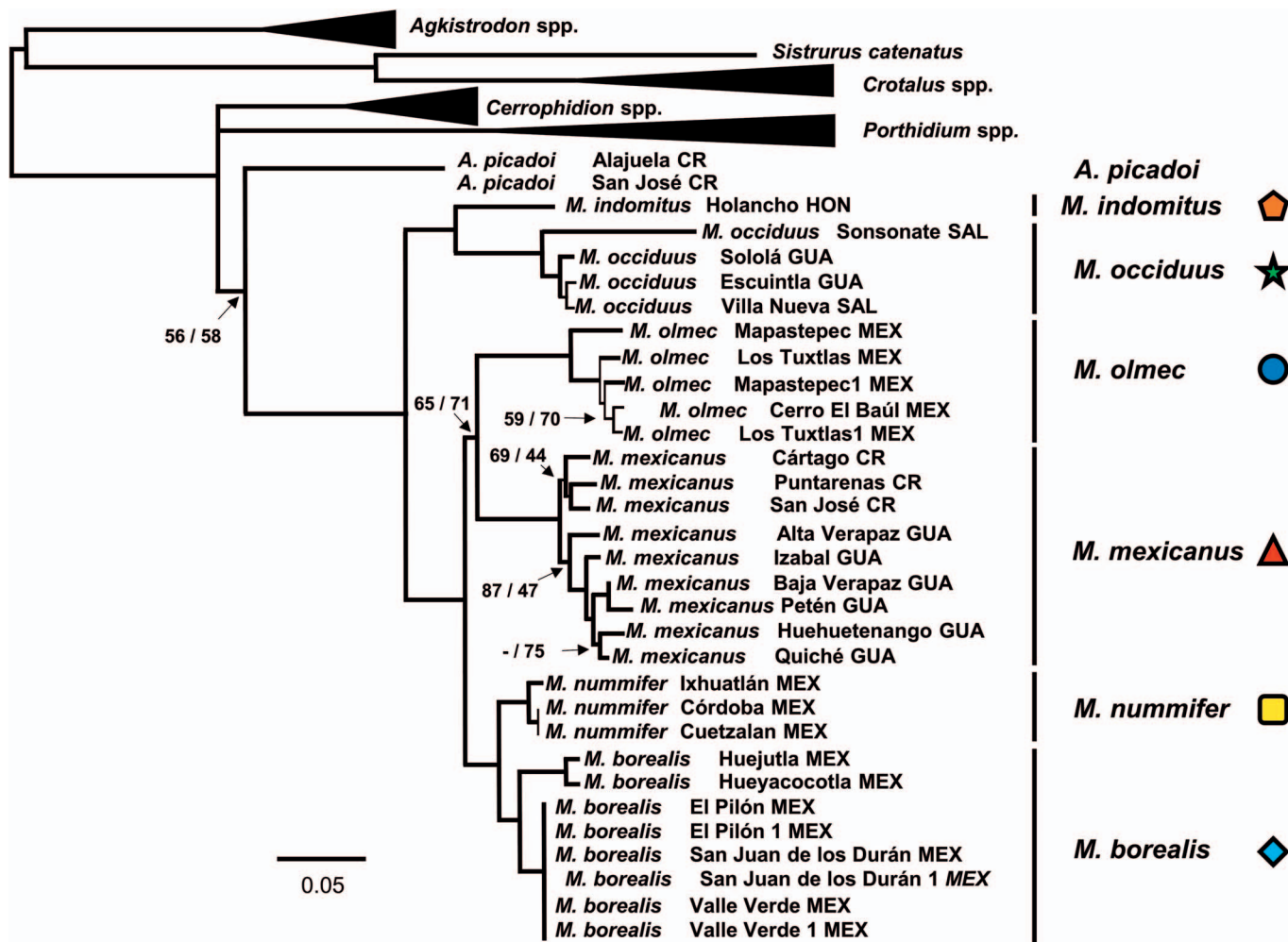


FIG. 5. Phylogenetic relationships of the genus *Metlapilcoatlus* obtained through Bayesian inference (IB) and maximum likelihood (ML) function with the RAxML software. The condensed nodes (black) correspond to the genera used in the external group. All nodes are shown with a subsequent probability (PP) ≥ 90 except those indicated with an arrow. The values obtained through IB are shown, followed by the values obtained through ML (IB/ML). Names of specimens at terminals correspond to IDs given in Table 1 and Supplemental Material 4.

369 (A), 378 (A); and three for ND4 at positions 192 (C), 405 (G), and 459 (T).

Externally, *M. borealis* can be distinguished from *M. indomitus* by having fewer ventral scales (130–132 vs. 133–142); in addition to the fact that the rows of scales in the mid part of the body do not exceed 25 (*M. indomitus* = 23–25, *M. mexicanus* = 22–27, *M. nummifer* = 23–28, *M. occiduus* = 23–27, *M. olmec* = 22–26); with regard to *M. mexicanus* and *M. olmec*, *M. borealis* can be distinguished because the supraocular scales are never divided, whereas in the other two species they can be; the postorbital stripe is narrower with respect to those of *M. mexicanus* and *M. nummifer*, covers a lower number of temporal scales (*M. borealis* = 3–5, *M. indomitus* = 4, *M. mexicanus* = 1–7, *M. nummifer* = 4–8, *M. occiduus* = 5–6, *M. olmec* = 3–6); has a lower average number of interocular scales (*M. borealis* = 2–3, *M. indomitus* = 4–5, *M. mexicanus* = 2–5, *M. nummifer* = 2–5, *M. occiduus* = 4–5, *M. olmec* = 4–6), and a higher number of scales in contact with the supraoculars (*M. borealis* = 4–6, *M. indomitus* = 4–5, *M. mexicanus* = 3–5, *M. nummifer* = 3–5, *M. occiduus* = 4, *M. olmec* = 3–5). *Metlapilcoatlus borealis* has a completely dark pigmentation in the last third of the body after the cloacal scale, as opposed to other species, where this region is black with light spots.

In addition to the differences in external morphology, the more distinctive traits are found in the hemipenial morphology, where *M. borealis* presents a strongly thickened *sulcus spermaticus* that turns ventro-diestrally to the apex of the lobes, while it is a thin structure that runs longitudinally along the center of the hemipenes sulcate side in the rest of the species of *Metlapilcoatlus*; the calyculate area in the interior region of the sulcate side of the hemipenis is laid out obliquely with respect to the spinous area, unlike in the rest of the species where the division of the spinous area and the calyculate area cuts transversally across the sulcate side of the hemipenis, forming a groove shaped like a “V”. The exception to this is *M. occiduus*, where there is no division of the calyculate area, since this species does not appear to have spines in the hemipenes; *M. borealis* has the smallest hemipenis of the entire genus, even among similarly sized males (*M. borealis* HL = 13.21, HW = 2.21; *M. indomitus* HL = 27 mm, HW = 10.5 mm; *M. mexicanus* HL \geq 20 mm, HW = 11.25 mm; *M. nummifer* = HL \geq 16 mm, HW = 9 mm; *M. occiduus* HL \geq 20 mm, HW = 7.5 mm; *M. olmec* HL = 20 mm, HW = 10 mm). *Metlapilcoatlus borealis* can be distinguished from *M. mexicanus*–*M. nummifer*–*M. olmec* by its higher number of spines at the base of the lobes (*M. borealis* = 6, *M. indomitus* = 13, *M. mexicanus* = 4, *M. nummifer* = 3, *M.*

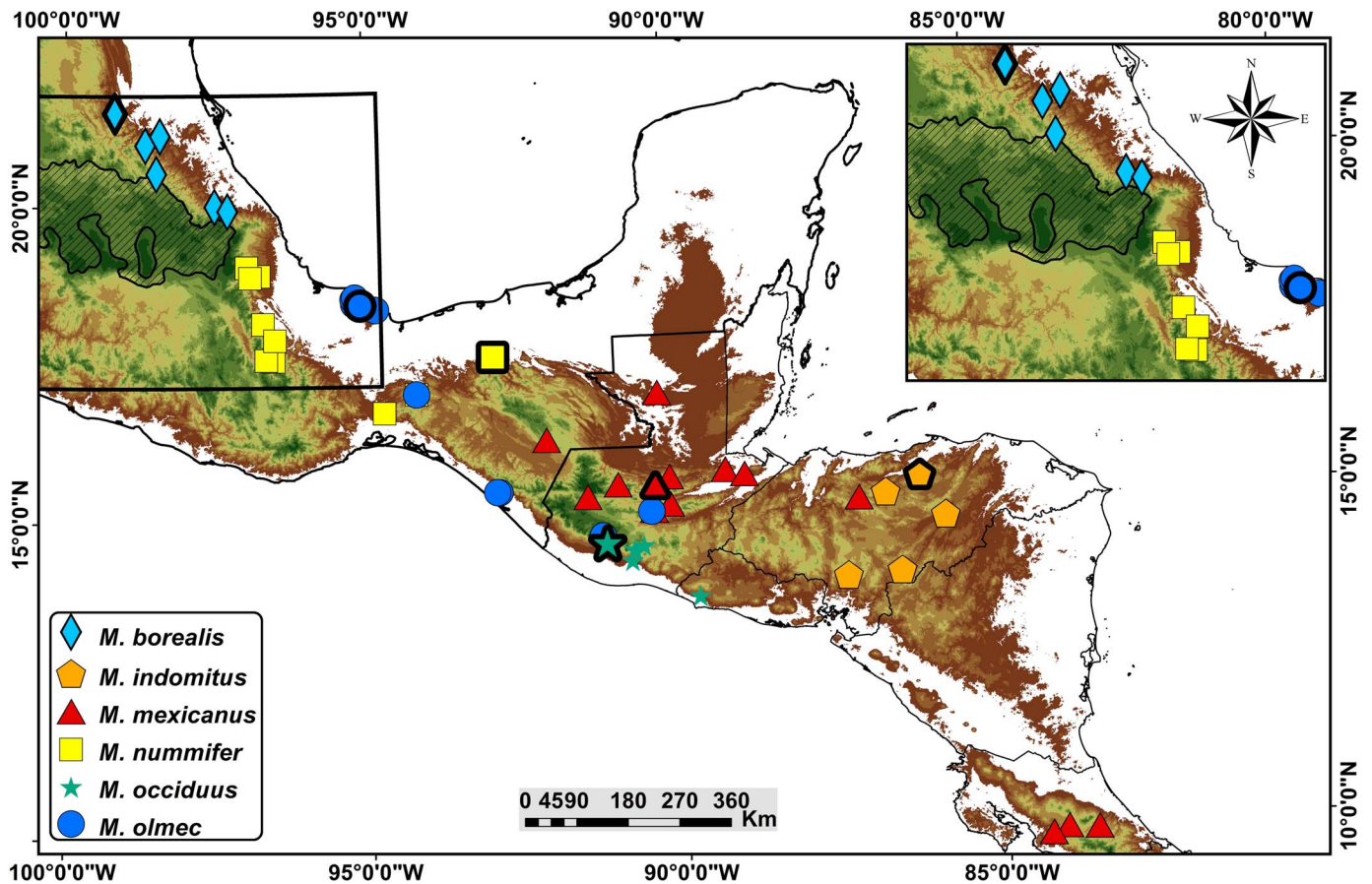


FIG. 6. Distribution of the specimens analyzed in this study. (A) Distribution of all species of the genus *Metlapilcoatlus* from Costa Rica to Mexico and location of the specimens used of the species *M. nummifer* (black box, solid line). (B) The two northernmost clades formed by the specimens of *M. borealis* and *M. nummifer* are showed in the black box. Shaded region represent the Transmexican Volcano Belt (TVB).



FIG. 7. (A) *Metlapilcoatlus borealis* specimen from the locality of San Juan de los Durán, in Jalpan de Serra, Querétaro, (B) dorsal view of the holotype of *M. borealis*, (C) ventral view of the holotype of *M. borealis*. All photographs correspond to MZFC-35381 from San Juan de los Durán, Municipality of Jalpan de Serra, Querétaro. Photographs by Óscar Flores-Villela (A) and Mauricio Tepos Ramírez (B and C).

occiduus = 0, *M. olmec* = 3) and a higher number of rows of calyces in the lobes (*M. borealis* = 6, *M. indomitus* = 13, *M. mexicanus* = 4, *M. nummifer* = 3, *M. occiduus* = 0, *M. olmec* = 3).

Holotype and Description.—Female adult holotype features are followed by male adult paratype variation in parentheses. Rostral wider than longer, 5.7 × 3.5 mm (7.2 × 3.1), concave, rounded apex reaching the canthal crest; four nasorostral scales (6) that allow extensive contact between rostral and nasal scale at both sides; prefoveals 4/6 (3/4); subfoveals (3/3), and postfoveals 2/2 (4/4); wide prelacular and extended towards the eye, in contact with both preoculars and loreals; prelacunar scale narrow in its inferior part, which grows wider and projects internally in the pit; loreals 1/1, in contact with superior preocular; preocular 2/2 (2/2), superior preocular enlarged, inferior elongated towards the anterior part of the head, reaching the half of the pit; suboculars 1/1 (1/1); postoculars 3/1 (4/3); supralabial 9/10 (9/10); mental wider than longer, 5 × 4 mm (5.5 × 3.8); infralabials 12/11 (9/10); canthals $\frac{3}{4}$ (3/3); simple, big, and narrow supraocular, 4.6/5 mm (5.7/4.5); head dorsal scales keeled; scales between the first canthals 4 (4); intersupraoculars 9 (8); interrials 25 (27); 3–4 pairs of gulars between the chin shields and first ventral; rows of dorsal scales 28–25–20 (24–25–20), keeled; ventrals 129; single anal scale; subcaudals 35 (38), not divided.

Coloration in Life.—Collected specimens of *M. borealis* presented a base coloration ranging from orange to dark brown. Dorsal and lateral blocks always darker than the base color, and sometimes the fringes of the blocks had darker colorations. Ventral coloration went from light yellow and white hues to yellow and orange in the gular region.

Etymology.—The specific epithet, *borealis*, references the northernmost distribution of this taxon with respect to the other taxa of *Metlapilcoatlus*.

Distribution and Ecology.—*Metlapilcoatlus borealis* is restricted to the tropical montane cloud forest in the confluence zone of the states of Hidalgo, San Luis Potosí, Querétaro and Veracruz.

Remarks.—*Metlapilcoatlus borealis* populations have differentiated from those of the group consisting of *M. mexicanus*–*M. nummifer*–*M. olmec* because of their isolation, which was likely brought about by two of the great biogeographical barriers that the TMVB and Sierra Madre Oriental pose to the genus *Metlapilcoatlus* distribution. However, it is possible that it is sympatric with *M. nummifer*, specifically in zones of mid and low elevations in central and south Veracruz, below the TMVB.

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