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RESOLUCIÓN TAXONÓMICA DE *Moxostoma austrinum* Bean, 1880 (Cypriniformes Catostomidae) A TRAVÉS DE UNA APROXIMACIÓN DE TAXONOMÍA INTEGRATIVA

TESIS

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RESUMEN

Moxostoma austrinum es una especie de pez de agua dulce ampliamente distribuida en las cuencas del centro de México, con una población disjunta en el norte de México. Actualmente, se han descrito tres taxones (*M. austrinum, M. milleri* y *M. mascotae*), y se han identificado dos linajes diferenciados, potencialmente nuevas especies: *Moxostoma* cf. *austrinum* del río Santiago y *Moxostoma* cf. *austrinum* del río Conchos. Sin embargo, el estatus taxonómico de algunas poblaciones (por ejemplo, *M. mascotae* y *M. milleri*) ha variado a lo largo de los años debido a la alta variación interpoblacional de *M. austrinum*, lo que genera una falta de caracteres diagnósticos que puedan ayudar a diferenciarlos con precisión. Por lo tanto, se realizó un análisis filogenético y de delimitación de especies utilizando genes mitocondriales y nucleares, incluyendo diferentes poblaciones de *M. austrinum*. Se utilizaron caracteres merísticos y morfométricos para identificar caracteres diagnósticos. Los análisis de delimitación de especies revelaron un patrón de diferenciación para *M*. cf. *austrinum* de Santiago, *M*. cf. *austrinum* de Conchos, *M. milleri* y *M. mascotae*; además, se encontraron caracteres diagnósticos (merísticos y morfométricos), respaldados significativamente por análisis univariados, para *M*. cf. *austrinum* de Santiago, *M*. cf. *austrinum* de Conchos y *M. mascotae*. Con base en lo anterior, se proponen *M*. cf. *austrinum* de Santiago y *M*. cf. *austrinum* de Conchos como nuevas especies, y *M. mascotae* es validada como especie.

Palabras clave: Delimitación de especies, genética, morfología, peces dulceacuícolas, evolución.

ABSTRACT

Moxostoma austrinum is a species of freshwater fish widely distributed through river basins of central Mexico with a disjointed population in northern Mexico. Currently, three taxa (*M. austrinum*, *M. milleri*, and *M. mascotae*) have been described, and two differentiated lineages, potentially new species, have been identified, *Moxostoma cf. austrinum* from the Santiago River and *Moxostoma cf. austrinum* from the Conchos River. However, the taxonomic status of some populations (e.g., *M. mascotae* and *M. milleri*) has varied over the years due to the high interpopulation variation of *M. austrinum*, which brings a lack of diagnostic characters that could help differentiate them accurately. Thus, a phylogenetic and species delimitation analysis was carried out using mitochondrial and nuclear genes, including different populations of *M. austrinum*. Meristic and morphometric characters were used to identify diagnostic characters. Species delimitation analyses revealed a differentiation pattern for *M. cf. austrinum* from Santiago, *M. cf. austrinum* from Conchos, *M. milleri*, and *M. mascotae*; additionally, diagnostic characters (meristic and morphometric) were found, significantly supported by univariate analyses, for *M. cf. austrinum* from Santiago, *M. cf. austrinum* from Conchos, and *M. mascotae*. Based on the above, *M. cf. austrinum* from Santiago and *M. cf. austrinum* from Conchos are proposed as new species, and *M. mascotae* is validated as a species.

Keywords: Species delimitation, genetics, morphology, freshwater fish, evolution.

INTRODUCTION

Taxonomy is the discipline responsible for identifying, describing, classifying, and naming new species and taxa. It provides the methodological foundations that allow the recognition and interpretation of biological diversity to delineate the different units in which living organisms are structured and organized (Iriondo, 2000; Dayrat, 2005). For taxonomy, the study of evolutionary biology is of utmost importance since the main objective of evolutionary biology is to elucidate the origin and natural history of living beings to analyze and understand the processes that have led to the emergence of new life forms (Perea, 2017). Understanding the causes that give rise to a taxon and determining its evolutionary trajectory allows us to delimit the various units in which living organisms are structured and organized more efficiently (Padial et al., 2010). That is how taxonomy expands its horizons towards integrative taxonomy, which seeks to prioritize identifying and delimitating new species over creating new species names (Dayrat, 2005). From the above, we can infer that one of the most important aspects of taxonomy and integrative taxonomy is the delimitation of the boundaries between different species. Species delimitation is the area that focuses on determining the boundaries between lineages and integrates various tools and methods to obtain data on different aspects of an individual's biology and thus discover new species (Dayrat, 2005; Padial et al., 2010).

Given the above, the use of phylogenetic systematics based on molecular characters together with other approaches, such as population genetics (Muñiz-Castro et al., 2020), analysis based on coalescent theory (Fujita et al., 2012), and comparative analysis using morphological characters, as well as other sources of evidence used in an integrative way, have proven to be an efficient strategy for delimiting morphologically cryptic species (Padial et al., 2010).

In this research, our model is *Moxostoma austrinum,* a freshwater fish, part of the southern group of the genus *Moxostoma* (Pérez-Rodríguez et al., 2016). which has a wide distribution and inhabits various basins in central and northern Mexico (Pérez-Rodríguez et al., 2016). Like other species of *Moxostoma,* the history of its taxonomic classification has been unstable and not very robust. From the classification based on meristic and linear morphological characters by Robins and Raney (1957), to the incorporation of genetic information, there have been changes and instability.

For instance, Robins and Raney (1957) considered populations from the Ameca River as *M. mascotae*, populations from the Lerma-Santiago hydrographic system, and the Mezquital River as *M. austrinum*. Additionally, they proposed two subspecies for *M. austrinum*: *M. austrinum austrinum* in the Lerma-Santiago system, and *M. austrinum milleri* in the Mezquital River. Later, Smith (1992) suggested considering the populations of Lerma River, Grande de Santiago River, Conchos River, and Mezquital River as *M. austrinum*.

However, studies based on molecular phylogenies suggest genetic differentiation among *M. austrinum* populations from the Conchos, Lerma, and Santiago rivers (Harris et al., 2002; Clements et al., 2012; Pérez-Rodríguez et al., 2016; Bagley et al., 2018). The remote and disjunct geographical location of the northern population of the Bravo-Conchos relative to the rest of the populations of *M. austrinum*, and its apparent morphological differentiation (Miller et al., 2005), along with its phylogenetic position that breaks the monophyly of *M. austrinum*, strongly question its taxonomic validity as *M. austrinum* (Clements et al., 2012; Pérez-Rodríguez et al., 2016; Bagley et al., 2018). Some studies recognize the Ameca River population as *M. mascotae* (Harris et al., 2002; Bagley et al., 2018), while other authors question its validity as a species (Miller et al., 2005). Regarding the population of the Mezquital River, considered as the subspecies *M. austrinum milleri* (Clements et al., 2012), it is recognized as a genetically differentiated lineage and recommended to be elevated to the species level as *M. milleri* (Pérez-Rodríguez et al., 2016). Finally, the population of Santiago River has shown high genetic differentiation compared to other members of *M. austrinum* (Harris et al. 2002; Clements et al. 2012; Pérez-Rodríguez et al. 2016). Additionally, the incorporation of new entities due to the increasing of geographic sampling has brought a lack of robustness, such as the population of Armería River, originally considered as *M. austrinum,* the sister group of *M. mascotae*, leading to the synonymization of *M. austrinum* (Clements, et al., 2012).

Based on the above, this study aims to conduct a taxonomic review of *M. austrinum* through an integrative taxonomy approach, incorporating phylogenetic analyses, population genetics, and species delimitation based on different genes (mitochondrial and nuclear) to determine whether the previously recorded genetic differentiation in populations and even subspecies allows for establishing species-level boundaries. Additionally, the study analyze morphological variations among different lineages to determine if the pattern of variation is consistent with species delimitation and propose a taxonomic arrangement that contributes to resolving the taxonomy of *M. austrinum*.

MATERIALS AND METHODS

Sampling

The sampling included individuals from all river basins comprising the entire distribution range of *M. austrinum* and *M.* cf. *austrinum* from Santiago and Conchos, which are well-differentiated lineages in central and northern Mexico: Lerma River, Grande de Santiago River, Ayuquila River and Conchos River (appendix S1; Fig. 1). In addition, *M. mascotae*, *M. milleri*, *M. albidum* and *M. congestum*, species closely related to *M. austrinum* were included (appendix S1). For the morphological analyses, only individuals of *M. austrinum* and their well-differentiated lineages were included (appendix S1; Fig. 1). Samples used on both the genetic and morphological analyses were obtained from the Colección de Peces de la Universidad Michoacana (CPUM), and supplemented with sequences from supplementary material of Bagley et al. (2018) and images from Robins and Raney (1957).

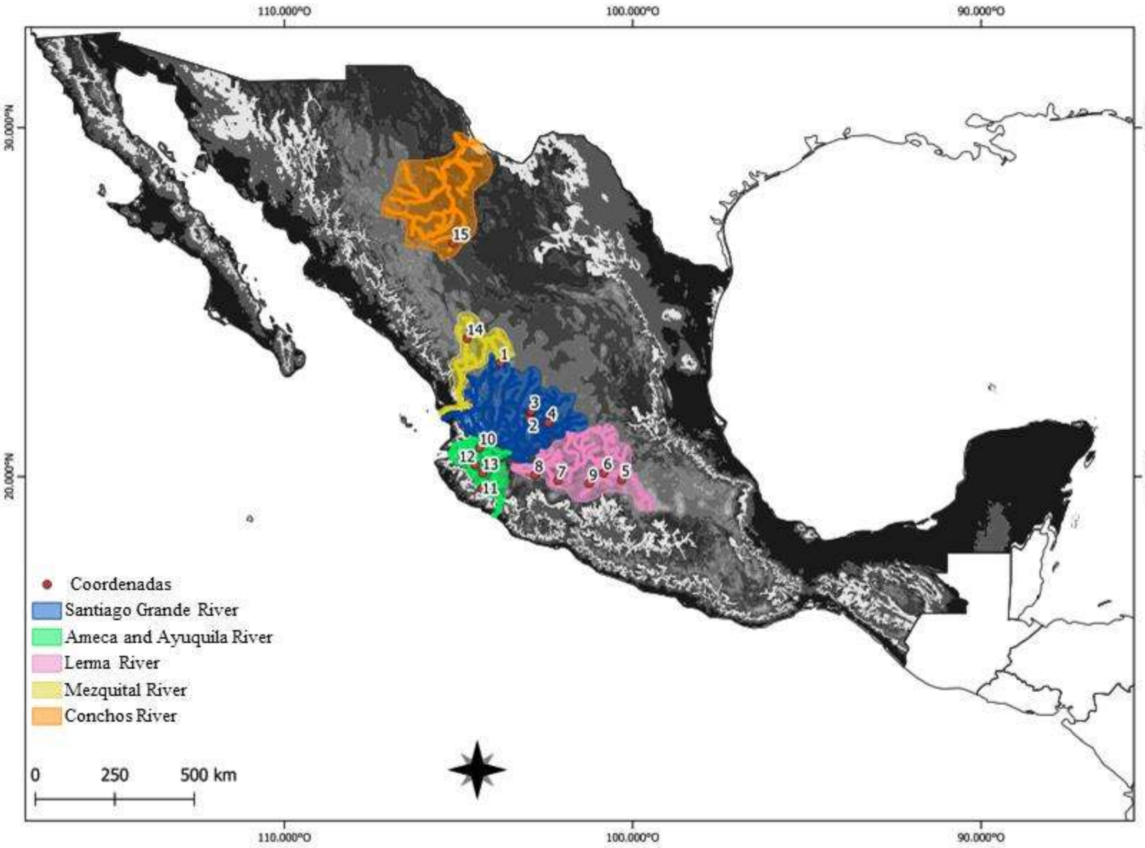

Figure 1. Sampling locations on the river basins comprising the distribution range of *M. austrinum* (Lerma and Ayuquila River) and including the well-differentiated lineages *M.* cf. *austrinum* from Santiago (Santiago River) and *M.* cf. *austrinum* from Conchos (Conchos River), and locations for the other taxa included *M. mascotae* (Ameca rivers), *M. milleri* (Mezquital River), *M. albidum* (San Felipe River and Soto la Marina River).

Sequencing

The genomic DNA of each individual was obtained using the Phenol-Chloroform extraction method, as described in Pérez-Rodríguez et al. (2016). The mitochondrial gene Cytochrome b (*cytb*), the nuclear ribosomal protein S7 intron 1 (*RPS7*), and the nuclear gene Growth Hormone Intron (*GHI*) were amplified using Polymerase Chain Reaction (PCR), following the same primers and protocols as tested for catostomids (Bagley et al., 2018). After gene amplification, the sequences were sent to Macrogen Korea for sequencing. In addition, sequences for *cytb, RPS7*, and *GHI* were retrieved from supplementary material of Bagley et al. (2018) for the outgroups.

Phylogenetic analyses

The sequences obtained were aligned using ClustalW (Thompson et al., 1994) as implemented in MEGA 7 software (Kumar et al., 2016), and were checked and manually corrected. The substitution models that best fit the mitochondrial and nuclear data sets were obtained by Jmodeltest 2 program (Darriba et al., 2012), considering the Bayesian information criterion (BIC). To obtain a phylogenetic hypothesis that includes a taxonomic sampling not considered by previous studies, a phylogenetic reconstruction analysis was carried out based on a concatenated matrix for the *cytb, RPS7,* and *GHI* genes, and using both methods, Bayesian inference and Maximum Likelihood. This hypothesis corresponds to the starting point to make the groupings to test with the different delimitation methods. Bayesian and Maximum Likelihood analyses were performed by Mr.Bayes v3.2 (Ronquist et al., 2012) and RAXML v. 8.2.12 (Stamatakis, 2014) as implemented in CIPRES (Miller et al., 2010). For both analyses, *Moxostoma albidum, M.* cf. *albidum* from Soto la Marina*, M congestum, M. poecilurum, M. duquesnei, M. valeniennesi, Scartomyzon rupiscartes, Thoburnia atripinnis, Hypentelium roanokense, Erimyzon tenuis, Myxocyprinus asiaticus* were used as outgroup.

Species delimitation

Three different species delimitation methods were used to assess the species-level ranking of the previously revealed well-differentiated lineages within *M. austrinum* (Clements et al., 2012; Pérez-Rodríguez et al., 2016; Bagley et al., 2018). The different lineages and related species recovered at the phylogenetic hypothesis were used as Operational Taxonomic Units (OUTs).

The first method involved a species tree constructed using multiple loci under the coalescent model in BEAST (StarBEAST v 1.8.4). This method assumes that the discordance among gene trees is due to incomplete lineage sorting, resolving this conflict by estimating each gene tree individually and importing this data to infer a species tree. This analysis was performed on BEAST 1.8.4 (Drummond et al., 2016), and the input file was prepared in BEAUTi v 1.8.4 (Drummond et al., 2016) with the following parameters: using a speciation tree prior (Yule Process); the independent lognormal relaxed clock was applied to each partition, for analyses we run 20×10^7 generations. Convergence was assessed with TRACER 1.7 (Rambaut et al., 2018). TreeAnnotator version 1.8.4 (Drummond et al., 2016) was used to build maximum clade credibility trees, after discarding the first 10% of generations by burn-in.

The third method was the Bayesian species delimitation analysis of multiple loci conducted on Bayesian Phylogenetics and Phylogeography (BPP) v3.4 (Yang, 2015). This method employs a multispecies coalescent model to compare the posterior probabilities of different species delimitation models and classifies lineages based on ancestral polymorphisms. The program generates posterior probabilities for each lineage and indicates if the previously assigned groups are different from each other. Two analyses were implemented: A10, a species delimitation analysis based on a guide tree (in this case, the species tree generated in BEAST), and A11, which performs species delimitation and with species tree inference or species delimitation without a guide tree (Yang and Rannala, 2010; Rannala and Yang, 2013). Different scenarios were tested by changing the values of ancestral population effective size (theta θ) and divergence time (tau τ), as previous studies have demonstrated, results can vary depending on the modification of these parameters (Pinacho-Pinacho et al., 2018). The tested scenarios were: large population effective size ($\theta=0.2$) and large divergence times ($\tau=0.4$); small population effective size ($\theta=0.002$) and small divergence times ($\tau=0.004$); large population effective size ($\theta=0.2$) and small divergence times ($\tau=0.004$); small population effective size ($\theta=0.002$) and large divergence times ($\tau=0.4$) (Yang and Rannala, 2010; Rannala and Yang, 2013).

Finally, a Species Tree and Classification Estimation Yarely, STACEY v.1.3.1 (Jones, 2017) was performed. The STACEY analysis is a Bayesian method based on the multispecies coalescent model (MSC) (Prebus, 2020), which uses MSC to infer a tree with the minimum number of species or clusters. This tree employs the birth-death-collapse parameter without the requirement of a guide tree and incorporates the birth-death-collapse model from the species delimitation method DISSECT (Jones et al., 2015). The input file was prepared in BEAUTi v 2.7.6 (Drummond et al., 2012) with the following set of parameters: strict clock was enforced and a birth-death-collapse tree prior; Collapse Height 1.0E-4; Birth Diff Rate 100; Relative Death Rate 0.5; Collapse Weight 0.5; Origin Height 100. The MCMC analysis was run for 15^7 generations, saving results every 95000 generations. The convergence and ESS values were checked in Tracer v 1.7. (Rambaut et al., 2018). The obtained files were processed in Species Delimitation Analyser with a Collapse Height $1*10^{-4}$ and a similarity cut-off of 1.0, after discharging the 10% of the analyses as burn-in. For all four analyses, *Moxostoma congestum* was used as outgroup.

Morphologic analysis

To find diagnostic characters, between *M. austrinum*, its well-differentiated lineages, and the taxonomically questioned *M. mascotae*, a morphological comparison was conducted using 10 meristic and 17 morphological (lineal measurements) characters (fig. 2, appendix S2) based on those used to diagnose species within the genus *Moxostoma* (Robins y Raney, 1957). To ensure only adult organisms were analyzed, only those individuals measuring ≤ 10 cm in standard length were considered (Robins & Raney, 1957). Stereoscopic magnifying glasses were used to count the meristic characters. Linear measurements were obtained with a Mitutoyo digital caliper (0.001 mm). To minimize the effect of the size of the specimens on the linear measurements, each measurement was standardized in proportion to the standard length (measurements/SL), calculating the relative value of each character to the standard length of the specimen and presenting the results as times in the standard length (TSL; appendix S2).

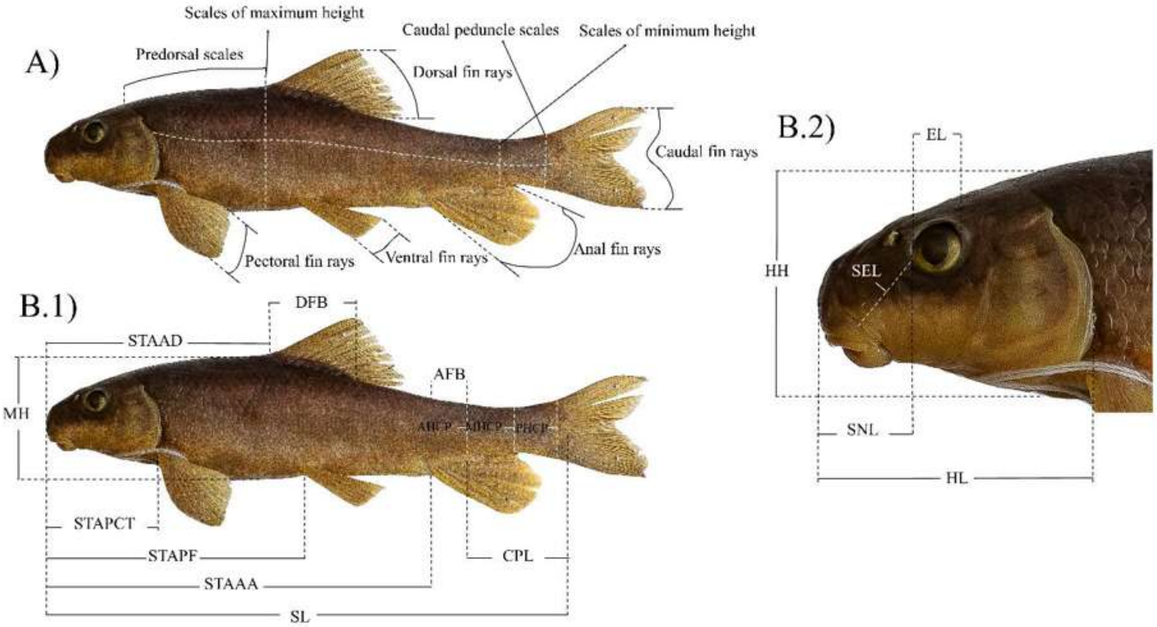

Figure 2. A) meristic characters; B.1) morphological measurements of the body, B.2) and head. Standard length (SL); maximum body height (MH); maximum head length (HL) and height (HH); eye diameter (EL); snout length (SNL); caudal peduncle length (CPL), anterior (AHCP), middle (MHCP), and posterior (PHCP) heights of the caudal peduncle; distance from the snout tip to the anterior edge of the pelvic (STAPF), pectoral (STAPCT), dorsal (STAAD), and anal fins (STAAA); dorsal fin base (DFB) and anal fin base (AFB); snout to eye length (SEL). Specimen: *M.* cf. *austrinum* from Juchipila; number of catalogue from CPUM: 5734.

To determine a differentiation pattern in each meristic and morphologic character a non-parametric Kruskal-Walli's test supported by the Mann-Whitney U test was carried out as well as descriptive statistics such as mean, standard deviation, maximum and minimum values. Statistical tests and graphs were carried out in Past 4.09 (Hammer et al. 2001).

RESULTS

Sequence data set

For each gene (*cytb*, *RPS7,* and *GHI*) 40 collected individuals and 11 from supplementary material of Bagley et al. (2018) were used (Table S1). Heterozygous individuals for degenerate bases were identified and included in the analyses (Table 1).

Table 1. Substitution models of evolution, number of heterozygous individuals, and gen size for *cytb*, *RPS7,* and *GHI.*

	cytb	*RPS7*	*GHI*
Length	1064	584	746
Substitution models	TN+F+I	F81+F	F81+F+I
number of heterozygous		13	11

Phylogenetic reconstruction and Species delimitation

The phylogenetic reconstruction using the concatened matrix using *cytb*, *RPS7,* and *GHI* revealed that *M. austrinum* does not correspond to a monophyletic group (Fig. 3). However, the main clade recovered seven groups reciprocally monophyletic. Maximum Likelihood Bootstrap values ranged from 73% to 100% and Bayesian posterior probability values ranged between 0.99 and 1, supporting the five well-differentiated OTUs of *M. austrinum* which supports all relationships between the seven clades (fig. 3). The phylogenetic hypothesis recovered the four species formally described, *M. albidum*, *M. milleri*, *M. austrinum,* and *M. mascotae*, as well as three well-differentiated lineages *M.* cf. *austrinum* from Santiago, M. cf. *austrinum* from Conchos, and *M.* cf. *albidum* from Soto la Marina (fig. 3).

With strong support at the nodes, *M.* cf. *austrinum* from Conchos is recovered in the basal position of the phylogenetic reconstruction, excluding the outgroups. Additionally, the Conchos River population is not within the *M. austrinum* clade. The populations from the Santiago River were divided into two clades: *M.* cf. *austrinum* from Juchipila and *M.* sp. cf. *austrinum* from Verde-Huaynamota. The populations from the Ayuquila River were recovered as part of *M. mascotae*; this species, in turn, was identified as a sister species to *M. austrinum*.

All the methods of species delimitation supported all four described taxa, *M. austrinum, M. mascotae, M. milleri,* and *M. albidum*, and the well-differentiated lineages *M.* cf. *austrinum* from Juchipila, *M.* cf. *austrinum* from Verde-Huaynamota, *M.* cf. *austrinum* from Conchos, and *M.* cf. *albidum* from Soto la Marina (fig. 3).

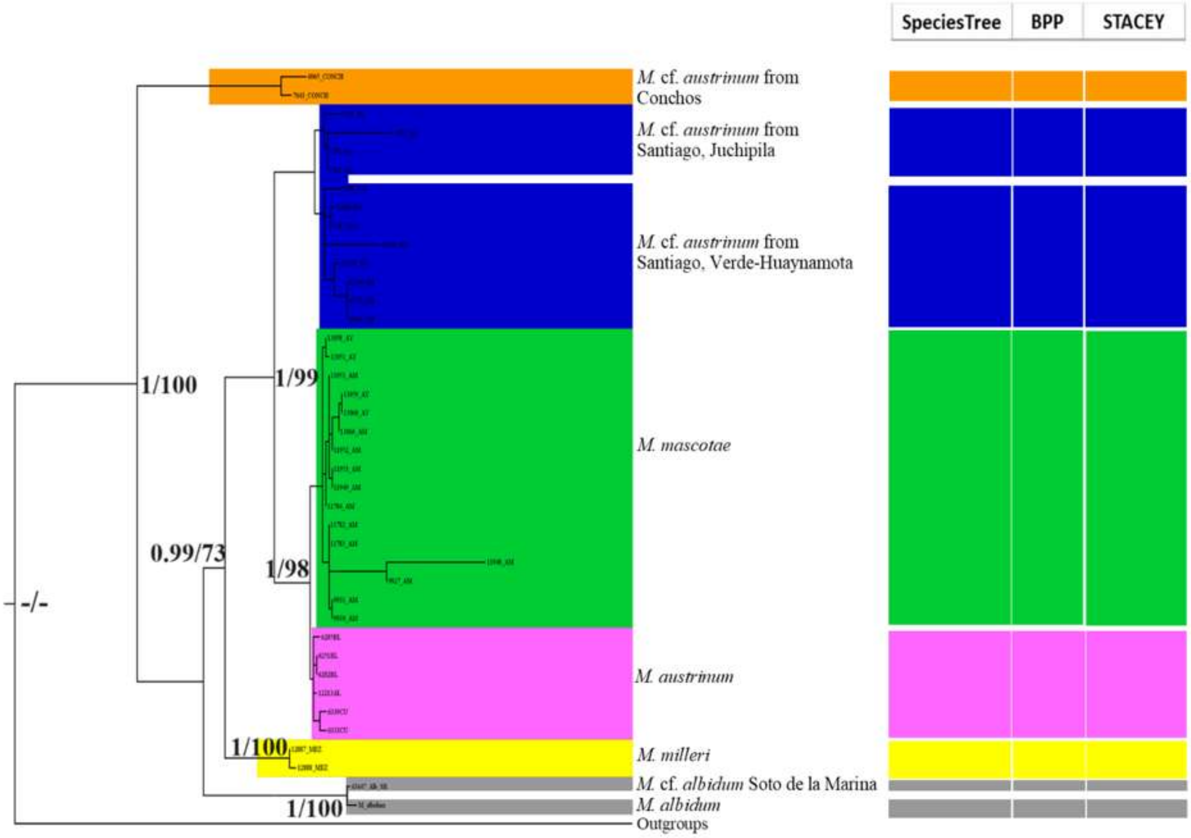

Figure 3. Bayesian Inference and Maximum Likelihood Phylogenetic Reconstruction of *M. austrinum* based on the concatened matrix (*cytb*, *RPS7,* and *GHI*). Nodal support values are displayed at the nodes (Bayesian posterior probabilities/bootstrap). The colored bars represent different groupings obtained for each species delimitation analysis using the genes *cytb*, *RPS7,* and *GHI*. The bars indicate the species delimitation analyses: Species Tree, BPP (A10 and A11), and STACEY.

In the present study, for the *BEAST species tree, the Bayesian posterior probability values ≥ 0.95 were considered highly supported, between 0.90 and 0.94 moderately supported, and < 90 poorly supported. Based on the above, all seven OTUs corresponding to the seven clades of the phylogenetic hypothesis resulted as moderately to highly supported (Fig. 4). According to the cladogenetic events dating, the divergence of the OTUs occurred approximately 6.71 million years ago (My) (fig. 3), while the most recent event corresponds to the divergence of *M.* sp. cf. *austrinum* from Juchipila and *M.* sp. cf. *austrinum* from Verde-Huaynamota, occurred approximately 0.32 My.

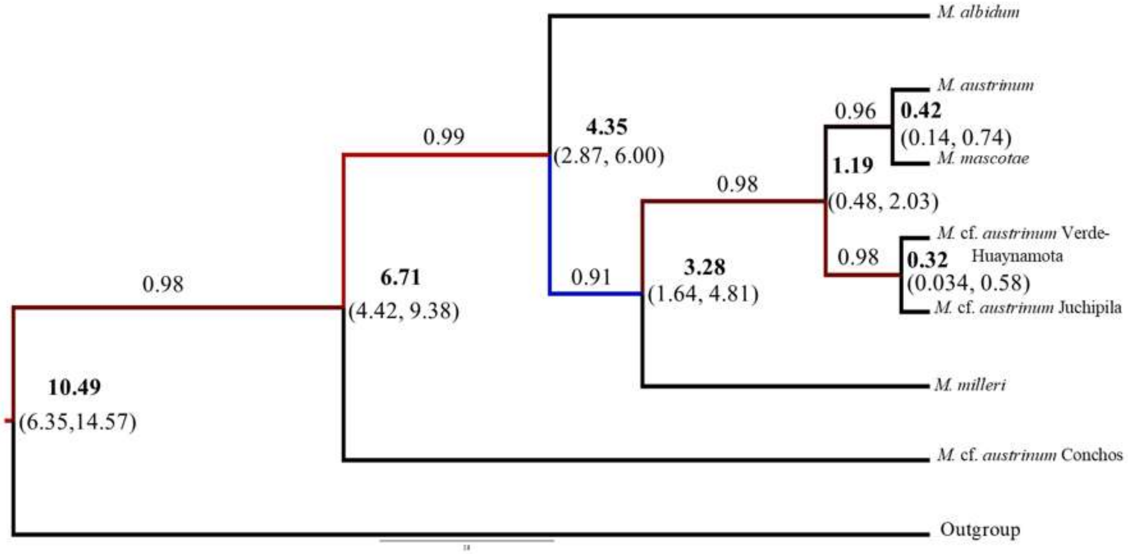

Figure 4. Species tree using the three loci (*cytb*, GHI, and S7). The numbers above the branches represent the posterior probability values for the species tree.

In the BPP, the A00 analysis revealed low effective population sizes for the *Moxostoma* OTUs (appendix S3). Therefore, considered supports for taking account are limited only to those scenarios involving a small θ (Table 2). However, posterior probability values in A10 and A11 analyses were significant for all OTUs in the scenario of a small population effective size (θ = 3,0.002) and small divergence times (θ = 3, 0.004) (Table 2). Results from STACEY for the global test, where we defined seven minimal clusters, according to the hypothesis obtained in both phylogenetic reconstructions, showed high support (PP = 0.99).

Table 2. Posterior probabilities for analyses A10 and A11, with bold values supporting each OTU. Mma: *M. mascotae*; Mau: *M. austrinum*; MsaJ: *M.* sp. cf. *austrinum* from Juchipila; MsaVH: *M.* sp. cf. *austrinum* from Verde-Huaynamota; Mco: *M.* sp. *cf. austrinum* Conchos; Mmi: *M. milleri*.

Analyses	***Scenarios***	*Mau*	*Mma*	MsaJ	*MsaVH*	*Mmi*
A10	θ(3, 0.2); τ(3, 0.4)	0.000	0.000	0.000	0.000	0.008
	θ(3,0.002); τ(3,0.004)	**1.000**	**1.000**	**0.904**	**0.904**	**1.000**
	θ(3, 0.2); τ(3, 0.004)	0.000	0.000	0.000	0.002	0.02
	θ(3,0.002); τ(3, 0.4)	**0.997**	**0.997**	**0.465**	**0.465**	**1.000**
A11	θ(3, 0.2); τ(3, 0.4)	0.005	0.000	0.005	0.002	0.000
	θ(3,0.002); τ(3,0.004)	**0.992**	**0.996**	**0.925**	**0.940**	**0.966**
	θ(3, 0.2); τ(3, 0.004)	0.010	0.008	0.001	0.001	0.000
	θ(3,0.002); τ(3, 0.4)	**0.885**	**0.994**	**0.501**	**0.549**	**0.846**

Morphological analyzes

The organisms sampled for each data set with their respective localities are shown in appendix 3. 200 samples were included from Robins and Raney (1957). From Grande de Santago River (Verde River) 90 individuals were included, from Ayuquila River 39 individuals, from Ameca River 47 individuals, and from Mezquital River 27 individuals.

Meristic variables

Univariate analyses Kruskal-Wallis and Mann-Whitney tests showed significant differences on four characters: (1) anal fin rays (*M.* sp. *cf. austrinum* Conchos has 7 to 8 rays, while the rest of the OTUs has 7 rays or fewer); (2) caudal peduncle scales, (*M.* sp. *cf. austrinum* Santiago has 7 to 8 rays, while the rest of the OTUs has 8 or more), (3) the minimum height (*M.* sp. *cf. austrinum* Santiago has 10 to 11 scales, *M.* sp. *cf. austrinum* Conchos 8 to 9 scales, and *M. mascotae* and *M. austrinum* have 9 to 10 scales), and (4) maximum height (*M. austrinum* has 14 to 16 scales, *M. mascotae* and *M.* sp. *cf. austrinum* Conchos have 15 to 17 scales, and *M.* sp. *cf. austrinum* Santiago has 16 to 17 scales) (Tables 3 and 4). No meristic data was obtained for populations of the Juchipila River from Grande de Santiago River, hence only *M.* sp. cf. *austrinum* from Verde-Huaynamota was included in the analysis.

Table 3. Counts of the meristic characters for the OTUs of *M. austrinum*. Mean ($\bar{X}$), standard deviation (SD), p-value from the Kruskal-Walli's test. Significant differences are shown in bold. Mma: *M. mascotae*; Mau: *M. austrinum*; MsaVH: *M.* sp. cf. *austrinum* from Verde-Huaynamota; Mco: *M.* sp. *cf. austrinum* Conchos; Mmi: *M. milleri*.

Character/OTU		***MsaVH***	***Mma***	***Mau***	***Mco***	***Mmi***	**Kruskal-Wallis**
Caudal fin rays	Range	18	18	18	18		N/A
	$\bar{X}$	18	18	18	18		
Anal fin rays	Range	7-6	8-7	7-6	8-7	7-7	**0.000**
	$\bar{X}$	6.98	7	6.94	7.2	7	
	SD	0.11	0.15	0.23	0.46	0	
Ventral fin rays	Range	11-8	10-8	10-8	10-8	9-9	0.710
	$\bar{X}$	9.13	9.14	9.16	9	9	
	SD	0.44	0.39	0.51	0.44	0	
Pectoral fin rays	Range	18-15	19-15	18-14	17-16	17-16	**0.001**
	$\bar{X}$	16.26	16.76	16.16	16.63	16.15	
	SD	0.82	0.91	0.98	0.50	0.37	
Dorsal fin rays	Range	12-10	11-11	13-11	11-11	12-11	**0.000**
	$\bar{X}$	11.06	11	11.44	11	11.81	
	SD	0.43	0	0.61	0	0.39	
Caudal peduncle scales	Range	10-7	10-7	11-8	9-7		**0.008**
	$\bar{X}$	7.9	8.68	8.66	8.54		
	SD	0.83	0.79	0.76	0.82		
Scales of minimum height	Range	11-9	10-8	10-8	9-7		**0.000**
	$\bar{X}$	10.14	9.18	9.05	8.36		
	SD	0.57	0.54	0.41	0.67		
scales of maximum height	Range	17-16	17-15	16-14	17-15		**0.000**
	$\bar{X}$	16.42	15.81	15.11	16.09		
	SD	0.50	0.91	0.67	0.83		
Predorsal scales	Range	19-16	20-17	20-16	20-17		0.240
	$\bar{X}$	17.85	18.37	18.5	18.36		
	SD	0.83	1.20	1.29	1.12		
Lateral line scales	Range	51-42	49-42	49-45	53-46	49-45	**0.001**
	$\bar{X}$	46.31	45.72	46.5	49.18	46.8	
	SD	1.82	1.70	0.92	2.40	1.48	

Table 4. Pairwise comparison between OTUs of *M. austrinum* OTUs, showing p-values from the Mann-Whitney test for meristic characters. Values with significant differences are indicated in bold. Mma: *M. mascotae*; Mau: *M. austrinum*; MsaVH: *M.* sp. cf. *austrinum* from Verde-Huaynamota; Mco: *M.* sp. *cf. austrinum* Conchos; Mmi: *M. milleri*.

Character/OTU		*Mma*	*Mau*	*Mco*	*Mmi*
Anal fin rays	*MsaVH*	0.143	0.283	**0.000**	0.861
	Mma		0.136	**0.007**	0.812
	Mau			**0.022**	0.723
	Mco				0.301
Pectoral fin rays	*MsaVH*	**0.000**	0.991	0.605	0.987
	Mma		0.020	0.990	**0.002**
	Mau			0.574	1.000
	Mco				**0.009**
Dorsal fin rays	*MsaVH*	0.312	**0.005**	0.610	**0.000**
	Mma		**0.000**	1	**0.000**
	Mau			**0.021**	**0.011**
	Mco				**0.000**
Caudal peduncle scales	*MsaVH*	**0.006**	**0.004**	**0.032**	
	Mma		0.717	0.866	
	Mau			0.817	
	Mco				
Scales of minimum height	*MsaVH*	**0.000**	**0.000**	**0.000**	
	Mma		0.414	**0.003**	
	Mau			**0.003**	
	Mco				
scales of maximum height	*MsaVH*	**0.027**	2.158E-06	0.269	
	Mma		0.034	0.414	
	Mau			0.004	
	Mco				
Lateral line scales	*MsaVH*	0.281	0.162	**0.000**	0.322
	Mma		0.124	**0.000**	0.218
	Mau			**0.004**	0.658
	Mco				0.085

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Morphometric characters

ANOVA test supported by Tukey's post hoc analysis shows significant differences for the 11 characters: four corresponding to the head (maximum head length and height, eye diameter, and snout length); and seven corresponding to the rest of the body (standard length, anterior, middle, and posterior heights of the caudal peduncle, distance from the snout tip to the anterior edge of the pelvic, and anal fins, dorsal fin base (Tables 5 and 6).

Table 5. Morphometric characters for the OTUs of *M. austrinum*. Mean (X̄), standard deviation (SD), and ANOVA p-values. Values with significant differences are shown in bold. All measurements were taken in mm. Standard length is presented in raw data, while the rest of the variables are presented standardized in proportion to standard length. Mma: *M. mascotae*; Mau: *M. austrinum*; MsaJ: *M.* sp. cf. *austrinum* from Juchipila; MsaVH: *M.* sp. cf. *austrinum* from Verde-Huaynamota; Mco: *M.* sp. *cf. austrinum* Conchos. Character code descriptions in figure 2.

Character/OTU		***MsaJ***	***MsaVH***	***Mma***	***Mau***	***Mco***	**ANOVA**
SL	Range	142.56-202.59	149.3-220.67	105.27-166.21	102.26-189.35	149.35-187.21	**0.000**
	X̄	168.56	192.14	126.65	139.44	170.6	
	SD	24.12	23	16.59	24.94	19.35	
MH	Range	3.74-4.29	3.54-4.51	3.72-4.54	3.94-4.77	3.88-4.14	0.380
	X̄	3.98	4.15	4.08	4.2	4.03	
	SD	0.22	0.21	0.27	0.22	0.13	
HL	Range	3.91-4.31	3.93-4.89	3.83-4.5	3.98-4.65	4.68-5.01	**0.000**
	X̄	4.13	4.32	4.2	4.32	4.84	
	SD	0.14	0.26	0.17	0.19	0.16	
HH	Range	5.29-6.19	5.33-5.97	5.26-6.13	5.48-6.71	5.44-5.97	**0.006**
	X̄	5.73	5.64	5.71	5.97	5.72	
	SD	0.32	0.17	0.25	0.31	0.26	
EL	Range	20.11-24.71	19.08-26.17	18.65-23.6	19.74-25.31	18.26-22.66	**0.004**
	X̄	22.18	21.75	20.22	22.14	20.47	
	SD	1.53	1.87	1.43	1.46	2.2	
SNL	Range	9.92-11.23	10.06-12.88	8.72-10.86	7.93-12.08	10.42-11.35	**0.000**
	X̄	10.67	11.27	9.8	10.15	10.82	
	SD	0.500	0.75	0.6	1.16	0.47	
CPL	Range	6.42-7.39	6.37-8.27	6.25-8.27	6.37-8.28	6.83-8.26	0.360
	X̄	7	7.35	7.01	6.99	7.42	
	SD	0.36	0.57	0.5	0.53	0.74	
AHCP	Range	9.09-10.71	8.23-9.69	8.4-9.54	8.79-10.68	8.58-9.01	**0.000**
	X̄	9.98	9	9.14	9.71	8.82	
	SD	0.55	0.41	0.31	0.51	0.22	
MHCP	Range	10-11.92	9.59-11.16	9.8-11.8	10.29-11.51	9.43-9.98	**0.000**
	X̄	10.94	10.28	10.39	10.88	9.75	

	SD	0.74	0.40	0.6	0.4	0.28	
PHCP	Range	9.06-9.91	8.55-9.47	7.98-9.85	8.72-11.19	8.59-9	**0.000**
	X̅	9.56	9.04	9.11	9.76	8.83	
	SD	0.27	0.30	0.49	0.57	0.21	
STAPF	Range	1.85-1.94	1.78-1.98	1.82-1.92	1.82-1.98	1.8-1.87	**0.039**
	X̅	1.89	1.85	1.85	1.89	1.83	
	SD	0.03	0.05	0.03	0.043	0.035	
STAPCT	Range	3.69-4.11	3.53-4.16	3.72-4.01	3.6-4.13	3.7-3.81	0.196
	X̅	3.89	3.78	3.86	3.88	3.75	
	SD	0.16	0.16	0.09	0.16	0.05	
STAAD	Range	2.05-2.16	1.96-2.19	1.96-2.16	1.93-2.22	1.99-2.08	0.138
	X̅	2.09	2.03	2.04	2.07	2.04	
	SD	0.04	0.07	0.05	0.07	0.04	
STAAA	Range	1.26-1.31	1.24-1.3	1.25-1.33	1.26-1.33	1.26-1.3	**0.045**
	X̅	1.28	1.27	1.28	1.29	1.28	
	SD	0.01	0.01	0.02	0.02	0.02	
DFB	Range	5.95-6.52	6.11-7.46	6.03-7.55	5.81-6.95	6.65-7.25	**0.000**
	X̅	6.32	6.77	6.8	6.32	6.9	
	SD	0.19	0.34	0.43	0.36	0.3	
AFB	Range	9.22-12.62	9.75-13.85	10.16-14.05	10.38-13.58	11.45-12.66	0.070
	X̅	11.07	12.35	11.57	11.96	12.22	
	SD	1.12	1.17	1.14	0.9	0.67	
SEL	Range	13.57-16.3	13.04-17.89	12.46-15.89	11.82-17.96	14.28-17.59	0.141
	X̅	14.71	15.65	14.41	14.74	15.83	
	SD	1.03	1.49	1.01	1.89	1.66	

Table 6. Pairwise comparison between OTUs of *M. austrinum* OTUs with Tukey's test for morphometric characters. Values with significant differences are indicated in bold Mma: *M. mascotae*; Mau: *M. austrinum*; MsaJ: *M.* sp. cf. *austrinum* from Juchipila; MsaVH: *M.* sp. cf. *austrinum* from Verde-Huaynamota; Mco: *M.* sp. *cf. austrinum* Conchos. Character codes description in figure 2.

Character/OTU		***MsaJ***	***Mma***	***Mau***	***Mco***
HL	*MsaVH*	0.115	**0.000**	**0.000**	0.536
	MsaJ		**0.000**	**0.000**	0.999
	Mma				**0.000**
	Mau				0.181
HL	*MsaVH*	0.249	0.465	1	**0.001**
	MsaJ		0.960	0.256	**0.000**
	Mma			0.475	**0.000**
	Mau				**0.001**
HH	*MsaVH*	0.919	0.922	**0.006**	0.987
	MsaJ		0.999	0.236	1
	Mma			0.06	1
	Mau				0.553
EL	*MsaVH*	0.970	0.081	0.958	0.723
	MsaJ		0.057	1	0.530
	Mma			**0.014**	0.999
	Mau				0.482
SNL	*MsaVH*	0.448	**0.000**	**0.002**	0.903
	MsaJ		0.135	0.590	0.998
	Mma			0.782	0.311
	Mau				0.696
AHCP	*MsaVH*	**0.000**	0.900	**0.000**	0.968
	MsaJ		**0.000**	0.619	**0.002**
	Mma			**0.006**	0.784
	Mau				**0.019**
MHCP	*MsaVH*	**0.037**	0.977	**0.015**	0.466
	MsaJ		0.122	0.998	**0.010**
	Mma			0.076	0.288
	Mau				**0.008**
PHCP	*MsaVH*	0.069	0.994	**0.000**	0.936
	MsaJ		0.151	0.827	0.120
	Mma			**0.001**	0.854
	Mau				**0.012**
STAAA	*MsaVH*	0.590	0.208	**0.021**	0.903
	MsaJ		0.997	0.798	1
	Mma			0.879	0.996
	Mau				0.897
DFB	*MsaVH*	**0.045**	0.999	**0.007**	0.975
	MsaJ		**0.029**	1	0.135
	Mma			**0.004**	0.991
	Mau				0.088

DISCUSSION

The species delimitation analyses including the members of the southern group of *Moxostoma* (Pérez-Rodríguez et al., 2016), support the presence of six well-differentiated lineages. Four of them correspond to the formally described species, *M. albidum*, *M. milleri*, *M. austrinum,* and *M. mascotae*, and the remaining three to undescribed species, *Moxostoma* sp. from Conchos, and two lineages from Santiago River basin, *Moxostoma* sp. 1 Santiago (from Verde and Huaynamota rivers), and *Moxostoma* sp. 2 Santiago (from Juchipila river) (Fig. 3). In addition to the species delimitation, diagnostic characters were determined from comparing different morphological variables between *M. austrinum sensu stricto* and the two potential undescribed species. However, because the Juchipila River was not sampled for morphological analysis, diagnostic characters could not be obtained for the two lineages found in the Río Grande de Santiago with the species delimitation analysis.

Taxonomic implications of the classification of M. austrinum

In this research, we used the species concept proposed by Queiroz (2007) "separately evolving metapopulation lineages", which takes into consideration the criteria of phylogenetic (through the monophyly) and diagnosability, considered operational criteria for recognizing species-level taxa (Aleixo, 2023) and that adjusted with the lines of evidence used herein. Although the present study included members of the southern group of *Moxostoma* (Pérez-Rodriguez et al., 2016), whose species were validated by the delimitation analyses, the relevant taxonomic findings were focused on the problematic classification of *M. austrinum*. Therefore, based on the main findings, the shortcomings of the classification of this taxon, such as the instability and the lack of robustness were solved as depicted ahead, 1) using phylogenetic and coalescent criteria, the validation of the four lineages well-differentiated, two corresponding to the species *M. austrinum sensu stricto* and *M. mascotae*, and the remaining one undescribed specie from Conchos River and two undescribed lineages from Grande Santiago River: *Moxostoma* sp. from Conchos, *Moxostoma* sp. 1 from Santiago, and *Moxostoma* sp. 2 from Santiago, and 2) the determination of potential diagnostic characters for these three undescribed lineages previously mentioned.

Distribution range and historical biogeography

By the geographic range of the southern group of *Moxostoma* genus, its members, including the described taxa and the undescribed species, are encompassed in two ichthyographic provinces from North America, the Grande River and Central Mexico (Burr and Mayden, 1999). The findings of this study contribute to the increase of richness and level of endemism in the two provinces. If well samples from the upper and lower parts of the Lerma River, as well as Lakes Chapala and Cutizeo, were included, our study lack of samples from the type locality of *M. austrinum* (Piedad in Morelia, Michoacán (Bean 1880 in Robins and Raney, 1957), which is located in the middle part of the Lerma River. Based on this geographic sampling, the species *M. austrinum* is limited to the Lerma-Chapala hydrographic system, including Cuitzeo Lake (fig. 1), which can be considered a sub-basin of the Lerma-Chapala system as they share a relatively recent history of connection (Miller et al., 2005).

For the formally described *M. mascotae,* Robins and Raney (1957) and previously limited to the Ameca River basin, our results of species delimitation supported it as a valid taxon, corroborating previous studies based on a mitochondrial marker: *cytb* (Harris et al. 2002), two mitochondrial markers: *cytb* and ND2, and three nuclear markers: IRBP, S7, and GHI (Bagley et al. 2018). In this regard, the main contribution of this study is that the population of Ayuquila-Armería River, previously considered as *M. austrinum* (Clements et al., 2012), was supported as a population of *M. mascotae*. With this validation as a species, the distribution range of *M. mascotae* expands, therefore it can no longer be considered endemic to the Ameca River basin. This distribution pattern is shared with other fish species such as *Ilyodon fursidens* (Beltrán-López et al., 2017) and *Poeciliopsis infans* (Miller, et al., 2005), supporting a recent connection between both basins. The divergence between *M. austrinum* and *M. mascotae* dates back to 0.46 My (0.12-0.80 My) and corresponds to the most recent complete speciation event within the populations of *M. austrinum*.

Regarding the one undescribed specie from Conchos River and two undescribed lineages from Grande Santiago River: 1) *Moxostoma* sp. from Conchos resulted restricted to the Conchos River basin in Chihuahua (Fig. 1). This proposed new taxon is added to the other cases of endemisms formally described as *Cyprinella panarcys, Cyprinodon julimes, Gambusia alvarezi, Notropis chihuahua, Lepomis cyanellus* (De la Maza Benignos, 2009) as well as the other undescribed well-differentiated lineages representing potential endemisms for Río Conchos (e. g., *Campostoma ornatum*, Domínguez-Domínguez et al., 2011 and Schonhuth et al., 2011; *Pantosteus plebeius*, Corona-Santiago et al., 2018); 2) *Moxostoma* sp. 1 from Santiago, its distribution is restricted to two tributaries of the Grande de Santiago River, Verde, and Huaynamota rivers. Finally, *Moxostoma* sp. 2 from Santiago, distribution is restricted to one tributary of the Grande de Santiago River to Juchipila River, other freshwater fish species are similarly restricted to Juchipila River, such as *Algansea monticola* (Barbour and Miller, 1994).

Speciation process

According to the divergence times (10.49 My), the speciation processes began approximately during the Miocene when a connection occurred between the Conchos River and the Grande River, allowing the common ancestor of the *M. austrinum* complex to expand its distribution range through a dispersal process. Subsequently, a vicariance event led to the isolation of *Moxostoma* sp. from Conchos.

The common ancestor of the *M. austrinum*, *M. mascotae*, *Moxostoma* sp. from Santiago expanded along the Pacific west slope, reaching central Mexico during the late Pliocene through the Santiago River. Once colonized various ancestral basins, a series of tectonic events associated with the extension of the Tepic-Zacoalco Rift during the Pleistocene possibly caused the disruption of populations in the different ancestral basins of the Santiago and Lerma-Ameca rivers (Pérez-Rodríguez et al., 2016). This likely led to the isolation of populations in the Santiago and Lerma-Ameca rivers due to various events, such as the formation of waterfalls in the Santiago River, the Ameca River, and elsewhere (Barbour, 1973), and the climatic changes caused by glacial and interglacial periods (Vázquez-Selem and Heine, 2011). Therefore, we can conclude that the speciation processes within this group appear to be strongly related to geographic and climatic processes that promote the connection and disconnection between basins (allopatric processes). Although a biogeographic analysis was not conducted, the pattern of allopatry, even among populations of some taxa, indicates that the primary driver of diversification within the *M. austrinum* complex was vicariance events, without discounting the contribution of dispersal, as previously revealed (Pérez-Rodríguez et al., 2016).

Conservation implications

It is important to highlight the conservation implications that this reclassification entails; the distribution range for *Moxostoma sp*. from Conchos is restricted only to the Conchos River, which represents new endemism for the basin; on the lineages found and restricted in the Río Grande de Santiago, *Moxostoma sp.* 1 from Santiago and *Moxostoma sp.* 2 from Santiago, represent two new endemisms for the basin. Due to the above, we consider that these three species should be protected, in addition to this providing new elements to determine areas designated for conservation in Mexico.

In the case of *Moxostoma mascotae*, it is a species classified as vulnerable by IUCN (Lyons, 2019), its distribution was reported only for the Ameca River, with our findings we consider that the distribution range of *M. mascotae* increases to the Ayuquila River, thus, *M. mascotae* should be considered as an endemic and vulnerable species of Mexico that lives in the Ameca River and Ayuquila River basins.

CONCLUSION

With the delimitation of the well-differentiated lineages that corresponded to the species formally described, and the determined diagnostic characters useful for the description of two new species as proposed herein: *Moxostoma* sp. from Conchos, and *Moxostoma* sp. from Santiago, this study contributed to solving the classification of the taxon *M. austrinum*. Also, the new species of the genus *Moxostoma* contributes to increasing the richness and endemism of two ichthyographic provinces of North America, Grande River and Central Mexico.

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Supplementary information

Appendix S1. Geographic distribution range of analyzed sequences. Samples from CPUM

Species	**Localityt**	**river basin**	**voucher**
Moxostoma sp. from *Santiago*	1. Atengo River, at Jiménez del Teul town, Zac., Mex.	Grande de Santiago-Huaynamota	42589
			42590
			42591
			42592
	2. Juchipila river, Jalpa town, Zac. Mex.	Grande de Santiago-Juchipila	5781
			5782
			5783
			5784
	4. Verde River, Belén del Refugio, Jal., Méx.	Grande de Santiago-Verde	5907
			5908
	Verde River, road Tepa-Yahualilca, Tepatitlán		51908
			51909
M. austrinum	5. Lerma river, Pateo town, Mich. Mex.	Lerma upper river	12213

	7. Celio River, Molino Viejo at Jacona town, Mich. Mex.	Lerma lower river	6251
	Lerma river, El Platanal town, Mich. Mex		6282
			6285
	9. Stream at Chiquimitio town, Morelia, Mich. Mex.	Cuitzeo lake	6333
			6339
M. mascotae	10. Chiquito river, Amatlan Town, Nay. Mex.	Ameca river	11948
	Potrero Grande, Jal., Mex.		9927
			9930
			9931
	Salado river, Tala town, Jal., Mex.		11782
			11783
			11784
	12. Atenguillo, Jal., Mex.	Ayuquila river	13051
			13058
			13059
			13060
	13. Atengo river at Ayutla Town, Jal. Mex.		13066
M. milleri	14. Tunal river, Tres Molinos, Durango, Dur. Mex.	Mezquital	12887
			12888
M. sp. *from Conchos*	Florido river, Villa Coronado, Jimenez, Chi. Mex.	Conchos	6965

	15. El Porvenir river, Balleza, Chi. Mex.		7043
Moxostoma cf. albidum	Santa Rosa, Tamaulipas, Mex.	Soto de la Marina	43447

Appendix S2. Geographic distribution range for morphological data. Samples from CPUM

Species	**Localityt**	**river basin**	**voucher**	**meristic**	**morphometrics**
Moxostoma sp. from *Santiago*	1. Atengo River, at Jiménez del Teul town, Zac., Mex.	Grande de Santiago-Huaynamota	13594	18	
	3. Juchipila River, under the bridge Balmines at 600 m at northwest of Tabasco, Mex.	Grande de Santiago-Juchipila	7650		8
	Verde River, road Tepa-Yahualilca, Tepatitlán, Jal., Mex	Grande de Santiago-Verde	13692	3	3
M. austrinum	5. Lerma River at 700 m west of Pateo town, Mich., Mex.	Lerma	4294	3	5
			16593		13
			2493	3	
			13861	3	
			7218	1	
	6. Celio River, Molino Viejo, Jacona town, Mich., Mex.		2325		2
	Duero River, El Platanal town, Mich., Mex.		2317		4

	9. Dam La Mintzita, Morelia, Mich., Mex.		7223	1	
			8439	1	
M. mascotae	10. Chiquito river, Amatlan Town, Nay. Mex.	Ameca	4335	5	10
	11. Ahuacapán town, Jal., Mex.	Ayuquila	4117	10	4
	13. Atengo River, Atengo town, Jali., Mex.	Ameca	2005	1	1
M. sp. *from Conchos*	15. El Porvenir river, Balleza, Durango, Mex.	Conchos	13612	3	3
	Florido river, Villa Coronado, Jimenez, Chi. Mex.		8411	8	

Appendix S3. Probabilistic values for analyses A00,. Mma: *M. mascotae*; Mau: *M. austrinum*; MsaJ: *M.* sp. cf. *austrinum* from Juchipila; MsaVH: *M.* sp. cf. *austrinum* from Verde-Huaynamota; Mco: *M.* sp. *cf. austrinum* Conchos; Mmi: *M. milleri*; Mal: *Moxostoma albidum*

	Theta_Mma	**Theta_MsaJ**	**Theta_MsaVH**	**Theta_Mmau**	**Theta_Mmi**	**Theta_Mco**	**Theta_albidum**	**lnl**
Mean	0.072	0.091	0.081	0.086	0.096	0.046	0.041	-5490.575
Median	0.049	0.069	0.060	0.066	0.073	0.040	0.036	-5490.319
S.D.	0.090	0.078	0.072	0.070	0.079	0.025	0.020	9.540
2.5%hpd	0.008	0.016	0.013	0.016	0.017	0.015	0.014	-5509.218
97.5%hpd	0.194	0.225	0.204	0.215	0.235	0.093	0.080	-5472.123
ESS*	253.900	4608.900	3314.900	1298.700	5185.000	24357.800	31986.100	176.700
EFF*	0.003	0.046	0.033	0.013	0.052	0.244	0.320	0.002

Identificación de reporte de similitud: oid:3117:371894917

NOMBRE DEL TRABAJO	AUTOR
RESOLUCIÓN TAXONÓMICA DEL COMPLEJO DE DESPECIES Moxostoma austrinum Bean, 1880 (Cypriniformes catost	Mariana Raya Aguiar

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Datos del solicitante

Nombre y firma	Mariana Raya Aguiar
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