Organisms Diversity & Evolution
https://doi.org/10.1007/s13127-019-00419-x

ORIGINAL ARTICLE

Phylogeographic patterns and species delimitation in the endangered silverside *"humboldtianum"* clade (Pisces: Atherinopsidae) in central Mexico: understanding their evolutionary history

Isai Betancourt-Resendes[1,2] · Rodolfo Perez-Rodríguez[3,4] · Irene De Los Angeles Barriga-Sosa[5] · Kyle R. Piller[6] · Omar Domínguez-Domínguez[3,4]

Received: 17 March 2019 / Accepted: 25 September 2019

Abstract

The Atherinopsidae is the second largest group of freshwater fishes occupying central Mexico and is one of biological, cultural, and economic importance. The *"humboldtianum"* clade (Genus *Chirostoma*) is a "species flock" of nine described species that inhabit lacustrine ecosystems in central Mexico. The high morphological polymorphism within the group makes species identification difficult and thereby limits the development of research and management projects focusing on this group. In this study, we used phylogeographic and coalescent-based methods to understand the evolution of genetic variation among these species. The results revealed taxonomic inaccuracies and genetic admixture among species. Genetic variation was structured geographically, rather than taxonomically, and five closely related genetic groups were recovered. Two evolutionary pathways were found. First, a novel geographical arrangement of haplotypes was recovered that gave rise to the five recently (Pleistocene, < 1 Myr) derived genetic groups. The second pathway showed a recent intra-lacustrine genetic differentiation that could be associated with sympatric or ecological speciation. The current classification of the group is revised and includes a reduction in the number of valid species in the *"humboldtianum"* clade. Moreover, this study provides new insight into the biogeography and evolutionary history of this important group of fishes.

Keywords Species flock · Phenotypic plasticity · Recent divergences · Taxonomic inaccuracy · Polymorphism

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13127-019-00419-x) contains supplementary material, which is available to authorized users.

✉ Omar Domínguez-Domínguez
goodeido@yahoo.com.mx

[1] Programa Institucional de Doctorado en Ciencias Biológicas, Facultad de Biología, Universidad Michoacana de San Nicolás de Hidalgo, Francisco J. Mujica S/N, 58030 Morelia, Michoacán, México

[2] CONACYT-Facultad de Ciencias Naturales, Universidad Autónoma de Querétaro, Av. De las Ciencias s/n, Santiago de Querétaro, México

[3] Laboratorio de Biología Acuática, Facultad de Biología, Universidad Michoacana de San Nicolás de Hidalgo, Francisco J. Mujica S/N, 58030 Morelia, Michoacán, México

[4] Laboratorio Nacional de Análisis y Síntesis Ecológica para la Conservación de Recursos Genéticos de México, Escuela Nacional de Estudios Superiores, Unidad Morelia, Universidad Nacional Autónoma de México, Morelia, Michoacán, México

[5] Laboratorio de Genética y Biología Molecular de la Planta Experimental de Producción Acuícola Departamento de Hidrobiología, Universidad Autónoma Metropolitana Unidad Iztapalapa, Av. San Rafael Atlixco 186 Col. Vicentina, Iztapalapa, CDMX 09340 México, México

[6] Department of Biological Sciences, Southeastern Louisiana University, Hammond, LA 70402, USA

Introduction

Species flock is a term coined to delimit a species-rich group that is endemic and reciprocally monophyletic and shows recent and rapid radiation in sympatry (Ribbink 1984; Schluter 2000; Schluter and Conte 2009; Seehausen 2004). Empirical data supports the concept of species flock in several fish groups, including the rapid radiation of African and Middle American lake cichlids (Barluenga et al. 2006; Wagner et al. 2012; Elmer et al. 2010; Mayer and Matschiner 2015). The high species richness and rapid radiation of these groups have been explained by adaptive radiation through the colonization of different environments (Yoder et al. 2010; Wainwright et al. 2012).

The high atherinopsid's species richness of central Mexico (*Chirostoma* spp.) is associated with the concept of flock speciation (Barbour 1973a; Echelle and Echelle 1984). Within *Chirostoma,* there is a monophyletic assemblage comprising nine species in the "*humboldtianum*" clade (Barbour 1973b; Bloom et al. 2009) that inhabit lacustrine habitats in central Mexico (Barbour 1973a; Bloom et al. 2009, 2013). This group has been supported with morphology and osteology (Barbour and Chernoff 1984), enzymatic characters (Echelle and Echelle 1984), and combined analyses based on morphological, meristic, and genetic data (Barriga-Sosa et al. 2005; Barriga-Sosa et al. 2002). The timing of diversification of this group has been dated to less than 1 Myr (Bloom et al. 2013; Campanella et al. 2015). Furthermore, some have argued that diversification of the group occurred through sympatric speciation due to trophic specialization or to some unknown intrinsic mechanisms of the species (Barbour 1973a, 1973b; Echelle and Echelle 1984; Bloom et al. 2013). However, other studies of trophic segregation in sympatric species of *Chirostoma* from Pátzcuaro and Chapala have failed to show a pattern of trophic differentiation among sympatric species (Moncayo-Estrada et al. 2012; García-de León et al. 2014; Mercado-Silva et al. 2015). The overlap in trophic ecology among sympatric silverside species from Chapala and Pátzcuaro raises questions about the evolutionary processes driving diversification of this morphologically diverse, but genetically and trophically similar, group of species (Mercado-Silva et al. 2015).

The supposed sympatric evolution in the "*humboldtianum*" clade contrasts with the evolutionary history proposed for other freshwater fish groups of central Mexico, in which allopatric speciation has been argued as the primary mechanism of diversification in the region (Doadrio and Domínguez 2004, Dominguez-Dominguez et al. 2006; Domínguez-Domínguez et al. 2008; Domínguez-Domínguez et al. 2010; Pérez-Rodríguez et al. 2009, 2015; Corona-Santiago et al. 2015; Beltrán-López et al. 2018). Additionally, recent molecular studies show that diversification in freshwater atherinopsids outside the genus *Chirostoma* is mainly associated with vicariance, due the emergence of barriers that act to limit gene flow (Bloom et al. 2013; Unmack et al. 2013; Campanella et al. 2015).

The high degree of morphological polymorphism found in the "*humboldtianum*" clade does not concur with the molecular data (Bloom et al. 2009). This incongruence between morphological and molecular data has made it challenging to understand the evolutionary history of the group. In the Atheriniformes, morphological differences within species have been closely linked to habitat adaptations (Fluker et al. 2011; Alarcón-Duran et al. 2017), restricted distributions, intense predation pressure (Unmack et al. 2013), and trophic specialization (Barbour 1973b; Barbour and Chernoff 1984; Echelle and Echelle 1984). Furthermore, the current classification of Atheriniformes has undergone changes at different taxonomic levels (Miller et al. 2005; Bloom et al. 2012; Unmack et al. 2013; Campanella et al. 2015; Betancourt-Resendes et al. 2018) due to inconsistencies between the morphological and genetic data. These changes have provided new insight into the phylogenetic, biogeographic, evolutionary, and diversification processes within the Atheriniformes (Bloom et al. 2012, 2013; Unmack et al. 2013; Campanella et al. 2015), including the "*humboldtianum*" group.

The effort to understand the evolutionary history of this group has previously employed a phylogenetic approach. However, examining the evolutionary process from a tokogenetic perspective and using coalescent-based methods may be useful for understanding the micro-evolutionary processes within species or between closely related species in silverside fishes (Avise 2000; Rannala and Yang 2003; Drummond et al. 2005; Yang and Rannala 2010, 2014; Maio et al. 2015). In the present study, therefore, mitochondrial and nuclear markers were used to address the question of whether the evolutionary history of the *"humboldtianum"* clade is linked to vicariance as a major diversification process or linked to intra-lacustrine speciation processes.

Materials and methods

Specimen collections

Specimens of all of the species in the *"humboldtianum"* clade, as well as several populations of some other species, were collected from fifteen localities in central Mexico (Fig. 1 and Table 1). Geographic units were established as discrete regions following Dominguez-Dominguez et al. (2006), Domínguez-Domínguez et al. 2010) and according to the watershed hydrography. Fin clips were obtained from each specimen and preserved in 96% ethanol. Voucher specimens were identified using the diagnostic morphological and meristic characters of Barbour (1973b), Barriga-Sosa et al. (2002, 2005), and Miller et al. (2005). These were preserved in the

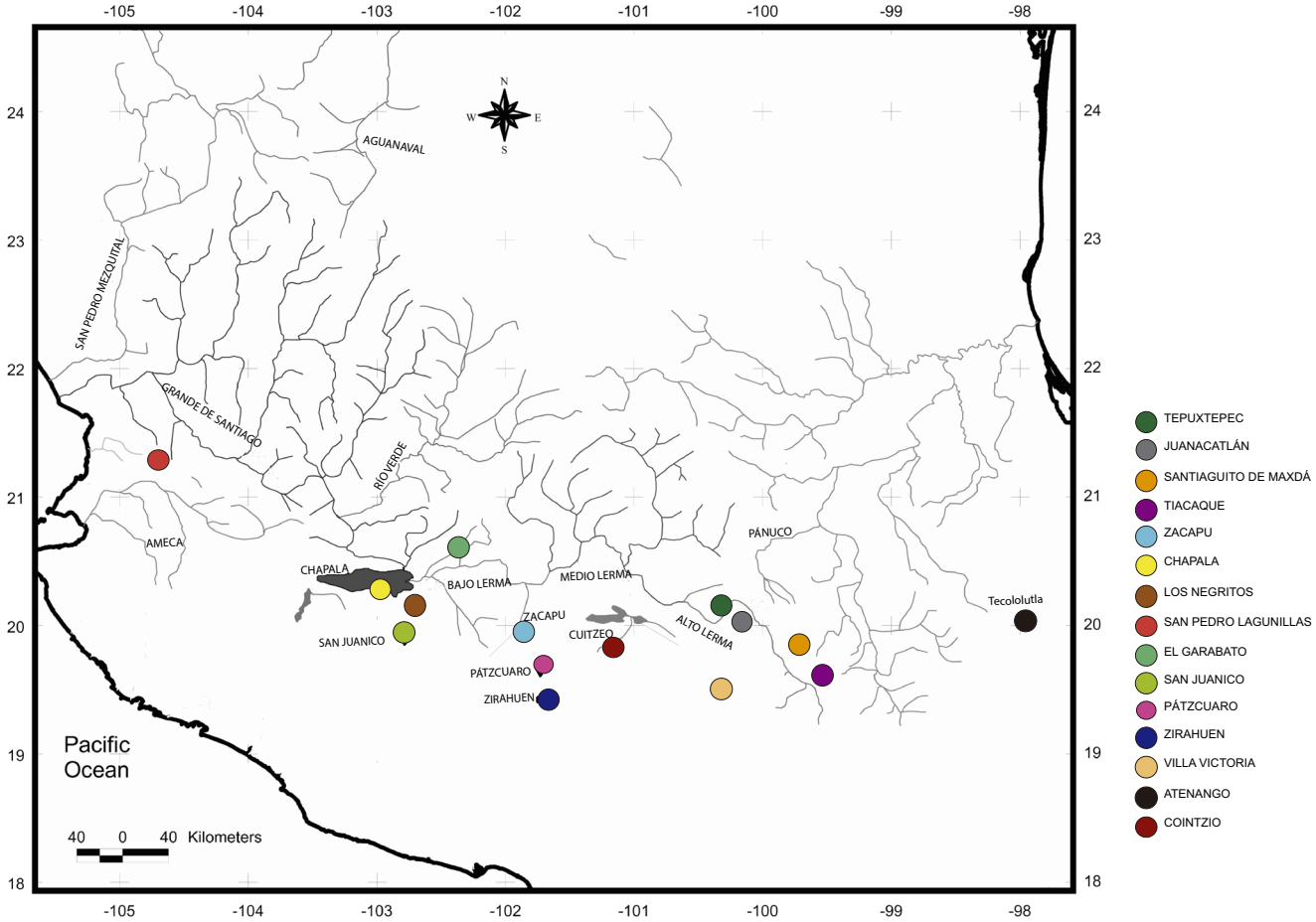

Fig. 1 Sample locations for species of the *"humboldtianum"* clade in central Mexico. Colored circles represent the localities described in the text

Colección de Peces de la Universidad Michoacana (CPUM) (Table 1). In addition, sequences were obtained from GenBank (Table 2).

Sequence data

Total genomic DNA was extracted from fin clips using a phenol-chloroform protocol (Sambrook et al. 1989). Two mitochondrial genes, cytochrome b (*Cytb*); and a fragment of the hypervariable control region (*D-loop*); and one nuclear locus, the first intron of the S7 ribosomal protein gene (*S7*); were amplified using the following primers: *Cytb* Glud-G (Palumbi et al. 1991) and H16460 (Perdices et al. 2002), *D-loop* RCA and RCE described in Lee et al. (1995), and S7 S7RPEX1F and S7RPEX1R, described in Chow and Hazama (1998). The amplification parameters used are provided in Appendix. Amplified products were sequenced in both directions by the high-throughput Genomics Center at Washington University, USA. Nucleotide sequences were edited and manually aligned with MEGA 6 (Tamura et al. 2013), using the complete mitochondrial genome of *Chirostoma humboldtianum* (GenBank reference NC_024883; Barriga-Sosa et al. 2014). The S7 was aligned by CLUSTAL_W 1.83 (Thompson et al. 1997). The alignment of *Cytb* was translated into amino acids to verify the absence of stop codons along the sequence. Nucleotide saturation was tested for *Cytb* using the software DAMBE v5 (Xia 2013), and non-recombination of S7 was performed with the software DNAsp (Librado and Rozas 2009). The sister species of the "*humboldtianum*" group, *C. attenuatum* Meek, was included in the phylogenetic inference (Bloom et al. 2013; Bloom et al. 2009; Campanella et al. 2015). Alignments for each locus were used to estimate and select the substitution model that best fitted the datasets, using partition settings performed in PartitionFinder v.1.1.0 (Lanfear et al. 2012), and the best partition was recovered by assigning a substitution model for each gene.

Haplotype network

Haplotype networks were generated to examine the intraspecific relationships within the *"humboldtianum"* clade. This was conducted in Network 4.6.1.3 using a median-joining algorithm. This method constructs networks from recombination-free population data and combines features of

Table 1 Sampling localities and sequence information for the three molecular markers *Cytb*, *D-loop,* and *S7* intro

Locality	Biogeographic region	Taxon	*Cytb* sequences	D-*loop* sequences	*S7* sequences
Zirahuén Lake	Zirahuén	*C. estor*	14	12	4
Pátzcuaro Lake	Pátzcuaro	*C. estor*	17	15	7
	Pátzcuaro	*C. grandocule*	10	10	10
	Pátzcuaro	*C. patzcuaro*	5	5	7
	Pátzcuaro	*Chirostoma* sp.	8	11	0
Chapala Lake	Chapala	*C. sphyraena*	44	33	8
	Chapala	*C. lucius*	15	15	10
	Chapala	*C. promelas*	8	9	5
	Chapala	*C. consocium*	10	10	10
	Chapala	*C. chapalae*	5	5	5
	Chapala	*Chirostoma* sp.	18	12	7
Zacapu Lake	Zacapu	*C. humboldtianum*	8	1	6
Santiaguito Maxda dam	Upper Lerma	*C. humboldtianum*	4	4	5
San Juanico dam	Balsas	*C. humboldtianum*	2	2	2
Garabato dam	Santiago	*C. consocium*	1	0	0
Negritos Lake	Chapala	*C. lucius*	3	3	2
San Pedro Lagunillas dam	Santiago	^*C. humboldtianum*	4	3	4
Juanacatlán dam	Upper Lerma	*C. humboldtianum*	5	3	4
Tepuxtepec dam	Upper Lerma	*C. humboldtianum*	9	2	7
Tiacaque dam	Upper Lerma	*C. humboldtianum*	4	1	4
Villa Victoria dam	Balsas	*C. humboldtianum*	3	3	2
Atenango dam	Tecololutla	*Chirostoma* sp.	3	5	3
Cointzio dam	Cuitzeo	*C. estor*	4	5	0
Total			*204*	*176*	*113*

[1] Discrete regions proposed for Domínguez-Domínguez et al. 2010. Based in goodeids endemism ^Samples given by PExPA (UAM-I)

Kruskal's algorithm for determination of minimum spanning trees by favoring short connections and Farris's maximum parsimony heuristic algorithm (Bandelt et al. 1999).

Species tree calibration and species delimitation analyses

To assess the different evolutionary scenarios, a multi-locus species tree was generated under a coalescent model in *BEAST v.1.8 (Heled and Drummond 2010). Four assignments were tested: (1) a morphological species criteria (Barbour 1973b; Barriga-Sosa et al. 2005; Bloom et al. 2013) and groupings based on the (2) *S7*, (3) *Cytb*, and (4) *D-loop* haplotype networks performed herein. A Yule process model species tree prior and a constant population size species model tree were chosen using an uncorrelated relaxed molecular clock. The 1% mutation rate for the *Cytb* gene was used according to the rate estimated for Atheriniformes (Unmack et al. 2013; Campanella et al. 2015). Since no mutation rate was found for the *D-loop* and *S7*, this mutation rate was estimated relative to the *Cytb* by choosing a log normal relaxed clock using a normal prior (0.005 ± 0.01). Two independent runs were conducted for 100,000,000 generations, sampling every 1000 generations. Convergence was evaluated in Tracer v.5, with –InL values and effective sample sizes > 200 (Drummond and Rambaut 2007). The maximum credibility tree was generated in TreeAnnotator v.1.8 and support values for the clades were evaluated by posterior probability values. Analyses were run in CIPRES Science Gateway v 3.3 (Miller et al. 2015).

In order to calculate and compare genetic segregation models of the four and five genetic groups found in the species tree analyses, a Bayes Factor (BF) test was performed using the marginal-likelihood value (MLE) approach. This method is useful for delimitation, by comparing the support for each model relative to the model with the highest ranking (Caviedes-Solis and Nieto-Montes de Oca 2018; Grummer, Bryson Jr, and Reeder Grummer et al. 2014; Leaché et al. 2014). Marginal-likelihood values were estimated using the path sampling (PS) (Lartillot and Philippe 2006) and stepping

Table 2 Sequences obtained for *"humboldtianum"* clade retrieve from GenBank database

Taxon	Accession number	Locality	Sequences
[1] *"Citocrome b"*			
Chirostoma grandocule	KC736369	Pátzcuaro Lake	1
Chirostoma estor	KJ921739	Pátzcuaro Lake	1
Chirostoma patzcuaro	JQ282029	Pátzcuaro Lake	1
Chirostoma chapalae	KC736397	Chapala Lake	1
Chirostoma consocium	KC736401	Chapala Lake	1
Chirostoma consocium	JQ282025	Chapala Lake	1
Chirostoma humboldtianum	KC736402	San Pedro Lagunillas lake	1
Chirostoma promelas	KC736368	Chapala Lake	1
Chirostoma humboldtianum	JQ282026	Zacapu Lake	1
Chirostoma sphyraena	KC736400	Chapala Lake	1
Total			**10**
[2] *"D-loop"*			
Chirostoma humboldtianum-SP	KF652026-KF652041	San Pedro Lagunillas Lake	15
Chirostoma humboldtianum-SJ	KF652014-KF652025	San Juanico dam	11
Chirostoma humboldtianum-Z	KF652006-KF652013	Zacapu Lake	7
Chirostoma humboldtianum-Tx	KF651996-KF652005	Tepuxtepec dam	7
Chirostoma humboldtianum-VV	KF651988-KF651995	Villa Victoria dam	7
Chirostoma humboldtianum-T	KF651987	Tiacaque dam	1
Total			**48**

[1] Reference Bloom et al. 2013

[2] Reference García-Martínez et al. 2014

stone (SS) (Xie et al. 2011) methods. Each assignment was run for a chain length of 100,000,000 generations for 200 path steps. The BF values were calculated with the equation BF = 2 (model 1-model 2) using the MLE for the two models and then compared and assessed using the framework of Kass and Raftery (1995). A negative BF value indicates support in favor of model 1, while a positive BF values indicates support in favor of model 2. The BF scale is as follows: 0 < ln (BF) < 1 is not worth more than a bare mention, 1 > ln (BF) < 3 is positive evidence, 3 > ln (BF) < 5 is strong support, and ln (BF) > 5 is decisive.

A Bayesian multi-locus species delimitation analysis was conducted in the program BPP v.3.1 (Yang 2015) to assess the same four evolutionary scenarios conducted in the multi-locus species tree analyses. This program uses a Bayesian framework and multispecies coalescent model to accommodate the gene tree-species conflict due to incomplete lineage sorting (Yang and Rannala 2010, 2014). Using a fixed guide tree, the following parameters were set up to account for variation in mutation rates among loci, a Dirichlet distribution ($\alpha = 2$) was specified; in order to discern the effective ancestral population size and time of divergence-influenced results, specifying the population size parameter Θ and root age $\tau 0$ of the species tree, in Θ $\alpha = 1$, $\beta = 10$, and $\tau 0$ $\alpha = 2$, $\beta = 2000$, a gamma prior (G) was used. Each analysis of 100,000 MCMC generations was run twice from different starting seeds with a burn-in period of 10% using algorithm 0 (default fine-tuning parameter, $e = 2$) and an estimated heredity ($a = 4$, $b = s4$). Convergence of chains was verified by effective sample size using Tracer.

Genetic structure

MEGA v.6 was used to calculate uncorrelated genetic distances (D_p) among the main groups found in the species tree analyses. Bootstrapping was performed with 1000 repetitions. Analysis of molecular variance (AMOVA) was performed using four population assignments based on the arrangement tested in the *Beast analysis: (1) morphological species, (2) *D-loop*, (3) *Cytb*, and (4) *S7* haplotype networks. Distributions were generated from 10,000 random permutations to estimate the significance of the variance components. Additionally, pairwise Φ_{ST} intra-lacustrine population differentiation was evaluated (Excoffier et al. 1992). The formation of subgroups (Lakes Chapala and Pátzcuaro) corresponded to the arrangement resolved by the haplotype network analysis (see Fig. 2). These analyses were carried out in ARLEQUIN 3.5.1.2 (Excoffier and Lischer 2010) and the significance values of Φ_{ST} were evaluated by performing a randomization test of 10,000 replications with a significance level of $\alpha = 0.05$.

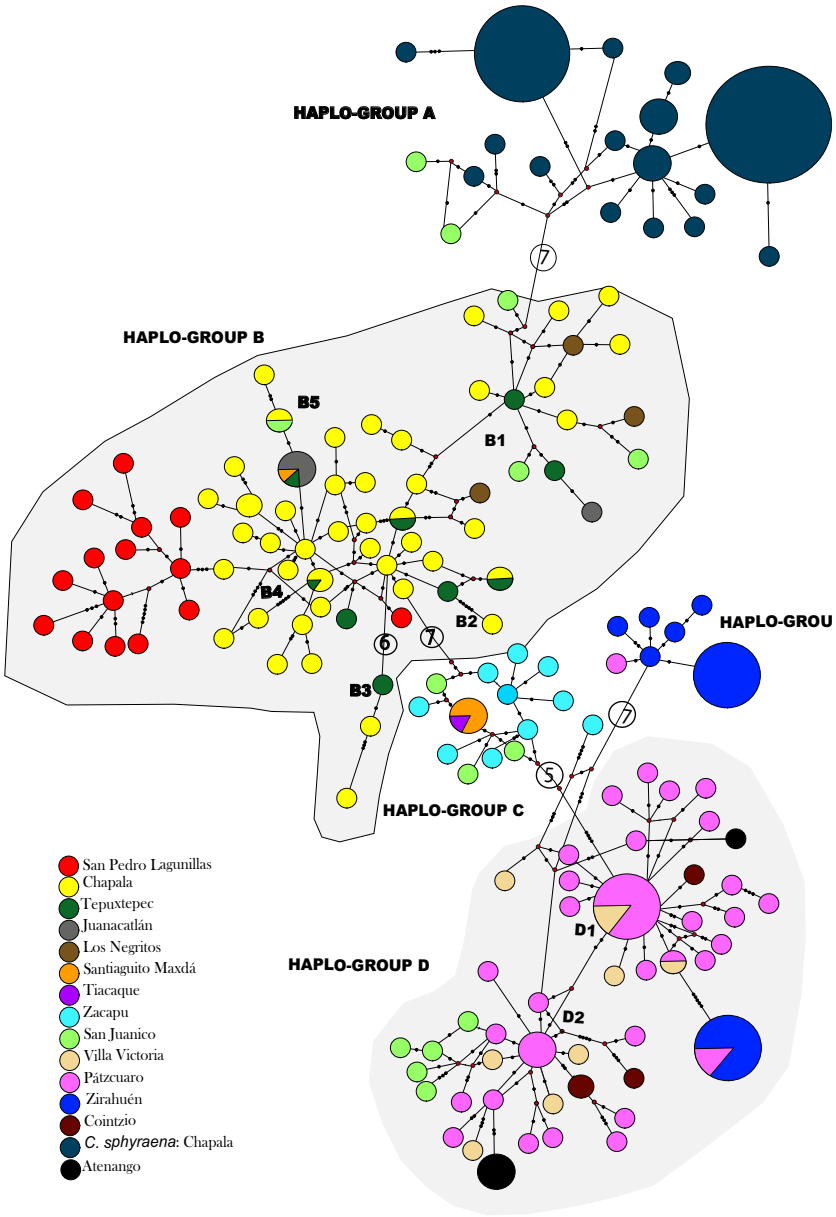

Fig. 2 Haplotype network based on *D-loop* (mtDNA). The major haplogroups are represented by A–E and intra-lacustrine groups are represented by letters B and D and numbers 1–5 and 1–2, respectively. Circles represent the haplotypes, and circle size is proportional to the haplotype frequency. The colors correspond to the localities shown in the associated legend and are in accordance with the colors depicted in Fig. 1. The small black points represent mutational steps. The small red points represent the median vectors

Results

Sequence data

Two hundred and four sequences of *Cytb*, 176 for *D-loop*, and 113 for *S7* were generated from 15 localities throughout central Mexico for all morphological species in the *"humboldtianum"* clade (Table 1 and Fig. 1). In addition, 10 sequences of *Cytb* of all members of the *"humboldtianum"* clade from Bloom et al. (2013) and 48 sequences of the species *Chirostoma humboldtianum* for *D-loop sensu* García-Martínez et al. (2015) were retrieved from GenBank and included in the analyses (Table 2). The alignment length for *Cytb* was 1087 bp, including 135 variables sites, of which 70 were parsimony informative. Nucleotide saturation was not detected (Iss < Iss.c, $P < 0.05$). The *D-loop* length was 284 bp and included 115 variables sites, of which 95 were parsimony informative. Finally, the *S7* alignment length was 671 bp, including 21 variable sites, of which 12 were parsimony informative. The best-fitting substitution models were T1M1+I+G, GTR+I+G, and TPM3 for *Cytb*, *D-loop*, and *S7*, respectively. The sequences generated during this study are as Electronic Supplementary Material (Cytb sequences: Dataset S1, D-loop sequences: Dataset S2, and S7 sequences: Dataset S3).

Haplotype network

The haplotype network shows a geographic structure for the mitochondrial genes (mtDNA). The *D-loop* network resulted in five major haplogroups (Fig. 2). Haplogroup A comprised all individuals identified as *C. sphyraena* from Lake Chapala and two individuals from San Juanico Dam (access numbers: KF652020, KF652023), identified by García-Martínez et al. (2015) as *C. humboldtianum*. This group was separated by 14 mutational steps from haplogroup B, which consisted of specimens identified as *C. promelas*, *C. lucius*, *C. consocium*, and *C. chapalae* from Lake Chapala, as well as specimens from the reservoirs Tepuxtepec, Juanacatlán, San Juanico, and San Pedro Lagunillas, also identified as *C. humboldtianum* by García-Martínez et al. (2015). Haplogroup C is separated by 11 mutational steps from the nearest haplogroup and includes specimens of *C. humboldtianum* from Zacapu, Tiacaque, and Santiaguito Maxda dam, and from San Juanico, Cointzio, and San Pedro Lagunillas. Haplogroup D, separated by 12 mutational steps from haplogroup C, groups all specimens sampled from Lake Pátzcuaro and identified as *C. estor*, *C. grandocule*, and *C. patzcuaro*, as well as those identified as *C. estor* from Cointzio dam, *C. humboldtianum* from the reservoirs of Villa Victoria and San Juanico, and *C. estor* from Zirahuén. Finally, haplogroup E was separated by 10 mutational steps and includes samples of *C. estor* from Lake Zirahuén and one specimen of *C. estor* from Lake Pátzcuaro (Fig. 2). Within haplogroup B, some haplotype segregation was found, with no geographic or species congruence, except for the San Pedro Lagunillas population, in which most samples are segregated by three mutational steps, although two haplotypes are shared with other major haplogroups. A haplotype segregation was also found within haplogroup D, with no geographic or species congruence, except for the samples from Zirahuén. This was represented by a unique haplotype separated by five mutation steps that grouped specimens of *C. estor* from Zirahuén and one individual of *C. estor* from Pátzcuaro (Fig. 2).

The *Cytb* network recovered four genetic groups that were consistent with the *D-loop* data, but with lower variation. The main difference of this network relative to the *D-loop* network is that *C. estor* specimens from Lakes Zirahuén and Pátzcuaro are included in the same haplogroup (Fig. 3). Like *D-loop*, the samples are grouped according to geographic location, with haplogroup A being the most divergent, and include all specimens identified as *C. sphyranea*. The central haplotype (h19) of haplogroup B is shared among *C. chapalae*, *C. consocium*, *C. promelas*, *C. lucius*, and *C. humboldtianum.* Haplotype h34 is shared among *C. lucius*, *C. promelas*, and *C. humboldtianum*. Haplotype h35 is shared between *C. consocium* and *C. humboldtianum*, and haplotype h38 is

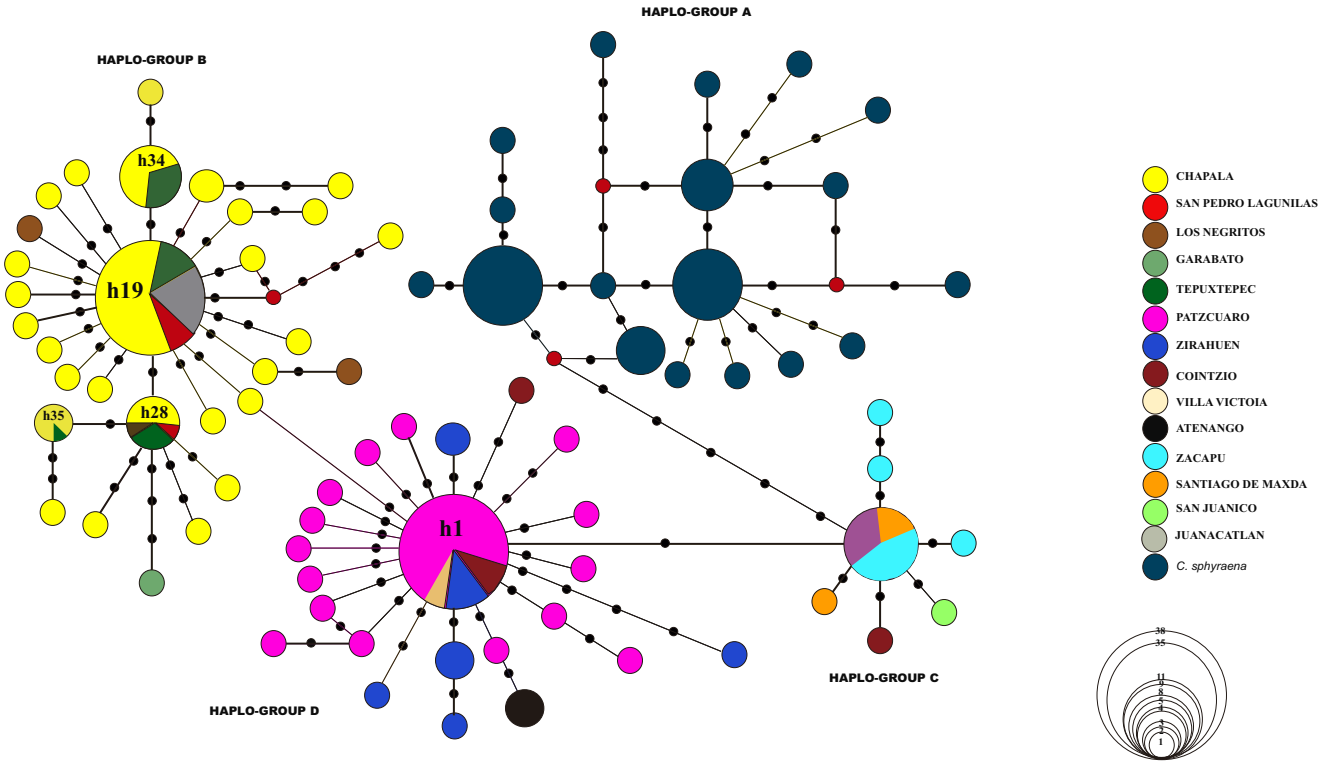

Fig. 3 Haplotype network based on *cytb* gene. The major haplogroups are represented by A–D. Circles represent unique haplotypes and circle size is proportional to the haplotype frequency. The legend within some haplotypes represents the haplotype number. The colors correspond to the localities shown in the associated legend and are in accordance with the colors depicted in Fig. 1. The small black points represent mutational steps. The small red points represent the median vectors

shared between *C. lucius* and *C. humboldtianum*. Haplogroup D also shows a shared haplotype among species, since haplotype h1 is shared among *C. patzcuaro*, *C. grandocule*, *C. estor*, and *C. humboldtianum* (Fig. 3).

The nuclear *S7* network recovered samples in two haplogroups, separated by two mutational steps. One group corresponds to samples of *C. sphyraena* from Lake Chapala, and the second group includes the rest of the species from central Mexico. Within the latter haplogroup, two central haplotypes with peripheral and unique haplotypes were found, but both lack geographic or species-level congruence (Fig. 4).

Species tree, Bayes factor test, and BPP analysis

The species tree and BPP analyses show that the hypotheses based on the currently recognized morphological species *C. sphyraena*, *C. promelas*, *C. lucius*, *C. chapalae*, *C. consocium*, *C. humboldtianum*, *C. patzcuaro*, *C. grandocule*, and *C. estor* were poorly supported (Fig. 5a). The arrangements based on the haplotype networks obtained by *S7*, *Cytb*, and *D-loop* that resolved two, four, and five differentiated genetic groups, respectively, showed highly supported branches in all cases (Fig. 5b–d). The first species hypothesis corresponds to the *S7* arrangement (Fig. 5b), which includes *C. sphyraena*, while all of the other lineages comprised the remaining morphological species of the *"humboldtianum"* clade. The second scenario corresponds to the *Cytb* arrangement (Fig. 5c), supporting four differentiated genetic groups related as follows: a Zacapu plus Lerma-Chapala group that is related to the Pátzcuaro group, and the three together were related to *C. sphyraena*. The Zacapu group includes *C. humboldtianum* from Zacapu and two localities from the upper Lerma basin (Tiacaque and Santiago de Maxda dam). The Lerma-Chapala group includes specimens of *C. humboldtianum* from two localities in the upper Lerma basin (Tepuxtepec and Juanacatlán dam) and samples identified as *C. consocium*, *C. chapalae*, *C. promelas*, and *C. lucius* from Lake Chapala. The Pátzcuaro group was comprised of *C. patzcuaro*, *C. grandocule*, and *C. estor* sampled in the Pátzcuaro and Zirahuén basins, as well as *C. estor* from Cointzio dam in the Cuitzeo drainage basin, an unidentified *Chirostoma* from Atenango dam of the Tecolutla basin, and *C. humboldtianum* from San Juanico and Villa Victoria dams in the Balsas drainage basin. The fourth scenario, corresponding to the *D-loop* arrangement (Fig. 5d), supported

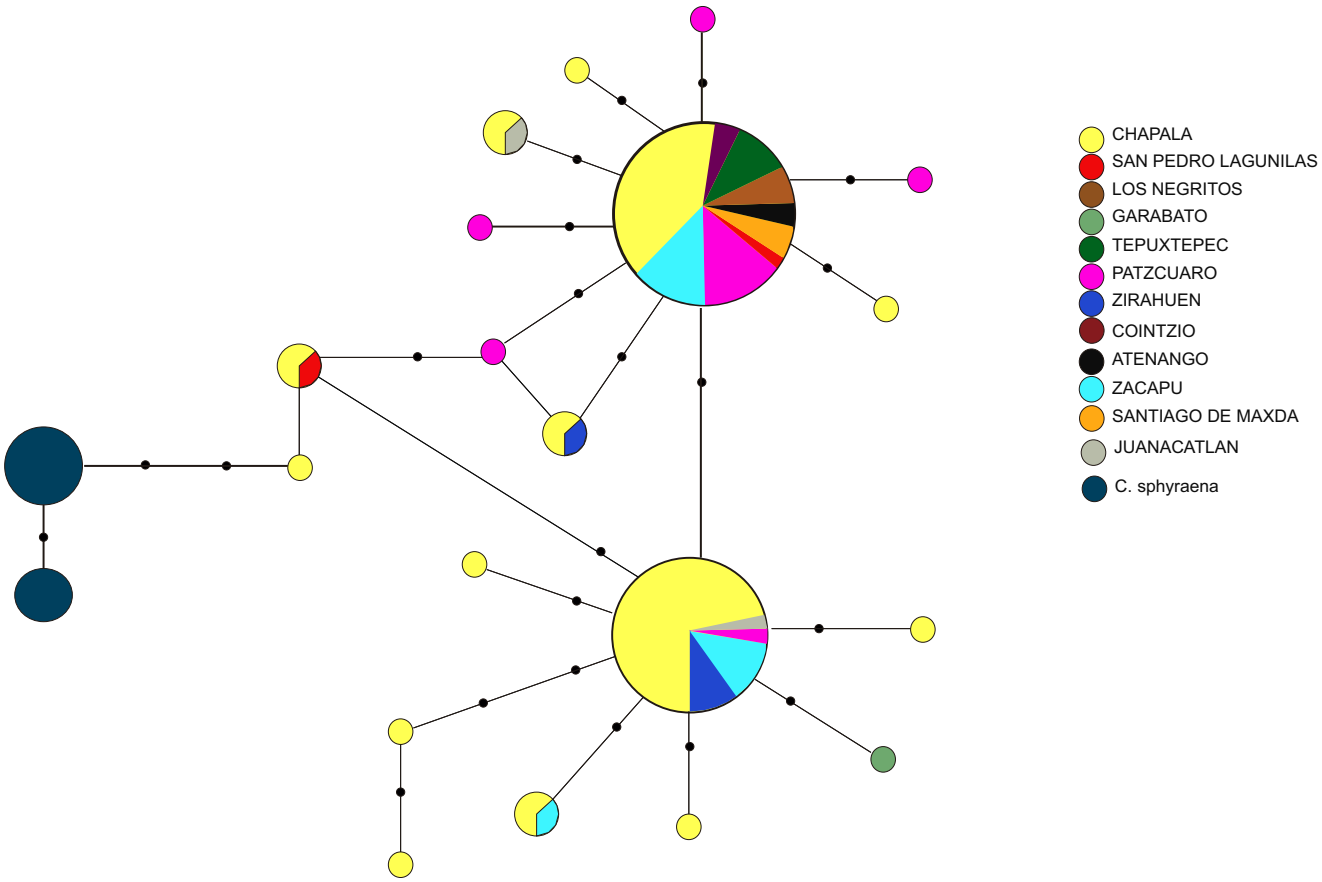

Fig. 4 Haplotype network based on the first intron of S7 ribosomal protein gene. Circles represent the haplotypes and circle size is proportional to the haplotype frequency. The colors correspond to the localities shown in the associated legend and are in accordance with the colors depicted in Fig. 1. The small black points represent mutational steps

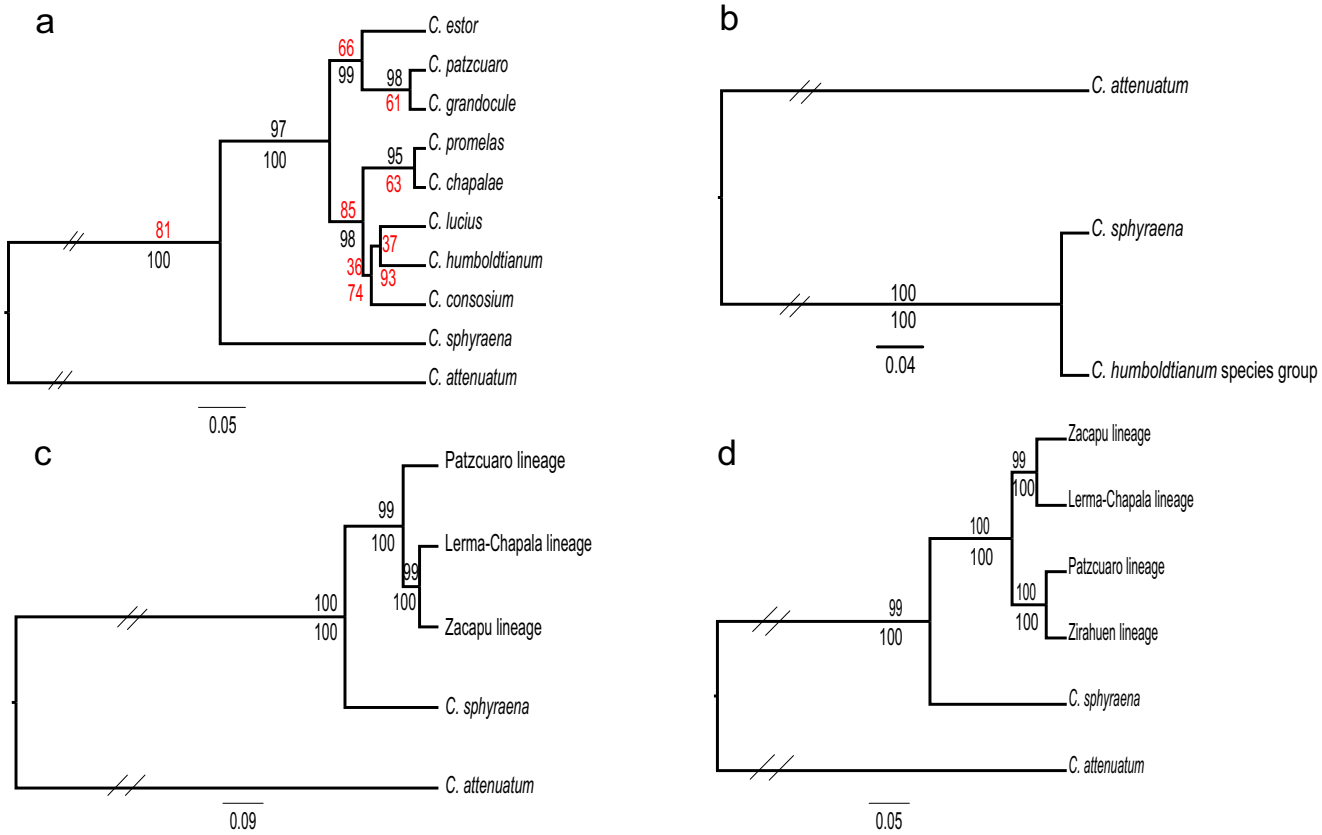

Fig. 5 Species tree and BPP analysis based on **a** the morphological species arrangement, and the haplogroups, **b** for *S7*, **c** for *cytb*, and **d** for *D-loop* markers for the *"humboldtianum"* clade. The numbers above the branches represent posterior probabilities of species tree analysis and the number under branches represents posterior probabilities of BPP analysis. The numbers in red are values with low support (< 85%)

five genetic groups, including the same four groups found in *Cytb*, but with the Pátzcuaro group divided into two sister groups: one included *C. estor* from Zirahuén, and the other consisted of all three species from Pátzcuaro and some individuals from Zirahuén.

The marginal likelihood of the S7 arrangement presented the lowest SS and PS values (SS = − 1207.41 and PS = − 1215.24), but this hypothesis of two species was highly supported in the BPP analysis (speciation probabilities > 0.95) (Fig. 5b). The *Cytb* and *D-loop* arrangements recorded higher and similar values in the marginal likelihood (SS = − 875.17; PS = − 875.18 and SS = − 875.47; PS = − 875.46, respectively), whereas these two arrangements were also supported by high speciation probabilities for all species in the guide trees (speciation probabilities > 0.95). The 2lnBf of the *Cytb* arrangement relative to the *D-loop* arrangement was 0.60 for SS and 0.57 for PS. Following Kass and Raftery (1995), these results are considered "not worth more than a bare mention", indicating a lack of support for choosing the best arrangement (Fig. 5c, d. Table 3).

Divergence times and genetic structure

The divergence time results obtained using four and five groups were the same, according to the high support found in the species tree analyses and BPP. The most common recent ancestor (MRCA) of the *"humboldtianum"* clade was estimated at ca. 0.58 Myr (HPD 95%, 0.25–1 Myr). The segregation of lineages from Pátzcuaro and Zirahuén, with respect to lineages from Lerma-Chapala and Zacapu, was estimated at ca. 0.265 Myr (HDP 95%, 0.13–0.45 Myr). The separation between Lerma-Chapala and Zacapu lineages was estimated

Table 3 Marginal likelihood estimates (ML) and empirical results for the Bayes factor value (2lnBf) test in the "*humboldtianum*" clade

Model	Stepping stone		Path sampling	
	MLE	2lnBF	MLE	2lnBF
4 genetic groups	-875.17	NA	875.18	NA
5 genetic groups	-875.46	0.60	-875.47	0.57

at ca. 0.127 Myr (HDP 95%, 0.06–0.21 Myr). Finally, the most recent split was between the Pátzcuaro and Zirahuén groups dated at ca. 0.076 Myr (HDP 95%, 0.133–0.033 Myr) (Fig. S4).

The uncorrelated mean genetic distance (D_p) between *C. sphyraena* with respect to the *"humboldtianum"* clade for the *Cytb* was 1.2%. The D_p values calculated among the Pátzcuaro and the Lerma-Chapala and Zacapu groups were 0.4% and 0.3%, respectively. The closest recorded group pair D_p values were 0.05% and 0.02% between Lerma-Chapala and Zacapu, and Pátzcuaro and Zirahuén groups, respectively. Whereas with the *D-loo*, the highest D_p was between *C. spyraena* with respect to "*humboldtianum"* clade recording 6.2% and the lowest D_p was between Pátzcuaro with respect to Zirahuén groups recording 4.2%.

The AMOVA results based on *Cytb* showed a significant value for the four genetic groups tested in the species tree analyses (nine, five, four, and two genetic groups, respectively). The greatest Φct value was when samples were grouped according to four and five genetic groups with same Φct value of 0.72, the two genetic groups arrangement showed a Φct value of 0.50, and the nine genetic groups showed a Φct value of 0.32 (Table 3). With *D-loop*, the greatest percentage of variation and significance values was recorded in the five genetic groups arrangement (Φct = 0.52), followed by the four and nine genetic group arrangements (Φct = 0.48, Φct = 0.37%), respectively. The two genetic group arrangement recorded the lowest value, which was not significant (Table 3). Finally, with the *S7* arrangement, four and five genetic groups arrangements also recovered the largest percentage of variation (Φct = 0.50), with the remaining two arrangements showing a non-significant Φct value (Table 4). The Φst value between the major genetic groups was high and significant (Table 5). The intra-lacustrine differentiation is significant for some of the subgroups resolved for *D-loop* by the haplotype network (see Fig. 2). For instance, in Lake Chapala, a high Φst value was recovered among the four subgroups (Φst value = 0.447 to 0.897), the Φst value of subgroup B1, with respect to the others (B2, B3, and B4), was high and significant (Φst = 0.721, 0.784, 0.458, respectively). Moreover, the subgroup B4 had a high and significant Φst value with respect to the subgroups B1, B2, and B3 (Φst = 0.0.458, 0.583, 0.447, respectively), while the Φst value of sub-group B2, with respect to B3, was not significant (Table 5). Within Lake Pátzcuaro, a significant Φst value (0.303) was recorded between both subgroups (D1 and D2) (Table 5).

Discussion

Identification of the species in the "*humboldtianum"* clade has been controversial because of the overlap in morphological features, making studies within the group challenging. The findings presented in this study, using phylogeographic and coalescent-based methods, as well as mitochondrial and nuclear markers, indicate that the species within the *"humboldtianum"* clade were not recovered as monophyletic and do not support the taxonomic species level as previously reported (Bloom et al. 2009, 2012, 2013). Instead, haplotypes were shared across recognized species. The results also show a correlation between

Table 4 Analysis of molecular variance (AMOVA) for the "*humboldtianum"* clade

Arrangement	Φct	Φst	Φsc	% of variation among groups	% of variation among population within groups	% of variation within populations
Cytb gene						
Morphological (nine genetic groups)	0.32*	0.71*	0.57*	31.82	39.52	28.65
Cytb (four genetic groups)	0.72*	0.76*	0.13*	72.80	3.74	23.45
D-loop (five genetic groups)	0.72*	0.75*	0.13*	72.12	3.65	24.23
S7 (two genetic groups)	0.50*	0.81*	0.62*	50.19	31.12	18.6
D-loop						
Morphological (nine genetic groups)	0.29*	0.58*	0.41*	29.10	29.64	41.25
Cytb (four genetic groups)	0.48*	0.60*	0.22*	48.35	11.30	40.35
D-loop (five genetic groups)	0.52*	0.59*	0.14*	52.18	6.66	41.15
S7 (two lineages)	0.26 ^{ns}	0.63*	0.50*	26.73	37.00	36.26
S7						
Morphological (nine genetic groups)	0.30 ^{ns}	0.45*	0.21*	30.75	14.84	54.41
Cytb (four genetic groups)	0.50*	0.53*	0.69*	49.68	3.45	46.87
D-loop (five genetic groups)	0.50*	0.53*	0.045 ^{ns}	50.43	2.27	47.31
S7 (two genetic groups)	0.75 ^{ns}	0.78*	0.13*	75.49	3.26	21.24

*P value < 0.01; **P value < 0.05; *ns* no significance

Table 5 Genetic differentiation (Φst pairwaise) between the major genetic groups and intra lacustrine genetic group segregation using the *D-loop* marker

Φst pairwaise between the major groups	Lerma-Chapala group	Zacapu group	Pátzcuaro group
Zacapu group	0.498**	0	
Pátzcuaro group	0.587**	0.561**	0
Zirahuén group	0.585**	0.555**	0.476**
Intra-lacustine differentiation			
Chapala Lake	Sub-group B1	Sub-group B2	Sub-group B3
Sub-group B2	0.721*	0	
Sub-group B3	0.784**	0.897 ns	0
Sub-group B4	0.458**	0.583**	0.447*
Pátzcuaro Lake	Sub-group D1		
Sub-group D2	0.303**	0	

*$P > 0.05$; **$P > 0.01$; *ns* no significance

geographic location and genetic signature, which suggests that allopatric speciation has played a substantial role in the diversification of the "*humboldtianum"* clade.

Phylogeographic patterns

Freshwater fishes display high levels of genetic differentiation and population subdivision compared with marine fishes, mainly due to the island-like model of speciation, related to the basin arrangements and hydrological complexity of freshwater systems (Ward et al. 1994; Seehausen 2004). However, the findings of this study provide evidence of a low level of genetic differentiation and an overestimation of specific level diversity within the *"humboldtianum"* clade of silverside fishes.

We recovered two, four, and five structured genetic groups in the haplotype networks with *S7*, *Cytb*, and *D-loop*, respectively. These genetic groups showed a mixture of haplotypes among species, but with a marked genetic segregation according to the hydrological basins of central Mexico. The arrangement of *S7*, clustering two differentiated genetic groups (*C. sphyraena* and other species within the *"humboldtianum"* clade; Fig. 5), was discarded following the results of the BF test, and the lack of significant genetic structure. The usual discordance recorded between nuclear and mitochondrial loci (Table 3) is mainly associated with the lower level of variation in nuclear genes. This lack of genetic variation resulted in a low level of resolution in recent cladogenetic events. Recently, Betancourt-Resendes et al. (2018) reported discordance between nuclear and mitochondrial genes in another group of silversides and attributed this to the low evolutionary rate and high coalescence time for nuclear loci in a recent diversification event. This proposal is supported by the low level of genetic distances (< 0.4%) and the recent divergence time (0.6 Myr) between the two lineages (Fig. S4).

The arrangement of five and four genetic groups is strongly supported, but the results of the BF (Fig. 5c, d; Table 3) and genetic structure (Tables 4 and 5) make it difficult to choose the best scenario of genetic differentiation. Based on these scenarios, *C. sphyraena* is the most divergent species, corresponding to the earlier divergence of the "*humboldtianum"* clade (ca. 0.6 Myr), which is consistent with the morphological data. *Chirostoma sphyraena* is considered the most morphologically differentiated species of *Chirostoma* from Lake Chapala (Barbour and Chernoff 1984). The remaining genetic groups detected within the *"humboldtianum"* clade show high geographic congruence, distinguishing at least five well-differentiated genetic groups, with *D-loop*, from the Lerma-Chapala basin, as well as Lakes Zacapu, Pátzcuaro, and Zirahuén (Figs. 2 and 5d and Tables 3 and 5).

Allopatry in the beginning of evolutionary history

The flock speciation model was previously proposed to explain the evolution of the *"humboldtianum"* clade (Barbour 1973b; Barbour and Chernoff 1984; Echelle and Echelle 1984). However, the genetic data from this study provides evidence that the early evolution of the *"humboldtianum"* clade was strongly associated with vicariant events, rather than being the result of an adaptive radiation scenario. Species within the "*humboldtianum"* clade are exclusively adapted to lacustrine ecosystems (Barbour 1973b; Barbour and Chernoff 1984; Echelle and Echelle 1984; Barriga-Sosa et al. 2005; Bloom et al. 2009); therefore, their low dispersal capacity through lotic systems may influence the isolation and genetic differentiation within the group, even with historic evidence of inter-basin connections via rivers and streams.

In central Mexico, tectonism, volcanism, and climatic fluctuations have configured the evolution of freshwater systems, promoting the high dynamism of genesis and destruction and change in hydrological basin configuration, with differential responses of the ichthyofaunal communities (Doadrio and Domínguez 2004; Dominguez-Dominguez et al. 2006, 2008; Pérez-Rodríguez et al.

2009; Corona-Santiago et al. 2015; García-Martínez et al. 2015; Piller et al. 2015; Beltrán-López et al. 2017, 2018, Betancourt-Resendes et al. 2018).

The colonization of the most recent common ancestor (MRCA) of the *"humboldtianum"* clade in central Mexico occurred with the invasion of a *C. humboldtianum*-like ancestor (*sensu* Barbour 1973a; Echelle and Echelle 1984). This colonization is believed to have occurred during the Miocene (Barbour 1973a). Nevertheless, the most recent molecular studies propose that the colonization age of the MRCA is less than 1 Myr (Bloom et al. 2013; Campanella et al. 2015). Our estimated divergence time for the split of *C. sphyraena*, with respect to other lineages within the *"humboldtianum"* clade, was dated around 0.58 Myr (Fig. S4) and is consistent with the hypothesis of recent diversification, as postulated by García-Martínez et al. (2015) (~ 0.72–0.83 Myr). The colonization route of the ancestor of the *"humboldtianum"* clade is unclear, but is most likely to have occurred though the Lerma-Chapala Basin, as proposed by Barbour (1973b).

The colonization of the Pátzcuaro basin could have been through an ancient connection with the Cuitzeo basin (Álvarez 1972), which is supported by geological (Moncayo-Estrada and Buelna-Osben 2001; Israde-Alcántara et al. 2005), as well as biological and fossil (Barriga-Sosa et al. 2002; Dominguez-Dominguez et al. 2006; Domínguez-Domínguez et al. 2008; Domínguez-Domínguez et al. 2010; Pérez-Rodríguez et al. 2009), evidence. The split between Zirahuén, with respect to Pátzcuaro groups, occurred ca. 0.076 Myr (HDP 95%, 0.133-0.033 Myr) (Fig. S1). Several connections and isolation events between both basins have been described (De Buen 1943; Israde-Alcántara et al. 2005; Garduño-Monroy et al. 2009). However, the divergence time between shared ichthyofauna is not synchronous (Domínguez-Domínguez et al. 2008; Corona-Santiago et al. 2015; Betancourt-Resendes et al. 2018).

The split between Zacapu and Lerma-Chapala groups was dated ca. 0.127 Myr ago (HDP 95% 0.06-0.21 Myr). The Zacapu Cienega was connected to the Lerma system in two independent ways: (1) the ancestral connection through the Angulo River, tributary of Middle Lerma (Moncayo-Estrada and Buelna-Osben 2001), and (2) the ancestral connection through the Cuitzeo basin for the Villa Morelos and the Chucandiro-Huaniqueo corridors. Both connections appear to have been produced by the activity of the Pliocenic northeast-southwest fault system of the area (Israde-Alcántara and Garduño-Monroy 1999). Segregation of the majority of the samples from San Pedro Lagunillas was found, separated by three mutation steps from the rest of samples within haplogroup B (*D-loop*), demonstrating that this population has had a long history of isolation. Barbour (1973b) suggested that these were peripheral relict populations that were isolated during the middle Pleistocene orogeny.

Genetic intra-lacustrine differentiation

Although the early diversification process of major genetic groups found in the "*humboldtianum*" clade is proposed to be the result of vicariance, our genetic data does not preclude the possibility of sympatric speciation in Lakes Chapala and Pátzcuaro. We found some genetic segregation within haplogroups B (Chapala-Lerma) and D (Pátzcuaro) (Fig. 2), with significant genetic structure (Table 5). Although more data or even more variable genetic markers are required in order to draw any robust conclusions, the genetic segregation process that promoted the separation of the four subgroups B1 to B4 within Lake Chapala (Fig. 2), and two subgroups D1 to D2 in Lake Pátzcuaro (Fig. 2), could be explained by the formation of intra-lacustrine barriers or ecological specialization, or both. Lake Chapala is the largest natural lake in Mexico, and several environmental and geological events have impacted the lacustrine basin. Some authors have proposed that changes in the paleoclimatic regime in the area promoted changes in water levels of the Chapala paleo-lake, resulting in the isolation and secondary contact of different regions within the large lake. These events could have promoted the genetic differentiation of groups of fish species, including *C. sphyraena*. This scenario has been proposed for goodeids (Domínguez-Domínguez et al. 2010) and for *C. grandocule* from Lake Pátzcuaro (Barriga-Sosa et al. 2004).

In the other case, the mechanisms involved in sympatric speciation or ecological segregation are specializations in response to ecological opportunity with subsequent reproductive isolation (Mayr 1942), as have been found in Nicaraguan cichlids (Barluenga et al. 2006), rift lake cichlids (Wagner et al. 2012; Seehausen and Wagner 2014), and *C. attenuatum* (Betancourt-Resendes et al. 2018). Studies focusing on the trophic ecology of sympatric species of silversides in Lakes Chapala and Pátzcuaro have not shown trophic overlap (García-de León et al. 2014; Mercado-Silva et al. 2015); however, the morphological differentiation in sympatric species of Chapala and Pátzcuaro is primarily related to the jaw bones, suggesting a relationship with trophic divergence (Lake et al. 1988; Soria-Barreto and Paulo-Maya 2005). Although the results of the mitochondrial *D-loop* marker could be considered evidence for intra-basin differentiation, this issue must be studied with more variable molecular markers, such as microsatellites or SNPs.

Species boundaries and taxonomic implications

The findings presented herein support the monophyly of the *"humboldtianum"* clade; nevertheless, the current classification based on morphological data (Barbour 1973b), electrophoretic assays (Echelle and Echelle 1984), and morphometric data (Barriga-Sosa et al. 2005; Barriga-Sosa et al. 2002)

was not supported by the two species delimitation analyses used herein, the validated species tree by marginal likelihood and BF testing, and the BPP. In relation with these analyses, the unique inconsistency between both methods consisted in that the *S7* arrangement, that clustered two differentiated lineages (*C. sphyraena* and *C. humboldtianum* species group; Fig. 4), showed the lowest SS and PS values (SS = − 1207.41 and PS = − 1215.24), while with the BBP analysis was highly supported (speciation probabilities > 0.95) (Fig. 5). In the case of four and five species arrangements, all of the analyses were highly supported. These inconsistencies between two vs. four and five species arrangements could be associated with the usual discordances between nuclear and mitochondrial loci, including lower variation in the nuclear genes. This lack of genetic variation resulted in a lower level of resolution, making it difficult to resolve recent cladogenetic events. It has been previously reported that discordance between these two genetic sources within *Chirostoma* is consistent with the low evolutionary rate and high coalescence time for nuclear loci (Betancourt-Resendes et al. 2018), which is revealed by the low genetic distances (< 0.4%) and the recent divergences recorded (< 0.265 Myr) between radiated lineages within the "*humboldtianum"* clade studied herein.

The main morphological characteristics used to differentiate among species of *Chirostoma* are related to features of the head region, presumably related to trophic specialization and swimming structures (Table 6) associated with occupancy of different strata of the water column (Barbour 1973b; Barbour and Chernoff 1984; Barriga-Sosa et al. 2004). The high variability in inter- and intra-specific morphological measurements in *Chirostoma* makes identification of the currently recognized species difficult. Furthermore, this variability has been associated with a variety of selective pressures, such as competition for food resources and space, as well as fishing pressure (Barriga-Sosa et al. 2002). Nevertheless, no evidence of trophic segregation has been recovered in sympatric species of *Chirostoma* from Lakes Pátzcuaro and Chapala, based on contemporary samples (Moncayo-Estrada et al. 2012; García-de León et al. 2014; Mercado-Silva et al. 2015). This contradicts the hypothesis of species flock radiation (Barbour 1973a; Echelle and Echelle 1984). In species of *Menidia* and *Chirostoma*, the meristic and morphometric characters are influenced by environmental conditions and geographic distribution (Miller et al. 2005; Alarcón-Duran et al. 2017), as is the case with the inland silverside *Menidia audens* and *M. beryllina* (Chernoff 1982).

Table 6 Diagnostic characters used to recognize between the nine morphological species and subspecies, and the genetic groups where these morph-species belong

Taxonomic unit	[1]Morphological diagnostic characters	[2]Genetic group recovery
Chirostoma chapalae	Jaw length 8.6–9.4%; median lateral scales, 44–55; mandible length 8.6–9.4; head length 23.8–25.5.	B
Chirostoma consocium	Median lateral scales, 52–68; predorsal scales 43–79; head length, 24.4–28; snout included by a slightly projecting lower jaw; anal fin base 20.1–26.2%.	B
Chirostoma estor estor	Median lateral scales, 69–90; predorsal scales 56–108; gill rakers, 23–28; lower jaw does not protrude beyond snout; teeth small.	D
Chirostoma copandaro	Predorsal scales 39–64; median lateral scales, 67–86; jaw length 10.3–12.2% (based in small specimens).	E
Chirostoma grandocule	Median lateral scales, 58–77; gill rakers, 28–34; anal rays, 18–22. Interior basin of Pátzcuaro Lake.	D
Chirostoma humboldtianum	Median lateral scales, 43–73; predorsal scales 24–50; anal fin base, 17.1–22.2	B; C
Chirostoma lucius	Predorsal scales, 50–117; lower jaw 13.1–17.9; snout included by lower jaw the projection of which beyond the snout may equal approximately one-half the interorbital distance. Teeth small.	B
Chirostoma patzcuaro	Predorsal scales of uniform size, scales behind the head not small or crowded; snout pointed; median lateral scales, 52–63.	D
Chirostoma promelas	Predorsal scales of not uniform size, scales behind the head small and crowded; lower jaw equal to or included by snout; snout pigmented black.	B
Chirostoma sphyraena	Predorsal scales, 56–111; snout length, 13.2–15.9%; pointed snout included by slightly projecting lower jaw; teeth large; body slender, barracuda-like.	A

[1] References: Barbour 1973b; Barbour and Chernoff 1984; Miller et al. 2005; Barriga-Sosa et al. 2002, 2005

[2] Genetic groups retrieved from the *D-loop* haplotype network (see Fig.2) and best supported by the delimitation test

The multispecies coalescent model used in the species tree analysis supports the occurrence of four (according to the *Cytb* arrangement) or five (according to *D-loop* arrangement) genetic groups (Figs. 5c and d), but with no species-level congruence, since several species show a mixture of haplotypes. According to the marginal likelihood and Bayes Factor results, it is not possible to support any of the two arrangements, with the five genetic group arrangement used for the taxonomic inference presented here. The existence of five genetic groups suggests an overestimation of species richness in the "*humboldtianum*" clade accepted with morphological data (9 species and 1 sub-species) (Table 6). This overestimation could be related to the high morphological variation due to the considerable phenotypic plasticity within the group, as has been found in other fish groups (Gulisija et al. 2016; Ornelas-García et al. 2008; Pérez-Miranda et al. 2017; Rosenblum et al. 2014; Tarvin et al. 2017; Zamudio et al. 2016; Alarcón-Duran et al. 2017).

In the case of Pátzcuaro and Zirahuén, the *D-loop* analyses show the segregation of some samples between these drainages, which is consistent with the current recognition of the two subspecies *Chirostoma estor* from Lake Pátzcuaro and *Chirostoma estor copandaro* from Lake Zirahuén (De Buen 1943; Barbour 1973b). In addition, the separation of *C. e. copandaro*, with respect to *C. e. estor*, is supported by other independent sources providing geological (Israde-Alcántara and Garduño-Monroy 1999), biogeographic (De Buen 1943; Álvarez 1972; Corona-Santiago et al. 2015), and biological (Corona-Santiago et al. 2015) evidence. Recently, Betancourt-Resendes et al. (2018), based on species tree analyses, found that *C. attenuatum* comprised two well-differentiated lineages, suggesting that both lineages should be elevated to the species level (*C. attenuatum* and *C. zirahuen*).

Another important taxonomic implication is that this genetic data supports the existence of only one species of the *"humboldtianum"* clade from Lake Pátzcuaro, and not three as recognized by the current taxonomy. In accordance with the principle of priority, the valid name for this single species of the *"humboldtianum"* clade is *C. estor*, over *C. patzcuaro* and *C. grandocule*, so the results presented here support the existence of two species in Pátzcuaro: *C. attenuatum* (see (Betancourt-Resendes et al. 2018) and *C. estor* (Table 7). The specimens morphologically identified as *C. humboldtianum* belong to different genetic groups. The type locality for this species is the endorheic Valle de México, and the species also is reported to occur in the Lerma Basin (Valenciennes [A.] in Cuvier and Valenciennes 1835). Although samples from Valle de México were not included in this study, samples from the Upper Lerma (Santiaguito Máxda and Tiacaque dams) and Zacapu Lake include specimens identified as *C. humboldtianum*, a genetic group well supported in the haplotype networks (Figs. 2, 3, 4) and *Beast analyses (Fig. 5c, d). These results suggest that *C. humboldtianum* may be more geographically restricted than was previously considered (García-Martínez et al. 2015).

Table 7. Summary of proposal taxonomy in *"humboldtianum"* clade based on the molecular data

Order Atheriniformes Rosen 1966
Family Atherinopsidae Fitzinger 1873
Subfamily Menidiinae Schultz 1948
Tribe Menidiini Schultz 1948
Genera *Chirostoma* Swaison 1839
Chirostoma sphyraena Boulenger 1900
Chirostoma estor Jordan 1879 (includes *Chirostoma grandocule* Steindachner 1894 and *Chirostoma patzcuaro* Meek 1902)
Chirostoma copandaro (species level of *C. estor copandaro* De Buen 1945)
Chirostoma chapalae Jordan & Snyder 1899 (includes *Chirostoma consocium* Jordan & Hubbs 1919, *Chirostoma lucius* Boulenger 1900 and *Chirostoma promelas* Jordan & Snyder 1899)
Chirostoma humboldtianum Valenciennes 1835.

Lake Chapala contains the highest species richness of *Chirostoma* (Barbour 1973b), including five species of the "*humboldtianum*" clade (Barbour 1973b; Echelle and Echelle 1984). However, the genetic results presented herein do not support recognition of the five species. Instead, the data support the occurrence of two well-differentiated groups. The first corresponds to specimens identified as *C. sphyraena*, which do not share haplotypes with any other species or genetic groups found in Lake Chapala. Furthermore, *C. sphyraena* is the most genetically and morphologically differentiated species, with no overlap in diagnostic characters with respect to other species within the "*humboldtianum*" clade (Barbour 1973b; Barbour and Chernoff 1984). The genetic data presented herein and previous morphological analyses support the validity of this species. The second genetic group found in Chapala includes four species (*C. chapalae*, *C. consocium*, *C. promelas*, and *C. lucius*), as well as individuals of *C. humboldtianum* from the Lerma (Tepuxtepec and Juanacatlán dam) and San Pedro Lagunillas dam. The genetic results presented herein support the existence of one species within this group, with *C. chapalae* the valid name in accordance with the principle of priority (Table 7).

These results reject the validity of the nine morphological species, confirming that, rather than distinct taxa, several morphs correspond to intra-specific polymorphisms, a possibility that was previously suggested as plausible (Echelle and Echelle 1984; Barbour and Chernoff 1984). One of the explanations proposed for the high morphological polymorphism within *Chirostoma* is hybridization (Alaye 1993, 1996). This process may predispose populations to the adaptive radiation (Seehausen and Wagner 2014) that occurs after colonization events. However, the low colonization capacity of silversides, due to the strong association of those species with lacustrine ecosystems, could be a barrier for dispersal following cladogenetic events (Betancourt-Resendes et al. 2018). A lack of resolution in the molecular markers used is less likely, since a previous study demonstrated the utility of these markers for

elucidating recent speciation events in *C. attenuatum* and *C. zirahuen* (Betancourt-Resendes et al. 2018). The discrepancies between molecular markers and morphological characters in the *"humboldtianum"* clade could therefore be related to high phenotypic plasticity, mainly related to habitat pressures, as has been demonstrated for *C. humboldtianum* (Alarcón-Duran et al. 2017) and to rapid adaptive divergences, as proposed for the related *C. grandocule* from Lake Pátzcuaro (Barriga-Sosa et al. 2004) and the inland silverside *Menidia beryllina* population in adjacent environmental regimes (Fluker et al. 2011). This has strong taxonomic implications, since it decreases the number of species within the genus *Chirostoma* (see Table 7).

Acknowledgements IBR would like to thank the Consejo Nacional de Ciencia y Tecnología (CONACyT) for granting a scholarship, and the CIPRES Cyberinfrastructure for Phylogenetic Research XSEDE for their computational support. Conacyt-CB-2009-1-130220 to IDLAB partially funded the specimen sampling. We thank the División de estudios de Posgrado de la Universidad Michoacana de San Nicolás de Hidalgo for funding the English editing of the manuscript. We thank B. García-Andrade, G. Beltrán-López, A. González-Alejo, D. Montejo-Diaz and F. Mar-Silva for their field support.

Funding information We thank the Coordinación de la Investigación Científica-UMSNH, of Mexico (CIC-2016-18 to ODD) and the National Science Foundation (NSF 1354930 to KRP) for financially funding the project.

Appendix

PCR Protocols

The amplification process was conducted in a reaction containing 50–100 ng DNA, 2.5 mM of 1× buffer, 1.5 mM $MgCl_2$, 2.5 μM dNTP mix (10 μM), 10 pmol of each primer, 1 unit of Taq DNA polymerase (Invitrogen), and distilled water to bring the reaction volume to 25 μL. The amplification conditions were the following: the *cytb* was an initial denaturalization at 94 °C for 2 min, 35 cycles of 94 °C for 45 s, 48 °C for 1 min, and 72 °C for 1 min, and final extension at 72 °C for 5 min. The *D-loop* amplification procedure was an initial denaturalization at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 52.4 °C for 45 s, and 72 °C for 1 min, and final extension at 72 °C for 5 min. The S7 was an initial denaturalization at 94 °C for 1 min, 35 cycles of 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 45 s and final extension at 72 °C for 5 min.

References

Alarcón-Duran, I., Castillo-Rivero, M. A., Figueroa-Lucero, G., Arroyo-Cabrales, J., Barriga-Sosa I. A., (2017). Diversidad morfológica en 6 poblaciones del pescado blanco *Chirostoma humboldtianum*. Revista Mexicana de Biodiversidad. 88:207-214. https://doi.org/10.1016/j.rmb.2017.01.018

Alaye, R. N. (1993). El pescado blanco (género *Chirostoma*) del lago de Pátzcuaro, Michoacán. Composición de especies. *Ciencia Pesquera, 9*, 113–128.

Alaye, R. N. (1996). Estudios del polimorfismo de la hemoglobina para identificar especies del género *Chirostoma* del lago de Pátzcuaro, Michoacán, México. *Ciencia Pesquera, 13*, 1–9.

Álvarez, J. (1972). Ictiología michoacana V. Origen y distribución del a ictiofauna dulceacuícola michoacana. *Anales. Escuela Nacional de Ciencias Biológicas, 19*, 155–161.

Avise, J. C. (2000). *Phylogeography: the history and formation of species*. Harvard University Press.

Bandelt, H. J., Forster, P., & Röhl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution, 16*, 37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036.

Barbour, D. (1973a). A biogeographical history of *Chirostoma* (Pisces: Atherinidae): a species flock from the Mexican Plateau. *Copeia., 3*, 533–556.

Barbour, D. (1973b). The systematics and evolution of the genus *Chirostoma* Swainson (Pisces, Atherinidae). *Talune Studies in Zoology and Botany., 18*(3), 97–140.

Barbour, C. D., & Chernoff, B. (1984). Comparative morphology and morphometric of the pescados blancos (genus *Chirostoma*) from Lake Chapala Mexico. In a Echelle & Kornfield (ed), *Evolution of fish species flock*. Univ Maine Orono, (pp 111-127).

Barluenga, M., Stölting, K. N., Salzburger, W., Muschick, M., & Meyer, A. (2006). Sympatric speciation in Nicaraguan crater lake cichlid fish. *Nature, 439*, 719–723. https://doi.org/10.1038/nature04325.

Barriga-Sosa, I., Ibáñez-Aguirre, A. L., & Arredondo-Figueroa, J. L. (2002). Morphological and genetic variation in seven species of the endangered *Chirostoma* "*humboldtianum* species group" (Atheriniformes: Atherinopsidae). *Revista de Biología Tropical, 50*, 199–216.

Barriga-Sosa, I., Eguiarte, L. E., & Arredondo-Figueroa, J. L. (2004). Low but significant subdivision among populations of *Chirostoma grandocule* from Lake Patzcuaro, México. *Biotropica, 36*, 85–98. https://doi.org/10.1111/j.1744-7429.2004.tb00299.x.

Barriga-Sosa, I., García-De Leon, F., & Del Río-Portillam M.A. (2014). The complete mitochondrial DNA of the endemic shortfin silverside, Chirostoma humboldtianum (Valenciennes, 1835). *Journal of Mitochondrial DNA Part A, 27:2*, 1545-1546. https://doi.org/10.3109/19401736.2014.953130.

Barriga-Sosa, I., Pérez-Ramírez, M. Y., Soto-Aguirre, F., Castillo-Rivera, M., & Arredondo-Figueroa, J. L. (2005). Inter-specific variation of the mitochondrial r16S gene among silversides, "Peces Blancos", (Atherinopsidae: Menidiinae) and its utilization for species identification. *Aquaculture, 250*, 637–651. https://doi.org/10.1016/j.aquaculture.2005.05.003.

Beltrán-López, R. G., Domínguez-Domínguez, O., Guerrero, J. A., Corona-Santiago, D. K., Mejía-Mojica, H., & Doadrio, I. (2017). Phylogeny and taxonomy of the genus Ilyodon Eigenmann, 1907 (Teleostei: Goodeidae), based on mitochondrial and nuclear DNA sequences. *Journal of Zoological Systematics and Evolutionary Research, 1907*. https://doi.org/10.1111/jzs.12175.

Beltrán-López, R. G., Domínguez-Domínguez, O., Pérez-Rodríguez, R., Kyle, P., & Doadrio, I. (2018). Envolving in the highlands: the case of the Neotropical Lerma live-bearing *Poeciliopsis infans* (Wolman, 1984) (Cyprinodontiformes: Poeciliidae) in Central Mexico. *BMC Evololutionary Biology, 18*, 56. https://doi.org/10.1186/s12862-018-1172-7.

Betancourt-Resendes, I., Pérez-Rodríguez, R., & Domínguez-Domínguez, O. (2018). Speciation of silverside *Chirostoma attenuatum* (Pisces: Atheriniformes) in Central Mexico. Journal of

Zoological Systematics and Evolutionary Research. 00:1-12. doi.org/https://doi.org/10.1111/jzs.12216.

Bloom, D. D., Piller, K. R., Lyons, J., Mercado-Silva, N., & Medina-Nava, M. (2009). Systematics and biogeography of the silverside tribe Menidiini (Teleostomi: Atherinopsidae) based on the mitochondrial ND2 gene. *Copeia., 2009*, 408–417. https://doi.org/10.1643/CI-07-151.

Bloom, D. D., Unmack, P. J., Gosztonyi, A. E., Piller, K. R., & Lovejoy, N. R. (2012). It's a family matter: molecular phylogenetics of Atheriniformes and the polyphyly of the surf silversides (family: Notocheiridae). *Molecular Phylogenetics and Evolution, 62*, 1025–1030. https://doi.org/10.1016/j.ympev.2011.12.006.

Bloom, D. D., Weir, J. T., Piller, K. R., & Lovejoy, N. R. (2013). Do freshwater fishes diversify faster than marine fishes? A test using state-dependent diversification analyses and molecular phylogenetics of new world silversides (atherinopsidae). *Evolution, 67*, 2040–2057. https://doi.org/10.1111/evo.12074.

Campanella, D., Hughes, L. C., Unmack, P. J., Bloom, D. D., Piller, K. R., & Ortí, G. (2015). Multi-locus fossil-calibrated phylogeny of Atheriniformes (Teleostei, Ovalentaria). *Molecular Phylogenetics and Evolution, 86*, 8–23. https://doi.org/10.1016/j.ympev.2015.03.001.

Caviedes-Solis, I. W., & Nieto-Montes de Oca, A. (2018). A multilocus phylogeny of the genus *Sarcohyla* (Anura: Hylidae), and an investigation of species boundaries using statistical species delimitation. *Molecular Phylogenetics and Evolution, 118*, 184–193. https://doi.org/10.1016/j.ympev.2017.09.010.

Chernoff, B. (1982). Character variation among populations and the analysis of biogeography. *American Zoology, 22*, 425–439.

Chow, S., & Hazama, K. (1998). Universal PCR primers for S7 ribosomal protein gene introns in fish. *Molecular Ecology, 7*, 1247–1263.

Corona-Santiago, D. K., Doadrio, I., & Domínguez-Domínguez, O. (2015). Evolutionary history of the live-bearing andemic *Allotoca diazi* species complex (Actinopterygii, Goodeinae): evidence of founder effect events in the Mexican Pre-hispanic period. *PLoS One, 10*, e0124138. https://doi.org/10.1371/journal.pone.0124138.

De Buen, F. (1943). Los lagos michoacanos. I. Caracteres generales el Lago de Zirahuén. *Revista de la Sociedad Mexicana de Historia Natural, 4*, 211–232.

Doadrio, I., & Domínguez, O. (2004). Phylogenetic relationships within the fish family Goodeidae based on cytochrome b sequence data. *Molecular Phylogenetics and Evolution, 31*, 416–430. https://doi.org/10.1016/j.ympev.2003.08.022.

Dominguez-Dominguez, O., Doadrio, I., & Perez-Ponce de León, G. (2006). Historical biogeography of some river basins in central México evidenced by their goodeine freshwater fishes: a preliminary hypothesis using secondary Brooks parsimony analysis. *Journal of Biogeography, 33*, 1437–1447. https://doi.org/10.1111/j.1365-2699.2006.01526.x.

Domínguez-Domínguez, O., Alda, F., Pérez-Ponce De León G., García-Garitagoitia, J. L, & Doadrio, I. (2008). Evolutionary history of the endangered fish *Zoogoneticus quitzeoensis* (Bean, 1898) (Cyprinodontiformes: Goodeidae) using a sequential approach to phylogeography based on mitochondrial and nuclear DNA data. BMC Evolutionary Biology, 8: 161. doi:https://doi.org/10.1186/1471-2148-8-161.

Domínguez-Domínguez, O., Pedraza-Lara, C., Gurrola-Sánchez, N., Perea, S., Pérez-Rodríguez, R., Israde-Alcántara, I., et al. (2010). Historical biogeography of the Goodeinae (Cyprinodontiforms). In: Uribe MC, Grier HJ. (Ed.) *Viviparous Fishes II.* (pp. 34–69). New life publications 2010.

Drummond, A. J., & Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology.* https://doi.org/10.1186/1471-2148-7-214.

Drummond, A. J., Rambaut, A., Shapiro, B., & Pybus, O. G. (2005). Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular Biology and Evolution, 22*, 1185–1192. https://doi.org/10.1093/molbev/msi103.

Echelle, A. A., Echelle, A. F. (1984). Evolutionary genetics of a "species flock" Atherinid fishes on mesa central of Mexico. In an Echelle & Kornfield. (Ed.) *Evolution of fish species flock*, (pp 93-110). University Maine Orono.

Elmer, K. R., Kusche, H., Lehtonen, T. K., & Meyer, A. (2010). Local variation and parallel evolution: morphological and genetic diversity across a species complex of neotropical crater lake cichids fishes. *Philosophical Transaction Royal Society B, 365*, 1763–1782. https://doi.org/10.1098/rstb.2009.0271.

Excoffier, L., & Lischer, H. E. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analysis under Linux and Windows. Molecular Ecology Resources, 10, 564–567. doi: org/https://doi.org/10.1111/j.1755-0998.2010.02847.x.

Excoffier, L., Smouse, P. E., & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distance among DNA haplotypes application to human mitochondrial DNA restriction data. *Genetics, 131*, 479–491.

Fluker, B. L., Pezold, F., & Minton, R. L. (2011). Molecular and morphological divergence in the inland silverside (*Menidia beryllina*) along a freshwater-estuarine interface. *Enviromental Biolology of Fishes, 91*, 311–325. https://doi.org/10.1007/s10641-011-9786-2.

García-de León, F. J., Ramírez-Herrejón, J. P., García-Ortega, R., & Hendrickson, D. A. (2014). Foraging patterns of four sympatric species of silversides (Atheriniformes: Atherinopsidae) in Lago de Pátzcuaro , Central México. *UNED Research Journal, 6*, 127–139.

García-Martínez, R. M., Mejía, O., García-De Leon, F. J., & Barriga-Sosa, I. (2015). Extreme genetics divergence in the endemic fish *Chirostoma humboldtianum* (Valenciennes , 1835): implications for its conservation. *Hidrobiológica, 25*, 95–106.

Garduño-Monroy, V. H., Chávez-Hernández, J., Aguirre-González, J., Vázquez-Rosas, R., Mijares, H., Israde-Alcántara, I., et al. (2009). Zonificación de los periodos naturales de oscilación superficial en la ciudad de Pátzcuaro, Mich. México, con base en microtremores y estudios paleosismología. *Revista Mexicana de Ciencias Geológicas, 26*, 623–637.

Grummer, J. A., Bryson Jr., R. W., & Reeder, T. W. (2014). Species delimitation using Bayes factors: simulations and application to the *Sceloporus scalaris* species group (Squamata: Phrynosomatidae). *Systematic Biology, 63*, 119–133. https://doi.org/10.1093/sysbio/syt069.

Gulisija, D., Kim, Y., & Plotkin, J. B. (2016). Phenotypic plasticity promotes balanced polymorphism in periodic environments by a genomic storage effect. *Genetics., 202*, 1437–1448. https://doi.org/10.1101/038497.

Heled, J., & Drummond, A. J. (2010). Bayesian inference of species trees from multilocus data. *Molecular Biology and Evolution, 27*, 570–580. https://doi.org/10.1093/molbev/msp274.

Israde-Alcántara, I., & Garduño-Monroy, V. H. (1999). Lacustrine record in a volcanic intra-arc setting: the evolution of the late Neogene Cuitzeo basin system (central-western Mexico, Michoacan). *Palaeogeography, Palaeoclimatology, Palaeoecology, 151*, 209–227.

Israde-Alcántara, I., Garduño-Monroy, V. H., Fisher, C. T., Pollard, H. P., & Rodríguez-Pascua, M. A. (2005). Lake level change, climate, and the impact of natural events: the role of seismic and volcanic events in the formation of the Lake Patzcuaro Basin, Michoacan, México. *Quaternary International, 135*, 35–46. https://doi.org/10.1016/j.quaint.2004.10.022.

Kass, R. E., & Raftery, A. E. (1995). Bayes factors. *Journal of the American Statistical Association, 90*, 773–795.

Lake, C., Rodriguez-ruiz, A., & Granado-lorencio, C. (1988). Características del aparato bucal asociadas al régimen alimenticio en cinco especies coexistentes del género *Chirostoma* (Lago de Chapala, México). *Revista Chilena de Historia Natural, 61*, 35–51.

Lanfear, R., Calcott, B., Ho, S. Y., & Guindon, S. (2012). PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyzes. *Molecular Biology and Evolution, 29*, 1695–1701.

Lartillot, N., & Philippe, H. (2006). Computing Bayes factors using thermodynamic integration. *Systematic Biology, 55*, 195–207.

Leaché, A. D., Fujita, M. K., Minin, V. N., & Bouckaert, R. R. (2014). Species delimitation using genome-wide SNP Data. *Systematic Biology, 63*, 534–542. https://doi.org/10.1093/sysbio/syu018.

Lee, W. J., Conroy, J., Huntting, W., & Kocher, T. D. (1995). Structure and evolution of teleost mitochondrial control regions. *Journal of Molecular Evolution, 42*, 54–66.

Librado, P., & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics, 25*, 1451–1452. https://doi.org/10.1093/bioinformatics/btp187.

Maio, N., De Wu. C., Reilly, K. M. O., & Wilson, D., (2015). New Routes to Phylogeography : A Bayesian Structured Coalescent Approximation. *PLoS Genet*ic, 1–22 doi:https://doi.org/10.1371/journal.pgen.1005421.

Mayer, B., & Matschiner, W. (2015). Molecular phylogenetics and evolution a tribal level phylogeny of Lake Tanganyika chichlid fishes based on a genomic multi-marker aproach. *Molecular Phylogentics and Evolution, 83*, 56–71. https://doi.org/10.1016/j.ympev.2014.10.009.

Mayr, E. (1942). *Systematics and the origin of species*. New York: Columbia University Press.

Mercado-Silva, N., Lyons, J., Moncayo-Estrada, R., Gesundheit, P., Krabbenhoft, T. J., Powell, D. L., & Piller, K. R., (2015). Stable isotope evidence for trophic overlap of sympatric Mexican Lake Chapala silversides (Teleostei: Atherinopsidae: *Chirostoma* spp.). *Neotropical Ichthyolgy*, 00–00. doi:https://doi.org/10.1590/1982-0224-20140079.

Miller, R. R., Minckley, W. L., & Norris, S. M. (2005). *Freshwater fishes of Mexico*. Chicago: The University of Chicago Press.

Miller, M. A., Schwartz, T., Pickett, B. E., He, S., Klem, E. B., Scheuermann, R. H., Passarotti, M., Kaufman, S., & O'Leary, M. A. (2015). A RESTful API for access to phylogenetic tools via the CIPRES Science Gateway. *Evolutionary Bioinformatics, 11*, 43–48. https://doi.org/10.4137/EBO.S21501.

Moncayo-Estrada, R., & Buelna-Osben, H. R. (2001). Fish fauna of Lake Chapala. In A. M. Hansen & M. van Afferden (Eds.), *The Lerma-Chapala watershed. Evaluation and management* (pp. 215–242). New York (NY): Kluwer Academic Press.

Moncayo-Estrada, R., Lyons, J., Escalera-Gallardo, C., & Lind, O. T. (2012). Long-term change in the biotic integrity of a shallow tropical lake: a decadal analysis of the Lake Chapala fish community. *Lake and Reservoir Management, 28*, 92–104. https://doi.org/10.1080/07438141.2012.661029.

Ornelas-García, C. P., Domínguez-Domínguez, O., & Doadrio, I. (2008). Evolutionary history of the fish genus *Astyanax* Baird & Girard (1854) (Actinopterygii, Characidae) in Mesoamerica reveals multiple morphological homoplasies. *BMC Evolutionary Biology, 8*, 340. https://doi.org/10.1186/1471-2148-8-340.

Palumbi, S., Martin, A., Romano, S., McMillan, W. O., Stice, L., Grabosky, G., & Stice, L. (1991). *Simple fool's guide to PCR, version 2*. Honolulu, HI: Department of Zoology, University of Hawaii.

Perdices, A., Bermingham, E., Montilla, A., & Doadrio, I. (2002). Evolutionary history of the genus *Rhamdia* (Teleostei: Pimelodidae) in Central America. *Molecular Phylogenetics and Evolution, 25*, 172–189. https://doi.org/10.1016/S1055-7903(02)00224-5.

Pérez-Miranda, F., Mejía, O., Soto-Galera, E., Espinosa-Pérez, H., Piálek, L., & Říčan, O. (2017). Phylogeny and species diversity of the genus *Herichthys* (Teleostei: Cichlidae). *Journla of Zoological Systematic and Evolutionary Research*. https://doi.org/10.1111/jzs.12197.

Pérez-Rodríguez, R., Domínguez-Domínguez, O., Doadrio, I., Cuevas-García, E., & Pérez-Ponce de Leon, G. (2015). Comparative historical biogeography of three groups of Neartic freshwater fishes across central Mexico. *Fish Biology 86*, 993-1015.

Pérez-Rodríguez, R., Domínguez-Domínguez, O., Pérez-Ponce de León, G., & Doadrio, I. (2009). Phylogenetic relationships and biogeography of the genus *Algansea* Girard (Cypriniformes: Cyprinidae) of central México inferred from molecular data. *BMC Evolutionary Biology*. https://doi.org/10.1186/1471-2148-9-223.

Piller, K. R., Kenway-Lynch, C. S., Camak, D. T., & Domínguez-Domínguez, O. (2015). Phylogeography and population structure of the imperiled redtail splitfin (Goodeidae: *Xenotoca eiseni*): Implication for conservation. *Copeia*. https://doi.org/10.1643/CI-14-067.

Rannala, B., & Yang, Z. (2003). Bayes estimation of species divergence times and ancestral population sizes using DNA sequences from multiple loci. *Genetics, 164*, 1645–1656.

Ribbink, A. J. (1984). Is the species flock concept tenable? In I. Kornfield (Ed.), *Echelle AA* (pp. 21–25). Orono: Evolution of fish species flocks. University of Maine at Orono Press.

Rosenblum, E. B., Parent, C. E., & Brandt, E. E. (2014). The molecular basis of phenotypic convergence. *Annual Review of Ecology, Evolution, and Systematics*. https://doi.org/10.1146/annurev-ecolsys-120213-091851.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual (2nd ed.)*. New York, NY: Cold Spring Harbor Laboratory.

Schluter, D. (2000). The ecology of adaptive radiation. Oxford Unversity Press.

Schluter, D., & Conte, G. L. (2009). Genetics and ecological speciation. *PNAS*. https://doi.org/10.1073/PNAS.0901264106.

Seehausen, O. (2004). Hybridization and adaptive radiation. *Trends in Ecology & Evolution, 19*, 198–207.

Seehausen, O., & Wagner, C. E. (2014). Speciation in freshwater fishes. *Annual Review of Ecology, Evolution, and Systematics*. https://doi.org/10.1146/annurev-ecolsys-120213-091818.

Soria-Barreto, M., & Paulo-Maya, J. (2005). Morfometría comparada del aparato mandibular en especies de *Chirostoma* Atheriniformes : Atherinopsidae del Lago de Pátzcuaro , Michoacán , México Morphometric comparison of the mandibular region in species of *Chirostoma* Atheriniformes : Atherinopsid. *Hidrobiologica, 15*, 161–168.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*. https://doi.org/10.1093/molbev/mst197.

Tarvin, R. D., Powell, E. A., Santos, J. C., Ron, S. R., & Cannatella, D. C. (2017). The birth of aposematism: high phenotypic divergence and low genetic diversity in a young clade of poison frogs. *Molecular Phylogenetics and Evolution*. https://doi.org/10.1016/j.ympev.2016.12.035.

Thompson, D. J., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The Clustal_X windows interface: flexible strategies for multiple sequences alignment aided by quality analysis tools. *Nucleic Acids Research*. https://doi.org/10.1093/nar/25.24. 4876.

Unmack, P. J., Allen, G. R., & Johnson, J. B. (2013). Phylogeny and biogeography of rainbowfishes (Melanotaeniidae) from Australia and New Guinea. *Molecular Phylogenetics and Evolution*. https://doi.org/10.1186/s12862-018-1172-7.

Wagner, C. E., Keller, I., Wittwer, S., Selz, O. M., Mwaiko, S., Greuter, L., Sivasundar, A., & Seehausen, O. (2012). Genome-wide RAD sequences data provide unprecedented resolution of species bundaries and relathionships in the Lake Victoria chichlid adaptaive radiation. *Molecular Ecolology*. https://doi.org/10.1111/mec.12023.

Wainwright, P., Smith, W., & Price, S. (2012). The evolution of pharyngognathy: a phylogenetic and functional appraisal of the

pharyngeal jaw key innovation in labroid fishes and beyond. *Systematic Biology*. https://doi.org/10.1093/sysbio/sys060.

Ward, R. D., Woodwark, M., & Skibinski, D. O. F. (1994). A comparison od genetic diversity levels in marine, freshwater, and anadramous fishes. *Journal of Fish Biology, 44*, 213–232.

Xia, X. (2013). DAMBE5: a comprehensive softwere package for data analysis in molecular biology and evolution. *Molecular Biology and Evolution*. https://doi.org/10.1093/molbev/mst064.

Xie, W. G., Lewis, P. O., Fan, Y., Kuo, L., & Chen, M. H. (2011). Improving marginal likelihood estimation for Bayesian phylogenetic model selection. *Systematic Biology*. https://doi.org/10.1093/sysbio/syq085.

Yang, Z. (2015). The BPP program for species tree estimation and species delimitation. *Current Zoology, 61*(5), 854–865. https://doi.org/10.1093/czoolo/61.5.854.

Yang, Z., & Rannala, B. (2010). Bayesian species delimitation using multilocus sequence data. *PNAS*. https://doi.org/10.1073/pnas.0913022107.

Yang, Z., & Rannala, B. (2014). Unguided species delimitation using DNA sequence data from multiple loci. *Molecular Biology and Evolution*. https://doi.org/10.1093/molbev/msu279.

Yoder, J. B., Clacey, E., Des Roches, S., Eastman, J. M., Gentry, L., Godsoe, W., Hagey, T. J., Jochimsen, D., Oswald, B. P., Robertson, J., Sarver, B. A. J., Schenk, J. J., Spear, S. F., & Harmon, L. J. (2010). Ecological opportunity and the origen of adaptative radiations. *Evolutionary Biology*. https://doi.org/10.1111/j.1420-9101.2010.02029.x.

Zamudio, K. R., Bel, R. C., & Mason, N. A. (2016). Phenotypes in phylogeography: species' traits, environmental variation, and vertebrate diversification. *PNAS*. https://doi.org/10.1073/pnas.1602237113.