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Phylogeny and taxonomy of the genus *Ilyodon* Eigenmann, 1907 (Teleostei: Goodeidae), based on mitochondrial and nuclear DNA sequences

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Abstract

Taxonomy of the live-bearing fish of the genus *Ilyodon* Eigenmann, 1907 (Goodeidae), in Mexico, is controversial, with morphology and mitochondrial genetic analyses in disagreement about the number of valid species. The present study accumulated a comprehensive DNA sequences dataset of 98 individuals of all *Ilyodon* species and mitochondrial and nuclear loci to reconstruct the evolutionary history of the genus. Phylogenetic inference produced five clades, one with two subclades, and one clade including three recognized species. Genetic distances in mitochondrial genes (*cytb*: 0.5%–2.1%; *coxI*: 0.5%–1.1% and *d-loop*: 2.3%–10.2%) were relatively high among main clades, while, as expected, nuclear genes showed low variation (0.0%–0.2%), with geographic concordance and few shared haplotypes among river basins. High genetic structure was observed among clades and within basins. Our genetic analyses, applying the priority principle, suggest the recognition only of *Ilyodon whitei* and *Ilyodon furcidens*, with *I. cortesae* relegated to an invalid species, the populations of which belong to *I. whitei*.

KEYWORDS
diversification, endemic fish, mitochondrial DNA, nuclear DNA, taxonomy

1 | INTRODUCTION

The geological history of Central Mexico is characterized by high tectonic and volcanic activity since the Miocene, at least 16 Mya that continues to the present, generating an ongoing process of hydrological reconfiguration (Ferrari, Conticelli, Vaggelli, Potrone, & Manetti, 2000). This dynamic geomorphology has been postulated as the primary cause of the complex evolutionary history of the freshwater fish fauna of Central Mexico, exceeding the effects of biological characteristics and the evolution of climate conditions (Barbour, 1973; Domínguez-Domínguez, Doadrio, Martínez-Meyer, Zambrano, & Pérez-Ponce de León, 2006; Domínguez-Domínguez et al., 2010;

Pérez-Rodríguez, Domínguez-Domínguez, Doadrio, Cuevas-García, & Pérez-Ponce de León, 2015; Smith, 1980). Of the nearly 100 described species of freshwater fish in Central Mexico, ~70% are endemic (Miller, Minckley, & Norris, 2005) as a result of paleogeological isolation processes, especially volcanism and tectonic events.

Studies of endemic and native freshwater fishes in Central Mexico have chiefly focussed on phylogeny based on DNA sequences or biogeographic aspects of complete groups of fishes in Mexico (Corona-Santiago, Doadrio, & Domínguez-Domínguez, 2015; Doadrio & Domínguez, 2004; Domínguez-Domínguez, Pérez-Rodríguez, Escalera-Vázquez, & Doadrio, 2009; Domínguez-Domínguez et al., 2010; Pérez-Rodríguez, Domínguez-Domínguez, Pérez-Ponce de León, & Doadrio, 2009; Pérez-Rodríguez et al., 2015; Schönhuth & Doadrio, 2003; Schönhuth, Doadrio, Domínguez-Domínguez, Hillis, & Mayden, 2008), while within-species phylogeographic studies are scarce (Domínguez-Domínguez, Alda, Pérez-Ponce de León, García-Garitagoitia, & Doadrio, 2008; Mateos, Sanjur, & Vrijenhoek, 2002), especially of species distributed in Central Mexico Pacific drainages (CMPD) (Domínguez-Domínguez et al., 2006; Mateos, 2005; Piller, Kenway-Lynch, Camak, & Domínguez-Domínguez, 2015). Phylogeographic and population studies are essential tools in understanding evolutionary patterns and provide useful information on genetic isolation of populations on a geographic and temporal scale. Phylogeographic data are especially relevant when the populations studied are under threat, as is the case for the Central Mexico endemic subfamily Goodeinae (Domínguez-Domínguez & Pérez Ponce de León, 2007). Phylogeographic studies can identify divergent populations and evolutionarily isolated lineages undetected by traditional taxonomy (Domínguez-Domínguez et al., 2008; Mateos, 2005; Piller et al., 2015).

The goodeids in Central Mexico include the endemic subfamily Goodeinae represented by approximately 19 genera, including *Ilyodon*, and 40 species of viviparous fishes with internal fertilization and matrotrophy (Doadrio & Domínguez, 2004; Domínguez-Domínguez & Pérez Ponce de León, 2007). Geographic distribution of *Ilyodon* is limited in the CMPD to the main basin of the Balsas River and the adjacent Coahuayana, Armería, Ameca, Purificacion, and Marabasco River basins (Figure 1).

Species of *Ilyodon* have long been taxonomically controversial. Five species have been described: *Ilyodon whitei* (Meek, 1904),

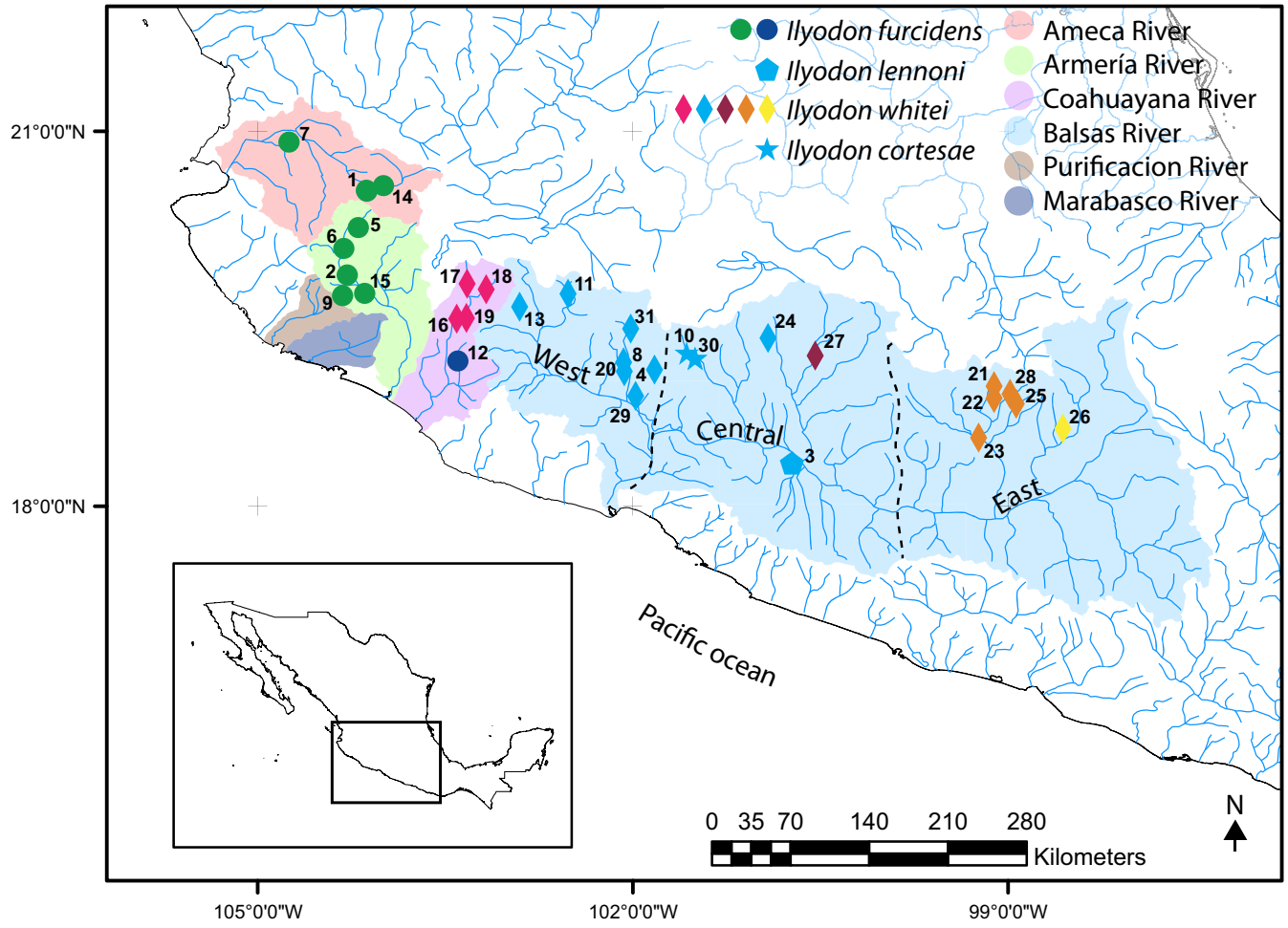

FIGURE 1 Sampling locations (symbols) and the hydrological basins (filled areas) where *Ilyodon* is distributed, numbers corresponded with Table 2. The lines of the Balsas basin show the west, central, and east and divisions considered in the present study. The colors of symbols correspond to the colors of the clades

Ilyodon furcidens (Jordan & Gilbert, 1882), *Ilyodon xantusi* (Hubbs & Turner, 1939), *Ilyodon lennoni* Meyer & Föerster, 1983, and *Ilyodon cortesae* Paulo-Maya & Trujillo-Jiménez, 2000. However, opinions have differed with regard to the number of valid species. The catalog of fishes (Eschmeyer, Fricke, & Van der Laan, 2016) includes four valid species (*I. whitei*, *I. furcidens*, *I. lennoni*, and *I. cortesae*), whereas taxonomy based on molecular studies has identified only *I. whitei* and *I. furcidens* (Doadrio & Domínguez, 2004). The results of a comprehensive study of Goodeidae, including all species of *Ilyodon*, using a single mitochondrial DNA gene, did not find monophyletic groups (Domínguez-Domínguez et al., 2010).

At the population level, morphological and genetic data also generate wide discussion of *Ilyodon* speciation *vs.* phenotypic plasticity in previously separated species or subspecies (Kingston, 1979; Turner & Grosse, 1980). Two trophic morphs found in sympatry in the Coahuayana and Armería River basins, described as *I. furcidens* and *I. xantusi*, have been considered the same species with an incipient signature of reproductive isolation, resulting from trophic differentiation (Grudzien & Turner, 1984a,b; Turner & Grosse, 1980).

The present study comprises a comprehensive report of *Ilyodon*, including all identified species throughout their distribution range and DNA sequences analyses of three mitochondrial and two nuclear markers. The use of combined mitochondrial and nuclear genes allows a better understanding of the evolution and taxonomy of *Ilyodon*. The aims of the study were to infer the evolutionary history of *Ilyodon* and elucidate the relationships among described species.

2 | MATERIALS AND METHODS

2.1 | Fish sampling

Ninety-eight specimens of *I. cortesae*, *I. furcidens*, *I. lennoni*, and *I. whitei* from throughout their distribution range were collected from the east, central, and west sub-basins of the Balsas River basin, as well as the Coahuayana, Armería, and Ameca River basins (Figure 1). *Ilyodon xantusi* has been described from a tributary of the Armería River, near Colima city. In this work, following Turner and Grosse (1980), we considered *I. xantusi* a junior synonym of *I. furcidens*.

Fish were captured by electrofishing and trawl nets and anesthetized with tricaine mesylate (MS-222). A fin fragment of each fish was preserved in 95% ethanol for DNA extraction. A maximum of five specimens from each locality were preserved in 5% formalin and deposited in the fish collection of several institutions, approved by the Ministry of Environment and Natural Resources for Mexico (SEMARNAT), with the permission number: SGPA/DGVS/08473/15. The remaining fish were released at the capture site. The fin clips were deposited in the fish collection at the Universidad Michoacana de San Nicolás de Hidalgo, México (SEMARNAT registration number MICH-PEC-227-07-09), the fish collection of the Universidad Autónoma del Estado de Morelos, México (SEMARNAT registration number MOR-CC-243-201), and the collection of the Museo Nacional de Ciencias Naturales, Spain (Table 1). Based on published reports and available samples, we used *Allodontichthys* as outgroup (Doadrio & Domínguez, 2004; Domínguez-Domínguez et al., 2010). Information on sampling is provided in Table 2.

2.2 | DNA Extraction, amplification, and sequencing

Total genomic DNA was isolated with the Qiagen Dneasy Tissue and Blood Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Fragments of three mitochondrial genes and two nuclear genes were amplified: *cytochrome b* (*cytb*: 533 bp), *cytochrome oxidase sub-unit I* (*coxI*: 626 bp), and control region (*d-loop*: 441 bp), for a total of 1600 bp from 98 individuals, and a fragment of the nuclear *β-actin* gene (*ACTB*: 979) and the exon 3 of the *recombination-activating* gene 1 (*RAG1*: 1453), for a total of 2432 bp from a subset of 51 individuals, representing the variation found in mtDNA haplotypes. Polymerase chain reactions (PCRs) were conducted in a reaction volume of 12.5 μl containing 4.25 μl ultrapure water, 0.5 μl of each 0.2 μM primer, 6.25 μl Dream Taq Green PCR Master Mix 2x (Thermo Scientific, Waltham, MA, USA), and 1 μl (ca 10–100 ng) of DNA template. The protocols for amplification are presented in Table S1. The PCR products were purified using ExoSAP-IT (USB Corp. Cleveland, OH, USA) and submitted to Macrogen Inc. (Netherlands) for sequencing. Nucleotide sequences were edited and aligned in Mega v. 6.06 (Tamura et al., 2013). The sequences of *ACTB* showed heterozygous positions defined by indels, and a manual reconstruction of the two allele phases was performed following the procedure described by Sousa-Santos, Robalo, Collares-Pereira, and Almada (2005). The *d-loop* and *ACTB* genes showed ambiguously aligned positions that are shown in Table S2. For the *RAG1* gene, the phase of heterozygous genotypes was resolved using DNAsp v. 5.10 (Librado & Rozas, 2009) and conducted with the algorithm provided by PHASE v. 2.0 (Stephens & Donelly, 2003). Recombination of nuclear genes was assessed with the phi test in Splitstree v. 4.13 (Huson & Bryant, 2006) and did not find significant evidence for recombination in either gene ($p = 1$ for both). Codification of amino acids was used to verify the alignment and the absence of stop codons. The obtained sequences were deposited in GenBank under accession numbers for *cytb*: KY204452-KY204540, for *coxI*: KY118827-KY118914, for *d-loop*: KY204628-KY204716, for *ACTB*: KY204717-KY204778, and for *RAG1*: KY204541-KY204627 (Table S3). All raw data: alignments of each one of the genes, are shown in Dataset S1, S2, S3, S4 and S5.

2.3 | Phylogeny based on DNA sequences and haplotype networks

The incongruence length difference test (partition homogeneity test; Farris, Källersjö, Kluge, & Bult, 1995) was conducted in Phylogenetic Analysis Using Parsimony* and other methods (PAUP*) v. 4.0b10 (Swofford, 2003) to evaluate the significance of conflict among datasets, using 1000 resampling of characters. Phylogenetic analyses were conducted for each gene, for the concatenated dataset for mitochondrial genes, and for the five genes combined. Model selection based on the Akaike information criterion and optimal partition-setting analyses, conducted using PartitionFinder v. 1.1.0 (Lanfear,

TABLE 1 Geographical information, collection where the tissue is deposited and the voucher number of the sample

Locality	GPS coordinates	Fish collection	Tissue voucher number
Río Ameca, puente la muerta	20°31′44″N, 104°7′47.3″W	MNCN	31943, 31944, 31945, 31946
Río Armería	19°51′1.3″N, 104°17′0.0″W	MNCN	31971
Arroyo Chacambero	18°20′44.53″N, 100°43′43.97″W	MNCN	32155, 32156, 32157, 32752
Río Las Trojes	19°05′38.8″N, 101°49′33.5″W	MNCN	32158, 32159
Presa Copales	20°13′49.2″N, 104°11′42.9″W	MNCN	33008
Presa Tacotán	20°3′42.78″N, 104°18′43.67″W	MNCN	64259
Río Las Rosas	20°54′51.27″N, 104°45′1.75″W	MNCN	4266, 64367, 64368
Río Cajones	19°9′13.59″N, 102°4′23.7″W	UMSNH	5179, 5180
Río Ahuacapan	19°39′54.21″N, 104°19′19.79″W	UMSNH	8842, 8843
Manantial Cutzaróndiro	19°11′0.6″N, 101°30′8.3″W	UMSNH MNCN	9162, 9164, 9166 64229, 64230
Manantial Tocumbo	19°42′7″N, 102°30′60″W	UMSNH	9260, 9984, 9986
Arroyo El Tule, Tuxpan	19°19′32.3″N, 103°22′18.8″W	UMSNH	9267, 9268, 9269
Los Horcones	19°35′48.1″N, 102°54′15.2″W	UMSNH	9393
Potrero Grande	20°31′15″N, 104°7′36″W	UMSNH	9938, 9940, 9942, 9946
Achacales	19°42′14.1″N, 104°8′37.9″W	UMSNH	11989, 11990, 11991
Atenquique, Tuxpan	19°31′46.35″N, 103°25′56.39″W	UMSNH	12018, 12019
San Jerónimo, Tuxpan	19°41′42.3″N, 103°21′8.2″W	UMSNH	12033
Los Pitayos, Tuxpan	19°45′35.3″N, 103°11′8.4″W	UMSNH	12056
Arroyo La Purisima, Tuxpan	19°31′19.8″N, 103°20′32.9″W	UMSNH	13042
Arroyo coróndiro, Nueva Italia.	19°4′46.7″N, 102°4′2.5″W	UMSNH	36420, 36421, 36422, 36423, 36424
Barranca de Cuernavaca, Morelos	18°52′0″N, 99°6′38.6″W	UMSNH	36434, 36435, 36436, 36437, 36438
Barranca San Andrés de la Cal	18°57′41.1″N, 99°7′46″W	UMSNH	36444, 36445, 36446, 36447, 36448, 36449
Río Apatlaco, Jojutla	18°33′0″N, 99°14′0″W	UAEM	51
Río Chinapa, Tzitzio	19°20′50.8″N, 100°55′6.7″W	UMSNH	36415,36416, 36417, 36418, 36419
Río Cuautla, el ojito	18°49′18.3″N, 98°56′0.1″W	UMSNH	36429, 36430, 36431, 36432, 36433
Río Rijo, Izúcar de Matamoros	18°37′23.8″N, 98°33′39″W	UAEM	52, 53, 54, 55
Río Zitacuaro, Tuzantla	19°12′18.3″N, 100°32′24.2″W	UMSNH	36406, 36407, 36408, 36409, 36450
Río Yautepec, Oaxtepec	18°53′55″N, 98°58′59″W	UMSNH	36439, 36440, 36441, 36442, 36443
Río Zicuirán, Zicuirán	18°53′1.4″N, 101°58′35.7″W	UMSNH	36410, 36411, 36412, 36413, 36414
Río Tacámbaro, Puruarán	19°11′0.6″N, 101°30′8.3″W	UMSNH	36425, 36426, 36427, 36428, 36451
Río Cupatitizio, Parque Uruapan	19°23′19″N, 102°0′51″W	MNCN UMSNH	33649 9584, 9585

UMSNH, Universidad Michoacana de San Nicolás de Hidalgo; UAEM, Universidad Autónoma del Estado de Morelos; MNCN, Museo Nacional de Ciencias Naturales

Calcott, Ho, & Guindon, 2012), suggested that optimal partition setting was obtained by assigning a substitution model for each gene (Table S4). Genetic trees were constructed using maximum likelihood and Bayesian inference. Maximum likelihood analyses were carried out using RAxMLGUI v.1.3.1 (Silvestro & Michalak, 2012; Stamatakis, 2014), with the substitution model GTR + gamma and 10,000 bootstrap replicates.

The relative stability of clades was evaluated by 1,000 nonparametric bootstrap replicates (Alfaro, Zooler, & Lutzoni, 2003). Bayesian analyses were implemented using MrBayes v. 3.2.1 (Ronquist et al., 2012). The analysis was run for 10 million of generations, with two independent runs implementing four Markov chain Monte Carlo (MCMC) processes, sampling every 100 generations. We evaluated the chain convergence with the log-likelihood (−lnL) values of the runs on Tracer v. 1.5 (Rambaut & Drummond, 2007), discarding 10% of generations as burn-in to construct the consensus tree ($\sigma = 0.0002$).

To determine the geographic correspondence with the genetic structure, a haplotype network for each gene was constructed using the median-joining algorithm as implemented in Network v. 4.6.1.3 (Bandelt, Forster, & Röhl, 1999).

2.4 | Genetic distances and structure

To analyze the genetic structure of populations of *Ilyodon* spp., we conducted analyses of molecular variance (AMOVA) and calculated

TABLE 2 Sampling locations

Site	Locality	Sub-basin	Basin	Species
1	Río Ameca, puente la muerta	Ameca	Ameca	*Ilyodon furcidens*
2	Río Armería	Ayuquila	Armería	*Ilyodon furcidens*
3	Arroyo Chacambero	Medio Balsas	Central Balsas	*Ilyodon lennoni*
4	Río Las Trojes	Cupatitzio	West Balsas	*Ilyodon whitei*
5	Presa Copales	Ayuquila	Armería	*Ilyodon furcidens*
6	Presa Tacotán	Ayuquila	Armería	*Ilyodon furcidens*
7	Río Las Rosas	Mascota	Ameca	*Ilyodon furcidens*
8	Río Cajones	Cupatitzio	West Balsas	*Ilyodon whitei*
9	Río Ahuacapan	Ahuacapan	Ameca	*Ilyodon furcidens*
10	Manantial Cutzaróndiro	Tacámbaro	Central Balsas	*Ilyodon cortesae*
11	Manantial Tocumbo	Tepalcatepec	West Balsas	*Ilyodon whitei*
12	Arroyo El Tule, Río Tuxpan	Tamazula	Coahuayana	*Ilyodon furcidens*
13	Los Horcones	Tepalcatepec	West Balsas	*Ilyodon whitei*
14	Potrero Grande	Ameca	Ameca	*Ilyodon furcidens*
15	Achacales	Ayuquila	Armería	*Ilyodon furcidens*
16	Atenquique, Río Tuxpan	Tamazula	Coahuayana	*Ilyodon whitei*
17	San Jerónimo, Río Tuxpan	Tamazula	Coahuayana	*Ilyodon whitei*
18	Los Pitayos, Río Tuxpan	Tamazula	Coahuayana	*Ilyodon whitei*
19	Arroyo La Purisima, Río Tuxpan	Tamazula	Coahuayana	*Ilyodon whitei*
20	Arroyo coróndiro, Nueva Italia.	Cupatitzio	West Balsas	*Ilyodon whitei*
21	Barranca de Cuernavaca, Morelos	Amacuzac	East Balsas	*Ilyodon whitei*
22	Barranca San Andrés de la Cal	Amacuzac	East Balsas	*Ilyodon whitei*
23	Río Apatlaco, Jojutla	Amacuzac	East Balsas	*Ilyodon whitei*
24	Río Chinapa, Tzitzio	Cutzamala	Central Balsas	*Ilyodon whitei*
25	Río Cuautla, el ojito	Amacuzac	East Balsas	*Ilyodon whitei*
26	Río Rijo, Izúcar de Matamoros	Atoyac	East Balsas	*Ilyodon whitei*
27	Río Zitacuaro, Tuzantla	Cutzamala	Central Balsas	*Ilyodon whitei*
28	Río Yautepec, Oaxtepec	Amacuzac	East Balsas	*Ilyodon whitei*
29	Río Zicuirán, Zicuirán	Cupatitzio	West Balsas	*Ilyodon whitei*
30	Río Tacámbaro, Puruarán	Tacámbaro	Central Balsas	*Ilyodon cortesae*
31	Río Cupatitizio, Parque Uruapan	Cupatitzio	West Balsas	*Ilyodon whitei*

the components of the fixation index Φ_{CT} (variation between groups), Φ_{ST} (variation within populations), and Φ_{SC} (variation among populations within groups) using Arlequin v. 3.5.1.3 (Excoffier & Lischer, 2010). The analyses were implemented for the five genes separately, as well as grouped, according to various criteria to estimate partitioning of the genetic variance at different hierarchical levels (Excoffier, Smouse, & Quattro, 1992). The first analysis considered each hydrological basin as a group. In the second analysis, each group comprised species that have been described and recognized as valid. Finally, in a third analysis all recovered groups within the main clades in phylogenetic inference were considered as groups. Analyses were performed using 10,000 permutations to significance values estimated in Arlequin v. 3.5.1.3.

The uncorrected genetic distances were calculated between the recovered groups in phylogenetic trees for each mitochondrial gene (*cytb*, *coxI* and *d-loop*) and between all individuals for *ACTB* and *RAG1* in Mega v.6.06 (Tamura et al., 2013), and a bootstrapping process was performed with 1,000 repetitions.

2.5 | Species delimitation test

Species tree analysis was conducted to obtain a guide tree and speciation model, using a multispecies coalescent model (Heled & Drummond, 2010) in BEAST v. 1.8.1 (Drummond, Suchard, Xie, & Rambaut, 2012), for implementation in the Bayesian species delimitation test using Bayesian phylogenetics and phylogeography (BPP v. 3.1; Yang & Rannala, 2010; Yang, 2015). For estimating the species tree model, the analysis was performed using the assumption that each clade recovered in the phylogenetic analyses represented a different species.

For the species tree ancestral reconstruction (StarBEAST) implemented in BEAST, the model parameters were unlinked across *cytb*,

coxI, *d-loop*, *ACTB*, and *RAG1* genes. The dataset consisted of one sequences for each gene (*cytb*, *coxI*, *d-loop*, *ACTB*, and *RAG1*), of each one of the clades and sub-clades. Substitution models were set according to the selected model for each gene by PartitionFinder v. 1.1.0 (Lanfear et al., 2012). We applied a lognormal relaxed clock (uncorrelated) model on branch length and calibrated the *cytb* partition using the mutation rate of *cytb* in teleosts of 0.76%–2.2%/million years (Machordom & Doadrio, 2001; Near & Benard, 2004; Zardoya & Doadrio, 1999). We estimated the evolutionary rate of the *coxI*, *d-loop*, *RAG1*, and *ACTB* genes relative to the *cytb* gene. We selected the tree prior-species Tree: Yule process model. Markov chain Monte Carlo analysis was run for 70 million generations, sampled every 1,000 generations. We evaluated the chain convergence with the −lnL values in Tracer v. 1.5 (Rambaut & Drummond, 2007) and summarized the results using TreeAnnotator v. 1.8.1 (Drummond et al., 2012).

For the BPP analyses of the five concatenated genes, we used the reversible-jump Markov chain Monte Carlo (rjMCMC) (Yang & Rannala, 2010) algorithm to determine whether to collapse or retain nodes throughout the phylogeny. Using the entire dataset coded by each gene, we tested with two algorithms: Analysis A10, in which the rjMCMC algorithm was used to move between species delimitation models that were compatible with a fixed guide tree (Rannala & Yang, 2013; Yang & Rannala, 2010), and Analysis A11 that explored species delimitation models and species phylogenies with the nearest neighbor interchange or sub-tree pruning and re-grafting used to change the species tree topology and test all species tree models from a fixed tree (Yang & Rannala, 2014).

To determine whether lineages could be considered distinct species under a general lineage species concept, the program assessed the probability of the node separating the species (De Queiroz, 2007). We used algorithm 0 with values of 5, 10, 15, 20 for the fine-tuning parameter to ensure that the rjMCMC mixed effectively in species delimitation models. We conducted analyses with priors θ and τ_0 (Leaché & Fujita, 2010) to discern how the effective ancestral population size and time of divergence influenced results. We initially set the gamma prior at θ and τ to the values $\alpha = 1$ and 2 and $\beta = 10$, 100, and 2000 and ran four analyses of each with different starting seeds for two independent chains of 500,000 generations with a burn-in of 50,000 and thinning every five generations. Finally, to test the robustness of the results, the analysis was repeated, randomizing individuals to either group to minimize the over-splitting effect and changing the speciation model according to the genetic results obtained (two to six species).

2.6 | Ancestral area reconstruction

The ancestral area reconstruction for the species of genus *Ilyodon* was estimated using the dispersal–extinction–cladogenesis (DEC) model of LAGRANGE (Ree, Moore, Webb, & Donoghue, 2005; Ree & Smith, 2008), implemented in RASP v. 3.2 software (Yu, Harris, Blair, & He,2015). The ultrametric and dichotomous tree obtained for the five concatenated genes in BEAST software was used as the tree topology on which mapping ancestral areas. The number of maximum areas was kept as 2. For this analysis, we divided the distribution area of *Ilyodon* into four according to hydrological regions of distribution: Ameca River, Armería River, Balsas River, and Coahuayana River.

3 | RESULTS

3.1 | Phylogenetic relationships

The incongruence length difference test did not show significant differences, indicating that all genes presented the same phylogenetic signal. The phylogenetic analyses for the mitochondrial (*cytb*, *coxI*, d-*loop*: 1,600 bp; Fig. S1) and the concatenated gene dataset (*cytb*, *coxI*, d-*loop*, *ACTB*, *RAG1*: 4032 bp), based on maximum likelihood and Bayesian methods, recovered the same topology. Five well-differentiated clades were geographically segregated but did not correspond to actual basin configuration, corresponding to Ameca–Armería (clade A), central and east Balsas (clade B), Coahuayana lower (clade C), Coahuayana upper (clade D), and west Balsas (clade E) watersheds. The phylogenetic relationships among the five clades were not resolved, appearing as a large basal polytomy (Fig. 2). Clade A clustered individuals identified as *I. furcidens* from Ameca and Armería basins. Clade B clustered individuals of the central and east Balsas basin identified as *I. whitei*. For clade B, two well-supported sub-clades were identified: B1 included the Atoyac sub-basin (east Balsas) specimens, and B2 included clustering samples from the Zitacuaro River of Cutzamala sub-basin (central Balsas) and the Amacuzac sub-basin (east Balsas). Clade C consisted of samples from the lower Coahuayana basin identified as *I. furcidens*. Clade D clustered samples of the upper Coahuayana basin identified as *I. whitei*. These two species were identified on the basis of the morphology of their mouth, teeth, head, and coloration patterns. Finally, clade E grouped individuals of the central and western Balsas, including the sub-basins Cutzamala, Tacámbaro, middle Balsas, Cupatitzio, and Tepalcatepec, comprising specimens identified as *I. whitei*, *I. lennoni*, and *I. cortesae*, the last two collected in the type locality. Phylogeny based on nuclear genes was unresolved and high polytomy was recovered, as expected, for genes with low variation in closely related species (Fig. S2).

The haplotype networks for the mitochondrial genes essentially showed the general pattern of the phylogenetic analyses, with no shared haplotypes among groups. Five corresponded to the main clades A, B, C, D, and E and two to the sub-clades B1 and B2 (Fig. S3). The number of mutation steps between haplogroups differed depending on the marker, with 14–31 for the *d-loop*, 5–12 for *cytb*, and 3–9 for the *coxI*. The six haplogroups found in mitochondrial networks were not recovered in the haplotype networks of the nuclear genes (Fig. S4), and shared haplotypes were observed in nuclear genes of the defined mitochondrial groups. For the nuclear *ACTB* gene, structure was found in Ameca, Armería, and lower Coahuayana specimens with shared haplotypes. They were separated from the Balsas specimens, with the exception of the samples from

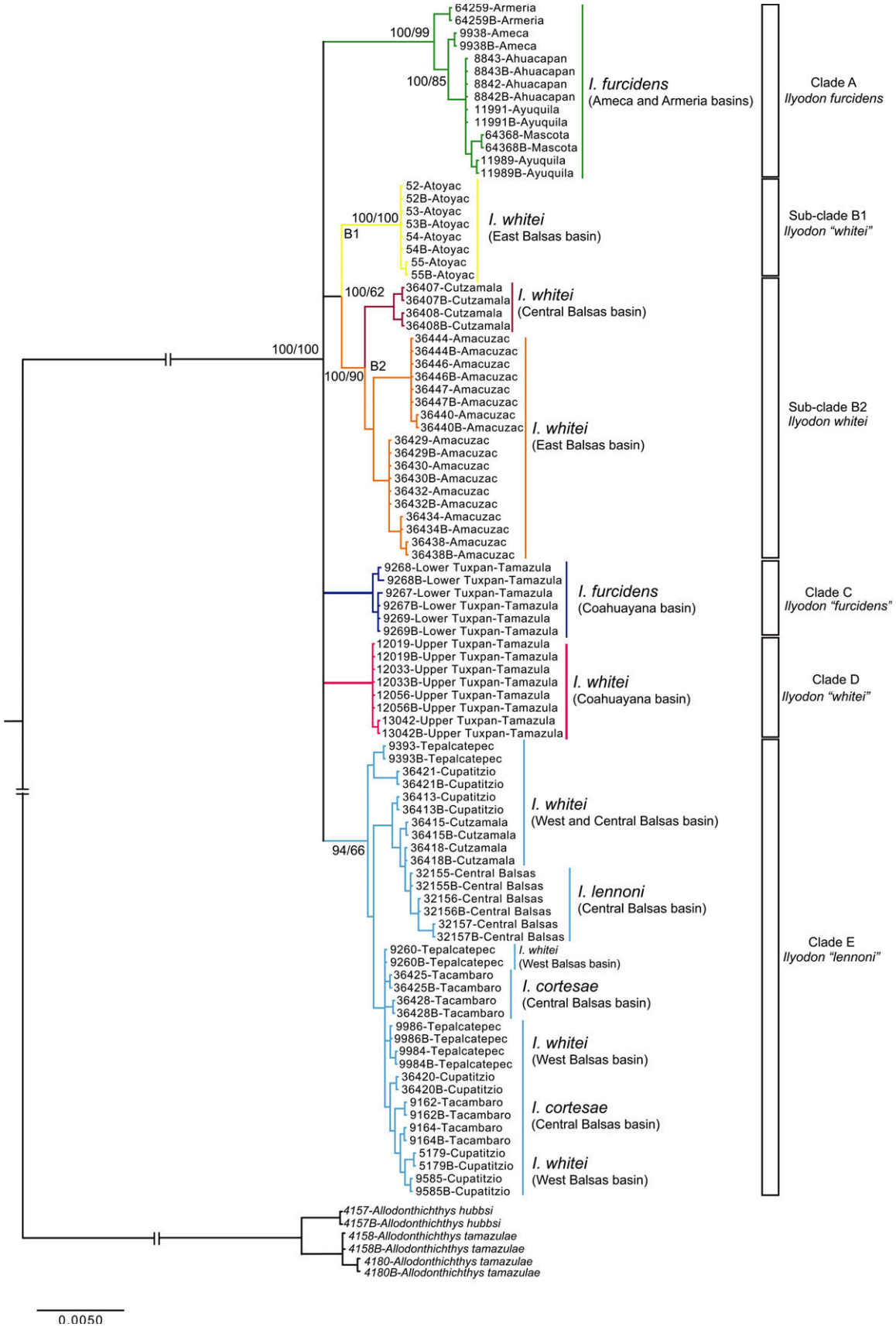

FIGURE 2 The Bayesian inference tree of *Ilyodon* species inferred from concatenated sequences of three mitochondrial genes (*cytb*, *coxI*, and *d-loop*; 1600 bp) and two nuclear genes (*ACTB* and *RAG1*; 2432 bp) concatenated. Bayesian posterior probability (>90%) and maximum likelihood bootstrap values (>80%) are indicated. Under the name of each clade, the taxonomic proposal of the present work is found

the Tepalcatepec sub-basin of the west Balsas basin, which were closely related to the upper Coahuayana samples. Samples from upper and lower Coahuayana showed no shared haplotypes. A single mutation step separated most of the samples from east-central Balsas from those to the west-central Balsas. The samples from the Zitacuaro River shared haplotypes with those of west Balsas, whereas the mtDNA was consistent with samples from the Amacuzac River, in the east Balsas. For *RAG1*, the same general pattern was observed, but haplotypes from Ameca, Armería, and the lower and upper Coahuayana showed more shared haplotypes than they did for the *ACTB* gene.

3.2 | Genetic distances and structure

The uncorrected mean genetic distances calculated between the main clades ranged from 3.7%–10.2% for the *d-loop*, 1%–2.1% for *cytb*, and 0.6%–1.1% for *coxI* (Tables S5, S6), and for nuclear genes ranged from 0%–0.2% (Table S7). Highest genetic distance for the *d-loop* and *cytb* genes was found between clade A and sub-clade B1, at 10.2% and 2.1% respectively. Based on *coxI*, the maximum genetic distances were observed in clades E and C with respect to sub-clade B2, 1.1% in both cases. Within the Balsas basin (clade B, east-central, and clade E, west-central), the mean genetic distances were 4.7% for the *d-loop*, 1.6% for *cytb*, and 1.1% for *coxI*. Sub-clades B1 and B2 showed mean genetic distances of 4.3% for the *d-loop*, 1.6% for the *cytb*, and 0.8% for the *coxI* genes. The genetic distances between clades C and D (lower and upper Coahuayana) were 0.5% for *cytb* and *coxI*, 2.3% for *d-loop*, and 0.1% for the nuclear genes.

In all analyzed genes, significant genetic structure was observed among *a priori* groups, among populations within groups, and within populations.

For the three mitochondrial genes, the highest ($p < .0001$) percent of variation among groups was when populations were grouped according to phylogenetic analyses (*cytb*, 72.9%; *cox*, 73.8%; *d-loop*, 78.6%; Table 3) and not according to hydrological basin (*cytb*, 35.5%; *coxI*, 29.9%; *d*-loop, 54.3%), or recognized species (*cytb*, 16.4%; *coxI*, 24.2%; *d-loop*, 31.1%). For nuclear genes, the highest ($p < .0001$) percent of variation was also among groups according to the phylogenetic analyses (*ACTB*, 80.09%; *RAG1*, 45.91%) (Table 4), but differences for *RAG1* were lower than found in mitochondrial genes and *ACTB* (Table 3).

3.3 | Species delimitation test

The speciation model based on the species tree estimate strongly supported the assumption of six species. In the tests of species delimitation implemented in BPP, we obtained strong support (posterior probability of 1) for the tested speciation model of six *a priori* defined species within *Ilyodon* (clade A: Ameca and Armería rivers; sub-clade B1: Atoyac sub-basin of the east Balsas River basin; sub-clade B2: Zitacuaro River of the Central Balsas and Amacuzac sub-basins of the east Balsas basin; clade C: lower Coahuayana basin; clade D: upper Coahuayana basin; and clade E: west Balsas River basin). However, in the posterior analyses conducted to minimize the over-splitting effect, reducing the number of species in the model and randomizing individuals or splitting populations to construct new clades, the posterior probability was 1 in all speciation models applied (Figure 3). The BPP was not sensitive for species delimitation, and no alteration of posterior probabilities of the speciation model was seen when we applied different values of root age (τ_0) and population size (θ), showing high posterior probabilities for models tested with both the A10 and A11 algorithms.

3.4 | Ancestral area reconstruction

Ancestral area reconstruction revealed a complex biogeographical history for *Ilyodon* species, with different events of dispersion and vicariance. The ancestral areas estimated for *Ilyodon* spp. were Armería and Balsas Rivers with a marginal probability of 0.315, followed by dispersion events to Ameca River, and one vicariance event in which Armería and Ameca Rivers were isolated from Balsas River. A second dispersal event was estimated from Balsas River toward Coahuayana River with a marginal probability of 0.769; inside of Balsas basin, several dispersal and vicariance events were estimated (Figure 4).

4 | DISCUSSION

In Central Mexico, the Pacific coast river drainages show a configuration in which the upper areas of the basins drain parts of the Mexican Plateau, while the low sections are in the Pacific Plain (Domínguez-Domínguez et al., 2006). This area is located in an active geological zone, with high volcanic activity during the late Pliocene and early Pleistocene (1.5–3.5 Mya) (Rosas-Elguera & Urrutia-Fucugauchi, 1998). Specifically, the activity in the triple junction (boundaries of the Tepic-Zacoalco, Chapala and Colima rifts), the Tenochtitlán fault system, and the Chapala–Oaxaca fault system (García-Palomo et al., 2002; Garduño-Monroy et al., 1998; Rosas-Elguera, Ferrari, Lopez-Martinez, & Urrutia-Fucugauchi, 1997), along with climate change at the pluvial–interpluvial period beginning *ca*. 0.9 Mya, had a strong influence on the configuration of river basins in the area and on distribution of freshwater fish populations (Hewitt, 2000; Smith et al., 2002; Webb & Bartlein, 1992).

The presence of the hard polytomy in the main clades recovered in the phylogenetic analyses makes the evolutionary history of *Ilyodon* difficult to interpret.

The phylogenetic, phylogeographic, and AMOVA results showed six well-differentiated groups, including main clades and sub-clades, that lack taxonomic and river basin configuration congruence: one distributed in the Armería and Ameca basins (clade A), two in the Coahuayana basin (clades C and D), and three belonging to the Balsas basin (B1, B2, and E).

At least four scenarios can be proposed for the genetic formation of the main groups: (i) the ancestor of *Ilyodon* evolved in the Armería or Balsas basins and later dispersed into the other basins, as is hypothesised by the DEC analysis and is partial supported by the

TABLE 3 Analyses of molecular variance for groups according to hydrological basin, recognized species, and those recovered in phylogenetic analyses [A = Ameca and Armería; B1 = east Balsas; B2 = east-central Balsas; C = lower Tuxpan River (Tamazula); D = upper Tuxpan River (Tamazula); E = west-central Balsas] for the mitochondrial genes

Testing assumptions	Source of variation	% of variance	Fixation index	*p*-value
Cytb				
Grouped according to hydrological basin [Ameca, Armería, Coahuayana and Balsas]	Among groups	35.46	Φ_{CT}: 0.35	ns
	Among populations within groups	51.85	Φ_{SC}: 0.80	<.0001
	Within populations	12.69	Φ_{ST}: 0.87	<.0001
	Total	100		
Grouped according to recognized species	Among groups	16.41	Φ_{CT}: 0.16	ns
	Among populations within groups	69.45	Φ_{SC}: 0.83	<.0001
	Within populations	14.14	Φ_{ST}: 0.85	<.0001
	Total	100		
Grouped according to recovered clades and sub-clades	Among groups	72.94	**Φ_{CT}: 0.72**	<.0001
	Among populations within groups	16.62	Φ_{SC}: 0.61	<.0001
	Within populations	10.44	Φ_{ST}: 0.89	<.0001
	Total	100		
cox1				
Grouped according to hydrological basin [Ameca, Armería, Coahuayana and Balsas]	Among groups	29.95	Φ_{CT}: 0.29	<.0001
	Among populations within groups	41.85	Φ_{SC}: 0.59	<.0001
	Within populations	28.20	Φ_{ST}: 0.71	<.0001
	Total	100		
Grouped according to recognized species	Among groups	24.24	Φ_{CT}: 0.24	ns
	Among populations within groups	66.78	Φ_{SC}: 0.88	<.0001
	Within populations	8.97	Φ_{ST}: 0.91	<.0001
	Total	100		
Grouped according to recovered clades and sub-clades	Among groups	73.87	**Φ_{CT}: 0.73**	<.0001
	Among populations within groups	21.52	Φ_{SC}: 0.82	<.0001
	Within populations	4.61	Φ_{ST}: 0.95	<.0001
	Total	100		
d-loop				
Grouped according to hydrological basin [Ameca, Armería, Coahuayana and Balsas]	Among groups	54.32	Φ_{CT}: 0.54	<.0001
	Among populations within groups	40.41	Φ_{SC}: 0.88	<.0001
	Within populations	5.27	Φ_{ST}: 0.94	<.0001
	Total	100		
Grouped according to recognized species	Among groups	31.10	Φ_{CT}: 0.31	ns
	Among populations within groups	62.36	Φ_{SC}: 0.90	<.0001
	Within populations	6.54	Φ_{ST}: 0.93	<.0001
	Total	100		
Grouped according to recovered clades and sub-clades	Among groups	78.64	**Φ_{CT}: 0.78**	<.0001
	Among populations within groups	15.34	Φ_{SC}: 0.71	<.0001
	Within populations	6.01	Φ_{ST}: 0.93	<.0001
	Total	100		

The highest values of percent of variation among groups are show in bold, while ns = not significant

results showed in Domínguez-Domínguez et al. (2010). This hypothesis was also supported by a study of helminth parasites of *Ilyodon* (Martínez-Aquino, Ceccarelli, Eguiarte, Vázquez-Domínguez, & Pérez-Ponce de León, 2014). (ii) Isolation of *Ilyodon* populations occurred, resulting in significant genetic structure in all analyzed genes, followed by secondary contact, supported by the shared haplotypes between drainages in the nuclear genes. This would involve a significant but low number of migrants, with genetic drift purifying the mitochondrial haplotype of migrants and acting on nuclear genes to a lesser extent than expected in large populations (Qu et al., 2012;

TABLE 4 Analyses of molecular variance of groups according to hydrological basin, recognized species, and groups recovered in phylogenetic analyses [A = Ameca and Armería; B1 = east Balsas; B2 = east-central Balsas; C = lower Tuxpan River (Tamazula); D = upper Tuxpan River (Tamazula); E = west-central Balsas] for the nuclear genes.

Testing assumptions	Source of variation	% of variance	Fixation index	*p*-value
ACTB				
Grouped according to hydrological basin [Ameca, Armería, Coahuayana and Balsas]	Among groups	75.81	Φ_{CT}: 0.75	<.0001
	Among populations within groups	9.97	Φ_{SC}: 0.41	<.0001
	Within populations	14.22	Φ_{ST}: 0.85	<.0001
	Total	100		
Grouped according to recognized species	Among groups	71.18	Φ_{CT}: 0.71	<.0001
	Among populations within groups	12.78	Φ_{SC}: 0.44	<.0001
	Within populations	16.05	Φ_{ST}: 0.83	<.0001
	Total	100		
Grouped according to clades and sub-clades	Among groups	80.09	**Φ_{CT}: 0.80**	<.0001
	Among populations within groups	1.64	Φ_{SC}: 0.08	Ns
	Within populations	18.27	Φ_{ST}: 0.81	<.0001
	Total	100		
RAG1				
Grouped according to hydrological basin [Ameca, Armería, Coahuayana and Balsas]	Among groups	46.90	**Φ_{CT}: 0.46**	<.0001
	Among populations within groups	15.13	Φ_{SC}: 0.28	<.0001
	Within populations	37.97	Φ_{ST}: 0.62	<.0001
	Total	100		
Grouped according to recognized species	Among groups	41.22	Φ_{CT}: 0.41	<.0001
	Among populations within groups	18.33	Φ_{SC}: 0.31	<.0001
	Within populations	40.46	Φ_{ST}: 0.59	<.0001
	Total	100		
Grouped according to recovered clades and sub-clades	Among groups	45.91	**Φ_{CT}: 0.46**	<.0001
	Among populations within groups	10.52	Φ_{SC}: 0.19	<.0001
	Within populations	43.57	Φ_{ST}: 0.56	<.0001
	Total	100		

The highest values of percent of variation among groups are show in bold, while ns = not significant

Sefc, Payne, & Sorenson, 2005). Possibly only males, which likely made up the bulk of migrants, reproduced, or selective pressures promoted the reproductive isolation of migrant females resulting in no shared haplotypes in mitochondrial genes (Qu et al., 2012). (iii) The genetic structure found in mitochondrial genes and the lack of resolution in nuclear genes could be due to relatively recent divergence of the main clades that shape *Ilyodon*, which resulted in the nuclear genes of the two most divergent groups (Ameca/Armería *vs*. central and east Balsas) showing no shared haplotypes, while divergent groups of the west Balsas basin and Coahuayana basin do exhibit shared haplotypes. This pattern in which nuclear genes resolve some structure but not to the extent of mitochondrial genes, due to recent diversification, has been reported for other freshwater fishes of Central Mexico (Pérez-Rodríguez et al., 2009). The last and more likely scenario for *Ilyodon* genetic groups evolution is (iv) a recent and simultaneous differentiation of the six genetic groups, that is supported by the similar values of genetic distance between them, the hard basal polytomy, and the lack of shared haplotypes in mitochondrial genes accompanied by incomplete lineage sorting in nuclear genes (Ballard, Chernoff, & James, 2002; Qu et al., 2012), and this also explains the low marginal probability found in the DEC analyses for the most plausible ancestral area. We suggest that our data are not enough for determining the ancestral area of *Ilyodon*. In any case, it is evident that the biogeographical history of *Ilyodon* is more complex than previously reported (Doadrio & Domínguez, 2004; Domínguez-Domínguez et al., 2010), mainly for lineage evolved in the Balsas basin, in which several events of dispersal and vicariance were estimated in DEC (Figure 4).

This complex history seems to be closely related to the complex hydrological system in the area. Genetic patterns related to connection and disconnection in Pacific slope drainages are partially supported by the goodeine species *Allodontichthys* spp., *Xenotoca eiseni*, and *Xenotoca melanosoma* (Domínguez-Domínguez et al., 2010; Piller et al., 2015; Webb, 2002) and other freshwater fish, such as *Algansea aphanea*, *Moxostoma* sp, and *Astyanax aeneus* (Ornelas-García, Domínguez-Domínguez, & Doadrio, 2008; Pérez-Rodríguez et al., 2009, 2015). Incomplete genetic data for most of these groups prevent accurate comparisons with *Ilyodon*. Certain geological events provide an independent line of evidence, such as the uplift of the Sierra de Manantlan and Cacoma, the volcanic activity of the Talpa–Mascota graben, dated 3.6 Mya (Carmichael, Lange, & Luhr, 1996), and the reactivation of the Colima and Tamazula graben in the Pliocene. These geologic events are related to the configuration of the river basin beds (Allan, 1986; Garduño-Monroy et al., 1998).

4.1 | Coahuayana groups

Two groups (clades C and D) were recovered in the specimens from Coahuayana River basin, one distributed in the lower and other in the upper Cohuayana basin. A genetic split between the upper and lower Coahuayana populations has been suggested, based on two cytotypes, one distributed in the upper and other in the lower Coahuayana (Turner, Grudzien, Adkisson, & Worrell, 1985). This is also supported for *Allodontichthys*, with two related species showing the same pattern: *Allodontichthys hubbsi* mainly distributed in the lower Coahuayana and *Allodontichthys tamazulae* in the upper Coahuayana, but with higher divergence than in *Ilyodon* (Doadrio & Domínguez, 2004; Domínguez-Domínguez et al., 2010; Webb, 2002).

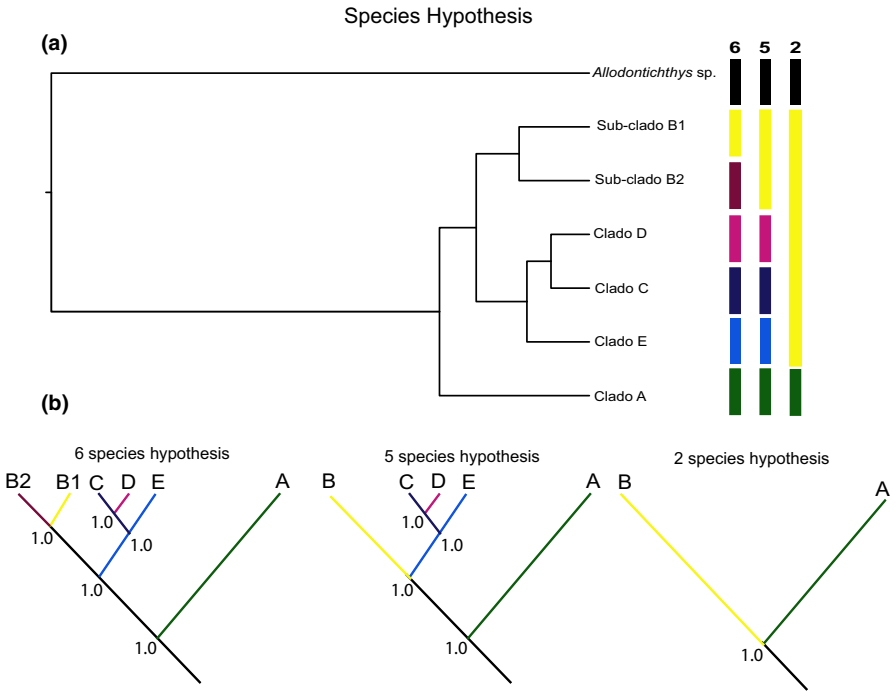

FIGURE 3 Alternative species hypothesis. (a) Guide topology based on the StarBEAST analysis. Bayesian species delimitation results for *Ilyodon* assuming six species, five species, and two species (b) guide trees. The speciation probabilities are provided for each node. We consider speciation probability values >0.95 as strong support for speciation event

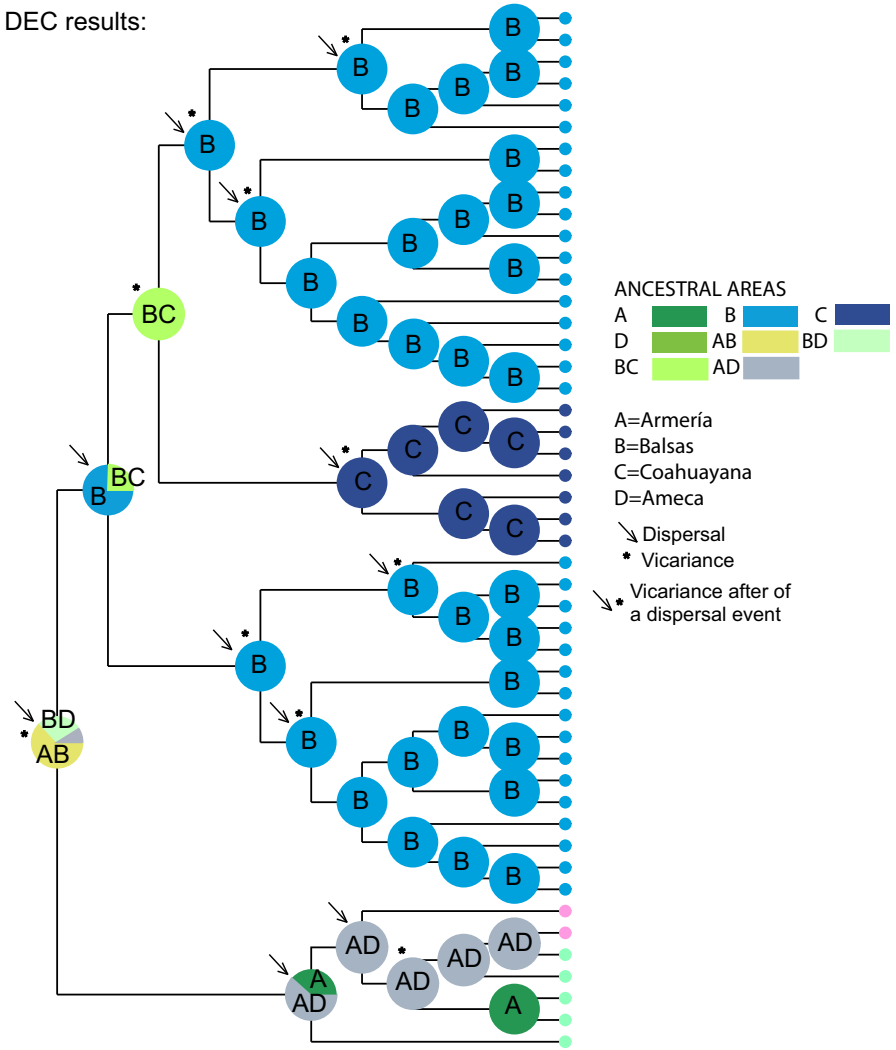

FIGURE 4 Ancestral area reconstruction with DEC for all species of genus *Ilyodon*, with the dichotomous tree obtained in BEAST and with the biogeographical regions Armería, Ameca, Coahuayana, and Balsas basins

The relationships within these groups were not resolved in the phylogenetic trees, and the haplotype networks indicate different relationships depending on the marker analyzed. Also, these two clades showed the lowest genetic distances among all the pairwise comparisons (Tables S5, S6 and S7). Mitochondrial and nuclear genes showed no shared haplotypes between clades C and D, with the exception of a single haplotype in *RAG1*. The nuclear *ACTB* showed the lower Coahuayana basin samples to share haplotypes with the Ameca–Armería clade, and the upper Coahuayana basin shared haplotypes with the west Balsas population. For the *RAG1* gene, the lower Coahuayana specimens possessed unique haplotypes, with the exception of one shared with the upper Coahuayana, Ameca, and Armería basins. The upper Coahuayana specimens shared haplotypes with lower Coahuayana, Ameca-Armería, and west Balsas. The most plausible scenario is a recent isolation event of the ancestor of these two Coahuayana groups, one in the upper and other in the lower Coahuayana basin, as could be indicated by the low genetic distances between them (2.3% for *d-loop* and 0.5% for *cytb* and *coxI*), and, by the DEC analysis, which showed several dispersal and vicariance events that separated these two groups inside Coahuayana River (marginal probability = 1.0). In this scenario, the relationships of the nuclear genes are a product of incomplete lineage sorting or of secondary contact between the lower Coahuayana and Armería–Ameca populations. Secondary contact is also supported by the occurrence of *Allodontichthys zonistius*, a species previously considered endemic to Armería drainages, in the lower reaches of the Coahuayana River, probably related to a river piracy event of nearby (15 km) tributaries (Webb, 2002). Cytogenetic data in *Ilyodon* show the cytotypes from the lower Coahuayana to be more closely related to the Armería population than those of the upper Coahuayana (Turner et al., 1985). Evidence of a founded population in the Ameca and Coahuayana drainages, the source of which was an Armería population, has also been suggested for *Allodontichthys* (Webb, 2002). In most of the genes analyzed, the upper Coahuayana population seems to be close related to west Balsas specimens and even the genetic distances are similar between lineage within Coahuayana than between upper Coahuayana and west Balsas. This may indicate that west Balsas specimens had secondary contact or that the upper Coahuayana population was founded by specimens from Balsas River as indicated by the DEC.

4.2 | Balsas basin

Three well-differentiated groups that show significant genetic structure and high divergence were identified within the Balsas River basin (Figure 2 and Fig. S3), the clades and sub-clades B1, B2, and E. This scenario of the formation of differentiated groups within the Balsas river basin is supported by the results of DEC, in which several dispersal and vicariance events have been promoted the actual distribution of these three groups (Figure 4). Isolation of other fish species in the Balsas watershed has been documented, including restriction of *Notropis boucardi* (Schönhuth & Doadrio, 2003) to the east Balsas and evidence for two divergent groups of *Astyanax*, one distributed in west and other in the east Balsas basin (Ornelas-García et al., 2008). The significant genetic structure and the relatively high genetic distances observed in the Balsas populations are at the same rank with that seen between populations of isolated drainages. This could be explained by ancient ecological or geological barriers within the basin, which is inferred in the mitochondrial genes. The shared haplotypes in *RAG1*, along with close relationships without shared haplotypes in *ACTB*, in the Balsas basin populations could be indicative of secondary contact and gene flow between previously isolated groups, as was previously suggested for the lower Coahuayana and Armería samples. Evidence of a gene flow process within Balsas populations was shown by the central Balsas specimens from Zitacuaro River (Figs. S1, S3 and S4), which showed a close relationship to an east Balsas population in mitochondrial genes, but a closer relationship to west Balsas populations in the *ACTB* gene. Gene flow previous to isolation and gene flow among *Ilyodon* populations have been suggested previously (Webb, 2002). These processes are as complex as the geological and climatic history of the Balsas depression. Geological activity has been suggested to have similarly affect on other endemic species, including spiders, butterflies, birds, amphibians, and reptiles, especially in a sector of the Amacuzac sub-basin (Escalante-Pliego, Navarro, & Peterson, 1993; Luna-Reyes, Llorente-Bousquets, & Luis-Martínez, 2008; Nieto-Castañeda, Pérez-Miguel, & García-Cano, 2014). Also, the Balsas depression is located between the Trans-Mexican Volcanic Belt and the Sierra Madre del Sur (Castañeda-Rico, León-Paniagua, Vázquez-Domínguez, & Navarro-Sigüenza, 2014; Ferrusquía-Villafranca, 1993) which has been active from the Eocene and Oligocene to the present (Yarza de De la Torre, 1992), specifically the Guerrero and Morelos platforms, the Tierra Caliente metamorphic complex and Guerrero terrane, the Taxco fault and Arcelia graben, the Tenochtitlan fault system, and the Chapala–Oaxaca fault system (García-Palomo et al., 2002; González-Torres et al., 2013).

4.3 | Taxonomic implications

The species delimitation tests did not resolve the species-level taxonomy of *Ilyodon*. We suggest that a speciation model based on different criteria (phylogenetic relationships, genetic structure, haplotype networks, genetic distances, and geography) is not informative when phylogenetic relationships are unresolved, low genetic divergences in nuclear genes are observed, or shared nuclear haplotypes are present, violating the algorithm assumptions in the species tree and species delimitation analyses. The BEAST analysis assumes a model in which the separation of species is complete, if this separation is not complete, can result an incorrectly specified guide tree or speciation model (Leaché & Fujita, 2010) that detects species before they are fully separated (incipient species) (Heled, Bryant, & Drummond, 2013). Eberle, Warnock, and Ahrens (2016) have shown that uncertainties in analyses implemented in BPP, such as guide tree inference, individual species assignment, and prior parameter choice, may impact the accuracy of results. However, in our tests, the use of different prior parameters (τ_0 and θ) did not affect the

results, which showed high values of posterior probability in all analyses (>0.95). We consider that basal hard polytomy obtained in the phylogenetic tree, and possible over-splitting, could explain the results obtained with the species delimitation tests (similar high posterior probabilities for two to six species of *Ilyodon*). Hence, we consider that our data do not meet the conditions and parameters necessary for the species delimitation test. It has been shown that both BEAST and BPP may be impacted by putative incomplete lineage sorting and are inadequate for delimiting very young species, which are difficult to distinguish on the basis of molecular or morphological data alone (Eberle et al., 2016).

Despite the failure of the species delimitation test, the finding of six well-differentiated lineages, together with the significant differentiation between them revealed by AMOVA, seemed to indicate a separate genetic identity of each group. The genetic distances calculated with mitochondrial genes are similar to those previously reported between *Ilyodon* species (Doadrio & Domínguez, 2004; Domínguez-Domínguez et al., 2010; Webb et al., 2004). The highest genetic distance was found between the *d-loop* and *cytb* gene of the Ameca–Armería population (clade A) with respect to the other clades (B1, B2, C, D, and E). For nuclear genes, the genetic distance between these clades ranged from 0.1%–0.2% (Table S7). Previous studies of other goodeine species showed similar genetic distances and lack of resolution in phylogenetic analyses, as did some species of *Allotoca* and *Goodea*, associated with recent isolations (<1 Mya) or secondary contact events promoted by river piracy or founder effect (Corona-Santiago et al., 2015; Doadrio & Domínguez, 2004; Domínguez-Domínguez et al., 2010).

Not all species within *Ilyodon* were identified as monophyletic in the phylogenetic results. The pattern of species or genera mixed in the phylogenetic tree has also been reported for other freshwater fishes of Mexico (Corona-Santiago et al., 2015; McMahan, Geheber, & Piller, 2010; Ornelas-García et al., 2008; Pérez-Rodríguez et al., 2009). The variation among *Ilyodon* with morphological recognized groups has been associated with trophic structure, with variation in the shape and arrangement of the head, mouth, and teeth and in fish size influenced by habitat and feeding (Grudzien & Turner, 1984a,b; Kingston, 1979; Turner & Grosse, 1980).

All currently recognized *Ilyodon* species were described morphologically (Paulo-Maya & Trujillo-Jiménez, 2000), with no descriptions based on molecular analyses. In the present study, results of molecular analyses of five genes disagree with the currently recognized taxonomy of *Ilyodon*. Our results showed lower genetic distance between most clades than the average found for all the recognized species of goodeines (1.7% in *cytb*), as well as shared haplotypes among most main clades in nuclear genes. We also found higher genetic divergence between the *I. whitei* sampled in west and east Balsas than between samples from the Balsas and Coahuayana basins, with evidence of interbreeding between highly divergent lineages. We found significant geographic structure in *Ilyodon*, but not concordance with the five previously recognized species *I. furcidens*, *I. xantusi*, *I. whitei*, *I. lennoni*, and *I. cortesae* showing polyphyletic relationships (Tables 3 and 4). Hence, taxonomic interpretations are difficult to assess. Further genetic and morphological analyses need to be conducted to provide a clearer picture of the taxonomy and evolution of genetically divergent populations of *Ilyodon*, but some suggestions can be made according to the priority principle and type locality.

All specimens belonging to clade A were identified as *Ilyodon furcidens*. Although the type locality was given as Cape San Lucas by Eigenmann (1907), later researchers state the type locality to be Río Colima, a tributary of Armería River basin (Hubbs & Turner, 1939). Although we did not include samples from the Colima River in the lower Armería basin, and because of high genetic differentiation found within other drainages, we provisionally designate the specimens of clade A as *I. furcidens*. Since the type locality for *I. xantusi* is the Colima River, in the Armería River basin, more samples from the lower Armería need to be examined to draw a robust conclusion.

The specimens of sub-clade B1 were identified as *Ilyodon whitei*, but this group showed high genetic divergence and significant structure with respect to other samples, so we considered this group as a differentiated group of *Ilyodon "whitei."*

Specimens belonging to sub-clade B2 were also identified as *I. whitei*. This group included specimens collected at its type locality (upper tributaries of Balsas, at Cuautla and Yautepec, in Morelos state), and we considered this clade as *I. whitei*. Within this clade, we found genetic flow between east Balsas (B2) and west Balsas (E) in nuclear genes.

Specimens belonging to clade C were identified as *I. furcidens*, but, because of the genetic divergence from other groups and the possibility of interbreeding with Armería populations, we considered this group a differentiated group of *Ilyodon "furcidens."*

Specimens belonging to clade D were identified as *I. whitei*, but, based on the divergence from other genetically identified *I. whitei*, and the high structure found, we considered this group a differentiated group of *Ilyodon "whitei."*

The specimens belong to clade E were identified as *I. whitei*, *I. cortesae*, and *I. lennoni*, with the latter two species collected from the type locality. Since specimens identified as *I. whitei* in the type locality (upper tributaries of Balsas, at Cuautla and Yautepec) belong to the sub-clade B2, the specimens of clade E must be considered *Ilyodon "lennoni,"* while *I. cortesae* was not considered a valid species.

Our results show more complex evolutionary and taxonomic history of *Ilyodon* than was previously revealed by molecular studies (Doadrio & Domínguez, 2004; Domínguez-Domínguez et al., 2010). In this scenario, and due to the high level of morphological differentiation, a broader taxonomic and systematic work for *Ilyodon* species is necessary to confirm the taxonomic status of each described species.

4.4 | Implications for conservation

We identified at least six genetic groups, with geographic correspondence, in analyses performed with mtDNA and with concatenated mtDNA + nDNA. Each of these groups should be considered an operational conservation unit (OCU), and effective protection of the

OCUs could guarantee the conservation and preservation of the genetic pool (Doadrio, Perdices, & Machordom, 1996) found within genus *Ilyodon*. *Ilyodon whitei* is catalogued since 1996 in the red list of endangered species as critically endangered (Contreras-Balderas & Almada-Villela, 1996), and *I. furcidens* is catalogued as threatened in the NOM-059 for the Ministry of Environmental and Natural Resources (SEMARNAT, 2010). Based on our genetic groups recovered for mitochondrial genes, we suggest a re-evaluation of the conservation status of the *Ilyodon* species or populations.

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