Neotropical Ichthyology

Original article https://doi.org/10.1590/1982-0224-2022-0101

Population level genetic divergence and phylogenetic placement of Mexican shortfin mollies (*Mollienesia*: Poecilia: Poeciliidae)

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Mexico is a megadiverse region with a complex geological history, but it remains unclear to what extent the distribution of freshwater fish has been influenced by geographic barriers. This study examines the population level genetic divergence and phylogenetic relationships of species in the shortfin group of the subgenus *Mollienesia* (genus *Poecilia*), a group of live-bearing fishes that are widely distributed across Mexico, with sampling at a small geographic scale. Samples from over 50 locations were analyzed for six species by using phylogenetic and haplotype network approaches to assess genetic diversity across geographic ranges and to refine the distributions of species in this group. The results indicate that Mexican species have diversified following multiple, independent invasions from Middle America. Two species found north of the Trans-Mexican Volcanic Belt (TMVB) and one transversal species exhibited weak phylogenetic structure, likely due to the lack of physiographic barriers, recent colonization, and high dispersal rates among regions. In contrast, three species found south of the TMVB exhibited strong phylogenetic structure, reflecting a longer presence in the area and multiple physiographic barriers that isolated populations. This study identified mechanisms driving divergence and speciation, expanded the known range of several species, and resolved taxonomic uncertainties of populations.

Keywords: Diversification, Fishes, Phylogenetics, Phylogeography, Trans-Mexican Volcanic Belt.

Submitted December 5, 2022
Accepted April 2, 2023
by Paulo Lucinda
Epub May 1, 2023

Online version ISSN 1982-0224
Print version ISSN 1679-6225

Neotrop. Ichthyol.
vol. 21, no. 2, Maringá 2023

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México es una región megadiversa con una historia geológica compleja, pero se desconoce el nivel de influencia de las barreras geográficas sobre las distribuciones de los peces dulceacuícolas. Este estudio examina las relaciones filogenéticas, a escala geográfica pequeña, de las especies del grupo de aletas cortas del subgénero *Mollienesia* (género *Poecilia*), un grupo de peces vivíparos ampliamente distribuidos en México. Se analizaron muestras de seis especies en más de 50 localidades, utilizando métodos filogenéticos y de redes de haplotipos, para evaluar la diversidad genética y precisar las distribuciones de especies en este grupo. Los resultados indican que las especies mexicanas se han diversificado a partir de múltiples invasiones independientes desde Mesoamérica. Se detectó estructura filogenética débil en dos especies distribuidas al norte del Eje Neovolcánico y una especie que atraviesa el Eje Neovolcánico, posiblemente debido a la ausencia de barreras fisiográficas, colonización reciente y altas tasas de dispersión entre regiones. En contraste, se detectaron niveles altos de estructura filogenética en tres especies distribuidas del Eje Neovolcánico, lo que refleja una presencia más prolongada en el área y la existencia de múltiples barreras fisiográficas que aislaron a las poblaciones. Este estudio identificó mecanismos que promueven la divergencia y la especiación, expandió el rango conocido de varias especies y resolvió incertidumbres taxonómicas de algunas poblaciones.

Palabras clave: Cinturón Volcánico Transmexicano, Diversificación, Filogenética, Filogeografía, Peces.

INTRODUCTION

Mexico's complex geological history has led to diverse ecosystems, making the region rich in flora and fauna. The historical geological activity is reflected in a landscape dominated by mountain chains that give rise to isolated hydrological units and regions with starkly different ecological conditions (Domínguez-Domínguez *et al*., 2006). In the north of the country, the mountain ranges of the Sierra Madre Oriental and the Sierra Madre Occidental extend along the eastern and western coast, respectively, and in the south, they join the Trans-Mexican Volcanic Belt (TMVB; Fig. 1), which transects the country at a latitude of about 20 °N and marks the southern edge of the North American plate (Ferrari *et al*., 1999). In the southern region of Mexico, the Sierra Madre del Sur and the Sierra Madre de Chiapas are separated by the Isthmus of Tehuantepec (Fig. 1). These mountain ranges produce a wide range of geographical landforms, including high mountains with permanent snow cover, highland plateaus, deep valleys, and coastal plains (Contreras-Balderas *et al*., 2008). Mexico is also unique in encompassing organisms from both the Neartic (North America south to the Mexican plateau) and Neotropical (Mexican central coasts to South America) biogeographic zones (Morrone, Márquez, 2001). The combination of the geological diversity and the interface of different biogeographic zones make Mexico a megadiverse region in terms of ecological habitats, species diversity, and levels of endemism (Conabio, 1998; Metcalfe *et al*., 2000; Hufnagel, Mics, 2021).

Mexico's high levels of biodiversity are also reflected in its freshwater ichthyofauna, which is comprised of 50 families, 155 genera, and 536 species (Miller *et al*., 2005; Mejía *et al*., 2012; Lyons *et al*., 2020), with 271 species being endemic to the country (Contreras-Balderas *et al*., 2008) and new species continually being described (Langerhans *et al*., 2012; De la Maza-Benignos *et al*., 2015; Ornelas-García *et al*., 2015; Domínguez-Domínguez *et al*., 2016; Matamoros *et al*., 2018; Conway *et al*., 2019). Previous phylogeographic studies have shown that some Mexican fishes have diversified, and their distribution patterns are in accordance with ancient geological events rather than modern hydrographic patterns (Mateos *et al*., 2002; Strecker *et al*., 2004; Huidobro *et al*., 2006; Agorreta *et al*., 2013; Zúñiga-Vega *et al*., 2014). However, population-level studies are rarely conducted, and there is a poor understanding of the microevolutionary dynamics that shape fish distributions and population structures in Mexico.

The most species-rich fish group in Mexico is the family Poeciliidae (Contreras-MacBeath *et al*., 2014), which includes small cyprinodontiform fishes inhabiting fresh, brackish, and coastal waters (Reis *et al*., 2003; Bragança *et al*., 2018). This group is characterized by a set of unique traits: adult males possess a modified anal fin, with rays 3, 4, and 5 forming a gonopodium for sperm transfer, and reproduction occurs via internal fertilization and matrotrophic or lecithotrophic embryonic development (Rosen, Bailey, 1963; Parenti, 1981; Greven, 2011). Recent studies have shed light on the systematics, phylogeography, and biodiversity of this family in Mexico and other areas of the Neotropics (Hrbek *et al*., 2007; Meredith *et al*., 2011; Agorreta *et al*., 2013; Reznick *et al*., 2017). Among the most diverse groups of poeciliids in Mexico is the subgenus *Mollienesia* LeSueur, 1821 (genus *Poecilia* Bloch & Schneider, 1801; see Tab. 1), which is represented by 15 species (Palacios *et al*., 2016). *Mollienesia* species are found in a variety of habitats, including streams and rivers, coastal marshes, volcanic lakes, cave systems, and hydrogen-sulfide-rich springs (Miller, 1975; Miller *et al*., 2005; Tobler, Plath, 2011; Palacios *et al*., 2016). Despite the wide distribution of these species in Mexico, the levels of intra-specific diversity and the distributional ranges of several species in this group remain poorly described (Bussing, 1976; Miller, 1983; Miller *et al*., 2005). Recent molecular studies of *Mollienesia* across its distribution in North, Central, and northern South America have revealed three species complexes (*Poecilia latipinna* LeSueur, 1821, *P. sphenops* Valenciennes, 1846, and *P. mexicana* Steindachner, 1863; Breden *et al*., 1999), a number of putatively undescribed species (Alda *et al*., 2013; Bagley *et al*., 2015; Ho *et al*., 2016; Palacios *et al*., 2016), and a complex history of colonization from nuclear Central America into North and South America, and back (Ho *et al*., 2016). In Mexico, some species are widespread and occur on both the Atlantic and Pacific slopes (*e.g*., *P. sphenops* and *P. mexicana*; Alda *et al*., 2013; Bagley *et al*., 2015; Ho *et al*., 2016; Palacios *et al*., 2016), while others are endemic to a few locations (*P. catemaconis* Miller, 1975, *P. chica* Miller, 1975, *P. sulphuraria* Álvarez, 1948, and *P. thermalis* Miller, 1975; Palacios *et al*., 2013, 2016). At a finer scale, previous studies have shown genetic differentiation among closely related lineages along environmental gradients (in *P. mexicana*, *P. sulphuraria*, and *P. thermalis*; Palacios *et al*., 2013) as well as diversification across physiographic barriers (in *P. butleri* Jordan, 1889, and *P. nelsoni,* Meek, 1904; Zúñiga-Vega *et al*., 2014). The variation of distribution patterns among species makes this subgenus a promising group to investigate phylogeographic patterns and microevolutionary processes. This study specifically applied phylogenetic and haplotype network analyses of seven species in the subgenus *Mollienesia* (*P. butleri*, *P. limantouri* Jordan & Snyder, 1899, *P. mexicana*, *P. nelsoni*, *P. sphenops*, *P. sulphuraria*, and *P. thermalis*) from 66 locations across Mexico to (1) determine the number of colonization events of Mexico from nuclear Central America, (2) assess patterns of phylogeography and haplotype diversity in different species, and (3) contribute to a better understanding of the distribution of species in this group.

MATERIAL AND METHODS

Specimen acquisition. We sampled specimens of the subgenus *Mollienesia* throughout the subgenus's distribution in Mexico (Fig. 1; see Tab. **S1** for locality data). Fish were captured using electrofishing, seines, cast and dip nets. Immediately after capture, some sampled fish were euthanized with buffered MS222; others were sampled and released (per permit restrictions). Regardless of sampling method, fin clips (right pectoral fin) were preserved in 95% ethanol for molecular analyses, and specimens were fixed in a 10% formaldehyde solution following protocols approved by the Texas A&M University and Oklahoma State University Committees on Use and Care of Animals (IACUC 2011-118 & ACUP AS10-15). Ethanol preserved tissues, DNA extractions, and formalin fixed specimens are housed in the Biodiversity, Research and Teaching Collections of the Department of Wildlife & Fisheries Sciences at Texas A&M University, College Station, Texas, USA, and the El Colegio de la Frontera Sur, San Cristóbal, Chiapas, Mexico. Voucher numbers for new specimens are provided in Tab. **S1**, where possible; many of our samples were fin clips (per permit restrictions) and some were used for life history dissections, and as such were not suitable as voucher specimens.

TABLE 1 | List of species from the *Poecilia sphenops* and *P. mexicana* species complexes present in Mexico. The table provides information about the slope on which a species occurs, a broad description of the distribution, the endemism status in Mexico, and phylogeographic breaks uncovered in this study. Asterisks indicate the inclusion of a species in phylogenetic analyses; crosses indicate focal species used for phylogeographic analyses based on Cyt *b*.

Species	Slope	Distribution	Endemic	Phylogeographic Break Cyt *b*
P. sphenops complex				
Poecilia catemaconis Miller, 1975*	Atlantic	Laguna Catemaco and surrounding rivers, Veracruz	X	Sierra de Tuxtlas1
Poecilia chica Miller, 1975*	Pacific	Three small rivers in Jalisco	X	Jalisco block
Poecilia maylandi Meyer, 1983	Atlantic	Rio Balsas, Guerrero	X	
Poecilia sphenops Valenciennes, 1846*†	Atlantic, Pacific	Tamaulipas, Veracruz, Guerrero, Oaxaca, Chiapas, and Guatemala		
P. mexicana complex				
Poecilia butleri Jordan, 1889*†	Pacific	Rio Fuerte, Sonora, Sinaloa, and Nayarit	X	Trans Mexican Volcanic Belt
Poecilia mexicana Steindachner, 1863*†	Atlantic	Nuevo Leon to Panama		
Poecilia limantouri Jordan, 1899*†	Atlantic	Rio Grande to N. Veracruz	X	
P. nelsoni (Meek, 1904) *†	Pacific	Colima to S. Guatemala	X	Trans Mexican Volcanic Belt, Balsas Depression
Poecilia orri Fowler, 1943*	Atlantic	Yucatan, Quintana Roo to Honduras		
Poecilia sulphuraria (Álvarez, 1948)*†	Atlantic	Sulfide springs in Tabasco and Chiapas	X	
Poecilia thermalis Steindachner, 1863*†	Atlantic	Sulfide springs in Chiapas	X	

Samples collected for this study were supplemented with sequence data obtained from GenBank to examine the relationship of *Mollienesia* species in Mexico to other members of the genus from North and Central America. We included sequence data for multiple genes (see below) for 18 out of 26 recognized species in the subgenus *Mollienesia* (Mateos, 2005; Alda *et al*., 2013; Ho *et al*., 2016; Palacios *et al*., 2016): *P. kykesis*, *P. latipinna*, *P. latipunctata* Meek, 1904, and *P. velifera* (Regan, 1914) (members of the sailfin molly clade); *P. catemaconis*, *P. chica*, *P. marcellinoi*, and *P. sphenops* (members of the *P. sphenops* species complex of the shortfin molly clade), as well as *P. butleri*, *P. gillii* (Kner, 1863), *P. hondurensis* Poeser, 2011, *P. mexicana*, *P. nelsoni*, *P. orri* Fowler, 1943, *P. petenensis* Günther, 1866 (sometimes referred to as *P. gracilis*), *P. salvatoris* Regan, 1907, *P. sulphuraria*, and *P. thermalis* (members of the *P. mexicana* species complex of the shortfin molly clade). In addition, we supplemented the cytochrome *b* dataset generated for our focal species with additional sequences. This expanded the distributional coverage for *P. butleri* and *P. nelsoni* (Zúñiga-Vega *et al*., 2014), spanning their entire ranges along the Pacific coast of Mexico. The distribution coverage was also extended north of our sampling for *P. limantouri* (Stöck *et al*., 2010) and south of our sampling in *P. mexicana* (Tobler *et al*., 2011).

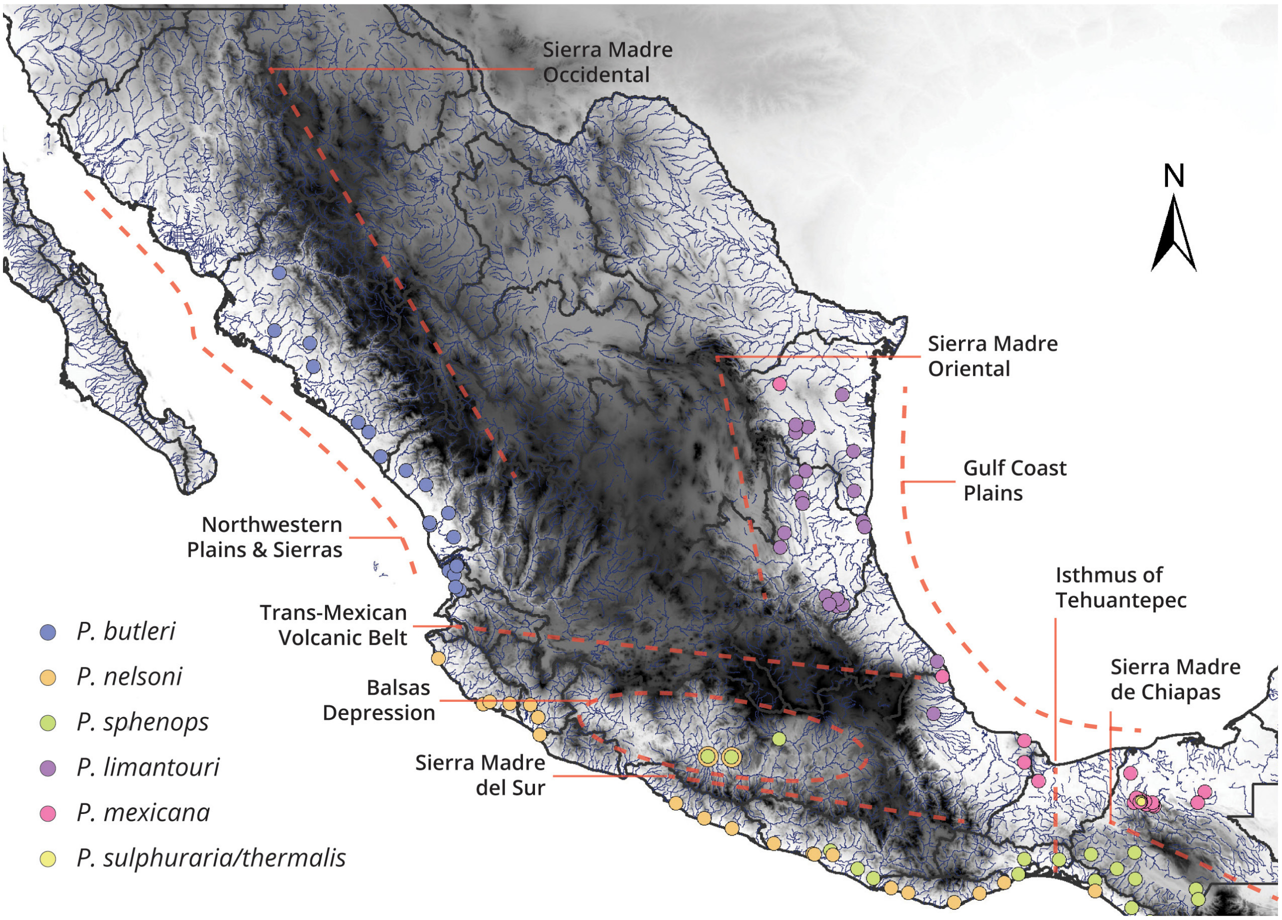

FIGURE 1 | Sampling localities of species in the subgenus *Mollienesia* in Mexico and the main physiographic barriers throughout the country. The main physiographic barriers in Mexico from north to south are Northwestern Plains and Sierras, Sierra Madre Occidental, Sierra Madre Oriental, Gulf Coast Plain, Trans Mexican Volcanic Belt, Balsas Depression, Sierra Madre del Sur, Isthmus of Tehuantepec, and Sierra Madre de Chiapas.

Molecular analyses. Total genomic DNA was extracted from ethanol-preserved fin clips with the DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol. The samples were amplified for several presumably neutral genes commonly used for phylogenetic reconstruction in fishes, including poeciliids (Hrbek *et al*., 2007; Meredith *et al*., 2010, 2011). Focal genes included the mitochondrial cytochrome *b* gene (cyt *b*, 1,140 base pairs) with LA and HA primers (Schmidt *et al*., 1998), the mitochondrial gene NADH subunit 2 (ND2, 1,047 bp) with ND2B-L (Broughton, Gold 2000) and ASN (Kocher *et al*., 1995) primers, and a nuclear gene, exon 3 of recombination activating gene-1 (RAG1, 1,561 bp) with the primers L2492 RAG1 and H4054 RAG1 (Hrbek *et al*., 2007). PCR protocols followed Palacios *et al*. (2016), and PCR products were purified with Exosap-IT enzyme reaction (GE Healthcare Bio-Sciences Corp., Piscataway, NT), directly sequenced with a dye-labeled terminator kit (Big Dye Terminator version 3.1, Applied Biosystems, Foster City, CA), and run on an ABI automated sequencer (Applied Biosystems, Foster City, CA). Sequence electrophenograms were edited with Sequencher version 4.8 (Gene Codes) and aligned with MAFFT v. 6.0 (Katoh, Toh, 2008).

Alignment and phylogenetic analyses. The data were partitioned into three datasets: (1) a concatenated dataset (cyt *b,* ND2, and RAG1, 3748 base pairs), (2) the nuclear gene alone (RAG1 1561 bp), and (3) cyt *b* alone (1140 bp). A strictly mitochondrial dataset (cyt *b* and ND2) was not analyzed because samples missing sequences for ND2 reduced the size of the dataset. All datasets were analyzed separately using FaBox online (Villesen, 2007) to determine the total number of haplotypes. The cyt *b* dataset was reduced from 953 sequences to 475 (139 haplotypes for the 6 species of interest). We used Partition Finder (Lanfear *et al*., 2012) to determine the best partition scheme and the most likely model of DNA substitution among 24 candidate models on a fixed BioNJ-JC tree based on the Bayesian information criterion (BIC; Tab. **S2**), separately for each gene. Phylogenetic analyses for the concatenated and the cyt *b* datasets were conducted with a maximum likelihood (ML) approach as implemented in RAxML GUI v. 1.0 (Stamatakis, 2006, 2008), with 500 Rapid Bootstrap searches followed by an ML search. The complex general time reversible (GTR) + Γ (gamma distribution for rate variation among sites) model was chosen for each partition. The bootstrap trees were summarized with a Sumtrees script that used a 50% percent majority rule consensus parameter in DendroPy v. 3.10.1 (Sukumaran, Holder, 2010), and the final tree was rooted and visualized in FigTree v. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). Bayesian analyses were conducted in MrBayes v. 3.2.1 (Ronquist, Huelsenbeck, 2003; Ronquist *et al*., 2012), implementing two runs with four chains under default parameters for 50 million generations, sampling every 10 generations. A 25% burn-in was applied and stable posterior probability values examined in Tracer v. 1.5 (Rambaut *et al*., 2018). Pairwise genetic distances were calculated under the Kimura-2 parameter model in MEGA v. 7 (Kumar *et al*., 2016) with pairwise deletion for missing data.

A haplotype network was generated with a statistical parsimony analysis of the cyt *b* gene dataset for the six focal species in TCS v. 1.2.1 (Clement *et al*., 2000), as implemented in the Popart program (Leigh, Bryant, 2015). This approach calculated the number of significant substitutions connecting haplotypes in a network by applying the algorithm developed by Templeton *et al.* (1992). A connection limit was set to the default of 95% to generate the haplotype networks between closely related sequences.

RESULTS

Phylogenetic relationships of Mexican mollies to other species in the subgenus *Mollienesia*. To determine the number of colonization events from nuclear Central America into Mexico, we generated a phylogenetic tree with 18 of the 26 recognized species in the subgenus *Mollienesia*, and overall, there was high concordance between maximum-likelihood and Bayesian analyses. The phylogeny of the concatenated dataset showed moderate to high support for the two clades representing the *P. sphenops* and *P. mexicana* species complexes (Fig. 2). Most of the phylogenetic signal came from the mitochondrial genes. Analysis of RAG1 alone resulted in poorly supported phylogenetic relationships due to the low levels of sequence variation in our samples (1–2 base pairs across the entire gene; results not shown). The genetic distances across the two complexes ranged from 6.9–10.5%, while they ranged from 1.0–8.2% within the *P. sphenops* species complex and 0.9–6.0% within the *P. mexicana* complex (Tab. **S3**).

The *P. sphenops* species complex was strongly (Bayesian, > 95%) or moderately (maximum-likelihood, 74%) supported and included three species from Mexico (*P. sphenops* and the two endemic species *P. chica* and *P. catemaconis*), which were interspersed among three species from Middle America (*P. marcellinoi* and two putatively undescribed species, *P.* "sp. Tipitapa" and *P.* "sphenops sp1"; Bagley *et al*., 2015) (Fig. 2). Within *P. sphenops*, our phylogeny did not provide support for the relationships among the sampled locations that cover both the Atlantic and Pacific versants of Mexico.

The *P. mexicana* species complex was strongly supported (>95% with both methods) and included five Mexican and five Middle American species (Fig. 2). *Poecilia hondurensis* and *P. orri* from Middle America represented the most basal divergences in the complex (Fig. 2). The next divergence in the complex was strongly supported and included two species from Mexico's Pacific slope, *P. butleri* and *P. nelsoni*. Next, our results recovered a strongly supported *P. limantouri* clade on the Atlantic slope of Mexico (which lacks resolution among different sampling sites) as sister to the sulfide spring endemics *P. sulphuraria* and *P. thermalis* (Fig. 2). The *P. limantouri*/*P. sulphuraria*-*P. thermalis* clade is sister to a clade comprised of two putatively undescribed lineages from Middle America (*P.* "sp 1" and *P.* "sp 2"; Palacios *et al*., 2016), *P. salvatoris* (also from Middle America), and the strongly/moderately supported *P. mexicana* clade. *Poecilia mexicana* is split into three clades. Two of these clades are of samples from Mexico, with one strongly supported clade comprised of the most northern sampling locations, from the Trans-Mexican Volcanic Belt north to the state of Nuevo León. The other clade includes the remaining samples, which come from south of the Trans-Mexican Volcanic Belt and span both sides of the Isthmus of Tehuantepec (Fig. 2). The two Mexican clades are not sisters, as a lineage consisting of samples from Honduras is more closely related to the southern clade.

Phylogeography of *Poecilia sphenops*. To assess patterns of phylogeography and haplotype diversity within different species, we generated haplotype networks and observed potential patterns of differentiation across known geographic barriers. The widespread species *P. sphenops* is found across the Atlantic and Pacific versants in Mexico, and genetic distances within this species ranged from 0 to 1.0% (Tab. **S4**). Our analyses revealed three divergent groups. The first of these (Texistepec) is represented by a single haplotype from the Rio Coatzacoalcos of the southern Atlantic coast (state

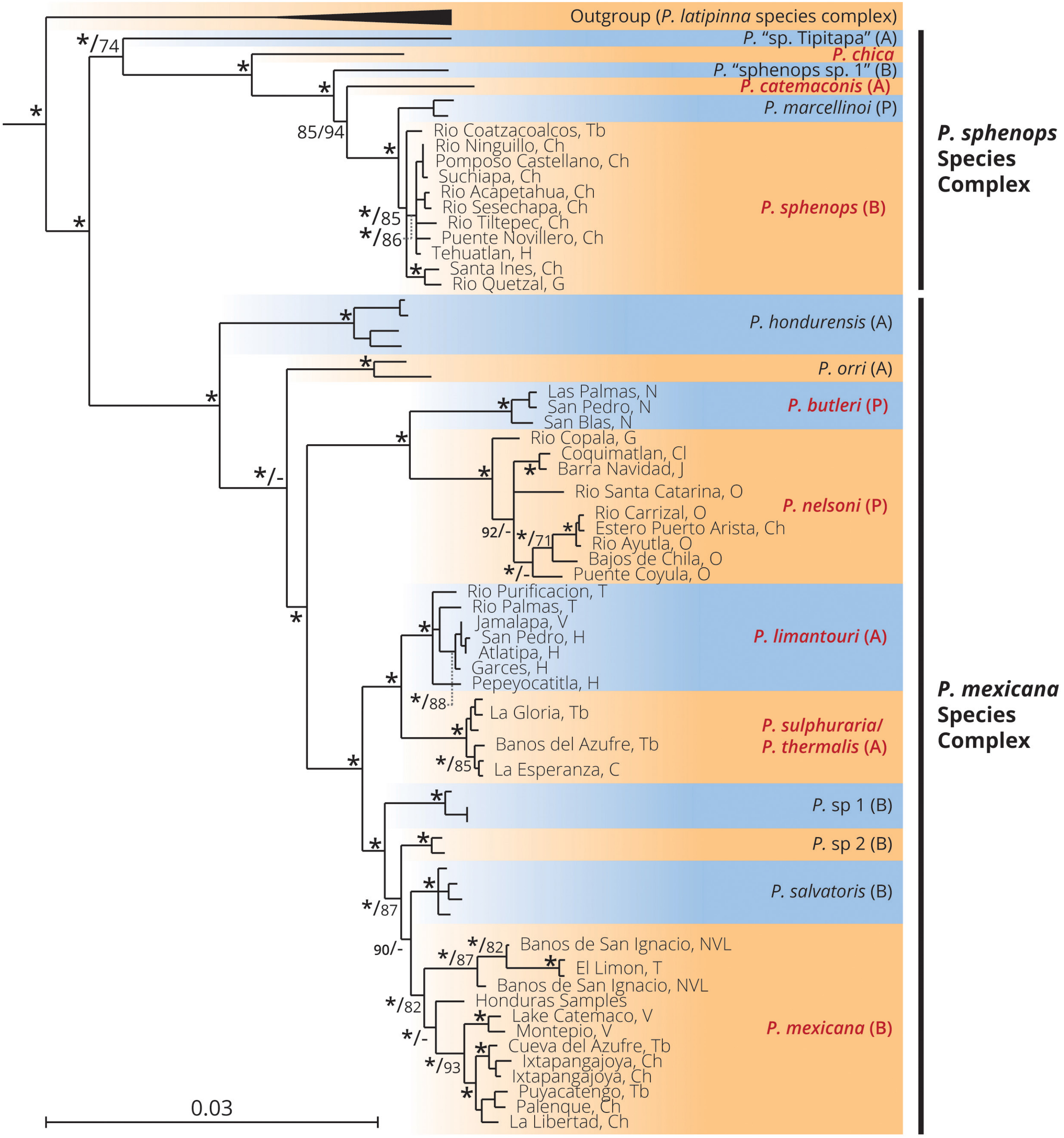

FIGURE 2 | Bayesian tree from the MrBayes partitioned analysis of *Poecilia* spp. for two mitochondrial genes (Cyt b and ND2, 2187 base pairs) and one nuclear (RAG1, 1561 base pairs) rooted with other poeciliid outgroups. Species names in black pertain to species outside of Mexico and species names in red are species found within Mexico. Species labels represent slope: (A) = Atlantic, (P) = Pacific, and (B) = Bi-coastal. Nodal support shown (left to right; respectively): Bayesian Posterior Probabilities followed by RAxML bootstrap support values. Asterisks denote nodal support of 95% or above for the two methods, and a single asterisk at a node indicates support values of 95% or above for both methods. Nodes with no values present either had low values or were of little interest for this study. The capital letters at the end of each sample represents the state of origin in Mexico, from North to South: T = Tamaulipas, NVL = Nuevo León, V = Veracruz, H = Hidalgo, M = Michoacan, G = Guerrero, O = Oaxaca, Tb = Tabasco, C = Chiapas.

of Veracruz), just west of the Isthmus of Tehuantepec (Fig. 3). The second group (clade A1) was composed of one Pacific slope haplotype from the Guerrero coast west of the Isthmus of Tehuantepec, and two haplotypes from the Atlantic versant in Chiapas west of the Isthmus of Tehuantepec (Fig. 3). The main *P. sphenops* clade (clade A2 in Fig. 3) included one widely distributed haplotype, found along the Atlantic coast north of the Trans-Mexican Volcanic Belt, in the Balsas depression, and along the Pacific coast east and west of the Isthmus of Tehuantepec. Clade A2 also exhibited three strongly supported sub-clades, the first consisting of one haplotype from the Balsas depression and one from a Pacific location at the Isthmus of Tehuantepec (Rio Ostuta on the Oaxacan coast). The second sub-clade consisted of four Pacific haplotypes from the Chiapas coast east of the Isthmus of Tehuantepec, and the third sub-clade was comprised of three haplotypes from the Atlantic versant east of the Isthmus of Tehuantepec in Chiapas (Fig. 3). Additionally, we found five other *P. sphenops* haplotypes with unresolved relationships; these samples were geographically dispersed across the Balsas depression and the Pacific versant on both sides of the Isthmus of Tehuantepec (Fig. 3).

The haplotype network of *P. sphenops* indicated that haplotypes were separated between 1–10 mutational steps. The most common haplotype included samples from the Pacific coast, including both sides of the Isthmus of Tehuantepec and the Balsas depression, and one sample from Atlantic versant north of the Trans-Mexican Volcanic Belt. The most divergent haplotypes in *P. sphenops* were found along the Pacific coast west of the Isthmus of Tehuantepec and in rivers of the Atlantic versant, both west (Rio Coatzocoalcos) and east (Chiapas highlands) of the Isthmus (Fig. 3). The remaining haplotypes, with two exceptions, were all found on the Pacific versant, including the Balsas depression. Overall, the lack of clear phylogeographic patterns and haplotype sharing across major biogeographic boundaries indicate low levels of differentiation within *P. sphenops*.

Phylogeography of *Poecilia butleri* and *P. nelsoni*. The sister taxa *P. butleri* and *P. nelsoni* are found on either side of the Trans-Mexican Volcanic Belt. The genetic distances within both *P. butleri* and *P. nelsoni* ranged from 0–2.0%, and the two species differed by 4.6% (Tab. **S4**).

Poecilia butleri was restricted to the Pacific versant north of the Trans-Mexican Volcanic Belt. The phylogenetic structure for this species showed an initial divergence represented by a single haplotype from our most northern sample in Sinaloa (Rio Choix). The next divergence (clade B2 in Fig. 4) included a clade composed of two haplotypes from the southernmost portion of the *P. butleri* range (San Blas and Rio El Palillo in southern Nayarit). Lastly, the strongly supported clade B1 contained the remaining haplotypes, which formed a five lineage polytomy with no obvious geographic structuring (Fig. 4). Within clade B1, two lineages represented single haplotypes, one from the northern (Sinaloa) and one from the southern portion (Nayarit) of *P. butleri*'s range. Two other lineages represented moderately to strongly supported sub-clades comprising six and three haplotypes from Sinaloa. The fifth lineage was a large weakly supported clade of individuals collected from both the northern and southern portions of the range. The haplotype network of *P. butleri* had a maximum of 17 mutational steps between haplotypes, with most haplotypes differing between 1–6 steps (Fig. 4). The most divergent haplotypes were from Rio Choix (192), the most northern sampling locality in Sinaloa, and from El Palillo (456), San Blas (399), and Acaponeta (408), all southern localities in Nayarit.

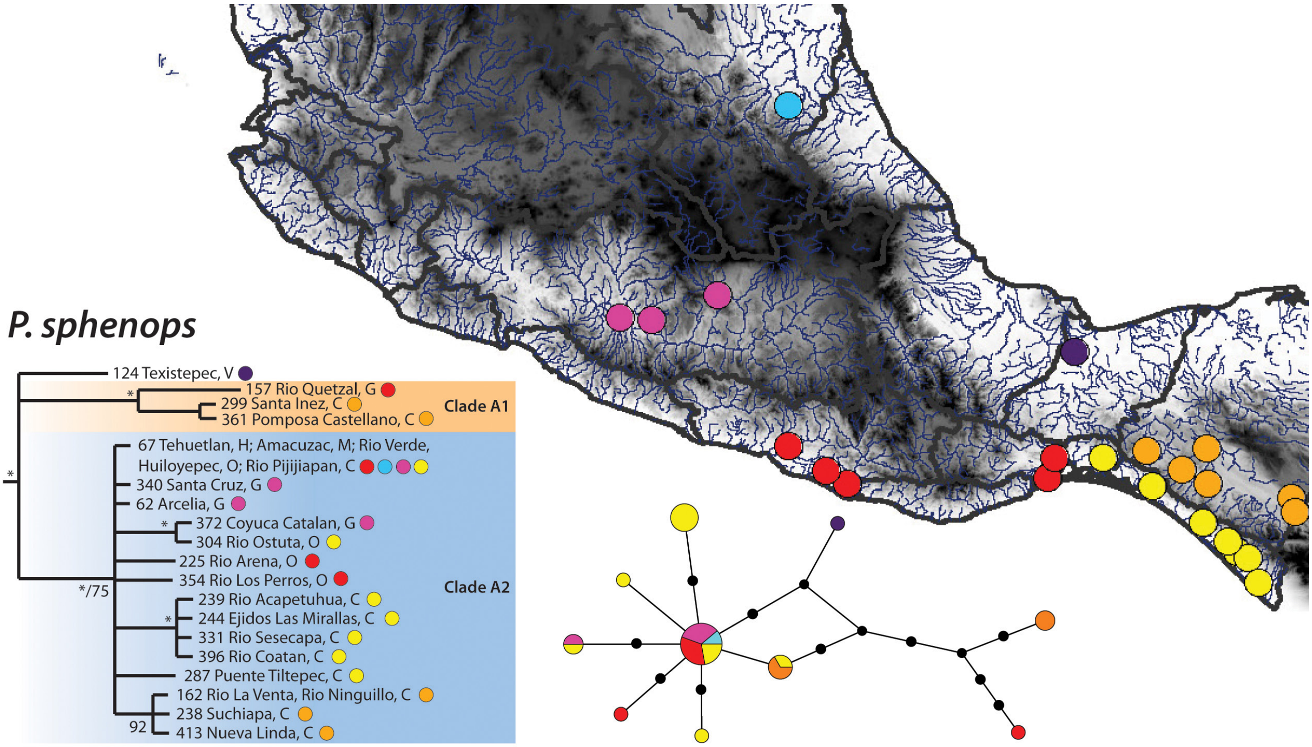

FIGURE 3 | Cytochrome *b* (1,140 bp) mitochondrial gene Bayesian phylogeny, parsimony haplotype network, and sampling distribution of *Poecilia sphenops* (aqua-Atlantic North of the Trans Mexican Volcanic Belt, pink- Balsas River Drainage, purple- Atlantic North of the Isthmus of Tehuantepec, red- Pacific North of the Isthmus of Tehuantepec, orange- Atlantic South of the Isthmus of Tehuantepec, and yellow- Pacific South of the Trans Mexican Volcanic Belt) across geographic barriers along the coasts of Mexico. The phylogeny has Bayesian posterior values followed by bootstrap values with asterisks representing support of 95% or above. The Parsimony network values correspond to the haplotype values and are colored according by geographic locations separated by barriers; circle sizes correspond to the number of individuals with that haplotype (larger circles reflect more individuals), and black circles indicate unsampled haplotypes. The capital letters at the end of each sample represents the state of origin in Mexico, from North to South: V = Veracruz, H = Hidalgo, M = Michoacan, G = Guerrero, O = Oaxaca, C = Chiapas.

Poecilia nelsoni is distributed on the Pacific versant south of the Trans-Mexican Volcanic belt, spanning both sides of the Isthmus of Tehuantepec. Although all *P. nelsoni* samples were recovered as monophyletic, the basal node consisted of a polytomy of six lineages (Fig. 4). Two lineages consisting of a single haplotype, a third lineage representing two haplotypes (clade C2), and a fourth lineage representing many haplotypes (clade C3) were mostly collected west of the Isthmus of Tehuantepec in Guerrero, with one individual from the Balsas depression (Fig. 4). Of the two remaining *P. nelsoni* lineages, one (clade C1) was composed almost entirely of individuals collected just south of the Trans-Mexican Volcanic Belt, including sites in Jalisco, Colima, and Michoacan, and one individual from the Balsas depression. Finally, clade C4 was composed of individuals collected west of the Isthmus of Tehuantepec in Oaxaca and individuals collected south of the Isthmus of Tehuantepec in Chiapas. The haplotype network for *P. nelsoni* underscored the phylogenetic results, in that multiple divergent haplotype groups were found at the northern extent of *P. nelsoni*'s range, and along the coast of Guerrero and Oaxaca. In addition, haplotypes were shared across the Isthmus of Tehuantepec, and the Balsas River samples were more closely associated with samples from other regions than with each other.

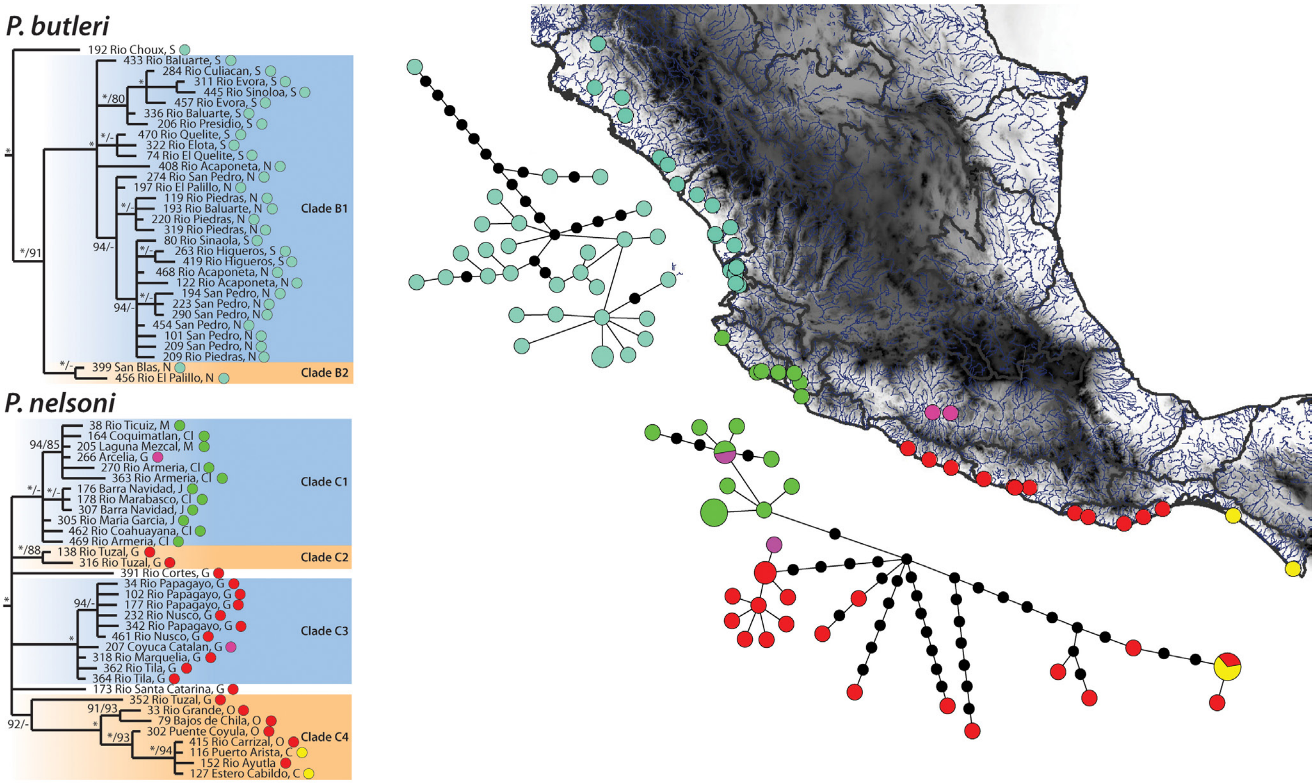

FIGURE 4 | Cytochrome *b* (1,140 bp) mitochondrial gene Bayesian phylogeny, parsimony haplotype network, and sampling distribution of the Pacific sister taxa *Poecilia butleri* (blue-North of the Trans Mexican Volcanic Belt) and *P. nelsoni* (lime green-South of the Trans Mexican Volcanic Belt, pink- Balsas River Drainage, red- North of the Isthmus of Tehuantepec, and yellow- South of the Trans Mexican Volcanic Belt) across geographic barriers along the coast of Mexico. The phylogeny has Bayesian posterior values followed by bootstrap values with asterisks representing support of 95% or above. The Parsimony network values correspond to the haplotype values and are colored according by geographic locations separated by barriers; circle sizes correspond to the number of individuals with that haplotype (larger circles reflect more individuals), and black cirles indicate unsampled haplotypes. The capital letters at the end of each sample represents the state of origin in Mexico, from North to South: S = Sinaloa, N = Nayarit, J = Jalisco, Cl = Colima, M = Michoacan, G = Guerrero, O = Oaxaca, C = Chiapas.

Phylogeography of *Poecilia mexicana sensu lato*. The closely related species *P. limantouri*, *P. thermalis* and *P. sulphuraria*, and *P. mexicana* formed, along with several Middle American species, the last major clade in the phylogeny (Figs. 2, 5). Among these species, the hydrogen-sulfide spring endemics *P. thermalis* and *P. sulphuraria* were most genetically similar (0–0.5% sequence divergence within each species), whereas *P. mexicana* (within species range: 0–2.0%) and *P. limantouri* (within species range: 0–1.7%) were more genetically diverse (Tab. **S4**). *Poecilia limantouri* was predominantly found north of the TMVB, but with a small range (represented by two sampling points) just south of this geographic barrier. Most haplotypes of *P. limantouri* across the distributional range did not exhibit any phylogenetic clustering or lacked support from the maximum likelihood analyses. The single sub-clade with strong support (D1) included two haplotypes: one from the state of Hidalgo and the other from the southern sampling points in the state of Veracruz (Fig. 5). The haplotype network exhibited between 1–6 mutational steps between haplotypes.

The species *P. sulphuraria* and *P. thermalis* from the states of Tabasco and Chiapas were recovered in two sub-clades with moderate support. The first (E1) included individuals of *P. sulphuraria* from the Baños del Azufre site and of *P. thermalis* from the La Esperanza spring complex. The second sub-clade (E2) was formed exclusively of haplotypes of *P. sulphuraria* from the La Gloria spring complex. The haplotype network showed almost no variation in these taxa, and interestingly the Baños del Azufre population of *P. sulphuraria* clustered more closely with *P. thermalis* (clade E1) than the La Gloria population of *P. sulphuraria* (clade E2; Fig. 5).

Finally, phylogenetic analyses recovered a monophyletic *P. mexicana*, with further phylogenetic structuring of varying support (Fig. 5). One sub-clade (0.91 Posterior Probability) was comprised of individuals from northern Mexico (in Nuevo León),

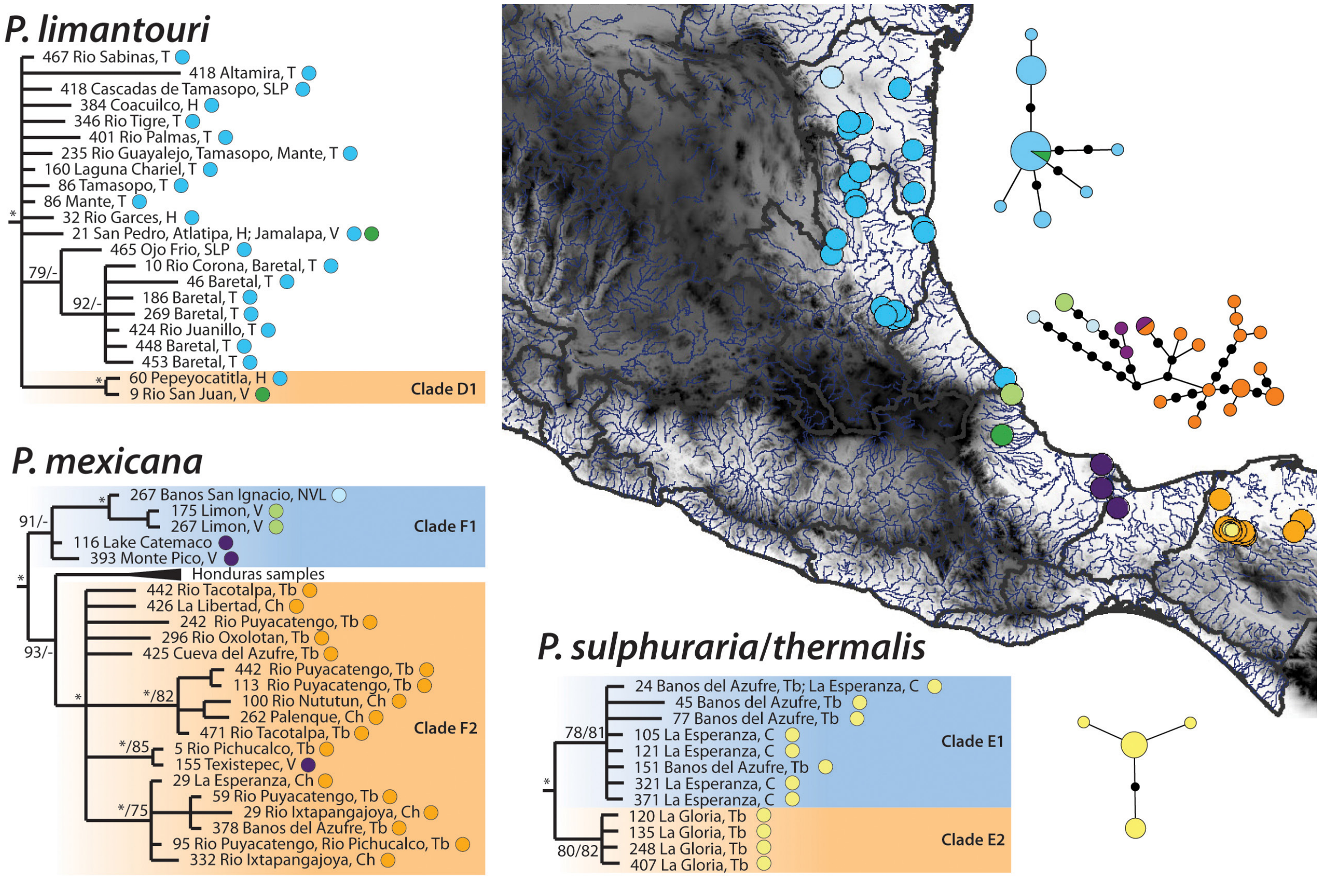

FIGURE 5 | Cytochrome *b* (1,140 bp) mitochondrial gene Bayesian phylogeny, parsimony haplotype network, and sampling distribution of Atlantic taxa *Poecilia limantouri* (Turqouise-North of the Trans Mexican Volcanic Belt, forest green-South of the Trans Mexican Belt), *P. sulphuraria*/*P. thermalis* (yellow-South of the Isthmus of Tehuantepec), and *P. mexicana* (baby blue-North of the Trans Mexican Volcanic Belt, light green-South of the Trans Mexican Volcanic Belt, purple-North of the Isthmus of Tehuantepec, orange-South of the Isthmus of Tehuantepec) across geographic barriers along the Atlantic coast of Mexico. The phylogeny has Bayesian posterior values followed by bootstrap values with asterisks representing support of 95% or above. The Parsimony network values correspond to the haplotype values and are colored according by geographic locations separated by barriers; circle sizes correspond to the number of individuals with that haplotype (larger circles reflect more individuals), and black circles indicate unsampled haplotypes. The capital letters at the end of each sample represents the state of origin in Mexico, from North to South: NVL = Nuevo Leon, T = Tamaulipas, V = Veracruz, H = Hidalgo, Tb = Tabasco, C = Chiapas.

individuals collected just south of TMVB, and two sites just west of the Isthmus of Tehuantepec in the Los Tuxlas region (clade F1 in Fig. 5). Another sub-clade represented individuals collected from the Atlantic versant of Honduras, and this sub-clade was recovered as sister to the last sub-clade (Clade F2), comprising individuals collected from Tabasco and Chiapas, except for one individual collected in Veracruz (Fig. 5). The haplotype network showed a range of variation with 1–15 mutational steps between haplotypes.

Our analyses indicated extensions of the documented ranges for *P. mexicana* and *P. limantouri*, which were previously believed to either occur only south and only north of the TMVB, respectively (Miller *et al*., 2005). Both species' distributions appear to span the TMVB (Fig. 5), and the northern haplotypes in *P. mexicana* represent a highly divergent lineage that may represent a yet unrecognized species that also differs in a series of morphological traits from *P. mexicana* south of the TMVB (M. Tobler, unpublished data). We also found the southern range of *Poecilia nelsoni* in Mexico to extend into southern Oaxaca and Chiapas.

DISCUSSION

The aim of this study was to provide a better understanding of the patterns of divergence and speciation across the geologically complex Mexican landscape in freshwater fishes of the subgenus *Mollienesia*. The overall phylogenetic patterns observed among species (Fig. 2) revealed multiple colonization events from lower Central America into Mexico and strong geographic structuring by physiographic barriers and by river basins. The exception was the widely distributed *P. sphenops*, which was distributed across all geographic barriers discussed here (Figs. 1, 3), thereby overlapping with the distribution of other species in the subgenus *Mollienesia*. In contrast, *P. nelsoni* and *P. mexicana* showed signals of population structure but not in relation to the Isthmus of Tehuantepec (Figs. 4, 5). This phylogeographic structure over small spatial scales, albeit weak at present, is likely the result of limited genetic exchange across smaller geologic barriers and disconnected river basins. Our results indicate that different species in the subgenus *Mollienesia*–despite their phenotypic similarities–vary in their vagility, and mechanistic studies are needed to understand how morphological, physiological, and behavioral differences among species may contribute to variation in dispersal.

Phylogenetic patterns of Mexican species. The phylogeny of the subgenus *Mollienesia* shows an interesting pattern with respect to the Mexican species in the *P. sphenops* and *P. mexicana* species complexes. Most Mexican species in the subgenus were directly derived from Central American lineages, demonstrating the complexity of the evolution of species in this group. In the *P. sphenops* species complex, there were three potential invasions that led to the speciation of Mexican species (*P. catemaconis*, *P. chica*, and *P. sphenops*). The differentiation of *P. catemaconis* on the Atlantic slope of Mexico from the ancestral population was a result of the geographic isolation as Lake Catemaco became geologically separated from other hydrological units by the Sierra de los Tuxtlas (Palacios *et al*., 2016). The Pacific *P. chica* possibly also evolved due to volcanic activity isolating the Jalisco block between the Pliocene and Quaternary (Rosas-Elguera *et al*., 1996).

In the *P. mexicana* species complex, Mexican species also showed multiple independent invasions from Central America. The Pacific slope species, *P. butleri* and *P. nelsoni*, evolved in Mexico due to the uplift of the Trans Mexican Volcanic Belt, isolating populations north and south of this barrier (Mateos, 2005; Zúñiga-Vega *et al*., 2014). On the Atlantic versant, the evolution of species was a combination of vicariance (*P. limantouri* from *P. petenensis* in Central America; Palacios *et al*., 2016) and adaptation to local environmental conditions in toxic, hydrogen-sulfide-rich springs (*P. sulphuraria* and *P. thermalis*; Palacios *et al*., 2013). Lastly, the most recent invasion of *P. mexicana* may have included two independent invasion events from Central America; one represented by lineages occurring in the northern distribution and the other into the southern portion in Mexico.

Within species, strong phylogeographic structuring was observed in two species (*P. nelsoni* and *P. mexicana*) over small spatial scales. This is likely due to the fragmentation and disruption of connections between drainages, potentially by the presence of physiographic barriers such as the Balsas Depression, thereby limiting dispersal and isolating populations. This pattern is common in other Neotropical fish species distributed in Mexico, such as the genera *Astyanax* Baird & Girard, 1854 (Coghill *et al*., 2014), *Profundulus* Hubbs, 1924 (Morcillo *et al*., 2016), and *Poeciliopsis* Regan, 1913 (Mateos *et al*., 2002), although other species, like *P. sphenops*, appear to be able to cross the very same barriers.

Geological barriers influencing diversification in Mexican mollies. The Trans Mexican Volcanic Belt (TMVB) is a physiographic feature that formed west to east between 25–2.5 million years ago (Miller *et al*., 2005). The TMVB is composed of ridges and volcanoes that decrease eastwardly in altitude toward Veracruz (Miller *et al*., 2005). This barrier is a phylogeographic break for both terrestrial and aquatic species (Huidobro *et al*., 2006), and our results suggest that the TMVB was associated with a vicariant speciation event on the Pacific versant, resulting in the evolution of sister species north (*P. butleri*) and south (*P. nelsoni*) of this barrier. This pattern has been previously observed in these sister taxa (Mateos, 2005; Zúñiga-Vega *et al*., 2014) and in other vertebrate species (Devitt, 2006; Blair, Sánchez-Ramírez, 2016; Light *et al*., 2016). However, the most common recent ancestors of several fish groups distributed across the TMVB originated north of the TMVB (Pérez-Rodríguez *et al*., 2015), whereas our study suggests an origin south of the TMVB because of the higher genetic variation in the populations of *P. nelsoni* (observed in most southern populations) and due to the phylogenetic structure pointing toward repeated colonization from Central America. A similar south to north crossing of the TMVB has recently been documented for *Poeciliopsis scarli* Meyer, Riehl, Dawes & Dibble, 1985 (Conway *et al*., 2019). Invasion by or isolation of populations of Neotropical freshwater fish to the north of the TMVB on the Pacific Coast has also been documented in fish of the genera *Astyanax* (Strecker *et al*., 2012) and *Poeciliopsis* (Mateos *et al*., 2002).

On the Atlantic slope, the eastern most point of TMVB is called Punta del Morro (PDM) and serves as a phylogeographic break (Contreras-Balderas *et al*., 1996). The uplift of this barrier has caused vicariant events evident in species pairs of various families of Neartic and Neotropical freshwater fish (*e.g.*, Lepisosteidae, Clupeidea, Cichlidae, Characidae, and Poeciliidae; Contreras-Balderas *et al*., 1996; Hulsey *et al*., 2004; Agorreta *et al*., 2013). The mountains at PDM serve as a filter for primary and secondary Neartic and Neotropical fishes (Obregon-Barboza *et al*., 1994), frequently setting distribution limits and influencing phenotypic differences (Hulsey *et al*., 2010). For *P. mexicana*, unique haplotypes exist north of the PDM, which may be indicative of such isolation. In contrast, our results for *P. sphenops* and *P. limantouri*, which exhibited shared haplotypes on either side of the TMVB, suggested that the PDM is not a strong isolating barrier for these species at this time. However, the presence of *P. sphenops* outside of the described range (Miller *et al*., 2005), especially north of the TMVB, may also be a result of human introductions (Espinosa Perez, Ramírez, 2015).

The Balsas River system is the largest hydrological system on the Pacific slope of Mexico and houses many endemic species. The high rate of endemism in the Balsas Depression (BD) is a result of the isolation due to the formation of the Trans Mexican Volcanic Belt and Sierra Madre del Sur (Ferrusquía-Villafranca, 1998; Ferrari *et al*., 2000). The Balsas River system has been linked to events of dispersals and colonization by Neartic fish from the Mesa Central, a high plateau in central Mexico (Domínguez-Domínguez *et al*., 2010), a pattern coinciding in fish parasites (Martínez-Aquino *et al*., 2014), or with isolation of populations due the uplift of the TMVB (Pérez-Rodríguez *et al*., 2015). In our case, the Balsas River Valley is a potential area of transition for the northern expansion of *P. nelsoni* and *P. sphenops.* For *P. nelsoni,* the southern distribution of our sampling in Chiapas showed higher genetic diversity, potentially in relation to older populations, and for *P. sphenops*, the BD formed part of the historical documented range (Miller *et al*., 2005). This idea is supported by the shared populations or sister taxa in surrounding areas of the BD for *Mollienesia* and other species and coincides with the most recent geological isolation of this area by Plio-Pleistocene volcanism (Marshall, Liebherr, 2000). Future fine scale sampling may reveal additional patterns in the BD, as the Sierra de Taxco divides the Balsas basin into western and eastern units (Cabral-Cano *et al*., 2000). Interestingly, this ichthyogeographical province (Miller, Smith, 1986; Miller *et al*., 2005) houses the native endemic species *P. maylandi* of the subgenus *Mollienesia*, but sampling efforts in and around the type locality (Meyer, 1983) were unsuccessful, instead finding only *P. nelsoni* and *P. sphenops*.

Finally, the Isthmus of Tehuantepec is a low elevation area recognized as a strong faunal barrier that emerged during the Pliocene and Pleistocene (Campbell, 1999; Marshall, Liebherr, 2000; Mulcahy *et al*., 2006). This potential barrier does not appear to have played a significant role in shaping inter-specific variation in *Mollienesia* taxa (Fig. 2). However, our results for *P. mexicana* suggest that the Isthmus may be a moderate barrier, with one sub-clade being distributed almost entirely south of the Isthmus of Tehuantepec (and this sub-clade was sister to Guatemalan samples), and another being distributed entirely north of the Isthmus of Tehuantepec (Fig. 5). The same pattern did not hold for either *P. sphenops* or *P. nelsoni*, as both species had shared haplotypes occurring across the Isthmus (Figs. 3, 4). Our results for these species contrast with a recent study of *Poeciliopsis pleurospilus* and *P. gracilis* which, based on ddRADseq data, showed that the Isthmus of Tehuantepec was a clear geographic barrier between them (Ward *et al*., 2022).

In summary, the phylogenetic patterns of Mexican species in the subgenus *Mollienesia* demonstrate several independent invasions from Central America and subsequent diversification of species associated with isolation, both because of major physiogeographic barriers and ecological selection. The phylogeographic patterns observed within Mexican species either indicated strong genetic structuring across a physiographic barrier (*P. nelsoni* and *P. mexicana*) or a lack of genetic structuring (*P. sphenops*, *P. butleri*, *P. limantouri*, and *P. sulphuraria/thermalis*), indicating that different species may vary significantly in their dispersal abilities. In fact, *P. sulphuraria* and *P. thermalis* are so closely related that they may represent the same species, but a formal taxonomic revision is required to determine whether *P. sulphuraria* should be treated as a synonym to *P. thermalis* (Palacios *et al*., 2013). Our analyses also indicated broader distributions than currently described for several species. This study also sheds insight into the taxonomic status of Pacific populations of *P. sphenops,* which was morphologically hypothesized as distinct based on the scale count around the peduncle (Miller *et al*., 2005). Since we find lack of genetic differentiation across the species' distribution, these morphological differences are likely indicative of geographic variation in phenotypes and not species differences.

ACKNOWLEDGMENTS

The authors thank C. Passow and Z. Culumber for help in the field. We thank G. Sosa with assistance in file transfer of analyses. Permits issued by the Municipio de Tacotalpa-Tabasco (DFET/23/2011), the Mexican Federal Agencies SEMARNAT (SGPA/DGVS/04315/11 for *Poecilia sulphuraria* and SGPA/DGVS/02076/12 for *P. nelsoni* (issued under *butleri*), CONAPESCA (DGOPA. 09004.041111.3088 for *Poecilia* sp.). Financial support came from Texas A&M University and the National Council for Science and Technology Exchange Program for Visiting Student Researchers in Mexico (to MP) and the National Science Foundation (IOS–1121832, IOS–1463720, and IOS–1557860 to MT; DEB–0743782 to Luis A. Hurtado and MM).

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ETHICAL STATEMENT

Protocols approved by the Texas A&M University and Oklahoma State University Committees on Use and Care of Animals (IACUC 2011–118 and ACUP AS10–15).

COMPETING INTERESTS

The author declares no competing interests.

HOW TO CITE THIS ARTICLE

- **Palacios M, González-Díaz AA, Rodriguez LA, Mateos M, Rodiles-Hernández R, Tobler M, Voelker G.** Population level genetic divergence and phylogenetic placement of Mexican shortfin mollies (*Mollienesia*: Poecilia: Poeciliidae). Neotrop Ichthyol. 2023; 21(2):e220101. https://doi.org/10.1590/1982-0224-2022-0101

Neotropical Ichthyology

Official Journal of the
Sociedade Brasileira de Ictiologia