# PHYLOGENETICS AND DIVERSIFICATION OF SAILFIN AND SHORTFIN MOLLIES

(*Mollienesia*, *Poecilia*, POECILIIDAE)

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2017

Major Subject: Wildlife & Fisheries Sciences

# ABSTRACT

Phylogeography aims to understand the formation of species across space and time. Freshwater fishes are studied because they strongly reflect historical and ecological changes in a region. The objective of this dissertation is to identify historic geological events and ecological factors that shaped the evolution of species in the subgenus *Mollienesia* (*Poecilia*, *Mollienesia*, Poeciilidae) within the geologically dynamic regions of Mexico, Central America, and the Caribbean. I investigate the evolutionary relationships in 19 of the 25 species by conducting phylogenetic, species trees with molecular clock estimates, and ancestral area estimates analyses on a multi-locus dataset. The phylogenetic and species trees results support three main groups: *Poeciia latipinna*, *P. sphenops*, and *P. mexicana* species complexes. The molecular clock estimates are inconclusive and ancestral area estimates indicate the diversity originated from the Maya and Chortis blocks. These findings uncover allopatric and ecological speciation events in the three main regions. I also analyze a three-gene dataset of fine scale sampling in Mexico of the *P. sphenops* and *P. mexicana* species complexes under phylogenetic and haplotype network. The phylogenetic results show that Mexican species are a result of independent invasions from Middle America with subsequent diversification. Haplotype network analyses demonstrate that within species, physiographic barriers or river basins cause strong phylogeographic structure of some species, while others show only weak structuring. These patterns are shared with other Neotropical freshwater fishes, mammals, amphibians, and birds. Lastly, I report on the phylogenetic, population genetics, and geometric morphometrics assessment of the sulfide spring rediscovered species *Poecilia thermalis* from Mexico, after 150 years of the type locality being unknown to

science. Phylogenetic analysis finds *P. thermalis* to be sister to one population of *P. sulphuraria* and not an independent lineage, complicating the systematics. Population genetic analyses shows *P. thermalis* to be genetically distinct from adjacent species found in freshwater and shape analyses finds unique morphological characteristics like an enlarged head and wide mouth to aid in the uptake of oxygen, a limited resource in an extreme habitat. This dissertation identified mechanisms of speciation in the subgenus *Mollienesia* and contributed a wealth of genetic information from previously unsampled regions.

# DEDICATION

I dedicate this body of work to my grandmother, Marcelina Garay. She was a humble woman with a will and a faith unmatched by any other. She left a great example for all who knew her. I admired her fearlessness, intelligence, patience and drive. You are dearly missed but we will be reunited again in heaven. I also dedicate this to my dear friend, Jose Adan Deras, a conservation biologist who had a passion for life and people.

# ACKNOWLEDGEMENTS

I would like to thank my chair, Dr. Gary Voelker and co-chair, Dr. Michael Tobler, my committee members, Drs. Kevin Conway and David Baumgardner, and collaborator Lenin Arias-Rodriguez for their guidance throughout the course of this research.

I would also like to thank my friends, Shuang Liu, Emma Lee, Jian Gong, Eli Borrego, Gaby Sosa, Alejandra Maldonado, Zaria Torres, Victoria Rodriguez, and other colleagues, the department faculty and staff for making my time at Texas A&M University a great experience. I thank my family in El Salvador and colleagues who assisted in the field.

Finally, I thank my mother, Maura del Rosario Mejia Garay, for her encouragement and support. I thank my father, Eriberto Reyes, for planting a seed to pursue a higher education. I thank my brother, Hector Palacios and his wife, Laura Palacios, my sister Jenny Argueta and her husband, David Argueta, and my brother, Douglas Vladimir Estrada and his wife, Helen Estrada for the emotional and financial support. A special thank you to my eight nieces and nephews, Nancy Estrada, Bryan Argueta, Amy Estrada, Yvette Argueta, Matthew Argueta, Josue Estrada, Hector Maximus Palacios, and Alexander Palacios. Thank you to Carrie Boren Headington who gave me the opportunity to go to college. Thank you to the Lord God Almighty, Jesus Christ and the Holy Spirit for granting me the wisdom, intelligence and patience to complete this work.

## CONTRIBUTORS AND FUNDING SOURCES

This work was supported by a dissertation committee consisting of Professor Dr. Gary Voelker from the Wildlife & Fisheries Sciences department, co-advisor Dr. Michael Tobler from the Biology department at Kansas State University, committee member Dr. Kevin Conway from the Wildlife & Fisheries Sciences department, and committee member Dr. David Baumgardner from the Biology department.

All work for the dissertation was completed by the student, in collaboration with the co-authors Drs. Lenin Arias Rodriguez, Martin Plath, Anton Lamboj, Hannes Lerp, Alfonso Gonzalez-Diaz, Mariana Mateos, Rocio Rodiles-Hernández, Michael Tobler, Gary Voelker, and Constanze Eifert (M.Sc.) and those acknowleged that appear or will appear in the journal publication.

Graduate study was supported by the Doctoral Diversity, Regents graduate, Hispanic Leaders in Agriculture and the Environment fellowship from Texas A&M University and a dissertation research fellowship from the Alfred P. Sloan Foundation. Funding for research was provided by the Society of Systematic Biology, L.T. Jordan Fellowship, Texas A&M University & National Council for Science and Technology Exchange Program for Visiting Student Researchers in Mexico, as well as the National Science Foundation (IOS-1463720, IOS-1557860, and DEB-0743782).

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# CHAPTER I

# GENERAL INTRODUCTION

Freshwater habitats house a wide range of endangered taxa and are therefore among the most threatened ecosystem on Earth (Revenga et al. 2005). Alterations to freshwater ecosystems include damming, habitat degradation, invasive species, over-exploitation, and pollution (Dudgeon et al. 2006; Darwall et al. 2008; Revenga et al. 2005; Arthington et al. 2010). Freshwater species account for six percent of total biodiversity on earth (Dudgeon et al. 2006) with freshwater fish totaling 40% of all fish species described (Lundberg et al. 2000). Unfortunately, the effects of modification and loss of freshwater ecosystems are occurring at a faster rate than freshwater fish species are discovered and described.

Freshwater fishes are interesting because their evolution frequently reflects the historical geological and ecological changes of a region due to their dependence on direct connections between river drainages for dispersal (Bermingham and Martin, 1998). As a result, freshwater fishes serve as great models for phylogeographic studies, which aim to understand the formation of species across space and time. Phylogeographic studies often uncover new genera and species of freshwater fish in historical geologically complex regions with extreme variations in topography and climates (Schönhuth et al. 2008). In this context, the regions of Mexico, Middle America, and the Caribbean are of interest because of the complex topography, diversity of habitats, high levels of biodiversity and endemism, and the threat of rapid human development (Campbell, 1999; Jackson et al., 1996). These areas have a unique freshwater fish community structure with a dominance of secondary (salinity

tolerant) freshwater fish consisting primarily of the families Poecillidae and Cichlidae (Miller, 1966).

The family Poeciliidae is diverse in the Neotropics (27 genera and over 250 species; Eschmeyer and Fong, 2012) and share three unique characteristics: adult males posses a specialized organ termed a gonopodium (a modified anal fin for sperm deposit; Parenti, 1981), reproduction via internal fertilization, and viviparity (livebearing-except for *Tomeurus gracilis*; Reis et al. 2003). The biodiversity and taxonomic classification of these subfamily Poeciliinae in the past was based on analyzing morphological characteristics (Ghedotti, 2000; Lucinda and Reis, 2003; Parenti, 1981; Rosen and Bailey, 1963), and recent molecular investigations indicated that we have underestimated the diversity or improperly diagnosed the relationships between and within groups (Alda et al. 2013, Bagley et al. 2015; Breden et al. 1999; Mateos et al. 2002; Meyer et al. 2013; Ptacek et al. 1999). Phylogeographic studies of this group have also recognized the importance of historical geologic events in the formation of species, with the presence of natural barriers such as mountains and catchment divides impeding the genetic exchange of species across regions (Agorreta et al., 2013; Hrbek et al. 2007; Meredith et al., 2011). Ecological variables have also contributed to the formation of new species in these areas such as salinity tolerance and hydrogen sulfide tolerance (Hankinson et al. 2006; Tobler et al. 2011). Phylogeographic studies help us to better understanding the mechanisms driving and maintaining species, detect unique lineages or new species, and make informed conservation decisions.

The objectives of this dissertation are to (1) establish the phylogeny for the subgenus *Mollienesia* (genus *Poecilia*) (2) better understand the diversification patterns of species across and within species in the historically geologic active regions of Mexico, Middle

America, and the Caribbean, (3) assess the biodiversity, and (4) contribute to the systematics of the group to provide make suggestions on the conservation of the species of this group.

Below I provide a brief summary of the background and main results from each chapter, beginning with macroevolutionary to microevolutionary processes shaping species within the subgenus *Mollienesia*.

In Chapter 2**,** I focus on establishing a phylogenetic framework for the subgenus *Mollienesia*, as this diverse group of freshwater fishes includes species that serve as important models across multiple biological disciplines. The phylogeny will also contribute to solving the conflictive and convoluted taxonomic history of this group. I conduct a comprehensive molecular phylogenetic analysis of the subgenus *Mollienesia* to identify taxonomic discrepancies and potentially identify undescribed species, estimate ancestral areas of origin and estimate dates of divergence, as well as explore biogeographical patterns. The results confirm the presence of three main clades, the *P. latipinna, P. sphenops*, and *P. mexicana* species complexes. The findings do not support the morphology-based hypothesis of Caribbean species grouping within the subgenus *Mollienesia*, but instead they are more closely related to species of the subgenus *Limia*. This study also revealed several taxonomic inconsistencies and distinct lineages in the *P. mexicana* species complex that may represent undescribed species. The diversity in the subgenus *Mollienesia* is a result of dynamic geologic activity leading to vicariant events, dispersal across geologic blocks, and ecological speciation.

In Chapter 3**,** I explore the subgenus *Mollienesia* in a phylogeographic context within Mexico. Mexico is a megadiverse region in ecosystems, species and endemics because of the country's complex geological history and geographic location at the interface of the Neartic

and Neotropical biogeographic zones. I examine the phylogeographic patterns of the subgenus *Mollienesia* (genus *Poecilia*) at a fine scale, particularly the shortfin mollies (*P. sphenops* and *P. mexicana* species complex), to assess haplotype diversity across their geographic distribution, identify phylogeographic breaks, and determine species' distributions. Over 50 locations throughout Mexico are sampled and analyzed with phylogenetic and haplotype network methods. The results demonstrate that the evolution of Mexican species is a result of several independent invasions from Middle America and subsequent diversification. The phylogeographic patterns observed within each species across Mexico showed grouping of haplotypes by river basin and physiographic barriers in some species (*P. nelsoni* and *P. mexicana*) or a lack thereof (*P. sphenops*, *P. butleri*, and *P. limantouri*). Interestingly, the distribution in relation to genetic information of each species contributed to expanding the range for two Atlantic species (*P. mexicana* and *P. limantouri*), resolve the taxonomic uncertainty of populations in another (*P. sphenops*), and provide an informed recommendation for the conservation status of a species (*P. butleri*). This study provides a better understanding of the mechanisms driving divergence and speciation across the geologically complex Mexican landscape in freshwater fishes of the subgenus *Mollienesia*. Future studies should focus on additional sampling in Mexico, which may reveal distinct genetic lineages or new species and monitor for the expansion of species in this group as invasives.

In Chapter 4, I focus on understanding the process of ecological speciation driving the evolution of locally adapted and reproductively isolated populations in response to divergent natural selection in the rediscovered species *P. thermalis*. In Southern Mexico, several lineages of the freshwater fish species of the genus *Poecilia* have independently colonized

toxic, hydrogen sulfide-rich springs. Even though ecological speciation processes are increasingly well understood in this system, aligning the taxonomy of these fish with evolutionary processes has lagged behind. While some sulfide spring populations are classified as ecotypes of *Poecilia mexicana*, others, like *P. sulphuraria*, have been described as highly endemic species. This study particularly focused on elucidating the taxonomy of the long described sulfide spring endemic, *Poecilia thermalis* Steindachner 1863, and investigates if similar evolutionary patterns of phenotypic trait divergence and reproductive isolation are present as observed in other sulfidic species of *Poecilia*. Three methodologies are applied: (1) a geometric morphometric approach to assess body shape similarity to other sulfidic and non-sulfidic fish of the genus *Poecilia*. (2) phylogenetic analyses to establish the phylogenetic relationships of *P. thermalis* and (3) population genetic analyses to determine levels of gene flow among *Poecilia* from sulfidic and non-sulfidic sites. Results indicate that *P. thermalis*' body shape has evolved in convergence with other sulfide spring populations in the genus. Phylogenetic analyses placed *P. thermalis* as most closely related to one population of *P. sulphuraria*, and population genetic analyses demonstrated that *P. thermalis* is genetically isolated from both *P. mexicana* ecotypes and *P. sulphuraria*. Based on these findings, we make taxonomic recommendations for *P. thermalis*. Overall, our study verifies the role of hydrogen sulfide as a main factor shaping convergent, phenotypic evolution and the emergence of reproductive isolation between *Poecilia* populations residing in adjacent sulfidic and non-sulfidic environments.

# CHAPTER II

# PHYLOGENETIC ANALYSES OF THE SUBGENUS MOLLIENESIA (POECILIA, POECILIIDAE, TELEOSTEI) REVEAL TAXONOMIC INCONSISTENCIES, CRYPTIC BIODIVERSITY, AND SPATIO-TEMPORAL ASPECTS OF DIVERSIFICATION IN MIDDLE AMERICA*

## 2.1. Introduction

Middle America is a geologically complex landmass that bridges the North and South American continents and separates the Atlantic and Pacific Ocean basins. It extends from the tropical region of Mexico southward to the Isthmus of Panama. Middle America lies at the active junction of four tectonic plates and is formed by the union of major geologic blocks (Fig. 2.1; Iturralde-Vinent, 2006; Marshall, 2007). The formation and closure of this narrow land bridge at the Isthmus of Panama had fundamental consequences for global ocean currents, climate patterns, and the evolution of tropical ecosystems (Haug et al., 2001; Schmidt, 2007). As a result, Middle America harbors a large number of endemic groups (Jackson et al., 1996) and a wide range of habitats supporting diverse ecological communities (Coates and Obando, 1996). The high levels of diversity along with the dynamic geological history are of particular interest to biogeographers, who have attempted to understand distribution patterns of organisms across spatio-temporal scales (Bacon et al., 2015). The

*Reprinted from Molecular Phylogenetics and Evolution. 103. Palacios, M., Voelker, G., Rodriguez, L. A., Mateos, M., & Tobler, M. Phylogenetic analyses of the subgenus *Mollienesia* (*Poecilia*, Poeciliidae, Teleostei) reveal taxonomic inconsistencies, cryptic biodiversity, and spatio-temporal aspects of diversification in Middle America. 230-244. 

formation of Middle America served as a land bridge and corridor for faunal exchange between Nearctic and Neotropical faunas, reshaping the biotic composition of both regions (Marshall, 1988; Marshall et al., 1982; Stehli and Webb, 1985). This historic exchange event is known as the Great American Biotic Interchange and is one of the most recognized biogeographic events in the western hemisphere (Webb, 1978).

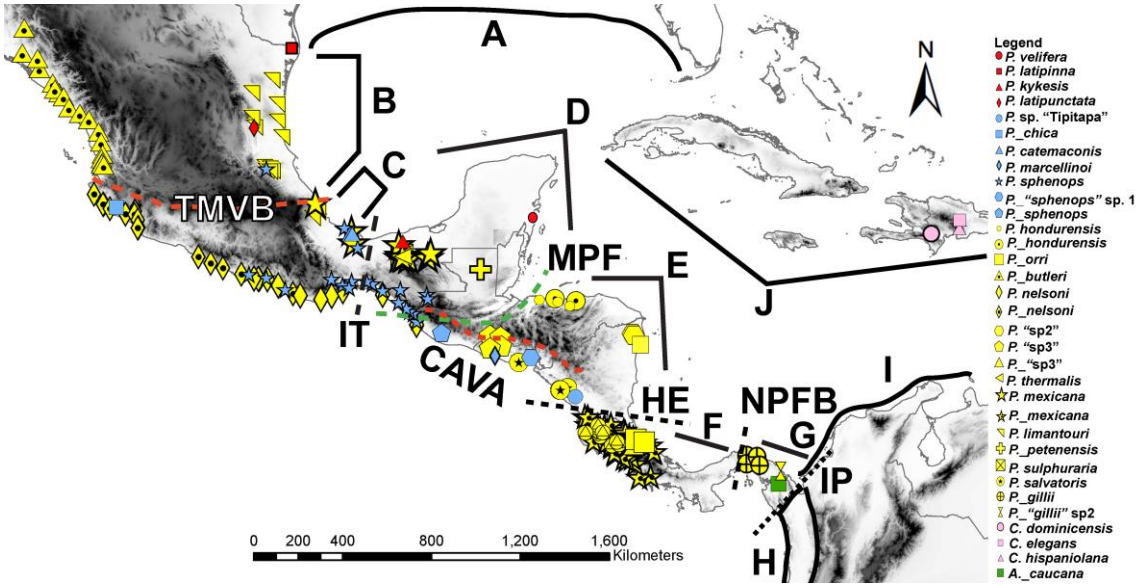


Figure 2.1 The major regions and geologic barriers in Middle America used to delineate regions in this study and the sampling of species in the subgenus *Mollienesia* used in the current study. The letters indicate the regions for the distribution as follows: (A) North America, (B) North of the Trans-Mexican Volcanic Belt, (C) South of the Trans-MexicanVolcanic Belt, (D) Maya, (E) Chortis, (F) Chorotega, (G) Choco North, (H) Choco South, (I) South America, and (J) Greater Antilles. The dashed lines are the geologic barriers and abbreviations are as follows: IT= Isthmus of Tehuantepec, MPF= Motagua-Polochic Fault, CAVA=Central American Volcanic Arc, HE=Hess Escarpment, NPFB= North Panama Fracture Belt, IP= Isthmus of Panama.

To explain the interaction between the geological and biotic history of the region, research on the biogeography of Middle America has historically focused on a variety of taxa, including mammals that mass migrated and left well documented fossil records (Webb, 1985), highly mobile taxa like birds (Barker, 2007; Smith and Klicka, 2010), herpetofauna

with water and temperature-dependent dispersal (Savage, 1966, 1982), and freshwater fish, whose dispersal abilities are dependent on direct connections between drainages (Briggs, 1984; Bussing, 1985; Miller, 1966; Myers, 1966; Rauchenberger, 1988; Rosen, 1975). The general patterns observed across these taxa indicate several waves of colonization from both North and South America coinciding with suitable climatological conditions (Bussing, 1976; Estes and Baez, 1985; Woodburne, 2010). The species that colonized the newly available habitats were identified as being ecologically adaptable, such as being generalists in their dietary resource use (Miller, 1966; Woodburne, 2010), capable of swimming across fragmented landscapes (Miller, 1966; Vences et al., 2003; Webb, 1976), and having broad physiological tolerances that allowed for persistence under novel environmental conditions (McDonald, 2005; Miller, 1966; Pyron et al., 2014). In recent decades, the use of molecular genetic data has expanded studies of the biogeographical patterns in an explicit phylogenetic context, both for plants (Lemes et al., 2010; Ornelas et al., 2013; Poelchau and Hamrick, 2010) and animals (Daza et al., 2010; Wang et al., 2008). Phylogeographical studies have contributed to understanding the processes that shaped patterns of biodiversity in the region by identifying vicariant events caused by geological processes (Cavers et al., 2003; Daza et al., 2010). In addition, phylogeographic studies have uncovered cryptic biodiversity by identifying genetic lineages that went undiscovered based on previous taxonomic analyses (Pinto-Sánchez et al., 2012; Martin and Bermingham, 2000).

Although Middle America does not house a single endemic freshwater fish family, it harbors unique fish communities composed of North and South American faunal elements (Myers, 1966). The most species-rich contributors to the freshwater fish fauna are two divergent groups of secondary freshwater fishes: cichlids and cyprinodonts (Miller, 1966).

Contrary to primary freshwater fishes with low salinity tolerances (Myers, 1949), secondary freshwater fishes can cope with elevated salinities allowing them to occasionally use coastal and marine waters for dispersal (Myers, 1966). This is important from a biogeographical perspective, because even in the presence of fragmented landscapes separated by marine waters, secondary freshwater fishes have the potential for the colonization of novel habitats. Phylogeographic studies of freshwater fishes suggest colonization of Middle America occurred ~3-7 Ma ago for both primary (Bermingham and Martin, 1998; Ornelas-García et al., 2008; Perdices et al., 2002) and secondary freshwater fishes (Bagley and Johnson, 2014; Perdices et al., 2005; Říčan et al., 2013). While some of these molecular dating estimates rely on mitochondrial datasets (Ornelas-García et al., 2008; Bagley and Johnson, 2014), which can result in overestimation of divergence times due to genetic divergences predating population divergences (Marko et al. 2015), colonization of Middle America roughly aligns with the geological upheavals associated with the rising Isthmus of Panama ~2.8-15 Ma ago (Jackson and O'Dea, 2013; Lessios, 2008). Previous studies have also documented genetic structuring between geological blocks and across the Atlantic and Pacific slopes of Middle America (Bermingham and Martin, 1998; Hrbek et al., 2007; Perdices et al., 2002; Perdices et al., 2005; Strecker et al., 2004). The major phylogeographic breaks for freshwater fishes and other taxa in this region include the Isthmus of Tehuantepec, the Motagua-Polochic Fault, the Central American Volcanic Arc, and the Isthmus of Panama (Fig. 2.1; Gutiérrez-García and Vázquez-Domínguez, 2013). Consequently, dispersal and vicariant events could both have contributed to generating diversity in this region; the relative contribution of these events, however, to the evolution of any particular freshwater fish group in the region has been poorly studied.

The family Poeciliidae (Cyprinodontiformes) is a group of secondary freshwater fish that has undergone a significant evolutionary radiation in Middle America (Hrbek et al., 2007) resulting in a multitude of endemic genera (Miller, 1966; Parenti, 1981; Rosen and Bailey, 1963). Three different processes have been hypothesized to play a major role in the diversification of poeciliids: dispersal over land bridges, dispersal across marine water barriers, and vicariance (Hrbek et al., 2007). Here, we focus on the subgenus *Mollienesia* (genus *Poecilia*), which represents an excellent group to contribute to the understanding of dispersal and vicariance in generating diversity in Middle America, because it consists of both geographically isolated species (e.g. *P. sulphuraria*, Tobler et al., 2008; *P. teresae*, Greenfield, 1990; *P. rositae*, Meyer et al., 2004) as well as widely distributed species with a strong capacity for dispersal (e.g. *P. mexicana* and *P. sphenops*, Alda et al., 2013). The wide distribution of the subgenus *Mollienesia* ranges from the southeastern United States, throughout Middle America, to northern South America. Depending on the classification scheme, the range of *Mollienesia* also includes the Antillean chains in the Caribbean (Table 2.1; Ptacek and Breden, 1998). Therefore, phylogeographic analyses of this group can provide insights into past historical processes that shaped diversity and distributional patterns in Middle America and adjacent regions. Establishing a sound phylogenetic hypothesis for the group is also of importance, because several species of the subgenus *Mollienesia* have served as models for different biological disciplines, including research on sexual selection (Pollux et al., 2014; Ptacek, 2005; Ptacek et al., 2011), adaptation and speciation (Palacios et al., 2013; Tobler et al., 2011), the evolution of sex (Schlupp, 2005), and cancer and infectious diseases (Schartl, 2014).

Table 2.1 List of species of the subgenus *Mollienesia*, including currently recognized species based on a morphological revision of the group (Poeser, 2011) and putatively undescribed linages from past and the present phylogenetic analyses. Species are categorized into three species complexes (*P. latipinna*, *P. sphenops*, and *P. mexicana* complexes). The table also includes species that have previously been associated with *Mollienesia*, and the current subgeneric assignment is provided in parentheses. The table indicates each species' previous inclusion in phylogenetic analyses, and asterisks designate species included in the present study. In addition, the table indicates the current knowledge of distributions as used for ancestral area estimations. Abbreviations are as follows: (A) North America, (B) North of the Trans-Mexican Volcanic Belt, (C) South of the Trans-Mexican Volcanic Belt, (D) Maya, (E) Chortis, (F) Chorotega, (G) Choco North, (H) Choco South, (I) South America, and (J) Greater Antilles.

| **Species** | **Previous studies** | **Distribution** | **Comments** |
|---|---|---|---|
| *P. latipinna* complex | | | |
| *Poecilia kykesis* Poeser, 2002 | 1, 9* | D | Formerly referred to as *P. petenensis* Günther, 1866 |
| *Poecilia latipinna* (LeSueur, 1821) | 1, 3, 8, 9* | AB | |
| *Poecilia latipunctata* Meek, 1904 | 1, 4, 5, 8* | B | |
| *Poecilia velifera* (Regan, 1914) | 1, 9* | D | |
| *P. sphenops* complex | | | |
| *Poecilia catemaconis* Miller, 1975 | 1, 7, 8, 11* | C | |
| *Poecilia chica* Miller, 1975 | 8* | C | |
| *Poecilia marcellinoi* Poeser, 1995 | * | E | |
| *Poecilia maylandi* Meyer, 1983 | | | |
| *Poecilia sphenops* Valenciennes, 1846 | 1, 7, 8, 9, 11* | CDE | |
| *Poecilia* "*sphenops"* sp. 1 | 7, 11* | E | Putatively undescribed lineage identified by Alda et al. (2013); may be the same distinct population discussed in Carr and Giovannoli, 1994 |
| *Poecilia* sp. "Tipitapa" | 11* | E | Putatively undescribed lineage identified by Bagley et al. (2015); may be *Poecilia dovii* Günther 1866, which is currently considered a synonym of *P. mexicana* |
| *P. mexicana* complex | | | |
| *Poecilia boesemani* Poeser, 2003 | | | |
| *Poecilia butleri* Jordan, 1889 | 1, 2, 6, 7, 8, 10 * | B | |
| *Poecilia formosa* (Girard, 1859) | | | Gynogenetic hybrid between *P. mexicana* and *P. latipinna* |
| *Poecilia gillii* (Kner, 1863) | 1, 3, 7, 9, 10, 11* | EFG | Currently thought to be widespread, but has restricted distribution based on Alda et al. (2013) |
| *Poecilia petenensis* Günther, 1866 | 9* | D | Referred to as *P. gracilis* Regan 1913 in some recent publications |
| *Poecilia hondurensis* Poeser, 2011 | 7, 11* | E | |
| *Poecilia koperi* Poeser, 2003 | 12 | | |

Table 2.1 Continued

| Species | Previous studies | Distribution | Comments |
|---|---|---|---|
| *Poecilia mechthildae* Meyer, Etzel & Bork, 2002 | | | |
| *Poecilia mexicana* Steindachner, 1863 | 1, 6, 7, 8, 9, 11* | CDE | |
| *Poecilia limantouri* Jordan & Snyder, 1900 | 6, 8, 11* | BC | Previously considered a subspecies of *P. mexicana* |
| *Poecilia nelsoni* (Meek, 1904) | 2, 9, 10* | CD | |
| *Poecilia orri* Fowler, 1943 | 1, 7, 11* | DEF | |
| *Poecilia rositae* Meyer, Schneider, Radda, Wilde & Schartl, 2004 | | | |
| *Poecilia salvatoris* Regan, 1907 | 10, 11* | EF | |
| *Poecilia sulphuraria* (Álvarez del Villar, 1948) | 1, 6, 8, 11* | D | |
| *Poecilia teresae* Greenfield, 1990 | | | |
| *Poecilia thermalis* Steindachner, 1863 | 8, 11* | D | |
| *Poecilia vandepolli* Van Lidth de Jeude, 1887 | 12 | | |
| *Poecilia wandae* | 12 | | Formerly assigned to *Allopoecilia*, assigned to *Mollienesia* by Ho et al. (2016) |
| *Poecilia* "*gillii*" sp. 2 | 7, 11, 12* | G | Putatively undescribed lineage identified by Alda et al. (2013) |
| *Poecilia sp. "Patuca"* | 11* | DEF | Putatively undescribed lineage identified by Bagley et al. (2015); treated as *P. orri* in this study |
| *Poecilia* "sp2" | * | EF | Putatively undescribed lineage identified in this study |
| *Poecilia* "sp3" | * | EF | Putatively undescribed lineage identified in this study |
| **Previously assigned to *Mollienesia*** | | | |
| *Poecilia* (*Allopoecilia*) *caucana* (Steindachner, 1880) | 1, 4, 5, 6, 7, 9, 12* | GHI | |
| *Poecilia* (*Allopoecilia*) *dauli* Meyer & Radda, 2000 | 12 | | |
| *Poecilia* (*Psychropoecilia*) *dominicensis* (Evermann & Clark, 1906) | * | J | |
| *Poecilia* (*Curtipenis*) *elegans* (Trewavas, 1948) | * | J | |
| *Poecilia* (*Psychropoecilia*) *hispaniolana* Rivas, 1978 | 7, 12* | J | |
| *Poecilia* (*Poecilia*) *vivipara* Bloch & Schneider, 1801 | 1, 4, 5, 6, 9, 12* | I | |

1. Ptacek & Breden 1998; 2. Mateos 2005; 3. Lee & Johnson 2009; 4. Meredith et al. 2010; 5. Meredith et al. 2011; 6. Tobler et al. 2011; 7. Alda et al. 2013; 8. Palacios et al. 2013; 9. Pollux et al. 2014; 10. Zuniga-Vega et al. 2014; 11. Bagley et al. 2015 12. Ho et al. 2016

Recent phylogenetic studies have established the phylogenetic relationships between *Mollienesia* and the other recognized subgenera (*Limia*, *Pseudolimia*, *Pamphorichthys*, *Micropoecilia*, *Poecilia*, and *Acanthophacelus*) in the genus *Poecilia* (Meredith et al. 2010, 2011; Fig. 2.2). However, species level taxonomy within the subgenus *Mollienesia* has a convoluted history because morphological revisions are complicated by meristic and morphometric characteristics often exhibiting more variation within species than between species (Poeser, 2003; Rosen and Bailey, 1963). In addition, hybridization has been documented in natural populations of *Mollienesia*, further complicating taxonomic revisions (Kittell et al., 2005). These complications have led to a long and winding taxonomic trail of species descriptions and synonymy (Poeser, 2003). Despite these issues, the most recent morphological assessment distinguished 26 species (see Table 2.1; Poeser, 2011). Molecular phylogenetic studies focusing on the subgenus *Mollienesia* have established an overall backbone of the species' relationships and indicated the presence of three broad species complexes (Fig. 2.2; Alda et al., 2013; Ptacek and Breden, 1998). In addition, they have revealed high genetic variation within single species (e.g. *P. mexicana*), events of hybridization, and series of putatively undescribed lineages (see Table 2.1; Alda et al. 2013; Bagley et al., 2015; Ho et al. 2016).

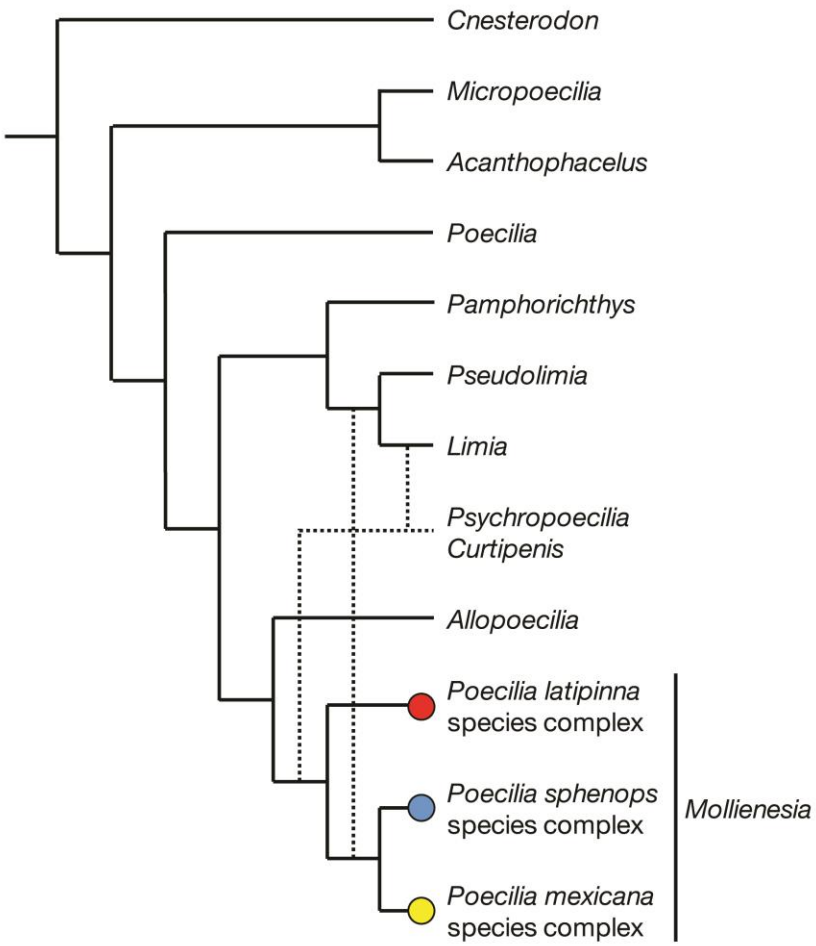


Figure 2.2 The evolutionary relationships among the main subgenera of the genus *Poecilia* relative to *Cnesterodon* (outgroup) based on past molecular phylogenetic studies (e.g., Meredith et al. 2011), with a particular emphasis on the species complexes within *Mollienesia* (Ptacek and Breden, 1998). Note that the relationship between Caribbean subgenera *Psychropoecilia* and *Curtipenis* as well as their phylogenetic placement within the genus largely remain unresolved (see main text). Based on phylogenetic, morphological, and biogeographic considerations, these subgenera have been hypothesized to be closely related to either *Mollienesia* or *Limia* (dashed lines). The color coding of species complexes reflects the color scheme of individual species in Figure 2.1.

Here, we build on these previous efforts with a more extensive sampling of both species and genes. To do so, we have collected previously unsampled species, increased sampling coverage particularly in the Northern range of the subgenus, and consolidated previously published data to maximize species and distribution coverage (Alda et al. 2013;

Bagley et al. 2015; Lee and Johnson, 2009; Pollux et al. 2014; Zuniga-Vega et al. 2014). The generation of a robust phylogeny of species within *Mollienesia* will allow for estimating timings of divergence, shed light into mechanisms on speciation processes, and provide an evolutionary context for comparative analyses for a broad array of disciplines. In addition, we have also sampled species that have previously been postulated to be part *Mollienesia* based on morphological characters (Myers,1931; Meyer, 1983). Specifically, this pertains to three Caribbean species occurring on the island of Hispaniola (*P. dominicensis* and *P. hispaniolana* from the formerly recognized subgenus *Psychropoecilia*, Myers, 1935 and *P. elegans* from the formerly subgenus *Curtipenis*, Rivas and Myers, 1950).

To obtain better insights into the evolution of *Mollienesia*, we asked the following questions: (1) Does the current morphology-based taxonomy agree with molecular phylogenetic hypotheses for the subgenus *Mollienesia*? (2) To what extent can phylogenetic analyses contribute to identifying cryptic diversity within the group? (3) What current and historical processes shaped the diversification and distribution of species in the subgenus *Mollienesia*? In addressing this last question, we ask whether the timings of lineage divergences in the group can allow us to assess the comparative roles that dispersal and vicariance processes have played in contributing to the current diversity and geographic distributions of the subgenus *Mollienesia*.

## 2.2. Materials and Methods

### *2.2.1. Sampling strategy and specimen acquisition*

Our sampling efforts focused on including species previously not sampled (Table 2.1) and covering areas where coverage was previously scarce (especially Mexico, El Salvador, Honduras, and Nicaragua). To maximize species and distribution coverage, we also included sequences from previously published data archived in Genbank in our analyses, and our overall sampling represents the highest number of species and broadest geographic sampling analyzed to date (19 of 25 recognized *Mollienesia* species *sensu* Poeser et al. 2011 and 4 undescribed lineages, Alda et al. 2013, Bagley et al. 2015; Fig. 2.1, Table 2.1, Table A1). Our samples were captured using electrofishing, seines, cast and dip nets. Immediately after capture, fish were euthanized with buffered MS222, fin clips (right pectoral fin) cut and preserved in 95% ethanol for molecular analyses, and specimens subsequently fixed in a 10% formaldehyde solution for morphological identification. Procedures followed protocols approved by the Texas A&M University (IACUC 2011-118) and Oklahoma State University (ACUP: AS10-15) Committees on Use and Care of Animals. Ethanol preserved tissues, DNA extractions, and formalin fixed specimens (currently in 70% ethanol) are housed in the Biodiversity Research and Teaching Collections of the Department of Wildlife & Fisheries Sciences at Texas A&M University or at the Division of Biology at Kansas State University (Table A1).

*2.2.2. Sequencing and alignment*

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 estimation in fishes (e.g., Hrbek et al., 2007; Li et al., 2007; Meredith et al., 2010; Meredith et al., 2011). Focal genes included the mitochondrial cytochrome *b* gene (cyt *b*, 1,140 base pairs [bp]) with LA and HA primers (Schmidt et al., 1998), the mitochondrial gene NADH subunit 2 (ND2, 1,047 bp) with ND2B-L (Broughton and Gold, 2000) and ASN (Kocher et al., 1995) primers. The nuclear genes amplified included exon 3 of recombination activating gene-1 (Rag1, 1,561 bp, Lopez et al. 2004), a portion of the 7 trans-membrane receptor region of Rhodopsin (Rh, 822 bp, Chen et al. 2003), exon 1 of myosin heavy polypeptide 6 (myh6, 767 bp), exon 2 of ectodermal-neural cortex 1 like protein (ENC1, 847 bp; Li et al. 2007), exon 2 of glycosyltransferase (Glyt, 886 bp; Li et al. 2007), exon 1 of SH3 and PX domain containing 3 (SH3PX3, 724 bp, Li et al. 2007), and two partial exons (8 and 10), all of exon 9, and two introns (8 and 9) of the tyrosine kinase gene (X-src, 581 bp, Meyer and Lydeard, 1993) following previously published protocols (Meredith et al., 2010; Meredith et al., 2011). 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 and Toh, 2008).

*2.2.3. Datasets*

Sequence data were assembled into several datasets: individual genes, mtDNA only (2187 bp), nDNA only (6188 bp), and a combined (8375 bp) dataset. The mitochondrial dataset covers a wider range in the distribution of the species and includes more species, because the dataset was enhanced by including sequences available on GenBank (Table A1). The nuclear and combined dataset was representative of the lineages of particular interest and included more molecular information. Besides members of the subgenus *Mollienesia*, our analyses include species of the previously recognized Caribbean subgenera *Curtipenis* (*P. elegans*) and *Psychropoecilia* (*P. hispaniolana, and P. dominicensis*), as well as members of all other recognized subgenera of the genus *Poecilia* (*Limia*, *Pseudolimia*, *Pamphorichthys*, *Micropoecilia*, *Poecilia*, and *Acanthophacelus*; Meredith et al. 2011). All phylogenetic and species tree analyses use two species of the genus *Cnesterodon* as outgroup taxa, because this genus has previously been found to be the closest relative of *Poecilia* (Hrbek et al., 2007).

*2.2.4. Phylogenetic and multi-species coalescent species tree analyses*

We used Partition Finder (Lanfear et al., 2012) to determine the best partition scheme and to determine the most likely model of DNA substitution among 24 candidate models on a fixed BioNJ-JC tree based on the Bayesian information criterion (BIC) (Table 2.2). For the x-src gene, we partitioned the gene into introns (8 and 9, GTR+G) and exons (8, K80+I; 9, TrNef+G; 10, K80+G). Overall, the alignments of all genes were similar to Meredith et al. (2011), with two exceptions: (1) Only coding portions of mitochondrial genes were included, and (2) the alignment of the tyrosine kinase marker required the addition of gaps adding total base pair read to that gene (572 vs 581 bp), the nuclear (6482 vs 6188 bp) and concatenated

dataset (8670 vs 8375 bp). In general, the mitochondrial markers had more complex models and the independent nuclear markers followed simpler models (Table 2.2).

Table 2.2 The genes partitioned by position and by gene for mitochondrial DNA and nuclear DNA for phylogenetic study of the subgenus *Mollienesia* are described, including the total length in base pairs and number of parsimony informative sites (PI). The best-fit substitution models under the Bayesian Information Criterion ran in the program Partition Finder is provided.

| Gene | Length, PI | Pos 1 | Pos 2 | Pos 3 | Gene |
|---|---|---|---|---|---|
| **X-src** | (581, 29) | See Text | | | K80+G |
| **Myh6** | (767, 29) | F81 | F81+I | K80+G | K80+I |
| **ENC** | (847, 24) | K80 | | K80+I+G | K80+I |
| **Glyt** | (886, 20) | HKY+I | | K80 | K80 |
| **SH3PX3** | (724, 25) | HKY | | K80+G | K80+I |
| **Rh** | (822, 34) | K80+I | HKY+I | HKY+G | HKY+I |
| **RAG1** | (1561, 52) | HKY+I | | K80+G | TrNef+G |
| **NADH2** | (1047, 309) | GTR+G | GTR+I+G | GTR+I+G | GTR+I+G |
| **Cytb** | (1140, 325) | SYM+I+G | HKY+I+G | GTR+I+G | GTR+I+G |

For maximum likelihood (ML) analyses, we used RAxML GUI version 1.0 (Stamatakis, 2006; Stamatakis et al., 2008) run to conduct 500 Rapid Bootstrap searches followed by an ML search. We ran the complex general time reversible (GTR) + Γ (Gamma distribution for rate variation among sites) model for each partition because RAxML does not implement simpler models with unlinked branch lengths. A 50% percent majority rule consensus was applied to summarize the in DendroPy 3.10.1 (Sukumaran and Holder, 2010).

Two independent Bayesian analyses were conducted in MrBayes version 3.2.1 (Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012), each implementing two runs with four chains under default parameters. Each analysis incorporated models of nucleotide

substitution uniquely defined for the partition of each data set (Table 2.2). A 25% burn-in was applied and stable posterior probability values examined in Tracer version 1.6 (Rambaut et al. 2014). Pairwise genetic distances were calculated under the Kimura-2 parameter in MEGA version 5 (Tamura et al., 2011) with pairwise deletion for missing data.

Since analyses implemented in MrBayes can result in high clade posterior probabilities in cases with known polytomies (star-tree paradox, Suzuki et al., 2002), we also ran an analysis that allows for polytomies in Phycas 2.2.0 (Lewis et al., 2015), which incorporates a polytomy prior (Lewis et al., 2005). The run was set for 50,000 generations, sampling trees and parameters every 10 generations. The sampled trees output file was summarized to form a 50% percent majority rule consensus in DendroPy 3.10.1 (Sukumaran and Holder, 2010).

We also analyzed our data using *BEAST v. 1.8.3 (Drummond and Rambaut, 2007; Suchard and Rambaut, 2009) to estimate a species tree and simultaneously generate molecular clock-based divergence estimates in CIPRES (Miller et al. 2010). Dating phylogenetic trees of *Poecilia* has been complicated by the scarcity of fossil calibration points as well as uncertainty associated with the timing of the emergence of geological barriers and putative vicariance events associated with these barriers (see Ho et al. 2016 for a discussion). Accordingly, we employed a dual approach by (1) applying universal mtDNA mutation rates for teleosts (Mateos et al. 2002), which makes estimated dates comparable to previous studies on *Poecilia*, and (2) constraining the monophyly of *Poecilia* with a range of dates based on Ho et al. 's (2016) application of a secondary fossil calibration of bony fish (Betancur et al. 2013), which circumnavigates arbitrary rates and uncertainties with the timing of geological barriers and associated vicariance events. For the first analysis, we

applied an uncorrelated relaxed molecular clock rate under a log-normal model that draws from the rate of each lineage independently from a log-normal distribution. As in previous analyses of poeciliid fishes (including *Poecilia*), we applied a 1.0-2.0%/My rate to cytochrome *b* (Mateos et al., 2002; Hrbek et al. 2007; Meredith et al. 2010; 2011), and the remaining rates were estimated under default parameters. We ran two replicate searches with chain lengths set to 200 million generations and parameters sampled every 2000 generations. Convergence parameters in the Markov chain Monte Carlo model were assessed by effective sample size in Tracer v. 1.6 (Rambaut et al. 2014) with all ESS values above 200. LogCombiner was used to reduce and combine the species trees files by applying a burn-in of 25%. The newly combined tree file was then entered into TreeAnnotator to generate the maximum clade credibility tree annotated with median node ages (final species tree with time estimates) following a post-burn-in of 50 %. For the second analysis, we applied an uncorrelated relaxed molecular clock rate under a log-normal model that draws from the rate of each lineage independently from a log-normal distribution. We set a prior for the monophyly of *Poecilia* and placed an initial time of divergence value of 6 Mya with a range of 2.5-9.5 Mya based on a secondary fossil calibration of actinopterygians (Ho et al. 2016). We ran three replicate searches with chain lengths set to 200 million generations and parameters sampled every 2,000 generations. Convergence parameters in the Markov chain Monte Carlo model were assessed by effective sample size in Tracer v. 1.6 (Rambaut et al. 2014). LogCombiner was used to reduce and combine the species trees files by applying a burn-in of 25%. The newly combined tree file was then entered into TreeAnnotator to generate the maximum clade credibility tree annotated with median node ages (final species tree with time estimates) following a post-burn-in of 50 %.

*2.2.5. Ancestral area estimation*

We used the program RASP v. 3.2 (Yu et al., 2015) for ancestral biogeographic estimation. RASP is based on Bayesian ancestral state estimation method that determines the probability of each ancestral area averaged over all sampled trees in the posterior distribution (Yu et al., 2015). We uploaded the species tree applying universal mtDNA mutation rates for teleosts estimated in BEAST v. 1.8.3 into RASP for analyses. We used 10 biogeographical regions that have previously been identified based on phylogeographic studies of various freshwater and terrestrial organisms (Gutiérrez-García and Vázquez-Domínguez, 2013): (A) North America, (B) North of the Trans-Mexican Volcanic Belt, (C) South of the Trans-Mexican Volcanic Belt, (D) Maya, (E) Chortis, (F) Chorotega, (G) Choco North, (H) Choco South, (I) South America, and (J) Greater Antilles (Fig. 2.1). We first identified species as operational taxonomic units based on phylogenetic clustering. We subsequently used a combination of published distribution maps (Miller et al., 2005; Poeser, 2003; Table A2), primary literature (Alda et al., 2013; Bagley et al., 2015; Table A2), and GPS coordinate mapping (Bagley et al., 2015) to determine whether a species or lineage should be coded as present or absent in each region. We ran the Bayesian Binary MCMC (BBM) method, which reconstructs ancestral distributions under a full hierarchical Bayesian approach accounting (Ronquist, 2004) and a null distribution (ancestral range contains none of the unit areas). The maximum number of areas set to three and the outgroups identified. The run was set for 10 chains for 1 million cycles in RASP, utilizing the F81 gamma model. This represents the most complex model and is expected to yield more realistic results in the ancestral area estimation. Sampling occurred every 100 cycles, and we discarded 100 samples (default) before the estimates of the partition frequencies were calculated.

## 2.3. Results

### *2.3.1. Multi-locus concatenated phylogeny*

The phylogenetic relationships among the subgenera within the genus *Poecilia* place the clade comprised of *Acanthophacelus* and *Micropoecilia* as sister to the remaining lineages with strong support (90% or above for bootstrap [BS] and Bayesian posterior probabilities [BPP]; Fig. 2.3 and Fig. A1). The next major clade consists of several subgenera; however, the relationships among these subgenera were only supported by Bayesian analyses (Fig. 2.3 and Fig. A1). This clade includes the subgenus *Poecilia* at a basal position; this subgenus is highly distinct and consists of a single species (*P. vivipara*). The next divergence groups together the subgenera *Pamporichthys +Psychropoecilia + Curtipenis +Pseudolimia + Limia*. The Caribbean species (classified in the subgenera *Psychropoecilia* and *Curtipenis*) are sister to the subgenera *Pseudolimia* and *Limia*.

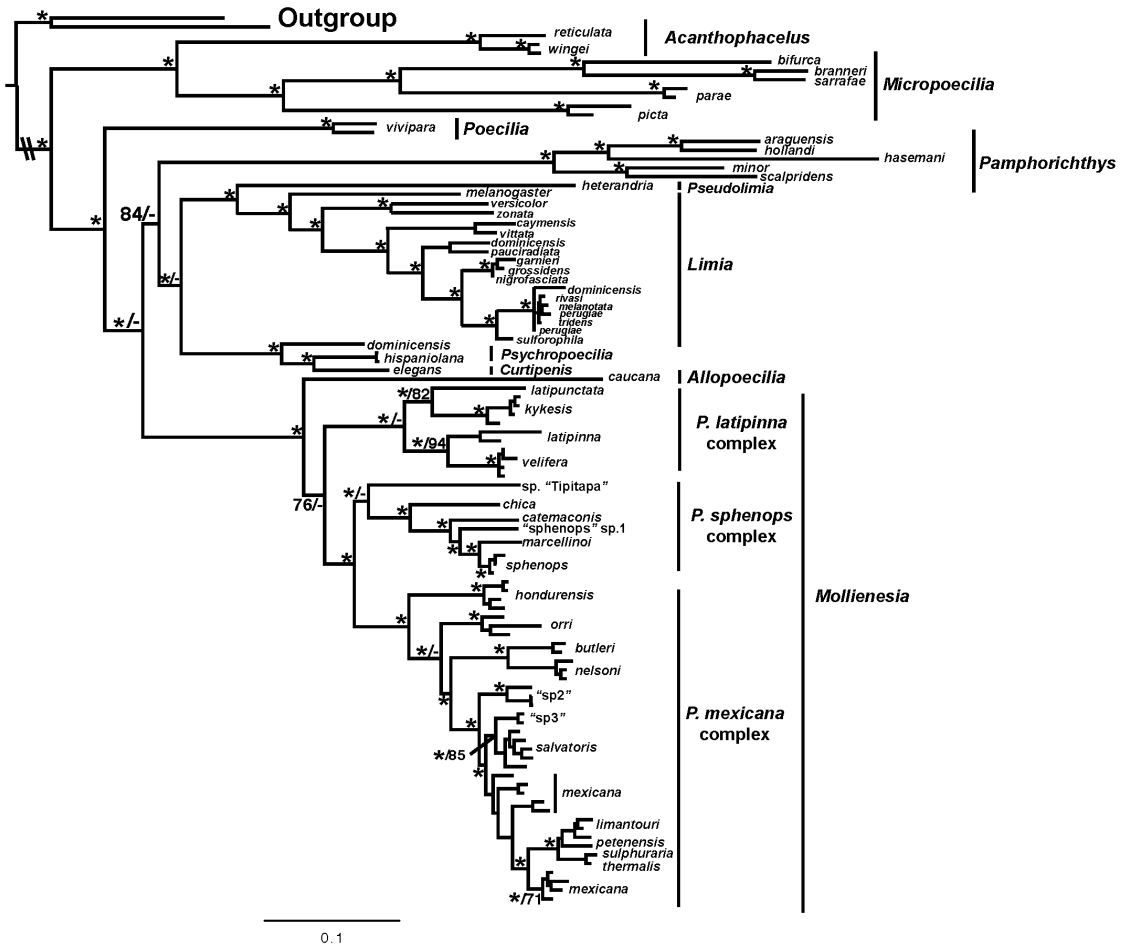


Figure 2.3 Bayesian tree from the MrBayes partitioned analysis of *Poecilia* spp. for the two mitochondrial and seven nuclear genes (8375 base pairs) rooted with species from the genus *Cnesterodon*. Nodal support shown Bayesian Posterior Probabilities in percent followed by RAxML bootstrap support values. Asterisks denote nodal support of 90% or above for the two methods. Nodes with no values present either had low values or were of little interest for this study.

Our results also found strong support for a clade that includes both the subgenera *Mollienesia* and *Allopoecilia* (Hubbs, 1924). In addition, the overall topology for the subgenus *Mollienesia* indicated three main clades (Fig. 2.3). However, the support for these relationships was moderate to low (76 BPP, >50% BS, Fig. 2.3), including the polytomy analyses (53 BPP, Fig. A1), resulting in lack of resolution between the subgenera *Allopoecilia* and *Mollienesia* and between the three clades within *Mollienesia.* Despite the

low support, the topology among clades of *Mollienesia* mirrored the relationships uncovered by a previous study based on two mitochondrial markers (Ptacek and Breden, 1998).

The first clade within *Mollienesia* represents the *Poecilia latipinna* species complex, which is composed of four species that form two species pairs: *P. latipinna* and *P. velifera*, and *P. latipunctata* and *P. kykesis*. Collectively, these four species are distributed along the Atlantic coast from the southern United States to Guatemala (Miller et al., 2005). The second clade is the *P. sphenops* species complex, which has seven lineages of which six were sampled here. Two lineages are widespread: *Poecilia sphenops* that has a wide bi-coastal range from Mexico south to the Lake Nicaragua district (Bagley et al., 2015), and *P.* "sphenops sp. 1," which is found on both coasts of nuclear Middle America (Alda et al., 2013; Bagley et al. 2015). The distributions of two lineages (*P. chica* and *P. marcellinoi*) are narrowly endemic to the Pacific coast, and two lineages are restricted to lake basins (*P. catemaconis* and *P.* sp. "Tipitapa"; Bagley et al., 2015).

The third *Mollienesia* clade is the *P. mexicana* species complex, which is comprised of 19 lineages, of which 12 are sampled here (including two putatively undescribed lineages recovered in this study, *P.* "sp2", and *P.* "sp3"; see Table 2.1). The majority of the sampled species in this clade are widely distributed along the Atlantic (*P. orri*), the Pacific coast (*P. butleri* and *P. nelsoni*), or both coasts (*P. gillii*; Alda et al., 2013; Bagley et al. 2015, *P. mexicana*, *P. salvatoris*, *P.* "sp2", and *P.* "sp3"). Two of the remaining species have restricted distributions to the Atlantic slope in Middle America (*P. hondurensis*, *P. petenensis*). Finally, two species are highly endemic, being found only in sulfidic springs in Mexico (*P. sulphuraria* and *P. thermalis*; Palacios et al., 2013).

*2.3.2. Mitochondrial gene phylogenies and mitochondrial concatenated phylogeny*

*2.3.2.1. The ND2 phylogeny*

The ND2 gene phylogeny had low resolution for the relationships among the subgenera, with support only being observed for the *Psychropoecilia + Curtipenis + Limia*, *Micropoecilia + Acanthophacelus*, and *Allopoecilia + Mollienesia* clades (Fig. A2). The inclusion of the published dataset from Alda et al. (2013) contributed to several findings. Samples of the Caribbean taxon *P. hispaniolana* from the Alda et al. (2013) grouped with samples we identified as the same species and reflected the same phylogenetic relationship sister (together with *P. elegans* and *P. dominicensis*) to the subgenus *Limia* (Fig. A2). For the subgenus *Allopoecilia*, which consists of a single species (*P. caucana*) present from lower Central America to northern South America, we found a deep divergence between representative samples from Venezuela (Meredith et al. 2011) and Panama (Alda et al. 2013), reflected by a large genetic distance (6.45%). The relationships among and within the species complexes of the subgenus *Mollienesia* were highly resolved and strongly supported (with the exception of some relationships within the *P. mexicana* species complex; Fig. A2). There was agreement in the phylogenetic relationship of the undescribed lineage *P.* "*gillii*" sp. 2 but not *P.* "*sphenops*" sp. 1. The latter species was found to be have an unresolved relationship to the *P. catemaconis* and the *P. marcellinoi* + *P. sphenops* clade (Fig. A2). Finally, samples identified as *P. mexicana* by Alda et al. 2013 grouped within clades we identified as *P.* "sp2" (samples from Nicaragua, Costa Rica, and Panama), *P.* "sp3" (2 samples from El Salvador), and *P. salvatoris* (one sample from Honduras).

*2.3.2.2. The Cytochrome* b *phylogeny*

The inclusion of previously published cytochrome *b* sequence data of fish identified as *Poecilia gillii* from Costa Rica (Lee and Johnson, 2009) revealed that none of the haplotypes actually fell within what is being considered to be *P. gillii* from the type locality (*sensu* Alda et al., 2013). Instead, haplotypes clustered with various other species or lineages within the *P. mexicana* species complex (cytochrome *b* phylogeny, Fig. A3). The phylogenetic placement of two haplotypes from the San Juan province clustered with *P. orri* (labeled orri [clade I] in Fig. A3). Clade III from Lee and Johnson (2009) was consistently recovered as a distinct group in our analyses (Fig. A3). The remaining haplotypes from Lee and Johnson (2009) grouped close to (clades II, V, VI, VII, VII) or within (clade IX) *P.* "sp2", except for haplotypes from clade IV that clustered within *P.* "sp3" or *P. salvatoris*.

We also included cytochrome *b* sequence data from Bagley et al. 2015 and there are similarities and differences in comparison to this study. There is agreement in the ambiguous evolutionary relationship of the undescribed lineage *P.* sp. "Tipitapa" based on the cytochrome *b* gene, however there is a marked difference in the species designation of other species. There is a discrepancy in the identification of the *P.* sp. "Patuca" clade, where we identified this clade to be *P. orri* (*sensu* Alda et al. 2013), which includes samples from Mexico, Honduras, Nicaragua, Costa Rica, and Panama. The identification of samples of *P. salvatoris* by our research team also differs and finds samples belonging to clade 8a in Bagley et al. 2015 to cluster within this species with a range of Guatemala, El Salvador, Honduras, Nicaragua, Costa Rica and Panama.

*2.3.2.3. Concatenated mitochondrial phylogeny*

The relationships among the subgenera of *Poecilia* were poorly resolved in the concatenated mitochondrial phylogeny, with the exception of the *Psychropoecilia + Curtipenis + Limia* clade (excluding *Pseudolimia*; Fig. A4). The relationships of the species within each of the subgenera were well resolved and highly supported, with the exception of *Micropoecilia* and *Mollienesia*. In *Mollienesia,* there was lack of resolution in the relationships of the three species complexes and lack of support for the relationships among species in the *P. mexicana* species complex (Fig. A4).

*2.3.2.4. Nuclear gene phylogenies and nuclear concatenated phylogeny*

The phylogenetic analyses based on individual nuclear genes were generally uninformative (Fig. A5), but all found support for the monophyly of the genus *Poecilia*. The relationships among species varied from gene to gene, and those within the subgenus *Mollienesia* were the least resolved.

The concatenated nuclear phylogeny (Fig. A6) for the most part was congruent with clades and relationships observed in the multi-locus phylogeny. However, several clade relationships were not supported, including *Pamphorichthys + Psychropoecilia + Curtipenis +Pseudolimia + Limia, Psychropoecilia + Curtipenis +Pseudolimia + Limia,* and the *P. sphenops + P. mexicana* species complexes. In addition, the relationships within the subgenus *Mollienesia* were not consistent with the other phylogenies.

*2.3.2.5. Species tree and molecular clock estimates*

Our species tree analyses corroborated the overall relationships among the subgenera that have been revealed by the multi-locus phylogeny with a few exceptions (Fig. 2.4a): (1) the position of the subgenus *Poecilia* in the species tree analyses is moderately supported to group with the clade composed of the subgenera *Psychropoecilia* + *Curtipenis*, *Pseudolimia*, and *Limia* and the clades of *Allopoecilia* + *Mollienesia*, (2) the relationships between the clades forming *Psychropoecilia* + *Curtipenis*, *Pseudolimia*, and *Limia* are not well resolved (Fig. 2.4), (3) within *Mollienesia*, the relationship of *butleri* + *nelsoni* precedes *orri* + all other taxa, and (4) within *Mollienesia*, *P. thermalis* + *sulphuraria* is sister to the *P. mexicana* clades, and not to the *P. petenensis* + *P. limantouri* clade (Fig. 2.4b).

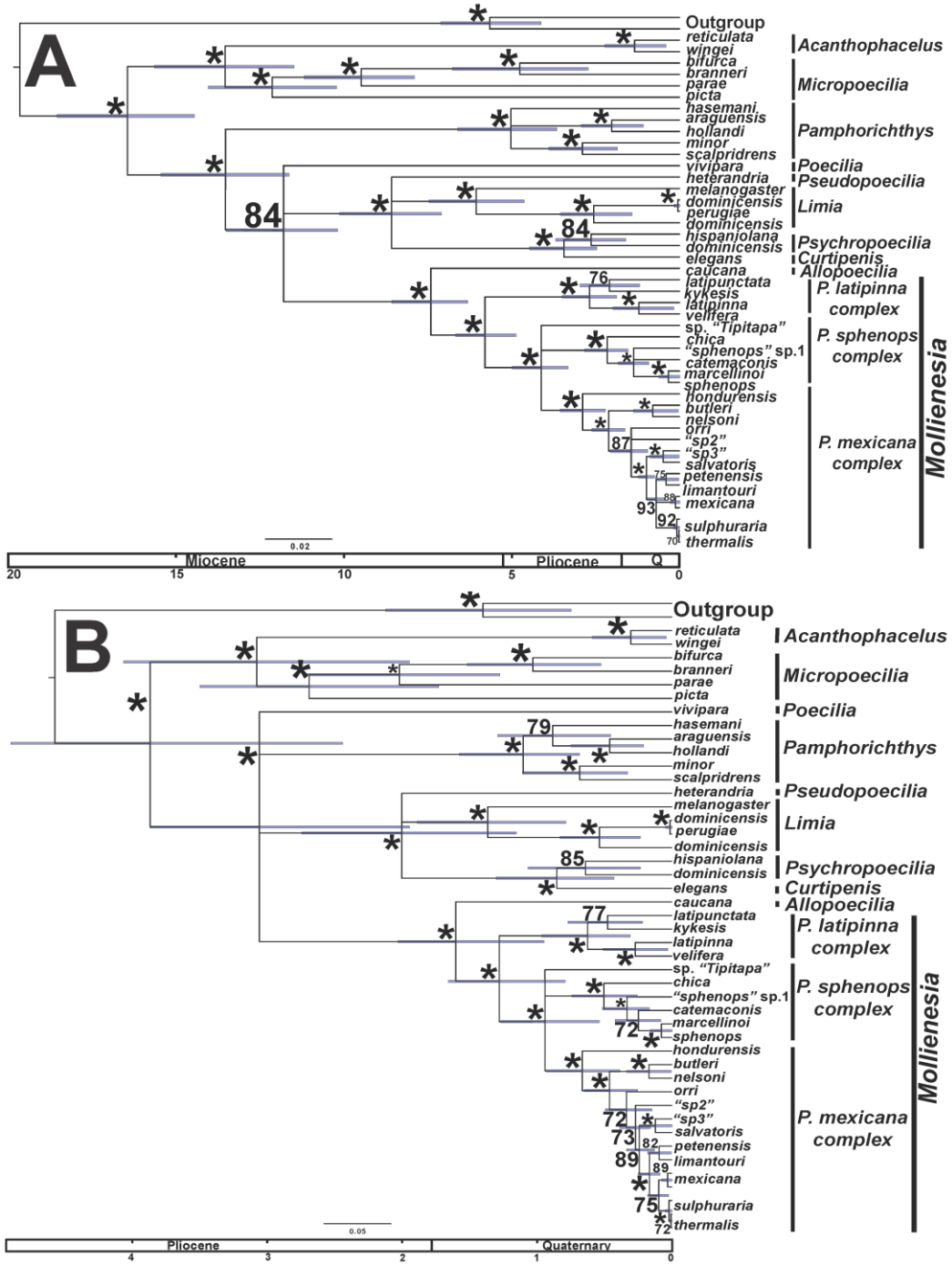


Figure 2.4 Time calibrated species tree phylogeny and divergence time estimates from the *BEAST analyses of *Poecilia* spp. for the two mitochondrial and seven nuclear genes (8375 base pairs) rooted with species from the genus *Cnesterodon*. (A) A molecular clock using a cytochrome *b* rate of 1.0-2.0% was applied. (B) A secondary fossil calibration was applied to the origin of the genus *Poecilia* set to 6 Mya and a range of 2.5-9.5 Mya. Nodal support shown represents Bayesian Posterior Probabilities in percent with asterisks denoting nodal support of 95%. Bars on nodes represent the 95% highest posterior density divergence-time estimates in Ma, million years ago.

The time estimates for the diversification of the genus *Poecilia* differed depending on the calibration method and were either placed in the Miocene (c. 16.4; 18.5-14.3 95% High Posterior Density [HPD]; Fig. 2.4a; fixed mtDNA mutation rate) or in the Pliocene (c. 3.87; 2.44-4.90 HPD; Fig. 2.4b; secondary fossil calibration). In general, employing the secondary fossil calibration yielded much more recent divergence estimates than applying a fixed mtDNA mutation rate. The subgenus *Mollienesia* is hypothesized to have diverged from *Allopoecilia* in the late Miocene at c. 5.80 Ma (4.88-6.68 HPD) or Pleistocene at c. 1.28 Ma (0.79-1.66 HPD), subsequently diversifying in to three species complexes. Additional major splits within *Mollienesia* occurred c. 4.13 Ma (3.33-4.19 HPD) or c. 0.9 (0.54-1.27 HPD), resulting in the *P. sphenops* and *P. mexicana* species complexes (see Fig. 2.4). The *P. sphenops* species complex diversified from the Pliocene (including *P.* sp. "Tipitapa," 3.61 Ma; 2.78-4.40 HPD) or Pleistocene (c. 0.86 Ma; 0.48-1.16 HPD) onwards, and the diversification of the *P. mexicana* species complex began in the Pliocene (c. 3.86 Ma; 3.59-2.20 HPD) or Pleistocene (c. 0.67 Ma; 0.39-0.93 HPD).

### *2.3.3. Ancestral area estimation*

The ancestral area estimations of the genus *Poecilia* indicated a South American origin for the group (Fig. 2.5). The group subsequently expanded from South America into both the Greater Antilles Islands of the Caribbean and Central America. The subgenus *Allopoecilia* represents the first northern expansion from northwestern South America (southern Choco block, H) into Lower Central America (northern Choco block, G; Fig. 2.5). The subgenus *Mollienesia* has the most likely ancestral area estimated in South America (area I). The *P.*

*latipinna* species complex was estimated to have its ancestral area in the Maya block (area D) and a complete absence South of the Trans-Mexican Volcanic belt (area C). Both the *P. sphenops* and *P. mexicana* species complexes have the most probable ancestral area identified to the Chortis block (area E; Fig. 2.5). In each of the latter complexes, there were independent colonization events of the areas both north and south of the Trans-Mexican Volcanic Belt (areas B and C; Fig. 2.5). In the *P. sphenops* complex, there are two events that reflect the disjunction of the Maya block (region D) to the South of the Trans-Mexican Volcanic Belt and Chortis regions (C and E; Fig. 2.5), with the occurrence of five speciation events (region E to C or region C to E; Fig. 2.5). The speciation events in the *P. mexicana* complex mostly pertain to the Maya, Chortis, and Chorotega blocks or a combination of these (D, E, and F respectively; Fig. 2.5).

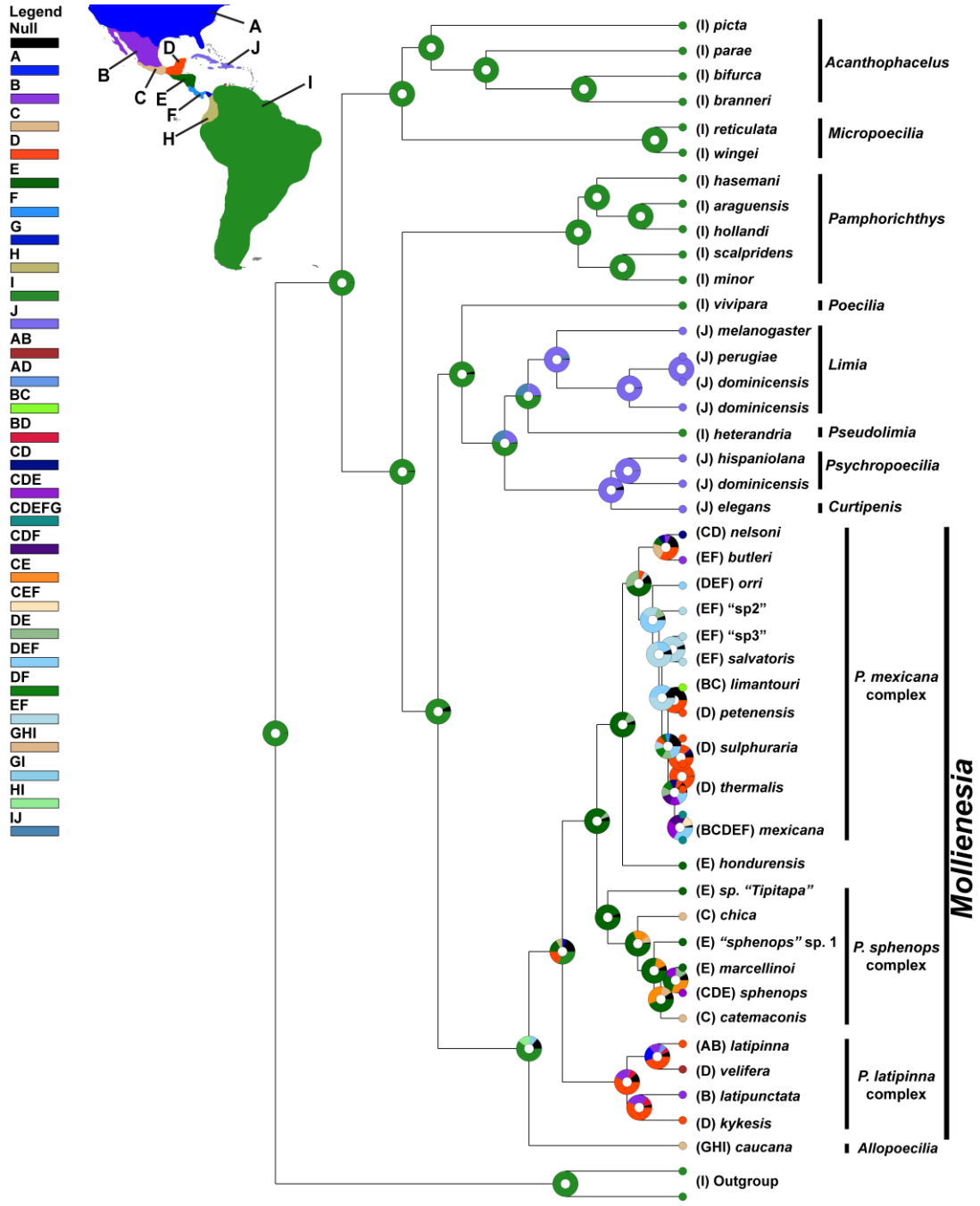


Figure 2.5 Estimation of ancestral areas for *Poecilia spp.* obtained in RASP based on Bayesian Binary Markov chain Monte Carlo (BBM) analysis. For each node, colors correspond to geographical areas, and proportions of each color correspond to the inferred ancestral area during the BBM analysis (black, null distribution; (A) North America, (B) North of the Trans-Mexican Volcanic Belt, (C) South of the Trans-Mexican Volcanic Belt, (D) Maya, (E) Chortis, (F) Chorotega, (G) Choco North, (H) Choco South, (I) South America, and (J) Greater Antilles. Abbreviations in parentheses are the region in which each species is found.

## 2.4. Discussion

We investigated the evolutionary relationships of species in the genus *Poecilia* using phylogenetic and species trees analyses. We particularly focused on the subgenus *Mollienesia*, including 19 of the 25 recognized species (*sensu* Poeser, 2011), 4 recently discovered undescribed lineages (Alda et al. 2013; Bagley et al. 2015), and a broad sampling throughout Middle America (see Table 2.1 and Table A1). Overall, our study found inconsistencies with current taxonomy, species identification, and uncovered genetically distinct lineages that putatively represent undescribed species. Our species trees, molecular clock estimates, and ancestral area estimations indicated that the majority of the diversity in the subgenus *Mollienesia* originated in upper and central Middle America. In the diverse *P. sphenops* and *P. mexicana* species complexes, diversification occurred from the Pliocene onwards. Speciation in these complexes was primarily driven by dispersal, but vicariant events and ecological processes may also have played a role.

### *2.4.1. Caveats: Unresolved relationships and molecular clock uncertainties*

Our study attempted to resolve the evolutionary relationships of species within the subgenus *Mollienesia* as well as the different subgenera in the genus *Poecilia* with a multi-locus dataset and applying various phylogenetic and species tree methods. Even though our approach has generally resulted in a well-supported phylogenetic hypothesis for the group, the relationships of some taxa were not completely resolved. Theoretically, this may be the result of hybridization (Alda et al., 2013; Bagley et al., 2015) or ancestral lineage sorting in the course of rapid radiations (Koblmüller et al. 2010). Previous analyses have indicated that inconsistencies among different phylogenetic hypothesis in *Mollienesia* are likely caused by

introgressive hybridization (Bagley et al., 2015), which has also beeen observed in other fishes of the family Poeciliidae (Cui et al., 2013). Although we did not test for introgressive hybridization or incomplete lineage sorting, we suspect one or both of these mechanisms, due to the conflicting results in the mitochondrial and nuclear phylogenies. Future studies should thoroughly test for introgressive hybridization and incomplete lineage sorting by increasing sampling and genetic data to assess hybridization and gene flow.

The molecular clocks and other calibration methods we applied have been criticized for the high rate of uncertainty in estimating the timescales at which evolutionary diversification has occurred (Warnock et al., 2012). This highlights a broader problem associated particularly with the dating of poeciliid phylogenies. On one hand, estimations of substitution rates are all but foolproof, particularly because the timing of the emergence of geological barriers is as uncertain as putative vicariance events associated with these barriers (e.g., the Trans-Mexican Volcanic belt: Mateos et al., 2002; Hrbek et al. 2007; Meredith et al. 2010; 2011; separation between Hispanola and Cuba: Alda et al. 2013). On the other hand, fossil calibration points are almost absent and often far removed from the core group of interest (Ho et al. 2016). When applying a universal mtDNA mutation rate to date our phylogeny, we recovered more recent estimates for diversification events in the genus *Poecilia* (~16 Ma *vs*. >25 Ma; Fig. 2.4a), both when compared to previous studies applying the same (Hrbek et al., 2007; Meredith et al., 2010; Meredith et al., 2011) and different calibrations (Alda et al., 2013; Bagley et al., 2015). Interestingly, a recent study that used a secondary calibration based on fossils also found substantially younger ages for the diversification of *Poecilia* (~6 Ma; Ho et al. 2016), and applying the same secondary calibration point to our dataset indicates a similar young age for *Poecilia* (~3.87 Ma; Fig.

2.4b). While the absolute timing of diversification events within the genus thus remains an unresolved issue, the recent analyses collectively suggest that we have likely been overestimating the age of *Poecilia*. We therefore acknowledge that the time estimates of divergence events hold some degree of uncertainty, and we thus limit major inferences about biogeographical patterns.

### *2.4.2. Molecular systematics of* Poecilia

Our phylogenetic and species tree analyses support the relationships among six of the recognized seven subgenera of *Poecilia* found in previous studies (Meredith et al., 2010; Meredith et al., 2011). The sole exception pertained to the monotypic subgenus *Poecilia* (*P. vivipara*), which we recovered forming part of the clade with the subgenera *Psychropoecilia*, *Curtipenis*, *Pseudolimia*, *Limia*, *Allopoecilia*, and *Mollienesia*. Although this relationship was only retrieved in the species tree analyses (Fig. 2.4a), this discrepancy may be a consequence of the constrained monophyly of the subgenera *Pamphorichthys*, *Limia*, and *Mollienesia* (including *Allopoecilia*) in previous analyses (Meredith et al., 2010; Meredith et al., 2011; also see Ho et al. 2016 that obtained the same result as our study). *Poecilia vivipara* is distributed along the coast from Venezuela to Lagunas Dos Patos in Brazil and on some islands of the Lesser Antilles (Poeser, 2003; Koerber and Litz, 2014). Previous morphological work distinguishes this species from members of the subgenus *Mollienesia* based on serrae found on gonopodial ray 4a (Miller, 1975) and postulated close phylogenetic relationships with *Mollienesia*, *Curtipenis*, and *Psychropoecilia* (but not *Pseudolimia* and *Limia*, Rivas, 1978).

Our analyses also resolved the phylogenetic placement of several species that have previously been associated with the subgenus *Mollienesia*. The three Caribbean species from the island of Hispaniola (*P. elegans, P. hispaniolana, and P. dominicensis*) were not actually closely related to species of the subgenus *Mollienesia* and instead grouped with the clades representing the subgenera *Pseudolimia* and *Limia* (the latter is also endemic to the islands of the Greater Antilles; Fig. 2.3 and 2.4). Recent molecular studies including samples of *P. hispaniolana* (Alda et al., 2013; Ho et al. 2016) also recovered a phylogenetic placement of the species outside of the monophyletic subgenus *Mollienesia*, but the phylogenetic clustering with *Limia* was doubted due to the small sample size (Fig. 2.2, Alda et al., 2013; but see Weaver, 2005). *Poecilia hispaniolana* and *P. dominicensis* are morphologically very similar to one another and were placed in the subgenus *Psychropoecilia* based on morphological revision (Rivas, 1978). Past taxonomic revisions placed *P. elegans* in the distinct subgenus *Curtipenis* based on the near absence of a palp on the gonopodial ray 3 (Rivas and Myers, 1950). Our molecular results (with exception of the multi-locus phylogeny and some poorly resolved nuclear gene phylogenies) are consistent with the previous assignment of these species to their own respective subgenera (Rivas and Myers, 1950; Rivas, 1978). Within the subgenus *Limia*, the species *P. dominicensis* was found to be polyphyletic (Fig. 2.3 and 2.4). One sample comes from Haiti and the other from the Dominican Republic, the two countries (which comprise the island of Hispaniola) from where this species is reported (Myers, 1931). However, without sampling the type locality (Santo Domingo), we cannot determine which sample represents “true” *P. dominicensis* and confirm the phylogenetic placement for this species.

The species *P. caucana* has been previously assigned to the subgenus *Allopoecilia* based on the morphological assessment of the gonopodium (Hubbs, 1924) and molecular genetic analyses (Figure 2.2, Ho et al. 2016; Ptacek and Breden, 1998). Our study supports this classification, reflected by the long branch in the phylogeny in relation to members of the subgenus *Mollienesia* (Fig. 2.3 & 2.4). The inclusion of *P. caucana* samples from two localities (Venezuela and Panama) for the mitochondrial ND2 gene analyses revealed a deep divergence between them (Fig. A2), with the possibility of the Venezuelan sample being *P. dauli* (Ho et al. 2016). This finding suggests the need for an assessment of intra-specific variation in *P. caucana*, including populations from Colombia, where the type specimens were collected (Meyer and Radda, 2000).

Within the subgenus *Mollienesia*, our analyses recovered three main species complexes (Figure 2.2, the *P. latipinna*, *P. sphenops*, and *P. mexicana* complexes), which were congruent with previous molecular studies (e.g., Alda et al., 2013; Ptacek and Breden, 1998; Fig. 2.3 and 2.4) but excluded South American species (see Ho et al. 2016). The *P. latipinna* species complex includes four species (*P. kykesis*, *P. latipinna*, *P. latipunctata*, and *P. velifera*), which are characterized by having a long dorsal fin and displaying courtship behavior (with the exception of *P. latipunctata* that secondarily lost these traits, see Ptacek, 2005). In contrast, members of the two short fin molly (*P. sphenops* and *P. mexicana*) species complexes share a short dorsal fin, but they differ in their dentition (Schultz and Miller, 1971; Alda et al. 2013).

The *P. sphenops* complex consists of five described species with tricuspid dentition, including four that were included in this study (*P. catemaconis*, *P. chica*, *P. marcellinoi*, and *P. sphenops*). This species complex also includes two distinct and previously recognized

genetic lineages: *P.* "sphenops sp. 1" (Alda et al., 2013), and *P.* sp. "Tipitapa" (Bagley et al., 2015). *Poecilia* "sphenops sp. 1" occurs along both slopes of Honduras and Nicaragua (Alda et al., 2013; Bagley et al. 2015). Given the sampling locality in Honduras for our sample, there is a possibility this species represents a previously recognized, morphologically distinct population of *P. sphenops* from the Choluteca River (see Carr and Giovannoli, 1994). *Poecilia* sp. "Tipitapa" may represent *P. dovii,* which has previously been considered a synonym to *P. mexicana* (Poeser, 2003) and *P. sphenops* (Reis et al., 2003; Rosen and Bailey, 1963). Previous studies have described this species as an abundant, large sized molly with a pale yellow coloration and a dark blotch at the base of the caudal fin (Astorqui, 1971; Quesada, 1971), which matches our field observations.

The *P. mexicana* species complex includes 18 described species, with 10 sampled for this study including *P. butleri, P. gillii* (*sensu* Alda et al., 2013), *P. hondurensis*, *P. mexicana*, *P. nelsoni, P. orri*, *P. petenensis, P. salvatoris*, *P. sulphuraria*, and *P. thermalis.* The complex also includes three distinct lineages recovered by previous studies (*P.* "gillii sp. 2": Alda et al., 2013; *P. sp.* "*Patuca*": Bagley et al. 2015; *P. limantouri*: Palacios et al., 2013; Tobler et al., 2011) and two additional distinct lineages recovered by the present study (*P.* "sp2" and *P.* "sp3"). *Poecilia limantouri* has long been considered a synonym (Poeser, 2003) or subspecies (Menzel and Darnell, 1973) of *P. mexicana* despite morphological (Menzel and Darnell, 1973) and genetic differences (Schartl et al., 1995). Our data support its recognition as a distinct species with genetic distance of 0.9% to the next closest lineage (*P. mexicana*) and an average of 2.39% to the remaining molly species (Table A2). It remains to be investigated whether the other two distinct genetic lineages (*P.* "sp2" and *P.* "sp3") exhibit unique phenotypic features and warrant recognition as distinct species.

Finally, we would like to emphasize that the present study has not only uncovered previously undescribed or unrecognized (due to synonymization) species, but it also illustrates the presence of taxonomic inconsistencies and challenges for accurately describing the diversity of this group. For example, Lee and Johnson (2009) presented a detailed biogeographic analyis of what phenotypically has been considered *P. gillii* (see Bussing, 2002). However, combining their data with samples from the type locality of *P. gillii* in Panama (Alda et al., 2013) and a broader taxon sampling from a wider geographic area (this study) revealed that all of the putative *P. gillii* haplotypes fell within the *P. mexicana* species complex (Figure A3, and none actually grouped with *P. gillii* (*sensu* Alda et al., 2013). Instead, haplotypes either grouped with *P. orri, P.* "CR lineage 1 and 2" (*sensu* Lee and Johnson, 2009), *P.* "sp2", *P. salvatoris,* and *P.* "sp3". These findings have implications for past and current collections of species of the subgenus *Mollienesia* sampled in Costa Rica, as *P. gillii* is thought to be ubiquitous throughout the country (Bussing, 2002; Lee and Johnson, 2009; Angulo et al. 2013), and for interpreting the appropriate phylogeographical structure of species in the subgenus *Mollienesia* (Bagley and Johnson, 2014). Taken as a whole, our study highlights the urgency for a taxonomic revision of the subgenus *Mollienesia,* which must include morphological, ecological, and molecular data, in order to improve species identification and distributional limits.

*2.4.3. Biogeographical patterns of diversification*

Based on the ancestral area estimations, the genus *Poecilia* originated in South America, aligning with the current distribution of six subgenera (*Micropoecilia, Acanthocephalus, Pamphorichthys, Poecilia*, *Pseudolimia,* and *Allopoecilia*; Fig. 2.5). The Caribbean and Central America subgenera (*Limia*, *Psychropoecilia*, *Curtipenis, Allopoecilia*, and *Mollienesia*) also originated in South America (Fig. 2.5). Our analyses provide three key insights into the colonization of both the Caribbean and Middle America.

First, our analyses indicated a more recent colonization of the Caribbean by members of the genus *Poecilia* (Fig. 2.4; also see Ho et al. 2016). This is in contrast with most other studies on *Poecilia* that did not include species from the subgenera *Curtipenis* and *Psychropoecilia* (Meredith et al., 2010; Meredith et al., 2011) or that used alternative calibration points (Alda et al., 2013). While older colonization estimates for *Poecilia* and other freshwater fishes (Chakrabarty, 2006) are consistent with the land bridge formation between northern South America to the emergent Greater Antilles and the movement of the Proto-Antilles between Central America and South America (Iturralde-Vinent, 2006; Holden and Dietz, 1972; Malfait and Dinkelmen, 1972), our data suggest dispersal through marine barriers as a likely the mechanism for colonization of the Greater Antilles, which is consistent with freshwater communities being strictly composed of secondary freshwater fish (Briggs, 1987; Burgess et al., 1994; Burgess and Franz, 1989). Interestingly, our analyses also indicated potentially reciprocal interactions between the South American and Caribbean blocks. At least in the species tree analyses, the South American species *P. heterandria* groups with the Caribbean subgenera *Limia*, *Curtipenis,* and *Psychropoecilia*, suggesting either a back colonization event to South America or two independent invasions from South

America into the Caribbean. Back colonization to ancestral blocks is also observed within the subgenus *Mollienesia*, where multiple South American species (not included in our analyses) were nested within Central American species of the *P. mexicana* species complex (Ho et al. 2016).

Second, the colonization of lower Central America (Choco North) by *P. caucana* (subgenus *Allopoecilia*; Fig. 2.5) may also be a result of dispersal. During time frame of the older time estimate (7.41 Ma), freshwater and marine corridors present between South America and lower Central America (Bacon et al., 2015; Coates and Obando, 1996; Gutiérrez-García and Vázquez-Domínguez, 2013). The dispersal mechanism and older molecular dating estimates agree with the phylogeographic patterns of another secondary freshwater fish (Synbranchidae: Perdices et al., 2005), but the dates are substantially more recent than those from studies employing alternative calibration points for *Allopoecilia* + *Mollienesia* (Alda et al., 2013). We obtain a younger time estimate of this event at 1.60 Ma, and our results thus add to previous work that has indicated multiple waves of invasion by freshwater fishes into Middle America (Abe et al., 2014; Bermingham and Martin, 1998; Picq et al., 2014).

Lastly, three species complexes represent the diversity within the subgenus *Mollienesia*, having an origin in South America (Fig. 2.5). The diversification within the *P. latipinna* species complex is dated to the Pliocene and Pleistocene (Fig. 2.4a and 2.4b) with an ancestral area in the Maya Block (Fig. 2.5). The diversification in this species complex is partially the result of adaptation to different ecological environments; with the sister taxa *P. latipinna* and *P. velifera* adapted to coastal habitats and elevated salinities (Hankison and Ptacek, 2008; Vega-Cendejas and de Santillana, 2004), and the sister taxa *P. latipunctata* and

*P. kykesis* exclusively occurring in freshwaters ecosystems (Hankison and Ptacek, 2008). The mechanisms of diversification within these ecological groups remain unclear, although it is noteworthy that both have one member north and south of the Trans Mexican Volcanic Belt. The endemicity of *P. latipunctata* and the older divergence time estimates align with other freshwater fishes that have evolved in the region (Agorreta et al., 2013; Langerhans et al. 2012; Mateos et al., 2002), which suggests speciation may be a result of Pliocene volcanic activity in the region (de Cserna, 1960).

Making biogeographic inferences for the *P. sphenops* and *P. mexicana* species complexes is complicated by gaps in current data availability. There are uncertainties surrounding the molecular clock estimates (see above) and major problems with the accuracy of species identifications and distributions (also see Alda et al., 2013; Bagley et al. 2015). In some instances, the distributions have been vastly overestimated (*P. gillii*) or underestimated (*P. mexicana*), requiring the reevaluation of current data and effectively preventing rigorous analyses of biogeographic patterns. Nonetheless, the presence of widespread species in both species complexes clearly indicates a high potential for dispersal even across major phylogeographic barriers. For example, both *P. sphenops* and *P. mexicana* occur on both the Atlantic and Pacific slopes, and their ability for dispersal and colonization of novel habitats may be mediated by high tolerance to environmental stress (see Hernández-Rodríguez and Bückle-Ramirez, 2010; Tobler et al. 2009; Trexler, 1986; Vega-Cendejas et al., 2013).

In addition, there are also potential examples of vicariant events, where patterns of divergence coincide with estimated dates of geological events or prominent phylogeographic breaks with other taxa. For example, in the *P. sphenops* species complex, the distribution of *Poecilia chica*, endemic to a few of Mexico's Pacific river drainages (Miller et al., 2005),

coincides with a phylogeographic break that has affected the diversification of other fishes and reptiles (Domínguez-Domínguez et al., 2006; Mateos et al., 2002; Reyes-Velasco et al., 2013; Zarza et al., 2008). Similarly, *P. catemaconis* is restricted to Lake Catemaco and surrounding rivers on the Atlantic coast of Mexico (Miller et al., 2005), which harbor a high level of freshwater fish species endemicity (McEachran and Dewitt, 2008). The divergence time estimates of *P. catemaconis* (c. 2.17 Ma and c. 0.25 Ma) are consistent with the estimated age of the Lake (Alva-Valdivia et al., 2001; Ferrari et al., 2005), which sits on the volcanic mountain range of the Sierra Los Tuxtlas, and implies a vicariance-driven speciation event. The estimated time of divergence is also in agreement with other endemic poeciliid fish in the volcanic lake (Agorreta et al., 2013; Mateos et al. 2002). Some evidence for vicariance is also available from the *P. mexicana* species complex. The species *Poecilia hondurensis*, which is distributed in the northeastern Caribbean drainage basins of Honduras (Poeser, 2011), has an estimated time of diversification in the Pliocene (ca 2.90 Ma, Fig. 2.4a) and Pliostecene (ca. 0.67 Ma), which coincides with tectonic activity influencing the direction of the rivers and active uplift of stream reaches in the North Coast Province of the North Chortis Terrane (Rogers and Mann 2007). Finally, the species pair *P. butleri* and *P. nelsoni*, likely evolved when an ancestral contiguous population in Central Pacific Mexico was separated by the emergence of the Trans-Mexican Volcanic Belt during the Quaternary (Mateos, 2005; Zúñiga-Vega et al., 2014).

Future studies need to rigorously address mechanisms underlying speciation within *Mollienesia*. This is particularly true for the *P. mexicana* species complex, which exhibits the highest species richness, and the short branches of the phylogeny suggest recent and rapid diversification. While there is evidence for ecological speciation in response to physico-

chemical stressors in some species (*P. thermalis* and *P. sulphuraria*, Palacios et al., 2013; Tobler et al., 2011), there are no obvious patterns of ecological divergence among other species. Future studies will also need to include additional endemic species such as *P. teresae* (which is restricted to a single drainage in southern Belize, Greenfield, 1990), *P. rositae* (from the highlands of Guatemala, Meyer et al., 2004), and *P. maylandi* (confined to the Rio Balsas system of Mexico, Meyer, 1983), as well as the species found in South America that have not yet been sampled (*P. mechthildae* and *P. boesemani*; Poeser 2011) to better understand speciation patterns of the subgenus *Mollienesia*.

# CHAPTER III

# THE PHYLOGEOGRAPHICAL PATTERNS OF SHORTFIN MEXICAN MOLLIES (MOLLIENESIA, POECILIA, POECILIIDAE)

## 3.1. Introduction

Phylogeographic studies aim to understand the history and formation of species at geographic and time scales. The application of population genetics and phylogeographic tools are used to correlate the evolutionary history of organisms to their current biogeographic patterns (e.g. Hickerson et al., 2010). Fish are the second most frequently studied taxon in this field (Beheregaray, 2008) with freshwater fish often reflecting strong phylogeographic structure in connection to the historical and ecological changes of a region (Bermingham and Avise, 1986; Bernatchez and Wilson, 1998). Phylogeographic studies of freshwater fishes can also result in drastic changes in the systematics and taxonomy of the species or group under study, such as erecting of a new genus, particularly in areas with complex geological histories and extreme variations in topography and climates (Schönhuth et al., 2008).

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 and volcanoes and an unusual ecological and hydrological history (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 each coast to join Trans Mexican Volcanic Belt. In the southern region of Mexico, the mountain chain

of Sierra Madre del Sur and the Sierra de Chiapas are separated by the Isthmus of Tehuantepec (Fig. 3.1). These mountain ranges produce a wide range of ecosystems including high mountains, deep valleys, plateaus, and coastal plains (Contreras-Balderas et al., 2008). Mexico is also unique in encompassing both the Neartic (North America south to the Mexican plateau) and Neotropical (Mexican central coasts to South America) biogeographic zones (Morrone and Márquez, 2001). The combination of the geologic formations and interface of biogeographic zones makes Mexico a megadiverse region in terms of ecological habitats (Metcalfe et al., 2000), species diversity, and supports high rates of endemism (CONABIO, 2008).

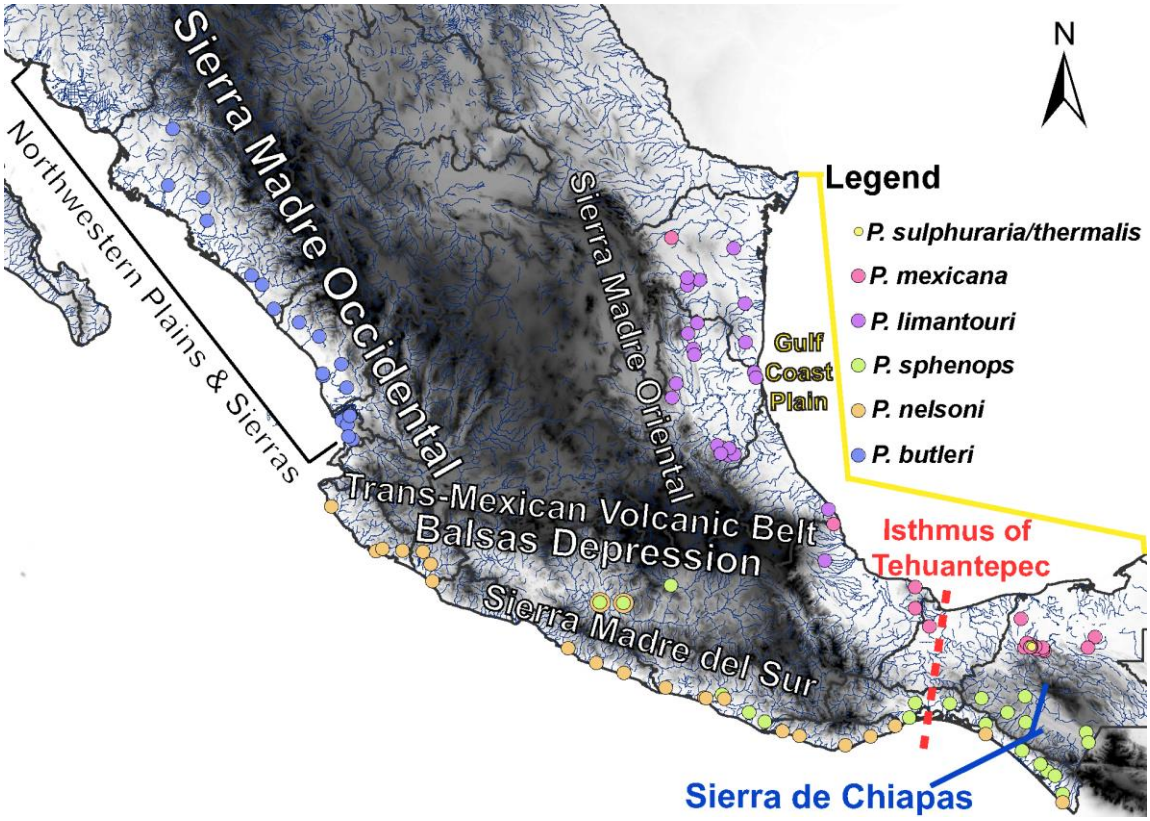


Figure 3.1 Sampling localities of species in the subgenus *Mollienesia* in Mexico.

In terms of the freshwater ichthyofauna, Mexico is comprised of 50 families, 155 genera and 540 species (Mejía et al., 2012), with 271 being endemic (Contreras-Balderas et al., 2008), and new species consistently being described (De la Maza-Benignos et al., 2015; Dominguez-Dominguez et al., 2016; Langerhans et al., 2012; Ornelas-García et al., 2015). Previous phylogeographic studies show Mexican fishes have diversified and distributed in accord to ancient geological events rather than modern hydrographic patterns (Agorreta et al., 2013; Huidobro et al., 2006; Mateos, 2005; Mateos et al., 2002; Strecker et al., 2004; Zúñiga- Vega et al., 2014). In contrast, population-level studies are rarely conducted and there is a poor understanding of the microevolutionary mechanisms shaping species in Mexico (but see Palacios et al., 2013; Strecker et al., 2003).

The largest fish family in Mexico is Poeciliidae, comprised of small and laterally compressed cyprinodontiform fishes found in fresh, brackish and coastal waters (Reis et al., 2003). The subfamily Poeciliinae is the most diverse (27 genera and over 250 species; Eschmeyer and Fong, 2012) and share three unique characteristics; adult males posses the specialized organ termed a gonopodium (a modified anal fin for sperm deposit; Parenti, 1981), reproduction via internal fertilization, and viviparity (livebearing-except for *Tomeurus gracilis*; Reis et al., 2003). There has been a recent interest in understanding the systematics, phylogeography, and biodiversity of this group, shedding light on the evolutionary mechanisms driving such high diversity (Agorreta et al., 2013; Meredith et al., 2011; Palacios et al., 2016). The subgenus *Mollienesia* (genus *Poecilia*) is represented by 14 freshwater fish species in Mexico and is found in a variety of climates and habitats ranging from marshes, volcanic lakes, cave systems, and hydrogen sulfide springs (Palacios et al., 2013; Palacios et al., 2016; Tobler et al., 2011). Despite the commonality of these species in

Mexico, the levels of intra-specific diversity and the distributional ranges of several species in this group are currently unknown (Bussing, 1976; Miller et al., 2005). Recent molecular studies of fish species of the subgenus *Mollienesia* across the distribution in Middle America and South America have revealed several species complexes (*P. latipinna*, *P. sphenops*, and *P. mexicana*), as well as several undescribed lineages (Alda et al., 2013; Bagley et al., 2015; Ho et al., 2016; Palacios et al., 2016), and interesting colonization events from nuclear Central America into Northern Central America and South America (Ho et al., 2016). In Mexico, some species demonstrate a widespread distribution (*P. sphenops*; Palacios et al., 2016; *P. mexicana*; Alda et al., 2013; Bagley et al., 2015; Ho et al., 2016), while others are endemic to a few locations (*P. catemaconis, P chica*, *P. sulphuraria*, *P. thermalis*; Palacios et al., 2013; Palacios et al., 2016). At a finer scale, previous studies have shown genetic differentiation among closely related lineages along environmental gradients (*P mexicana*, *P. sulphuraria*, and *P. thermalis*; Palacios et al., 2013) as well as diversification across physiographic barriers (*P. butleri* and *P. nelsoni*; Zúñiga- Vega et al., 2014). The intricacy of the distribution patterns between species of different complexes makes this a promising group to investigate phylogeographic and microevolutionary processes in relation to systematics in Mexico.

This study aims to examine fine scale sampling of six species (*P. butleri*, *P. limantouri*, *P. mexicana*, *P. nelsoni*, *P. sphenops*, and *P. sulphuraria/thermalis*) of the subgenus *Mollienesia* in Mexico to: (1) assess haplotype diversity across geographic distribution (2) identify phylogeographic breaks and (3) determine species' distributions. We hypothesize that species with high colonization capabilities will have less differentiation between haplotypes across geographic regions, whereas more ecologically restricted species

will have higher differentiation across geographic regions. We sample over 50 locations across Mexico and analyze haplotype data from mitochondrial and nuclear DNA sequences through phylogenetic and haplotype network methods to identify genetic groups across the species' geographical range and shared phylogeographical patterns to co-distributed species.

## 3.2. Materials and Methods

### *3.2.1. Specimen acquisition*

Specimens of the subgenus *Mollienesia* were captured using electrofishing, seines, cast and dip nets. Immediately after capture, fish were euthanized with buffered MS222, with fin clips (right pectoral fin) cut and preserved in 95% ethanol for molecular analyses and subsequently fixed in a 10% formaldehyde solution following protocol (IACUC 2011-118 & ACUP: AS10-15) approved by the Texas A&M University and Oklahoma State University Committee on Use and Care of Animals. Ethanol preserved tissues, DNA extractions, and formalin fixed specimens, currently in 70% ethanol are housed in the Biodiversity, Research and Teaching Collections of the Department of Wildlife & Fisheries Sciences at Texas A&M University or the El Colegio de la Frontera Sur, Tuxtla Gutierrez (see Table 3.1).

Table 3.1 List of species from the *P. sphenops* and *P. mexicana* complex from the subgenus *Mollienesia* present in Mexico. A= Atlantic; P= Pacific

| Species | Slope | Distribution | Endemic | Phylogeographic Break Cyt *b* |
|---|---|---|---|---|
| *P. sphenops* complex | | | | |
| *Poecilia catemaconis** Miller, 1975 | A | Lake Catemaco and rivers, Veracruz | X | Sierra de Tuxtlas |
| *Poecilia chica* * Miller, 1975 | P | Three small rivers in Jalisco | X | Jalisco block |
| *Poecilia maylandi* Meyer, 1983 | A | Rio Balsas, Guerrero | X | |
| *Poecilia sphenops*^ Valenciennes, 1846 | AP | Tamaulipas, Veracruz, Guerrero, Oaxaca, Chiapas | | |
| *P. mexicana* complex | | | | |
| *Poecilia butleri*^ Jordan, 1889 | P | Rio Fuerte, Sonora, Sinaloa, and Nayarit | X | Trans Mexican Volcanic Belt |
| *Poecilia mexicana*^ Steindachner, 1863 | A | Nuevo Leon to Panama | | |
| *Poecilia limantouri* Jordan & Schneider, 1900 | A | Rio Grande to N. Veracruz | X | |
| *P. nelsoni*^ (Meek, 1904) | P | Colima to S. Guatemala | X | Trans Mexican Volcanic Belt, Balsas Depression |
| *Poecilia orri** Fowler, 1943 | A | Yucatan, Quintana Roo to Honduras | | |
| *Poecilia sulphuraria*^ (Álvarez, 1948) | A | Sulfide springs in Tabasco | X | |
| *Poecilia thermalis*^ Steindachner, 1863 | A | Sulfide springs, Chiapas | X | |

* Included in phylogenetic analyses, ^ Included in cyt *b* phylogeographic analyses

The multi-gene analyses (2 mitochondrial and 1 nuclear marker) incorporated species for which all three genes were available on Genbank to determine the relationship between Mexican samples (see Table 3.1) to the predominantly Middle American samples of the subgenus *Mollienesia*. This study included 18 (*P. kykesis, P. latipinna, P. latipunctata, P. velifera, P. catemaconis, P.chica, P.marcellinoi, P. sphenops, P. butleri, P. gillii, P. hondurensis, P. mexicana, P. nelsoni, P. orri, P. petenensis, P. salvatoris, P. sulphuraria,* and *P. thermalis*) out of the 26 species (Ho et al., 2016; Palacios et al., 2016) in the subgenus *Mollienesia*, with 8 species (*P. boesemani, P. formosa, P. koperi, P. maylandi, P. mechthildae, P. rositae, P. teresae, P. vandepolli*) remaining absent from the analyses.

A more in depth analyses was conducted on the Mexican samples of the subgenus *Mollienesia* for the cytochrome *b* gene because of the abundance of data for this group on Genbank. The inclusion of these sequences expanded the distribution coverage to cover all of *P. butleri* and *P. nelsoni's* range along the Pacific coast of Mexico. In addition, the distribution coverage was extended north of our sampling for *P. limantouri* and south of our sampling in *P. mexicana* for a more informative picture of the phylogeographic patterns.

### *3.2.2. Sequencing and alignment*

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 (e.g., Hrbek et al., 2007; Li et al., 2003; Meredith et al., 2010; Meredith et al., 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 and Gold, 2000) and ASN (Kocher et al., 1995) primers and the single 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 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 and Toh, 2008).

*3.2.3. Datasets, haplotypes, and partition*

The data was partitioned into four datasets: (1) a concatenated dataset (3748 base pairs) (2) the coding portion of cyt *b* (1140 bp) (3) coding portion of ND2 (1047 bp) and the combined mitochondrial DNA (2187 bp). The cyt *b* dataset includes sequences available on Genbank of *P. limantouri* from Stöck et al., 2010 and *P. butleri* and *P. nelsoni* from Zúñiga- Vega et al., 2014. The ND2 dataset includes sequences available on Genbank of *P. butleri* and *P. nelsoni* from Mateos, 2005 and *P. butleri, P. sphenops, P. mexicana, and P. sulphuraria* from Alda et al., 2013. All datasets were then run in FaBox online (Villesen, 2007) to determine the total number of haplotypes for each dataset. The cyt *b* dataset was reduced from 953 sequences to 475 (139 haplotypes for the 6 species of interest), the ND2 dataset from 257 to 190 (60 haplotypes for the 6 species of interest), and the combined mitochondrial dataset was reduced from 144 to 139 (85 haplotypes for the 6 species of interest). We used Partition Finder (Lanfear et al., 2012) to determine the best partition scheme and to find the determine the most likely model of DNA substitution among 24 candidate models on a fixed BioNJ-JC tree based on the Bayesian information criterion (BIC) (Table A3). Partitions of the datasets were followed in our analyses where possible. We focus on the analysis of the concatenated and cyt *b* datasets below.

*3.2.4. Phylogenetic and haplotype network analyses*

Both the concatenated and cyt *b* datasets were phylogenetically analyzed. Phylogenetic analysis under maximum likelihood (ML) analyses was conducted in RAxML GUI version 1.0 (Stamatakis, 2006; Stamatakis et al., 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 with a 50% percent majority rule consensus parameter in DendroPy 3.10.1 (Sukumaran and Holder, 2010), with the final tree figure rooted and generated in Fig Tree v. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). Bayesian analyses was conducted in MrBayes version 3.2.1 (Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012), implementing two runs with four chains under default parameters for 50 million generations. A 25% burn-in was applied and stable posterior probability values examined in Tracer version 1.5 (Rambaut and Drummond, 2007). Pairwise genetic distances were calculated under the Kimura-2 parameter in MEGA version 7 (Kumar et al., 2016) with pairwise deletion for missing data.

A haplotype network was made under statistical parsimony analysis of the cyt *b* gene dataset for the six species of interest in TCS v. 1.2.1 (Clement et al., 2002) within the Popart program (http://popart.otago.ac.nz), which calculates the significant number of substitutions connecting haplotypes in a network applying the algorithm developed by (Templeton et al., 1992). The species were designated and a connection limit was set to the default of 95% to generate the haplotype networks between closely related sequences.

## 3.3. Results

*3.3.1 Phylogenetic patterns of Mexican mollies to other species in the subgenus Mollienesia*

The phylogeny of the concatenated dataset shows high/moderate support for the two clades representing the *P. sphenops* and *P. mexicana* species complexes (Fig. 3.2). The *P. sphenops* species complex is strongly supported with the 3 represented species from Mexico (2 endemic-*P. chica* and *P. catemaconis,* and *P. sphenops*) interspersed between the 3 species from Middle America (2 undescribed species, *P.* "sp Tipitapa" and *P.* "*sphenops* sp .1", and *P. marcellinoi*). The species of interest, *P sphenops*, has low resolution for the relationships among the sampled locations that cover both Atlantic and Pacific versants of Mexico. The *P. mexicana* species complex is strongly supported and represented by 5 Mexican distributed and 5 Middle American species (Fig. 3.2). *Poecilia hondurensis* and *P. orri* from Middle America represent the most basal lineages in the complex (Fig. 3.2). The next divergence in the complex represents a sister relationship between the Mexican Pacific slope species, *P. butleri* and *P. nelsoni*, which is strongly supported, and *P. nelsoni* shows poor intra-specific resolution. Next, our results recover a strongly supported Atlantic slope Mexican *P. limantouri* clade (which lacks resolution among Atlantic slope sampling sites) as sister to the sulfide spring inhabiting species *P. sulphuraria* and *P. thermalis* (Fig. 3.2). Subsequent divergences include two unidentified lineages from Middle America (*P.* sp 1 and *P.* sp 2), *P. salvatoris* (also from Middle America), and lastly, the strong/moderately-supported *P. mexicana.* Lastly, the strong/moderately-supported species of *P. mexicana* is split into two strong/moderately-supported clades and an unresolved lineage of samples from Honduras. One clade is composed of the most northern sampling location north of the Trans Mexican Volcanic Belt (TMVB) and the most northern sample south of the TMVB. The other clade is

formed from the remaining samples, which come from West and East of the Isthmus of Tehuantepec (WIT, EIT; Fig. 3.2).

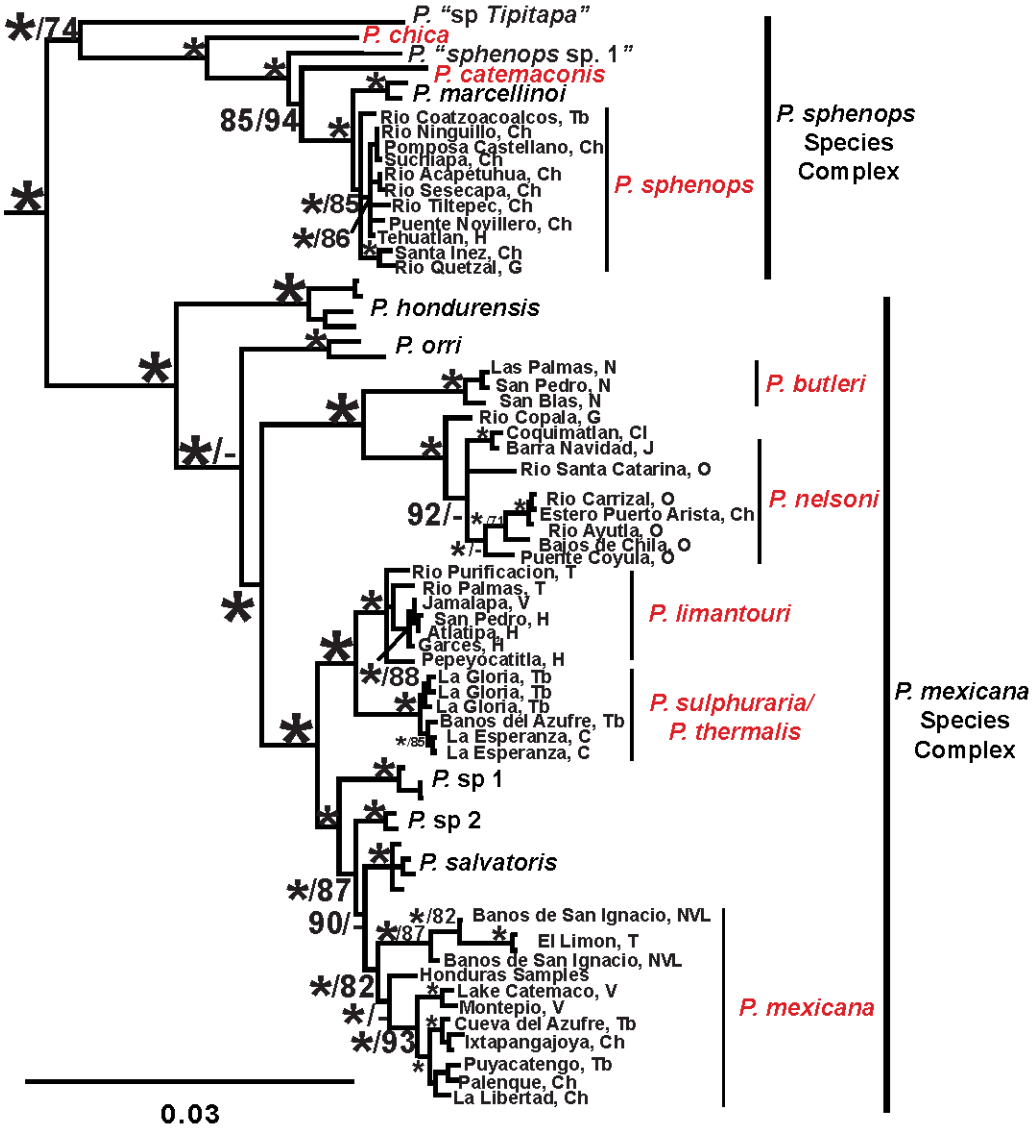


Figure 3.2 Bayesian tree from the MrBayes partitioned analysis of *Poecilia* spp. for two mitochondrial genes (Cyt b and ND2, 2187 base pairs) and one nuclear (RAG1, 1561 base pairs) rooted with other poeciliid outgroups. Species names in black pertain to species outside of Mexico and species names in red are species found within Mexico. Nodal support shown (left to right; respectively): Bayesian Posterior Probabilities in percent followed by RAxML bootstrap support values. Asterisks denote nodal support of 95% or above for the two methods. Nodes with no values present either had low values or were of little interest for this study.

*3.3.1.2. Phylogeographic patterns in P. sphenops*

The widespread species *Poecilia sphenops* is found across both the Atlantic and Pacific versants in Mexico and phylogenetic analyses revealed two lineages and one main clade (Fig. 3.3). One lineage represents a single haplotype from the southern Atlantic coast, in the state of Veracruz where it meets the Isthmus of Tehuantepec (Fig. 3.3). The other small lineage (Clade A) is composed of one Pacific slope haplotype found west of the Isthmus of Tehuantepec (WIT) from the Guerrero coast, and two haplotypes from east of the Isthmus of Tehuantepec (EIT) from the Atlantic versant in Chiapas. The main *P. sphenops* clade (Clade B) includes the widely distributed haplotype 67, found in the Atlantic coast north of the TMVB, Balsas Depression (BD), and along the Pacific coast WIT and EIT. This also has three strongly supported sub-clades, the first consisting of one haplotype from the BD and one from a Pacific EIT location (Oaxacan coast). The second sub-clade consists of four Pacific EIT haplotypes from the Chiapas coast, and the third sub-clade is comprised of three haplotypes from the Atlantic versant EIT Chiapas (Fig. 3.3). Additionally, the *P. sphenops* clade has five other haplotypes with undefined relationships; these samples are geographically dispersed across the BD, Pacific WIT, and Pacific EIT (Fig. 3.3).

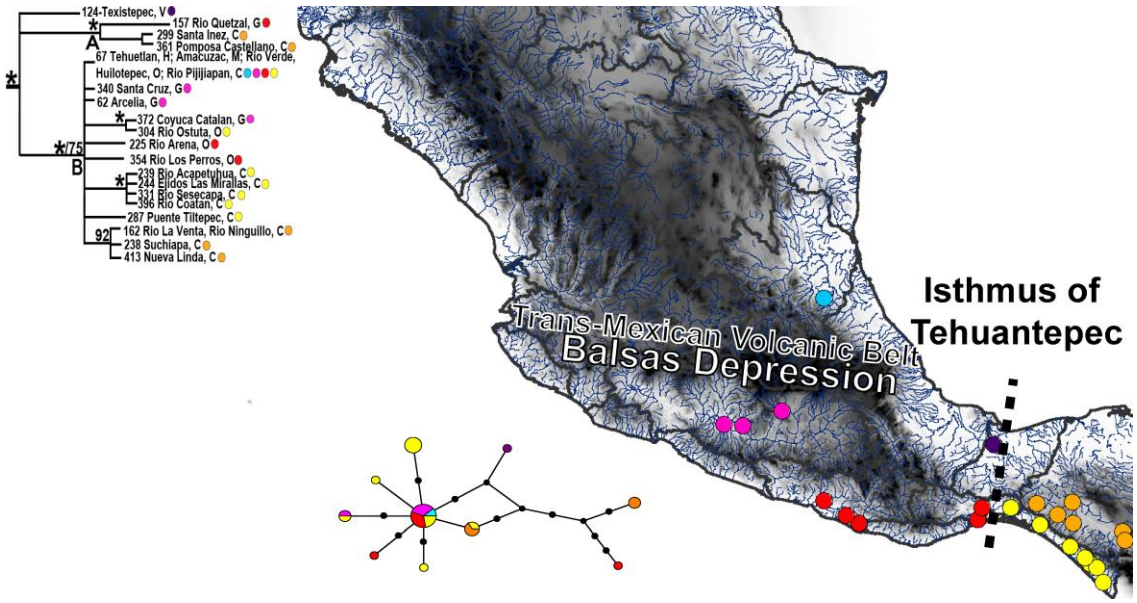


Figure 3.3 Cytochrome *b* (1,140 bp) mitochondrial gene Bayesian phylogeny, parsimony haplotype network, and sampling distribution of *Poecilia sphenops* (aqua-Atlantic North of the Trans Mexican Volcanic Belt, pink- Balsas River Drainage, purple- Atlantic North of the Isthmus of Tehuantepec, red- Pacific 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.

The haplotype network of *P. sphenops* has haplotypes separated between 0-7 mutational steps with the most divergent haplotypes being from the Pacific WIT, along the northern Guerrero coast and Atlantic EIT from the state of Chiapas (Fig. 3.3). The next most divergent haplotype is the single individual collected from WIT from the southern Atlantic coast, on the Isthmus of Tehuantepec. The most frequent haplotype is distributed largely on the Pacific coast in BD, WIT and EIT, with a single Atlantic coast sample being found north of the TMVB. Remaining haplotypes are, with just a few exceptions, also from the Pacific coast in BD, WIT and EIT (Fig. 3.3).

*3.3.1.3. Phylogeographic patterns in the P. butleri/nelsoni clade*

The sister taxa *Poecilia butleri* and *P. nelsoni* are found north and south of the Trans-Mexican Volcanic Belt (TMVB) and form clade C and F in the phylogenetic tree, respectively (Fig. 3.4). The phylogenetic structure for *P. butleri* shows an initial divergence represented by a single haplotype from the most northern sample, collected in northern Sinaloa. The next divergence (D) includes a clade composed of two haplotypes from the southernmost portion of the *P. butleri* range (southern Nayarit). Lastly, clade E comprises a strongly supported clade of the remaining haplotypes, which form a five lineage polytomy (Fig. 3.4). Two of these lineages represent single individuals, one from the northern (Sinaloa) and one from the southern portion (Nayarit) of the range. Two other lineages represent moderately to strongly supported sub-clades comprising six and three individuals, respectively; these are also from the northern portion of the *P. sphenops* range in Sinaloa. The fifth lineage represents a large weakly supported clade of individuals collected from both the northern and southern portions of the range. The haplotype network of *P. butleri* has a maximum of 14 mutational steps between haplotypes with most haplotypes differing between 0-6 steps (Fig. 3.4). The most divergent haplotypes are from Rio Choix (H192), the most northern sampling locality in Sinaloa, and haplotypes from El Palillo, Nayarit (H456), San Blas, Nayarit (H399), and Acaponeta, Nayarit (H408).

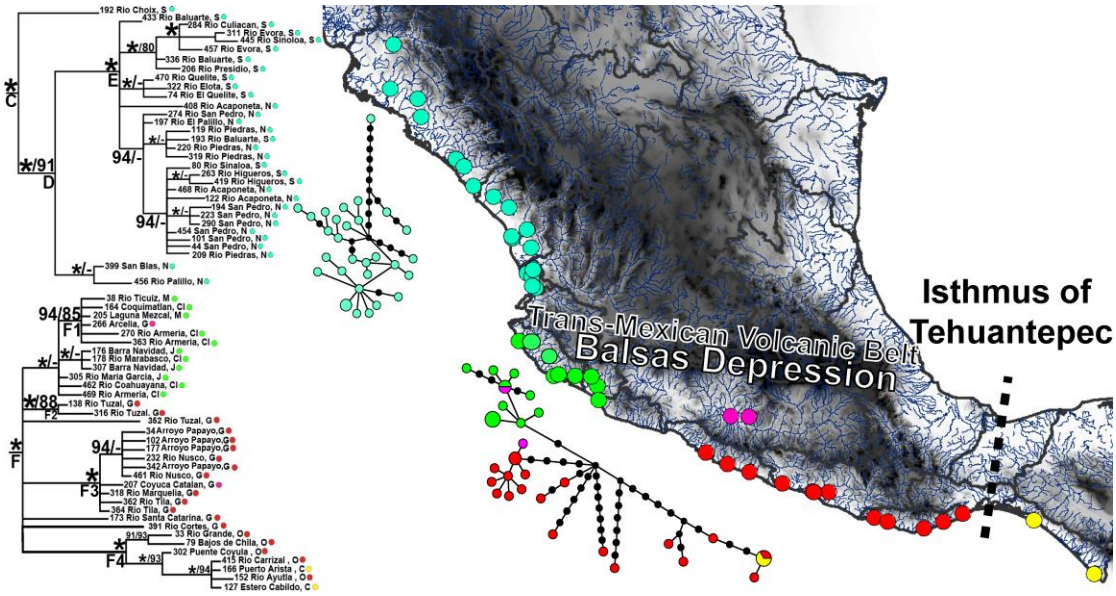


Figure 3.4 Cytochrome *b* (1,140 bp) mitochondrial gene Bayesian phylogeny, parsimony haplotype network, and sampling distribution of the Pacific sister taxa *Poecilia butleri* (blue-North of the Trans Mexican Volcanic Belt) and *P. nelsoni* (lime green-South of the Trans Mexican Volcanic Belt, pink- Balsas River Drainage, red- North of the Ithmus 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.

The Pacific lowland species *Poecilia nelsoni,* found south of the TMVB, is recovered as monophyletic, but the basal node consists of a polytomy of seven lineages (Fig. 3.4). Three of these lineages represent single individuals, all collected from the Pacific slope WIT, in Guerrero (Fig. 3.4). A fourth lineage consists of two WIT individuals from Guerrero (Clade F2), and the fifth (F3) consists of a large number of individuals from WIT, and with one exception (collected in BD) all are from Guerrero. Of the two remaining *P. nelsoni* lineages, one is composed almost entirely of individuals collected south of the TMVB along the coasts of Jalisco, Colima, and Michoacan; the exception is one individual from Guerrero. Lastly, Clade F4 is composed of individuals collected WIT in Oaxaca, and individuals collected EIT in Chiapas.

The haplotype network for *P. nelsoni* serves to underscore the phylogenetic results, in that multiple highly divergent haplotypes are found in close geographic proximity in Guerrero and Oaxaca, and haplotypes are shared across the Isthmus of Tehuantepec, and the Balsas River samples are more closely associated with other areas than they are each other (Fig. 3.4).

*3.3.1.4. Phylogeographic patterns in the P. mexicana species complex*

The closely related species *P. limantouri*, *P. thermalis*, *P. sulphuraria*, and *P. mexicana*, form clades G (*P. limantouri*), H (including both *P. thermalis* and *P.sulphuraria*), and I and J (*P. mexicana*), respectively from the phylogeny (Fig. 3.5). *P. limantouri* is predominantly found north of the TMVB, with a small range (represented here by two sampling points) south of this barrier. Most haplotypes across the distribution do not demonstrate phylogenetic clustering, or lack support from the maximum likelihood analyses. The single sub-clade with strong support (G1) includes two haplotypes: one from the state of Hidalgo and the other from the southern sampling points in the state of Veracruz (Fig. 3.5). The haplotype network has between 0-6 mutational steps with the most distinct haplotype coming from a coastal sampling point in the middle of the range.

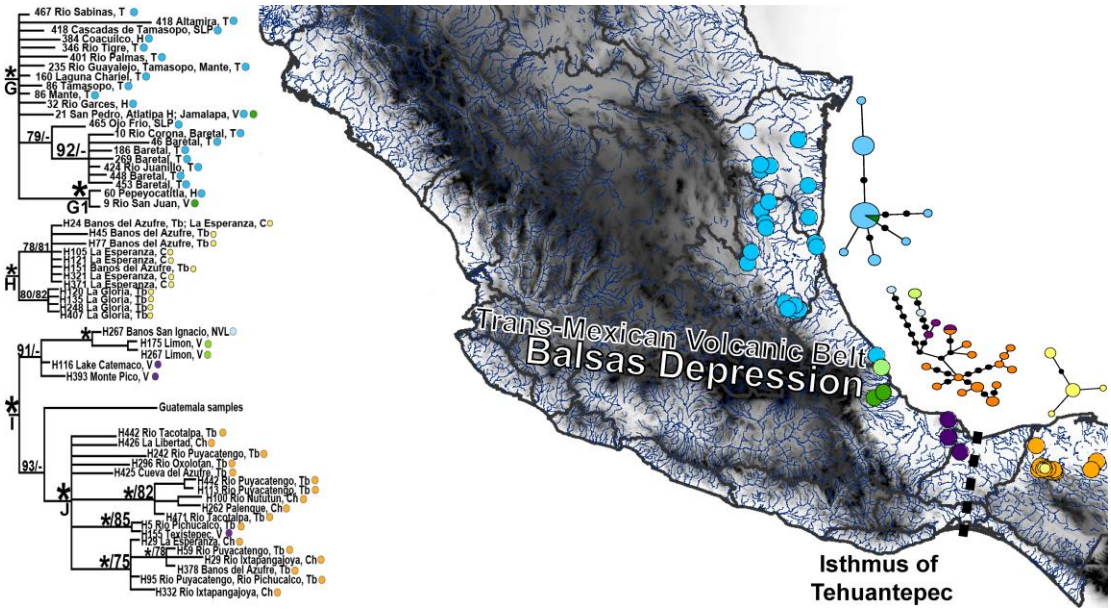


Figure 3.5 Cytochrome *b* (1,140 bp) mitochondrial gene Bayesian phylogeny, parsimony haplotype network, and sampling distribution of Atlantic taxa *Poecilia limantouri* (green blue-North of the Trans Mexican Volcanic Belt, forest green-South of the Trans Mexican Belt), *P. sulphuraria*/*P.* thermalis (yellow-South of the Isthmus of Tehuantepec), and *P. mexicana* (baby blue-North of the Trans Mexican Volcanic Belt, green, 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.

The species *P. sulphuraria* and *P. thermalis* (Clade H) from the states of Tabasco and Chiapas, are recovered in two sub-clades with moderate support. The first includes individuals of *P. sulphuraria* from the Banos del Azufre site and of *P. thermalis* from La Esperanza. The second sub-clade is formed exclusively of haplotypes of *P. sulphuraria* from the La Gloria. The haplotype network shows almost no variation in these taxa (Fig. 3.5).

Phylogenetic analysis recovered a monophyletic *P. mexicana* (Clade I), with further phylogenetic structuring of varying support (Fig. 3.5). One sub-clade (0.91 Posterior Probability) comprises individuals from northernmost Mexico (in Nuevo Leon) as well as individuals collected just south of TMVB, and two sites just west of the Isthmus of

Tehuantepec (Fig. 3.5). Another sub-clade represents individuals collected from the Atlantic versant of Guatemala. The last sub-clade (Clade J) comprises individuals collected from Tabasco and Chiapas, except for one individual collected in Veracruz (Fig. 3.5). The haplotype network shows a lot of variation with 0-14 mutational steps between the least and most distinct haplotypes.

### 3.4. Discussion

The purpose of this study is to provide a better understanding of the mechanisms driving divergence and speciation across the geologically complex Mexican landscape in freshwater fishes of the subgenus *Mollienesia*. The overall phylogenetic and haplotype patterns observed among species (Figs. 3.3, 3.4, 3.5) revealed strong geographic structuring by physiographic barrier and by river basins. The exception was the widely distributed *P. sphenops*, which was distributed across all geographic barriers discussed here (Fig. 3.3), thereby overlapping the distribution of most other *Poecilia* taxa. The haplotype pattern for *P. sphenops* shows a very weak signal of regional sorting (restricted to both EIT sampling areas). In contrast, *P. nelsoni* and *P. mexicana* show similar signals of population structure both WIT and EIT (Figs. 3.5, 3.6). This phylogeographic structure, albeit weak at present, over small spatial scales of populations within the species of the subgenus *Mollienesia* is almost certainly the result of limited genetic exchange set by geologic barriers and disconnected river basins. As such, these isolated populations may, over time, lead to the evolution of new species within the subgenus *Mollienesia*, just as these isolating barriers appear to have done for extant *Poecilia* species.

*3.4.1. Phylogenetic and phylogeographic patterns of Mexican species*

The phylogeny of the subgenus *Mollienesia* shows an interesting pattern with respect to the Mexican species of the *P. sphenops* and *P. mexicana* species complexes. Most species derive from Middle American lineages, demonstrating the complexity of the evolution of species in this group. In the *P. sphenops* species complex, the three Mexican species (*P.chica, P. catemaconis,* and *P. sphenops*) diversified from a Middle American species and each represents an invasion into Mexico. The diversification of *P. catemaconis* on the Atlantic slope of Mexico is a result of the geologic isolation by the Sierra de Tuxtlas (Palacios et al., 2016), the Pacific *P. chica* also possibly evolved due to volcanic activity isolating the of Jalisco block between the Pliocene and Quaternary (Rosas-Elguera et al., 1996), and the diversification of *P. sphenops* remains unknown. In the *P. mexicana* species complex, the Mexican species also show at least two invasions from Middle America, with the first invasion leading to the evolution of four of the five species in this group present in Mexico. The Pacific slope species, *P. butleri* and *P. nelsoni,* evolved in Mexico due to 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 is a combination of vicariance (*P. limantouri* from *P. gracilis* in Middle America) and environmental divergence (*P. sulphuraria*/*thermalis* (Palacios et al., 2013)). Lastly, the most recent invasion of *P. mexicana* may include 2 independent events from Middle America; one represented by lineages in the northern distribution and the other in the one southern portion in Mexico.

Within each species strong phylogeographic structure is observed in two species (*P. nelsoni* and *P. mexicana*) in Mexico over small spatial scales due to fragmentation and disruptions of connections of drainages by the presence of physiographic barriers of the Trans Mexican Volcanic Belt and the Balsas Depression limiting dispersal and isolating populations. This pattern is common in other Neotropical fish species distributed in Mexico, such as the genus *Astyanax* (Coghill et al., 2014), *Profundulus* (Morcillo et al., 2016), and *Poeciliopsis* (Mateos et al., 2002).

### *3.4.2. Geologic barriers as evolutionary drivers of diversity in Mexican Mollies*

#### *3.4.2.1. Trans Mexican Volcanic Belt*

The Trans Mexican Volcanic Belt (TMVB) is a physiographic feature that formed west to east between 25-2.5 mya, composed of ridges and volcanoes that decrease eastwardly toward Veracruz (Miller et al., 2005). This barrier has been observed to be a phylogeographic break for both terrestrial and aquatic species (Huidobro et al., 2006). In our study, the TMVB on the Pacific versant led to a vicariant event and the evolution of the sister species north (*P. butleri*) and south (*P. nelsoni*) of this barrier. This pattern has been previously observed in this sister taxa (Mateos, 2005; Zúñiga- Vega et al., 2014) and in many other vertebrate species (e.g. (Blair and Sánchez-Ramírez, 2016; Devitt, 2006; Light et al., 2016). The most common recent ancestor of Neartic fish found 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). Invasion or isolation of populations by Neotropical freshwater

fish north of the TMVB on the Pacific Coast has also been documented in *Astyanax* (Strecker et al., 2012), *Mayaheros* (Choudhury et al., 2016), 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 served as a vicariant event evident in the various species pairs in various families of Neartic and Neotropical freshwater fish (e.g. Lepisosteidea, Clupeidea, Cichlidae, Characidae, and Poeciliidae; (Agorreta et al., 2013; Contreras-Balderas et al., 1996; Hulsey et al., 2004). The mountains of the PDM serve as a filter for primary and secondary Neartic and Neotropical fishes (Obregón-Barboza et al., 1994), setting a limit and driving phenotypic differences (Hulsey et al., 2010). Our results for *P. sphenops* and *P. limantouri*, where a shared haplotype is distributed on either side of the TMVB, suggest the PDM is not a strong isolating barrier for these species at this time. For *P. mexicana*, unique haplotypes exist north of the PDM, which may be indicative of isolation.

*3.4.2.2. Balsas Depression*

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 by the formation of the Trans Mexican Volcanic Belt and Sierra Madre del Sur (Ferrari et al., 2000; Ferrusquía-Villafranca, 1998). The Balsas river system has been linked to events of dispersals and colonization by Neartic fish from the Mesa Central (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 possibly represents an area of

transition for northern expansion of *P. nelsoni* and *P. sphenops*. This is supported by the shared species in surrounding areas of the BD and coincides with the most recent geologic isolation of this area by Plio-Pleistocene volcanism (Marshall and 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 icthyogeographical province (Miller et al., 2005; Miller and Smith, 1986) houses the native endemic species *P. maylandi* of the subgenus *Mollienesia* but sampling efforts in and around the type locality (Meyer, 1983) were unsuccessful, finding instead *P. butleri* and *P. sphenops*.

*3.4.2.3. Isthmus of Tehuantepec & Sierra Madre de Chiapas*

The Isthmus of Tehuantepec is low elevation area recognized as strong faunal barrier during the Pliocene and Pleistocene (e.g. Campbell, 1999; Marshall and Liebherr, 2000; Mulcahy et al., 2006). The Sierra de Chiapas is composed of four geological domains (Andreani and Gloaguen, 2016) with geologic changes occurring as late as the Pleiostocene (Molina-Garza et al., 2015). These potential barriers do not appear to play a significant role in driving inter-specific variation in *Mollienesia* taxa (Fig. 3.2). However, our results for *P. mexicana* suggest that the Isthmus may be a moderate barrier, with one sub-clade being distributed almost entirely EIT (and this sub-clade is sister to Guatemalan samples), and another being distributed entirely WIT (Fig. 3.5). The same does not hold for either *P. sphenops* or *P. nelsoni*, as both species have shared haplotypes occurring across the Isthmus (Figs. 3.3, 3.4).

### 3.5. Conclusion and Future Research

The phylogenetic patterns of Mexican species in the subgenus *Mollienesia* demonstrate several independent invasions from Middle America and subsequent diversification of species associated with isolation because of a vicariant event or ecological driver. The phylogeographic patterns observed within Mexican species either reflected strong genetic structuring by physiographic barrier (*P. nelsoni* and *P. mexicana*) or a lack of genetic structuring (*P. sphenops, P. butleri, P. limantouri, and P. sulphuraria/thermalis*). This study also sheds insight into the taxonomic status of Pacific populations of *P. sphenops,* which was morphologically designated as distinct based on the scale count around the peduncle (Miller et al., 2005), where we find lack of genetic distinction across the species' distribution. The most interesting discovery was the range extension of *P. mexicana* and *P. limantouri,* north and south of the Trans Mexican Volcanic Belt, respectively in areas that may yield the discovery of a new species (Clements et al., 2012). We also determined the southern range of *Poecilia nelsoni* in Mexico to extend into southern Oaxaca and Chiapas, where the species is found in salinities of 0-34 ppt. Additionally, we recommend delisting *P. nelsoni* as endangered by the Mexican authorities based on the quantities of fish observed in the field. However, all freshwater fish are under threat by pollution, water shortages, and invasive species in Mexico (Contreras-Balderas et al., 2008). A recent checklist list identified four species of *Mollienesia* (*P. butleri*, *P. lati pinna*, *P. latipunctata*, and *P. mexicana*) as invasive, introduced either accidentally or for ornamental purposes (*P. butleri* needs verification; Perez and Ramírez, 2015) outside of their native habitats in Mexico. Interestingly, the tolerant dispersing species *P. sphenops* is not reported potentially due to the misidentification of *P. sphenops* for *P. mexicana* (Gómez-Márquez et al., 2016) and the

common confusion in identifying species in this group (Palacios et al., 2016). Future studies should evaluate species in the subgenus *Mollienesia* as invasive as the hearty nature and high density makes them difficult to eradicate once populations are established, disrupting the integrity and stability of the fish communities they are anthropogenically introduced into.

# CHAPTER IV

# THE REDISCOVERY OF A LONG DESCRIBED SPECIES REVEALS ADDITIONAL COMPLEXITY IN SPECIATION PATTERNS OF POECILIID FISHES IN SULFIDE SPRINGS*

## 4.1. Introduction

Divergent natural selection, often mediated by environmental variation, is a key driver of phenotypic evolution. Its effects can lead to the emergence of locally adapted populations that exhibit unique traits and occupy habitats with distinct combinations of environmental factors (Kawecki and Ebert, 2004). Depending on the strength of selection and rates of gene flow, such local adaptation may also cause the emergence of reproductive isolating barriers among diverging populations as a byproduct, a process known as ecological speciation (Rundle and Nosil, 2005; Schluter, 2000, 2001). Such speciation driven by adaptation has been documented in a variety of organisms and in response to a diversity of selective forces (e.g., resource exploitation (Grant and Grant, 1989; Schluter and McPhail, 1992), habitat use (Losos, 1992; Lu and Bernatchez, 1999), and predation (Langerhans et al., 2004; Vamosi and Schluter, 2004). Current research efforts focus on elucidating the range of conditions under which local adaptation is likely to translate into reproductive isolation, particularly because speciation does not appear to be an inevitable consequence of divergent selection even when

*Reprinted with permission from Palacios M, Arias-Rodriguez L, Plath M, Eifert C, Lerp H, Lamboj A, Voelker G, Tobler M. The rediscovery of a long described species reveals additional complexity in speciation patterns of poeciliid in fishes sulfide springs. PLoS One. 2013 Aug 16;8(8):e71069. doi:10.1371/journal.pone.0071069.

phenotypic differentiation is pronounced (Hendry et al., 2007; Magurran and Magurran, 1998; Nosil, 2008). The fact that phenotypic divergence is not necessarily tied to the emergence of reproductive isolation, as well as the dynamic nature of species boundaries, pose a challenge for efforts in biological systematics and taxonomy, which attempt to describe and organize biological diversity (Hey et al., 2003).

Historically, taxonomists catalogued species solely based on morphological trait variation (i.e., they applied a morphological species concept) without considering the underlying mechanisms contributing to phenotypic variation and speciation. Darwin (Darwin, 1859) himself conceded that the recognition of species was often left to the opinion, experience, and expertise of naturalists. Confounding evolutionary processes and taxonomy has led to much confusion for both taxonomists and evolutionary biologists (Padial et al., 2010). While the Modern Synthesis consolidated the definition of a biological species to being based on reproductive isolation (Dobzhansky and Dobzhansky, 1937; Mayr, 1942), a wide variety of other species concepts is currently being applied and newly developed depending on the objective at hand (Hausdorf, 2011). Consequently, there is a perpetual struggle to align the works of earlier biologists with a more modern understanding of phenotypic evolution and speciation (Hausdorf, 2011), particularly in diverse and taxonomically difficult groups with a wealth of available species names, many of which currently are considered junior synonyms of older epithets (Gaston and Mound, 1993). Reexamination of long described taxa is critical to accurately assess biodiversity and align taxonomy with evolutionary processes leading to diversity, because phenotypic variation due to developmental plasticity (Pfennig et al., 2010), genetically based intraspecific

polymorphisms (Olvido and Mousseau, 2012), and large-scale geographic trait variation are not necessarily tied to reproductive isolation.

The genus *Poecilia*, which is part of the livebearer family Poeciliidae, represents an excellent example of the difficulties in aligning evolutionary processes and taxonomy. *Poecilia* is a diverse group of freshwater fish species that are distributed from the southeastern United States and Middle America to parts of South America and the Greater Antilles (Rosen and Bailey, 1963). Taxonomic confusion is particularly prevalent in the *P. sphenops* (short fin molly) species group, which occurs from northern Mexico to Venezuela (Poeser, 2011). On one hand, these fish show tremendous variability in phenotypic traits, which has lead to the description of numerous species (many of which are currently considered synonyms) (Reis et al., 2003). Despite clear phylogenetic structuring (Alda et al., 2013) and a long list of available names (Reis et al., 2003), species designation often remains unclear. This is predominantly caused by pronounced eco-morphological and geographic variation (Poeser, 2003b), intra-specific trait variability that appears to regularly exceed the inter-specific differences (Hubbs, 1926; Regan, 1913; Rosen and Bailey, 1963), uncertainty about whether and how morphological differences are tied to reproductive isolation (Seda, 2010; Zúñiga-Vega et al., 2011), potential hybridization and introgression among lineages (Alda et al., 2013; Kittell et al., 2005; Parzefall, 1989), and sometimes unclear type localities of available names (Reis et al., 2003). On the other hand, there is some well documented cases of phenotypically divergent and reproductively isolated species; yet these lineages often do not appear to be phylogenetically distinct, presumably because divergence occurred relatively recently (Tobler et al., 2011). Therefore, evaluation of species in this group in an

evolutionary context is crucial for the resolution of taxonomy and for a better understanding of the historical and current processes shaping the species' evolution.

Here, we attempt to clarify the status of one such long described species, *Poecilia thermalis* Steindachner 1863 (Fig. 4.1), which has had a vivid taxonomic history (Table 4.1). The species was originally described based on specimens that C. B. Heller collected in a sulfidic spring (La Esperanza) located in the Ixtapangajoya river drainage of Chiapas, Mexico, in 1848 (Steindachner, 1863). The species description particularly emphasized the large head size in the available specimens. Shortly after the species description, Günther (Günther, 1866) thought to recognize *P. thermalis* in samples from El Salvador, but this has been largely viewed as a misidentification (Poeser, 2003a). Subsequently, the species was subjected to various nomenclatural re-assignments by taxonomists (i.e., it has predominantly been viewed as a synonym to *P. sphenops*; see Table 4.1).

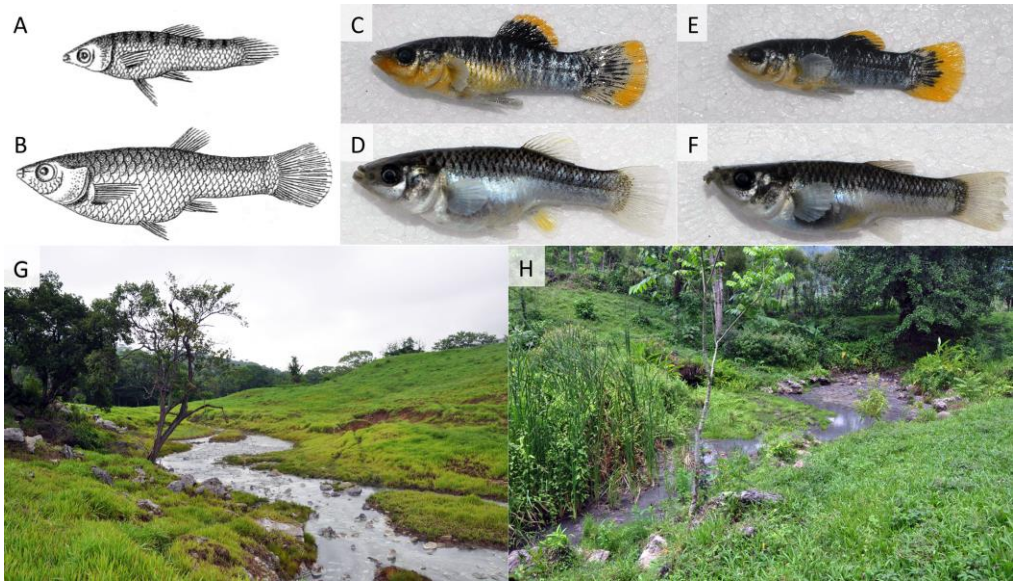


Figure 4.1. *Poecilia thermalis* and its natural habitat. A.-F. Representative specimens of *Poecilia thermalis* (males on top, females below). A. & B. represent artistic depictions of the species from the original description by Steindachner [35]. C.-F. are photos of freshly collected specimens of the *P. thermalis* from the large La Esperanza spring in 2012. G. The type locality of *P. thermalis* at La Esperanza (large spring), Chiapas, Mexico, a typical sulfidic spring habitat. H. A second, smaller sulfide spring (La Esperanza small spring) in close proximity to the type locality, which is also inhabited by *P. thermalis*.

Table 4.1. Summary of the taxonomic history of *Poecilia thermalis* and *P. sulphuraria* in chronological order.

| Author | Year | Action taken | Species examined | Location of examined specimens |
|---|---|---|---|---|
| Steindachner | 1863 | Original description of *P. thermalis* | *P. thermalis* | La Esperanza, Chiapas, Mexico |
| Günther | 1866 | Misidentified *P. thermalis* | *Poecilia cf. salvatoris* | Thermal Springs, El Salvador |
| Regan | 1907 | Revised misidentified *P. thermalis* and synonymized it with *P. salvatoris* | *Poecilia cf. salvatoris* | Thermal Springs, El Salvador |
| Rosen & Bailey | 1963 | Synonymized *P. thermalis* with *P. sphenops* | *Poecilia cf. salvatoris* | Thermal Springs, El Salvador |
| Alvarez | 1947 | Original description of *P. sulphuraria* | *Poecilia sulphuraria* | Baños del Azufre, Tabasco, Mexico |
| Miller | 1983 | Synonymized *P. thermalis* with *P. mexicana* | *Poecilia cf. salvatoris* | Thermal Springs, El Salvador |
| Poeser | 2003 | Revalidated *P. thermalis* | None | |
| Poeser | 2011 | Validated *P. thermalis* | None | |

Interestingly, all prior nomenclatural acts revolving around this species have either been based solely on the consultation of the misidentified specimens (Miller, 1983; Regan, 1907; Rosen and Bailey, 1963) or on information given in the species description (without actually examining specimens (Poeser, 2003a; Poeser, 2011). Because the species has not

been collected since 1848, we revisited the type locality of *P. thermalis* based on descriptions in Heller's (Heller, 1853) autobiographical account of his travels in southern Mexico to investigate the status of this enigmatic species. Our interest was fueled by the sulfidic nature of *P. thermalis*' habitat, which was emphasized both in the species description and Heller's field accounts (Heller, 1853; Steindachner, 1863). In drainages adjacent to the Río Ixtapangajoya (namely the Tacotalpa and Puyacatengo drainages to the east, and the Pichucalco drainage to the west), evolutionarily independent *Poecilia* lineages have colonized springs with high concentrations of toxic hydrogen sulfide (H2S)(Tobler et al., 2011). These sulfide spring inhabitants are phenotypically distinct from the closely related *P. mexicana* in non-sulfidic environments within the same drainage and are characterized by morphological, physiological, behavioral, and life history adaptations that show strong signals of convergent evolution across drainages (Tobler et al., 2011). In particular, sulfide spring fishes are characterized by enlarged heads and correlated increases in gill surface area, which facilitates oxygen acquisition in hypoxic sulfide spring environments and directly affects survival (Plath et al., 2007; Tobler et al., 2011), as well as physiological and biochemical adaptations that reduce the impacts of sulfide toxicity (Pfenninger et al. 2009). In conjunction with adaptive trait divergence, the sulfide spring populations are reproductively isolated from adjacent populations from non-sulfidic waters despite small geographic distances (in some instances <100 meters) and a lack of physical barriers that would prevent fish migration (Plath et al., 2010a; Plath et al. 2013). Reproductive isolation appears to be mediated particularly by natural and sexual selection against immigrants (Plath et al. 2013). The taxonomic status of sulfide spring populations varies across drainages; sulfide spring residents in the Tacotalpa and Puyacatengo drainages are considered ecotypes

of the widespread *P. mexicana* despite clear morphological differences and strong reproductive isolation, while sulfide spring residents in the Pichucalco drainage have been described as a distinct species, *Poecilia sulphuraria* (Alvarez del Villar, 1947).

Consequently, this study investigated the re-discovered sulfide spring species *Poecilia thermalis* to examine whether it shows similar evolutionary patterns (i.e., phenotypic trait divergence and reproductive isolation) as other sulfidic populations in the region and to shed light on its taxonomy. Specifically, we used morphological, phylogenetic, and population genetic approaches to address three major questions: (1) How do specimens from the type locality of *Poecilia thermalis* phenotypically compare to other *Poecilia* populations from sulfidic and non-sulfidic spring habitats in the region? Using a geometric morphometric approach, we tested for potential morphological convergence between *P. thermalis* and other sulfide spring fish in southern Mexico. We also explored the similarity of body shape between historical samples of *P. thermalis* (Steindachner, 1863) to current populations of sulfidic and non-sulfidic spring fish, including recently collected *P. thermalis* from the type locality. (2) What is the phylogenetic relationship of *P. thermalis* to other mollies? Based on mitochondrial and nuclear markers from a broad taxonomic sampling of *Poecilia*, we elucidated the phylogenetic position of *P. thermalis*. We were particularly interested in determining whether the species represents a unique sulfide-adapted lineage within *Poecilia*. (3) Is *P. thermalis* genetically isolated from adjacent *Poecilia* populations? Sulfide spring populations of *Poecilia* consistently exhibit a high degree of reproductive isolation from non-sulfidic populations despite the small spatial distance and a lack of migratory barriers (Plath et al., 2006; Tobler et al., 2008; Tobler et al., 2009b). Using a population genetic approach based on microsatellites, we quantified gene flow between the

endemic sulfide-adapted species of *P. thermalis* and *P. sulphuraria*, and *P. mexicana* populations from adjacent non-sulfidic habitats.

## 4.2. Materials and Methods

All procedures conducted for this study were approved by the Institutional Animal Care and Use Committee at Oklahoma State University (ACUP: AS10-15) and permits issued by the Municipio de Tacotalpa-Tabasco (DFET/23/2011), as well as the Mexican Federal Agencies SEMARNAT (SGPA/DGVS/04315/11 for *Poecilia sulphuraria*) and CONAPESCA (DGOPA.09004.041111.3088 for *Poecilia sp*.). Details about the selection of focal populations are given below for each section separately.

### *4.2.1. Study site and sampling*

Study sites and sample collection Our study area lies in the foothills of the Sierra Madre de Chiapas in the northeastern part of the state of Chiapas, where the mountains meet the wide floodplains of the state of Tabasco. Here, four tributaries of the Río Grijalva, the Tacotalpa, Puyacatengo, Ixtapangajoya, and Pichucalco (from east to west), provide a system of naturally replicated non-sulfidic and adjacent sulfidic habitats (see Figure 2). The presence of high concentrations of hydrogen sulfide (H2S) in the sulfidic springs is a result of a nearby, active volcano, El Chinchón, and bacterial activity (Armienta and De la Cruz-Reyna, 1995; Rosales Lagarde, 2012; Rosales Lagarde et al., 2006). Besides the presence of H2S, the sulfidic spring environments differ from non-sulfidic habitats in a variety of environmental factors, including reduced oxygen concentration, structural habitat differences, reduced species richness, and reduced photoautotrophic primary production (Roach et al., 2011;

Tobler et al., 2011; Tobler et al., 2006). In all cases, sulfidic habitats drain directly into non-sulfidic habitats in the same drainage, and there are no major physical barriers preventing movement of fish, especially during high flow periods. The distances between sulfide spring habitats to the closest freshwater habitat range from <50 m to about 500 m.

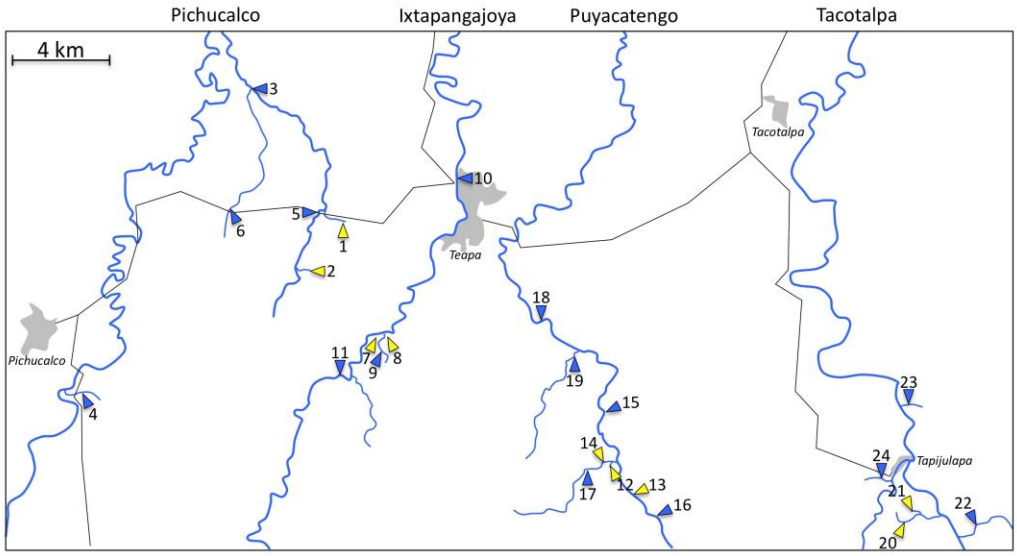


Figure 4.2. Overview of the study area and sampling localities of three *Poecilia* species in southern Mexico. Sampling of *P. thermalis*, *Poecilia mexicana mexicana*, and *P. sulphuraria* in southern Mexico. The colors represent sulfidic (yellow) and non-sulfidic (blue) sites and the numbers represent localities as described in Table 4.2. For orientation purposes, we included black lines representing major roads and gray areas representing major towns in the region.

While abiotic environmental parameters of other sites investigated here have been published in detail before (Tobler et al., 2011), the type locality of *P. thermalis* at 'La Esperanza' has not been resampled since Heller's expedition. Exploration of the general area in May and June of 2012 revealed not one, but two proximate sulfide springs, a larger one (site 7) and a smaller one (site 8) separated by a freshwater tributary (site 9) of the Río Ixtapangajoya (Fig. 4.2). Based on descriptions in his autobiography, it is evident that Heller collected the type specimens from the larger spring (hence this larger spring should be

considered as the type locality), which represents a cluster of relatively high discharge sulfide springs that forms a distinct tributary flowing over gravelly bottom for about 200 meters before merging with the Río Ixtapangajoya over a small drop. In this habitat, we measured the following water parameters (N=4 for all measurements): sulfide concentration: 216 ± 38 μM; dissolved oxygen: 1.31 ± 0.39 mg/l; pH: 6.9 ± 0.0; specific conductivity: 4.132 ± 0.083 mS/cm; temperature: 28.7 ± 0.9 °C. The smaller spring represents a group of lower discharge springs in a swampy area with dense reeds. The site is merely a shallow pool (about 15 × 20 m) with a narrow outflow that—at least during our visit at the end of the dry season—eventually disappeared into pasture grounds (i.e., there was no direct, permanent connection to the Río Ixtapangajoya less than 150 m away). Here, we measured the following water parameters (N=4 for all measurements): sulfide concentration: 41 ± 3 μM; dissolved oxygen: 1.67 ± 0.21 mg/l; pH, 7.0 ± 0.1; specific conductivity: 2.979 ± 0.071 mS/cm; temperature: 27.2 ± 0.3 °C. Overall, the physiochemical conditions in the two La Esperanza springs aligned well with data collected over multiple years in sulfide springs of other drainages (Tobler et al., 2011).

All specimens for this study were collected using seines, euthanized with buffered MS222 immediately after capture, and fixed in a 10% formaldehyde solution for geometric morphometric analyses. All specimens are housed in the Department of Zoology, Oklahoma State University. In addition, we took fin clips (right pectoral fin) that were preserved in 95% ethanol and stored at 4 °C for molecular analyses.

*4.2.2. Geometric morphometrics*

The specimens sampled for the geometric morphometric analyses broadly covered the study region, including all currently known sulfide springs inhabited by *Poecilia* species. Our sampling included the southern subspecies of *P. mexicana* mexicana from a variety of non-sulfidic habitats in all drainages, sulfidic ecotypes of *P. m. mexicana* in the Tacotalpa and Puyacatengo drainages as well as the previously described sulfide spring endemics *P. thermalis* (Ixtapangajoya drainage) and *P. sulphuraria* (Pichucalco drainage; see Table 4.2 for an overview). We analyzed a total of 1099 specimens from the four drainages for a total of 23 localities.

Table 4.2. Overview of samples used in this study for morphometric and population genetic analyses.

| No | Site | $H_2S$ | Latitude, longitude | Species | *N* body shape lateral (males/females) | *N* body shape dorsal (males/females) | *N* population genetics |
|---|---|---|---|---|---|---|---|
| **Río Pichucalco drainage** | | | | | | | |
| 1 | Baños del Azufre | + | 17.552, −92.999 | *P. sulphuraria* | 54/54 | 52/53 | 25 |
| 2 | La Gloria | + | 17.532, −93.015 | *P. sulphuraria* | 29/28 | 28/30 | 24 |
| 3 | Río Pichucalco | − | 17.605, −93.036 | *P. mexicana* | 22/30 | 23/30 | |
| 4 | Arroyo Rosita | − | 17.485, −93.104 | *P. mexicana* | 27/29 | 27/28 | 25 |
| 5 | Río El Azufre, west branch | − | 17.556, −93.008 | *P. mexicana* | 36/53 | 46/47 | 55 |
| 6 | Arroyo Raphael | − | 17.558, −93.043 | *P. mexicana* | | | 16 |
| **Río Ixtapangajoya drainage** | | | | | | | |
| 7 | La Esperanza, large spring | + | 17.511, −92.983 | *P. thermalis* | 24/30 | 24/30 | 36 |
| 8 | La Esperanza, small spring | + | 17.511, −92.980 | *P. thermalis* | 31/29 | 30/30 | 24 |
| 9 | Tributary to Rio Ixtapangajoya | − | 17.510, −92.980 | *P. mexicana* | 3/26 | 2/26 | 18 |
| 10 | Río Teapao | − | 17.555, −92.952 | *P. mexicana* | 6/11 | 6/11 | 25 |
| 11 | Río Ixtapangajoya | − | 17.495, −92.998 | *P. mexicana* | 39/32 | 44/32 | 24 |
| **Río Puyacatengo drainage** | | | | | | | |
| 12 | La Lluvia, small spring | + | 17.464, −92.895 | *P. mexicana* | 40/25 | 40/25 | |
| 13 | Puyacatengo springs | + | 17.458, −92.889 | *P. mexicana* | 13/4 | 13/4 | |
| 14 | La Lluvia, big springs | + | 17.464, −92.896 | *P. mexicana* | 5/11 | 4/10 | |
| 15 | Río Puyacatengo road crossing | − | 17.470, −92.896 | *P. mexicana* | 8/7 | 8/8 | |
| 16 | Río Puyacatengo upstream | − | 17.456, −92.888 | *P. mexicana* | 2/11 | 2/11 | |
| 17 | La Lluvia upstream | − | 17.461, −92.897 | *P. mexicana* | 5/14 | 5/15 | |
| 18 | Río Puyacatengo at Vincente Guerrero | − | 17.510, −92.914 | *P. mexicana* | 31/45 | 31/45 | |
| 19 | Tributary to Río Puyacatengo | − | 17.504, −92.909 | *P. mexicana* | 12/24 | 12/24 | |
| **Río Tacotalpa drainage** | | | | | | | |
| 20 | El Azufre II | + | 17.438, −92.775 | *P. mexicana* | 11/30 | 10/30 | |
| 21 | El Azufre I | + | 17.442, −92.775 | *P. mexicana* | 47/47 | 46/47 | |
| 22 | Arroyo Bonita | − | 17.427, −92.752 | *P. mexicana* | 14/24 | 12/25 | |
| 23 | Arroyo Tres | − | 17.484, −92.776 | *P. mexicana* | 9/22 | 9/22 | |
| 24 | Arroyo Tacubaya | − | 17.454, −92.785 | *P. mexicana* | 15/30 | 15/30 | |

We conducted a geometric morphometric analysis of body shape both from the lateral and dorsal view, as body shape has been shown to be a reliable indicator for convergent evolution in sulfide spring environments (Tobler and Hastings, 2011; Tobler et al., 2011). For all specimens, lateral and dorsal photographs were taken using a Nikon D90 camera mounted on a copy stand. We digitized 16 lateral and 9 dorsal landmark points using tpsDig2 (Rohlf, 2010) (see Figure C1A, 1B for details on landmark locations). We analyzed lateral and dorsal landmarks separately and performed a geometric morphometric analysis based on the coordinates of the digitized landmarks (Zelditch et al., 2012). Landmark coordinates were aligned using least-square superimposition as implemented in the program tpsRelw (Rohlf, 2007) to remove effects of translation, rotation, and scale. Based on the aligned coordinates, we calculated centroid size and partial warp scores with uniform components (weight matrix) for each individual. Unless otherwise stated, all statistical analyses were performed using SPSS 20 (IBM Inc.). All raw data used for the analyses described below are archived on http://datadryad.org under the DOI number associated with this publication.

The weight matrices obtained from the geometric morphometric analyses were first subjected to principal components analyses (PCA) using a covariance matrix to reduce data dimensionality. We retained 9 PC axes with an eigenvalue greater than 1 for the lateral dataset (explaining >95% of variation) and 8 PC axes for the dorsal dataset (explaining >96% of variation). Individual PC axis scores were used as dependent variables in multivariate analyses of covariance (MANCOVA). Assumptions of multivariate normal error and homogeneity of variances and co-variances were met for all analyses performed. F-values were approximated using Wilks' lambda and effect strengths by use of partial eta squared ($\eta p2$). We also calculated the relative variance as the partial variance for a given term

divided by the maximum partial variance value in a model (Langerhans and DeWitt, 2004). We included sex, habitat type (H2S present or not), drainage, and site (nested within the H2S × drainage interaction) as well as all interaction terms as independent variables. Centroid size was included in the models as a covariate to control for multivariate allometry.

Since random nested factors are not applicable for MANCOVAs, and the use of fixed effects can inflate type I error rates when nested terms are significant, we also analyzed shape variation using a mixed-model nested analysis of covariance (ANCOVA) (Langerhans, 2009). To do so, we calculated divergence scores for each individual along the sulfide/non-sulfide gradient based on a divergence vector as defined by Langerhans (Langerhans, 2009). Individual divergence scores were used as dependent variables in ANCOVAs with the same model structure as outlined above, except that site was designated a random factor to account for the fact that only a random subset of sites where *Poecilia* occurs was analyzed for this study. Shape variation along the first two PC axes and along the sulfide/non-sulfide divergence axes was visualized with thin-plate spline transformation grids using tpsRegr (Rohlf, 2005).

To fully examine the multidimensional affinities of different *Poecilia* populations relative to each other, including information from both the lateral and the dorsal projection, weight matrices for both projections were combined and subjected to a principal components analysis, from which we retained 14 axes with an Eigenvalue >1. Population-specific estimated marginal means for each axis were calculated using a MANCOVA model as detailed above and used to create a dissimilarity matrix that was subjected to a hierarchical cluster analysis using the neighbor-joining algorithm (Saitou and Nei, 1987).

Finally, we tested whether the type specimens of *P. thermalis* collected in 1848 by Heller clustered with specimens we collected from the two La Esperanza springs in 2012. To do so, lateral photographs were taken from the available syntypes in the collection of the Natural History Museum in Vienna (N=18), and we digitized the same lateral landmarks as for all other specimens. We compared the museum specimens to the samples obtained from the two La Esperanza sulfide springs, the two most proximate non-sulfidic locations in the same drainage (tributary to Río Ixtapangajoya and Río Ixtapangajoya proper), and—given the phylogenetic affinity of *P. thermalis* to *P. sulphuraria* (see below)—to the specimens obtained from the Baños del Azufre and La Gloria sulfide springs. Landmark coordinates from said collections were aligned separately. The weight matrix was then subjected to PCA, and the effects of sex and allometry removed from the dataset by using the residuals of a preparatory MANCOVA, in which the principal component scores were used as dependent variables, centroid size as a covariate, and sex as an independent variable. We then conducted a discriminant function analysis (DFA) to elucidate whether museum specimens were classified to the Esperanza sulfide springs based on body shape data. We used a cross-validation technique where discriminant functions were generated based on the data of contemporary samples (training data), and classification probabilities of museum specimens (testing data) to any of the six populations were calculated based on the established functions.

*4.2.3. Phylogenetic analyses*

To establish the phylogenetic relationships of *P. thermalis,* we sequenced a set of genes in specimens from select non-sulfidic and sulfidic habitats included in the morphometric analyses (Table C1). In addition, we broadened our taxon sampling by adding several other species of the subgenus *Mollienesia* (including the endemic sulfide spring species *P. sulphuraria,* the Southern and Northern Mexican subspecies *P. m. mexicana* and *P. m. limantouri*, as well as *P. butleri*, *P. sphenops*, *P. latipinna*, and *P. caucana*) and more distant groups in the genus *Poecilia* (sensu lato; including, *Limia vittata*, *L. dominicensis*, *L. melanogaster*, *Acanthophacelus reticulata*, *A. wingei*, *Micropoecilia bifurca*, *M. parae*, *Pamphorichthys hollandi*, and *P. minor*). The distantly related species *Cnesterodon decemmaculatus* and *C. hypselurus* were used as outgroups to root phylogenetic trees. A complete list of all taxa examined, along with locality information and GenBank Accession numbers, is provided in the Appendix in Table C1.

The 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. 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 and Gold, 2000) and ASN (Kocher et al., 1995) primers. The nuclear genes amplified included exon 3 of recombination activating gene-1 (Rag1, 1,561 bp), a portion of the 7 trans-membrane receptor region of Rhodopsin (Rh, 822 bp), and exon 1 of myosin heavy polypeptide 6 (myh6, 767 bp) with the primers and protocol following previously published PCR protocols

(Meredith et al., 2010; Meredith et al., 2011). 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 and Toh, 2008).

We tested for incongruence between mitochondrial (mtDNA) and nuclear (nDNA) markers to determine evidence of introgression. Given the agreement between both datasets (data not shown), we used a concatenated dataset for further analyses. We used MrModeltest version 2.3 (Nylander, 2004) to determine the most likely model of DNA substitution among 24 candidate models on a fixed BioNJ-JC tree based on the Akaike information criterion (AIC) (Table C2). We also compared likelihood scores between Bayes runs of 12,000,000 generations of mtDNA as a single unit, partitioned by gene and by position to determine the most informative partition. The best likelihood score was observed in the codon partition dataset and used for analyses where possible.

For maximum likelihood (ML) analyses, we used RAxML GUI version 1.0 (Stamatakis, 2006; Stamatakis et al., 2008) run to conduct 500 Rapid Bootstrap searches followed by an ML search. We ran the complex general time reversible (GTR) + Γ (Gamma distribution for rate variation among sites) model because RAxML does not implement simpler models. We also used GARLI version 2.0 (Zwickl, 2006) to perform ML bootstrap searches (500 replicates) on the concatenated datasets under the corresponding best model selected (Table C2). The bootstrap trees were summarized with a Sumtrees script with a 50%

percent majority rule consensus parameter in DendroPy 3.10.1 (Sukumaran and Holder, 2010).

Bayesian analyses were run twice independently in MrBayes version 3.2.1 (Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012), under models of nucleotide substitution uniquely defined for the partition of each data set (Table C2) implementing two runs with four chains under default parameters. Appropriate "burn-in" (i.e., samples discarded prior to reaching a stationary posterior distribution) was determined based on small and stable average standard deviation of the split frequencies, potential scale reduction factor close to 1 (see MrBayes manual), and stable posterior probability values examined in Tracer version 1.5 (Rambaut and Drummond, 2007). Pairwise genetic distances based on the concatenated dataset were calculated under the Kimura-2 parameter in MEGA version 5 (Tamura et al., 2011) with pairwise deletion for missing data.

### *4.2.4. Population genetics*

Besides the two populations of *P. thermalis*, samples for population genetic analyses included two proximate non-sulfidic sites within the Ixtapangajoya drainage to test for gene flow between adjacent populations from sulfidic and non-sulfidic waters. Given the phylogenetic clustering of *P. thermalis* with *P. sulphuraria* from the Pichucalco drainage (see below), we also included both known *P. sulphuraria* populations as well as adjacent *P. mexicana* samples from that drainage into our analyses (Table 4.2). We used 17 previously developed microsatellite markers (Slattery et al., 2012; Tiedemann et al., 2005) to genotype a total of 272 samples and arranged the microsatellites into three multiplex reactions (Plath et al. 2013). Data from 80 specimens (Baños del Azufre and Puente El Azufre II) were re-analyzed

from a previous study (Plath et al. 2013). All raw data used for the population genetic analyses are archived on http://datadryad.org under the DOI number associated with this publication.

We extracted DNA using the NucleoSpin®Tissue kit (Macherey-Nagel). Microsatellites were amplified with the Type-it Microsatellite PCR kit from Qiagen (Hilden, Germany). The PCR protocol included an initial denaturation step for 5:00 min at 95°C, 30 cycles of 1:30 min at 60°C, and 0:30 min at 72°C, followed by a final extension step for 30:00 min at 60°C. The 5 µl reaction mix included 2.5 µl Type-it master mix, 0.4 µl primer mix, 0.4 µl Q-solution, 0.9 µl RNase-free water, and 0.8 µl template DNA. PCR products were analyzed on a CEQ2000 sequencer (Beckman) Coulter; denaturation at 90°C for 2 min, injection at 2.0 kV for 30 s, separation at 6.0 kV for 45 min) along with the manufacturer's internal size standard. Samples were screened using Genome Lab GeTX 10.2 software (Beckman Coulter) and alleles were called manually.

We employed the software STRUCTURE 2.3.4 (Pritchard et al., 2000) to identify the number of genetically distinct clusters (K) and then used the method of Evanno et al. (2005) and the web-based software STRUCTURE HARVESTER 0.6.93 (Earl and vonHoldt, 2012) to detect the uppermost level of population differentiation. In addition, we calculated pairwise FST-values between all population pairs and conducted a Principal Component Analysis (PCA) to further examine genetic distinctiveness between populations using GenAlEx 6.5 (Peakall and Smouse, 2006).

## 4.3. Results

### *4.3.1. Phenotypic variation*

For the lateral projection (N= 1099 individuals), body shape varied in the position of the anal fin along PC axis 1 and the head size along PC axis 2 (Fig. 4.3). ‘Sex’ and the ‘presence of H2S’ explained the majority of body shape variation in our dataset (Table 4.3). ‘Sex’ particularly accounted for variation along the first PC axis (males have a more anterior anal fin position, as this fin is modified into a copulatory organ, the gonopodium). ‘Presence of H2S’ explained variation along the second PC axis (with sulfide spring fish having larger heads than fish from non-sulfidic habitats). All other factors and the interaction terms also had significant effects on body shape, but only ‘centroid size’, ‘drainage’, ‘site’, and the interaction of ‘ H2S × drainage’ explained an appreciable amount of variation (relative variance >0.1; Table 4.3).

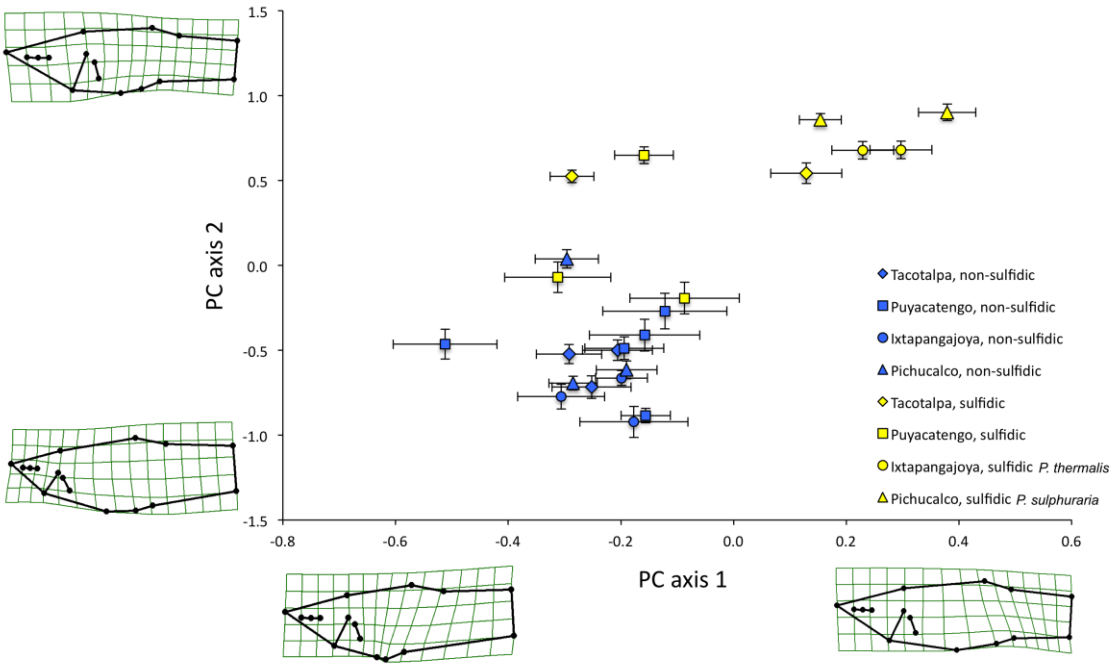


Figure 4.3. Body shape variation of *Poecilia* species in the lateral projection. Depicted are mean principal component scores along the first two principal component axes for each site for *P. thermalis* (yellow circles), *P. sulphuraria* (yellow triangles), as well as sulfidic and non-sulfidic populations of *P. mexicana* across the 23 study sites in southern Mexico. The thin-plate spline transformation grids represent shape variation along each principal component axis.

Table 4.3. Results of a multivariate analysis of covariance on lateral body shape of *Poecilia* from sulfidic and non-sulfidic habitats.

| Effect | *F* | Hypothesis *df* | Error *df* | *P* | Partial Eta Squared | Relative variance |
|---|---|---|---|---|---|---|
| Intercept | 143.786 | 9.0 | 1059.0 | <0.001 | 0.550 | 0.672 |
| Centroid size | 148.242 | 9.0 | 1059.0 | <0.001 | 0.557 | 0.680 |
| **Sex** | **531.555** | **9.0** | **1059.0** | **<0.001** | **0.819** | **1.000** |
| **$H_2S$** | **301.533** | **9.0** | **1059.0** | **<0.001** | **0.719** | **0.878** |
| Drainage | 65.127 | 27.0 | 3093.5 | <0.001 | 0.355 | 0.433 |
| Site ($H_2S$ × Drainage) | 12.579 | 135.0 | 8254.7 | <0.001 | 0.149 | 0.182 |
| Sex × $H_2S$ | 7.581 | 9.0 | 1059.0 | <0.001 | 0.061 | 0.074 |
| Sex × Drainage | 4.703 | 27.0 | 3093.5 | <0.001 | 0.038 | 0.046 |
| $H_2S$ × Drainage | 46.531 | 27.0 | 3093.5 | <0.001 | 0.282 | 0.344 |
| Sex × $H_2S$ × Drainage | 2.843 | 27.0 | 3093.5 | <0.001 | 0.024 | 0.029 |

These general patterns, and particularly the strong differentiation between ecotypes from sulfidic and non-sulfidic springs, were confirmed in the analysis of divergence vector scores with 'site' being treated as a random factor (Table 4.4). Visualization of shape variation along the sulfide-non-sulfide divergence vector corroborated head size as the primary difference between ecotypes (Fig. 4.4). Inspection of divergence scores indicated that *P. thermalis,* just like *Poecilia* from the Tacotalpa and the Pichucalco drainages, exhibits a body shape typical for sulfide spring populations. In the Puyacatengo drainage, differentiation between sulfidic and non-sulfidic ecotypes was less clear-cut, with some populations exhibiting more intermediate body shapes (Fig. 4.4).

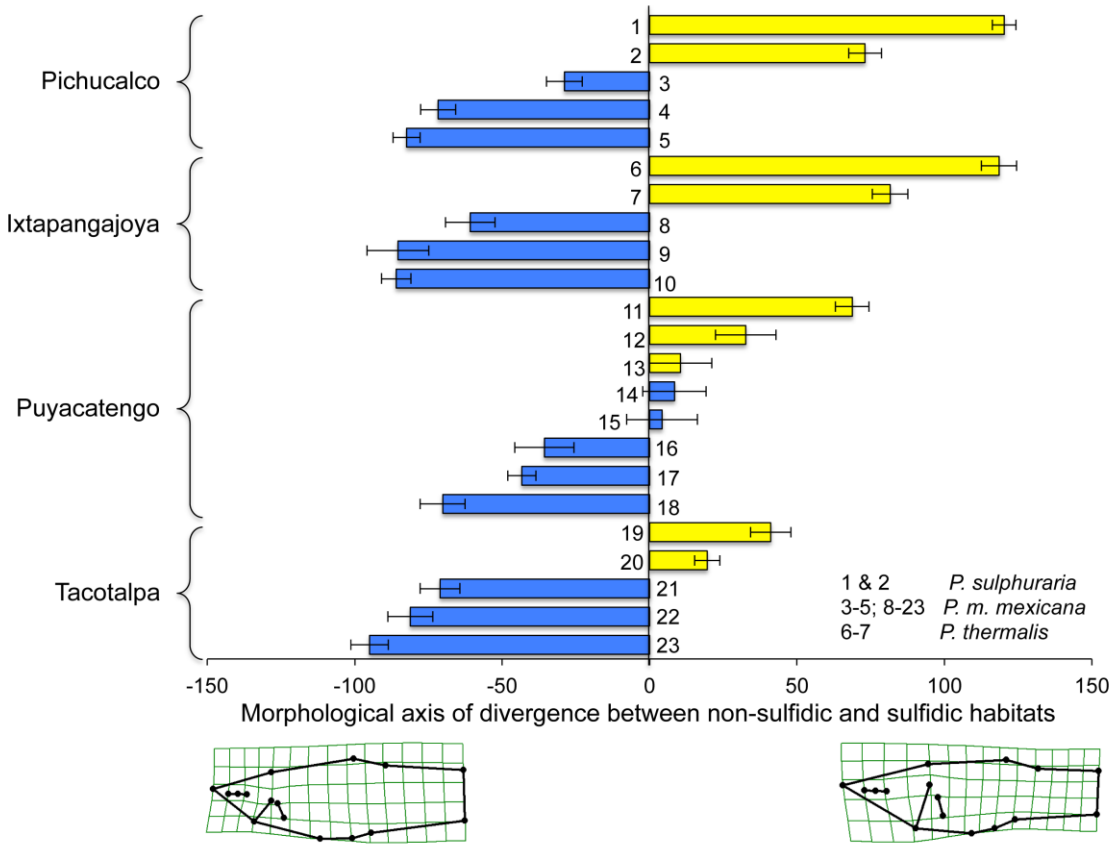


Figure 4.4. Convergent changes in body shape of *Poecilia* species from sulfidic and non-sulfidic habitats in the lateral projection. Depicted are the mean divergence scores (± SEM; derived from the $H_2S$ term in the MANCOVA) for each site for the three formal species (*P. thermalis*, *P. sulphuraria*, and *P. mexicana*) across the 23 sites in southern Mexico from sulfidic (yellow) and non-sulfidic (blue) populations including. The numbers correspond to sites as described in Table 4.2.

Table 4.4. Results of a univariate analysis of covariance on the lateral body shape divergence vector scores between *Poecilia* from sulfidic and non-sulfidic habitats.

| Effect | *F* | Hypothesis *df* | Error *df* | *P* | Partial Eta Squared | Relative variance |
|---|---|---|---|---|---|---|
| Intercept | 0.530 | 1.0 | 93.3 | 0.468 | 0.006 | 0.007 |
| Centroid size | 0.547 | 1.0 | 1067.0 | 0.460 | 0.001 | 0.001 |
| Sex | 90.425 | 1.0 | 1067.0 | <0.001 | 0.078 | 0.088 |
| **$H_2S$** | **123.415** | **1.0** | **16.2** | **<0.001** | **0.884** | **1.000** |
| Drainage | 3.092 | 3.0 | 15.1 | 0.059 | 0.381 | 0.431 |
| Site ($H_2S$ × Drainage) | 15.411 | 15.0 | 1067.0 | <0.001 | 0.178 | 0.201 |
| Sex × $H_2S$ | 33.671 | 1.0 | 1067.0 | <0.001 | 0.031 | 0.035 |
| Sex × Drainage | 0.232 | 3.0 | 1067.0 | 0.874 | 0.001 | 0.001 |
| $H_2S$ × Drainage | 4.092 | 3.0 | 15.0 | 0.026 | 0.450 | 0.509 |
| Sex × $H_2S$ × Drainage | 0.247 | 3.0 | 1067.0 | 0.863 | 0.001 | 0.001 |

For the dorsal projection (N=1093), analyses revealed that body shape particularly varied in head length and width, mouth width, and body width at the insertion of the pelvic fins (see Fig. 4.5). 'Presence of H2S' explained most variation in body shape, with ecotypes from sulfidic and non-sulfidic habitats particularly segregating along the first PC axis. As for the lateral projection, all other factors and the interaction terms were also significant predictors of body shape, but only 'sex', 'drainage', 'site', and the interaction of 'H2S × drainage' had relative variance >0.1 (Table 4.5).

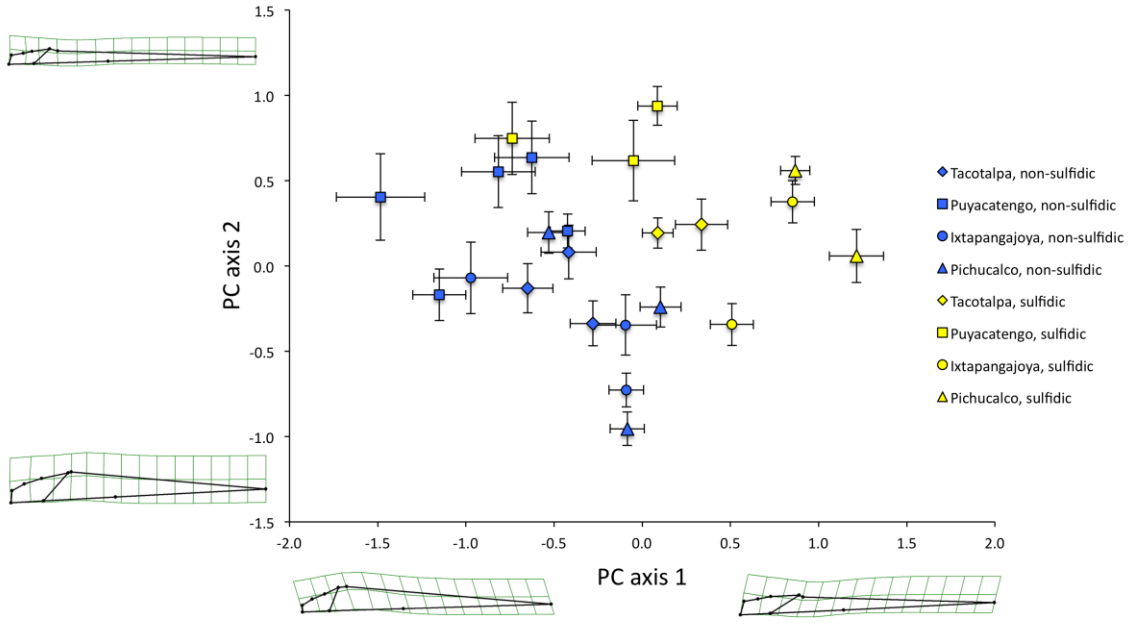


Figure 4.5. Body shape variation of *Poecilia* species in the dorsal projection. Depicted are mean principal component scores along the first two principal component axes for each site for *P. thermalis* (yellow circles), *P. sulphuraria* (yellow triangles), as well as sulfidic and non-sulfidic populations of *P. mexicana* across the 23 study sites in southern Mexico. The thin-plate spline transformation grids represent shape variation along each principal component axis.

Table 4.5. Results of a multivariate analysis of covariance on dorsal body shape of *Poecilia* from sulfidic and non-sulfidic habitats.

| Effect | *F* | Hypothesis *df* | Error *df* | *P* | Partial Eta Squared | Relative variance |
|---|---|---|---|---|---|---|
| Intercept | 6.221 | 8.0 | 1039.0 | <0.001 | 0.046 | 0.086 |
| Centroid size | 6.154 | 8.0 | 1039.0 | <0.001 | 0.045 | 0.084 |
| Sex | 15.988 | 8.0 | 1039.0 | <0.001 | 0.110 | 0.206 |
| **$H_2S$** | **148.306** | **8.0** | **1039.0** | **<0.001** | **0.533** | **1.000** |
| Drainage | 33.562 | 24.0 | 3014.0 | <0.001 | 0.205 | 0.385 |
| Site ($H_2S$ × Drainage) | 6.632 | 120.0 | 7409.6 | <0.001 | 0.087 | 0.163 |
| Sex × $H_2S$ | 2.605 | 8.0 | 1039.0 | 0.008 | 0.020 | 0.038 |
| Sex × Drainage | 3.191 | 24.0 | 3014.0 | <0.001 | 0.024 | 0.045 |
| $H_2S$ × Drainage | 17.269 | 24.0 | 3014.0 | <0.001 | 0.117 | 0.220 |
| Sex × $H_2S$ × Drainage | 3.105 | 24.0 | 3014.0 | <0.001 | 0.023 | 0.043 |

The strong differentiation between ecotypes in dorsal body shape was confirmed in the analysis of divergence vector scores with site being treated as a random factor (Table 4.6). Visualization of shape variation along the sulfide-non-sulfide divergence vector indicated that sulfide spring fish had longer heads, wider mouths, but narrower bodies (Fig. 4.6). Differentiation between ecotypes from sulfidic and non-sulfidic springs was highly significant for all sites and drainages investigated, including both *P. thermalis* populations. The only exception was site 14 (Río Puyacatengo road crossing), which exhibited an intermediate morphology (Fig. 4.6).

Table 4.6. Results of a univariate analysis of covariance on the dorsal body shape divergence vector scores between *Poecilia* from sulfidic and non-sulfidic habitats.

| Effect | *F* | Hypothesis *df* | Error *df* | *P* | Partial Eta Squared | Relative variance |
|---|---|---|---|---|---|---|
| Intercept | 17.959 | 1.0 | 311.3 | <0.001 | 0.055 | 0.060 |
| Centroid size | 18.768 | 1.0 | 1046.0 | <0.001 | 0.018 | 0.020 |
| Sex | 16.682 | 1.0 | 1046.0 | <0.001 | 0.016 | 0.018 |
| **$H_2S$** | **167.011** | **1.0** | **15.9** | **<0.001** | **0.913** | **1.000** |
| Drainage | 1.469 | 3.0 | 15.0 | 0.263 | 0.227 | 0.249 |
| Site ($H_2S$ × Drainage) | 7.703 | 15.0 | 1046.0 | <0.001 | 0.099 | 0.108 |
| Sex × $H_2S$ | 2.391 | 1.0 | 1046.0 | 0.122 | 0.002 | 0.002 |
| Sex × Drainage | 0.491 | 3.0 | 1046.0 | 0.689 | 0.001 | 0.001 |
| $H_2S$ × Drainage | 3.775 | 3.0 | 15.1 | 0.033 | 0.429 | 0.470 |
| Sex × $H_2S$ × Drainage | 1.756 | 3.0 | 1046.0 | 0.154 | 0.005 | 0.005 |

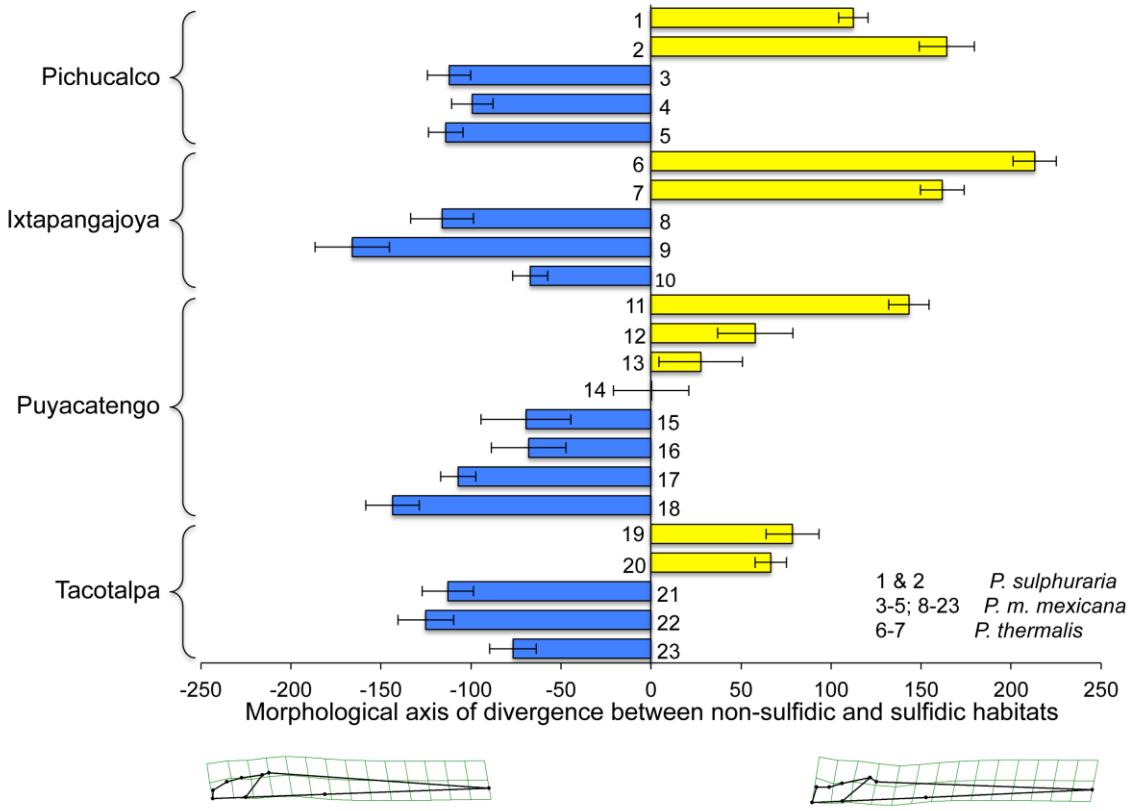


Figure 4.6. Convergent changes in body shape of *Poecilia* species from sulfidic and non-sulfidic habitats in the dorsal projection. Depicted are mean divergence scores (± SEM; derived from the $H_2S$ term in the MANCOVA) for each site for the three formal species (*P. thermalis*, *P. sulphuraria*, and *P. mexicana*) across the 23 sites in southern Mexico from sulfidic (yellow) and non-sulfidic (blue) populations. Numbers correspond to sites as described in Table 4.2.

The cluster analysis based on the combined lateral and dorsal datasets grouped 7 of 9 populations from sulfidic habitats together in a discrete cluster, highlighting a strong convergent pattern of body shape evolution in sulfide springs (Fig. 4.7). The two notable exceptions were fish from the La Lluvia big spring and the Puyacatengo springs (both in the Puyacatengo drainage), which were nested within non-sulfidic populations (Fig. 4.7). Most importantly, however, the *P. thermalis* samples from both La Esperanza springs in the

Ixtapangajoya drainage formed a cluster with the *P. sulphuraria* samples from the Baños del Azufre and the La Gloria springs in the Pichucalco drainage.

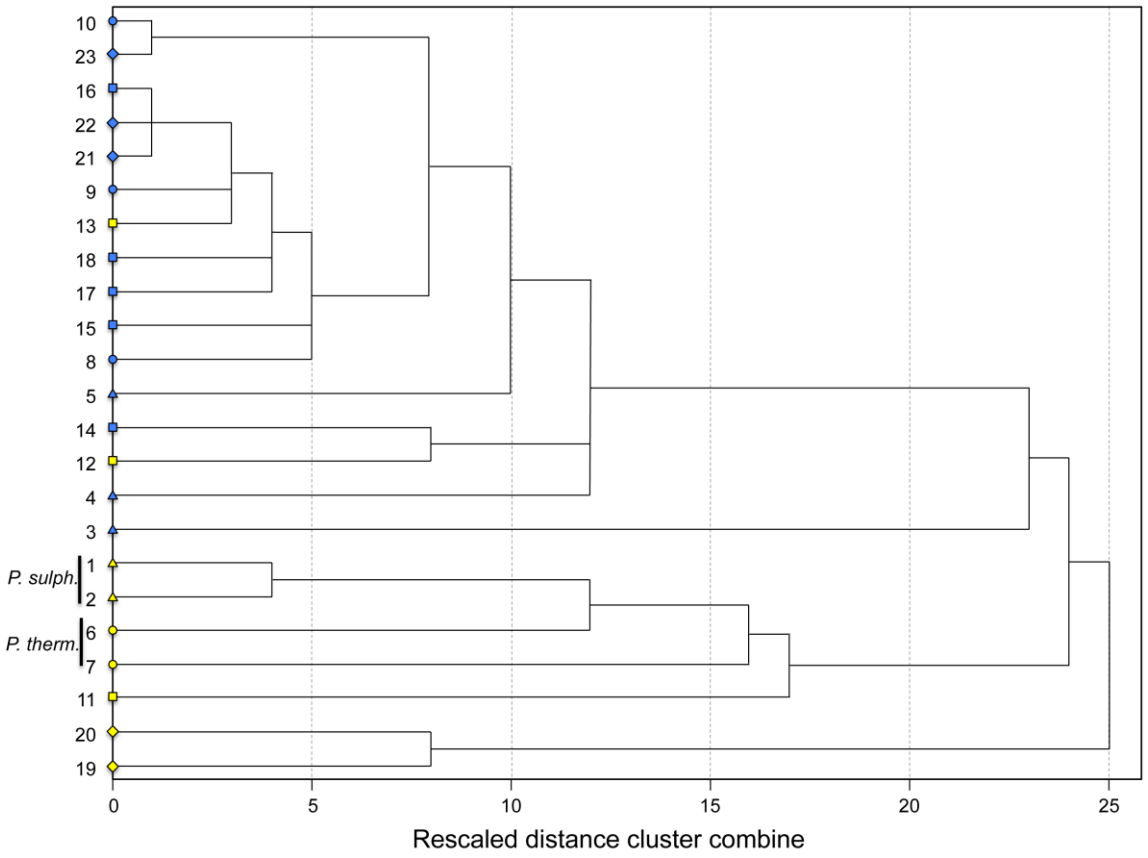


Figure 4.7. Hierarchical cluster analysis of *Poecilia* populations from sulfidic and non-sulfidic environments based on body shape variation in the lateral and dorsal projections. Colors denote sulfide-adapted (yellow) and non-adapted (blue) populations of three species, *Poecilia thermalis* (yellow circles), *P. sulphuraria* (yellow triangles), and *P. mexicana*. The shapes represent the drainages (diamonds- Tacotalpa, squares- Puyacatengo, circles- Ixtapangajoya, and triangles- Pichucalco) and the numbers correspond to sites as described in Table 4.2.

Finally, the DFA (subset of N=397) clearly grouped the 18 type specimens, for which we were able to obtain lateral body shape data, with contemporary sulfide spring samples, not with proximate non-sulfidic samples. Nonetheless, only 72.2 % of samples were assigned to the large La Esperanza spring (Fig. C2; Table C3), with the remaining individuals grouped

either with *P. sulphuraria* samples from the La Gloria springs (16.7 %) and Baños del Azufre (11.1 %). It should be noted at this point that the sample size for historical specimens was relatively low, such that within population variation in body shape may be underestimated. The examination of additional syntypes, which were not available at the time of our study, consequently could lead to a lower overall classification success.

*4.3.2. Phylogenetic analyses*

The evolutionary relationships in our phylogenetic analyses corroborate previously observed relationships in *Poecilia* among the main lineages of *Acanthophacelus*, *Micropoecilia*, *Limia*, *Pamphorichthys*, and *Mollienesia* (Meredith et al., 2010; Meredith et al., 2011). The results also show similar patterns previously observed in the relationships among species within the subgenus *Mollienesia*, with the sailfins, *P. latipinna* and *P. latipunctata*, forming a monophyletic group (Fig. 4.8; 100% BSS; 100% BPP). They are closely related to the monophyletic the shortfin group (100% BSS; 100% BSP (Tobler et al., 2011)) with an average genetic divergence of 7.6% (Table C4). Within the shortfin mollies, there is a separation among the *P. sphenops* clade (*P. catemaco* and *P. sphenops*; 100% BSS; 100% BSP) and the *P. mexicana* clade (*P. butleri*, *P. sulphuraria*, *P. thermalis*, *P m. mexicana*, *P. m. limantouri*; 100% BSS; 100% BSP) with an average genetic divergence of 6.7%. Phylogenetic analyses strongly (100% BSS and 100% BPP) indicate that *P. mexicana*, *P. sulphuraria*, and *P. thermalis* represent a monophyletic group. However, we did not find *P. sulphuraria* to be monophyletic, as *P. thermalis* is most closely related to the *P. sulphuraria* from the Baños de Azufre population, a relationship that is highly supported (87% BSS; 100% BSP). Genetic divergence between *P. thermalis* and this population of *P. sulphuraria*

was generally low (0.1%) and only slightly less than the divergence to the La Gloria population (0.2%; Table C4).

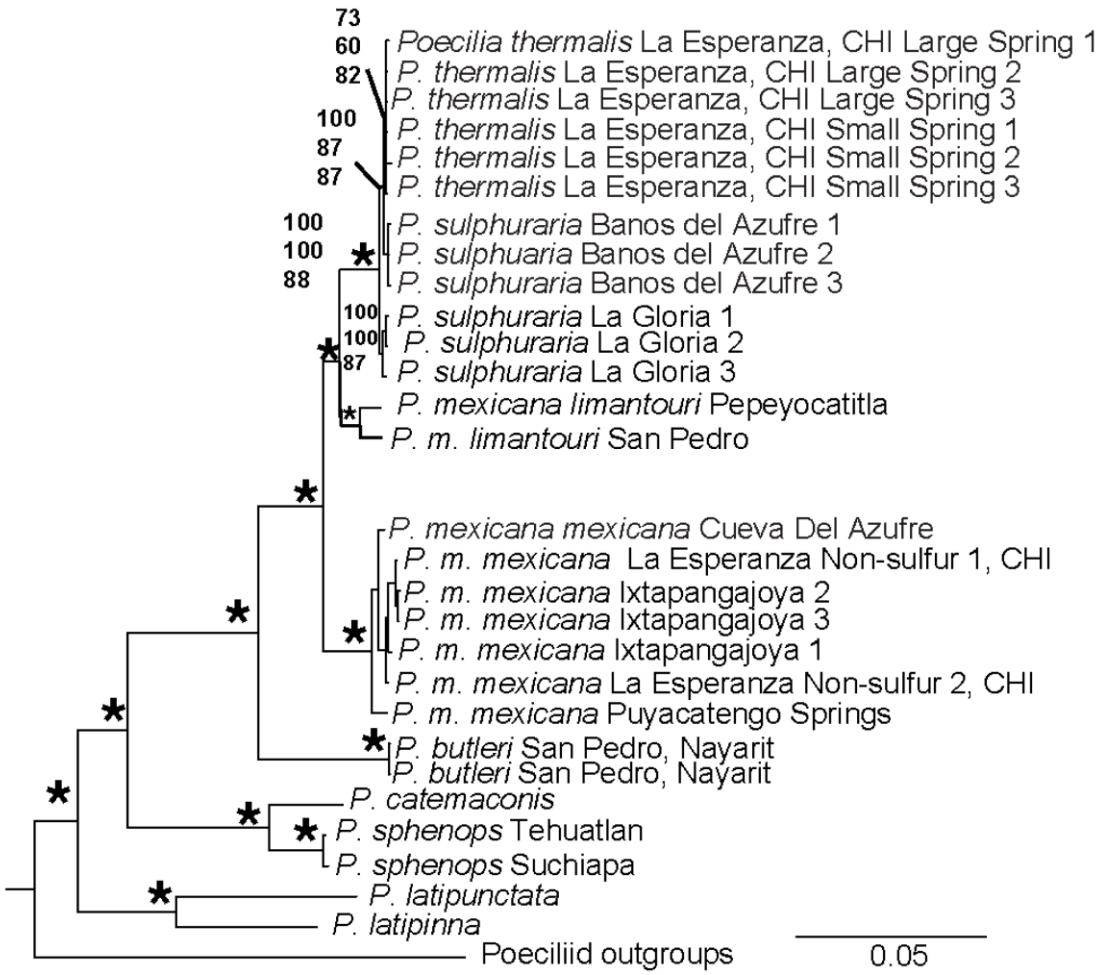


Figure 4.8. Bayesian tree from phylogenetic analysis of *Poecilia* species for five markers rooted with poeciliid outgroups. Phylogenetic analyses of two mitochondrial and three nuclear genes (5337 base pairs) yielded nodal support values (in percent) represent (from top to bottom) Bayesian Posterior Probabilities, as well as RAxML, and GARLI bootstrap support values. Asterisks denote nodal support of ≥95% for all three methods. Nodes with no values present either had low values or were of little interest for this study.

*4.3.3. Population genetic analyses*

Our Bayesian clustering analysis uncovered K= 2 as the uppermost hierarchical level of population structure. *Poecilia sulphuraria* (*sensu lato*; i.e, including both the Baños del Azufre and the La Gloria population) and *P. thermalis* together formed one genetic cluster that was distinct from all *P. mexicana* populations (Fig. 4.9). The only exception was one animal caught in the small Esperanza spring, which was assigned to *P. mexicana*, not *P. thermalis*. Another peak for ln P (X|K) —i.e., the second most likely level of population structure according to Evanno et al. (2005)— was obtained for K= 7. In addition to detecting population genetic structure within and between drainages in *P. mexicana* a clear separation between *P. thermalis* and *P. sulphuraria* (s.l.) became apparent (Fig. 4.9), indicating low recurrent gene flow between them. Pairwise FST-values (Table C5) revealed significant genetic differentiation between populations of *P. thermalis* and *P. sulphuraria* (0.086-0.103) as well as between populations within each species (*P. thermalis*, 0.031; P. *sulphuraria*, 0.082). Further support for genetic differentiation between the two species was obtained from the individual-based PCA (Fig. C3).

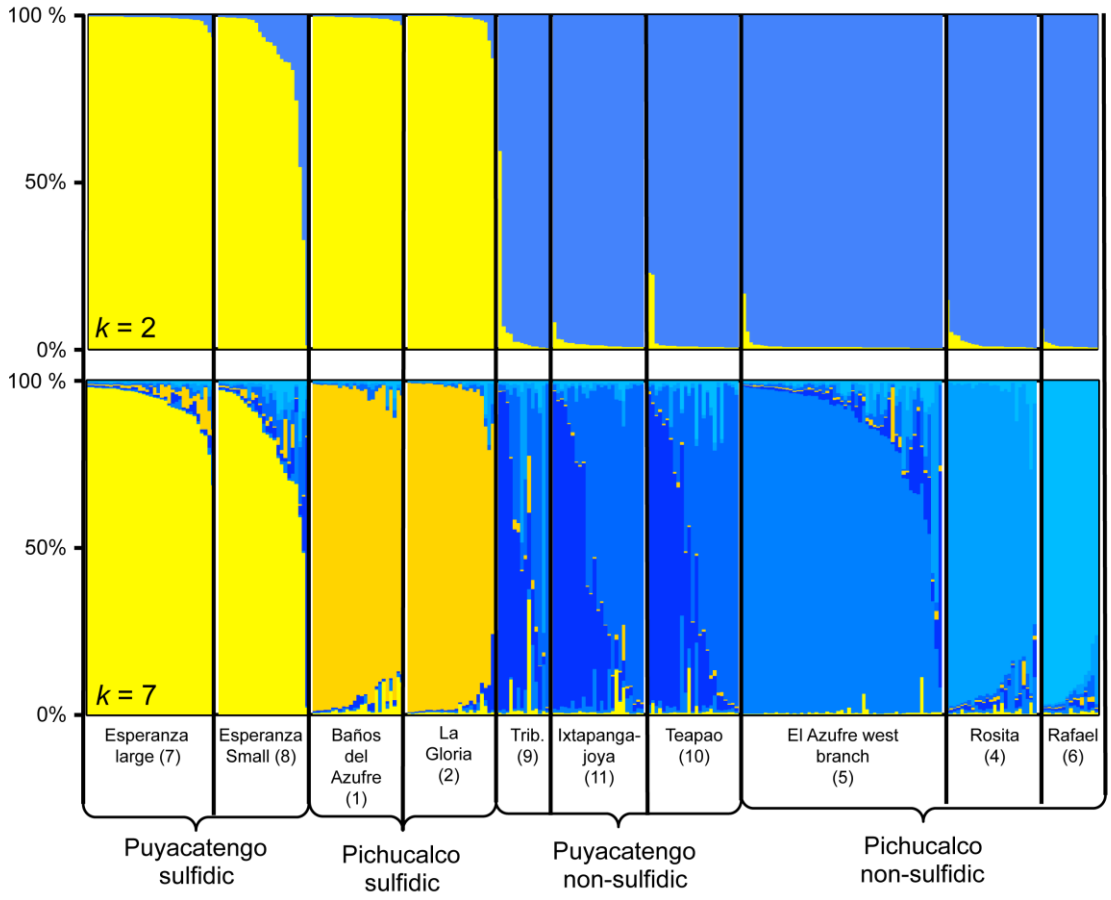


Figure 4.9. Genetic structure among populations based on microsatellites analysis in *N* = 272 individuals from 10 populations. The top panel is a bar plot showing the assignment scores of individuals by STRUCTURE with *K = 2* with yellow representing *P. thermalis* and *P. sulphuraria* as a cluster from the Puyacatengo and Pichucalco sulfidic drainages and blue representing *P. m. mexicana* from non-sulfidic sites in the same drainage. The bottom panel is a bar plot showing the assignment scores clustering at *K = 7*, the second most likely number of distinct groups.

### 4.4. Discussion

The re-discovered species, *Poecilia thermalis*, is a highly endemic sulfide spring fish found to inhabit two proximate sulfidic springs in the Ixtapagajoya drainage of southern Mexico. Morphological features exhibited by *P. thermalis* closely resemble those of other sulfide spring fishes, independent of their phylogenetic relationship, highlighting strong patterns of convergent morphological evolution in sulfidic environments. Phylogenetic analyses placed

*P. thermalis* as sister taxon to one (Baños del Azufre) of the two populations of the sulfidic spring endemic *P. sulphuraria* (Alvarez del Villar, 1947) from the Pichucalco drainage with low — albeit significant — genetic distance. In addition, population genetic analyses detected little or no current gene flow between *P. thermalis* and *P. m. mexicana* from adjacent non-sulfidic habitats or *P. sulphuraria* from sulfidic springs in the Pichucalco drainage, respectively, indicating an independent evolutionary trajectory of *P. thermalis*.

*4.4.1. Morphological variation*

In general, body shape analyses of sulfidic and non-sulfidic fishes in southern Mexico demonstrated a clear differentiation between habitat types across drainages, except for the Puyacatengo drainage. In the latter, phenotypic differentiation—particularly in the lateral projection—was less pronounced and more gradual, which could be driven by the higher spatial heterogeneity in the presence of H2S (see Plath et al. 2013 for a discussion). Our results support previous analyses suggesting that fish in the Puyacatengo drainage were not as differentiated along a speciation continuum as sulfide spring fish from other drainages, because either they colonized sulfide springs more recently or gene flow across habitat types constrains phenotypic divergence (Plath et al. 2013). Despite the strong patterns of convergence, we also found significant differences in body shape between sulfidic fish among drainages, indicating that there is both convergent and non-convergent aspects trait differentiation in response to sulfide exposure.

The phenotypic analyses of lateral body shape indicated that fishes from sulfidic and non-sulfidic habitats primarily differ in head size, with populations from sulfidic habitats having significantly larger heads. As such, our study validated previous findings from the

genus *Poecilia* (Tobler et al., 2011) and other poeciliids (Tobler and Hastings, 2011) with the most comprehensive sampling of sulfide spring mollies to date. Previous studies have shown that head size is positively correlated gill surface area, which is adaptive in sulfidic environments because sulfide springs exhibit low oxygen concentrations and sulfide detoxification requires additional oxygen (Tobler et al., 2011). Modification of respiratory morphological traits in conjunction with changes in respiratory behavior represent a critical adaptation mediating survival in the sulfidic and hypoxic environments (Plath et al., 2007; Tobler et al., 2009a).

Our study is the first to examine body shape variation in the dorsal projection and found sulfide spring fishes to exhibit longer and wider heads, wider mouths, and narrower bodies. Sulfidic spring fishes are known to rely on aquatic surface respiration (ASR), i.e., they skim the water from the air-water interface (with higher dissolved oxygen concentrations) using their gills (Plath et al., 2007; Tobler et al., 2009a). Wider heads and mouths likely are adaptive, because they maximize the uptake of surface water as reported in neotropical characids (Winemiller, 1989) and serrasalmids (Scarabotti et al., 2009; Scarabotti et al., 2011), which exhibit temporary dermal swellings of the lower jaw allowing for an increased efficiency of ASR when exposed to hypoxic conditions. The decrease in body width may be associated with a reduced body condition previously documented in fish from sulfidic habitats (Plath et al., 2005; Tobler, 2008; Tobler et al., 2006).

Our morphological analyses also provided critical insights about the rediscovered P. *thermalis*. Most importantly, specimens collected by Heller in 1848 mostly grouped with samples from our 2012 survey, suggesting we have visited the locality described in Heller's autobiography (Heller, 1853). We found *P. thermalis* to exhibit a typical sulfide spring body

shape and to be phenotypically similar to *P. sulphuraria*. Qualitatively, this is also the case for color patterns (pronounced turquoise highlights on the abdomen with a relatively dark dorsal coloration, which are not found in sulfidic *P. mexicana* populations) and lateral lip appendages on the lower jaw (authors, personal observation), which are mentioned in the species description of *P. sulphuraria* (Alvarez del Villar, 1947). Note, however, that such lip appendages are not a diagnostic trait for *P. sulphuraria* (s. l.), as specimens from the La Gloria population do not exhibit this morphological trait (Tobler and Plath, 2009). Despite the close morphological affinity of *P. thermalis* to *P. sulphuraria* (as compared to sulfidic and non-sulfidic populations of *P. mexicana*), our analyses indicated significant differences in body shape between the two species both in the lateral and dorsal projection.

*4.4.2. Phylogenetic analyses and population genetics*

The broad phylogenic relationships uncovered in our study match the patterns of other studies (Alda et al., 2013; Meredith et al., 2010; Meredith et al., 2011; Tobler et al., 2011). We found *P. thermalis* collected in both La Esperanza springs (Ixtapangajoya drainage) to be sister to P. *sulphuraria* from the Baños del Azufre population (Pichucalco drainage), which together were sister to *P. sulphuraria* from La Gloria (also Pichucalco drainage) and formed a monophyletic group. This group (*P. thermalis* and *P. sulphuraria* together) was more closely related to the northern Mexican subspecies of *P. m. limantouri* than the southern P. *m. mexicana* populations from adjacent non-sulfidic sites, corroborating earlier investigations (Tobler et al., 2011). This suggests that colonization of sulfide springs in the Ixtapangajoya and Puyacatengo drainages by the *P. m. limatouri*-like ancestor shared by *P. thermalis* and *P. sulphuraria* occurred earlier than sulfide spring colonization by *P. m. mexicana* in the other

drainages. This is reflected in higher genetic divergences between sulfide spring and adjacent non-sulfidic populations in the Ixtapangajoya and Pichucalco drainages (2.0-2.2% in mitochondrial genes) compared to the Puyacatengo and Tacotalpa drainages (0.1-0.4%; see Tobler et al., 2011 for a discussion).

Our results indicate that sulfide springs in the Pichucalco and the Ixtapangajoya drainages were not colonized independently, but rather *P. sulphuraria* and *P. thermalis* are of a single evolutionary origin despite their current distribution in independent drainages, which in the area of the sulfide springs are separated by mountainous terrain reaching more than 500 meters above the surrounding elevation. This can be explained by the dynamic nature of the courses of major river systems in southern Mexico (Psuty, 1965; West et al., 1969). Historically, the Grijalva River was an independent deltaic system that followed the course of Ixtapangajoya river (Böse, 1905), presenting an opportunity for connections between currently independent tributaries, particularly during periods of heavy rain and flooding between tributaries. However, considering the reduced viability of sulfide adapted fish in non-sulfidic environments (Plath et al. 2013; Plath et al., 2010b) and consequently the absence of sulfide-adapted ecotypes even in proximate freshwater habitats (Plath et al., 2010a), it remains unclear how colonization through stretches of unsuitable habitats was possible even in the presence of potential connections among drainages. Hence, the alternative hypothesis is that colonization of different springs in the two drainages could have occurred independently by a once widespread ancestor (a lineage with close affinities to extant northern Mexican *P. m. limantouri*) with standing genetic variation for traits adaptive to sulfidic springs. Such a scenario was recently supported in stickleback, where low rates of gene flow from freshwater to marine populations maintain freshwater alleles in the marine

environments at low frequency, such that selection upon colonization of a new freshwater system can rapidly reassemble freshwater ecotypes based on allelic variants already present in the ancestral population (Jones et al., 2012; Schluter and Conte, 2009). The currently available data does not allow for rigorously testing these contrasting hypotheses, and additional research including a more thorough analysis of the northern Mexican *P. m. limantouri* is required to elucidate historical patterns of sulfide spring colonization in the Ixtapangajoya and Puyacatengo drainages. Nonetheless, our data indicate that sulfide spring colonization may not have occurred independently in different drainages, adding an additional layer of complexity in the analysis of speciation patterns in sulfidic spring fishes.

Our population genetic analyses largely supported the phylogeny in that the uppermost level of population differentiation included two clusters distinguishing between populations in sulfidic (*P. sulphuraria* and *P. thermalis*) and non-sulfidic (*P. mexicana*) environments irrespective of the drainage of origin. As such, the results generally support previously uncovered patterns of strong reproductive isolation between sulfide spring residents and fish from adjacent non-sulfidic sections of the same drainage (Plath et al. 2013). Reproductive isolation among ecotypes is at least partially mediated by natural and sexual selection against immigrants, where migrant individuals from the opposite habitat type have reduced survivability and are discriminated against during mate choice (Plath et al., 2006; Plath et al. 2013; Schluter and Conte, 2009). Our analyses also found strong support for genetic structure with K=7 divergent clusters. At this finer scale, the two *P. thermalis* populations from the Ixtapangajoya drainage were clearly distinct from the two *P. sulphuraria* populations in the adjacent Pichucalco drainage, reflecting the absence of gene flow due to the lack of contemporary connections between the two drainages.

*4.4.3. Taxonomic considerations and conclusions*

The taxonomic history of *Poecilia thermalis* remained uncertain since its original description by Steindachner in 1863. Our study revisited the status of *P. thermalis* based on recently collected material from the type locality and museum specimens using morphological, phylogenetic, and population genetic approaches. Based on our findings, we can clearly reject the previously prevalent notion that *P. thermalis* Steindachner 1863 is synonymous to either *P. salvatoris* (Regan, 1907), *P. sphenops* (Gordon and Rosen, 1962), or P. *mexicana* (*sensu stricto*, Miller, 1983). However, in relation to the sulfide spring populations from the Pichucalco drainage, currently denominated *P. sulphuraria* Alvarez 1947, taxonomic change can proceed in the form of two alternatives. Sulfide spring populations from the Ixtapangajoya (*P. thermalis*) and the Pichucalco (*P. sulphuraria*) could be considered as derivatives from the same evolutionary lineage and therefore considered the same species. In this case, the older names takes precedence (IUCN, 2012) and *P. sulphuraria* (Alvarez del Villar, 1947) would be designated as a junior synonym of *P. thermalis*. Alternatively, *P. thermalis* could be designated as a valid, distinct species restricted to the Ixtapangajoya drainage, which would require the name *P. sulphuraria* to be restricted to the type locality (Baños del Azufre) and the sulfide spring population at La Gloria (currently a population of *P. sulphuraria*) to be considered a distinct species awaiting formal description. This interpretation is supported by reciprocal monophyly, significant population genetic differentiation as evident from FST values and principal components analysis, and significant differences in body shape among all three groups. Examination of additional characters, especially meristic traits and the structure of the male copulatory organ (gonopodium),

commonly used in poeciliid systematics will hopefully lead to the resolution of the taxonomic conundrum surrounding *P. thermalis*.

Regardless of the taxonomic conclusions, our study has direct implications for the conservation of the sulfide spring populations in the Ixtapangajoya and Pichucalco drainages. Currently, P. s*ulphuraria* is listed as threatened and federally protected by the Mexican government (Sedesol, 2010). In addition, the IUCN has listed the species as critically endangered because of a very limited distribution (IUCN, 2012), and the species is threatened by deforestation, farming, recreational activities, and more recently by extensive palm oil plantations (Tobler and Plath, 2009). Despite these concerns, no conservation measures have been implemented to mitigate these effects (Tobler and Plath, 2009). Potential taxonomic changes will require according changes in the list of endangered species in Mexico. Whether all three populations will be designated as *P. thermalis* or as three distinct species in the future, they clearly represent unique evolutionary lineages with highly restricted distributions meriting separate management and a high priority for conservation (Ryder, 1986).

Overall, this study confirms the role of hydrogen sulfide in shaping convergent, phenotypic evolution in sulfide spring fishes and causing reproductive isolation between populations residing in proximate sulfidic and non-sulfidic environments. It also illustrates how an integrative, mechanistic approach to studying phenotypic evolution and speciation can inform taxonomy.

# CHAPTER V

# GENERAL CONCLUSIONS

This dissertation explored the evolutionary and ecological drivers of diversification in the subgenus *Mollienesia*, identifying vicariant, dispersal, and ecological events. The main findings of chapter II include discovering that Caribbbean taxa previously assigned to the subgenus *Mollienesia* are in fact more closely related to other Caribbean subgenera. In addition, I identified two distinct lineages in the subgenus *Mollienesia* that may represent new, undescribed species upon further investigation, and I found large discrepancies in the identification of species within the subgenus *Mollienesia* based on the phylogenetic results. This chapter also uncovered uncertainties surrounding the application of molecular clock estimates applying a fossil and gene rate of evolution calibration points, leading to inconclusive results in the timing of diversification among species of the subgenus *Mollienesia*. Despite the uncertainties of the time estimates, the uplift of several geologic formations was attributed to the isolation and subsequent evolution of several species, which are in agreement with other organisms present in the same areas. Lastly, the ancestral area estimations indicate that the origin of the genus *Poecilia* was located in South America, with multiple subsequent dispersal events into the Caribbean and Lower Central America. The origins of the main clades in the subgenus *Mollienesia* include the Maya and the Chortis blocks due to the isolation of these areas by faults and geologic activity.

In chapter III, I found that the diversity of *Mollienesia* from Mexico in a result of multiple independent invasions from Middle America with subsequent speciation. Two species found north of the Trans Mexican Volcanic belt (*Poecilia butleri* and *P. limantouri*) and one transversal species (*P. sphenops*) have weak phylogeographic structuring, whereas three species (*P. nelsoni, P. mexicana, P. sulphuraria/thermalis*) found south of the Trans Mexican Volcanic Belt exhibit strong phylogeographic structure. The weak phylogeographic structure in some species is likely a consequence the lack of physiographic barriers, recent colonization, and high dispersal rates among regions. In contrast, species with strong phylogeographic structure have inhabited the area for a longer period of time and the presence of multiple physiographic barriers created disjunctions among river basins.

In chapter IV, the main research discoveries include the rediscovery of the sulfide spring endemic species *P. thermalis* after 150 years. I document the presence of convergent morphological evolution of *P. thermalis* with other sulfide spring fishes, having longer and wider heads for aquatic surface respiration. The systematics of *P. thermalis* and its close relative *P. sulphuraria* is complicated based on their phylogenetic placement, despite genetic isolation at the population level indicating they may be distinct, valid species. The study recommends the implementation of conservation actions for sulfide spring habitats, which are currently threatened by agriculture.

Overall, this dissertation also highlighted the vulnerability of some species in the subgenus *Mollienesia* to becoming extinct because of their limited distribution and habitat specificity (*P. chica, P. catemaconis*, *P. sulphuraria,* and *P. thermalis*). Therefore, there is a need to conserve their habitats to ensure the protection of these species and future loss of biodiversity. Despite the major threats of habitat degradation by pollution, invasive species,

and fragmentation of freshwater habitats, two species (*P. sphenops* and *P. mexicana*) have demonstrated resilience and adaptability. The findings of this study highlight the need for more phylogeographic investigations of freshwater fish to better account for the biodiversity, understand the mechanisms driving the origin of species, improving systematics in order to make informed decisions on the conservation of both species and ecosystems.

In order to advance our understanding of research on species in the subgenus *Mollienesia*, a taxonomic revision needs to be conducted based on morphological, systematic, and ecological information. Future studies should establish clear species delineations, further assess the biodiversity, and determine accurate distribution ranges. This will require investigating specimens from historical type localities and the designation of neotypes in cases where original collections are unclear, morphological comparisons among museum and newly collected specimens, and assessing the ecological characteristics of each species. Based on field sampling, up to four species co-occur in the same habitat (e.g. Lake Nicaragua), and future research should investigate niche partitioning among these sympatric species by assessing habitat use, trophic resource partitioning, and linking them to eco-morphological differences. The species within the subgenus *Mollienesia* can also drastically change their coloration to adapt to ambient the environmental conditions and future research could focus on understanding color background responses and the mechanisms driving such changes. In addition, there are several color variant patterns, particularly within the *P. mexicana* species complex that should be further investigated for genetic inheritance. Lastly, the distribution of species in the subgenus *Mollienesia* as a common aquarium pet is contributing to the increase of releasing species into non-native environments worldwide, which requires evaluation as invasive species.

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# APPENDIX A

Table A1. Gene accession numbers for sequences used in this study.

| Taxa | Locality[a] | Genes | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | X-src | Myh6 | ENC1 | Glyt | SH3PX3 | Rh | Rag1 | NADH2 | Cyt b |
| *Cnesterodon decemmaculatus*^ | Canãda, Artigas, Uruguay | GU179152 | GU179243 | GU179168 | GU179197 | GU179214 | GU179271 | EF017427 | EF0175791 | EF017529 |
| *Cnesterodon hypselurus*^ | Cilada, Paraná, Brazil | GU179153 | GU179244 | GU179169 | GU179198 | GU179215 | GU179272 | GU179260 | GU179231 | GU179185 |
| *Poecilia (Acanthophacelus) reticulata*^ | Turure River, Trinidad | GU179162 | GU179253 | GU179178 | GU179207 | GU179224 | GU179281 | EF017434 | GU179238 | GU179192 |
| *Poecilia (Acanthophacelus) wingei* | Aquarium stock | GU179163 | GU179254 | GU179179 | GU179208 | GU179225 | GU179282 | GU179267 | GU179239 | GU179193 |
| *Poecilia (Acanthophacelus) wingei*^ | Cumaná, Venezuela | GU179164 | GU179255 | GU179180 | GU179209 | GU179226 | GU179283 | GU179268 | GU179240 | GU179194 |
| *Poecilia (Allopoecilia) caucana*^ | Panama | U02355 | GU179258 | GU179183 | GU179212 | GU179229 | GU179286 | EF017437 | EF017589 | EF017540 |
| *Poecilia (Psychropoecilia) dominicensis*^ | Dominican Republic | KP943136* | KP943214* | KP943282 | KP943257* | - | KP943175* | KP943194* | KP943308* | KP943160* |
| *Poecilia (Curtipenis) elegans*^ | Dominican Republic | KP943137* | KP943215* | KP943283* | KP943258* | KP943234* | KP943176* | KP943195* | KP943309* | KP943161* |
| *Poecilia (Psychropoecilia) hispaniolana*^ | Dominican Republic | KP943138* | KP943216* | KP943284* | KP943259* | KP943235* | - | KP943196* | KP943310* | KP943162* |
| *Poecilia (Limia) caymenensis* | | KJ697601 | KJ697122 | KJ696902 | KJ697012 | KJ697495 | KJ697385 | KJ697291 | AF353192 | KJ696810 |
| *Poecilia (Limia) dominicensis*^ | River Picot, Pont Salomon, Haiti | GU179154 | GU179245 | GU179170 | GU179199 | GU179216 | GU179273 | EF017431 | EF017582 | EF017533 |
| *Poecilia (Limia) dominicensis*^ | Dominican Republic | KP943134* | KP943212* | KP943280* | KP943255* | KP943232* | - | KP943192* | KP943306* | - |
| *Poecilia (Limia) garnieri* | | KJ697602 | KJ697123 | KJ696903 | KJ697013 | KJ697496 | KJ697386 | KJ697292 | NA | KJ696811 |
| *Poecilia (Limia)* | | KJ697603 | KJ697124 | KJ696904 | KJ697014 | KJ697497 | KJ697387 | KJ697293 | NA | KJ696812 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *grossidens* | | | | | | | | | | |
| *Poecilia (Limia) heterandria*^ | Puerto Cabello, Venezuela | HQ857432 | HQ857456 | HQ857468 | HQ857462 | HQ857420 | HQ857438 | HQ857444 | HQ857450 | HQ857426 |
| *Poecilia (Limia) melanogaster*^ | Aquarium stock | GU179155 | GU179246 | GU179171 | GU179200 | GU179217 | GU179274 | EF017432 | EF017583 | EF017534 |
| *Poecilia (Limia) melanotata* | | KJ697604 | KJ697125 | KJ696905 | KJ697015 | KJ697498 | KJ697388 | KJ697294 | AF353197 | KJ696813 |
| *Poecilia (Limia) nigrofasciata* | | KJ697605 | KJ697126 | KJ696906 | KJ697016 | KJ697499 | KJ697389 | KJ697295 | AF031391 | KJ696814 |
| *Poecilia (Limia) pauciradiata* | | KJ697606 | KJ697127 | KJ696907 | KJ697017 | KJ697500 | KJ697390 | KJ697296 | AF353196 | KJ696815 |
| *Poecilia (Limia) perugiae* | | KJ697607 | KJ697128 | KJ696908 | KJ697018 | KJ697501 | KJ697391 | KJ697297 | AF031392 | KJ696816 |
| *Poecilia (Limia) perugiae*^ | Dominican Republic | KP943135* | KP943213* | KP943281* | KP943256* | KP943233* | KP943174* | KP943193* | KP943307* | KP943159* |
| *Poecilia (Limia) rivasi* | | KJ697608 | KJ697129 | KJ696909 | KJ697019 | KJ697502 | KJ697392 | KJ697298 | NA | KJ696817 |
| *Poecilia (Limia) sulphorophila* | | KJ697609 | KJ697130 | KJ696910 | KJ697020 | KJ697503 | KJ697393 | KJ697299 | NA | KJ696818 |
| *Poecilia (Limia) tridens* | | KJ697610 | KJ697131 | KJ696911 | KJ697021 | KJ697504 | KJ697394 | KJ697300 | EF017584 | EF017535 |
| *Poecilia (Limia) versicolor* | | KJ697611 | KJ697132 | KJ696912 | KJ697022 | KJ697505 | KJ697395 | KJ697301 | AF353193 | KJ696819 |
| *Poecilia (Limia) vittata* | | KJ697612 | KJ697133 | KJ696913 | KJ697023 | KJ697506 | KJ697396 | KJ697302 | AF353201 | KJ696820 |
| *Poecilia (Limia) zonata* | | KJ697613 | KJ697134 | KJ696914 | KJ697024 | KJ697507 | KJ697397 | KJ697303 | AF353194 | KJ696821 |
| *Poecilia (Mollienesia) butleri*^ | San Pedro, Nayarit, Mexico | KP943148* | KF276643 | KP943294* | KP943269* | KP943244* | KF276730 | KF276701 | KF276672 | KF276614 |
| *Poecilia (Mollienesia) catemaconis* | Lake Catemaco, Veracruz, Mexico | KP943144* | KF276639 | KP943290* | KP943265* | KP943240* | KF276726 | KF276697 | KF276668 | KF276610 |
| *Poecilia (Mollienesia) chica*^ | Jalisco, Mexico | KJ697628 | KJ697149 | KJ696929 | KJ697039 | KJ697522 | KJ697412 | KJ697311 | KJ697230 | KJ696830 |
| *Poecilia (Mollienesia) hondurensis* | Rio Lancentilla, Honduras | KP943147* | KP943223* | KP943293* | KP943268* | KP943243* | KP943183* | KP943203* | KP943315* | KP943168* |
| *Poecilia (Mollienesia) kykesis*^ | Tabasco, Mexico | KP943141* | KP943218* | KP943287* | KP943262* | KP943237* | KP943178* | KP943198* | KP943311* | KP943164* |
| *Poecilia (Mollienesia) latipinna*^ | North Carolina, USA | KP943140* | KF276638 | KP943286* | KP943261* | KP943236* | KF276725 | KF276696 | KF276667 | KF276609 |
| *Poecilia (Mollienesia) latipunctata*^ | Ciudad Mante, Tamaulipas, Mexico | GU179167 | GU179259 | GU179184 | GU179213 | GU179230 | GU179287 | EF017436 | EF017588 | EF017539 |
| *Poecilia (Mollienesia) marcellinoi*^ | La Libertad, El Salvador | KP943145* | KP943221* | KP943291* | KP943266* | KP943241* | KP943181* | KP943201* | KP943314* | KP943167* |

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|---|---|---|---|---|---|---|---|---|---|---|
| *TCWC 16336.01* | | | | | | | | | | |
| *Poecilia (Mollienesia) limantouri*^ | Baretal, Mexico | KP943153* | KP943226* | KP943300* | KP943274* | KP943249* | KP943186* | KP943206* | HQ677845 | HQ677873 |
| *Poecilia (Mollienesia) mexicana* ^ | Tabasco, Mexico | KP943157* | KP943230* | KP943304* | KP943278* | KP943253* | KP943190* | KP943210* | HQ677857 | HQ677897 |
| *Poecilia (Mollienesia) mexicana* ^ | Tabasco, Mexico | KP943158* | KP943231* | KP943305* | KP943279* | KP943254* | KP943191* | KP943211* | HQ677854 | HQ677894 |
| *Poecilia (Mollienesia) nelsoni*^ | Barra de Navidad, Jalisco | KP943149* | KP943224* | KP943295* | KP943270* | KP943245* | KP943184* | KP943204* | KP943295* | KP943169* |
| *Poecilia (Mollienesia) orri* | Prinzapolka, Nicaragua | KP943150* | KP943225* | KP943296* | KP943271* | KP943246* | KP943185* | KP943205* | KP943296* | KP943170* |
| *Poecilia (Mollienesia) petenensis*^ | | KJ697630 | KJ697151 | KJ696931 | KJ697041 | KJ697524 | KJ697414 | KJ697313 | KJ697231 | KJ696832 |
| *Poecilia (Mollienesia) "sp3" TCWC 16349.01* | Rio Paso Hondo, El Salvador | KP943156* | KP943229* | KP943303* | KP943277* | KP943252* | KP943189* | KP943209* | KP943319* | KP943173* |
| *Poecilia (Mollienesia) sp. "Tipitapa" TCWC 16369.01* | Lake Nicaragua, Nicaragua | KP943142* | KP943219* | KP943288* | KP943263* | KP943238* | KP943179* | KP943199* | KP943312* | KP943165* |
| *Poecilia (Mollienesia) "sp2" TCWC 16366.01* | Puerto Cabezas, Nicaragua | KP943154* | KP943227* | KP943301* | KP943275* | KP943250* | KP943187* | KP943207* | KP943317* | KP943171* |
| *Poecilia (Mollienesia) salvatoris TCWC 16367.01* | Lake Xiloa, Nicaragua | KP943155* | KP943228* | KP943302* | KP943276* | KP943251* | KP943188* | KP943208* | KP943318* | KP943172* |
| *Poecilia (Mollienesia) sphenops* | Pomposa Castellano, Mexico | KP943146* | KP943222* | KP943292* | KP943267* | KP943242* | KP943182* | KP943202* | HQ677862 | HQ677899 |
| *Poecilia (Mollienesia) "sphenops 1"*^ | Choluteca, Honduras | KP943143* | KP943220* | KP943289* | KP943264* | KP943239* | KP943180* | KP943200* | KP943313* | KP943166* |
| *Poecilia (Mollienesia) sulphuraria*^ | La Gloria, Mexico | KP943152* | KF276656 | KP943299* | KP943273* | KP943248* | KF276742 | KF276713 | KF276685 | KF276627 |
| *Poecilia (Mollienesia) sulphuraria*^ | Banos del Azufre, Mexico | - | KF276652 | KP943298* | - | - | KF276739 | KF276710 | KF276681 | KF276623 |
| *Poecilia (Mollienesia) thermalis*^ | La Esperanza, Mexico | KP943151* | KF276646 | KP943297* | KP943272* | KP943247* | KF276734 | KF276705 | KF276676 | KF276618 |
| *Poecilia (Mollienesia) velifera*^ | Yucatan, Mexico | KP943139* | KP943217* | KP943285* | KP943260* | - | KP943177* | KP943197* | - | KP943163* |
| *Poecilia (Micropoecilia) bifurca*^ | Coropina Creek, Republiek district, Suriname | GU179156 | GU179247 | GU179172 | GU179201 | GU179218 | GU179275 | GU179261 | GU179232 | GU179186 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Poecilia (Micropoecilia) branneri*^ | João Alves Stream, Pará State, Brazil | GU179157 | GU179248 | GU179173 | GU179202 | GU179219 | GU179276 | GU179262 | GU179233 | GU179187 |
| *Poecilia (Micropoecilia) parae*^ | Rowa, French Guyana | GU179158 | GU179249 | GU179174 | GU179203 | GU179220 | GU179277 | GU179263 | GU179234 | GU179188 |
| *Poecilia (Micropoecilia) parae* | Leliendaal or Emmastraat, Suriname | GU179159 | GU179250 | GU179175 | GU179204 | GU179221 | GU179278 | GU179264 | GU179235 | GU179189 |
| *Poecilia (Micropoecilia) picta*^ | Orinoco River Delta, Venezuela | GU179160 | GU179251 | GU179176 | GU179205 | GU179222 | GU179279 | GU179265 | GU179236 | GU179190 |
| *Poecilia (Micropoecilia) picta* | Marianne River, Trinidad | GU179161 | GU179252 | GU179177 | GU179206 | GU179223 | GU179280 | GU179266 | GU179237 | GU179191 |
| *Poecilia (Pamphorichthys) araguaiensis* ^ | Cristalino River, Mato Grosso, Brazil | GU179165 | GU179256 | GU179181 | GU179210 | GU179227 | GU179284 | GU179269 | GU179241 | GU179195 |
| *Poecilia (Pamphorichthys) hasemani*^ | Máximo Lake, Amazonas, Brazil | GU179166 | GU179257 | GU179182 | GU179211 | GU179228 | GU179285 | GU179270 | GU179242 | GU179196 |
| *Poecilia (Pamphorichthys) hollandi*^ | Cavalos Lagoon, Brazil | HQ857434 | HQ857458 | HQ857470 | HQ857464 | HQ857422 | HQ857440 | HQ857446 | HQ857452 | HQ857428 |
| *Poecilia (Pamphorichthys) minor*^ | Máximo Lake, Amazonas, Brazil | GU179166 | GU179257 | GU179182 | GU179211 | GU179228 | GU179285 | GU179270 | GU179242 | GU179196 |
| *Poecilia (Pamphorichthys) scalpridens*^ | Santarém, Brazil | HQ857435 | HQ857459 | HQ857471 | HQ857465 | HQ857423 | HQ857441 | HQ857447 | HQ857453 | HQ857429 |
| *Poecilia (Poecilia) vivipara*^ | Rio De Janeiro, Brazil | HQ857436 | HQ857460 | HQ857472 | HQ857466 | HQ857424 | HQ857442 | HQ857448 | HQ857454 | HQ857430 |
| *Poecilia (Poecilia) vivipara* | Trinidad | HQ857437 | HQ857461 | HQ857473 | HQ857467 | HQ857425 | HQ857443 | HQ857449 | HQ857455 | HQ857431 |

**New sequences are denoted by * and samples used in species trees analyses denoted by ^**

*Poecilia* (*Mollienesia*) *gillii* OTU= *P. mexicana*, Lee and Johnson, 2009; Cytochrome *b* Accession numbers: FJ446154- FJ446413
*Poecilia* (*Mollienesia*) *sp.* Tobler et al. 2011; Cytochrome *b* Accession numbers: HQ677869- HQ677897
*Poecilia* (*Mollienesia*) *sp.* Palacios et al. 2013; Cytochrome *b* Accession numbers: KF276615- KF276635
*Poecilia* (*Mollienesia*) *butleri* OTU= *P. butleri* and *P. nelsoni*, Zuniga-Vega et al. 2014; Cytochrome *b* Accession numbers: JN368082- JN368131
*Poecilia* (*Mollienesia*) *sp.* Bagley et al. 2015; Cytochrome *b* Accession numbers: KP699837-KP700403
*Poecilia* (*Mollienesia*) *butleri* Palacios et al. 2015 (this study) Cytochrome *b* Accession numbers: KT626860-KT626891

*Poecilia* (*Mollienesia*) *sp.* Alda et al. 2013; NADH2 Accession numbers: JX968697- JX968742
*Poecilia* (*Mollienesia*) *butleri* Mateos et al.; NADH2 Accession numbers: AY743242- AY74325

Table A2. Genetic distances (in %) for the concatenated dataset based on the mean Kimura-2 parameter model of evolution. Pair-wise comparisons are between the main lineages of *Mollienesia.*

| | *P. cau* | *P. ltpnct* | *P. kyk* | *P. lpna* | *P. vel* | *P. chi* | *P. cat* | *P. mar* | *P. sph* | *P. hon* | *P. orr* | *P. but* | *P. nel* | *P. salv* | *P. mex* | *P. lim* | *P. sulph* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. caucana* | | | | | | | | | | | | | | | | | |
| *P. latipunctata* | 4.1 | | | | | | | | | | | | | | | | |
| *P. petenensis* | 3.9 | 1.7 | | | | | | | | | | | | | | | |
| *P. latipinna* | 4.1 | 2.2 | 2.1 | | | | | | | | | | | | | | |
| *P. velifera* | 4.4 | 2 | 2.1 | 1.4 | | | | | | | | | | | | | |
| *P. chica* | 4 | 3.1 | 3 | 2.9 | 3 | | | | | | | | | | | | |
| *P. catemaconis* | 4.1 | 3.2 | 3 | 2.9 | 3.3 | 1.6 | | | | | | | | | | | |
| *P. marcellinoi* | 3.9 | 3 | 3 | 3 | 3.2 | 1.5 | 1.1 | | | | | | | | | | |
| *P. sphenops* | 4 | 3.1 | 3 | 3 | 3.3 | 1.5 | 1 | 0.4 | | | | | | | | | |
| *P. hondurensis* | 3.9 | 3 | 3 | 3 | 3.2 | 2.4 | 2.4 | 2.4 | 2.5 | | | | | | | | |
| *P. orri* | 4.4 | 3.5 | 3.4 | 3.4 | 3.3 | 3.1 | 3 | 2.9 | 3 | 2.1 | | | | | | | |
| *P. butleri* | 4.2 | 3.5 | 3.5 | 3.3 | 3.6 | 3.1 | 3 | 2.6 | 2.7 | 2.1 | 2.2 | | | | | | |
| *P. nelson* | 4.2 | 3.4 | 3.3 | 3.3 | 3.6 | 3.1 | 3 | 2.7 | 2.9 | 2.1 | 2.2 | 1.3 | | | | | |
| *P. salvatoris* | 4.2 | 3.3 | 3.3 | 3.3 | 3.5 | 3.1 | 2.9 | 2.8 | 2.9 | 2.0 | 1.8 | 1.8 | 1.6 | | | | |
| *P. mexicana* | 4.2 | 3.3 | 3.3 | 3.3 | 3.5 | 3 | 2.8 | 2.8 | 2.9 | 1.9 | 1.7 | 2 | 1.9 | 0.9 | | | |
| *P. limantouri* | 4.2 | 3.5 | 3.4 | 3.2 | 3.5 | 3 | 2.9 | 2.9 | 3 | 1.9 | 1.5 | 2 | 2 | 1.2 | 0.9 | | |
| *P. sulphuraria* | 4.4 | 3.4 | 3.3 | 3.2 | 3.5 | 3 | 2.9 | 2.9 | 3 | 1.9 | 1.8 | 2.1 | 2 | 1.3 | 0.9 | 0.9 | |
| *P. thermalis* | 4.2 | 3.5 | 3.3 | 3.2 | 3.5 | 2.9 | 2.8 | 2.8 | 3.1 | 1.9 | 1.7 | 1.9 | 1.9 | 1.3 | 0.7 | 0.7 | 0.1 |

Table A3. Distribution and reference of *Mollienesia* species for ancestral area estimation under BBM analyses in RASP.

| Species | BBM code | Reference | Comments |
|---|---|---|---|
| *kykesis* | D | Miller, 2005 | |
| *latipinna* | AB | Miller, 2005 | |
| *latipunctata* | B | Miller, 2005 | |
| *velifera* | D | Miller, 2005 | |
| *catemaconis* | C | Miller, 2005; this study | Range extended vicinity |
| *chica* | C | Miller, 2005 | |
| *marcellinoi* | E | Poeser, 2003; this study | |
| *sphenops* | CDE | Miller, 2005; Alda et al. 2013; Bagley et al. 2015; this study | Clade 2b in Bagley et al. 2015 |
| "*sphenops*" sp. 1 | E | Alda et al. 2013; Bagley et al. 2015 | |
| sp. "Tipitapa" | E | Bagley et al. 2015 | |
| *butleri* | B | Miller, 2005; Zuniga-Vega et al. 2014; this study | |
| *gillii* | EFG | Alda et al. 2013; Bagley et al. 2015 | Clade 5c in Bagley et al. 2015, included Honduras |
| *hondurensis* | E | Poeser, 2011; Alda et al. 2013; Bagley et al. 2015 | |
| *mexicana* | BCDEF | Miller, 2005; Bagley et al. 2015; this study | |
| *limantouri* | BC | Tobler et al. 2011; this study | |
| *nelsoni* | CD | Poeser, 2003; Miller, 2005; Zuniga-Vega et al. 2014; this study | |
| *orri* | DEF | Miller, 2005; Alda et al. 2013; Bagley et al. 2015; this study | Combined with *P*. sp. "Patuca" |
| *petenensis* | D | Poeser, 2003; Bagley et al. 2015 | |
| *salvatoris* | EF | Miller, 1994; Bagley et al. 2015; this study | *P. mexicana* in Bagley et al. 2015 (Clade 8a) |
| *sulphuraria* | D | Miller, 2005; Tobler et al. 2008; Palacios et al. 2013 | |
| *thermalis* | D | Poeser, 2003; Palacios et al. 2013 | Clade 5c in Bagley et al. 2015, included Honduras |
| "*gillii*" sp. 2 | E | Alda et al. 2013; Bagley et al. 2015 | |
| sp. "Patuca" | DEF | Bagley et al. 2015; this study | Treated as *P. orri* in this study |
| "sp2" | EF | this study | Clade 8m in Bagley et al. 2015 |
| "sp3" | EF | this study | Clade 8b in Bagley et al. 2015 |

Figure A1. The phylogeny for the multilocus analyses in PHYCAS.

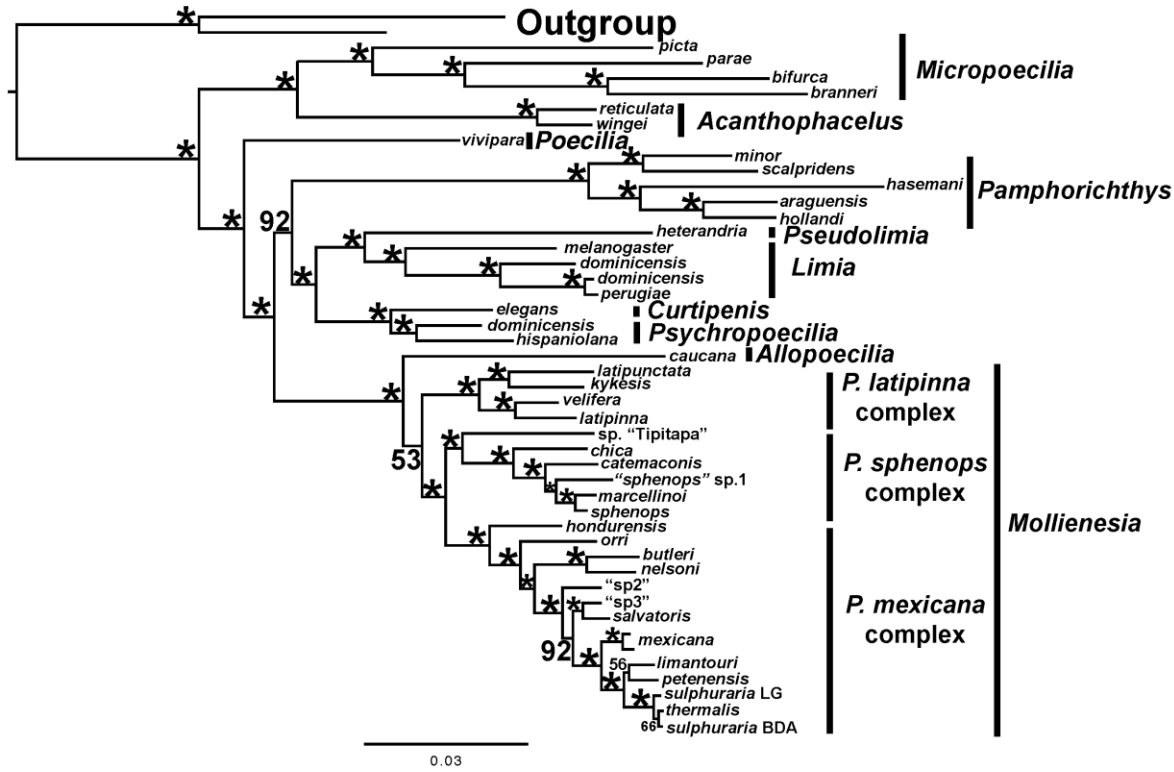


Figure A2. The phylogeny for the NADH2 gene for *Poecilia spp.*

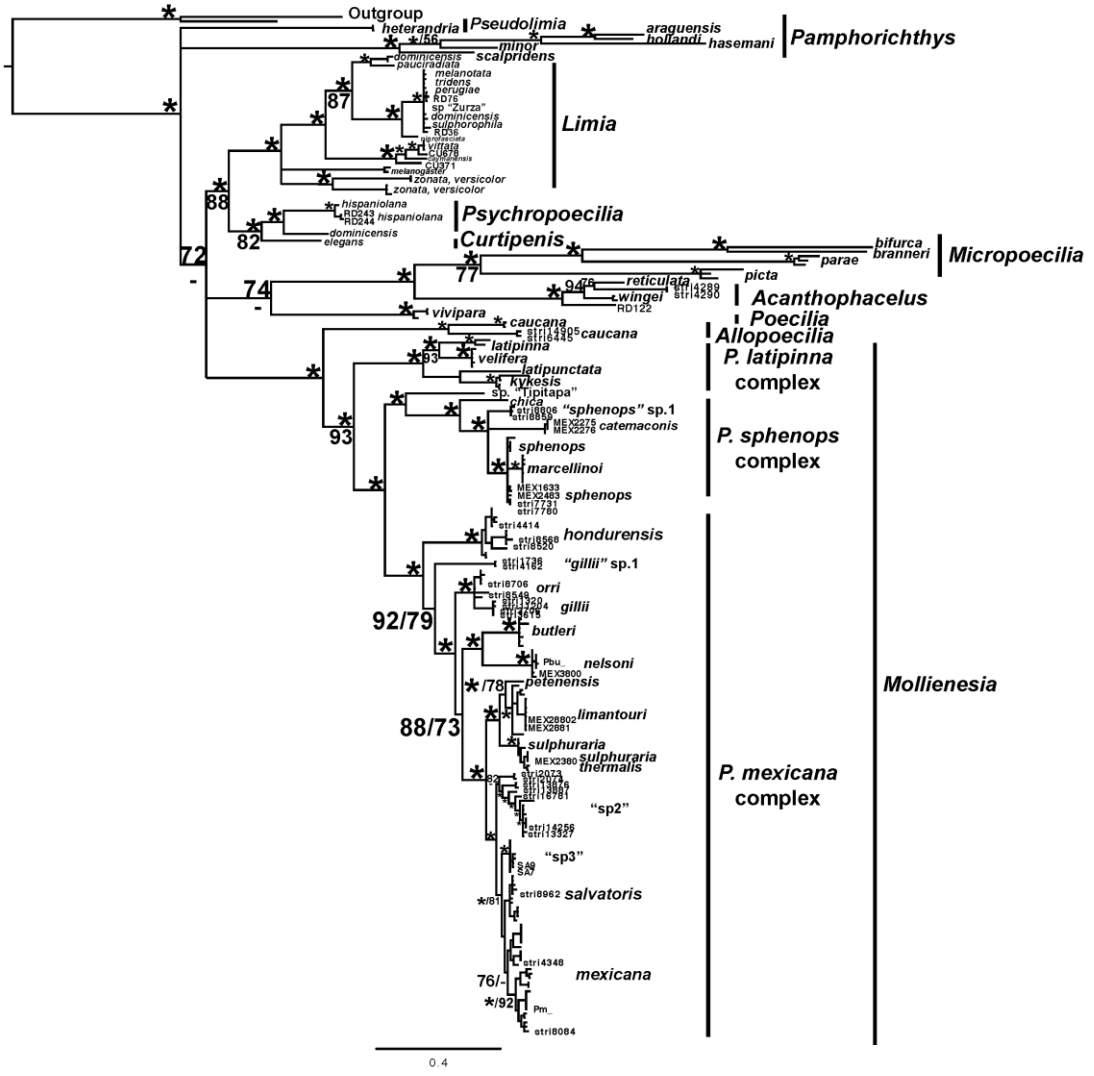


Figure A3. The phylogeny for the Cytochrome *b* gene for *Poecilia spp.*

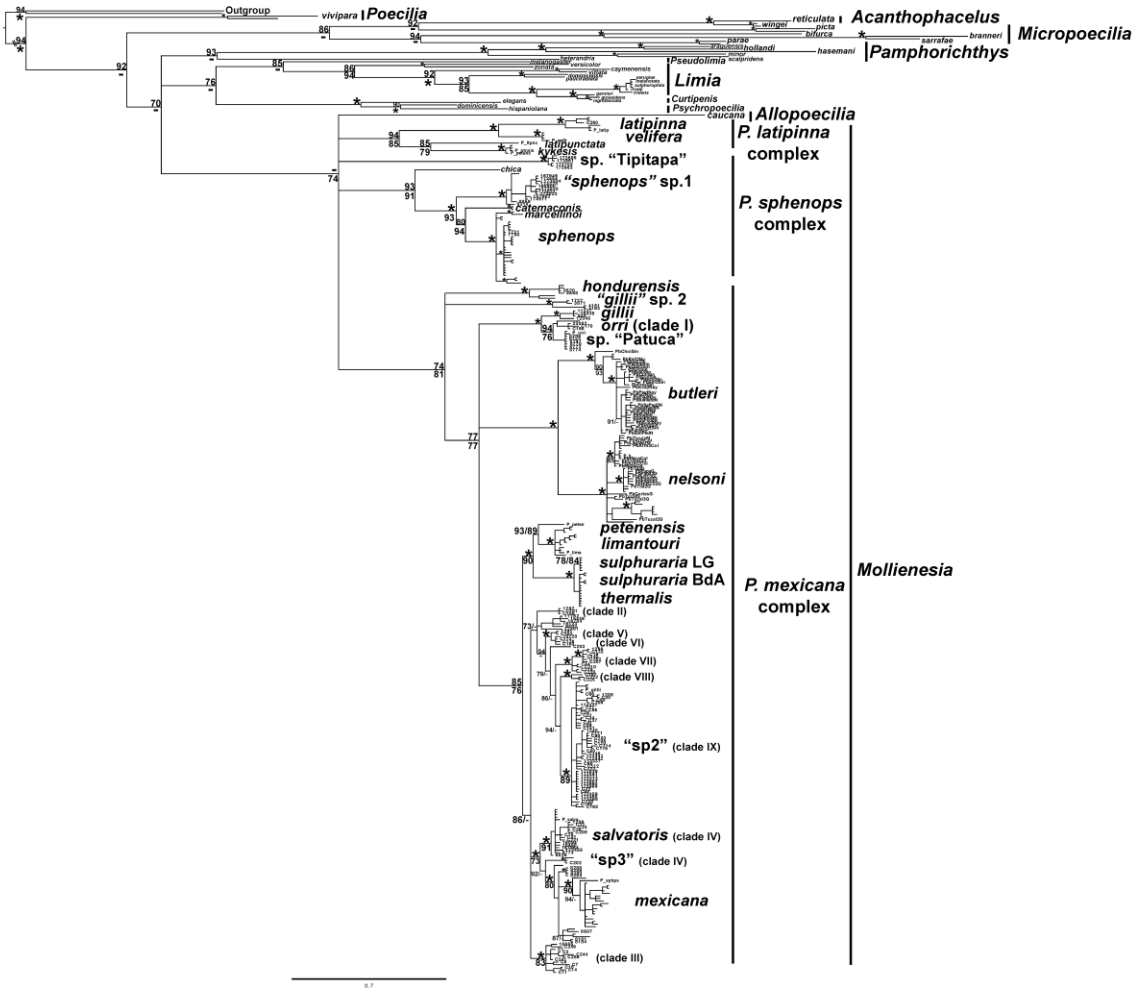


Figure A4. The phylogeny for the combined mtDNA genes for *Poecilia spp.*

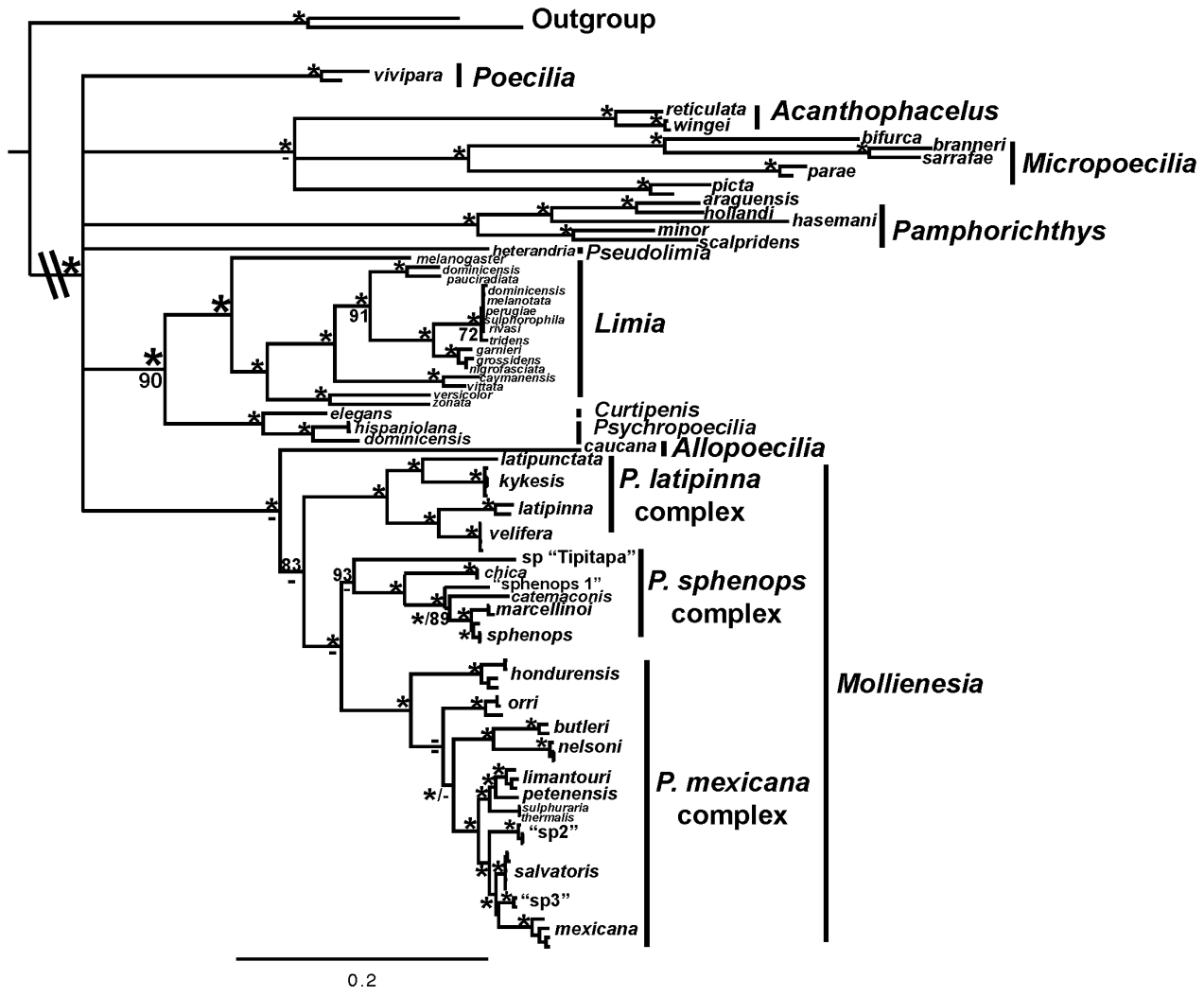


Figure A5. The phylogeny for each nuclear gene for *Poecilia spp.*

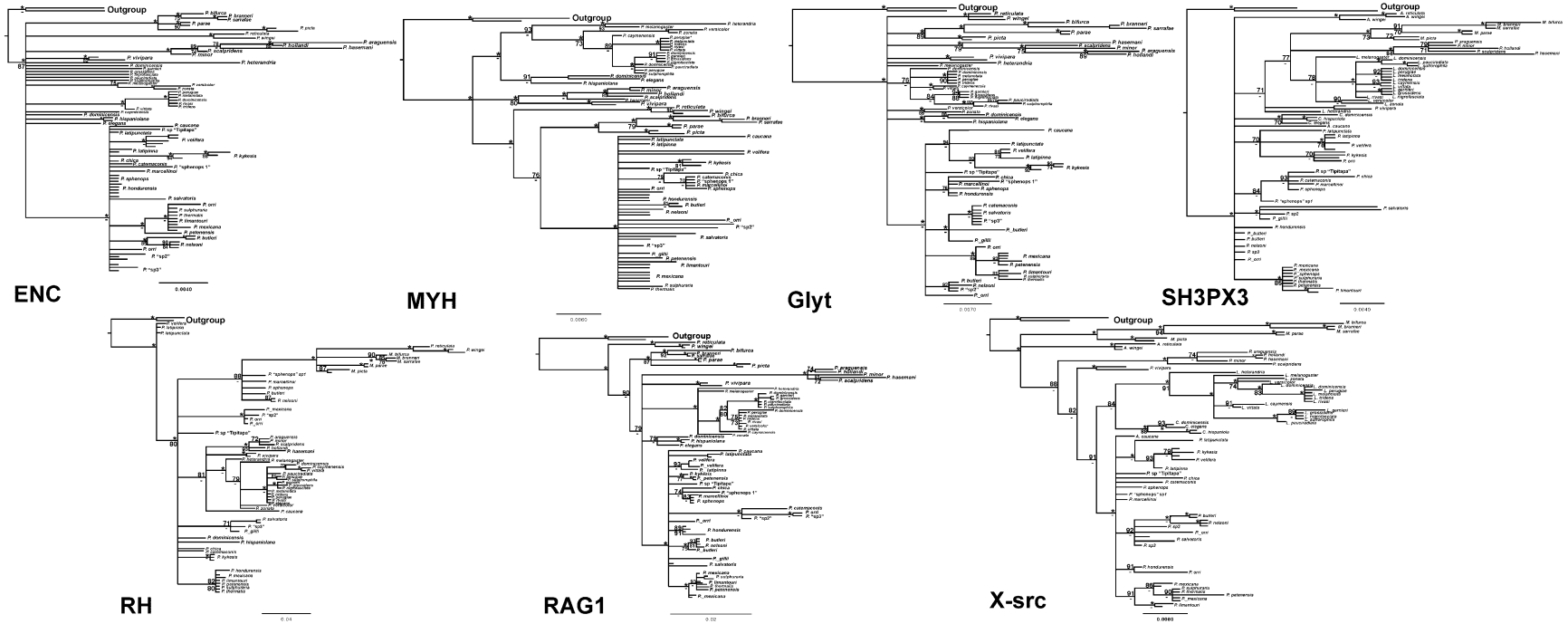
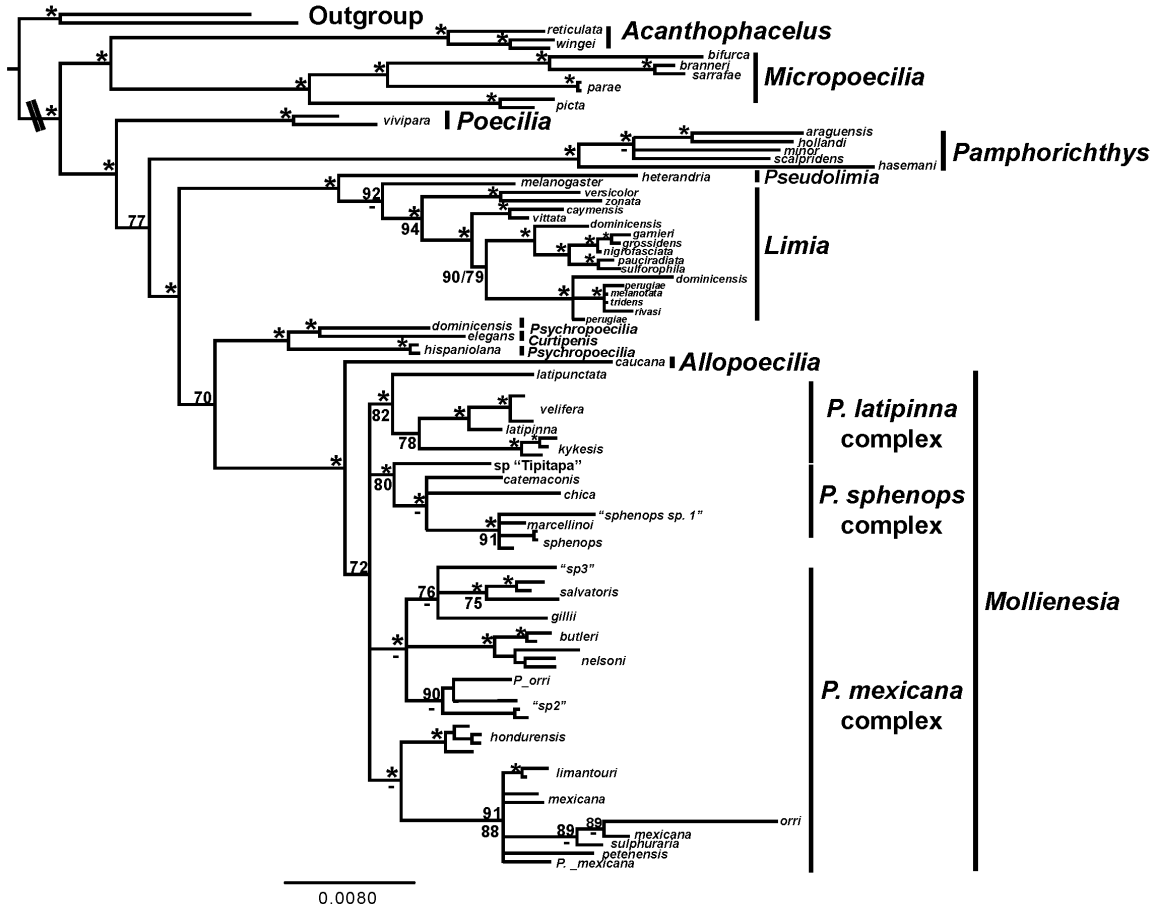


Figure A6. The phylogeny for the concatenated nuclear genes for *Poecilia spp.*



# APPENDIX B

Table B1. Species, sampling locations, genes, and Genbank sequence IDs.

| Taxa | Locality | Rag1 | NADH2 | Cyt b |
|---|---|---|---|---|
| *Poecilia (Mollienesia) butleri** | San Pedro, Nayarit, Mexico | KF276701 | KF276672 | KF276614 |
| *Poecilia (Mollienesia) butleri** | Las Palmas, Nayarit, Mexico | KY769373 | AY743247 | KT626888 |
| *Poecilia (Mollienesia) butleri** | San Blas, Nayarit, Mexico | KY769374 | AY743245 | KT626889 |
| *Poecilia (Mollienesia) catemaconis** | Lake Catemaco, Veracruz, Mexico | KF276697 | KF276668 | KF276610 |
| *Poecilia (Mollienesia) chica** | Jalisco, Mexico | KJ697311 | KJ697230 | KJ696830 |
| *Poecilia (Mollienesia) hondurensis** | Rio Lancentilla, Honduras | KP943203 | KP943315 | KP943168 |
| *Poecilia (Mollienesia) marcellinoi** TCWC 16336.01 | La Libertad, El Salvador | KP943201 | KP943314 | KP943167 |
| *Poecilia (Mollienesia) limantouri** | Rio Purificacion, Baretal, Mexico | KP943206 | HQ677845 | HQ677873 |
| *Poecilia (Mollienesia) limantouri** | Rio Palmas, Soto La Marina, Mexico | KY769377 | HQ677844 | HQ677872 |
| *Poecilia (Mollienesia) limantouri** | Jamalapa, Veracruz, Mexico | KY656814 | KY656783 | KY656742 |
| *Poecilia (Mollienesia) limantouri** | Pepeyocatitla, Hidalgo, Mexico | KY656815 | KY656784 | KY656743 |
| *Poecilia (Mollienesia) limantouri** | San Pedro, Hidalgo, Mexico | KY656816 | KY656785 | KY656744 |
| *Poecilia (Mollienesia) limantouri** | Rio Garces, Hidalgo, Mexico | KY656817 | KY656786 | KY656745 |
| *Poecilia (Mollienesia) limantouri* | Coacuilco, Hidalgo, Mexico | | KY656787 | KY656746 |

| | | | | |
|---|---|---|---|---|
| *Poecilia (Mollienesia) limantouri** | Atlatipa. Hidalgo, Mexico | KY656818 | KY656789 | KY656747 |
| *Poecilia (Mollienesia) mexicana** | Cueva del Azufre, Tabasco, Mexico | KP943210 | HQ677857 | HQ677897 |
| *Poecilia (Mollienesia) mexicana** | Rio Puyacatengo, Tabasco, Mexico | KP943211 | HQ677854 | HQ677894 |
| *Poecilia (Mollienesia) mexicana** | Banos de San Ignacio, Nuevo Leon, Mexico | KY775388<br>KY775389 | KY769378<br>KY769379 | KY769384<br>KY769385 |
| *Poecilia (Mollienesia) mexicana** | Creek between Lake Catemaco, Veracruz, Mexico | KY656819 | KY656791 | KY656749 |
| *Poecilia (Mollienesia) mexicana** | Creek after Monte Pio, Veracruz, Mexico | KY656820 | KY656792 | KY656750 |
| *Poecilia (Mollienesia) mexicana* | Porvenir, Veracruz, Mexico | | KY656793 | KY656751 |
| *Poecilia (Mollienesia) mexicana** | El Limon, Mexico | KY656823<br>KY656824 | KY656796<br>KY656797 | KY656754<br>KY656755 |
| *Poecilia (Mollienesia) mexicana* | Texistepec, Veracruz, Mexico | | KY656790 | KY656748 |
| *Poecilia (Mollienesia) mexicana** | Palenque, Chiapas, Mexico | KY656822 | KY656795 | KY656753 |
| *Poecilia (Mollienesia) mexicana** | Palenque/La Libertad, Chiapas, Mexico | KY656821 | KY656794 | KY656752 |
| *Poecilia (Mollienesia) nelsoni** | Barra de Navidad, Jalisco, Mexico | KP943204 | AY743250 | KT626891 |
| *Poecilia (Mollienesia) nelsoni** | Coquimatlan, Rio Armeria, Colima, Mexico | KY769375 | AY743252 | KT626890 |
| *Poecilia (Mollienesia) nelsoni ECOSC 7120* | Estero Cabildo, Chiapas, Mexico | KY769376 | KY656776 | KT626860 |
| *Poecilia (Mollienesia) nelsoni* ECOSC 7356* | Estero Antes de Puerto Arista, Chiapas, Mexico | KY656806 | KY656777 | KT626861 |
| *Poecilia (Mollienesia) nelsoni* ECOSC 7126* | Rio Carrizal, Mazatlan, Oaxaca, Mexico | KY656807 | KY769380 | KT626862 |
| *Poecilia (Mollienesia) nelsoni** | Rio Ayutla, Oaxaca, Mexico | KY656809 | KY769381 | KT626863 |

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|---|---|---|---|---|
| *Poecilia (Mollienesia) nelsoni** *ECOSC 7128* | Puente Coyula, Oaxaca, Mexico | KY656808 | KY769382 | KT626864 |
| *Poecilia (Mollienesia) nelsoni** *ECOSC 7129* | Puente Bajos de Chila, Oaxaca, Mexico | KY656810 | KY769383 | KT626865 |
| *Poecilia (Mollienesia) nelsoni** *ECOSC 7130* | Rio Santa Catarina, Guerrero, Mexico | KY656812 | KY656779 | KT626866 |
| *Poecilia (Mollienesia) nelsoni* | Rio Marquelia, Guerrero, Mexico | | KY656780 | KT626867 |
| *Poecilia (Mollienesia) nelsoni** | Rio Copala, Guerrero, Mexico | KY656813 | KY656781 | - |
| *Poecilia (Mollienesia) nelsoni* | Coyuca de Catalan, Guerrero, Mexico | | KY656782 | KT626868 |
| *Poecilia (Mollienesia) nelsoni* | Canal en Santa Cruz, Guerrero, Mexico | | | KT626869 |
| *Poecilia (Mollienesia) orri** | Prinzapolka, Nicaragua | KP943205 | KP943296 | KP943170 |
| *Poecilia (Mollienesia) petenensis* | | KJ697313 | KJ697231 | KJ696832 |
| *Poecilia (Mollienesia) salvatoris** *TCWC 16349.01* | Rio Paso Hondo, El Salvador | KP943209 | KP943319 | KP943173 |
| *Poecilia (Mollienesia) "sp 2"** *TCWC 16366.01* | Puerto Cabezas, Nicaragua | KP943207 | KP943317 | KP943171 |
| *Poecilia (Mollienesia) "sp 3"** *TCWC 16367.01* | Lake Xiloa, Nicaragua | KP943208 | KP943318 | KP943172 |
| *Poecilia (Mollienesia) sphenops** | Tehuatlan, Hidalgo | KF276698 | KF276669 | KF276611 |
| *Poecilia (Mollienesia) sphenops* | Pomposa Castellano, Mexico | KP943202 | HQ677862 | HQ677899 |
| *Poecilia (Mollienesia) sphenops** | Suchiapa, Chiapas, Mexico | KF276699 | KF276670 | KF276612 |
| *Poecilia (Mollienesia) sphenops** | Rio Coatzoacoalcos, Mexico | KY769372 | HQ677861 | HQ677898 |

| | | | | |
|---|---|---|---|---|
| *Poecilia (Mollienesia) sphenops** | Rio Ninguillo, Mexico | KY769371 | HQ677862 | HQ677899 |
| *Poecilia (Mollienesia) sphenops** *ECOSC 7131* | Pomposa Castellano, Mexico | KF276698 | KY656770 | KT626877 |
| *Poecilia (Mollienesia) sphenops** *ECOSC 7363* | Santa Inez, Chiapas, Mexico | KY656801 | KY656757 | KT626870 |
| *Poecilia (Mollienesia) sphenops* *ECOSC 7361* | Nueva Linda, Chiapas, Mexico | | | KT626871 |
| *Poecilia (Mollienesia) sphenops* | Rio Coatan, Chiapas, Mexico | | KY656758 | KT626872 |
| *Poecilia (Mollienesia) sphenops* *ECOSC 7364* | Ejido Las Mirallas, Chiapas, Mexico | | KY656759 | KT626873 |
| *Poecilia (Mollienesia) sphenops** *ECOSC 7121* | Rio en Acapetuhua, Chiapas, Mexico | KY656799 | KY656760 | KT626874 |
| *Poecilia (Mollienesia) sphenops** *ECOSC 7122* | Rio Sesecapa, Chiapas, Mexico | KY656800 | KY656761 | KT626875 |
| *Poecilia (Mollienesia) sphenops* *ECOSC 7123* | Rio Pijijiapan, Chiapas, Mexico | | KY656762 | KT626876 |
| *Poecilia (Mollienesia) sphenops** *ECOSC 7356* | Puente Tiltepec, Chiapas, Mexico | KY656802 | KY656763 | KT626878 |
| *Poecilia (Mollienesia) sphenops** | Puente Novillero, Oaxaca, Mexico | KY656802 | KY656764 | - |
| *Poecilia (Mollienesia) sphenops* *ECOSC 7368* | Rio Ostuta, Oaxaca, Mexico | | KY656765 | KT626879 |
| *Poecilia (Mollienesia) sphenops* *ECOSC 7125* | Rio Los Perros, Oaxaca, Mexico | | KY656766 | KT626880 |
| *Poecilia (Mollienesia) sphenops* *ECOSC 7355* | Huilotepec, Oaxaca, Mexico | | KY656767 | KT626881 |
| *Poecilia (Mollienesia) sphenops* ECOSC 7362 | Rio Verde, Oaxaca, Mexico | | KY656768 | KT626882 |

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|---|---|---|---|---|
| *Poecilia (Mollienesia) sphenops ECOSC 7359* | Rio Arena, Oaxaca,Mexico | | | KT626883 |
| *Poecilia (Mollienesia) sphenops* ECOSC 7360* | Rio Quetzal, Guerrero, Mexico | KY656804 | KY656769 | KT626884 |
| *Poecilia (Mollienesia) sphenops* | Rio Cuirio, Guerrero, Mexico | | KY656771 | KT626885 |
| *Poecilia (Mollienesia) sphenops* | Canal en Santa Cruz, Guerrero, Mexico | | KY656772 | KT626887 |
| *Poecilia (Mollienesia) sphenops* | Canal Presa San Vicente, Guerrero, Mexico | | KY656774 | KT626886 |
| *Poecilia (Mollienesia) sphenops* | Huajintlan, Morelos, Mexico | | KY656756 | KY656741 |
| *Poecilia (Mollienesia) "sphenops 1"** | Choluteca, Honduras | KP943200 | KP943313 | KP943166 |
| *Poecilia (Mollienesia) sulphuraria** | La Gloria, Mexico | KF276715 | KF276685 | KF276627 |
| *Poecilia (Mollienesia) sulphuraria** | La Gloria, Mexico | KF276713 | KF276684 | KF276626 |
| *Poecilia (Mollienesia) sulphuraria** | La Gloria, Mexico | KF276714 | KF276686 | KF276628 |
| *Poecilia (Mollienesia) sulphuraria** | Banos del Azufre, Mexico | KF276710 | KF276681 | KF276623 |
| *Poecilia (Mollienesia) sp. "Tipitapa"* TCWC 16369.01* | Lake Nicaragua, Nicaragua | KP943199 | KP943312 | KP943165 |
| *Poecilia (Mollienesia) thermalis** | La Esperanza, Mexico | KF276705 | KF276676 | KF276618 |
| *Poecilia (Mollienesia) thermalis** | La Esperanza, Mexico | KF276707 | KF276678 | KF276707 |

Table B2. Genetic distances (in %) for the Cytb dataset based on the mean Kimura-2 parameter model of evolution. Pair-wise comparisons are between the lineages of focus in *Mollienesia.*

| | sph | but | nel | thr | BDA | LG | lim | mx |
|---|---|---|---|---|---|---|---|---|
| sph | 0-0.98 | | | | | | | |
| but | 8.03 | 0-1.99 | | | | | | |
| nel | 7.75 | 4.62 | 0-1.99 | | | | | |
| thr | 8.99 | 6.02 | 6.17 | 0.00 | | | | |
| BDA | 8.61 | 5.66 | 5.83 | 0.33 | 0.32 | | | |
| LG | 9.18 | 6.20 | 6.35 | 0.16 | 0.49 | 0.00 | | |
| lim | 8.93 | 5.96 | 6.19 | 2.02 | 2.19 | 1.68 | 0-1.65 | |
| mx | 8.09 | 5.43 | 6.14 | 2.48 | 2.65 | 2.17 | 2.56 | 0-1.99 |

Table B3. Genetic distances (in %) for the ND2 dataset based on the mean Kimura-2 parameter model of evolution. Pair-wise comparisons are between the lineages of focus *Mollienesia.*

| | sph | but | nel | thr | BDA | LG | lm | mx |
|---|---|---|---|---|---|---|---|---|
| sph | 0-0.77 | | | | | | | |
| but | 10.7 | 0-0.77 | | | | | | |
| nel | 11.7 | 4.45 | 0-0.97 | | | | | |
| thr | 10.5 | 5.45 | 5.54 | 0.00 | | | | |
| BDA | 10.4 | 5.61 | 5.49 | 0.24 | 0.00 | | | |
| LG | 10.5 | 5.45 | 5.42 | 0.39 | 0.00 | 0.28 | | |
| lm | 10.8 | 5.08 | 5.51 | 2.18 | 2.13 | 1.99 | 0-1.1 | |
| mx | 10.8 | 5.44 | 5.53 | 3.42 | 3.37 | 3.31 | 2.92 | 0-1.6 |

Table B4. Genetic distances (in %) for the CYTBND2 dataset based on the mean Kimura-2 parameter model of evolution. Pair-wise comparisons are between the lineages of focus in *Mollienesia.*

| | sph | but | nel | thr | BDA | LG | lm | mx |
|---|---|---|---|---|---|---|---|---|
| sph | 0-0.9 | | | | | | | |
| but | 9.05 | 0.1-0.7 | | | | | | |
| nel | 9.62 | 3.44 | 0-1.6 | | | | | |
| thr | 9.33 | 4.82 | 5.34 | 0.10 | | | | |
| BDA | 9.29 | 4.94 | 5.31 | 0.19 | 0.00 | | | |
| LG | 9.33 | 4.98 | 5.35 | 0.22 | 0.19 | 0.10 | | |
| lm | 9.25 | 4.57 | 4.92 | 2.12 | 2.08 | 2.12 | 0-0.9 | |
| mx | 9.73 | 5.02 | 5.17 | 3.27 | 3.23 | 3.27 | 2.64 | 0-1.4 |

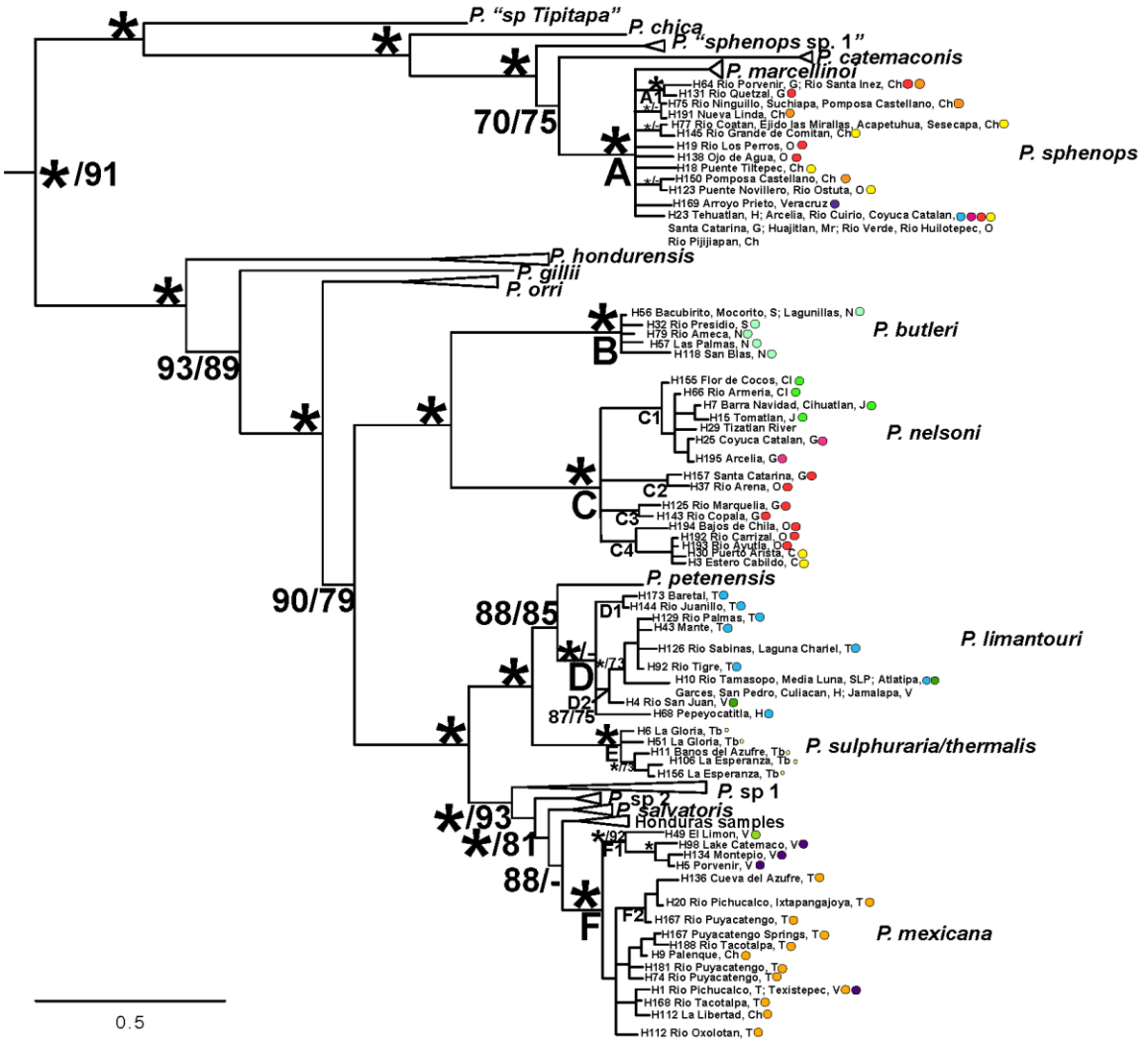


Figure B1. Bayesian tree from the MrBayes partitioned analysis of the subgenus *Mollienesia* for the ND2 mitochondrial gene (1140 base pairs) rooted with other poeciliid outgroups. Nodal support shown (left to right; respectively): Bayesian Posterior Probabilities in percent followed by RAxML bootstrap support values. Asterisks denote nodal support of 95% or above for the two methods. Nodes with no values present either had low values or were of little interest for this study.

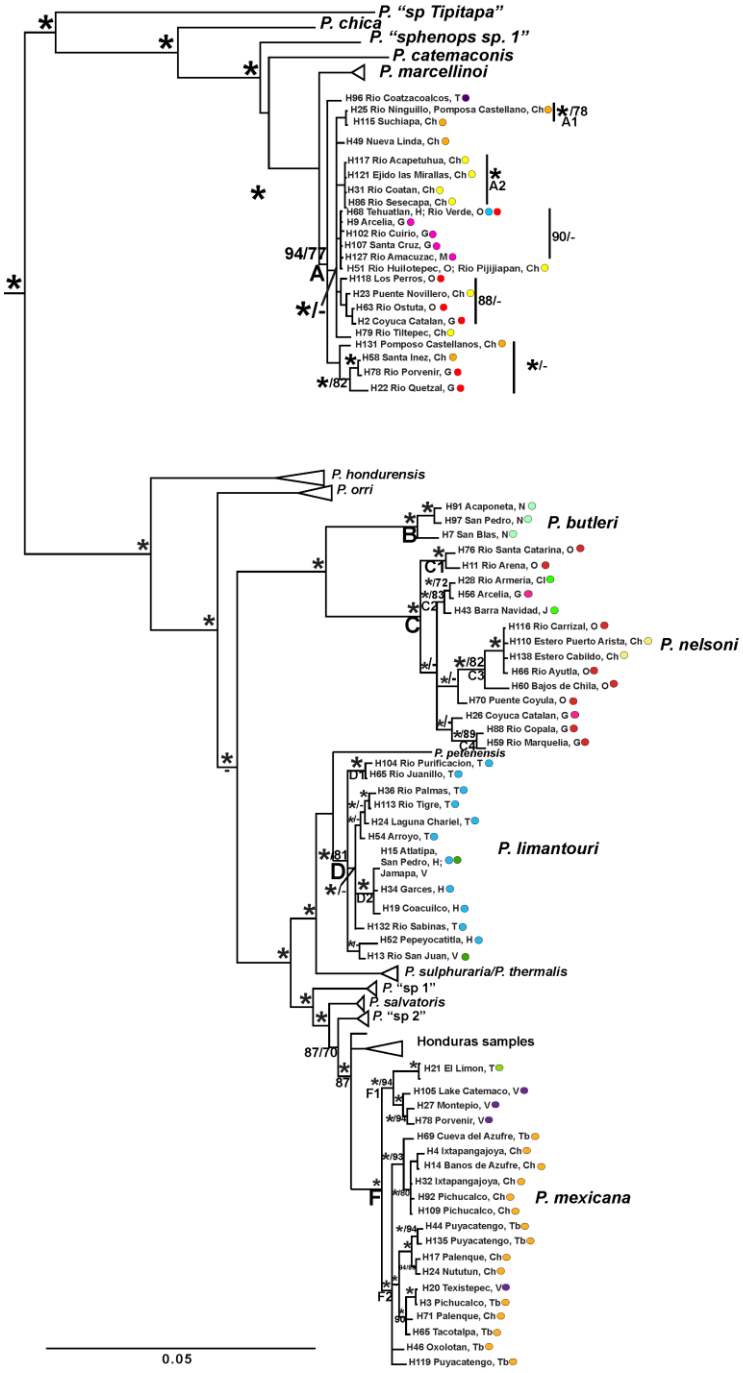


Figure B2. Bayesian tree from the MrBayes partitioned analysis of the subgenus *Mollienesia* for the combined mitochondrial dataset (Cytb, 1140 base pairs and ND2, 1047 base pairs) rooted with a poeciliid outgroups. Nodal support shown (left to right; respectively): Bayesian Posterior Probabilities in percent followed by RAxML bootstrap support values. Asterisks denote nodal support of 95% or above for the two methods. Nodes with no values present either had low values or were of little interest for this study.

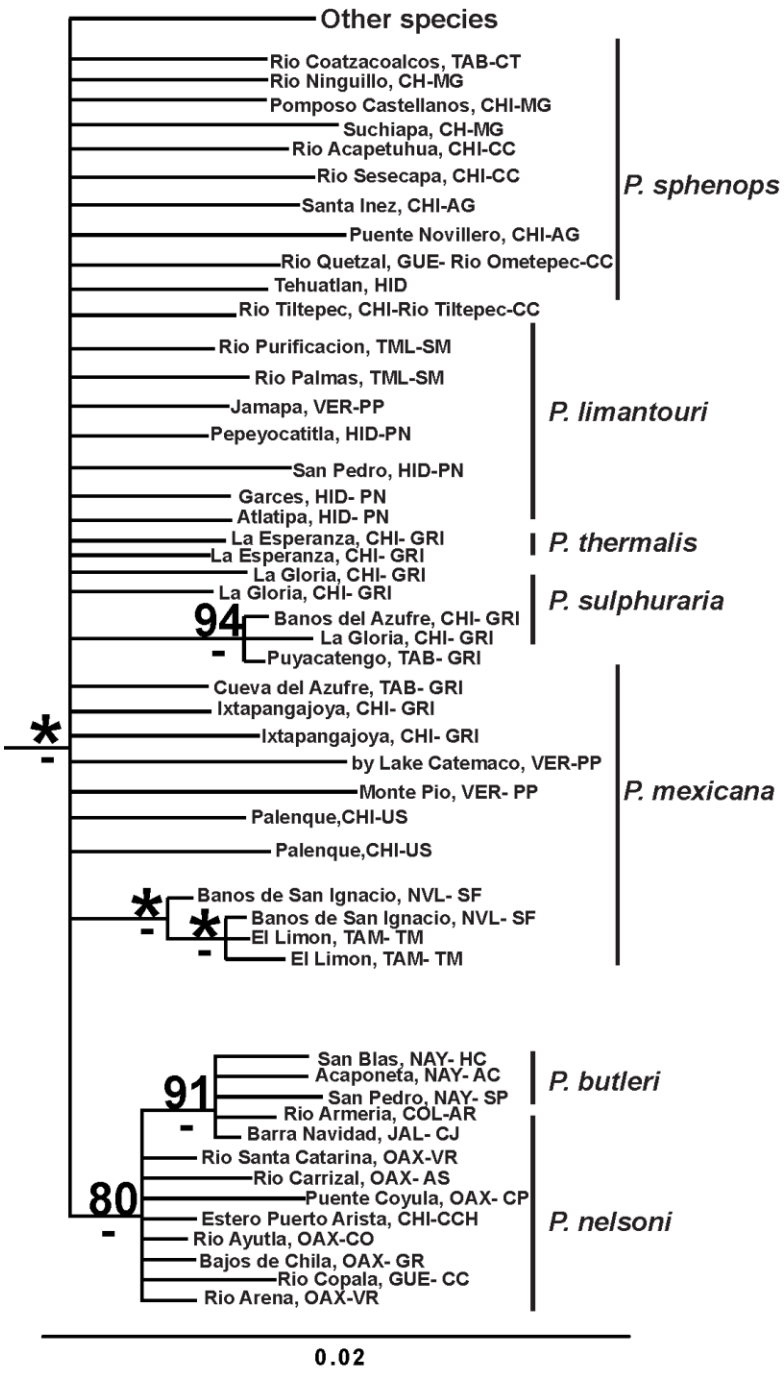


Figure B3. Bayesian tree from the MrBayes partitioned analysis of the subgenus *Mollienesia* for the RAG1 nuclear gene (1051 base pairs) rooted with other poeciliid outgroups. Nodal support shown (left to right; respectively): Bayesian Posterior Probabilities in percent followed by RAxML bootstrap support values. Asterisks denote nodal support of 95% or above for the two methods. Nodes with no values present either had low values or were of little interest for this study

APPENDIX C

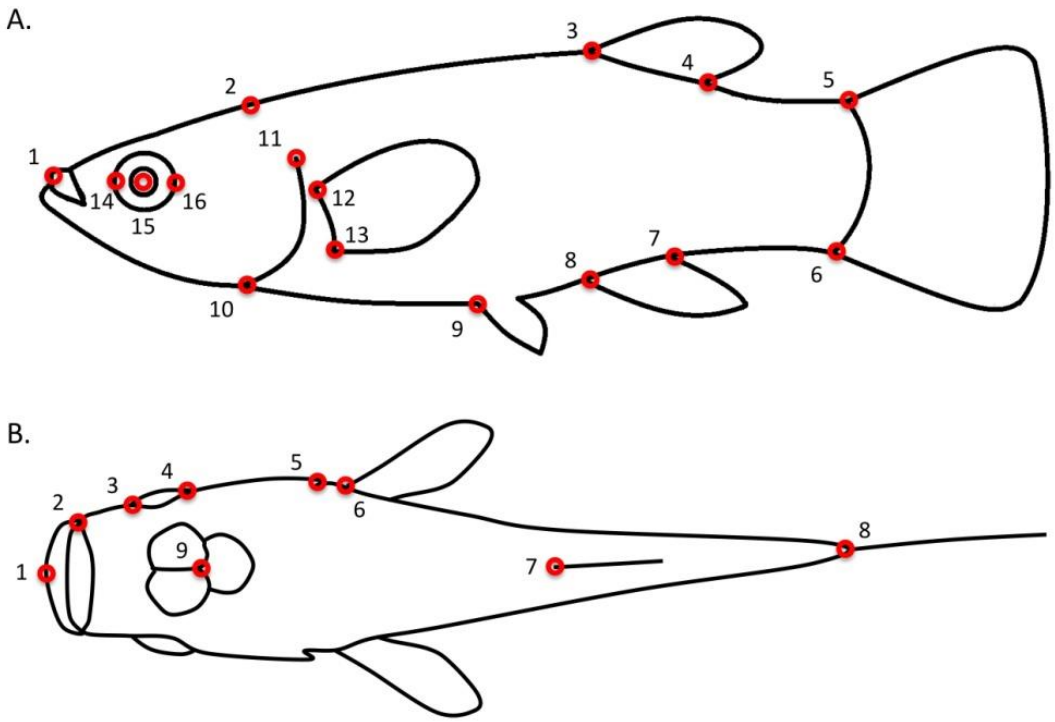


Figure C1. The 16 lateral and the 9 dorsal landmarks used for geometric morphometric analysis. A. The 16 lateral landmarks for the lateral pictures and included (1) the tip of the upper jaw, (2) the postero-dorsal corner of the head, (3) the anterior and (4) posterior insertions of the dorsal fin, (5) the dorsal and (6) ventral insertions of the caudal fin, (7) the posterior and (8) anterior junctions of the anal fin, (9) the anterior junction of the pelvic fin, (10) the bottom of the head where the operculum breaks away from the body outline, (11) the dorsal endpoint of the opercular bone, (12) the dorsal and (13) ventral insertions of the pelvic fin, as well as (14) the anterior edge, (15) the center, and the posterior edge of the eye orbit. B. The 9 dorsal landmarks included (1) center of the lower jaw, (2) the corner of the mouth where the lower and upper jaws meet, (3) the anterior and (4) posterior corners of the eye, (5) the posterior edge of the operculum, (6) the anterior insertion of the pectoral fin, (7) the anterior insertion of the dorsal fin, (8) the dorsal insertion of the caudal fin, and (9) the intersection of the last two head and the first dorsal body scales.

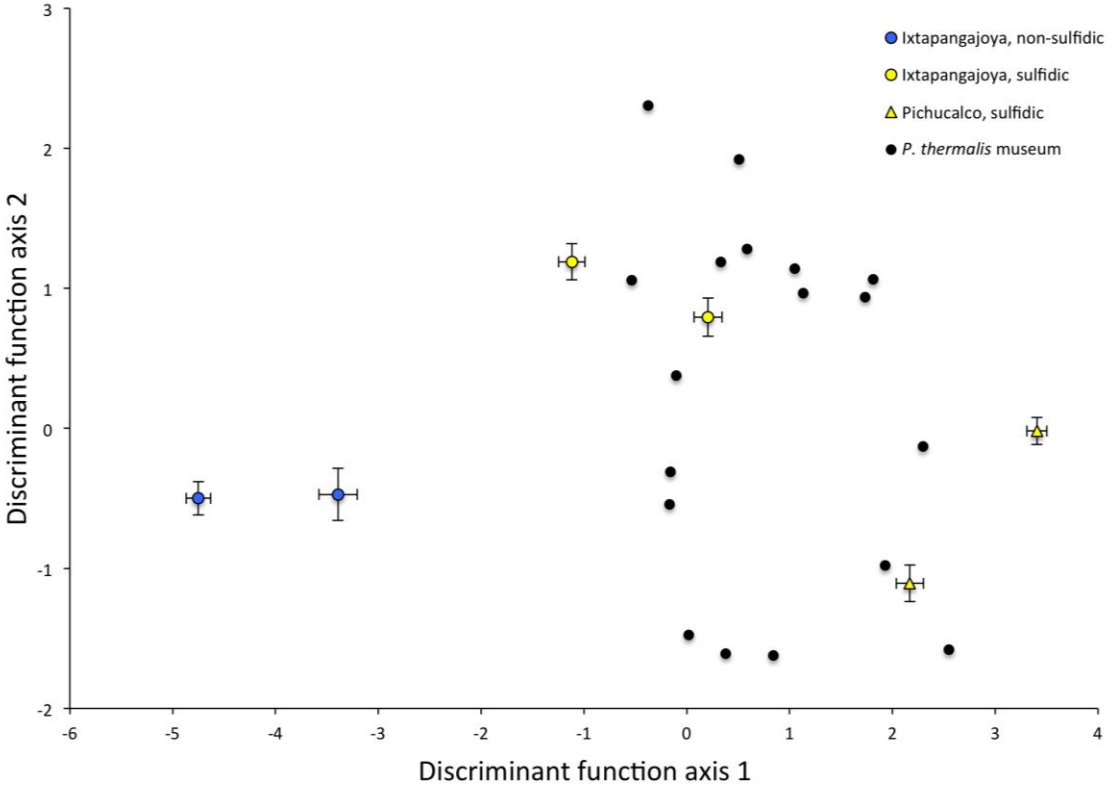


Figure C2. Results of discriminant function analysis (DFA) based on lateral body shape analysis of three *Poecilia* species. Note that historical samples of *P. thermalis* clearly cluster with contemporary samples of *P. thermalis* and *P. sulphuraria*, not with *P. m. mexicana* from adjacent non-sulfidic environments.

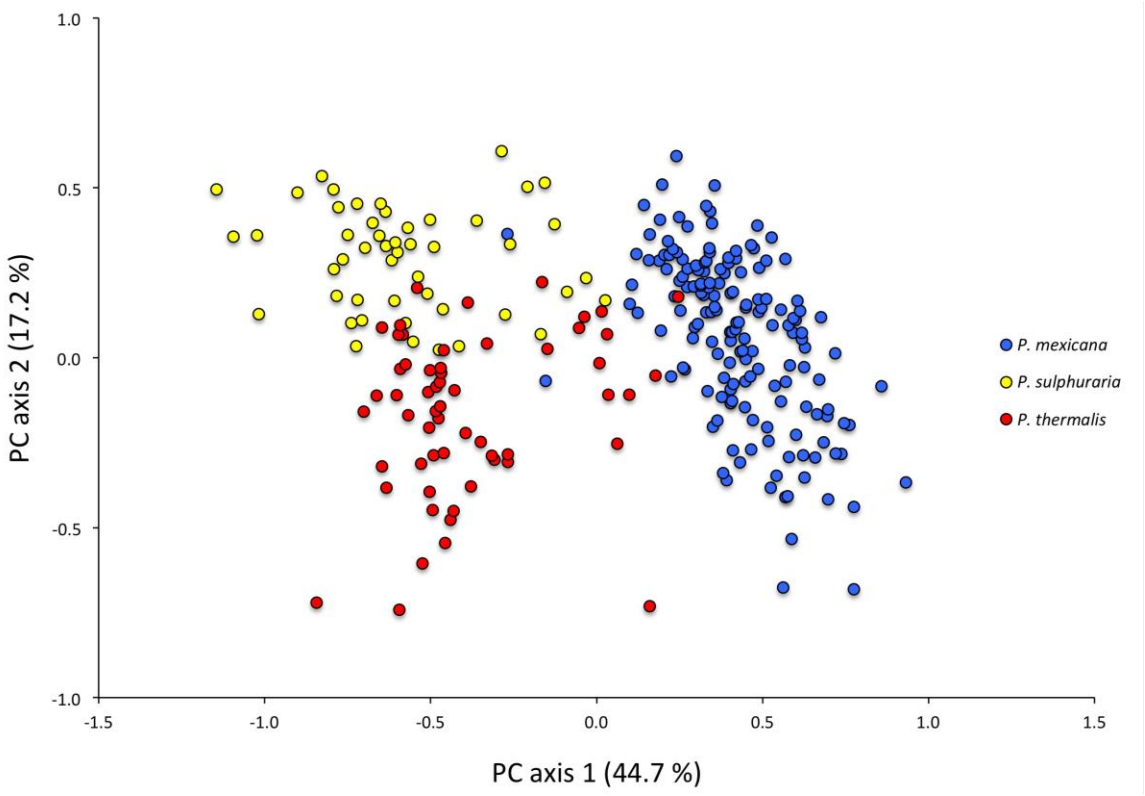


Figure C3. Principle component analysis based on the microsatellite dataset used for population genetic analyses.

Table C1. Specimens investigated for phylogenetic analyses including locality, genes, and accession numbers. Asterisks indicate samples that were sequenced specifically for the present study. For samples collected by the authors, we also included the catalog numbers of voucher specimens (MT numbers).

| Taxon | Location | Drainage | State | Country | Cyt b | ND2 | Myh6 | RAG1 | Rh |
|---|---|---|---|---|---|---|---|---|---|
| *P. thermalis* (MT12-30-1) | La Esperanza, large spring | Ixtapangajoya | Chiapas | Mexico | *KF276617 | *KF276675 | *KF276646 | *KF276704 | *KF276733 |
| *P. thermalis* (MT12-30-2) | La Esperanza, large spring | Ixtapangajoya | Chiapas | Mexico | *KF276618 | *KF276676 | *KF276647 | *KF276705 | *KF276734 |
| *P. thermalis* (MT12-30-3) | La Esperanza, large spring | Ixtapangajoya | Chiapas | Mexico | *KF276619 | *KF276677 | *KF276648 | *KF276706 | *KF276735 |
| *P. thermalis* (MT12-32-1) | La Esperanza, small spring | Ixtapangajoya | Chiapas | Mexico | *KF276620 | *KF276678 | *KF276649 | *KF276707 | *KF276736 |
| *P. thermalis* (MT12-32-2) | La Esperanza, small spring | Ixtapangajoya | Chiapas | Mexico | *KF276621 | *KF276679 | *KF276650 | *KF276708 | *KF276737 |
| *P. thermalis* (MT12-32-3) | La Esperanza, small spring | Ixtapangajoya | Chiapas | Mexico | *KF276622 | *KF276680 | *KF276651 | *KF276709 | *KF276738 |
| *P. sulphuraria* (MT12-37-1) | Baños del Azufre | Pichucalco | Tabasco | Mexico | *KF276623 | *KF276681 | *KF276652 | *KF276710 | *KF276739 |
| *P. sulphuraria* (MT12-37-2) | Baños del Azufre | Pichucalco | Tabasco | Mexico | *KF276624 | *KF276682 | *KF276653 | *KF276711 | *KF276740 |
| *P. sulphuraria* (MT12-37-3) | Baños del Azufre | Pichucalco | Tabasco | Mexico | *KF276625 | *KF276683 | *KF276654 | *KF276712 | *KF276741 |
| *P. sulphuraria* (MT-12-36-1) | La Gloria | Pichucalco | Tabasco | Mexico | *KF276626 | *KF276684 | *KF276655 | *KF276713 | *KF276742 |
| *P. sulphuraria* (MT-12-36-2) | La Gloria | Pichucalco | Tabasco | Mexico | *KF276627 | *KF276685 | *KF276656 | *KF276714 | *KF276743 |
| *P. sulphuraria* (MT-12-36-3) | La Gloria | Pichucalco | Tabasco | Mexico | *KF276628 | *KF276686 | *KF276657 | *KF276715 | *KF276744 |
| *P. mexicana mexicana* (MT12-31-1) | Tributary to the Rio Ixtapangajoya | Ixtapangajoya | Chiapas | Mexico | *KF276629 | *KF276687 | *KF276658 | *KF276716 | *KF276745 |
| *P. mexicana mexicana* (MT12-31-2) | Tributary to the Rio Ixtapangajoya | Ixtapangajoya | Chiapas | Mexico | *KF276630 | *KF276688 | *KF276659 | *KF276717 | *KF276746 |
| *P. mex. mexicana* (MT12-39-1) | Rio Ixtapangajoya | Ixtapangajoya | Chiapas | Mexico | *KF276631 | *KF276689 | *KF276660 | *KF276718 | *KF276747 |
| *P. mex. mexicana* (MT12-39-2) | Rio Ixtapangajoya | Ixtapangajoya | Chiapas | Mexico | *KF276632 | *KF276690 | *KF276661 | *KF276719 | *KF276748 |
| *P. mex. mexicana* (MT12-39-3) | Rio Ixtapangajoya | Ixtapangajoya | Chiapas | Mexico | *KF276633 | *KF276691 | *KF276662 | *KF276720 | *KF276749 |
| *P. mex. mexicana* (MT10-35-1) | Cueva del Azufre | Tacotalpa | Tabasco | Mexico | *KF276644 | *KF276673 | *KF276644 | *KF276702 | *KF276731 |
| *P. mex. mexicana* (MT09-10-1) | Puyacatengo Springs | Puyacatengo | Tabasco | Mexico | *KF276645 | *KF276674 | *KF276645 | *KF276703 | *KF276732 |
| *P. mex. limantouri* (MT10-16-1) | Pepeyocatitla | Panuco | Hidalgo | Mexico | *KF276634 | *KF276692 | *KF276663 | *KF276721 | *KF276750 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *P. mex. limantouri* (MT10-12-1) | San Pedro | Panuco | Hidalgo | Mexico | *KF276635 | *KF276693 | *KF276664 | *KF276722 | *KF276751 |
| *P. butleri* (1) | San Pedro | San Pedro | Nayarit | Mexico | *KF276613 | *KF276671 | *KF276642 | *KF276700 | *KF276729 |
| *P. butleri* (4) | San Pedro | San Pedro | Nayarit | Mexico | *KF276614 | *KF276672 | *KF276643 | *KF276701 | *KF276730 |
| *P. sphenops* (MT10-15-1) | Tehuatlan | Panuco | Hidalgo | Mexico | *KF276611 | *KF276669 | *KF276640 | *KF276698 | *KF276727 |
| *P. sphenops* (MT09-21-1) | Suchiapa | Grijalva | Chiapas | Mexico | *KF276612 | *KF276670 | *KF276641 | *KF276699 | *KF276728 |
| *P. catemaconis* (MT10-23-1) | Lake Catemaco | Papaloapan | Veracruz | Mexico | *KF276610 | *KF276668 | *KF276639 | *KF276697 | *KF276726 |
| *P. latipinna* | Wilmington | Cape Fear | North Carolina | U.S. | *KF276609 | *KF276667 | *KF276638 | *KF276696 | *KF276725 |
| *P. latipunctata* | Ciudad Mante | Panuco | Tamaulipas | Mexico | EF017539 | EF017588 | EF017436 | GU179287 | GU179259 |
| *P. caucana* | Unknown | | | Mexico | EF017540 | EF017589 | EF017437 | GU179286 | GU179258 |
| *Acanthophacelus reticulate* | Cumaná | Litoral Caribe | Sucre | Venezuela | GU179192 | GU179238 | EF017434 | GU179281 | GU179253 |
| *A. wingei* | Aquarium Stock | | | | GU179193 | GU179239 | GU179267 | GU179282 | GU179254 |
| *Micropoecilia bifurca* | Coropina Creek | | Republiek district | Suriname | GU179186 | GU179232 | GU179261 | GU179275 | GU179247 |
| *M. parae* | Rowa | | | French Guyana | GU179188 | GU179234 | GU179263 | GU179277 | GU179249 |
| *Limia dominicensis* | River Picot | | Pont Salomon | Haiti | EF017533 | EF017582 | EF017431 | GU179273 | GU179245 |
| *L. melanogaster* | Aquarium stock | | | | EF017534 | EF017583 | EF017432 | GU179274 | GU179246 |
| *Pamphorichthys araguaiensis* | Cristalino River | | Mato Grosso | Brazil | GU179195 | GU179241 | GU179269 | GU179284 | GU179256 |
| *P. minor* | Máximo Lake | | Amazonas | Brazil | GU179196 | GU179242 | GU179270 | GU179285 | GU179257 |
| *Cnesterodon hypselurus* | Cilada | | Paraná | Brazil | GU179185 | GU179231 | GU179260 | GU179272 | GU179244 |
| *C. dessemaculatus* | Canãda | | Artigas | Uruguay | EF017529 | EF0175791 | EF017427 | GU179271 | GU179243 |

Table C2. The genes partitioned by position for mitochondrial DNA and by gene for nuclear DNA for phylogenetic analyses of *Poecilia* species are described including the total length and number of parsimony informative sites in parentheses. The best-fit substitution models for each data partition for the concatenated dataset are provided as well as the likelihood score.

| | **Cyt b *p. 1*** | **Cyt b *p. 2*** | **Cyt b *p. 3*** | **ND2 p. 1** | **ND2 p. 2** | **ND2 p. 3** | **Myh6** | **RAG1** | **Rh** |
|---|---|---|---|---|---|---|---|---|---|
| | (380, 70) | (380, 11) | (380, 282) | (349, 88) | (349, 29) | (349, 252) | (767, 45) | (1561, 101) | (822, 44) |
| **AIC** | SYM+I+G | HKY+I | GTR+I+G | GTR+G | GTR+G | GTR+G | GTR+I | GTR+G | HKY+I |
| | -1455.33 | -634.12 | -4252.63 | -1751.21 | -967.27 | -3919.00 | -1695.87 | -3715.77 | -1729.11 |

Table C3. Results of the discriminant function analysis (DFA). Cross validation (i.e., assignment of museum specimens to sampling sites based on discriminant functions generated based from contemporary collections) indicated that 72.2 % of *P. thermalis* individuals were assigned to the large La Esperanza spring, 16.7 % to the La Gloria springs, and 11.1 % to the springs at the Baños del Azufre.

| | **Function 1** | **Function 2** | **Function 3** | **Function 4** | **Function 5** |
|---|---|---|---|---|---|
| PC 1 | 0.157 | -0.190 | 0.219 | -0.052 | -0.254 |
| PC 2 | 1.093 | 0.169 | 0.092 | 0.026 | -0.052 |
| PC 3 | -0.389 | 0.760 | 0.547 | 0.329 | -0.159 |
| PC 4 | 0.268 | 0.877 | -0.098 | 0.222 | -0.382 |
| PC 5 | 0.146 | 0.024 | 0.220 | 0.043 | 0.872 |
| PC 6 | 0.276 | 0.288 | -0.410 | 0.375 | 0.261 |
| PC 7 | -0.119 | -0.375 | 0.639 | 0.248 | 0.080 |
| PC 8 | 0.172 | -0.519 | -0.197 | 0.666 | 0.038 |
| PC 9 | 0.074 | -0.299 | -0.133 | 0.311 | -0.083 |
| Eigenvalue | 9.471 | 0.571 | 0.286 | 0.072 | 0.016 |
| % of variance | 90.9 | 5.5 | 2.7 | 0.7 | 0.2 |
| Cumulative % | 90.9 | 96.4 | 99.2 | 99.8 | 100.0 |
| Canonical correlation | 0.951 | 0.603 | 0.471 | 0.259 | 0.125 |
| Wilks' lambda | 0.043 | 0.455 | 0.714 | 0.918 | 0.984 |
| $\chi^2$ | 1162.181 | 292.006 | 124.763 | 31.703 | 5.877 |
| *Df* | 45 | 32 | 21 | 12 | 5 |
| *P* | <0.001 | <0.001 | <0.001 | 0.002 | 0.318 |

Table C4. Genetic distances (in %) for the concatenated dataset based on the mean Kimura-2 parameter model of evolution. Pair-wise comparisons are between the main lineages of *Poecilia* (asterisks indicate more than one species) and an out-group. The range within each lineage – when applicable – is shown in the diagonal.

| | *P. thermalis* | *P. sulphuraria* (s.l.) | *P. m. limantouri* | *P. m. mexicana* | *P. butleri* | *P. sphenops** | Saifin clade* | *P. caucana* |
|---|---|---|---|---|---|---|---|---|
| *P. thermalis* | (0-0.001) | | | | | | | |
| *P. sulphuraria* (s.l.) | 0.200 | (0-0.300) | | | | | | |
| *P. mex. limantouri* | 1.500 | 1.500 | (0.700) | | | | | |
| *P. mex. mexicana* | 2.200 | 2.200 | 2.000 | (0-0.200) | | | | |
| *P. butleri* | 3.900 | 3.900 | 3.800 | 3.800 | - | | | |
| *P. sphenops*-clade* | 6.800 | 6.800 | 6.700 | 6.700 | 6.600 | (0.1-2.200) | | |
| Sailfin-clade* | 7.600 | 7.500 | 7.700 | 7.700 | 7.900 | 7.300 | (5.200) | |
| *P. caucana* | 9.600 | 9.600 | 9.200 | 9.300 | 9.300 | 9.400 | 9.300 | - |
| Outgroup | 15.60 | 15.600 | 15.40 | 15.30 | 15.40 | 15.100 | 15.10 | 15.00 |

Table C5. Pairwise $F_{ST}$-values calculated using GenAlEx 6.5 based on 17 microsatellite markers analyzed in 10 populations of *Poecilia* from Southern Mexico. Numbers after population names correspond to site numbers listed in Table 4.2. Bolded sites represent the highly endemic sulfide springs populations: Baños del Azufre and La Gloria represent populations of *P. sulphuraria* (*s. l.*) in the Pichucalco drainage and Esperanza Large and Small indicate populations of *P. thermalis* in the Ixtapangajoya drainage. The remaining populations represent *P. m. mexicana* populations from adjacent non-sulfidic habitats of both drainages. Asterisks denote significant ($P \leq 0.01$) $F_{ST}$-values based on randomization tests with $10^5$ permutations.

| | **Baños (1)** | **La Gloria (2)** | Rosita (4) | El Azufre West (5) | Rafael (6) | **Esperanza Large (7)** | **Esperanza Small (8)** | Tributary (9) | Teapao (10) |
|---|---|---|---|---|---|---|---|---|---|
| **La Gloria (2)** | **0.082*** | | | | | | | | |
| Rosita (4) | 0.214* | 0.194* | | | | | | | |
| El Azufre West (5) | 0.171* | 0.152* | 0.084* | | | | | | |
| Rafael (6) | 0.196* | 0.181* | 0.062* | 0.078* | | | | | |
| **Esperanza Large (7)** | **0.093*** | **0.103*** | 0.214* | 0.172* | 0.195* | | | | |
| **Esperanza Small (8)** | **0.086*** | **0.092*** | 0.143* | 0.114* | 0.125* | **0.031*** | | | |
| Tributary (9) | 0.156* | 0.143* | 0.070* | 0.049* | 0.099* | 0.188* | 0.112* | | |
| Teapao (10) | 0.139* | 0.133* | 0.071* | 0.032* | 0.071* | 0.137* | 0.083* | 0.019 | |
| Ixtapangajoya (11) | 0.154* | 0.143* | 0.080* | 0.043* | 0.095* | 0.183* | 0.111* | 0.015 | 0.013 |