1 **Phylogenetic analyses of the subgenus *Mollienesia* (*Poecilia*,**
2 ***Poeciliidae*, Teleostei) reveal taxonomic inconsistencies, cryptic**
3 **biodiversity, and spatio-temporal aspects of diversification in**
4 **Middle America**

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Abstract

The subgenus *Mollienesia* is a diverse group of freshwater fishes, including species that have served as important models across multiple biological disciplines. Nonetheless, the taxonomic history of this group has been conflictive and convoluted, in part because the evolutionary relationships have not been rigorously resolved. We conducted 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. Our findings confirm the presence of three main clades composed of the *P. latipinna*, *P. sphenops*, and *P. mexicana* species complexes. Unlike previously hypothesized morphology-based analyses, species found on the Caribbean Islands are not part of *Mollienesia*, but are more closely related to species of the subgenus *Limia*. Our 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.

Key words

Mollienesia; Middle America; Phylogeny; Biogeography; Ecology; Evolution

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. 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 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).

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 and Bermingham, 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

82 fauna are two divergent groups of secondary freshwater fishes: cichlids and cyprinodonts
83 (Miller, 1966). Contrary to primary freshwater fishes with low salinity tolerances (Myers,
84 1949), secondary freshwater fishes can cope with elevated salinities allowing them to
85 occasionally use coastal and marine waters for dispersal (Myers, 1966). This is important
86 from a biogeographical perspective, because even in the presence of fragmented
87 landscapes separated by marine waters, secondary freshwater fishes have the potential for
88 the colonization of novel habitats. Phylogeographic studies of freshwater fishes suggest
89 colonization of Middle America occurred ~3-7 Ma ago for both primary (Bermingham
90 and Martin, 1998; Ornelas-García et al., 2008; Perdices et al., 2002) and secondary
91 freshwater fishes (Bagley and Johnson, 2014; Perdices et al., 2005; Řičan et al., 2013).
92 While some of these molecular dating estimates rely on mitochondrial datasets (Ornelas-
93 García et al., 2008; Bagley and Johnson, 2014), which can result in overestimation of
94 divergence times due to genetic divergences predating population divergences (Marko et
95 al. 2015), colonization of Middle America roughly aligns with the geological upheavals
96 associated with the rising Isthmus of Panama ~2.8-15 Ma ago (Jackson and O'Dea, 2013;
97 Lessios, 2008). Previous studies have also documented genetic structuring between
98 geological blocks and across the Atlantic and Pacific slopes of Middle America
99 (Bermingham and Martin, 1998; Hrbek et al., 2007; Perdices et al., 2002; Perdices et al.,
100 2005; Strecker et al., 2004). The major phylogeographic breaks for freshwater fishes and
101 other taxa in this region include the Isthmus of Tehuantepec, the Motagua-Polochic Fault,
102 the Central American Volcanic Arc, and the Isthmus of Panama (Fig. 1; Gutiérrez-García
103 and Vázquez-Domínguez, 2013). Consequently, dispersal and vicariant events could both
104 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 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).

Recent phylogenetic studies have established the phylogenetic relationships between *Mollienesia* and the other recognized subgenera (*Limia*, *Pseudolimia*, *Pamphorichthys*, *Micropoecilia*, *Poecilia*, and *Acanthophaeus*) in the genus *Poecilia* (Meredith et al. 2010, 2011; Fig. 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 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; 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 Table1; Alda et al. 2013; Bagley et al., 2015; Ho et al. 2016).

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. Materials and Methods

2.1 Sampling strategy and specimen acquisition

Our sampling efforts focused on including species previously not sampled (Table 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. 1, Table 1, SI Table S1). 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 S1).

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 (Kato and Toh, 2008).

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 (SI Table S1). 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 *Acanthophaelus*; 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).

2.4 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). 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).

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

249 Two independent Bayesian analyses were conducted in MrBayes version 3.2.1
250 (Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012), each implementing two runs
251 with four chains under default parameters. Each analysis incorporated models of
252 nucleotide substitution uniquely defined for the partition of each data set (Table 2). A
253 25% burn-in was applied and stable posterior probability values examined in Tracer
254 version 1.6 (Rambaut et al. 2014). Pairwise genetic distances were calculated under the
255 Kimura-2 parameter in MEGA version 5 (Tamura et al., 2011) with pairwise deletion for
256 missing data.

257 Since analyses implemented in MrBayes can result in high clade posterior
258 probabilities in cases with known polytomies (star-tree paradox, Suzuki et al., 2002), we
259 also ran an analysis that allows for polytomies in Phycas 2.2.0 (Lewis et al., 2015), which
260 incorporates a polytomy prior (Lewis et al., 2005). The run was set for 50,000
261 generations, sampling trees and parameters every 10 generations. The sampled trees
262 output file was summarized to form a 50% percent majority rule consensus in DendroPy
263 3.10.1 (Sukumaran and Holder, 2010).

264 We also analyzed our data using *BEAST v. 1.8.3 (Drummond and Rambaut,
265 2007; Suchard and Rambaut, 2009) to estimate a species tree and simultaneously
266 generate molecular clock-based divergence estimates in CIPRES (Miller et al. 2010).
267 Dating phylogenetic trees of *Poecilia* has been complicated by the scarcity of fossil
268 calibration points as well as uncertainty associated with the timing of the emergence of
269 geological barriers and putative vicariance events associated with these barriers (see Ho
270 et al. 2016 for a discussion). Accordingly, we employed a dual approach by (1) applying
271 universal mtDNA mutation rates for teleosts (Mateos et al. 2002), which makes estimated
272 dates comparable to previous studies on *Poecilia*, and (2) constraining the monophyly of
273 *Poecilia* with a range of dates based on Ho et al. 's (2016) application of a secondary
274 fossil calibration of bony fish (Betancur et al. 2013), which circumnavigates arbitrary
275 rates and uncertainties with the timing of geological barriers and associated vicariance
276 events. For the first analysis, we applied an uncorrelated relaxed molecular clock rate
277 under a log-normal model that draws from the rate of each lineage independently from a
278 log-normal distribution. As in previous analyses of poeciliid fishes (including *Poecilia*),
279 we applied a 1.0-2.0%/My rate to cytochrome *b* (Mateos et al., 2002; Hrbek et al. 2007;
280 Meredith et al. 2010; 2011), and the remaining rates were estimated under default
281 parameters. We ran two replicate searches with chain lengths set to 200 million
282 generations and parameters sampled every 2000 generations. Convergence parameters in
283 the Markov chain Monte Carlo model were assessed by effective sample size in Tracer v.
284 1.6 (Rambaut et al. 2014) with all ESS values above 200. LogCombiner was used to
285 reduce and combine the species trees files by applying a burn-in of 25%. The newly
286 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 %.

2.5 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. 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 S2), primary literature (Alda et al., 2013; Bagley et al., 2015; Table S2), 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.

3. Results

3.1 Multi-locus concatenated phylogeny

The phylogenetic relationships among the subgenera within the genus *Poecilia* place the clade comprised of *Acanthophaelus* and *Micropoecilia* as sister to the remaining lineages with strong support (90% or above for bootstrap [BS] and Bayesian posterior probabilities [BPP]; Fig. 3 and SI Fig. S1). The next major clade consists of several subgenera; however, the relationships among these subgenera were only supported by

Bayesian analyses (Fig. 3 and SI Fig. S1). 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*.

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. 3). However, the support for these relationships was moderate to low (76 BPP, >50% BS, Fig. 3), including the polytomy analyses (53 BPP, SI Fig. 1), 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. catemacónis* 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 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).

3.2 Mitochondrial gene phylogenies and mitochondrial concatenated phylogeny

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* + *Acanthophaelus*, and *Allopoecilia* + *Mollienesia* clades (SI Fig. S2). 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* (SI Fig. S2). 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 *Mollienesis* were highly resolved and strongly supported (with the exception of some relationships within the *P. mexicana* species complex; SI Fig. S2). 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 have an unresolved relationship to the *P. catemaconis* and the *P. marcellinoi* + *P. sphenops* clade (SI Fig. S2). 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).

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, SI Fig. S3). The phylogenetic placement of two haplotypes from the San Juan province clustered with *P. orri* (labeled *orri* [clade I] in SI Fig. S3). Clade III from Lee and Johnson (2009) was consistently recovered as a distinct group in our analyses (SI Fig. S3). 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.

3.2.1 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*; SI Fig. S4). 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 (SI Fig. S4).

3.3 Nuclear gene phylogenies and nuclear concatenated phylogeny

The phylogenetic analyses based on individual nuclear genes were generally uninformative (SI Fig. S5), 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 (SI Fig. S6) 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.

3.4 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. 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. 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. 4b).

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. 4a; fixed mtDNA mutation rate) or in the Pliocene (c. 3.87; 2.44-4.90 HPD; Fig. 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. 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).

3.5 Ancestral area estimation

The ancestral area estimations of the genus *Poecilia* indicated a South American origin for the group (Fig. 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. 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. 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. 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. 5), with the occurrence of five speciation events (region E to C or region C to E; Fig. 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. 5).

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 1 and Table S1). 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

494 Middle America. In the diverse *P. sphenops* and *P. mexicana* species complexes,
495 diversification occurred from the Pliocene onwards. Speciation in these complexes was
496 primarily driven by dispersal, but vicariant events and ecological processes may also
497 have played a role.

499 *4.1 Caveats: Unresolved relationships and molecular clock uncertainties*

500 Our study attempted to resolve the evolutionary relationships of species within the
501 subgenus *Mollienesia* as well as the different subgenera in the genus *Poecilia* with a
502 multi-locus dataset and applying various phylogenetic and species tree methods. Even
503 though our approach has generally resulted in a well-supported phylogenetic hypothesis
504 for the group, the relationships of some taxa were not completely resolved. Theoretically,
505 this may be the result of hybridization (Alda et al., 2013; Bagley et al., 2015) or ancestral
506 lineage sorting in the course of rapid radiations (Koblmüller et al. 2010). Previous
507 analyses have indicated that inconsistencies among different phylogenetic hypothesis in
508 *Mollienesia* are likely caused by introgressive hybridization (Bagley et al., 2015), which
509 has also been observed in other fishes of the family Poeciliidae (Cui et al., 2013).
510 Although we did not test for introgressive hybridization or incomplete lineage sorting, we
511 suspect one or both of these mechanisms, due to the conflicting results in the
512 mitochondrial and nuclear phylogenies. Future studies should thoroughly test for
513 introgressive hybridization and incomplete lineage sorting by increasing sampling and
514 genetic data to assess hybridization and gene flow.

515 The molecular clocks and other calibration methods we applied have been
516 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. 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. 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.

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. 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. 3 and 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

Mollienesis, but the phylogenetic clustering with *Limia* was doubted due to the small sample size (Figure 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. 3 and 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, 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 *Mollienesis* (Fig. 3 & 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 (SI Fig. S2), 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 *Mollienesis*, our analyses recovered three main species complexes (Figure 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. 3 and 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, 2003; Rosen and Bailey, 1963). Previous studies have described

608 this species as an abundant, large sized molly with a pale yellow coloration and a dark
609 blotch at the base of the caudal fin (Astorqui, 1971; Quesada, 1971), which matches our
610 field observations.

611 The *P. mexicana* species complex includes 18 described species, with 10 sampled
612 for this study including *P. butleri*, *P. gillii* (*sensu* Alda et al., 2013), *P. hondurensis*, *P.*
613 *mexicana*, *P. nelsoni*, *P. orri*, *P. petenensis*, *P. salvatoris*, *P. sulphuraria*, and *P.*
614 *thermalis*. The complex also includes three distinct lineages recovered by previous
615 studies (*P.* “*gillii* sp. 2”: Alda et al., 2013; *P. sp.* “*Patuca*”: Bagley et al. 2015; *P.*
616 *limantouri*: Palacios et al., 2013; Tobler et al., 2011) and two additional distinct lineages
617 recovered by the present study (*P.* “sp2” and *P.* “sp3”). *Poecilia limantouri* has long been
618 considered a synonym (Poeser, 2003) or subspecies (Menzel and Darnell, 1973) of *P.*
619 *mexicana* despite morphological (Menzel and Darnell, 1973) and genetic differences
620 (Schartl et al., 1995). Our data support its recognition as a distinct species with genetic
621 distance of 0.9% to the next closest lineage (*P. mexicana*) and an average of 2.39% to the
622 remaining molly species (SI Table S2). It remains to be investigated whether the other
623 two distinct genetic lineages (*P.* “sp2” and *P.* “sp3”) exhibit unique phenotypic features
624 and warrant recognition as distinct species.

625 Finally, we would like to emphasize that the present study has not only uncovered
626 previously undescribed or unrecognized (due to synonymization) species, but it also
627 illustrates the presence of taxonomic inconsistencies and challenges for accurately
628 describing the diversity of this group. For example, Lee and Johnson (2009) presented a
629 detailed biogeographic analysis of what phenotypically has been considered *P. gillii* (see
630 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 S3, 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.

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. 5). The Caribbean and Central America subgenera (*Limia*, *Psychropoecilia*, *Curtipenis*, *Allopoecilia*, and *Mollienesia*) also originated in South America (Fig. 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. 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. 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. 5). The diversification within the *P. latipinna* species complex is dated to the Pliocene and Pleistocene (Fig. 4a and 4b) with an ancestral area in the Maya Block (Fig. 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 freshwater 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 endemism 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).

699 Making biogeographic inferences for the *P. sphenops* and *P. mexicana* species
700 complexes is complicated by gaps in current data availability. There are uncertainties
701 surrounding the molecular clock estimates (see above) and major problems with the
702 accuracy of species identifications and distributions (also see Alda et al., 2013; Bagley et
703 al. 2015). In some instances, the distributions have been vastly overestimated (*P. gillii*) or
704 underestimated (*P. mexicana*), requiring the reevaluation of current data and effectively
705 preventing rigorous analyses of biogeographic patterns. Nonetheless, the presence of
706 widespread species in both species complexes clearly indicates a high potential for
707 dispersal even across major phylogeographic barriers. For example, both *P. sphenops* and
708 *P. mexicana* occur on both the Atlantic and Pacific slopes, and their ability for dispersal
709 and colonization of novel habitats may be mediated by high tolerance to environmental
710 stress (see Hernández-Rodríguez and Bückle-Ramírez, 2010; Tobler et al. 2009; Trexler,
711 1986; Vega-Cendejas et al., 2013).

712 In addition, there are also potential examples of vicariant events, where patterns
713 of divergence coincide with estimated dates of geological events or prominent
714 phylogeographic breaks with other taxa. For example, in the *P. sphenops* species
715 complex, the distribution of *Poecilia chica*, endemic to a few of Mexico's Pacific river
716 drainages (Miller et al., 2005), coincides with a phylogeographic break that has affected
717 the diversification of other fishes and reptiles (Domínguez-Domínguez et al., 2006;
718 Mateos et al., 2002; Reyes-Velasco et al., 2013; Zarza et al., 2008). Similarly, *P.*
719 *catemacensis* is restricted to Lake Catemaco and surrounding rivers on the Atlantic coast
720 of Mexico (Miller et al., 2005), which harbor a high level of freshwater fish species
721 endemism (McEachran and Dewitt, 2008). The divergence time estimates of *P.*

catemacensis (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. 4a) and Pliostocene (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 *Mollienesis*.

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Figures and Tables

Table 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 lineages 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
<u><i>P. latipinna</i> complex</u>			
<i>Poecilia kykesis</i> Poeser, 2002	1, 9*	D	Formerly referred to as <i>P. petenensis</i> Günther, 1866
<i>Poecilia latipinna</i> (LeSueur, 1821)	1, 3, 8, 9*	AB	
<i>Poecilia latipunctata</i> Meek, 1904	1, 4, 5, 8*	B	
<i>Poecilia velifera</i> (Regan, 1914)	1, 9*	D	
<u><i>P. sphenops</i> complex</u>			
<i>Poecilia catemacensis</i> Miller, 1975	1, 7, 8, 11*	C	
<i>Poecilia chica</i> Miller, 1975	8*	C	
<i>Poecilia marcellinoi</i> Poeser, 1995	*	E	
<i>Poecilia maylandi</i> Meyer, 1983			
<i>Poecilia sphenops</i> Valenciennes, 1846	1, 7, 8, 9, 11*	CDE	
<i>Poecilia</i> “ <i>sphenops</i> ” sp. 1	7, 11*	E	Putatively undescribed lineage identified by Alda et al. (2013); may be the same distinct population discussed

<i>Poecilia</i> sp. “Tipitapa”	11*	E	in Carr and Giovannoli, 1994 Putatively undescribed lineage identified by Bagley et al. (2015); may be <i>Poecilia dovii</i> Günther 1866, which is currently considered a synonym of <i>P. mexicana</i>
<u><i>P. mexicana</i> complex</u>			
<i>Poecilia boesemani</i> Poeser, 2003			
<i>Poecilia butleri</i> Jordan, 1889	1, 2, 6, 7, 8, 10 *	B	
<i>Poecilia formosa</i> (Girard, 1859)			Gynogenetic hybrid between <i>P. mexicana</i> and <i>P. latipinna</i>
<i>Poecilia gillii</i> (Kner, 1863)	1, 3, 7, 9, 10, 11*	EFG	Currently thought to be widespread, but has restricted distribution based on Alda et al. (2013)
<i>Poecilia petenensis</i> Günther, 1866	9*	D	Referred to as <i>P. gracilis</i> Regan 1913 in some recent publications
<i>Poecilia hondurensis</i> Poeser, 2011	7, 11*	E	
<i>Poecilia koperi</i> Poeser, 2003	12		
<i>Poecilia mechtildae</i> Meyer, Etzel & Bork, 2002			
<i>Poecilia mexicana</i> Steindachner, 1863	1, 6, 7, 8, 9, 11*	CDE	
<i>Poecilia limantouri</i> Jordan & Snyder, 1900	6, 8, 11*	BC	Previously considered a subspecies of <i>P. mexicana</i>
<i>Poecilia nelsoni</i> (Meek, 1904)	2, 9, 10*	CD	
<i>Poecilia orri</i> Fowler, 1943	1, 7, 11*	DEF	
<i>Poecilia rositae</i> Meyer, Schneider, Radda, Wilde & Scharl, 2004			
<i>Poecilia salvatoris</i> Regan, 1907	10, 11*	EF	
<i>Poecilia sulphuraria</i> (Álvarez del Villar, 1948)	1, 6, 8, 11*	D	
<i>Poecilia teresae</i> Greenfield, 1990			
<i>Poecilia thermalis</i> Steindachner, 1863	8, 11*	D	
<i>Poecilia vandepolli</i> Van Lidth de Jeude, 1887	12		
<i>Poecilia wandae</i>	12		Formerly assigned to <i>Allopoecilia</i> , assigned to <i>Mollienesis</i> by Ho et al. (2016)
<i>Poecilia</i> “gillii” sp. 2	7, 11, 12*	G	Putatively undescribed lineage identified by Alda et al. (2013)
<i>Poecilia</i> sp. “Patuca”	11*	DEF	Putatively undescribed lineage identified by Bagley et al. (2015); treated as <i>P. orri</i> in this study
<i>Poecilia</i> “sp2”	*	EF	Putatively undescribed lineage identified in this study
<i>Poecilia</i> “sp3”	*	EF	Putatively undescribed lineage identified in this study

Previously assigned to *Mollienesia*

<i>Poecilia (Allopoecilia) caucana</i> (Steindachner, 1880)	1, 4, 5, 6, 7, 9, 12*	GHI
<i>Poecilia (Allopoecilia) dauli</i> Meyer & Radda, 2000	12	
<i>Poecilia (Psychropoecilia) dominicensis</i> (Evermann & Clark, 1906)	*	J
<i>Poecilia (Curtipenis) elegans</i> (Trewavas, 1948)	*	J
<i>Poecilia (Psychropoecilia) hispaniolana</i> Rivas, 1978	7, 12*	J
<i>Poecilia (Poecilia) vivipara</i> 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

Table 2. The genes partitioned by position and by gene for mitochondrial DNA and nuclear DNA for phylogenetic study of the Subgenus *Mollienesis* 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

Figure 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 *Mollienesisia* 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-Mexican Volcanic 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.

Figure 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 *Mollienesisia* (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 *Mollienesisia* or *Limia* (dashed lines). The color coding of species complexes reflects the color scheme of individual species in Figure 1.

Figure 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.

Figure 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.

Figure 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.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5