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EVOLUTION IN EXTREME ENVIRONMENTS: REPLICATED PHENOTYPIC DIFFERENTIATION IN LIVEBEARING FISH INHABITING SULFIDIC SPRINGS

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We investigated replicated ecological speciation in the livebearing fish *Poecilia mexicana* and *P. sulphuraria* (Poeciliidae), which inhabit freshwater habitats and have also colonized multiple sulfidic springs in southern Mexico. These springs exhibit extreme hypoxia and high concentrations of hydrogen sulfide, which is lethal to most metazoans. We used phylogenetic analyses to test whether springs were independently colonized, performed phenotypic assessments of body and gill morphology variation to identify convergent patterns of trait differentiation, and conducted an eco-toxicological experiment to detect differences in sulfide tolerances among ecotypes. Our results indicate that sulfidic springs were colonized by three different lineages, two within *P. mexicana* and one representing *P. sulphuraria*. Colonization occurred earlier in *P. sulphuraria*, whereas invasion of sulfidic springs in *P. mexicana* was more recent, such that each population is more closely related to neighboring populations from adjacent nonsulfidic habitats. Sulfide spring fish also show divergence from nonsulfidic phenotypes and a phenotypic convergence toward larger heads, larger gills, and increased tolerance to H_2S. Together with previous studies that indicated significant reproductive isolation between fish from sulfidic and nonsulfidic habitats, this study provides evidence for repeated ecological speciation in the independent sulfide spring populations of *P. mexicana* and *P. sulphuraria*.

KEY WORDS: Ecological speciation, hydrogen sulfide, hypoxia, local adaptation, morphological differentiation, mtDNA phylogeny, natural selection, *Poecilia*.

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Divergent natural selection is an evolutionary force that not only drives adaptive trait divergence along environmental gradients, but also has the potential to drive the evolution of reproductive isolating barriers (Schluter 2000; Rundle and Nosil 2005). Evidence for such ecological speciation is mounting not only from theoretical studies (Gavrilets et al. 2007; Thibert-Plante and Hendry 2009), but also from empirical studies in the wild (Funk 1998; Rundle et al. 2000; Langerhans et al. 2007) and in the laboratory (Kilias et al. 1980; Dodd 1989; Dettman et al. 2007, 2008).

Predictable differentiation along replicated selective gradients provides strong evidence for a central role of natural selection in driving adaptive trait divergence (Clarke 1975; Endler 1986; Wood et al. 2005). Systems where populations of the same species evolved reproductively isolated ecotypes in convergence are therefore particularly valuable to study mechanisms of ecological speciation (Schluter and Nagel 1995; Johannesson 2001; see Arendt and Reznick 2008 for a disambiguation of the term "convergence"). Examples of convergent ecological speciation have been documented in organisms inhabiting different habitat types (skinks: Richmond and Reeder 2002; monkey flowers: Lowry et al. 2008; marine snails: Johannesson et al. 2010), exploiting different resources (sticklebacks: Rundle et al. 2000; walking stick insects: Nosil et al. 2002; island finches: Ryan et al. 2007), and experiencing different predator regimes (mosquito fish: Langerhans et al. 2007).

We investigated potential convergent patterns of ecological speciation in fish inhabiting freshwater springs with high concentrations of H_2S. Hydrogen sulfide is a potent respiratory toxicant with effects similar to cyanide (Lovatt Evans 1967; Petersen 1977; National Research Council 1979; Reiffenstein et al. 1992). Most organisms are able to detoxify H_2S to some extent, as sulfide is present in the atmosphere in very low concentrations and also produced endogenously by eukaryotic cells as a product of cysteine catabolism (Kimura 2002; Szabo 2007). However, only a few organisms are able to withstand continued exposure to higher concentrations of H_2S (Bagarinao 1992; Reiffenstein et al. 1992). Sulfide-tolerant metazoans can either be found in aquatic habitats where sulfide concentrations peak intermittently, usually because of bacterial decomposition of organic matter in severely oxygen deprived environments (e.g., in salt marshes: Bagarinao and Vetter 1993; or aquatic sediments: Grieshaber and Völkel 1998), or in environments with sustained and high concentrations of sulfide, where H_2S is of geological origin (e.g., in cold seeps and hydrothermal vents: Van Dover 2000). Freshwater systems with continuously high concentrations of H_2S also exist as sulfide springs commonly associated with volcanism (Rosales Lagarde et al. 2006). Such springs have been colonized by various cyprinodont fishes (particularly livebearing species of the family Poeciliidae) in different parts of the United States and the Neotropics (Tobler and Plath 2011).

Patterns of trait divergence and reproductive isolation between fish inhabiting sulfide springs and adjacent nonsulfidic habitats have so far only been studied in the southern Mexican Cueva del Azufre system, where sulfidic springs occur in surface as well as cave habitats (Tobler et al. 2006). In this system, *Poecilia mexicana* (Poeciliidae) from sulfide-rich springs diverged in a number of traits including body shape and gill morphology (Tobler et al. 2008a), life-history strategies (Riesch et al. 2010), and behavior (Plath and Schlupp 2008). Respiratory adaptations are a key for surviving in sulfidic environments, as supplementary oxygen is required for sulfide detoxification, and at the same time oxygen availability is limited in sulfidic environments because of the spontaneous reaction of sulfide and oxygen in aqueous solution (Cline and Richards 1969; Chen and Morris 1972). Hence, morphological changes of the gills (e.g., enlarged gill surface area), along with a behavioral response elicited by the presence of sulfide (aquatic surface respiration, ASR, where the fish skim the more oxygen-rich air-water interface using their gills), facilitate efficient oxygen acquisition in the hypoxic and sulfidic environment and have direct effects on fish survival (Plath et al. 2007b; Tobler et al. 2009a).

In addition to adaptive trait divergence, gene flow between *P. mexicana* populations inhabiting adjacent sulfidic and nonsulfidic habitats is low despite the absence of major physical barriers preventing fish migration (Plath et al. 2007a, 2010a; Tobler et al. 2008a). Reproductive isolation seems to be mediated at least in part by natural and sexual selection against immigrants, that is, reduced survival of nonadapted fish and female mate choice against males of the respective "opposite" ecotype (Tobler et al. 2009b). Taken together, these previous results suggest that evolutionary patterns present in the Cueva del Azufre system are consistent with ongoing ecological speciation (Plath and Tobler 2010; Tobler and Plath 2011).

Mollies of the genus *Poecilia*, however, not only colonized sulfidic springs in the Cueva del Azufre system, which is part of the Río Tacotalpa drainage, but also in at least two other tributaries of the Río Grijalva in southern Mexico (Fig. 1). *Poecilia mexicana* type fish are present in sulfidic springs of the Puyacatengo drainage, and *P. sulphuraria*, a species endemic to sulfidic springs, has been described from a spring in the Pichucalco drainage (Alvarez del Villar 1948; Tobler et al. 2008b). These independent sulfide springs offer the possibility to test for convergent patterns of adaptive trait divergence and ecological speciation along multiple sulfidic/nonsulfidic environmental gradients. In this study, we addressed three basic questions: (1) Is there evidence for independent colonization of different sulfur-spring complexes? (2) Do we find support for convergent phenotypic divergence among drainages? (3) Are there consistent differences in physiological performance between fish from sulfide springs and nonsulfidic habitats when exposed to H_2S? We addressed these

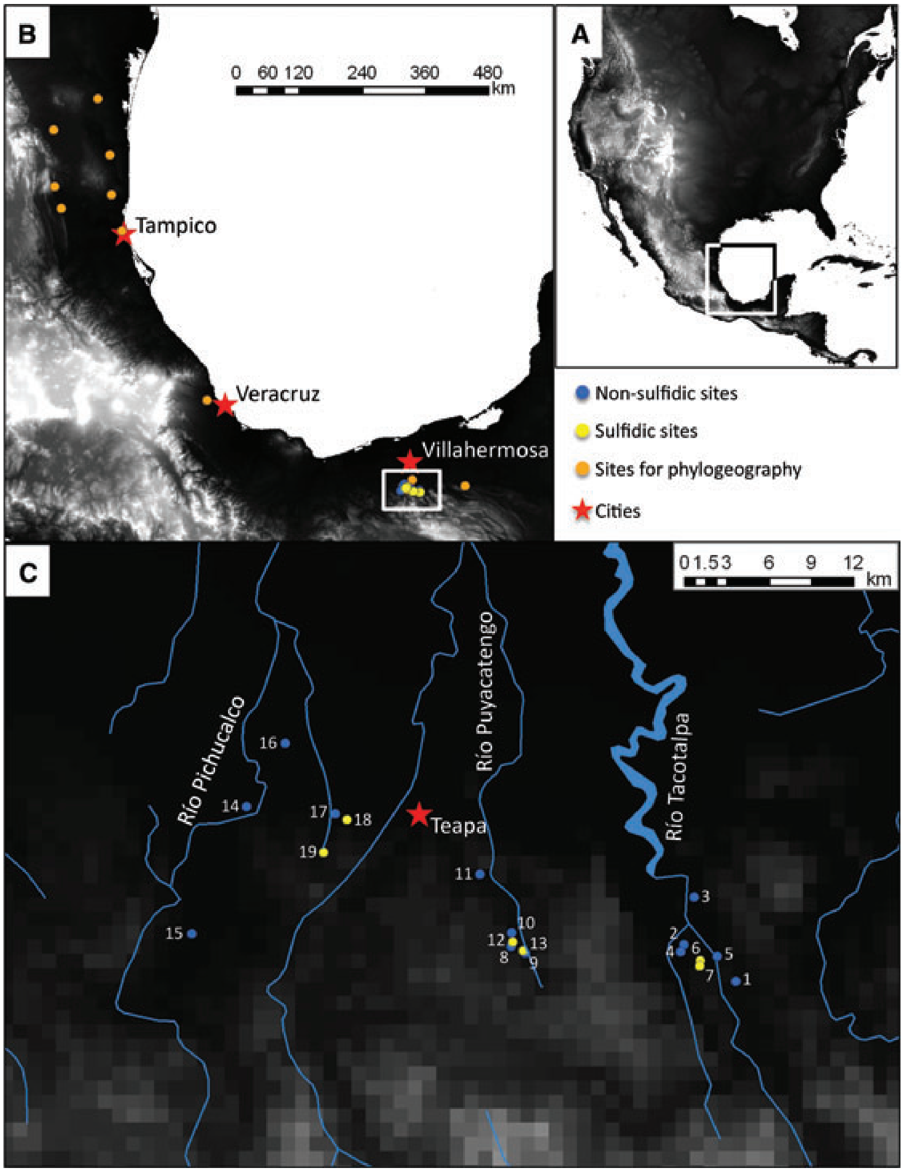

Figure 1. (A) Overview of the general study area. (B) Detailed view of the Atlantic versant of Mexico indicating the collection sites of *Poecilia mexicana* and *P. sulphuraria*. (C) Magnification of the study area in southern Mexico, where the sulfidic springs are located. See Table S2 for locality names.

questions by combining phylogenetic, morphological, and ecophysiological approaches.

Materials and methods

STUDY SITES AND SAMPLE COLLECTIONS

For this study, *Poecilia* spp. were collected in sulfidic springs and adjacent nonsulfidic habitats in the Mexican states of Tabasco and Chiapas, particularly in the region around the city of Teapa. Here, the mountains of the Sierra Madre de Chiapas meet the wide floodplains of northern Tabasco. The six spring complexes known to be inhabited by *Poecilia* are located in the foothills of the Sierra Madre and are distributed across three major tributaries of the Río Grijalva. In the upper reaches where the sulfidic springs are located, these tributaries (Tacotalpa, Puyacatengo, and Pichucalco from east to west; Fig. 1) are separated by mountains, while they all eventually join the Río Grijalva and are widely interconnected in the lowlands at least during the wet season (Miller 1966). The major trajectory of ecological differences among the sites examined is due to the presence of hydrogen sulfide in the water (Tobler et al. 2006). H_2S in this region is likely associated with volcanic activity (Chichón Volcano; Rosales Lagarde et al. 2006). Sulfidic habitats on average exhibit sulfide concentrations between 23 and 190 μM depending on the spring (see Table S1 for data on physical and chemical water characteristics for each site). Although spatial variation in sulfide concentrations is relatively pronounced in all springs, with concentrations decreasing with increasing distance from the spring heads due to volatilization (Cooper et al. 1989) and oxidation of sulfide molecules (Cline and Richards 1969; Chen and Morris 1972), temporal variation in both sulfidic and nonsulfidic habitats is minimal by comparison (Table S1; Plath et al. 2010a). Besides the presence of H_2S, sulfidic habitats also differ from nonsulfidic habitats in exhibiting higher temperatures and higher specific conductivities, as well as lower pH and lower dissolved oxygen concentrations (Table S1).

Each of the tributaries harbors two spring complexes from which we obtained samples. For comparison, we also collected specimens from a variety of habitats that do not contain H_2S throughout the tributaries (Fig. 1C, Table S2). All specimens were collected using seines, euthanized with buffered MS222 immediately after capture, and fixed in a 10% formaldehyde solution. In addition, we took fin clips that were preserved in 90% ethanol and stored at 4°C. Table S2 summarizes the material collected and examined in the different analyses.

For the phylogenetic analysis, we used previously published and newly sequenced haplotypes (see Table S3 for GenBank accession numbers). We also incorporated samples of *P. mexicana* collected throughout the Atlantic versant of Mexico (Fig. 1B). These samples included *P. mexicana* from northern Mexico that differ from the southern populations in morphological traits and have been described as a distinct subspecies (*P. m. mexicana* from the Río Cazones basin on south, *P. m. limantouri* to the north [Menzel and Darnell 1973]). From southern Mexico, samples included multiple individuals of *P. mexicana* from a variety of nonsulfidic sites as well as *P. mexicana* and *P. sulphuraria* from all known sulfidic springs (including previously published haplotypes from the Tacotalpa system [Tobler et al. 2008a]).

For the subgenus *Mollienesia* (within the genus *Poecilia*), we additionally included a single specimen each of *P. catemaconis*, *P. latipunctata*, and *P. caucana*, as well as multiple individuals from multiple localities of *P. sphenops, P. butleri,* and *P. latipinna.* As outgroups, we used *Cnesterodon decemmaculatus* and *C. hypselurus*, as well as representatives of the other subgenera in the genus *Poecilia* (sensu lato) found outside *Mollienesia* (Breden et al. 1999; Hrbek et al. 2007): *Poecilia* (sensu stricto), *Limia, Micropoecilia*, and *Pamphorichthys.*

PHYLOGENETIC ANALYSES

To elucidate relationships among sulfide spring populations, total genomic DNA was extracted from ethanol-preserved fins with the DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol. The samples were amplified for the mitochondrial cytochrome *b* gene (cyt *b*, 1140 bp) with the LA and HA primers (Schmidt et al. 1998). A subset of samples was also amplified for the mtDNA gene NADH subunit 2 (ND2, 1047 bp) with the ND2B-L (Broughton and Gold 2000) and ASN (Kocher et al. 1995) primers following previously published polymerase chain reaction (PCR) protocols (Mateos et al. 2002). 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), initially aligned with Clustal X version 2.0 (Larkin et al. 2007), and edited manually. None of the sequences had premature stop codons or frame shifts, suggesting that they do not represent pseudogenes. All new sequences have been deposited in GenBank (see Table S3).

For data analysis, we chose three different approaches: (A) cyt *b* gene only; (B) ND2 gene only; and (C) concatenated cyt *b* and ND2. The cyt *b* only dataset contained 85 haplotypes and included those from Tobler et al. (2008a). The ND2 gene was only amplified in a subset of individuals and consequently contained fewer sequences (32). The concatenated dataset contained only distinct haplotypes for which both sequences were available (34). We used jModeltest version 0.1.1 (Posada 2008) to determine the most appropriate model of DNA substitution among 88 candidate models on a fixed BioNJ-JC tree based on the Akaike information criterion (AIC), corrected AIC(c), and Bayesian information criterion (BIC).

For maximum likelihood (ML) analyses, we used RAxML version 7.2.5 (Stamatakis 2006, 2008) as implemented on the Cyberinfrastructure for Phylogenetic Research (CIPRES) server and version 7.2.6 run on a computer to conduct 200 Rapid Bootstrap searches followed by an ML search. We used the complex general time reversible (GTR: Tavaré 1986) + G (Gamma distribution for rate variation among sites) model, with and without I (proportion of invariable sites), because of potential problems with estimating $G + I$ simultaneously (see RaxML manual and Yang 2006). RaxML does not implement simpler models. We also used GARLI version 0.96 (Zwickl 2006) to perform ML bootstrap searches (200 replicates) on the three datasets under their corresponding best model(s) selected (see Results), also with and without parameter I.

Bayesian analyses were run on the parallel version of MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003), under the GTR + G + I and GTR + G models, implementing two runs with four chains each for 50,000,000 generations sampled every 5000 generations (all other parameters were default). 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).

To test hypotheses about the phylogenetic origin of sulfide spring mollies, we used the approximately unbiased (AU) test (Shimodaira 2002) as implemented in CONSEL (Shimodaira and Hasegawa 2001) based on the cyt *b* and the cyt *b* and ND2 concatenated dataset. We compared the following hypotheses: (1) maximum likelihood (ML) tree (i.e., based on an ML unconstrained search); (2) the most likely tree containing the monophyly of all haplotypes of sulfur-inhabiting *P. m. mexicana* (i.e., single origin within *P. mexicana*); and (3) the most likely trees containing the monophyly of haplotypes of *P. sulphuraria* and all sulfur-inhabiting *P. m. mexicana* (i.e., single origin of all sulfide spring mollies). To obtain trees (2) and (3), we performed ML heuristic searches constrained to the respective monophyly. These ML searches were performed in PAUP* version 4b10 (Swofford 1998), with TBR branch swapping and 20 random additions, and assuming the models selected above and with parameter values estimated on the unconstrained topology.

GEOMETRIC MORPHOMETRICS

To quantify phenotypic differentiation between populations from sulfidic and nonsulfidic habitats, we performed a geometric morphometric analysis. For all specimens, lateral radiographs were taken with a Hewlett-Packard (Palo Alto, CA) Faxitron cabinet X-ray system. We digitized 13 landmark points on each image (see Fig. 2) using the software program tpsDig (Rohlf 2004). Based on the coordinates of the digitized landmarks, a geometric morphometric analysis was performed. Landmark coordinates were aligned using least-squares superimposition as implemented in the program tpsRelw (Rohlf 2007) to remove effects of translation, rotation, and scale. In addition, eye diameter was measured using calipers. This distance was halved and used to position two reference points anterior and posterior to the center of the orbit (with the same y-coordinate). After realignment, we calculated centroid size and partial warp scores with uniform components (weight matrix) for each individual. The weight matrix was subjected to a principal component analysis based on a covariance matrix to reduce data dimensionality. Principal axes with eigenvalues below 1 were dropped from the analysis and the remaining axes were retained as shape variables (seven axes accounting for 92.9% of variance). Unless stated otherwise, all statistical analyses were performed using SPSS 17 (SPSS, Inc., 2008).

To test for phenotypic differentiation (i.e., body shape variation) among sites, we used multivariate analyses of covariance (MANCOVA). Assumptions of multivariate normal error and homogeneity of variances and covariances were met for all analyses performed. F-values were approximated using Wilks' lambda and effect strengths by use of partial eta squared (η_p^2). We also calculated the relative variance as the partial variance for a given term divided by the maximum partial variance value in a model. We tested for effects of centroid size to control for multivariate allometry and included sex, H_2S (present or absent), drainage, and site as independent variables in a nested model following Langerhans and DeWitt (2004). Shape variation along the first two principal component axes and along factors included in the MANCOVA was visualized with thin-plate spline transformation grids using tpsRegr (Rohlf 2005).

As random nested factors are not applicable for MANCOVAs, and the use of fixed effects can inflate type I error rates if nested terms are significant, we also conducted a mixed-model nested analysis of covariance (ANCOVA; see Langerhans 2009). To do so, we calculated divergence scores for each individual along the sulfide/nonsulfide gradient based on a divergence vector as defined by Langerhans (2009). We then analyzed these scores with a model identical to the one described above, while site was designated a random factor.

In addition, discriminant function analysis (DFA) was conducted within each drainage to determine the percentage of specimens that could be correctly classified to sulfidic and nonsulfidic habitats solely based on body shape. To facilitate the DFA, we first removed the effects of sex and allometry by using the residuals of a preparatory MANCOVA, in which the seven principal components were used as dependent variables, centroid size as a covariate, and sex as an independent variable.

GILL MORPHOLOGY

To quantify variation in gill morphology, we measured total gill filament length (TGFL) as an index for oxygen uptake. We selected TGFL rather than another commonly measured parameter, gill surface area, because it is more likely to reflect spatial constraints of large gills, and because previous studies have found that populations with larger TGFL are characterized by a larger gill surface area in the poeciliid *P. latipinna* (Timmerman and Chapman 2004) as well as the cichlids *Pseudocrenilabrus multicolor* (Chapman et al. 2000; Chapman et al. 2008; Crispo and Chapman 2010) and *Astatoreochromis alluaudi* (Chapman et al. 2007).

To quantify TGFL, each of the four gill arches from the left side of the branchial basket was separated and laid flat on a microscope slide (Fig. S1). For each hemibranch, the length of every fifth gill filament was measured, and two successive measurements were averaged to estimate length of intermediate filaments. Filament lengths were summed for the eight hemibranchs and were multiplied by 2 to yield an estimate of TGFL. Data were analyzed using ANCOVA; TGFL was log-transformed prior to analysis. Body mass (log-transformed) was used as a covariate and sex, H_2S (present or absent), drainage, as well as their interactions were used as factors. The three-way interaction between sex, H_2S, and drainage was not significant ($F_{1,82} = 1.18$, $P = 0.281$, $\eta_p^2 = 0.014$) and thus removed from the final model.

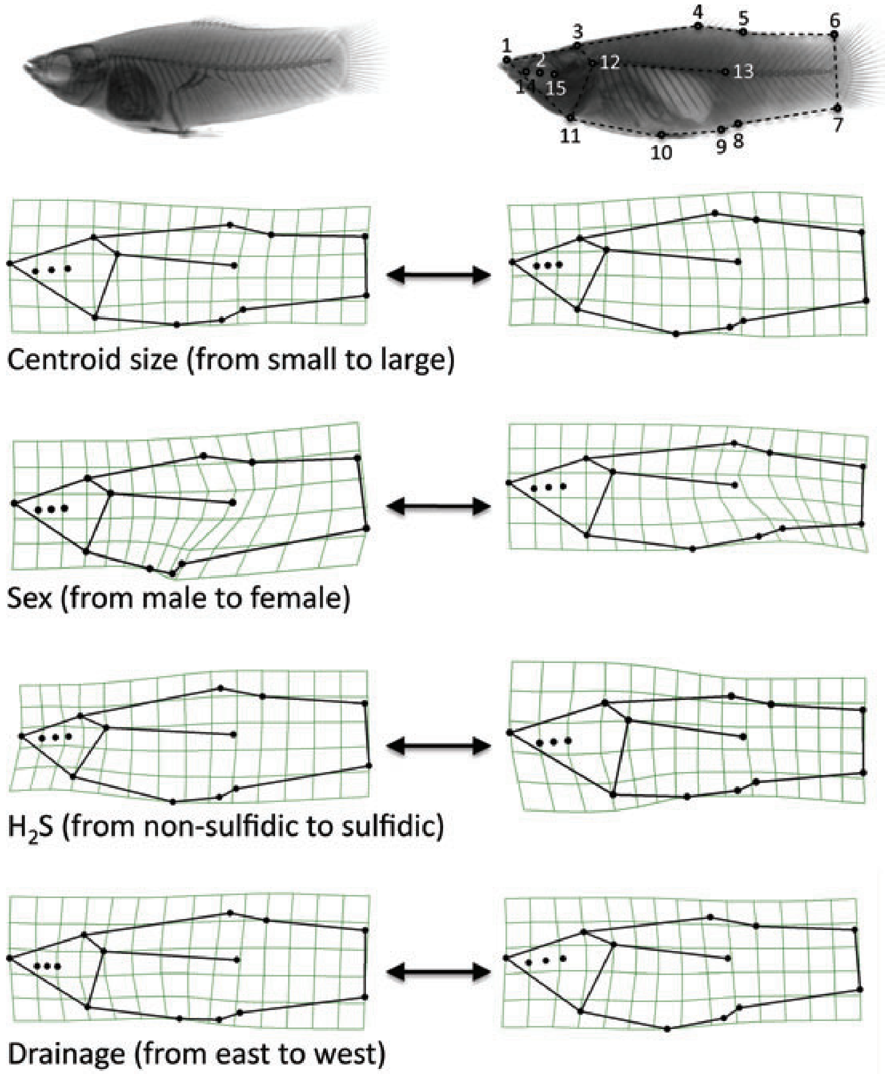

Figure 2. The top panel shows radiographs of a male (left) and a female *Poecilia mexicana* (right; both nonsulfidic ecotype). Landmarks included the tip of the upper jaw (1); the center of the orbit (2); the posterodorsal tip of the supraoccipital crest (3); the anterior (4) and posterior (5) junction of the dorsal fin with the dorsal midline; the junction of the caudal fin with the dorsal (6) and ventral midline (7); the posterior (8) and anterior (9) junction of the anal fin with the ventral midline; the anterior junction of the pelvic fins and the ventral midline (10); the bottom of the head where the operculum breaks away from the body outline (11); the center of the first rib-bearing vertebra (12); the center of the third vertebra with a hemal arch (13); and the anterior (14) and posterior (15) edge of the eye. Landmarks are connected as in the thin-plate spline transformation grids used throughout the article. The lower four panels show visualizations of shape variation accounted for by the terms having the highest effect size ($\eta_p^2 > 0.3$; see Table 2) in the MANCOVA. Note that sulfidic ecotypes are characterized by having larger heads than nonsulfidic ecotypes.

SULFIDE TOLERANCE

To test for differences in sulfide tolerance, we subjected wild-caught fish from different populations to increasing concentrations of sulfide. Upon collection, fish were kept in insulated coolers with water from the collection sites. To standardize experimental conditions, the water from the collection sites was gradually replaced with aerated well water that did not contain H_2S over the first 12 h after capture, and fish were allowed to acclimatize to these conditions for about 24 h. During this time, the water was continuously aerated and filtered, and fish were not fed.

To compare the tolerance to sulfide between fish from different sites, we obtained sulfidic water from a spring at the Baños del Azufre that exhibits high H_2S concentrations (mean ± SD from 2008 and 2009: 0.904 ± 0.012 mM). For the experiment, individual fish were placed in containers with 100 mL water from the stock tanks, and 10 mL of sulfidic water was added at 2-min intervals. This resulted in a logarithmic increase in concentration to 0.540 mM sulfide over 30 min (Fig. 3A). We measured the time to the initial loss of motion control. Then fish were removed from the container, measured for standard length, weighed, and placed

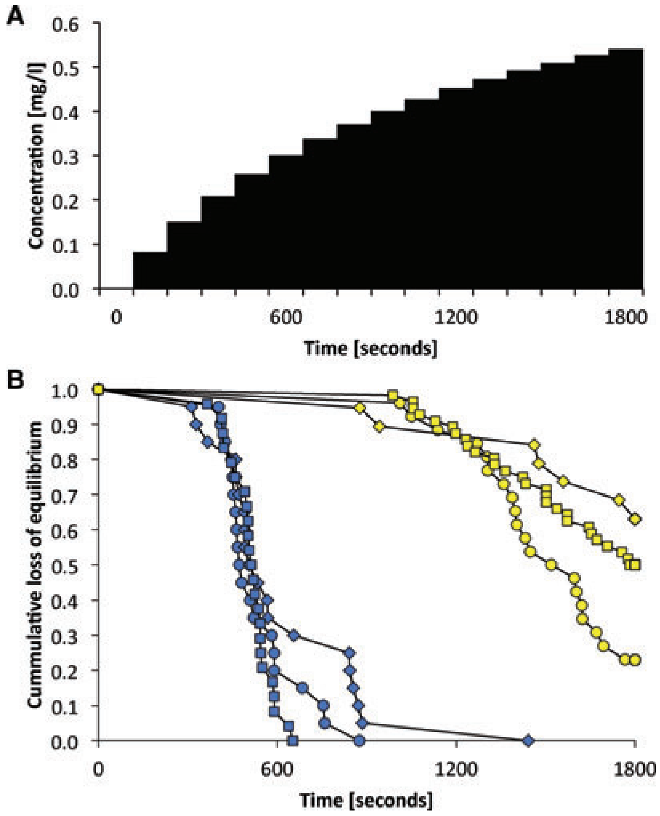

Figure 3. (A) Increase in H_2S concentration over 30 min in the experiment on sulfide resistance. (B) Survival curves of *Poecilia* from different sites. Yellow symbols indicate fish from sulfidic habitats, blue symbols fish from nonsulfidic reference habitats, and symbol shapes stand for the drainage of origin: Tacotalpa (●), Puyacatengo (◆), and Pichucalco (■).

in a heavily aerated recovery container. All fish survived these tolerance tests. Data were analyzed using a survival analysis (Cox regression) in which time until loss of motion control was used as dependent variable. Individuals unaffected by the addition of sulfidic water at the end of the experiment were censored. Drainage, H_2S (present or absent), sex, mass (log-transformed) as well as their interactions were used as independent variables while using a backwards elimination approach. The model with the highest support retained H_2S, sex, mass, and the interaction between drainage and H_2S in the final analysis. *P. sulphuraria* populations from the Baños del Azufre and La Gloria were pooled for this analysis because Cox regression does not allow for nested terms.

Results

PHYLOGENETIC ANALYSES

Technical aspects of the results of phylogenetic analyses and a discussion of general relationships of taxa examined can be found in the Supporting information. Our analyses indicated that all of our samples of *P. mexicana* and *P. sulphuraria* formed a well-supported monophyletic group (97–100%; see Figure 4 for a phylogenetic tree based on cyt *b* and Fig. S2 for a tree based on the combined dataset). Within this group, three major highly supported lineages were recovered, corresponding to *P. sulphuraria*, *P. m. mexicana,* and *P. m. limantouri*, respectively. However, *P. mexicana* was not monophyletic. Instead, *P. m. limantouri* was most closely related to *P. sulphuraria*, a relationship supported by at least 92% support. Our data thus indicate that *P. sulphuraria* evolved independently from the *P. m. mexicana* sulfide spring populations. Relationships among the *P. m. mexicana* mollies, including the sulfur spring haplotypes, are not well resolved, however. Except for two cyt *b* haplotypes (green in Fig. 4), all haplotypes recovered were unique to either sulfidic springs (red) or nonsulfidic waters. Cyt *b* analyses recovered a moderately supported (77–100%) lineage including all of the sulfide spring haplotypes found in springs of the Puyacatengo drainage (Puyacatengo springs and La Lluvia springs). The combined dataset analyses, too, recovered a strong monophyly of these (91–100%). The AU test significantly rejected ($P < 0.001$; Table 1) the monophyly of all sulfide spring *P. m. mexicana* haplotypes + the *P. sulphuraria* clade (15 equally likely topologies) for both datasets (i.e., cyt *b* only and cyt *b* plus ND2). The cyt *b*-only AU test failed to reject the monophyly of all sulfide spring *P. m. mexicana* haplotypes ($P = 0.506$; Table 1), but this was probably due to lack of sufficient phylogenetic information. This was enhanced by inclusion of the ND2 sequences, and monophyly was significantly rejected by the AU test on the concatenated dataset ($P = 0.021$). Thus, our results with the concatenated dataset suggest at least three independent colonizations of sulfidic habitats by mollies in southern Mexico: one by *P. sulphuraria* and two within *P. m. mexicana.* Although the concatenated dataset contains a subset of the cyt *b* haplotypes, this subset is representative of most of the observed cyt *b* haplotype clades (marked with in Fig. 4). Thus, it is unlikely that a larger sample of concatenated haplotypes would have affected our AU test results.

GEOMETRIC MORPHOMETRICS

In total, we examined 629 individuals from 19 sites (Table S2). Body shape significantly and strongly ($\eta_p{}^2 \geq 0.597$) differed between nonsulfidic and sulfidic ecotypes as well as between the sexes (Table 2A). This pattern persisted even when treating site as a random factor in the analysis (Table 2B). Inspection of a PCA plot indicates that fish from sulfidic habitats occupy a distinct area in morphospace, with more negative scores along principal axis 1 and especially axis 2 (Fig. 5A).

Thin-plate spline transformation grids visualizing the shape differences along these axes indicate that fish inhabiting sulfidic habitats differ from those from nonsulfidic ones primarily in head shape, caudal peduncle proportions, and position of dorsal and anal fins along axis 1, as well as in head shape and body depth

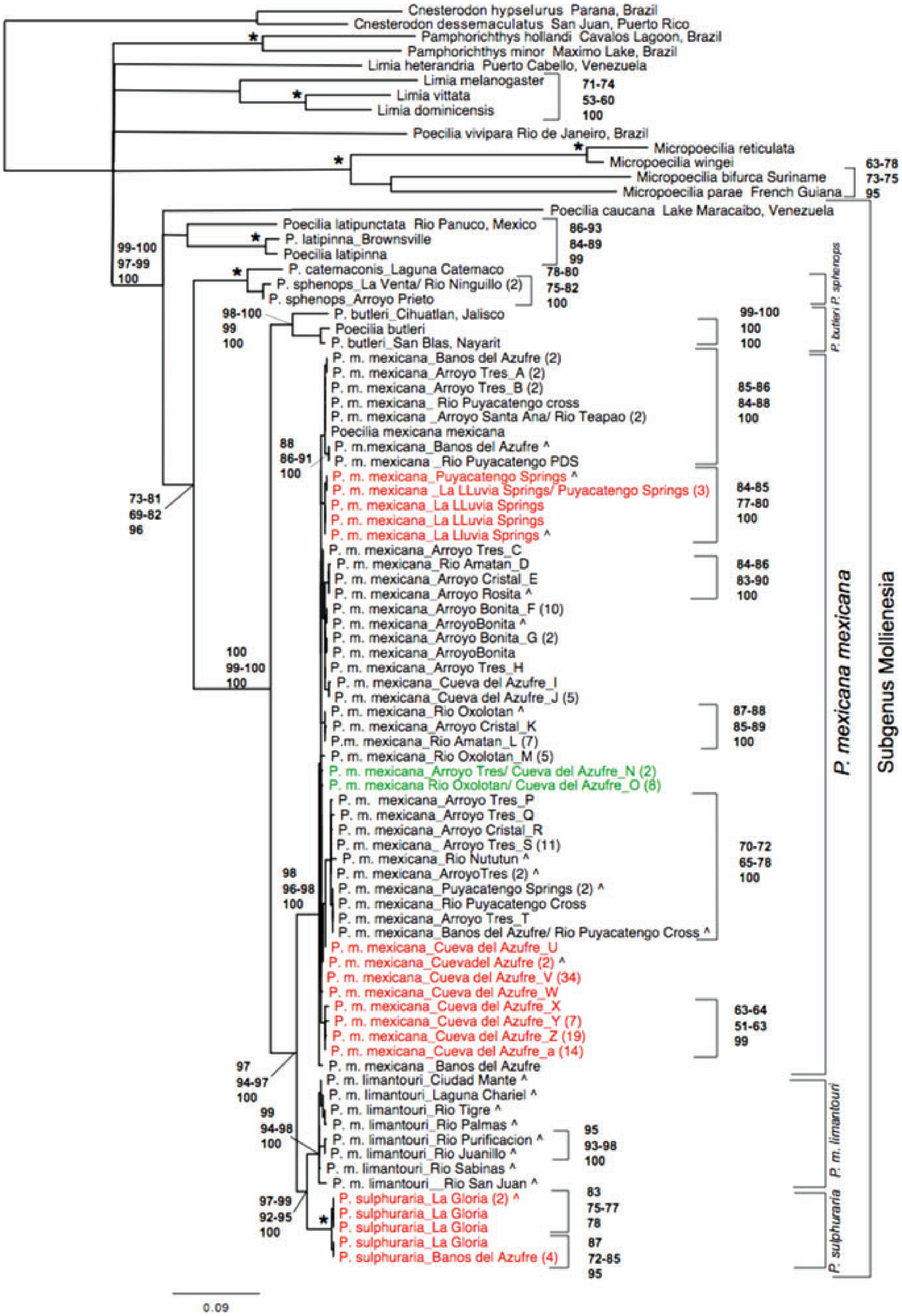

Figure 4. Maximum likelihood tree from the RAxML analysis of *Poecilia* spp. for the mitochondrial cytochrome *b* gene, rooted with other poeciliid outgroups. Nodal support shown (top to bottom; respectively): RaxML bootstrap support, GARLI bootstrap support, and Bayesian Posterior Probabilities. Asterisks denote nodal support of 100% for all three methods. Samples highlighted in red represent sulfur habitat individuals and samples highlighted in green represent haplotypes found in both sulfur and nonsulfur habitats. Nodes with no values present either had low values or were of little interest for this study. ˆ indicates presence in concatenated phylogenetic tree.

Table 1. **Approximately unbiased test for comparison of the following hypotheses: (1) Maximum likelihood (ML) tree; (2) the most likely tree containing the monophyly of all *P. mexicana* sulfide spring inhabiting haplotypes; and (3) the most likely trees containing the monophyly of *P. sulphuraria* and *P. mexicana* sulfide spring inhabiting haplotypes. AU test conducted on two datasets: cyt *b* only; and cyt *b* and ND2 concatenated.**

Rank[a]	Tree	AU test *P*-value
Cyt b only dataset		
1	ML cyt *b* ML unconstrained	0.495
2	Monophyly: *Poecilia m. mexicana* sulfur haplotypes	0.506
3	Monophyly: *Poecilia m. mexicana* and *P. sulphuraria* sulfur haplotypes (15 equally likely topologies)	<0.001
Cyt b and ND2 dataset		
1	ML cyt *b* and ND2 ML unconstrained	0.979
2	Monophyly: *Poecilia m. mexicana* sulfur haplotypes	0.021
3	Monophyly: *Poecilia m. mexicana* and *P. sulphuraria* sulfur haplotypes	<0.001

[a]Best to worst based on ML score.

along axis 2. Visualizing the shape effect of the H_2S term from the MANCOVA (Fig. 2) highlights that the shared differences between all sulfidic and all nonsulfidic populations primarily involve head shape, and fish from sulfidic habitats generally have larger heads than fish from nonsulfidic habitats.

Differentiation between sulfidic and nonsulfidic ecotypes was significant in each drainage (Fig. 5B), and DFA classified the majority of specimens to the habitat type of origin based on body shape alone (percentage of correctly classified individuals, Tacotalpa: 91.7%; Puyacatengo: 91.0%; Pichucalco: 97.3%; whereby classification success is expected to be 50% under a null hypothesis of no correct site-assortment based on the morphological features considered here).

The primary difference between sexes was the position of the anal fin (Fig. 2). In males, the anal fin is modified into a copulatory organ (gonopodium) characteristic for the subfamily Poeciliinae, which is typically located more anterior than the female anal fin (Rosen and Bailey 1963). Although centroid size and drainage also accounted for a good proportion of the variation in body shape (see Fig. 2 for the nature of shape effects), the factor site and all interaction terms involving site, although being significant, generally had low effect sizes ($\eta_p^2 \leq 0.159$).

GILL MORPHOLOGY

We measured TGFL for 94 fish from seven sites. TGFL was strongly and positively correlated with body mass (Table 2C). More importantly, fish from sulfidic habitats consistently had larger gills than fish from nonsulfidic habitats (Fig. 6), even though the magnitude of difference varied across drainages (see significant Drainage × H_2S interaction in Table 2C). We also found significant sexual dimorphism in gill morphology, and males generally exhibited smaller gills than females (estimated marginal means ± SEM of TGFL, males: 470.4 ± 17.0 mm, females: 502.4 ± 7.1 mm). This sex effect, however, needs to be interpreted with caution, as the statistical power is low due to low sample sizes of males in two of the three drainages. In addition, we quantified four complementary metrics of gill size (total number of gill filaments, average gill filament length, total hemibranch perimeter, and total hemibranch area), which showed the same pattern of variation as TGFL (see Supporting information).

SULFIDE TOLERANCE

Sulfide tolerance was strongly dependent on an individual's body mass, and smaller individuals exhibited higher tolerance than large ones (Table 3). Furthermore, the experiment revealed a significant interaction term between drainage and H_2S, which appears to be driven mainly by variation in sulfide tolerance of sulfidic ecotypes from different drainages (Fig. 3B). Overall, however, fish from sulfidic habitats exhibited consistently higher tolerances as compared to fish from nonsulfidic habitats. There was also a tendency for males to lose motion control earlier than females ($P = 0.070$).

Discussion

In southern Mexico, fish of the genus *Poecilia* have invaded several sulfide springs. Our study indicates that sulfide spring inhabitants consistently differ from conspecifics from adjacent nonsulfidic habitats in body shape, gill morphology, as well as sulfide tolerance. Phylogenetic analyses based on DNA sequences of two mitochondrial genes further indicate independent colonization events in three different drainages. Phenotypic similarities among sulfide spring fish from different drainages thus originated through convergent evolution along replicated sulfide/nonsulfide environmental gradients.

PHYLOGENETIC RELATIONSHIPS AND THE INDEPENDENCE OF COLONIZATION EVENTS

Phylogenetic analyses revealed three basic clades of "*P. mexicana*-type" fish including two clades of *P. mexicana* and a monophyletic clade of *P. sulphuraria*. Essentially, *P. sulphuraria* is sister to a clade of *P. mexicana* from northern Mexico, rendering *P. mexicana* paraphyletic. This northern clade of *P. mexicana* corresponds to a previously described subspecies, *P. m. limantouri* (see Menzel and Darnell 1973). Together, the northern *P. m. limantouri* and the *P. sulphuraria* clades are sister to a clade

Table 2. Results of (multivariate) analyses of covariance examining body shape and gill morphology variation of *P. mexicana* and *P. sulphuraria* from different sites. Terms with a partial variance ≥ 0.3 are given in bold.

Effect	F	Hypothesis df	Error df	P	Partial variance	Relative variance
(a) Body shape (MANCOVA)						
Centroid size	**36.9**	**7.0**	**597.0**	**<0.001**	**0.302**	**0.364**
Sex	**413.2**	**7.0**	**597.0**	**<0.001**	**0.829**	**1.000**
H_2S	**126.2**	**7.0**	**597.0**	**<0.001**	**0.597**	**0.720**
Drainage	**53.4**	**14.0**	**1194.0**	**<0.001**	**0.385**	**0.464**
Site (Drainage × H_2S)	7.1	91.0	3730.0	<0.001	0.132	0.159
Drainage × H_2S	16.1	14.0	1194.0	<0.001	0.159	0.192
Drainage × Sex	20.6	14.0	1194.0	<0.001	0.195	0.235
H_2S × Sex	1.9	7.0	597.0	0.067	0.022	0.027
Drainage × H_2S × Sex	2.2	14.0	1194.0	0.008	0.025	0.030
(b) Body shape (ANCOVA)						
Centroid size	20.0	1.0	603.0	<0.001	0.032	0.038
Sex	10.9	1.0	603.0	0.001	0.018	0.021
H_2S	**77.8**	**1.0**	**14.7**	**<0.001**	**0.841**	**1.000**
Drainage	2.0	2.0	14.0	0.174	0.222	0.264
Site (Drainage × H_2S)	13.4	13.0	603.0	<0.001	0.224	0.266
Drainage × H_2S	1.1	2.0	13.9	0.348	0.141	0.168
Drainage × Sex	1.5	2.0	603.0	0.232	0.005	0.006
H_2S × Sex	4.3	1.0	603.0	0.039	0.007	0.008
Drainage × H_2S × Sex	0.4	2.0	603.0	0.673	0.001	0.001
(c) Gill morphology (ANCOVA)						
Mass (log)	**938.8**	**1.0**	**83.0**	**<0.001**	**0.919**	**1.000**
Sex	10.7	1.0	83.0	0.002	0.114	0.124
H_2S	**164.6**	**1.0**	**83.0**	**<0.001**	**0.665**	**0.724**
Drainage	3.0	2.0	83.0	0.053	0.068	0.074
Drainage × H_2S	7.9	2.0	83.0	0.001	0.160	0.174
Drainage × Sex	0.9	2.0	83.0	0.400	0.022	0.024
H_2S × Sex	0.8	1.0	83.0	0.369	0.010	0.011

comprised of all southern Mexican *P. mexicana*, which correspond to the subspecies *P. m. mexicana*. These results, along with previously published morphological data (Menzel and Darnell 1973), indicate that the two clades of *P. mexicana* may represent two distinct species, *P. mexicana* (Steindachner 1863) and *P. limantouri* (Jordan and Snyder 1899), corresponding to the previously recognized subspecies. However, further research, including the analysis of nuclear genes and a broader taxon sampling, will be required to conclusively resolve the relationship within *P. mexicana* sensu lato.

Based on the phylogenetic analyses, we could reject the hypothesis of a single colonization event of sulfide springs in southern Mexico. The first colonization event, namely the colonization of the springs in the Pichucalco drainage (La Gloria and Baños del Azufre) that correspond to *P. sulphuraria*, must have occurred relatively early, as these sulfide spring fish are more closely related to northern Mexican *P. mexicana* (i.e., *P. m. limantouri*), occurring some 400 km further northwest, than to *P. mexicana* in adjacent nonsulfidic habitats. Furthermore, *P. sulphuraria* is reciprocally monophyletic and relatively divergent (Kimura 2 Pairwise distance = 1.86–1.95%) from *P. m. limantouri*. Similar vicariant distributions have been documented in characids of the genus *Astyanax* and have been related to recurrent range expansions caused by climatic changes during the Pleistocene (Strecker et al. 2004; Ornelas-Garcia et al. 2008).

Within the southern *P. m. mexicana* lineage, we could reject a single colonization event of the sulfide environment based on the concatenated cyt *b* and ND2 dataset, so colonization of the Puyacatengo drainage (La Lluvia and Puyacatengo springs) appears to have occurred independently from the colonization of the Cueva del Azufre system in the Tacotalpa drainage. These independent colonizations must have occurred much more recently than that of *P. sulphuraria*, considering the small divergences between sulfide and nonsulfide haplotypes (Kimura 2 Pairwise distance = 0.23–0.69%). Independent colonization is also suggested by the topography of the region. The springs of the Puyacatengo and

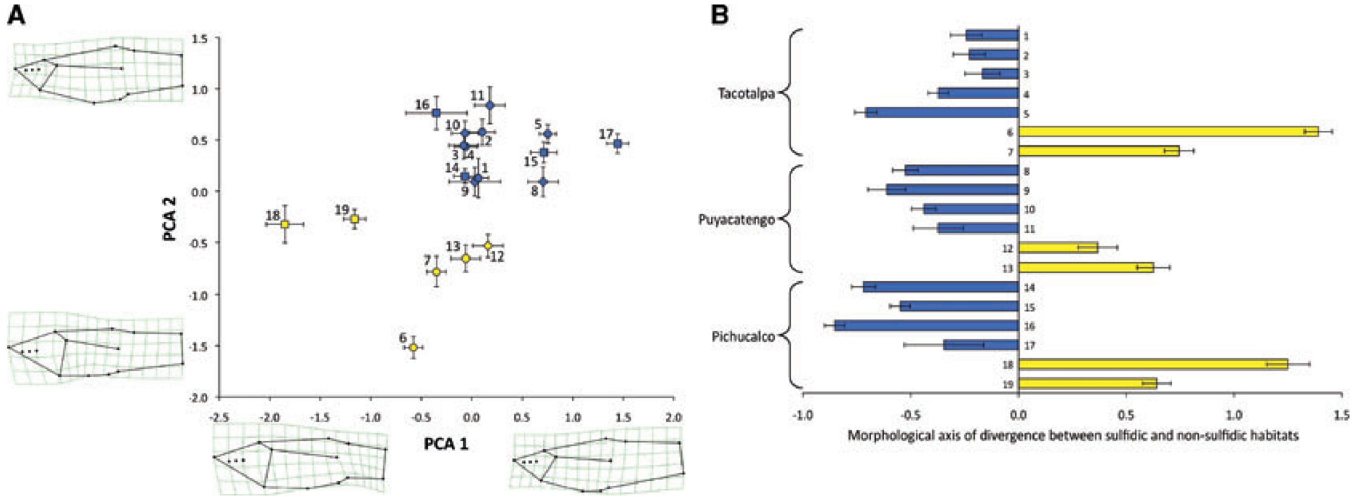

Figure 5. (A) Phenotypic variation in *P. mexicana* and *P. sulphuraria* from different sites investigated. Depicted are mean (± SEM) residual principal component scores (corrected for allometric effects and sex differences by use of a preparatory MANCOVA) for each site. Yellow symbols indicate fish from sulfidic habitats, blue symbols fish from nonsulfidic reference habitats. Symbol shapes stand for the drainage of origin: Tacotalpa (●), Puyacatengo (◆), and Pichucalco (■). The thin-plate spline transformation grids represent shape variation along each principal component axis. (B) Body shape divergence between sulfidic (yellow) and nonsulfidic (blue) populations from all three drainages investigated. Depicted are mean divergence scores (± SEM; derived from the H_2S term in the MANCOVA) for each site. Numbers correspond to sites as described in Table S2.

Tacotalpa drainages are separated by mountains, and direct exchange of fish between springs even during rare stream capture events seems unlikely.

Our analysis indicated very little geographic differentiation within the southern *P. mexicana* clade, even among specimens from different major rivers (i.e., from the Río Grijalva vs. Río Usumacinta drainage), which is in accordance with results form other taxa (Ornelas-Garcia et al. 2008). This is not surprising considering that rivers of Tabasco are well connected in the lowlands particularly during the wet season (Miller 1966), and that *P. mexicana* is very abundant and exhibits broad environmental tolerances like many other cyprinodont fish (Nordlie 2006), potentially allowing for admixture throughout the coastal plains of southern Mexico.

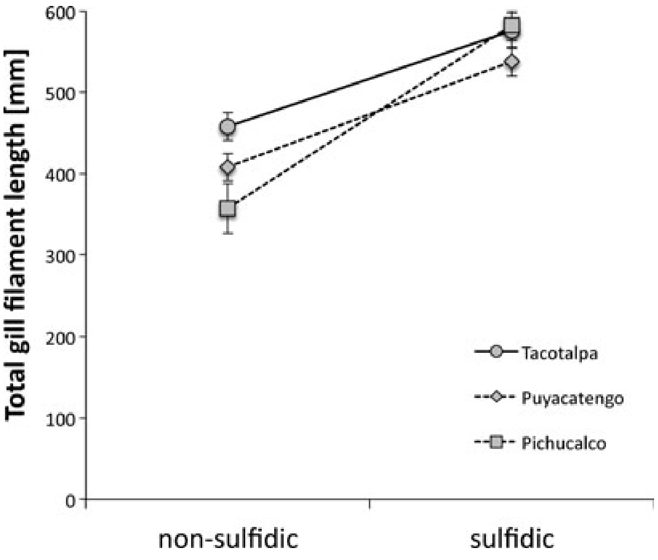

Figure 6. Visualization of the H_2S by drainage interaction term from the ANCOVA (see Table 2C) for total gill filament length. Depicted are estimated marginal means (± SEM).

CONVERGENT PATTERNS OF PHENOTYPIC DIFFERENTIATION

Phenotypic analyses indicated convergent patterns of phenotypic differentiation among all sulfur spring fish. Along with sex, habitat type was the best predictor of body shape and gill morphology. As documented in previous studies (Tobler et al. 2008a; Fontanier and Tobler 2009), fish from sulfidic habitats are consistently characterized by having enlarged heads. This increase in head size is correlated with a significant increase in gill size for all gill

Table 3. Survival analysis (Cox regression) of *Poecilia* exposed to increasing sulfide concentrations. −2 Log Likelihood = 873.2; χ^2 = 254.3; df = 4, $P < 0.001$. Significant effects are in bold.

	B	SE	Wald	df	*P*	Exp(*B*)
H_2S	**5.017**	**0.515**	**94.967**	**1**	**<0.001**	**150.959**
Sex	−0.450	0.248	3.282	1	0.070	0.638
Mass	**1.177**	**0.163**	**51.968**	**1**	**<0.001**	**3.243**
Drainage × H_2S	**−0.446**	**0.174**	**6.582**	**1**	**0.010**	**0.640**

morphometric traits analyzed. Such increases in gill size have been shown in various aquatic organisms exposed to hypoxia, including fish (Chapman et al. 1999, 2000; Chapman and Hulen 2001), amphibians (Bond 1960; Burggren and Mwalukoma 1983), and invertebrates (Astall et al. 1997; Roast and Jones 2003), and highlight the importance of respiratory adaptations and efficient oxygen acquisition in sulfidic habitats. Such habitats are not only notoriously hypoxic, but oxygen is also required for oxidative H_2S detoxification within the body (Curtis et al. 1972; Bagarinao 1992; Ip et al. 2004). In addition to the morphological modifications in sulfidic waters, most fish also rely on alternative respiratory strategies when exposed to H_2S, particularly air-breathing, which also minimizes the uptake of sulfide through the gills (Bagarinao and Vetter 1989; Brauner et al. 1995; Affonso and Rantin 2005). In contrast, *Poecilia* rely on aquatic surface respiration, where fish skim the water from the air-water interface using their gills, when exposed to sulfidic water (Plath et al. 2007b; Tobler et al. 2009a).

Although traits associated with respiratory adaptations appear to have evolved in convergence, there are also differences in body shape among drainages (drainage term in the MANCOVA), potentially reflecting effects of different evolutionary histories (see Langerhans and DeWitt 2004) and unique aspects of differentiation in body shape and gill morphology between sulfidic and nonsulfidic ecotypes in each drainage (interaction term between drainage and H_2S in the MANCOVA for body shape). For example, sulfidic ecotypes in the Pichucalco drainage (*P. sulphuraria*) are characterized by a unique posterior shift of both anal and dorsal fins. These drainage-specific and unique aspects of trait variation indicate that sulfide spring populations in different drainages, and maybe even different spring complexes within the same drainage, are on independent evolutionary trajectories. Factors potentially influencing these independent trajectories may include different genetic backgrounds of original invaders, stochastic processes such as genetic drift in the relatively small sulfide spring populations, and as well undetected environmental variation among spring complexes within and among drainages (Mani and Clarke 1990; Ricklefs and Schluter 1993; Price et al. 2000; Blount et al. 2008; Beatty 2010).

Convergent patterns of differentiation are also reflected in the results of sulfide tolerance experiments. Fish from sulfidic springs exhibit significantly higher tolerances than conspecifics from adjacent nonsulfidic habitats, indicating that sulfide tolerance is not simply a trait present in putative ancestral populations. These results, however, have to be interpreted with caution, as many physiological traits can be highly inducible. Because we have performed our experiments on wild-caught fish after relatively short laboratory acclimation, it cannot be ruled out that sulfide tolerance of nonsulfidic fish may increase upon gradual preexposure to sublethal levels of H_2S. Our tolerance experiments hence highlight the need for laboratory common garden experiments to elucidate the mechanisms underlying the expression of divergent morphological and physiological traits. Although there is limited evidence for heritable differentiation in body shape (Tobler et al. 2008a) and sulfide tolerance in fish from the Tacotalpa drainage (Peters et al. 1973; reanalyzed in Plath and Tobler 2010), it is currently unknown how short-term acclimation responses, developmental plasticity, and heritable differentiation interact to produce phenotypes observed in natural habitats. It is important to note, however, that even if certain traits such as sulfide tolerance prove to be highly inducible, there may still be significant variation in acclimation responses among ecotypes (e.g., Timmerman and Chapman 2004 for physiological responses to hypoxia).

EVIDENCE FOR REPRODUCTIVE ISOLATION

This study not only adds to the understanding of phenotypic differentiation in sulfide spring fish, but also to the potential consequences of adaptation for gene flow among populations residing in different habitats. Previous studies in the Tacotalpa system have indicated strong reductions of gene flow between adjacent habitats with different environmental conditions based on frequencies of cyt *b* haplotypes and nuclear microsatellites (Plath et al. 2007a, 2010a; Tobler et al. 2008a). Here, we found strong divergence also between sulfide spring fish (*P. sulphuraria*) and nonsulfidic ecotypes in the Pichucalco system, indicating strong reproductive isolation despite the lack of physical barriers that would prevent fish movement. This is despite the fact that hybridization among more distantly related taxa are known to produce fertile offspring in other poeciliid fish, including other mollies (Hubbs 1959; Ptacek 2002; Dries 2003; Rosenthal et al. 2003). Based on the present data, however, we cannot exclude introgression of nuclear genes across habitat borders, and further population genetic studies are still needed, especially in the little investigated Puyacatengo drainage. Reproductive isolation in the Tacotalpa and Pichucalco drainages at least in part seems to be mediated by natural and sexual selection against immigrants (Tobler et al. 2009b; Plath et al. 2010b). To date, it is unknown whether there are also postzygotic isolating mechanisms (like intrinsic or ecologically dependent reductions of hybrid fitness) present in any of the three systems (see Hatfield and Schluter 1999; Via et al. 2000; Russell and Magurran 2006; Gow et al. 2007).

If population differences in sulfide-tolerance are heritable, a major open question remains why genes responsible for increased sulfide tolerance are not sweeping throughout *P. mexicana* populations residing in nonsulfidic habitats. Obviously, sulfide resistance and adaptation to sulfide springs must incur some costs. Most organisms are able to detoxify H_2S to some extent, and an enzyme that plays a key role in sulfide homeostasis, sulfide-quinone oxidoreductase (SQR, Marcia et al. 2009), occurs in most domains of life (Theissen et al. 2003; Shahak and Hauska 2008). SQR is tied to the mitochondrial respiratory chain and binds sulfide molecules that are then processed by additional enzymes to form thiosulfate, which then is excreted (Hildebrandt and Grieshaber 2008). In nontolerant species, sulfide detoxification ceases at higher ambient sulfide concentrations, when inhibition of cytochrome *c* oxidase by sulfide eventually blocks electron flow through the respiratory chain (Whitehead et al. 2010). However, little is yet known about modification of enzymes or alternate pathways that allow coping with elevated sulfide concentrations in tolerant species. When comparing sulfide oxidation in rats and sulfide-tolerant lungworms, Hildebrandt and Grieshaber (2008) found the lungworms to have an SQR with higher activity and a sulfur transferase specialized in processing persulfides. It is not inconceivable that modification of enzymes involved in sulfide detoxification could results in trade-offs, as they are also involved in other aspects of sulfur metabolism. Trade-offs may also arise in terms of differential expression of antioxidant pathways in the hypoxic sulfidic environments (see Tobler et al. 2009b for a discussion) or adaptation to correlated ecological factors (Tobler and Plath 2011). For example, *Poecilia* residing in sulfidic and nonsulfidic habitats differ in trophic resource use due to altered resource availability and competitive regimes (M. Tobler et al. unpubl. data). Furthermore, the modified morphology of sulfide spring populations may reduce streamlining and increase energy expenditure during swimming and susceptibility to predation particularly in nonsulfidic habitats that are generally characterized by higher water flow and more diverse predator communities (see Tobler and Plath 2011 for a discussion).

CONCLUSIONS

Overall, this and previous studies document convergent patterns of trait divergence and the evolution of reproductive isolation in replicated systems of sulfidic and nonsulfidic habitats in southern Mexico. As such, these fish provide an ideal model system to study mechanisms involved in replicated adaptive divergence and ecological speciation. As physiochemical stressors, in the form of sulfide and chronic hypoxia, appear to be key drivers in the evolution of sulfide spring ecotypes, sulfide spring fish may further provide an ideal model system to start elucidating genes underlying adaptation and speciation, because prior knowledge of the physiologically and biochemically explicit effects of selective forces should facilitate identifying potential candidate genes. Bridging sources of selection in natural habitats, through divergent traits involved in reproductive isolation, to underlying genomic changes remains a key—but yet unresolved—problem in evolutionary biology (Schluter 2009; Hohenlohe et al. 2010; Stapley et al. 2010; Wolf et al. 2010).

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Supporting Information

The following supporting information is available for this article:

Figure S1. Gill arch of *P. mexicana* from a nonsulfidic habitat.

Figure S2. Maximum likelihood tree from the RAxML analysis of *Poecilia* spp. relationships for the combined dataset of the mitochondrial genes Cytochrome *b* (cyt *b*) and NADH2 (ND2), rooted with poeciliid outgroups.

Figure S3. Visualization of the H_2S by drainage interaction term from the MANCOVA (see Table S4) for total number of filaments (A), average gill filament length (B), total hemibranch perimeter (C), and total hemibranch area (D).

Table S1. Water chemistry data from different sites investigated.

Table S2. Overview of sample sites of *P. mexicana* and *P. sulphuraria* used for the morphological analyses and sulfide tolerance tests.

Table S3. Accession numbers of samples used for phylogenetic analyses retrieved from GenBank and additional samples collected by the authors with locality data.

Table S4. Results of multivariate analysis of covariance examining additional gill morphometric variables of *P. mexicana* and *P. sulphuraria* from different sites.

Table S5. Description of the three datasets used for phylogenetic analyses and the corresponding models determined by jModeltest version 0.1.1 (Posada, D. 2008. j Model Test: phylogenetic model averaging. Mol. Biol. Evol. 25:1253-1256).

Supporting Information may be found in the online version of this article.