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TOXIC HYDROGEN SULFIDE AND DARK CAVES: PHENOTYPIC AND GENETIC DIVERGENCE ACROSS TWO ABIOTIC ENVIRONMENTAL GRADIENTS IN *POECILIA MEXICANA*

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Divergent natural selection drives evolutionary diversification. It creates phenotypic diversity by favoring developmental plasticity within populations or genetic differentiation and local adaptation among populations. We investigated phenotypic and genetic divergence in the livebearing fish *Poecilia mexicana* along two abiotic environmental gradients. These fish typically inhabit nonsulfidic surface rivers, but also colonized sulfidic and cave habitats. We assessed phenotypic variation among a factorial combination of habitat types using geometric and traditional morphometrics, and genetic divergence using quantitative and molecular genetic analyses. Fish in caves (sulfidic or not) exhibited reduced eyes and slender bodies. Fish from sulfidic habitats (surface or cave) exhibited larger heads and longer gill filaments. Common-garden rearing suggested that these morphological differences are partly heritable. Population genetic analyses using microsatellites as well as cytochrome *b* gene sequences indicate high population differentiation over small spatial scale and very low rates of gene flow, especially among different habitat types. This suggests that divergent environmental conditions constitute barriers to gene flow. Strong molecular divergence over short distances as well as phenotypic and quantitative genetic divergence across habitats in directions classic to fish ecomorphology suggest that divergent selection is structuring phenotypic variation in this system.

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A fundamental question in evolutionary biology is how populations adapt to heterogeneous environments (Levins 1968; Bohonak 1999; Schluter 2000). When populations are exposed to spatially divergent selection there are three typical evolutionary outcomes (Kawecki and Ebert 2004). These scenarios are not mutually exclusive but rather constitute the extremes of a spectrum: (1) A single specialist optimally adapted to one habitat (usually the more common or productive one) and poorly adapted to others may evolve. In this case, source–sink dynamics are expected, whereby persistence in habitats to which the species is not adapted depends on migration from habitats in which the species is better adapted (Dias 1996; Dias and Blondel 1996; Day 2000; Holt et al. 2004). (2) Generalists adapted to tolerate multiple habitat types may evolve. Generalists can be phenotypically uniform intermediates (Van Tienderen 1991; Palaima 2007) or express alternate phenotypes under different environmental conditions (i.e., phenotypic plasticity: West-Eberhard 1989; Pigliucci 1996; DeWitt and Scheiner 2004). In the case of a generalist, bidirectional migration between habitat types may occur (Wilson and Yoshimura 1994; Sultan and Spencer 2002). (3) Multiple specialists may be locally adapted to alternative habitat types (Levene 1953). Hence, one expects divergent specialized phenotypes that maximize fitness in a given habitat and do not migrate between habitat types, which results in limited gene flow among populations (Kawecki and Ebert 2004; Hays 2007).

Depending on the pattern of environmental heterogeneity, populations of organisms respond evolutionarily by evolving intermediate generalist phenotypes, phenotypic plasticity, or local adaptation, and either exhibit considerable or minimal migration. Local adaptation is hindered by gene flow because gene flow homogenizes allele frequencies among populations and prevents divergent selection from creating genetic divergence (Storfer and Sih 1998; Lenormand 2002; Moore et al. 2007). However, if divergent selection is sufficiently strong it can maintain population differentiation even when gene flow is present and can cause local adaptation on small spatial scales (Jimenez-Ambriz et al. 2006; Hays 2007; Manier et al. 2007).

If the response to environmental heterogeneity is heritable, local adaptation can proceed to speciation and adaptive radiation from a single ancestor (Schluter 2000; Streelman and Danley 2003). Ecological speciation occurs when divergent selection, in addition to driving trait divergence among populations, also leads to evolution of reproductive isolation. In traditional models of ecological speciation, reproductive isolation evolves incidentally as a byproduct (Schluter 2000, 2001; Dieckmann et al. 2004; Rundle and Nosil 2005); but whenever divergent natural selection occurs among populations, there may be direct selection for premating isolation (i.e., reinforcement: Schluter 2001; Rodriguez et al. 2004). Evidence for ecological speciation in the wild is mounting (Funk 1998; McPeek and Wellborn 1998; Rundle et al. 2000; Jiggins et al. 2001; Nosil et al. 2002; McKinnon et al. 2004; Boughman et al. 2005; Langerhans et al. 2007b). In animals, a variety of—mostly biotic—selective agents have been documented to lead to reproductive isolation, including reproductive interference (Servedio and Noor 2003), resource use (Funk 1998; Ryan et al. 2007), interspecific resource competition (Pfennig and Rice 2007; Tyerman et al. 2008), predation (Nosil and Crespi 2006; Langerhans et al. 2007b), and parasitism (Blais et al. 2007). Adaptive divergence in response to divergent abiotic conditions is predominantly known from plants exposed to different soil types or elevation gradients (Macnair and Christie 1983; Wang et al. 1997; Rajakaruna et al. 2003; Silvertown et al. 2005; Antonovics 2006).

In the present study, we examined phenotypic and genetic divergence in the livebearing fish *Poecilia mexicana* (Atlantic molly, Poeciliidae). This species has colonized habitats differing in abiotic conditions in the Cueva del Azufre system in southern Mexico. Habitats in this system are characterized by the presence or absence of naturally occurring hydrogen sulfide (H_2S) and/or light (i.e., cave versus surface habitats), providing a natural 2 × 2 factorial design of these two environmental conditions (Tobler et al. 2006, 2008a). Both the presence of H_2S and the absence of light are potential sources of divergent natural selection. H_2S is correlated with extreme hypoxia in aquatic environments and is a potent respiratory toxicant lethal for most metazoans even in micromolar amounts (Evans 1967; Bagarinao 1992; Grieshaber and Völkel 1998). In the Cueva del Azufre system, H_2S is present in acutely toxic concentrations of up to 300 μM (Tobler et al. 2006). Similarly, the absence of light in caves inhibits the use of visual senses, and cave-dwellers are under selection to cope with darkness, especially if they evolved from a diurnal surface-dwelling form like in *P. mexicana* (Poulson and White 1969; Howarth 1993; Culver et al. 1995; Langecker 2000; Plath et al. 2004). Therefore these environmental axes should provide two divergent natural selection gradients along which to test for phenotypic divergence and patterns of gene flow in the absence of vicariance.

In this study, we ask four major questions: (1) Is phenotypic differentiation in *P. mexicana* populations evident across

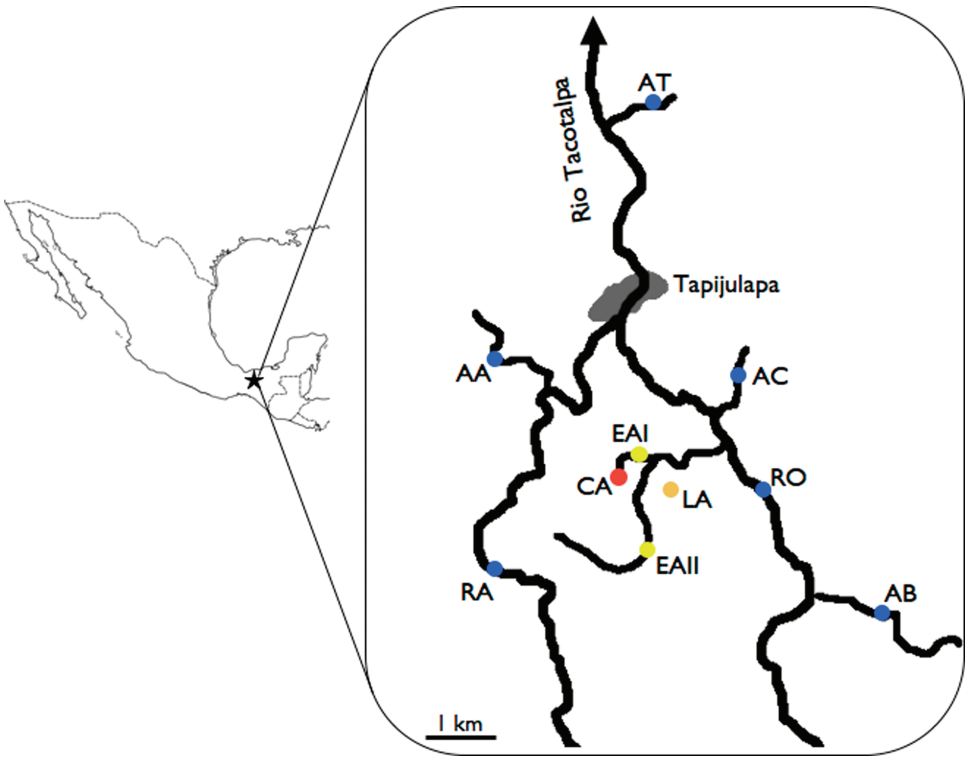

Figure 1. **Map of the collection sites around the village of Tapijulapa (Tabasco, Mexico). Color coded are nonsulfidic surface habitats in blue, sulfidic surface habitats in yellow, the entrance to the Cueva Luna Azufre (nonsulfidic) in orange, and the entrance to the Cueva del Azufre (sulfidic) in red. Abbreviations follow Table 1.**

two environmental gradients? (2) Does divergence correspond to ecomorphological expectations? (3) Do divergent traits have a heritable component? (4) Is gene flow limited among *P. mexicana* from different habitats? We address these questions by surveying morphology across environmentally diverse sites, comparing diversification with previous studies on ecomorphology, analyzing phenotypes of fish from alternative populations when raised in a common garden, and analyzing marker genetics among sites.

Materials and Methods

POPULATIONS

Poecilia mexicana is common in freshwater habitats on the Atlantic side of Central America from northern Mexico to Costa Rica (Miller 2005). Our study sites are located near the village of Tapijulapa in the southern Mexican state of Tabasco (Fig. 1; Table 1). We sampled four different habitat types that are characterized by the presence or absence of H_2S and/or light (online Supplementary Table S1). All sites are within 10 km of each other (river distance), and the average distance between sites is about 3.5 km (online Supplementary Table S2). Sites sampled include normal (nonsulfidic, surface) rivers ($N = 6$ sites), sulfidic surface rivers ($N = 2$ sites), a nonsulfidic cave ($N = 1$ site), and a sulfidic cave in which we sampled from multiple ($N = 5$) cave chambers. The two caves investigated are the only known subterranean habitats inhabited by *P. mexicana*.

- The Cueva del Azufre is a sulfidic cave. The cave is structured into different chambers, the nomenclature of which follows Gordon and Rosen (1962). The front chambers obtain some dim light, whereas the rearmost cave chambers are completely dark. The cave is drained by a creek fed by a number of springs throughout the cave, most of which contain high levels of dissolved H_2S (Tobler et al. 2006). *Poecilia mexicana* occur throughout the cave, and for this study they were collected in chambers II, V, X, XI and XIII.
- Despite its name, the Cueva Luna Azufre is a nonsulfidic cave (Tobler et al. 2008a). It is smaller than the Cueva del Azufre, and *P. mexicana* occur at lower densities. Although the two caves are in close proximity, they are located within different hills that are separated by a surface valley. The creek in the Cueva Luna Azufre is also fed by springs, however, these do not contain H_2S (Tobler et al. 2008a). *Poecilia mexicana* were collected south of the Entrada Marabunda.
- The El Azufre is a sulfidic surface habitat. It is fed by multiple independent sulfidic as well as nonsulfidic springs and flows through the valley that separates the two caves. Both caves drain into the El Azufre, which eventually joins the Río Oxolotan.

Table 1. List of the collection sites (abbreviations as used throughout the article in brackets following the name), their location (latitude, longitude), and the number of individuals (males/females for the morphological studies) examined from each site in the different parts of the study.

Site	Location	Geometric morphometrics (field)	Geometric morphometrics (laboratory-reared)	Gills	Microsatellites	Cytochrome *b*
Nonsulfidic surface habitats						
Arroyo Bonita (AB)	17.42706, −92.75194	15/29		0/2	24	10
Arroyo Cristal (AC)	17.45063, −92.76369	4/3			20	10
Arroyo Tacubaya (AA)	17.45355, −92.78449	14/24				
Arroyo Tres (AT)	17.48368, −92.77627	0/18		0/1	19	10
Río Amatan (RA)	17.43331, −92.79293	26/29		0/3	24	11
Río Oxolotan (RO)	17.44444, −92.76293	23/20	8/5	0/1	24	9
Sulfidic surface habitats						
El Azufre I (EAI)	17.44225, −92.77447	21/28		0/2	40	20
El Azufre II (EAII)	17.43852, −92.77475	19/34		0/4	20	11
Nonsulfidic cave habitat						
Cueva Luna Azufre (LA)	17.44171, −92.77312[1]	23/43	5/4	0/6	19	10
Sulfidic cave habitat						
Cueva del Azufre, chamber II (II)	17.44234, −92.77542[1]	10/14		0/3		
Cueva del Azufre, chamber V (V)	17.44234, −92.77542[1]	26/27		0/3	19	21
Cueva del Azufre, chamber X (X)	17.44234, −92.77542[1]	15/20	5/6	0/2	20	10
Cueva del Azufre, chamber XI (XI)	17.44234, −92.77542[1]	6/7			19	10
Cueva del Azufre, chamber XIII (XIII)	17.44234, −92.77542[1]				21	10

[1]Location of cave entrance is provided.

Hydrogen sulfide concentrations are comparable to those in the Cueva del Azufre. *Poecilia mexicana* were collected upstream around some sulfidic springs as well as downstream near the resurgence of the Cueva del Azufre.

• Six nonsulfidic surface habitats were sampled. These habitats include large rivers like the Río Oxolotan (most proximate to the other habitat types; Fig. 1) and the Río Amatan, as well as some of their tributaries that are similar in size and structure to the El Azufre.

Fish were collected in January 2006 and May 2007. Because habitat structures differed between sampling sites, different methods were employed. In the caves, where the water is very shallow and low ceilings preclude seining, fish were caught with dip nets (13 × 14 cm, 1 mm mesh-width). In the other habitats, fish were caught using a seine (4 m long, 4 mm mesh-width). All specimens were euthanized using MS222 immediately after capture and fixed in a 10% formaldehyde solution. Fin clips for extraction of DNA were stored in 96% ethanol at 4°C. Table 1 summarizes the material collected and examined in the different analyses.

Heritability of traits was estimated by analysis of a population-level common garden rearing experiment (Weir 1996). Laboratory stocks of fish were available from three populations: the sulfidic cave, the nonsulfidic cave, and a nonsulfidic surface habitat (Río Oxolotan). Fish from sulfidic surface habitats were not available in the laboratory. All stocks were founded in January 2006 and maintained as randomly out-bred populations in 1000-L tanks in a greenhouse at the Aquatic Research Facility of the University of Oklahoma. All stocks were exposed to identical environmental conditions (i.e., natural light cycle and no H_2S). Algae, detritus, and invertebrates were present in the stock tanks, and the diet was supplemented with commercial flake food twice a week. Random samples of fish from these stocks were collected in May 2007 (Table 1). At this point the stocks were established in the laboratory for multiple generations. As for the wild-caught fish, specimens were euthanized using MS222 and fixed in a 10% formaldehyde solution.

MORPHOMETRICS

We investigated divergence in *P. mexicana* morphology across habitat types as well as similarity of laboratory-reared and

wild-caught fish using a geometric morphometric analysis of body shape. Due to the hypoxic nature of sulfidic habitats, we further analyzed gill morphology of wild-caught fish from different habitats.

Geometric morphometics

For geometric morphometric analyses, lateral radiographs were taken with a Hewlett-Packard (Palo Alto, CA) Faxitron cabinet x-ray system. We digitized 13 landmark points on each image using the software program tpsDig (Rohlf 2004). Landmarks included the tip of the upper jaw (1); the center of the orbital (2); the posterodorsal tip of the skull (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 (7) midline; the anterior (8) and posterior (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 vertebra (12); and the center of the third vertebra with a hemal arch (13).

Based on the coordinates of the digitized landmarks, we performed a geometric morphometric analysis (e.g., Zelditch et al. 2004). Data were translated to NTS format using tpsUtil (Rohlf 2006). Landmark coordinates were aligned using least-squares superimposition as implemented in the program tpsRelw (Rohlf 2007) to remove effects of translation, rotation, and scale. Eye diameter was measured to the nearest 0.01 mm with calipers. This distance was halved, and used to position two reference points anterior (14) and posterior (15) to the orbit landmark (with the same y-value).

The aligned coordinates plus reference points were subjected to eigendecomposition (principal component analysis) to reduce the data to true dimensionality. The last seven eigenvalues were null; four due to superimposition (two for translation, one for rotation, one for scaling) and three due to deficiency (sensu Bookstein 1991) of the two reference points. Null dimensions were dropped from the analysis and the remaining principle axes were retained as shape variables. Body shape variation (23 principle components) was analyzed using multivariate analyses of covariance (MANCOVA). Assumptions of multivariate normal error and homogeneity of variances and covariances were met for all analyses performed. Effect strengths were estimated using partial eta squared (η_p^2). For wild-caught fish, we tested for effects of centroid size to control for multivariate allometry, and sex as well as presence or absence of H_2S and light as independent variables. Shape variation along the two environmental gradients was visualized using thin-plate spline transformation grids (Zelditch et al. 2004; Rohlf 2005). To provide a quantitative basis for the nature of shape effects, we calculated correlations of superimposed landmark coordinates with the shape gradients. This was done by creating a score for each specimen on the focal shape axis. To wit, we multiplied the eigenvector of the effect SSCP matrix by the principle components block to yield a column of scores. Correlation was then calculated between these scores and superimposed coordinate values.

For the comparison of wild-caught and laboratory fish, we used centroid size as a covariate, and sex, habitat type, as well as treatment (i.e., wild-caught or laboratory-reared) as independent variables. If morphological variation were entirely caused by environmentally induced phenotypic plasticity, differences among fish from alternative habitat types should disappear in laboratory stocks housed under identical conditions. Likewise, if morphological differences were principally heritable, no differences between laboratory raised and wild-caught individuals would be expected. An intermediate result would suggest that the traits under investigation have a heritable basis, but phenotypic plasticity also plays a role.

To provide another intuitive measure of effect strength, we conducted heuristic discriminant function analyses (DFA) to determine the percentage of specimens that could be correctly classified to the population of origin based on body shape. To facilitate the DFAs we first removed the effects of sex and allometry by using the residuals of preparatory MANCOVAs. In these MANCOVAs, the 23 principle components were used as dependent variables, centroid size as a covariate, and sex as an independent variable. DFA on the pooled laboratory and wild-caught fish also allowed us to test whether laboratory-reared individuals clustered with wild-caught specimens from their original habitat type. All statistical analyses were performed using SPSS 16 (SPSS, Inc., 2007).

Gill morphometrics

Total gill filament length (TGFL) was measured as a proxy for the gill surface area in a subsample of individuals. TGFL is correlated with gill surface area in the closely related *Poecilia latipinna* (Timmerman and Chapman 2004) and other fish (Chapman et al. 2000; Langerhans et al. 2007a). To determine TGFL, each of the four gill arches from the left branchial basket was removed in a random sub-sample of individuals from each habitat type. Arches were placed on a microscope slide, and a picture was taken from both sides using a Spot Insight digital camera (Sterling Heights, MI) mounted on an Olympus stereomicroscope (Center Valley, PA). For each hemibranch, the length of every fifth filament was measured using an image analysis program (Spot Advanced 4.5, Diagnostic Instruments 2005). The mean of successive measurements was calculated to estimate the length of intermediate filaments. Then, filament lengths were summed for the eight hemibranches and multiplied by two to produce an estimate of TGFL. Variation in TGFL among habitats differing in abiotic environmental conditions was examined using an analysis of covariance (ANCOVA),

in which TGFL (log-transformed) was used as a dependent variable, body mass of the individual (log-transformed) as a covariate (to control for allometry, see Timmerman and Chapman 2004; Graham 2005), and presence of H_2S, as well as presence of light as independent variables. Homogeneity of slopes was observed for this analysis.

GENETIC ANALYSES

We used a population genetic approach to distinguish between the evolutionary scenarios outlined in the Introduction. If a single specialist adapted to nonsulfidic surface habitats was present, we would expect little genetic differentiation between different habitat types and primarily unidirectional migration patterns from nonsulfidic surface habitats to sink populations residing in the other habitats. If *P. mexicana* is a generalist equally adapted to multiple habitat types, genetic differentiation among populations is also expected to be low if not absent, but bidirectional migration across gradients should occur. Alternatively, *P. mexicana* in each habitat type may be locally adapted to the respective abiotic conditions. In this case genetic differentiation among populations from different habitat types would be expected, and migration events may be more common between sites with similar abiotic conditions. To test these alternative hypotheses, we performed a population genetic study using microsatellite markers and cytochrome *b* gene sequence variation.

Microsatellite analysis

DNA was extracted from tissue samples using the DNeasy DNA Extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Ten microsatellite loci were amplified according to previously described cycling parameters using approximately 400 ng of genomic DNA as a template (Tiedemann et al. 2005; Plath et al. 2007a). Fragment sizes were determined on an ABI 3100 automatic sequencer using GENESCAN 2.1 and an internal size standard (GeneScan-500 LIZ, Applied Biosystems, Foster City, CA). Data for $N = 99$ individuals from a previous study were reanalyzed (Plath et al. 2007a).

We checked for the independent inheritance of all loci (linkage disequilibrium) with a likelihood ratio test using GENEPOP on the internet (http://wbiomed.curtin.edu.au/genepop/). FSTAT (Goudet 2002) was used to calculate allelic richness. GenAlEx (Peakall and Smouse 2001) was employed to calculate observed (H_O) and expected heterozygosity (H_E). GENEPOP was also used to conduct a probability test for deviation from Hardy–Weinberg equilibrium (HWE). For all tests, we used 1000 dememorization steps and 100 batches with 10,000 iterations each.

We calculated pairwise genetic distances (F_{ST}) using Arlequin (Schneider et al. 2000). *P*-values were based on 1000 permutations. The same program was also used to test for overall differentiation among populations using analysis of molecular variance (AMOVA). We tested whether genetic differentiation would be greater among sites of a different habitat type compared to sites of the same habitat type by subjecting the pairwise F_{ST} values to a partial Mantel test with 2000 randomizations as implemented in FSTAT (Goudet 2002). Predictor matrices were based on habitat type (same or different) and distance between sites as a covariate (to test for an effect of isolation by distance). STRUCTURE (version 2.1: Pritchard et al. 2000) was used to identify the number of genetically distinct clusters (k) according to HWE and linkage equilibrium with the method presented by Evanno et al. (2005). For each value of k ($k = 1$ through 12), three iterations were run using the admixture model with a burn-in period of 100,000 iterations followed by the same number of iterations for the collection phase. Each simulation was performed using an ancestry model incorporating admixture, a model of correlated allele frequencies, and the prior population information.

To estimate the number of first-generation migrants, we used GENECLASS2 (Piry et al. 2004). We used the L_home likelihood computation, the Bayesian method of classification (Rannala and Mountain 1997), and a threshold *P*-value of 0.05. We used a partial Mantel test with 2000 randomizations to compare the number of migrants (square root-transformed) between pairwise sites (see Crispo et al. 2006). Predictor matrices were based on distance between sites and habitat type (same or different) as well as difference in habitat types with respect to the presence of H_2S (-1: movement form a sulfidic to a nonsulfidic habitat; 0: no change; $+1$: from nonsulfidic to sulfidic) and the absence of light (-1: movement from a cave to a surface habitat; 0: no change; $+1$: from surface to cave).

Cytochrome b *sequencing*

The complete mitochondrial cytochrome *b* gene was sequenced using the primers LA15058 and HA16249 (Schmidt et al. 1998). Approximately 800 ng of genomic DNA was used as a template for each PCR. The annealing temperature was $T_a = 47^{\circ}C$, otherwise PCRs were performed according to Feulner et al. (2005), but using GoTaq Flexi (Promega, Mannheim, Germany) as polymerase. PCR products were purified using the QIA-quick PCR purification kit (Qiagen). Cytochrome *b* was then sequenced in both directions with the primers used for amplification using the BigDye v3.1 Terminator Cycle-sequencing Kit (Applied Biosystems). The Multiscreen-HV (Millipore, Bedford, MA) purified products were analyzed on an ABI 3100 multicapillary automatic sequencer (Applied Biosystems). All sequences are available on GenBank (Accession numbers: EU269039-EU269065).

A network analysis was performed to estimate gene genealogies using the TCS program (Clement et al. 2000), which implements the Templeton et al. (1992) statistical parsimony. To summarize the degree of genetic differentiation, we calculated pairwise F_{ST} values using *F*-statistics (Weir and Cockerham

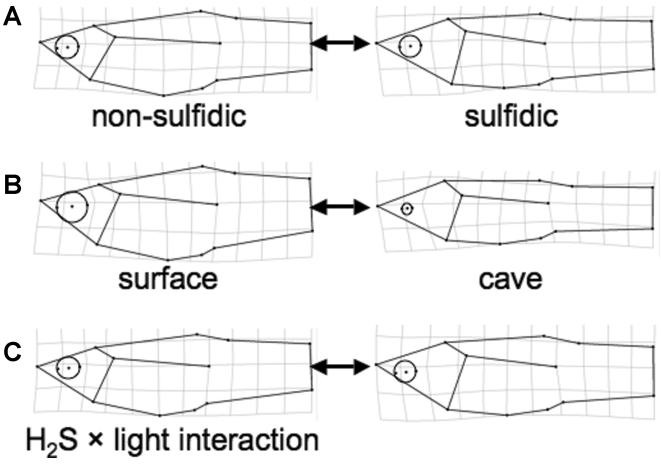

Figure 2. Morphological variation of *P. mexicana* along the two environmental gradients. Independent variation is explained along each environmental gradient (nonsulfidic to sulfidic, A; surface to cave, B), and there is also a significant interaction effect (H_2S × light, C). See online Supplementary Table S3 for correlations of superimposed landmark coordinates along these shape gradients. Effects have not been magnified in these visualizations.

1984). The significance of F_{ST} was tested by permutation analysis, and AMOVA (Excoffier et al. 1992) was conducted as implemented in Arlequin (Schneider et al. 2000). We tested whether genetic differentiation would be greater among sites of a different habitat type compared to sites of the same habitat type by subjecting the pairwise F_{ST} values to a partial Mantel test with 2000 randomizations as implemented in FSTAT (Goudet 2002). Predictor matrices were based on habitat type (same or different) and distance between sites as a covariate (to test for an effect of isolation by distance).

Table 2. Results of multivariate analyses of covariance (MANCOVA) examining body shape variation of *P. mexicana* from field collections (A) and using both field-collected and laboratory-reared animals (B). *F*-ratios were approximated using Wilks' Lambda. (C) Analysis of covariance (ANCOVA) results examining the total gill filament length (TGFL) of *P. mexicana* from different habitat types.

Effect	*F*	df	*P*	η_p^2
A. Geometric morphometrics: wild-caught fish				
centroid size	36.99	23, 466	**<0.001**	**0.646**
sex	230.88	23, 466	**<0.001**	**0.919**
H_2S	29.22	23, 466	**<0.001**	**0.591**
light	69.48	23, 466	**<0.001**	**0.774**
sex × H_2S	2.83	23, 466	**<0.001**	0.123
sex × light	2.78	23, 466	**<0.001**	0.121
H_2S × light	18.42	23, 466	**<0.001**	**0.476**
sex × H_2S × light	3.23	23, 466	**<0.001**	0.137
B. Geometric morphometrics: wild-caught and laboratory-reared fish				
centroid size	28.31	23, 393	**<0.001**	**0.624**
sex	67.66	23, 393	**<0.001**	**0.798**
habitat	11.67	46, 786	**<0.001**	**0.406**
treatment	5.69	23, 393	**<0.001**	**0.250**
sex × habitat	1.61	46, 786	**0.007**	0.086
sex × treatment	3.78	23, 393	**<0.001**	0.181
habitat × treatment	5.90	46, 786	**<0.001**	**0.257**
sex × habitat × treatment	1.26	46, 786	0.117	0.069
C. Gill morphometrics				
log(mass)	27.74	1, 22	**<0.001**	**0.545**
H_2S	33.76	1, 22	**<0.001**	**0.611**
light	0.50	1, 22	0.488	0.013
H_2S × light	0.91	1, 22	0.350	0.040

Effect sizes were estimated with partial Eta squared (η_p^2). Significant *P*-values and $\eta_p^2 \geq 0.2$ are given in boldface.

Results

MORPHOLOGICAL ANALYSES

Geometric morphometrics

A total of 497 wild-caught individuals were analyzed (Table 1). Body shape differed significantly and strongly (i.e., $\eta_p^2 > 0.5$) along both environmental gradients as well as between the sexes (Fig. 2, Table 2A, and online Supplementary Table S3). Fish from cave habitats had smaller eyes and were also more shallow bodied than fish from surface habitats, irrespective of whether the habitat of origin contained H_2S or not. Fish from sulfidic habitats were characterized by an increase in head size, irrespective of whether the habitat was located in a cave or at the surface. Consequently, *P. mexicana* in nonsulfidic surface habitats were high bodied with large eyes but small heads; in sulfidic surface habitats, fish were high bodied with large eyes and large heads; in the nonsulfidic cave they were shallow bodied with small eyes and small heads; and in the sulfidic cave, they were shallow bodied with small eyes and large heads (online Supplementary Fig. S1). The primary difference among sexes was in the position of the anal fin. In males, the anal fin is modified into a copulatory organ (the gonopodium, characteristic of the subfamily Poeciliinae), which is typically more anterior than the female anal fin (Rosen and Bailey 1963). Although all effects in the model were significant, the interaction effects were generally weak ($\eta_p^2 < 0.2$), with the exception of the H_2S × light interaction ($\eta_p^2 \approx 0.5$). Over 91% of the specimens (compared to the expected 25% under a null hypothesis of no pattern) could be assigned to the habitat type of origin based on shape data only (Fig. 3A, online Supplementary Table S4A).

Laboratory-raised fish differed significantly from wild-caught specimens, indicating that body shape was to some extent

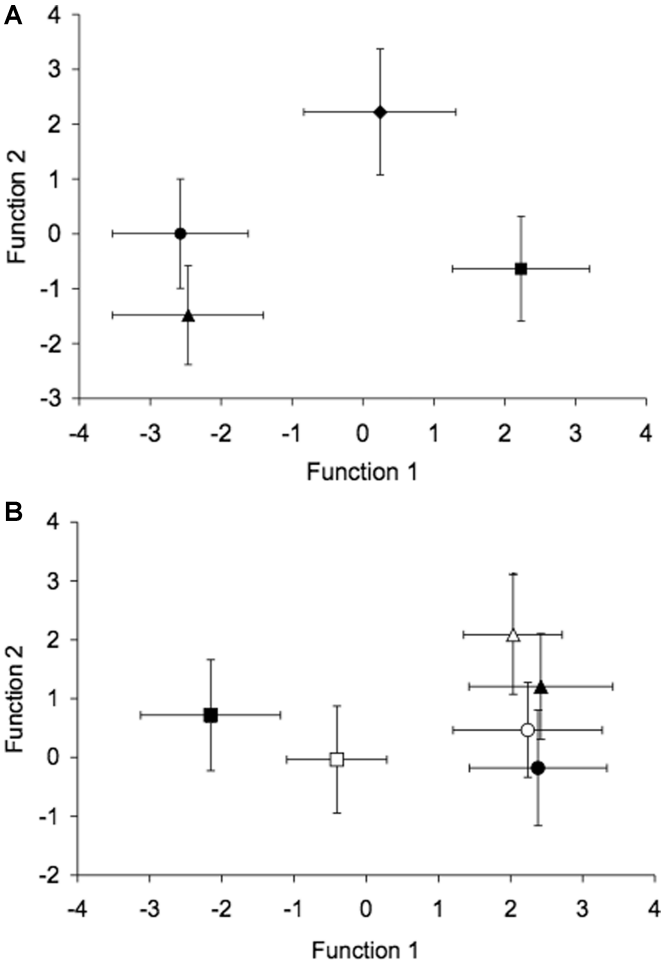

Figure 3. Discriminant function plots (group centroids ± SD for the first two discriminant functions) for the analyses presented in Table 3. (A) Analysis of wild-caught individuals from all four habitat types. (B) Comparison between wild-caught and laboratory-reared individuals. Nonsulfidic surface habitats (▲), sulfidic surface habitats (♦), nonsulfidic cave (■), and sulfidic cave (•). Closed symbols represent wild-caught individuals, open symbols represent laboratory-reared individuals.

phenotypically plastic (Table 2B). This effect can be seen in Figure 3B as the laboratory-reared fish multivariate centroids do not superimpose directly on those for wild-caught specimens. Although laboratory fish were raised under identical conditions and never encountered H_2S or permanent darkness, they arrayed geometrically like (clustered with) wild-caught individuals from their habitat type of origin (Fig. 3B). Our result does not allow for an estimation of narrow sense heritability, but it shows that divergent body morphologies have a heritable basis. Although phenotypic plasticity (via tank effects) and maternal effects (if persistent for multiple generations) could have created some of the population difference, it is improbable those effects could completely replicate the geometry of difference in laboratory-reared fish in conformation with that observed for wild-caught fish. The DFA

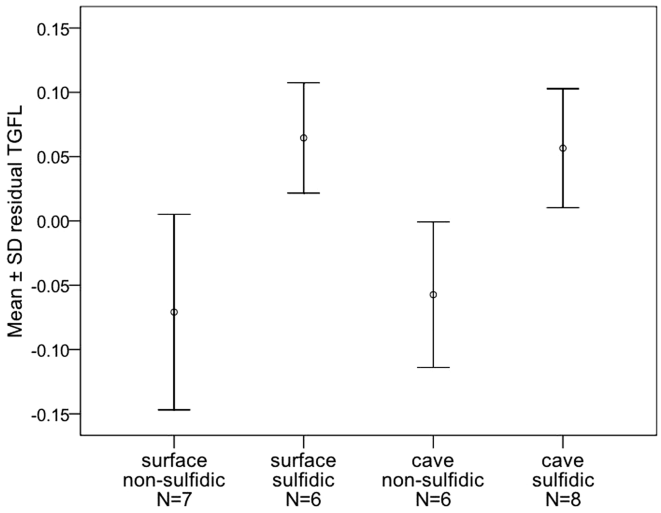

Figure 4. Mean (± standard deviation) residual total gill filament length (TGFL) for *P. mexicana* of different habitat types. Residuals were obtained using a linear regression with log (TGFL) as dependent variable and log (body mass) as independent variable. *N* represents the sample size.

classified over 92% of the specimens to the correct habitat type (compared to the expected 33% under a null hypothesis of no pattern; online Supplementary Table S4B).

Gill morphometrics

TGFL increased with increasing body mass, and fish from sulfidic habitats had a longer TGFL than fish from nonsulfidic habitats (Table 2C, Fig. 4). This effect was not dependent on whether specimens were collected in a cave or a surface habitat.

GENETIC ANALYSES

Microsatellite analysis

Overall, we genotyped 269 specimens (Table 1). A total of 225 alleles were found within 10 loci, ranging from 4 to 48 per locus (for descriptive statistics see online Supplementary Table S5). We observed strong genetic differentiation among populations (AMOVA: overall $F_{ST} = 0.198$; $P < 0.001$), and 19.8% of variation was assigned to variability among sites. The partial Mantel test explained 46.1% of the variance in pairwise F_{ST}. Pairwise F_{ST} values were significantly lower between sites of the same habitat type (mean pairwise $F_{ST} \pm$ SD: 0.074 ± 0.069) than between sites of a different habitat type (0.241 ± 0.088; Table 3; $r = 0.678$, $P < 0.001$). Distance between sites did not have a significant influence on genetic differentiation ($r = 0.038$, $P = 0.75$). The assignment test (STRUCTURE) found most support for $k = 5$ clusters (Fig. 5). Clusters corresponded with habitat types in all but one case. The *P. mexicana* collected in Arroyo Cristal were genetically distinct from their conspecifics in other nonsulfidic surface habitats, even though some of the individuals

Table 3. Pairwise genetic divergence (F_{ST}-values) among 12 populations of *P. mexicana*.

	RA	RO	AB	AC	AT	EA I	EA II	V	X	XI	XIII	LA
RA	–	**0.160**	**0.196**	0.130	0.075	**0.332**	**0.473**	**0.387**	**0.368**	**0.326**	**0.196**	**0.239**
RO	0.016	–	**0.231**	0.163	0.107	**0.370**	**0.528**	**0.427**	**0.415**	**0.369**	**0.231**	**0.277**
AB	**0.023**	0.001	–	**0.200**	0.144	**0.397**	**0.552**	**0.453**	**0.444**	**0.400**	**0.267**	**0.311**
AC	**0.104**	**0.092**	**0.096**	–	0.078	**0.339**	**0.486**	**0.395**	**0.378**	**0.333**	**0.200**	**0.244**
AT	**0.034**	0.011	0.015	**0.137**	–	**0.289**	**0.431**	**0.345**	**0.322**	**0.278**	0.144	0.189
EA I	**0.156**	**0.181**	**0.215**	**0.286**	**0.183**	–	**0.620**	**0.533**	**0.541**	**0.507**	**0.397**	**0.435**
EA II	**0.159**	**0.170**	**0.217**	**0.287**	**0.185**	0.027	–	**0.668**	**0.724**	**0.681**	**0.552**	**0.595**
V	**0.230**	**0.248**	**0.278**	**0.346**	**0.254**	**0.059**	**0.127**	–	**0.594**	**0.560**	**0.453**	**0.489**
X	**0.214**	**0.233**	**0.271**	**0.336**	**0.230**	**0.068**	**0.149**	**0.108**	–	**0.578**	**0.444**	**0.489**
XI	**0.276**	**0.289**	**0.321**	**0.389**	**0.293**	**0.072**	**0.152**	0.022	**0.096**	–	**0.400**	**0.444**
XIII	**0.290**	**0.300**	**0.334**	**0.404**	**0.305**	**0.092**	**0.178**	0.052	**0.105**	0.022	–	**0.311**
LA	**0.318**	**0.322**	**0.359**	**0.425**	**0.325**	**0.175**	**0.274**	**0.234**	**0.168**	**0.229**	**0.198**	–

Microsatellite data (below diagonal) and cytochrome *b* sequence data (above diagonal). Statistically significant values are shown in bold ($\alpha' = 0.0007$). Abbreviations follow Table 1.

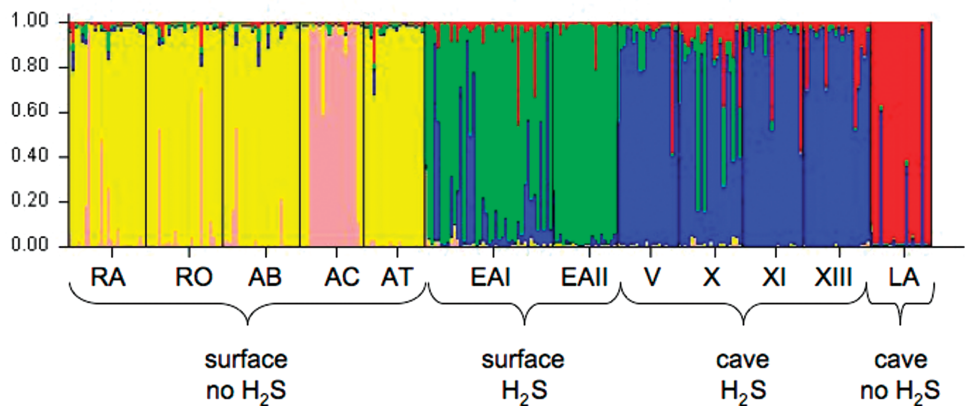

Figure 5. Population differentiation (STRUCTURE) for $k = 5$ clusters. Abbreviations follow Table 1.

were genetically similar to conspecifics from other nonsulfidic surface habitats.

The genetic differentiation among *P. mexicana* was also reflected in the analysis of first-generation migrants. The partial Mantel test explained 35.2% of variance in the number of migrants between sites. Migration events were more common between sites of the same habitat type than between sites of different habitat types (Table 4; $r = -0.568$, $P < 0.001$). There was no significant effect of distance between sites on the number of migrants ($r = -0.154$, $P = 0.18$). Furthermore, we found no evidence that migration is more common from sulfidic to nonsulfidic ($r = 0.005$, $P = 0.70$) or from cave to surface habitats ($r = -0.076$, $P = 0.38$).

Table 4. Mean (± standard deviation, SD) number of first-generation migrants between different habitat types (based on microsatellite data) as determined by GENECLASS2.

ΔH_2S	Δlight	Mean±SD
from sulfidic to nonsulfidic	from cave to surface	0.00±0.00
from sulfidic to nonsulfidic	no change	0.21±0.58
from sulfidic to nonsulfidic	from surface to cave	0.00±0.00
no change	from cave to surface	0.92±1.44
no change	no change	2.88±2.83
no change	from surface to cave	0.00±0.00
from nonsulfidic to sulfidic	from cave to surface	0.50±0.71
from nonsulfidic to sulfidic	no change	0.07±0.27
from nonsulfidic to sulfidic	from surface to cave	0.00±0.00

ΔH_2S indicates a change in the presence of hydrogen sulfide; Δlight indicates a change in the presence of light.

Cytochrome b *sequencing*

We sequenced the cytochrome *b* gene of 142 individuals. The minimum spanning network (Fig. 6) showed a central haplotype, shared by most populations on the plateau on which El Azufre and the two caves are located (sulfur plateau), along with a set of haplotypes that formed a star-like topography around the central haplotype that were found throughout the various nonsulfidic

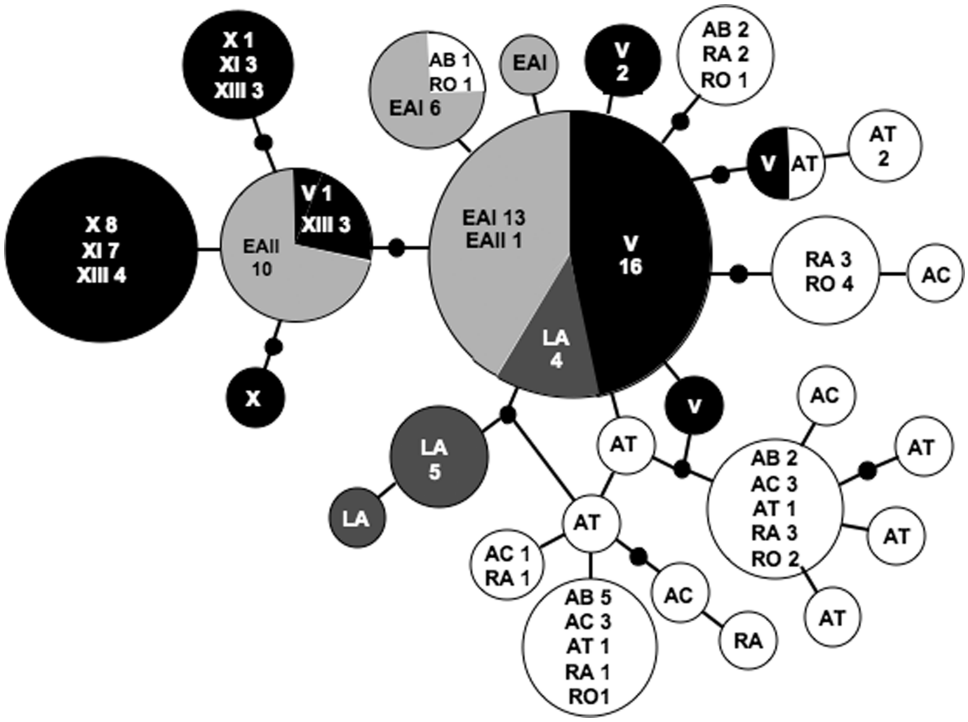

Figure 6. Minimum spanning network showing the relationship of different cytochrome *b* haplotypes. Different habitat types are coded using a gray-scale (white: surface, nonsulfidic; light gray: surface, sulfidic; dark gray: cave, nonsulfidic; black: cave, sulfidic). Abbreviations indicate field sites (see Table 1), numbers represent the number of individuals carrying the respective haplotype, whereby no number after the population code refers to only one individual showing this haplotype.

surface sites. Although both cave populations and the sulfidic surface habitats still share the central haplotype, each habitat type also harbored at least two private haplotypes. Altogether, this suggests a close relationship of the fish on the sulfur plateau. Both caves have likely been colonized from the El Azufre.

The AMOVA assigned 39.5% of variation to variability among sites and indicated a high genetic differentiation among populations (Table 3; overall $F_{ST} = 0.395$; $P < 0.001$). The partial Mantel test explained 37.8% of variance in pairwise F_{ST}. Pairwise genetic differentiation was slightly lower between sites of the same habitat type (mean pairwise $F_{ST} \pm$ SD: 0.324 ± 0.202) than between sites of different habitat types (0.393 ± 0.130; $r = 0.201$. $P = 0.059$) and there was no isolation by distance ($r = -0.581$; $P < 0.001$). In fact, genetic distance decreased significantly with increasing distance between sites. This negative relationship was driven by high genetic differentiation in *P. mexicana* from sites on the sulfur plateau—both within as well as between habitat types—even at low spatial scales (< 1 km).

Discussion

In southern Mexico, *P. mexicana* has colonized four different habitat types characterized by the presence or absence of toxic H_2S and/or light providing a "natural experiment" with a fully factorial 2 × 2 design of the two abiotic selective agents. The results of our study indicate that fish diverged morphologically and genetically in each habitat despite the close spatial proximity of the sites, which is most consistent with a local adaptation scenario as outlined in the Introduction. Patterns of heritable phenotypic differentiation and low gene flow among habitats exclude the single-specialist scenario, as there appear to be no sink populations maintained by migration. Likewise the single generalist scenario is not supported by our results because each habitat has a clearly defined phenotype having a heritable basis.

MORPHOLOGICAL DIFFERENTIATION

Independent morphological variation occurs along each of the two environmental gradients. *Poecilia mexicana* from cave habitats are characterized by reduced eye size and more shallow bodies compared to fish from surface habitats, independent of whether H_2S is present in their habitat. Reduced eye size has previously been reported for *P. mexicana* from the Cueva del Azufre (Walters and Walters 1965; Peters et al. 1973; Plath et al. 2007a), but unlike in other cave organisms (Porter and Crandall 2003), the eyes in cave-dwelling *P. mexicana* are still functional (Plath et al. 2004; Körner et al. 2006). The adaptive value of eye reduction in subterranean organisms is still under debate with two major opposing theories. The "neutral mutation hypothesis" posits that eye regression is caused by the accumulation of mutations in eye-forming genes under relaxed selection in darkness (Culver 1982; Wilkens 1988).

In contrast, the "adaptation hypothesis" suggests that eye reduction provides fitness benefits in the cave environment (Poulson 1963; Poulson and White 1969). Different versions of the adaptation hypothesis attribute the regression of visual senses to energy economy, emphasizing the costs of making and maintaining an eye (Culver 1982), or to pleiotropic effects, in which structures beneficial to survival in the cave environment are enhanced at the expense of eyes (Barr 1968). Recent evidence for the adaptation hypothesis comes from studies investigating the genetic and developmental mechanisms of eye degeneration in cave-dwelling *Astyanax mexicanus* (Jeffery 2005; Protas et al. 2007; but see Wilkens 2007). It is argued that pleiotropic effects act on eye degeneration while enhancing traits that are adaptive in the cave environment, such as nonvisual sensory structures (Jeffery 2001, 2005). *Poecilia mexicana* from cave habitats have previously been shown to posses a hyperdeveloped cephalic lateral line system and an increased number of taste buds (Walters and Walters 1965; Parzefall 1970, 2001), but the developmental pathways of eye development/degeneration and their potential pleiotropic linkage to the development of nonvisual sensory structures remain to be studied in this species.

Poecilia mexicana from cave habitats are also more shallow bodied than fish from surface habitats, a trait commonly observed in cave organisms (Langecker 2000). The reduction in body height is not due to poor nutritional condition of cave populations because we find equally low storage lipid levels in both cave populations and surface fish from sulfidic habitats (Tobler, in press). Shallow bodies in caves could be driven by divergent predatory regimes, which have been shown to induce phenotypically plastic morphological changes in prey organisms (Spitze 1992; DeWitt 1998; Relyea 2001) as well as heritable morphological differences among prey populations (McPeek 1995; Nosil and Crespi 2006; Johnson et al. 2007). In the two caves, piscine and avian predators are absent (Tobler et al. 2006; Tobler et al. 2007a), and the only predator in the Cueva del Azufre is a giant water-bug of the genus *Belostoma* (Tobler et al. 2007b; Tobler et al., 2008c). A lower body height (especially for the caudal peduncle) has been reported for other fish living in low predation environments (crucian carp: Brönmark and Miner 1992; Western mosquitofish: Langerhans et al. 2004; guppy: Hendry et al. 2006; perch and roach: Eklöv and Jonsson 2007; Bahamas mosquitofish: Langerhans et al. 2007b).

Poecilia mexicana from nonsulfidic and sulfidic habitats diverged primarily in head size and TGFL (irrespective of whether the habitat was located at the surface or within a cave), which is consistent with findings in fish (Chapman et al. 1999, 2000; Chapman and Hulen 2001; Timmerman and Chapman 2004; Langerhans et al. 2007a), amphibians (Bond 1960; Burggren and Mwalukoma 1983), and invertebrates (Astall et al. 1997; Roast and Jones 2003) living in other types of hypoxic environments. This highlights the importance of respiratory adaptations facilitating efficient oxygen acquisition for survival in sulfidic habitats (McMullin et al. 2000; Van Dover 2000; Affonso and Rantin 2005; Plath et al. 2007b). Sulfide detoxification in organisms capable of tolerating high and sustained concentrations of H_2S is primarily achieved through its oxidation to less toxic sulfur species and subsequent excretion (Curtis et al. 1972; Bagarinao 1992; Ip et al. 2004). Due to the hypoxic conditions in sulfidic habitats, however, oxygen available for respiration is generally limited, but at the same time oxygen is required for coping with the toxic effects of H_2S. Some fish species rely on air-breathing to cope with low oxygen availability in sulfidic habitats (Bagarinao and Vetter 1989; Brauner et al. 1995; Affonso and Rantin 2005), but *P. mexicana* from the Cueva del Azufre rely on compensatory behavior (aquatic surface respiration), where the fish exploit the more oxygen-rich air–water interface using their gills (Plath et al. 2007b).

The significant $H_2S \times$ light effect on body shape is characterized by a shift of eye position (and to a lesser degree a decrease in eye size) as head size increases. The interaction effect either indicates developmental constraints on morphological evolution or correlational selection (Brodie 1992; Sinervo et al. 2001; DeWitt and Langerhans 2003). Selection for an increase in gill size is generally thought to impose morphological trade-offs and to indirectly affect other head characteristics such as brain size (Chapman and Hulen 2001) or trophic morphology (Chapman et al. 2000).

Morphological differences among *P. mexicana* from different habitat types do not seem to be entirely caused by environmentally induced phenotypic variation, because laboratory stocks maintained under identical conditions clustered morphologically with wild-caught fish from the respective habitat types. This result indicates that these axes of morphological variation are at least partially heritable. Variation in body morphology (Greenfield et al. 1982; Greenfield and Wildrick 1984; Ptacek 2002; Langerhans et al. 2004; Langerhans et al. 2005) as well as gill morphometrics (Timmerman and Chapman 2004) have been shown to have a heritable component in other poeciliid fish. Likewise, other aspects of divergent morphological (Peters and Peters 1968; Parzefall 2001) as well as behavioral traits (Plath et al. 2004; Plath et al. 2006; Plath 2008) of *P. mexicana* from different habitat types in the Cueva del Azufre system have a heritable basis. However, heritability of body shape in this study may have been overestimated, if epigenetic (e.g., maternal) effects influenced morphology (Holtmeier 2001; Keller et al. 2001), or underestimated, if laboratory conditions (exposure to light and lack of H_2S) exerted strong selection on body shape of fish naturally occurring in cave or sulfidic habitats leading to a rapid evolutionary change in the stock populations. Hence, future studies need to estimate narrow-sense heritability as well as the degree of phenotypic plasticity of

morphological traits when fish are exposed to continuous darkness and/or H_2S.

GENETIC DIFFERENTIATION AND MIGRATION

Genetic differentiation of *P. mexicana* in the Cueva del Azufre system parallels the observed morphological differentiation. Both marker systems used (microsatellites and cytochrome *b* sequences) indicate that each habitat type harbors a distinct population, and genetic distance is lower among *P. mexicana* from sites of the same than from different habitat types. No evidence for isolation by distance was uncovered.

Likewise, contemporary dispersal between sites predominantly occurred within the same habitat type. This suggests that the divergent abiotic conditions indeed constitute strong (albeit not insurmountable) barriers to migration. Support for the hypothesis that migration events between different habitat types should be more common from harsh to benign environments than vice versa (see Railsback et al. 1999; Caskey et al. 2007) was not evident. Notably, there was no migration from surface to cave habitats, but low rates of migration were detected from nonsulfidic to sulfidic habitats. Thus, either the absence of light is a stronger selective agent than the presence of H_2S, or the shallow passages with swift flow at the cave resurgences constitute stronger physical barriers for the movement of *P. mexicana* than previously thought. The latter hypothesis, however, seems unlikely, because bidirectional migration over potential physical barriers (waterfalls) were detected at least among populations from nonsulfidic surface habitats (e.g., between Arroyo Bonita and Río Oxolotan); and cave resurgences seem less likely as barriers than waterfalls.

The mitochondrial haplotypes recorded in the Cueva del Azufre system differ by few mutation steps, suggesting that fish from different habitat types are closely related and have diverged only recently. The fish on the sulfur plateau share a common haplotype, which suggests common ancestry. The two caves were probably colonized independently from the sulfidic surface creek. Overall, evidence hints toward a parapatric divergence of *P. mexicana* populations in different habitat types, because physical separation of the divergent populations is not evident.

SPECIATION ALONG ABIOTIC GRADIENTS?

Divergent natural selection has been shown to shape population genetic structure in several other studies (Turgeon et al. 1999; Steiniger et al. 2002; Martel et al. 2003; Dhuyvetter et al. 2007; Quesada et al. 2007). Abiotic gradients commonly structure phenotypic variation and perhaps facilitate speciation in plants (e.g., Donohue et al. 2001; McDonald et al. 2003; Swenson and Enquiest 2007). However, abiotic gradients seem either to exert less influence, or perhaps just get less play in literature on animals. A recent study investigating possible genetic differentiation along a similar environmental gradient as addressed here (hypoxic versus normoxic habitats) did not find any effect of the oxygen regime on the population genetic structure in a cichlid fish (Crispo and Chapman 2008). In that system, phenotypic plasticity is thought to play a central role for phenotypic differentiation of fish across habitat types (Crispo and Chapman 2008). It seems likely that the high levels of toxic hydrogen sulfide in our study system represent a stronger selection factor for aquatic animals than hypoxia alone.

In our study we found strong phenotypic and genetic divergence across two abiotic gradients. The isolating mechanisms leading to genetic differentiation among populations of *P. mexicana* from different habitat types are unclear. Obvious physical barriers or significant distances among populations are lacking. It is unlikely that populations in the divergent habitats are genetically incompatible (i.e., not interfertile), because there is no intrinsic postzygotic reproductive isolation known even in more distantly related poeciliid species (Hubbs 1959; Schartl 1995; Ptacek 2002; Dries 2003; Rosenthal et al. 2003; Alexander and Breden 2004; Kittell et al. 2005). Likewise, isolation due to genetically based preferences for separate habitat types (Rice and Salt 1990; Johnson et al. 1996), which are common for radiations in phytophagous insects (Berlocher and Feder 2002), is unlikely at least for the separation between surface- and cave-dwelling populations. Like surface-dwelling fish (El Azufre population), *P. mexicana* from the Cueva del Azufre exhibit photophilic behavior (Parzefall et al. 2007).

We propose that divergent natural selection caused by the abiotic gradients, in combination with local adaptation in *P. mexicana*, limits gene flow across habitat types (Räsänen and Hendry 2008). Correspondence between morphological variation along environmental gradients and ecomorphological expectations suggests local adaptation through divergent natural selection. The patterns of migration and genetic differentiation also point to abiotic conditions creating divergence among populations through divergent natural selection. Several isolating mechanisms, which may act in synchrony, seem possible in this system. (1) Selection could act directly on immigrants from divergent populations causing premating isolation (Nosil et al. 2005). For example, *P. mexicana* from nonsulfidic habitats are highly susceptible to the toxic effects of H_2S (Tobler et al. 2008b). (2) *Poecilia mexicana* from different habitat types may be less attracted to conspecifics from divergent habitat types, which may cause prezygotic isolation (Schluter 2000; Rundle and Nosil 2005). (3) Divergent selection could act against hybrids of *P. mexicana* from different habitats (Hatfield and Schluter 1999; Schluter 2000). To date no empirical evidence for the latter two mechanisms is available.

Future studies will need to pay careful attention to the evolutionary forces causing the observed small-scale population differentiation in the Cueva del Azufre system to test whether parapatric ecological speciation is occurring. Regardless, the strong divergence observed along two abiotic gradients in the present study,

and a potentially growing literature on divergence along abiotic gradients in animals (e.g., Schilthuizen et al. 2005; Fuller et al. 2007) suggests that abiotic factors may be potentially more important in animal diversification than is currently thought. These factors may be complex. We not only found effects of multiple environmental factors (see also Langerhans et al. 2007a), but also a significant interaction between the two abiotic gradients. It would be no stretch of imagination to expect such interactions also between biotic and abiotic environmental factors. A key to understanding biological diversity will often be to embrace the complexity of nature and admit it conceptually and empirically into our investigations.

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Supplementary Material

The following supplementary material is available for this article:

Figure S1. Independent shape variation along each environmental gradient gives rise to unique phenotypes in each habitat type.

Table S1. Water chemistry data from different sites investigated.

Table S2. Distance matrix between sites in km.

Table S3. Correlations of superimposed landmark coordinates with the shape gradient between fish from sulfidic and nonsulfidic habitats, surface and cave habitats as well as the interaction between the two environmental factors.

Table S4. Discriminant function analyses (DFA) of the morphology in *P. mexicana* from different habitat types.

Table S5. Descriptive statistics of the microsatellite analysis in *Poecilia mexicana*.

This material is available as part of the online article from:
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Supplementary material

Figure S1. Independent shape variation along each environmental gradient gives rise to unique phenotypes in each habitat type. Effects have not been magnified in these visualizations.

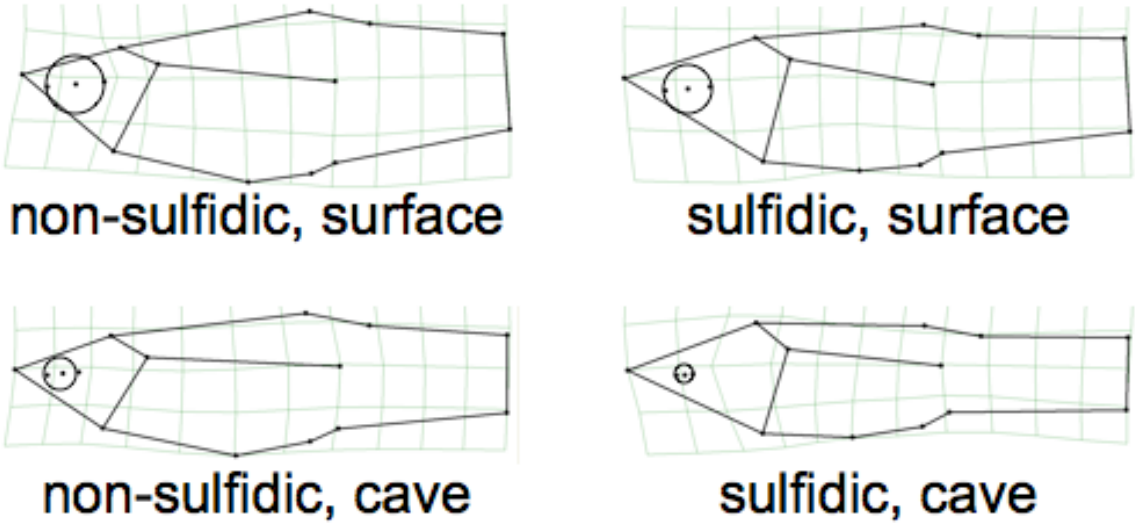

Table S1. Water chemistry data from different sites investigated. Temperature, specific conductivity, pH, and oxygen content were measured using a Hydrolab Multisonde 4A (Hach Environmental). Measurements and calibration of probes were conducted according to the manufacturer's recommendations. For the measurement of H_2S concentrations, 1 ml of water was injected into a vial containing 1 ml of zinc acetate (0.12 M with 0.5 ml NaOH 1.5 M in a N_2-atmosphere) using a syringe. The vials were stored at room temperature, and photometric measurements were conducted in the laboratory according to Cline (1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnology and Oceanography 14:454-458.). At least three Hydrolab readings and one H_2S sample was taken at each site and visit. If data from multiple years were available, values of each variable were averaged for each year, and mean and standard deviation among years are shown. For sites only sampled once, mean and standard deviation of the replicates taken are shown.

Site	Years sampled	Temperature [°C]	Specific conductivity [mS/cm]	pH	O_2 [% saturation]	O_2 [mg/l]	H_2S [µM]
Non-sulfidic surface habitats							
Arroyo Bonita	3	24.2±3.5	0.341±0.010	8.0±0.4	68.3±18.6	5.41±0.91	-1.1±1.9[1]
Arroyo Cristal	3	24.6±1.7	0.372±0.029	8.1±0.3	69.3±14.5	5.36±0.63	-4.2±6.2[1]
Arroyo Tacubaya	2	25.8±3.1	0.301±0.038	7.7±0.2	53.6±3.8	4.14±0.32	1.2±1.1[1]
Arroyo Tres	3	24.1±4.2	0.313±0.061	7.4±0.5	22.5±19.5	1.89±1.73	-1.9±4.8[1]
Río Amatan	1	29.5±0.2	0.387±0.084	7.8±0.4	75.3±14.7	5.68±1.16	0.2±1.0[1]
Río Oxolotan	4	26.8±2.3	0.510±0.041	8.1±0.2	88.8±8.8	6.28±0.04	1.7±1.2[1]
Sulfidic surface habitats							
El Azufre I	4	28.0±0.4	4.059±0.226	7.2±0.0	16.5±2.2	1.17±0.05	18.3±17.4
El Azufre II	3	27.6±1.2	4.022±0.537	6.8±0.1	17.2±4.0	1.35±0.37	151.5±113.2
Non-sulfidic cave habitat							
Cueva Luna Azufre	3	29.9±0.3	4.563±0.692	7.0±0.1	25.6±5.0	1.86±0.31	3.2±1.0[1]
Sulfidic cave habitat							
Cueva del Azufre, chamber II	4	28.2±0.1	4.142±0.255	7.0±0.0	11.7±1.9	0.77±0.08	24.0±21.6
Cueva del Azufre, chamber V	4	28.0±0.1	4.139±0.178	7.1±0.0	20.6±5.2	1.48±0.41	28.4±15.3
Cueva del Azufre, chamber X	4	28.1±0.0	4.150±0.159	6.9±0.0	21.2±1.3	1.54±0.26	91.5±32.3
Cueva del Azufre, chamber XI	4	28.1±0.1	4.417±0.077	7.0±0.5	48.2±22.3	3.66±1.76	53.6±51.2
Cueva del Azufre, chamber XIII	4	28.2±0.1	4.163±0.194	7.4±0.2	59.4±17.2	4.10±1.55	1.2±1.3[1]

[1]The spectrophotometric measurement of sulfide has an error margin of about ±5 µM. Consequently, these samples likely do not contain any H_2S.

Table S2. Distance matrix between sites in km. Distances were estimated by plotting GPS coordinates of the collection site into GoogleEarth, and then measuring the river-distance between sites with the measurement tool.

	AB	AC	AT	RA	RO	EA1	EA2	LA	V	X	XI
AB											
AC	3.36										
AT	8.50	5.78									
RA	9.85	7.13	8.27								
RO	2.52	0.82	5.98	7.33							
EAI	4.10	1.77	6.72	8.07	1.58						
EAII	4.60	2.27	7.22	8.57	2.08	0.50					
LA	4.04	1.72	6.66	8.01	1.52	0.30	0.80				
V	4.26	1.93	6.88	8.23	1.74	0.16	0.66	0.46			
X	4.36	2.03	6.98	8.33	1.84	0.26	0.76	0.56	0.10		
XI	4.40	2.07	7.02	8.37	1.88	0.30	0.80	0.60	0.14	0.04	
XIII	4.41	2.08	7.03	8.38	1.89	0.31	0.81	0.61	0.15	0.05	0.01

Table S3. Correlations of superimposed landmark coordinates with the shape gradient between fish from sulfidic and non-sulfidic habitats, surface and cave habitats as well as the interaction between the two environmental factors. Correlations ≥ |0.5| are given in bold.

Trait	H_2S	light	$H_2S \times$ light
X1	-0.417	0.298	-0.413
Y1	0.066	0.304	0.213
X2	-0.064	0.300	**-0.506**
Y2	-0.049	0.175	-0.180
X3	0.438	-0.101	0.072
Y3	0.424	-0.135	**0.538**
X4	-0.094	0.089	-0.051
Y4	-0.118	**0.605**	0.159
X5	-0.222	0.035	0.067
Y5	-0.021	**0.645**	-0.036
X6	0.182	-0.190	-0.311
Y6	0.140	0.279	-0.171
X7	0.204	-0.081	-0.358
Y7	-0.073	-0.087	-0.326
X8	-0.129	-0.037	0.226
Y8	-0.044	**-0.686**	0.137
X9	-0.104	-0.008	0.173
Y9	-0.061	**-0.588**	0.110
X10	-0.052	0.161	0.147
Y10	0.119	**-0.523**	-0.014
X11	**0.561**	-0.192	0.232
Y11	**-0.546**	-0.066	**-0.561**
X12	0.401	-0.021	0.143
Y12	0.371	0.199	0.278
X13	-0.302	0.007	-0.067
Y13	-0.077	-0.047	0.226
X14	-0.034	**0.560**	**-0.556**
Y14	-0.049	0.175	-0.180
X15	-0.034	**0.560**	**-0.556**
Y15	-0.049	0.175	-0.180

Table S4. Discriminant function analyses (DFA) of the morphology in *P. mexicana* from different habitat types. (A) Analysis of wild-caught individuals from all four habitat types. (B) Comparison between wild-caught and laboratory-reared individuals.

	A. Wild-caught fish			B. Wild-caught and lab-reared fish	
	Function 1	Function 2	Function 3	Function 1	Function 2
X1	0.413	-0.363	0.143	-0.324	0.126
Y1	0.102	0.148	-0.108	-0.106	0.053
X2	0.049	-0.245	0.412	0.067	-0.175
Y2	0.117	-0.034	0.176	-0.180	-0.207
X3	0.300	-0.121	0.420	-0.276	-0.292
Y3	0.117	-0.034	0.176	-0.182	-0.207
X4	0.506	0.027	0.357	-0.542	-0.328
Y4	0.117	-0.034	0.176	-0.184	-0.207
X5	-0.216	0.322	0.086	0.275	-0.193
Y6	0.329	0.160	-0.277	0.320	0.043
X6	0.074	-0.052	0.060	0.009	-0.006
Y6	-0.256	0.375	-0.297	-0.328	0.135
X7	0.071	-0.106	-0.184	-0.014	0.236
Y7	0.365	0.179	-0.060	-0.375	0.021
X8	-0.127	-0.056	0.383	0.140	-0.279
Y8	0.189	0.159	0.151	-0.162	-0.311
X9	-0.059	-0.020	0.530	0.048	-0.459
Y9	0.038	-0.190	0.247	-0.024	-0.214
X10	-0.078	-0.041	-0.460	0.033	0.398
Y10	-0.419	-0.230	-0.048	0.372	0.246
X11	-0.034	-0.008	-0.271	-0.009	0.228
Y11	-0.320	-0.186	-0.045	0.303	0.248
X12	0.087	0.083	-0.106	-0.106	0.096
Y12	-0.271	-0.079	0.098	0.272	0.020
X13	-0.329	0.419	-0.017	0.398	-0.156
Y13	0.187	-0.559	0.302	-0.065	0.067
X14	-0.138	0.301	-0.004	0.171	-0.059
Y14	-0.051	0.371	-0.107	0.114	-0.090
X15	0.125	-0.259	-0.193	-0.115	0.289
Y15	-0.031	0.004	-0.160	-0.016	0.282
Canonical correlation	0.906	0.772	0.623	0.991	0.664
Eigenvalue	4.565	1.479	0.635	4.871	0.787
% Variance	68.3	22.1	9.5	86.1	13.9
Chi-square	1503.51	675.29	237.23	973.01	240.23
Df	69	44	21	46	22
P	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Table S5. Descriptive statistics of the microsatellite analysis in surface- and cave-dwelling Atlantic mollies (*Poecilia mexicana*). For each population and locus, observed (H_O) and expected (H_E) heterozygosity and allelic richness (A) are given. Zero-values indicate that the locus is monomorphic in this population. Non-sulfidic surface habitats: RA, Río Amatan; RO, Río Oxolotan; AB, Arroyo Bonita; AC, Arroyo Cristal; AT, Arroyo Tres. Sulfidic surface habitats: EA I, El Azufre I; EA II, El Azufre II. Sulfidic cave: V-XIII, cave chambers V-XIII of the Cueva del Azufre. Non-sulfidic cave: LA, Cueva Luna Azufre.

Locus	No. of alleles	Range of allele size	Test	RA N=24	RO N=24	AB N=24	AC N=20	AT N=19	EA I N=40	EA II N=20	V N=19	X N=20	XI N=19	XIII N=21	LA N=19	Mean across populations
GAI29B	16	217-255	H_O	0.42	0.46	0.17*	0.35	0.53	0.25	0.65	0.00	0.20	0.15	0.00	0.00	0.26
			H_E	0.49	0.53	0.23	0.31	0.51	0.29	0.51	0.00	0.18	0.10	0.00	0.00	0.26
			A	8.00	6.38	4.94	5.60	7.74	2.00	2.90	1.00	2.90	2.90	1.00	1.00	3.86
GAIV42	48	180-466	H_O	0.74	0.79	0.92	0.70	0.95	0.53	0.70	0.58	0.65	0.42	0.52	0.11	0.63
			H_E	0.90	0.90	0.92	0.90	0.90	0.70	0.68	0.72	0.72	0.65	0.49	0.10	0.72
			A	16.47	16.21	17.62	16.08	20.16	6.98	5.82	7.89	8.59	5.89	4.85	1.99	10.72
GTII33	14	167-231	H_O	0.48	0.75	0.63	0.30	0.53	0.03	0.00	0.05	0.00	0.00	0.00	0.00	0.23
			H_E	0.63	0.69	0.62	0.60	0.65	0.03	0.00	0.05	0.00	0.00	0.00	0.00	0.27
			A	5.35	5.50	4.69	5.70	5.84	1.45	1.00	1.95	1.00	1.00	1.00	1.00	2.96
GAII41	13	122-150	H_O	0.88	0.83	0.67	0.65	0.72	0.53	0.50	0.58	0.05	0.21	0.14	0.21	0.50
			H_E	0.69	0.64	0.58	0.66	0.65	0.64	0.56	0.43	0.05	0.19	0.28	0.27	0.47
			A	6.25	6.19	6.18	5.70	5.00	4.88	3.89	2.95	1.90	2.00	2.00	2.95	4.16
GAI29A	18	137-259	H_O	0.83	0.83	0.67	0.50	0.72	0.73	0.80	0.79	0.70	0.58	0.62	0.79	0.71
			H_E	0.66	0.72	0.66	0.53	0.71	0.77	0.74	0.68	0.65	0.57	0.52	0.56	0.65
			A	6.99	8.12	7.63	3.80	9.00	5.45	5.00	4.90	6.60	3.95	2.86	3.95	5.69
GAV18	18	114-154	H_O	0.82	0.88	0.96	0.90	0.79	0.43*	0.35*	0.42	0.35	0.47	0.52	0.47	0.61
			H_E	0.86	0.85	0.88	0.88	0.89	0.58	0.47	0.50	0.49	0.52	0.49	0.48	0.66
			A	9.58	10.12	11.42	12.67	11.94	4.15	2.99	2.00	2.90	2.95	5.68	2.00	6.53

GTI49	12	130-168	H_O	0.55	0.46	0.57	0.50	0.32	0.13	0.10	0.05	0.00	0.00	0.00	0.00	0.22
			H_E	0.71	0.55	0.67	0.64	0.36	0.12	0.10	0.05	0.00	0.00	0.00	0.00	0.27
			A	5.82	4.98	7.64	6.69	3.95	3.05	1.99	1.95	1.00	1.00	1.00	1.00	3.34
GAI26	45	167-295	H_O	0.91	0.83	0.96	0.75	0.74	0.35*	0.20*	0.21	0.80	0.05*	0.19*	0.32	0.53
			H_E	0.89	0.92	0.89	0.94	0.86	0.69	0.53	0.24	0.89	0.10	0.34	0.68	0.66
			A	14.68	16.55	17.25	21.75	13.58	11.63	7.60	4.84	15.18	2.90	6.39	4.90	11.44
GAIII28	33	193-265	H_O	0.73	0.92	0.86*	0.30*	0.68	0.63*	0.68	0.58	0.70	0.63	0.48	0.00	0.60
			H_E	0.91	0.88	0.82	0.71	0.78	0.89	0.87	0.74	0.82	0.74	0.54	0.00	0.72
			A	16.27	13.15	12.76	8.78	9.79	13.69	11.79	10.73	10.59	10.73	9.84	1.00	10.76
GTI13B	4	217-237	H_O	0.21*	0.29	0.21	0.10	0.00	0.03	0.00	0.11	0.00	0.00	0.00	0.00	0.08
			H_E	0.26	0.32	0.26	0.41	0.00	0.03	0.00	0.10	0.00	0.00	0.00	0.00	0.12
			A	3.87	3.00	2.99	2.90	1.00	1.45	1.00	2.00	1.00	1.00	1.00	1.00	1.85
Mean across loci			H_O	0.66	0.70	0.66	0.50	0.60	0.36	0.40	0.34	0.35	0.25	0.25	0.19	
			H_E	0.70	0.70	0.65	0.66	0.63	0.47	0.44	0.35	0.38	0.29	0.27	0.21	
			A	9.32	9.02	9.31	8.97	8.80	5.47	4.41	4.02	5.17	3.43	3.56	2.08	

*indicates significant deviations from Hardy-Weinberg Equilibrium after Bonferroni adjustment at an experiment-wise error rate of α=0.05.