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Conservation genetics of threatened Mexican axolotls (*Ambystoma*)

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Abstract

The loss of genetic diversity in small or isolated populations can increase inbreeding, decrease fitness and adaptive potential and increase a species' probability of extinction. In species with life histories that naturally result in small populations and/or low levels of gene flow, patterns of anthropogenically induced genetic erosion can be obscured by evolutionary history; yet these species may still be susceptible to genetic loss. We assess genetic diversity among populations of *Ambystoma* salamanders endemic to Mexico, including populations that are facultatively or obligately paedomorphic, to test whether paedomorphic lineages have lower genetic diversity than metamorphic ones, and whether gene flow contributes to the maintenance of diversity in divergent forms with either life history. We also test the utility of our markers in assigning illegally harvested individuals to populations of origin. We found reduced genetic diversity in paedomorphic compared with some, but not all, metamorphic populations. Populations of both forms showed genetic signatures of bottlenecks, underscoring that factors other than paedomorphosis contribute to historical reductions in population size. In general, *Ambystoma* populations have low interpopulation gene flow and admixture, but paedomorphic populations have higher within-population relatedness than most metamorphic populations. We discuss historical and current landscape attributes that impact populations and their connectivity, the implications of our findings for ongoing captive propagation programs and the prospects for continued genetic health of *Ambystoma* in México.

Introduction

The loss of genetic diversity in small or isolated populations is a concern for threatened species isolated by natural or anthropogenic barriers (Daniels, Triddy & Walters, 2000; Sherwin & Moritz, 2000). This genetic erosion will be exacerbated in cases where isolated populations are small (Keller & Waller, 2002) as is often the case with threatened species in disturbed habitats (Andersen, Fog & Damgaard, 2004; Stow & Briscoe, 2005). Species with life history attributes that naturally result in small populations and/or limited dispersal often show the genetic signatures of low intrapopulation genetic diversity and limited interpopulation genetic exchange (Shaffer & Breden, 1989), which can confound signatures of anthropogenically induced genetic erosion.

The *Ambystoma* species endemic to México are members of the widespread, polytypic, *Ambystoma tigrinum* complex (Shaffer, 1984a; Shaffer & McKnight, 1996; Recuero *et al*., 2010). Previous morphological (Shaffer, 1984b; Webb, 2004) and phylogenetic studies using allozymes (Shaffer, 1984a), mtDNA (Shaffer & McKnight, 1996; Recuero *et al*., 2010) and nuclear sequences (Weisrock *et al*., 2006) have examined species boundaries and divergences among regional groups of populations. Independent of the molecular markers used, these studies corroborate that divergence of the Mexican forms, especially the narrow endemics, restricted to the Mexican Transvolcanic Belt (TVB), are very recent in origin. Lineages within the *A. tigrinum* complex are polymorphic for life history (Routman, 1993) and obligate paedomorphism has evolved multiple times in the endemic populations in México (Shaffer, 1984a; 1993). Based on this polymorphic life history trait, and their limited distribution in one or two lakes, some of these forms (but not all) have been named as independent species, but with no attention to relationships among taxa, reciprocal monophyly, and sometimes, the availability of diagnostic characters. Divergences among Mexican *Ambystoma* are generally shallow, resulting in low support for monophyletic

clades (Shaffer & McKnight, 1996). Comparing independent nuclear and mitochondrial markers additionally suggests that continued gene flow and introgression among independent species, and possibly lineage sorting during the recent evolutionary histories of species, result in non-monophyly of independent species. Surprisingly, this introgression is evident even between paedomorphic and metamorphosing populations, suggesting that barriers to reproduction resulting from this life history shift are incomplete. Combined, these phylogenetic patterns suggest that relationships among species and populations of Mexican *Ambystoma* are recent and dynamic, and the taxonomic status of most forms is ambiguous, at best.

The obligate paedomorphic *Ambystoma* (*Ambystoma mexicanum, Ambystoma dumerilii, Ambystoma andersoni* and *Ambystoma taylori*) are listed as critically endangered by the international union for conservation of nature (IUCN), because of water quality, pollution and other altered environmental conditions in their small ranges. Most critically threatened is the Mexican axolotl, *A. mexicanum,* a species endemic to the Xochimilco lake system, which is now reduced to an area only 1% of its historical size (Fox, 1965). Recent surveys show that the remaining population decreased substantially due to overexploitation, introduced species and habitat modification (Zambrano *et al*., 2007). Long-term isolation and reduction in population size leads to genetic erosion (O'Grady *et al*., 2006; Vilas *et al*., 2006), but the magnitude of these effects depends necessarily on the ancestral genetic composition of the species. Genetic erosion can occur gradually, and thus may not threaten populations in the short term, especially those that historically showed reduced population genetic diversity (Lande, 1995; Lynch, Conery & Burger, 1995). However, inbreeding can act swiftly if small populations are composed primarily of related individuals (Keller & Waller, 2002).

Life history shifts can alter the degree and direction of migration and, in some cases, impose limits on population sizes, mechanisms that will affect genetic drift and gene flow in and among populations. A comparative survey of genetic variation in metamorphic and paedomorphic salamanders shows that on average, larval reproducers are less genetically variable than metamorphosing species (Shaffer & Breden, 1989), a pattern attributed to differences in population structure correlated with life history aspects. Paedomorphosis requires the colonization of aquatic habitats that are typically less persistent than terrestrial habitats; the bottlenecks associated with repeated extinction/colonization events can reduce effective population sizes. In addition, gene flow among discreet aquatic environments will be more difficult than in continuous terrestrial habitats. Given these consequences of larval reproduction, we expect paedomorphic populations to show the signatures of reduced gene flow, smaller populations and higher genetic drift.

Species conservation requires a clear understanding of genetic diversity within and among populations (Frankham, Ballou & Briscoe, 2002). Here, we characterize the genetic diversity of two paedomorphic *Ambystoma* and compare them with populations of facultative and obligate transforming species to test whether: (1) paedomorphic species have lower genetic diversity attributable to historical or recent population bottlenecks, drift and/or lack of gene flow; (2) paedomorphic species show evidence of high within-population relatedness; (3) gene flow among transforming and paedomorphic populations contributes to the maintenance of diversity in species with either life history; (4) we can use molecular markers to assign illegally harvested individuals to their populations of origin. Characterizing genetic diversity of Mexican *Ambystoma* will identify mechanisms with the largest positive effects on remaining populations, prioritize conservation efforts, and provide a method for identifying populations threatened by illegal harvest. We interpret our results in light of what is known about historical (Shaffer, 1993; Shaffer & McKnight, 1996) and recent changes (Zambrano *et al*., 2007) in population size and habitat availability in this highly disturbed biodiversity hotspot (Flores-Villela, Canseco-Márquez & Ochoa-Ochoa, 2010).

Materials and methods

Population sampling and laboratory protocols

We sampled 298 *Ambystoma* from eight populations (Fig. 1; Table 1) including five populations of four metamorphic species (*Ambystoma velasci*, *Ambystoma granulosum*, *Ambystoma rivulare* and *A. altamirani*), and three populations of two obligate paedomorphic species (*A. mexicanum* and *A. andersoni*). We sampled *A. mexicanum* at two sites: the type locality of the species (Xochimilco) and a second introduced population in a lake in Parque Chapultepec, a recreational area in Mexico City (Recuero *et al*., 2010).

We focused on populations for which we could obtain sufficient sample sizes [mean (SD) = 33.1 (12.0, range 21–57)] and included in the final dataset only individuals with complete genotypes for at least five loci (Table 1). We also genotyped 38 individuals purchased at a market in Morelia, Michoacán. These individuals were purchased as large aquatic forms (body size > 25 cm) but were of unknown origin and/or species identity. Tissue samples (liver or tail/gill clips) were incubated in 250 µl 5% Chelex and 0.20 mg proteinase K (Roche, Indianapolis, IN, USA) at 55°C for 180 min, followed by 10 min at 99°C (Sambrook & Russell, 2001). Genomic DNA was used as template for amplification of nine loci (Atig52.143, Atig52.115, At52.1, At60.3, At52.2, At52.20, At52.6, At52.10, At52.34) following published protocols (Parra-Olea, Recuero & Zamudio, 2007). Amplified products were multiplexed on an ABI 3100 Genetic Analyzer and sized with a LIZ-500 standard in GeneMapper v. 3.5 (Applied Biosystems, Foster City, CA, USA).

Characterizing population genetic variability

For each population, we estimated the rarefied number of alleles (A) and number of private alleles (P) in the program

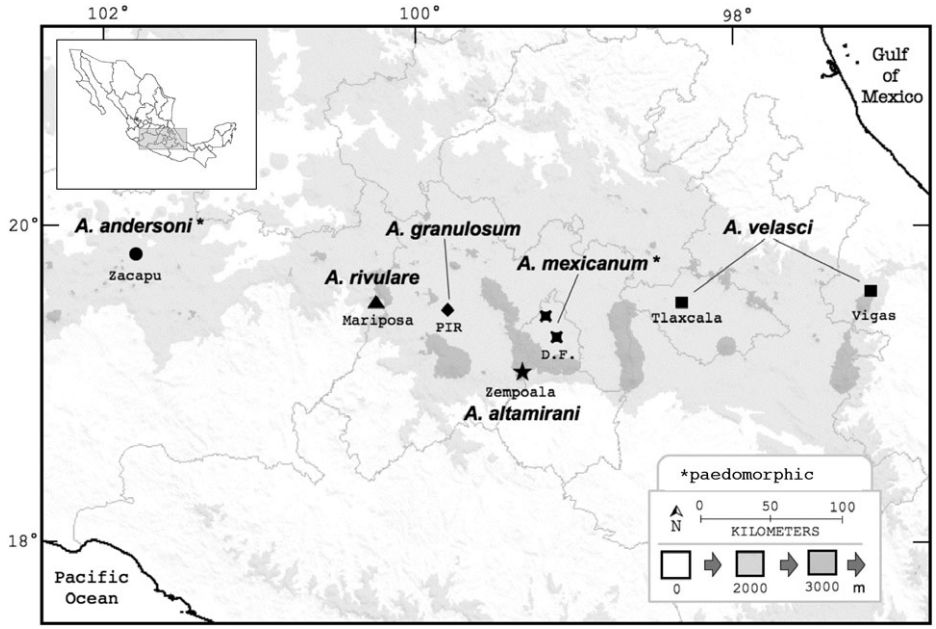

Figure 1 Topographic map of the Trans-Mexican Volcanic Belt, with localities for the *Ambystoma* populations sampled for this study.

Table 1 Populations of *Ambystoma* sampled for this study. We sampled a total of 298 individuals from eight wild populations and six species, including two obligate paedomorphic species (*A. andersoni* and *A. mexicanum*). The 'market' samples were purchased at a public market in Morelia, Michoacán; these samples are of unknown origin and species identity

Locality	State	GPS coordinates	Species	Paedomorphic	Sample
Lagunas de Zempoala	Morelos	19.052999, –99.310997	*A. altamirani*	No	33
Presa Ignacio Ramírez (PIR)	México	19.448, –99.788002	*A. granulosum*	No	57
Reserva de la Mariposa Monarca	Michoacán	19.523001, –100.252998	*A. rivulare*	No	26
Laguna San Fernando, Hueyotlipan	Tlaxcala	19.509359, –98.303998	*A. velasci*	No	21
Road to Microondas station 'Las Lajas' near Las Vigas	Veracruz	19.57333, –97.09833	*A. velasci*	No	30
Laguna de Zacapu	Michoacán	19.824433, –101.787685	*A. andersoni*	Yes	46
Lago Xochimilco	Distrito Federal	19.288, –99.102997	*A. mexicanum*	Yes	23
Laguna Vieja, Parque Chapultepec	Distrito Federal	19.422001, –99.184998	*A. mexicanum*	Yes	24
San Juan Market, Morelia, Michoacán	–	–	Unknown	Unknown	38

GPS, Global Positioning System.

HP-RARE (Kalinowski, 2005) to account for the potential bias resulting from different sample sizes among populations. We estimated observed (Ho) and expected (He) heterozygosities in GENALEX v. 6 (Peakall & Smouse, 2006) or GenePop on the Web v. 3.4 (Raymond & Rousset, 1995). We tested for deviation from Hardy–Weinberg equilibrium using an exact test (10 000 dememorization steps, 1000 batches, 10 000 iterations/batch; Guo & Thompson 1992). Statistical significance values were corrected using sequential Bonferroni (Rice, 1989) for a table-wide significance value of 5%. After an initial analysis, heterozygous deficiency was detected at one locus (At52.10), but not at the other eight markers, suggesting the possible existence of a null allele at that marker (Brookfield, 1996; Hedrick, 1999). The data were then analysed using MICRO-CHECKER v. 2.2.1 (van Oosterhout *et al.*, 2004) for the presence of null alleles at each of the nine loci. For loci with significant probability of null alleles, we followed corrective procedures using the program FREENA (Chapuis & Estoup, 2007). The corrected frequencies were then used to recalculate the number of homozygotes and heterozygotes in each population sample, for comparison with values derived from the original dataset. The corrected and 'null allele free' dataset was used to estimate F_{ST} (Chapuis & Estoup, 2007). Comparing F_{ST} values derived from both datasets indicates the effects of presumptive null allele at these loci on estimates of genetic diversity. We tested for linkage disequilibrium between all pairs of loci across all populations in our sample using an exact test (Raymond & Rousset, 1995) implemented in GenePop on the Web v.3.4, with 10 000 dememorization steps and 1000 batches of 10 000 iterations per batch.

Genetic divergences among pairs of populations were estimated using F_{ST} (Weir & Cockerham, 1984). Pairwise F_{ST} significance tests were performed by permutation and resampling of multilocus genotypes among pairs of samples

in the program ARLEQUIN v. 3.0 (Excoffier, Laval & Schneider, 2005). Performing 90 000 randomizations allowed for a table-wide significance at the 5% nominal level after Bonferroni corrections (adjusted P-value = 0.00066). We tested for isolation by distance (excluding the Market sample) using a Mantel test (Mantel, 1967) in GENALEX v. 6 (Peakall & Smouse, 2006).

Population structure of paedomorphic and metamorphic populations

We used STRUCTURE v. 2.2 (Pritchard, Stephens & Donnelly, 2000) to infer the number of genetic clusters (K) diagnosable in our sample. We tested $K = 1$ through $K = 15$, in 20 independent runs of 3 000 000 iterations (following a burn-in period of 1 000 000). We plotted *log P(D|M)* against K, and sought the range of K along the inflection point of the curve (Pritchard, Wen & Falush, 2007). Within this range is the smallest value of K that captures the most structure in the data. Second, we applied the ΔK method (Evanno, Regnaut & Goudet, 2005) that estimates the plateau in *log P(D|M)* by comparing second derivatives for various values of K. Individual membership coefficients were graphed in the program DISTRUCT v. 1.0 (Rosenberg, 2004).

Bottlenecks, relatedness and potential for inbreeding

A number of different procedures exist for estimating or detecting population size changes interpreted as past bottlenecks (Cornuet & Luikart, 1996; Beaumont, 1999; Pybus, Rambaut & Harvey, 2000; Storz & Beaumont, 2002; Drummond *et al.*, 2005; Chapuis & Estoup, 2007). However, most models assume that samples must be obtained from populations that can be approximated by a Wright–Fisher model, which assumes panmixia and demographic stationarity (Chikhi *et al.*, 2010). Inferences on population size change can be drastically affected, generating spurious bottleneck signals when population subdivision, genetic differentiation, gene flow, genetic diversity and the sampling scheme are not properly accounted for (Chikhi *et al.*, 2010; Peter, Wegmann & Excoffier, 2010). We used BOTTLENECK (Cornuet & Luikart, 1996) and M-ratio tests (Garza & Williamson, 2001) to detect bottlenecks in our populations. We used 1 000 000 replicates, assuming a two-phase mutation model (TPM) with 90% one-step and 10% multistep mutations (Di Rienzo *et al.*, 1994). Significant heterozygosity excess or deficit was estimated by comparison with a simulated null distribution. We also used BOTTLENECK to examine mode shifts in allele frequency distributions (Luikart & Cornuet, 1998).

M-ratios were calculated in M_P_VAL (Garza & Williamson, 2001). Three parameters are needed for this program: theta ($\theta = 4*N_e*\mu$), percentage of mutations greater than one step (P_s), and average size of mutations that are not one-step (Δ_g). The significance of an observed M-value is determined by comparing it with a distribution of M-values calculated from theoretical populations in mutation-drift equilibrium. The test is significant if more than 95% of the simulated values are superior to the observed value. The critical value of M (Mc) is set at the lower 5% tail of this distribution. The program CRITICAL_M (Garza & Williamson, 2001) generates critical values M_C. Because N_e and μ are typically unknown, most studies base their significance criteria on a wide range of biologically plausible θ values (Abdelkrim, Pascal & Samadi, 2005; Busch, Waser & DeWoody, 2007). We chose to use general and species-specific estimates of θ. First, we estimated θ for each site using MIGRATE 2.1.3 (Beerli & Felsenstein, 1999, 2001). We used stepwise mutation model (Ohta & Kimura, 1973) with the following conditions: 10 short chains of 1000 genealogies sampled every 200 trees, followed by five short chains of 10 000 genealogies sampled every 50 trees. The first 10 000 trees were discarded as burn-in. This gave us the smaller value of θ used for the estimation of M. We also used three generic N_e values (1500, 3000 and 5000) and microsatellite mutation rate (5.0×10^{-4} mutants/generation/locus; Garza & Williamson, 2001). Substituting these values in θ gives a broad range of 3–10, plus the individual θ estimated for each population by MIGRATE. We used default values for the remaining two parameters needed for the TPM ($P_s = 0.12$ $\Delta_g = 2.8$) (Garza & Williamson, 2001). We excluded the market population from both bottleneck tests.

Finally, we estimated the within-population coefficient of genetic relatedness, r (Queller & Goodnight, 1989) in GENALEX v. 6 (Peakall & Smouse, 2006). We bootstrapped allelic data within populations 999 times to derive 95% confidence intervals for r estimates; localities with nonoverlapping bootstrap intervals are statistically distinct. We also permuted genotypes from all populations 999 times and derived upper and lower 95% confidence intervals (CIs) for r based on all populations. These intervals represent the range of r expected if random mating occurs across all populations. Population r-values that fall above the upper bound of the 95% CI indicate that reproductive skew, inbreeding or drift are increasing relatedness, despite potential gene flow among localities.

Results

Characterizing population genetic variability

Three of the transforming populations (*A. rivulare* and two *A. velasci* populations) and the market population were polymorphic at all loci. In contrast, the three paedomorphic populations (*A. andersoni* and two *A. mexicanum* populations) and two transforming populations (*A. altamirani* and *A. granulosum*) were monomorphic at one or two of the loci genotyped. Averaging across all loci, transforming species generally show higher genetic diversity (Fig. 2), with the exception of the populations in Zempoala (*A. altamirani*) and Vigas (*A. velasci*), which are more similar to paedomor-

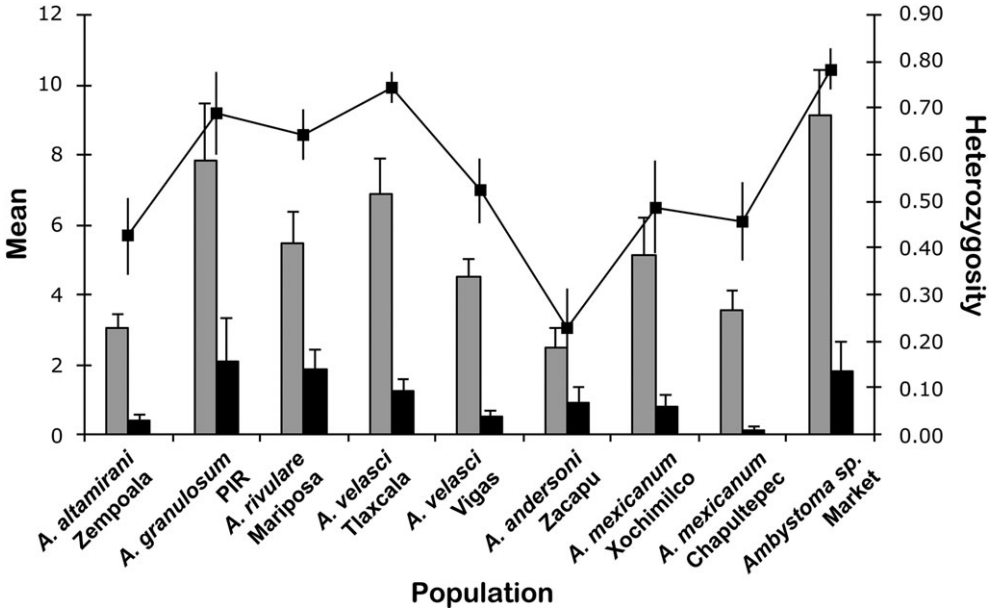

Figure 2 Patterns of allelic richness and heterozygosity in nine sampled populations of *Ambystoma*. Bars represent rarified mean ± SD number of alleles (grey bars) and private alleles (black bars), adjusted for the number of alelles present in the population with the smallest sample size. Mean ± SD heterozygosities for each population (across all loci) are represented by the black line.

phic species. Our market sample shows the highest genetic diversity, suggesting it may contain a mixture of individuals from different populations.

Randomization tests of Hardy–Weinberg equilibrium indicated heterozygous deficit at one locus (At52.10) for four of the natural populations (Supporting Information Table S1). The market population showed evidence of departure from equilibrium at eight of nine loci, likely as a result of the pooled distinct genetic demes in that sample. Tests for null alleles using MICRO-CHECKER were significant at loci At52.10 (for six populations) and At52.115 (for five populations). For those two loci, we derived the corrected frequencies from FREENA and used those to recalculate the number of homozygotes and heterozygotes in each population sample, for comparison with values derived from the original dataset. Comparing F_{ST} values derived from both datasets (Table 2) indicates that the presumptive null allele at these loci has only a marginal effect on estimates of genetic diversity; therefore, we used the original dataset for all subsequent analyses.

Population structure of paedomorphic and metamorphic populations

All pairwise F_{ST} values were significant (range 0.04–0.64; Table 2). The paedomorphic species *A. andersoni* exhibited the greatest divergences from other populations (range 0.37–0.64). The lowest divergence ($F_{ST} = 0.04$) was between the paedomorphic populations from Xochimilco and Chapultepec, corroborating the identity of the Chapultepec population as *A. mexicanum* (Recuero *et al.*, 2010). Mantel tests showed a nonsignificant correlation ($r = 0.410$, $P = 0.104$) between pairwise F_{ST} and geographical distances; thus, distance alone is not responsible for the distribution of genetic differentiation among *Ambystoma* populations in the TVB.

Bayesian assignment tests corroborated high population divergences among populations. The probability of the data under models with increasing K showed an inflection point in the range of $K = 8 – 11$, and the ΔK method confirmed eight genetic clusters in our sample (Fig. 3). Most clusters corresponded to sampling localities, underscoring the independent history of many *Ambystoma* populations. All individuals sampled from wild populations showed high cluster membership coefficients (mean membership coefficients range = 0.932 to 0.980). The three paedomorphic populations had the highest membership coefficients and lowest variances among individuals in genetic admixture (*A. mexicanum* Chapultepec: $Q = 0.974 \pm 0.040$; *A. mexicanum* Xochimilco: $Q = 0.951 \pm 0.110$; *A. andersoni*: $Q = 0.979 \pm 0.034$). Two transforming species showed similarly high levels of cluster membership: *A. altamirani* from Zempoala ($Q = 0.980 \pm 0.021$) and *A. velasci* from Las Vigas ($Q = 0.934 \pm 0.108$), underscoring that even among metamorphic forms, populations show genetic patterns consistent with prolonged isolation.

Bayesian assignment of the market samples confirmed their mixed origin. In the model with eight genetic clusters, the market sample showed low membership coefficients to two independent demes (Q1 = 0.390 and Q2 = 0.547). Some individuals show genetic similarity to *A. granulosum* individuals from Presa Ignacio Ramirez; the other genetic deme is unique to the market sample, and thus likely represents an unsampled population (Fig. 3).

Table 2 Pairwise estimates of population differentiation for nine populations of paedomorphic and transforming *Ambystoma* species and/or populations. We estimated pairwise F_{ST} (below diagonal) for each pair of populations and tested for significance using permutation tests in the program FSTAT v. 2.9.3.2 (1000 randomization) (Goudet 1995). Numbers above diagonal are pairwise F_{ST} values of the null allele-free dataset after corrective procedures described in Chapuis & Estoup (2007) in the program FREENA

		1	2	3	4	5	6	7	8	9
1	*A. altamirani* Zempoala	–	0.3215	0.3970	0.3571	0.4400	0.6573	0.5514	0.5702	0.2946
2	*A. granulosum* Presa Ignacio Ramírez	**0.2950**	–	0.2242	0.1331	0.2361	0.4262	0.3509	0.3587	0.1111
3	*A. rivulare* Mariposa	**0.4011**	**0.2418**	–	0.2021	0.3214	0.5221	0.3648	0.3854	0.1487
4	*A. velasci* Tlaxcala	**0.3518**	**0.1515**	**0.2201**	–	0.2280	0.4922	0.2758	0.3031	0.1148
5	*A. velasci* Las Vigas	**0.4302**	**0.2490**	**0.3350**	**0.2397**	–	0.4960	0.4173	0.4296	0.2381
6	*A. andersoni* Zacapu	**0.6488**	**0.4341**	**0.5467**	**0.5085**	**0.51202**	–	0.6204	0.5952	0.3909
7	*A. mexicanum* Xochimilco	**0.5506**	**0.3753**	**0.3888**	**0.2886**	**0.4334**	**0.6407**	–	0.0653	0.2759
8	*A. mexicanum* Chapultepec	**0.5532**	**0.3541**	**0.3900**	**0.2945**	**0.4281**	**0.6111**	0.0523	–	0.2903
9	*Ambystoma* sp. Market	**0.2458**	**0.1126**	**0.1392**	**0.1273**	**0.2389**	**0.3782**	**0.2948**	**0.2812**	–

PIR, Presa Ignacio Ramírez.

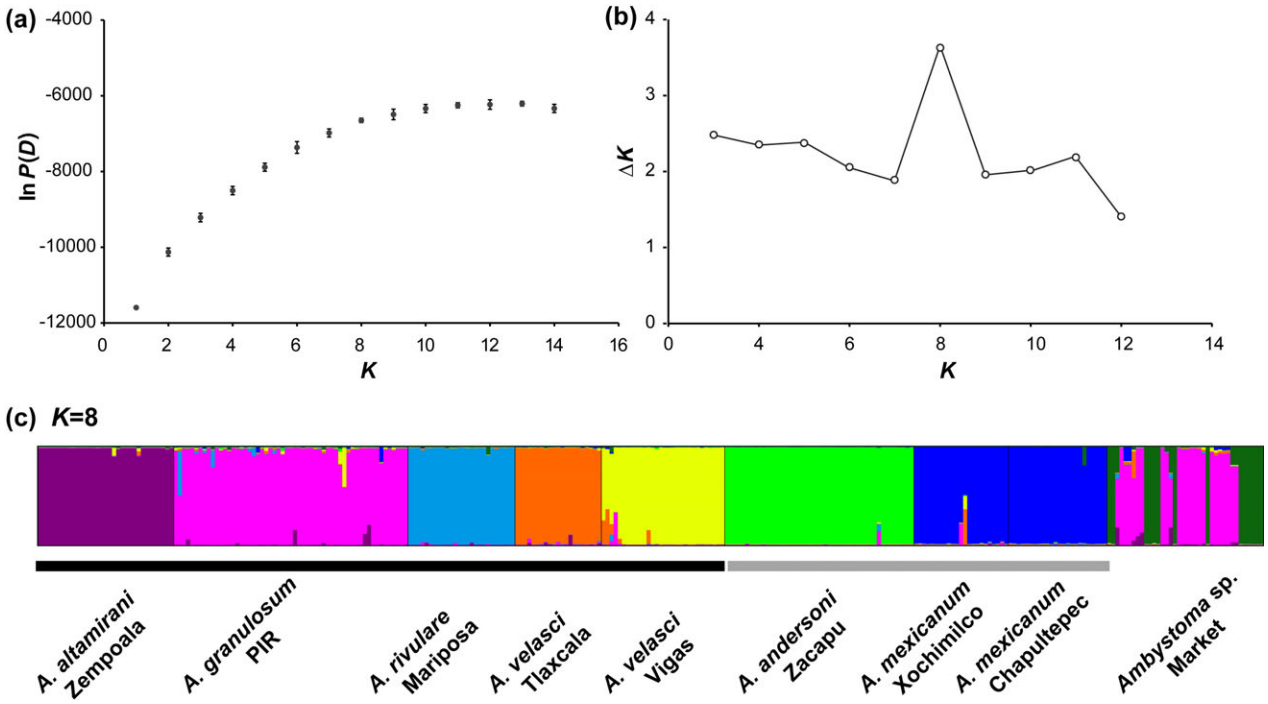

Figure 3 Population structure inferred by Bayesian assignment of 298 individuals of paedomorphic and metamorphic populations of *Ambystoma* endemic to Mexico. (a) Mean ln *P*(*D*) and SD of 20 independent runs for $K = 1–14$. (b) Value of ΔK as a function of $K = 3–12$. (c) Distribution of the eight genetic clusters inferred for our sample; assignment graphs represent the mean membership coefficient for each individual to each genetic cluster. Populations marked with a grey bar are obligate paedomorphs and show significantly lower admixture than most transforming populations (black bar).

Bottlenecks, relatedness and potential for inbreeding

BOTTLENECK did not detect excess of heterozygosity or allele frequency mode shifts in any of the sampled populations (Table 3). In contrast, *M*-ratio values for all populations were lower than the critical value for individual sites (ranging from 0.4164 to 0.6805, independent of the θ assumed in the model; Table 3), indicating historical reduction of effective population sizes.

Average pairwise relatedness (r) within populations was generally high for all populations, but was significantly higher in paedomorphic species (Fig. 4). The paedomorphic *A. andersoni* had the highest relatedness (mean $r = 0.825$, CI = 0.817–0.832), followed by the two paedomorphic and isolated populations of *A. mexicanum* [Xochimilco: 0.571 (CI 0.550–0.592); Chapultepec: 0.626 (CI 0.611–0.643)]. Mean relatedness across paedomorphic populations (0.674 ± 0.133) was significantly higher than that of the five metamorphosing populations (0.4 ± 0.202). However, two populations of transforming *Ambystoma* show unexpectedly high levels of intrapopulation relatedness: *A. altamirani* from Zempoala and the *A. velasci* from Las Vigas show relatedness comparable with paedomorphic species (Fig. 4), suggesting that the same processes increasing within-population relatedness might be acting in isolated metamorphic populations as well. Finally, relatedness among individuals in the market sample was lower than in any other sample (0.098, CI 0.074–0.123) and approximates values expected assuming panmictic breeding among all populations (Fig. 4).

Discussion

Genetic diversity, bottlenecks and relatedness

Our results are consistent with the hypothesis that paedomorphic species have overall low genetic diversity (Shaffer & McKnight, 1996); however, a few transforming species show a similar pattern (Fig. 2). Thus, paedomorphosis certainly contributes to lower genetic diversity, but does not fully explain the variance in genetic diversity among *Ambystoma* populations; other microevolutionary processes such as bottlenecks and limited genetic exchange may shape metamorphic populations as well. We found the signature of genetic bottlenecks in both paedomorphic and metamorphic forms, but those results were inconsistent among tests. Methods classically used to detect genetic bottlenecks rely on different statistics and can favor different time scales for detection. The *M*-ratio method is expected to detect older events because of the longer time needed for the *M*-statistic to reach equilibrium (Garza & Williamson, 2001); this method detected a significant reduction in population size for all *Ambystoma* populations (Garza & Williamson, 2001). In contrast, tests based on heterozygosity did not support a bottleneck in any of the sampled populations. Heterozygosity-based tests measure genetic changes that occur immediately after the bottleneck (Nei, Maruyama & Chakraborty, 1975; Cornuet & Luikart, 1996; Luikart & Cornuet, 1998) and are expected to disappear after a few

Table 3 Results of heterozygosity excess tests, allele frequency analyses and *M*-ratio tests. For heterozygosity excess tests, we used a two-phase mutation model with 10% multistep mutations. Ex HE: expected number of loci with heterozygosity excess. Obs HE: observed number of loci with heterozygosity excess. Wilcoxon test probability (one tail for H excess). Normal allele frequency means that the majority of alleles had frequencies less than 0.1. *M*-ratio averages were calculated across loci with the program M_Val_ P. Mc is the critical *M*-value calculated through the M-crit program developed by Garza & Williamson (2001). θ^* is the effective population sizes estimated by MIGRATE for each population. $\theta\hat{} = 10$ is the largest generic value used when Ne = 5000 for all populations. *P*-values are significant for all analyses except for *A. mexicanum* from Xochimilco when using the θ^* generated by MIGRATE

	Expected HE	Observed HE	*P*-value	Allele freq	*M*	Mc	θ^*= (*P*-value)	*P*-value ($\theta\hat{} = 10$)
A. altamirani Zempoala	4.28	4.0	0.32031	Normal	0.6180	0.8302	0.211 (0.0)	0.03
A. granulosum PIR	4.75	3	0.87500	Normal	0.4664	0.816	0.503 (0.0)	< 0.0001
A. rivulare Mariposa	5.05	6	0.12500	Normal	0.5456	0.8304	0.314 (0.0)	< 0.0001
A. velasci Tlaxcala	5.34	8	0.06445	Normal	0.4939	0.8333	0.211 (0.0)	< 0.0001
A. velasci Las Vigas	5.21	4	0.71484	Normal	0.4164	0.8388	0.212 (0.0)	< 0.0001
A. andersoni Zacapu	3.50	2	0.94531	Normal	0.4694	0.8238	0.267 (0.0)	< 0.0001
A. mexicanum Xochimilco	4.46	3	0.87500	Normal	0.6805	0.8191	0.300 (0.71)	0.03
A. mexicanum Chapultepec	4.55	4	0.37109	Normal	0.465	0.8229	0.279 (0.0)	< 0.0001

HE, heterozygosity excess; PIR, Presa Ignacio Ramírez.

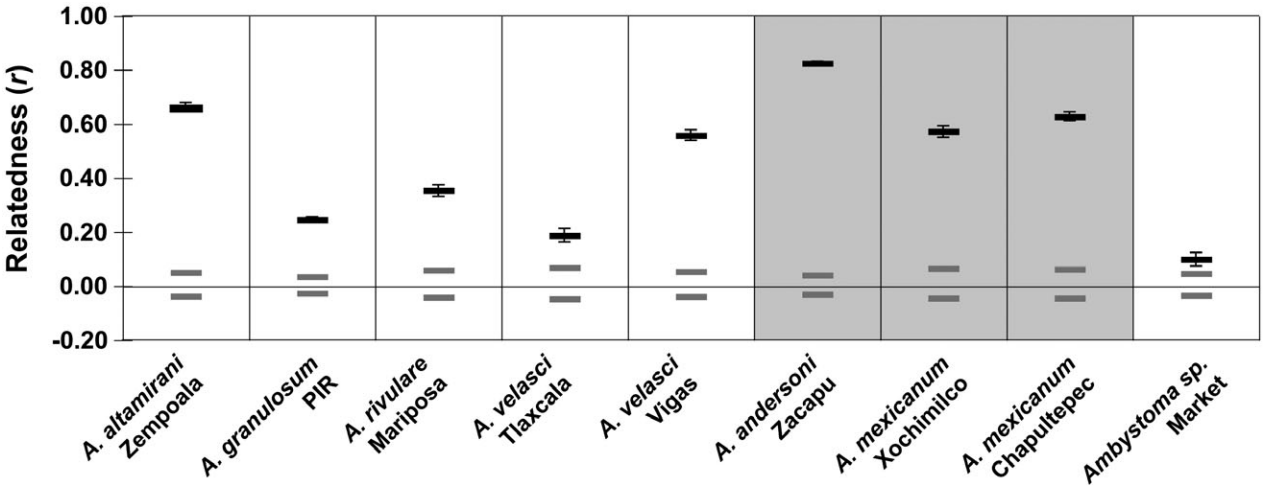

Figure 4 Mean within-population pairwise relatedness for nine *Ambystoma* populations. Grey bars are 95% upper and lower expected values for a null distribution generated from 999 permutations of data from all populations, and enclose the values expected if breeding were panmictic across all populations; relatedness in all sampled populations fell outside of the range expected under panmixia. Black bars represent the observed mean relatedness in each population or species, and the upper and lower bootstrap value for each population. The three populations shaded in grey are the obligate paedomorphic species/populations with the highest degree of inbreeding. Populations of *A. altamirani* (Zempoala) and *A. velasci* (Las Vigas) show surprisingly high levels of intrapopulation relatedness for metamorphic populations.

generations following the reduction in population size (Keller *et al*., 2001; Williamson-Natesan, 2005). In combination, these results show that many *Ambystoma* populations have suffered decreases in population size, but that those changes likely are historical, not recent. Further evidence for historical changes comes from comparison of the two paedomorphic forms in our study, which are threatened to different degrees by anthropogenic change. The Mexican axolotl has suffered drastic range reductions and population sizes (Zambrano *et al*., 2007); in contrast, *A. andersoni* still persists in relatively high numbers in its native range (Huacuz, 2001), but both species show similar low diversity and heterozygosity.

We found evidence for high relatedness within paedomorphic populations, suggesting a high potential for inbreeding within those populations. Although inbreeding can lead to reduced fitness, the degree to which populations suffer from inbreeding depression can vary widely depending on population history, lineage effects and the environment (Keller & Waller, 2002). Deleterious alleles may have higher chances of being removed by natural selection in populations where inbreeding accumulates gradually than in populations where all individuals are the result of breeding between close relatives (Keller & Waller, 2002). The paedomorphic species *A. mexicanum* and *A. andersoni* have high levels of relatedness among individuals, but we have no evidence of decreased fitness due to inbreeding depression. Gradual increases in relatedness within these populations may make them less susceptible to inbreeding depression (Keller & Waller, 2002), but this does not mean that inbreeding does not threaten these species. Overexploitation of paedomorphic populations that significantly reduces population sizes could potentially increase inbreeding to a critical threshold causing reduced population fitness.

The lakes where Mexican paedomorphic species of *Ambystoma* are found are isolated from each other by considerable distances of inhospitable terrestrial habitat. Therefore, gene flow, if it occurs, could be mediated by introgression with facultatively paedomorphic forms rather than direct movement of paedomorphic species among lakes. Our data show clearly that the paedomorphic species in our sample have negligible admixture with other species, corroborating data from other markers showing that paedomorphic populations have independent evolutionary histories (Shaffer, 1993). Paedomorphic populations are more genetically diverged from neighboring populations, consistent with predictions of low interpopulation connectivity.

Two populations of the metamorphic species *A. velasci* (Las Vigas) and *A. altamirani* (Zempoala) are unique in being similar to paedomorphs in population genetic patterns. We infer that population dynamics in these two species somehow restrict genetic exchange and limit population sizes. *Ambystoma velasci* is widely distributed from north-western Chihuahua to central Veracruz. Current molecular data suggest that *A. velasci* is a paraphyletic complex of metamorphic populations, with some populations more closely related to paedomorphic species than to other *A. velasci* populations (Shaffer & McKnight, 1996). The Las Vigas population is the eastern and southernmost population of the genus *Ambystoma* in Mexico, and the population genetic signatures we identified may be the result of isolation at the edge of the species' distribution. In fact, the second *A. velasci* population from Tlaxcala shows the high genetic diversity we expect from large populations of metamorphic forms with higher population connectivity. Comparing other central and range edge *A. velasci* populations will further elucidate how gene flow and drift shape genetic diversity in metamorphic species in this complex

landscape. *Ambystoma altamirani* is distributed in the highlands of the state of México, southern Distrito Federal and north-western Morelos. The creeks surrounding the Lagunas de Zempoala harbor a facultatively transforming population of *A. altamirani* (Aguilar-Miguel *et al.*, 1997; Castro-Franco *et al.*, 2006), which was once described as *Ambystoma zempoalense* (Taylor & Smith, 1945) but later synonymized with *A. altamirani* (Reilly & Brandon, 1994). The sample we obtained from that locality had the highest assignment values and relatedness of all the populations we sampled. Further population genetic comparisons among populations of *A. altamirani* will help guide conservation efforts for this species.

Our results highlight the utility of high-resolution markers in efforts to curb illegal harvest of endangered *Ambystoma*. We could not identify the exact origin of the *Ambystoma* from the Morelia market; however, given high assignments probabilities for the obligate paedomorphic species, we can conclude that the market individuals were not *A. mexicanum* or *A. andersoni*. We also found that the market individuals represented a mixed sample collected from at least two independent genetic demes. The resolution of our markers to distinguish finer-scale population differentiation should be tested with more reference populations and species, but it is clear from our results that they can clearly differentiate among some populations tested thus far.

Conservation implications for Mexican paedomorphic *Ambystoma*

Currently, the largest threat to wild populations of paedomorphic salamanders is anthropogenic habitat change, including altered hydrology, pollution, exotic species, overexploitation and diseases. The axolotl (*A. mexicanum*) was once abundant in the Xochimilco and Chalco lakes despite the man-made landfill mounds created for agriculture that began with the Aztecs. Increased modification of hydrological patterns over the last 50 years further reduced the lake system and exchange among different channels (Mazari-Hiriart *et al.*, 2006). The original lake system occupied an area of 240 km^2 in the Mexico City valley, and this area has been reduced to 2.3 km^2, or 1% of its original size (Fox, 1965). As a consequence, *A. mexicanum* populations have dramatically declined over 5 years from 0.006 individuals/m^2 to 0.0012 individuals/m^2 and have high probabilities of extinction in the wild by 2019 (Zambrano *et al.*, 2007). Combined, habitat changes reduce and isolate populations, and because paedomorphic populations harbor lower genetic diversity, they may be especially vulnerable to the effects of drift and inbreeding, resulting in extinction in these rapidly changing environments.

Two other obligate paedomorphic species, *A. andersoni* (Zacapu Lake, Michoacán) and *A. dumerilii* (Pátzcuaro Lake, Michoacán) suffer from many of the same threats as *A. mexicanum* in Xochimilco. Population growth in the towns around Zacapu and Pátzcuaro has been exponential in the last decades (Fernández & Miranda, 1998), causing influx of sewage and waste from local agriculture into the lakes (Fernández & Miranda, 1998; Huacuz, 2001). As a result, paedomorphic *Ambystoma* are today some of Mexico's most threatened species and are listed as critically endangered species on the IUCN red data list of species, each with a current population area of less than 10 km (Griffiths & Bride, 2005).

Our results have important implications for conservation of Mexican *Ambystoma*. Lower historical population genetic diversity, yet high levels of relatedness among individuals in remaining paedomorphic populations, is a serious concern for populations surviving in highly impacted habitats. Our results confirm that these remaining species are genetically distinguishable, reflecting their independent evolution within this radiation, and that the genetic demes representing those species are not introgressed or admixed with other *Ambystoma* species. Thus, any local population declines or extinctions will not benefit from the influx of new genes via gene flow. Also, although our results show that historical changes in population size have occurred in paedomorphic *Ambystoma*, the historical population sizes of this species must have been exceedingly large, and composed of connected regional populations, when they occupied the entire Valley of Mexico. We hypothesize that the large ancestral populations protected the species from negative effects of drift and inbreeding. The current highly reduced and isolated remaining population no longer has the benefit of a diverse and outbreeding population, and any negative effects of inbreeding will be more extreme.

We have no direct evidence that the high relatedness observed today among individuals in paedomorphic populations confers negative effects in individual fitness. However, high relatedness could also result in low diversity in genes that enhance survival when wild populations are challenged by new infectious diseases (Bernatchez & Landry, 2003). *Ambystoma* are susceptible to chytridiomycosis (Frías-Álvarez *et al.*, 2008) and to a number of parasites (Recuero *et al.*, 2010); thus, lowered population genetic diversity exacerbated by inbreeding could increase susceptibility to outbreaks. Ongoing captive-breeding efforts are a potential avenue for restoring extirpated populations in the future (Valiente *et al.*, 2010). Given the high level of divergence we observed within and among species of *Ambystoma*, these efforts would benefit from assessing the genetic composition of breeding stock, and maximizing genetic diversity of *ex situ* populations. Our data provide the first benchmark for genetic diversity in wild populations of six species in the TVB.

Current protection for widespread metamorphic taxa such as *A. velasci* and *A. altamirani* is not as stringent as for paedomorphic species. However, our data show that many populations of widespread species show limited genetic diversity and low population connectivity. If each population is threatened because of anthropogenic factors, then a species-wide collapse could occur in the absence of any rescue effects (Waite *et al.*, 2005). Our results underscore that we have insufficient knowledge of population genetic patterns in wide-ranging metamorphic species, and those data will be important for their conservation management.

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Supporting information

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

Table S1. Tests of deviation from Hardy–Weinberg equilibrium at each locus in the nine sampled populations of *Ambystoma* from Central Mexico. Observed and expected heterozygosity (Ho/He) and associated *P*-values (in parentheses) are reported for each locus and population. *P*-values in bold exceed the Bonferroni-corrected value ($P < 0.00066$, 75 comparisons) for a table-wide significance level of 0.05.

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