

Urban aquatic habitats and conservation of highly endangered species: the case of *Ambystoma mexicanum* (Caudata, Ambystomatidae)

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Received 27 May 2009, revised version received 7 Jan. 2010, accepted 2 Feb. 2010

Recuero, E., Cruzado-Cortes, J., Parra-Olea, G. & Zamudio, K. R. 2010: Urban aquatic habitats and conservation of highly endangered species: the case of *Ambystoma mexicanum* (Caudata, Ambystomatidae). — *Ann. Zool. Fennici* 47: 223–238.

Species with highly restricted distributions are vulnerable to extinction, and modification of natural habitats within their small ranges is a primary threat to their persistence. Expansion of urban development significantly impacts natural habitats and, therefore, threatens local diversity. The Mexican axolotl, *Ambystoma mexicanum*, is a strictly aquatic species that persists currently in two highly threatened and isolated populations. The current habitat remaining for these species are remnants of a historically extensive lacustrine system that occupied the entire Valley of Mexico, but has been destroyed by the growth of Mexico City. Unexpectedly, a third viable population of axolotls has been found in Chapultepec Park, a public recreational area in the heart of Mexico City. Phylogenetic and haplotype network analyses of mitochondrial DNA sequences confirmed low genetic differentiation and a recurrent lack of monophyly in many of the taxa belonging to the *Ambystoma tigrinum* species group, including *A. mexicanum*, but clustered the Chapultepec samples with other *A. mexicanum* samples. Our data revealed higher haplotypic diversity in *A. mexicanum* populations than previously recorded, due to new haplotypes from Chapultepec Park. We found high incidence of parasites and deformities among individuals in this population, which could negatively impact their viability. Our results emphasize the important role that artificial or semi-natural urban habitats can play in the conservation of highly threatened species.

Introduction

Amphibian populations are declining worldwide (Houlahan *et al.* 2000, Stuart *et al.* 2004, Young

et al. 2004) and various factors such as climate change, emergent diseases, and introduced species have been implicated as threats to remaining species and populations (Alford & Richards

1999, Blaustein & Kiesecker 2002, Lips *et al.* 2006, Pounds & Puschendorf 2004, Pounds *et al.* 2006). Habitat loss and fragmentation are two important factors threatening amphibians (Dodd & Smith 2003, Stuart *et al.* 2004) because they potentially lead to reduced population sizes, isolation and loss of metapopulation dynamics, habitat split, and increased susceptibility to edge effects (Petranka *et al.* 1993, Marsh & Trenham 2001, Andersen *et al.* 2004, Homan *et al.* 2004, Cushman 2006, Gagné & Fahrig 2007, Becker *et al.* 2007). Urban development, especially in cities with high population densities, eliminates habitat required for local species and reduces chances for dispersal and recolonization of habitat patches that remain (Marzluff 2005, Husté & Boulinier 2007, Hahs *et al.* 2009). Aquatic habitats are especially vulnerable, because in addition to becoming isolated, they also often serve as final disposal points for urban pollutants that affect the viability of biological communities (Wolter *et al.* 2000). The threat of extinction due to urban development will be particularly high for locally endemic aquatic species that have small geographic ranges and therefore no assurance of population survival outside of the urbanized region. Management of narrow endemic species is a difficult task, and requires preservation of as much native habitat as possible, restoration of historical habitats, careful planning of new urban development, and attention to potential alternate solution such as the use of non-native habitats and *ex-situ* conservation programs (Gordon *et al.* 2009, Hostetler & Drake 2009).

The Valley of México is an inland hydrographic basin that was partially covered by a widespread complex of several large, interconnected lakes (Armillas 1971, Berres, 2000). These lakes were the original habitat of a rich biological community that has been exploited by humans since prehistoric times (Niederberger 1979). Modification of the lacustrine complex started in pre-Hispanic times with the construction of dykes separating Lago de México from Lagos de Xochimilco, Chalco and the saline Lago Texcoco (Armillas 1971, Alcocer-Durand & Escobar-Briones 1992). Several artificial drainages were constructed from the 17th to the 20th centuries in attempts to dry the lake systems for urban and

agricultural development and to prevent frequent floods in the area (Aréchiga Córdoba 2004). Currently, the lakes are reduced to only a few small and highly perturbed remnants and are isolated by Mexico City, one of the world's largest metropolitan areas. Not surprisingly, the native biota of the valley lakes is now impoverished; habitat loss and alterations have led to the extinction of approximately one third of the aquatic plant species in Xochimilco (Novelo & Gallego 1988) as well as endemic taxa such as four species of the cyprinid fish in the genus *Evarra* (Méndez-Sánchez *et al.* 2002) and Tlaloc's Leopard Frog, *Lithobates tlaloci* (Santos-Barrera & Flores-Villega 2004). Chapultepec Park is the largest green area in Mexico City, occupying approximately 670 hectares. Chapultepec harbors three artificial lakes (Alcocer *et al.* 1988), and one of them, Lago Viejo, still harbors a substantial number of species of the historical aquatic biota of the Valley of México (Alcocer-Durand & Escobar-Briones 1992, Ceballos *et al.* 2005), including several protected species including the atherinid *Chirostoma jordani*, the goodeid *Girardinichthys viviparus*, and the amphibians *Rana montezumae* and *Ambystoma* salamanders (Alcocer & Lugo 1995).

Ambystoma mexicanum is an obligate paedomorphic species, endemic to the Valley of México. It is widely used as a model organism in evolutionary and developmental biology, and is thus commonly maintained in captivity, with several breeding colonies around the world (Malacinski & Able 1989). Currently, only two wild populations persist: one in the channels of Xochimilco and a second in the remnants of Lago de Chalco, both in southern México City (Fig. 1; Zambrano *et al.* 2004). The continued persistence of this species in the wild is uncertain due to population isolation, water pollution and eutrophication, introduction of exotic species and overharvesting. In addition, hormonal disruption caused by chemical contaminants can cause abnormal reproductive development in amphibians that disrupt recruitment in natural populations (Hayes *et al.* 2002, Reeder *et al.* 1998, 2005, Petterson & Berg 2007). Hormonal disruption has been proposed as a cause of sex ratio biases in the Xochimilco population (Griffiths *et al.* 2003). Water pollution can also

negatively affect amphibian immune systems (Gilbertson *et al.* 2003) leading to higher levels of parasitism and susceptibility to infectious diseases. Combined, these factors have raised concerns for the persistence of the Mexican axolotl and led to various initiatives to preserve remaining natural populations (Graue *et al.* 1998, Griffiths *et al.* 2003, 2004, Zambrano 2006, Bride *et al.* 2008). In an effort initiated in 2004, the Mexico City government began a program to eradicate exotic species from Chapultepec Park (Ceballos *et al.* 2005). Lago Viejo was drained and 26 adults and three clutches of *Ambystoma* were found. The adults were identified as *A. mexicanum* based on morphology (Taylor 1939). The presence of *Ambystoma* in the Chapultepec lakes had been previously reported (Alcocer-Durand & Escobar-Briones 1992); however, species identity was uncertain, and the specimens were tentatively assigned either to *A. mexicanum* or to the widespread *A. velasci* (Zambrano *et al.* 2006, Stuart *et al.* 2008).

Here, we characterize the *Ambystoma* population from Chapultepec Park on the basis of mtDNA sequence data, and examine the genetic relationships among individuals in this artificial lake and the two remaining natural populations. We also report on deformities and parasites found in the Chapultepec population. Our study highlights the persistence of a highly threatened species in an artificial and highly urbanized environment. We discuss the possible role of this and other similar populations as reservoirs that guard against species extinction and/or reductions in the genetic diversity of wild populations.

Material and methods

Study site

Chapultepec Park is located in the western section of Mexico City (between 19°24' and 19°26'N, 99°11' and 99°13'W) at an elevation of 2240 m (Fig. 1). Currently, three lakes exist in the park: Lago Viejo, with a surface area of 6 ha and a maximum depth of 1.8 m; Lago Mayor, which is 5.8 ha in area and with a 1.3 m maximum depth; and Lago Menor, which is 2.8 ha in area and with a 1.2 m maximum depth

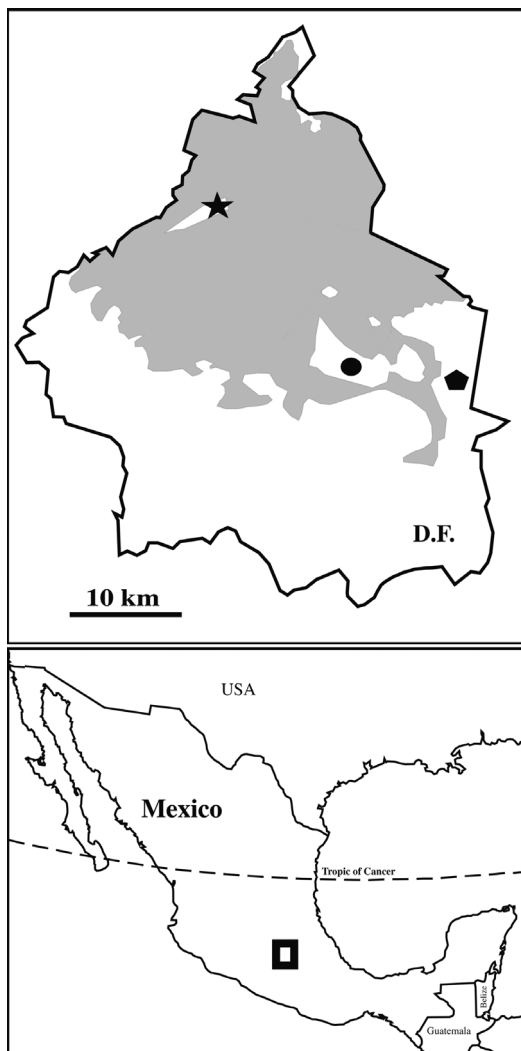


Fig. 1. Location of the three remaining populations of *Ambystoma mexicanum*. Lower map indicates the position of Distrito Federal, México (square). Upper map shows the political boundaries of the Distrito Federal and the location of the three known *Ambystoma mexicanum* populations: Xochimilco (circle), Chalco (pentagon), and Chapultepec (star). The extent of urban development in México City is represented by the gray shaded area.

(Lugo *et al.* 1998). These three lakes are widely used for recreational purposes and are mainly filled with treated wastewater, resulting in high degree of eutrophication (Alcocer-Durand & Escobar-Briones 1992, Lugo *et al.* 1998). The three Chapultepec lakes are approximately 27 and 20 km distant from Lago de Chalco and

Xochimilco, respectively, the two sites that harbor natural populations of *A. mexicanum*. Although the Chapultepec Lakes are artificial, they occur very near the historical range of the lacustrine system that occupied the Valley of México and still harbor a number of aquatic organisms that were present in the ancient lake complex (Alcocer-Durand & Escobar-Briones 1992).

Sampling

All individuals in this study were collected from Lago Viejo in the same series of lake drainages in 2005. The lake was partially drained multiple times for cleaning and removal of non-native fauna. Native vertebrate and macroinvertebrate species were captured with nets and maintained in outdoor tanks. Once the lake was cleaned and refilled, the animals were released at the site of capture. Tissues were collected from adult *Ambystoma* by clipping a small portion of the tip of the tail.

Mitochondrial DNA amplification and sequencing

We extracted total genomic DNA from 11 *A. mexicanum* individuals from Chapultepec and six individuals from Xochimilco. We digested ethanol-preserved tissues in lysis buffer and Proteinase K, followed by standard organic purification with phenol-chloroform (Sambrook & Russell 2001). Polymerase chain reaction (PCR) was used to amplify a fragment of 1029 base pairs, including the tRNA-proline and the mitochondrial control region (D-loop), using the primers THR and 651 (Shaffer & McKnight 1996). PCR reactions were performed in a total volume of 25 μ l, including one unit of *Taq* polymerase (Biotools, 5 U ml⁻¹), 2.5 μ M of each primer, 0.4 mM of dNTPs, 1.5 mM of MgCl₂, and 67 mM of PCR buffer (Tris-HCl, pH = 8.3, Biotools). PCR reactions consisted of 35 cycles with a denaturing temperature of 94 °C (1 min), an annealing temperature of 50 °C (1 min) and an extension temperature of 72 °C (1 min). Double-stranded templates were cleaned using

sodium acetate and ethanol precipitation, and PCR products were then re-suspended in 22 μ l of ddH₂O. Sequencing of the amplified segments followed Martínez-Solano *et al.* (2006). The resulting sequences were deposited in GenBank (accession nos. HM142769–HM142771).

Sequence alignment and phylogenetic reconstruction

Sequences were read and aligned by eye using Sequence Navigator™ ver. 1.0.1 (Applied Biosystems). In our phylogenetic analyses, we also included published sequences from 17 additional *Ambystoma* species from GenBank (number of haplotypes included in the analyses are given in parentheses): *Ambystoma mexicanum* (4), *A. velasci* (14), *A. ordinarium* (9), *A. flavipiperatum* (1), *A. taylori* (1), *A. andersoni* (2), *A. amblycephalum* (1), *A. granulosum* (2), *A. lermaense* (1), *A. altamirani* (2), *A. rivulare* (2), *A. dumerilii* (3), *A. rosaceum* (2), *A. tigrinum* (4), *A. m. mavortium* (4), *A. mavortium melanostictum* (4) and *A. mavortium nebulosum* (6). The California tiger salamander, *A. californiense*, was used as an outgroup (Table 1).

We applied a neighbor-joining (NJ) analysis under the Minimum Evolution objective function with uncorrected (“p”) distances; ties were broken systematically. We used nonparametric bootstrapping (1000 pseudoreplicates) to assess the stability of internal branches in the resulting topologies. Neighbor-joining analyses were performed with PAUP* 4.0b10 (Swofford 2002). We also performed Bayesian phylogenetic analyses using MrBayes ver. 3.1.2 (Ronquist & Huelsenbeck 2003). We initiated the analysis with random starting trees and ran four Metropolis coupled Monte Carlo Markov chains (three heated, one cold) for 10⁷ generations, sampling every 1000 generations. We checked for stationarity and convergence of the chains with Tracer ver. 1.3 (Rambaut & Drummond 2004) and discarded 10⁴ trees as burn-in. Posterior clade probabilities were used to assess nodal support. In addition, we constructed a haplotype network from mtDNA sequences using the software TCS ver. 1.18 (Clement *et al.* 2000), which implements the statistical parsimony algorithm

Table 1. Species, collection localities, and sequences included in this study. Numbered localities, and GenBank accession numbers for each haplotype are from Shaffer and MacKnight (1996), Weisrock *et al.* (2006), Arnason *et al.* (2004), and Samuels *et al.* (2005) indicated in the table's locality and reference columns with A, B, C, and D, respectively. Haplotype numbers are those represented in Figs. 2–4.

| Species | Locality | Haplotype | GenBank accession number | References |
|----------------------------|---|---------------|------------------------------------|------------|
| <i>A. altamirani</i> | Locality 64 (A): México, México | XLIII | DQ241130 | A, B |
| <i>A. altamirani</i> | Locality 65 (A): Morelos, México | XLII | DQ241131 | A, B |
| <i>A. amblycephalum</i> | Locality 58 (A): Michoacán, México | XII | DQ241132 | A, B |
| <i>A. andersoni</i> | Locality 55 (A): Michoacán, México | XXIII, XXIV | DQ241134, DQ241133 | A, B |
| <i>A. californiense</i> | Locality 2 (A): California, USA | LXIV | DQ241127 | A, B |
| <i>A. dumerilii</i> | Lake Patzcuaro, Michoacán, México | XLVIII, L, LI | DQ241203, DQ241202, DQ241137 | A, B |
| <i>A. flavipiperatum</i> | Locality 54 (A): Jalisco, México | XVII | DQ241138 | A, B |
| <i>A. granulorum</i> | Locality 61 (A): México, México | XLV | DQ241139 | A, B |
| <i>A. granulorum</i> | Locality 62 (A): México, México | XL | DQ241140 | A, B |
| <i>A. lermaense</i> | Locality 63 (A): México, México | XLI | DQ241142 | A, B |
| <i>A. m. mavortium</i> | Locality 32 (A): Nevada, USA | XXXVI | DQ241143 | A, B |
| <i>A. m. mavortium</i> | Locality 33 (A): Colorado, USA | XXXV | DQ241144 | A, B |
| <i>A. m. mavortium</i> | Locality 34 (A): Nuevo Mexico, USA | XXXVII | DQ241145 | A, B |
| <i>A. m. mavortium</i> | Locality 35 (A): Texas, USA | XXXVIII | DQ241146 | A, B |
| <i>A. m. melanostictum</i> | Locality 15 (A): California, USA | XXXVI | DQ241147 | A, B |
| <i>A. m. melanostictum</i> | Locality 16 (A): Washington, USA | XXXII | DQ241148 | A, B |
| <i>A. m. melanostictum</i> | Locality 18 (A): Montana, USA | XXXIII | DQ241150 | A, B |
| <i>A. m. melanostictum</i> | Locality 25 (A): Wyoming, USA | XXXIV | DQ241154 | A, B |
| <i>A. m. nebulosum</i> | Locality 11 (A): Colorado, USA | XXX | DQ241163 | A, B |
| <i>A. m. nebulosum</i> | Locality 6 (A): Utah, USA | XXVIII | DQ241158 | A, B |
| <i>A. m. nebulosum</i> | Locality 7 (A): Utah, USA | XXXI | DQ241159 | A, B |
| <i>A. m. nebulosum</i> | Locality 8 (A): Utah, USA | XXVII | DQ241160 | A, B |
| <i>A. m. nebulosum</i> | Locality 9 (A): Arizona, USA | XXIX | DQ241161 | A, B |
| <i>A. mexicanum</i> | captive animals, source population unknown | II, III | AJ584639, AY659991 | C, D |
| <i>A. mexicanum</i> | Locality 68 (A): Chalco, D.F., México | II | DQ241155, DQ241156 | A, B |
| <i>A. mexicanum</i> | Lago Viejo, Parque Chapultepec, D.F., México | I, IV, V | HM142769–HM142771 | this paper |
| <i>A. mexicanum</i> | Lago Xochimilco, D.F., México | I, IV | HM142769–HM142770 | this paper |
| <i>A. nebulosum</i> | Locality 14 (A): Colorado, USA | LIX | DQ241167 | A, B |
| <i>A. ordinarium</i> | Locality 1 (B): Michoacán, México | XLVII | DQ240926 | B |
| <i>A. ordinarium</i> | Locality 15 (B): Michoacán, México | VI | DQ241073 | B |
| <i>A. ordinarium</i> | Locality 16 (B): Michoacán, México | VII | DQ241084 | B |
| <i>A. ordinarium</i> | Locality 17 (B): Michoacán, México | VIII | DQ241096 | B |
| <i>A. ordinarium</i> | Locality 18 (B): Michoacán, México | IX | DQ241106 | B |
| <i>A. ordinarium</i> | Locality 19 (B): Michoacán, México | LIV | DQ241115 | B |
| <i>A. ordinarium</i> | Locality 20 (B): Michoacán, México | XV | DQ241125 | B |
| <i>A. ordinarium</i> | Locality 4 (B): Michoacán, México | XLIX | DQ240967 | B |
| <i>A. ordinarium</i> | Locality 60 (A): Michoacán, México | LIII | DQ241169 | A, B |
| <i>A. rivulare</i> | Res. Mariposa Monarca, Michoacán, México | XLIV, XLVI | DQ241217, DQ241215 | B |
| <i>A. rosaceum</i> | Locality 51 (A): Durango, México | LXII | DQ241170 | A, B |
| <i>A. rosaceum</i> | Locality 52 (A): Chihuahua, México | LXIII | DQ241171 | A, B |
| <i>A. taylori</i> | Locality 76 (A): Puebla, México | XXII | DQ241173 | A, B |

continued

Table 1. Continued.

| Species | Locality | Haplotype | GenBank accession number | References |
|--------------------|--|-----------|--------------------------|------------|
| <i>A. tigrinum</i> | Locality 41 (A): Tennessee, USA | LVI | DQ241179 | A, B |
| <i>A. tigrinum</i> | Locality 42 (A): South Carolina, USA | LVIII | DQ241180 | A, B |
| <i>A. tigrinum</i> | Locality 43 (A): Georgia, USA | LVII | DQ241181 | A, B |
| <i>A. tigrinum</i> | Locality 44 (A): Florida, USA | LV | DQ241182 | A, B |
| <i>A. velasci</i> | Locality 45 (A): Chihuahua, México | XXV | DQ241183 | A, B |
| <i>A. velasci</i> | Locality 46 (A): Chihuahua, México | LX, LXI | DQ241184, DQ241185 | A, B |
| <i>A. velasci</i> | Locality 47 (A): Durango, México | XIV | DQ241186 | A, B |
| <i>A. velasci</i> | Locality 48 (A): Nuevo León, México | XIX | DQ241187 | A, B |
| <i>A. velasci</i> | Locality 49 (A): San Luis Potosí, México | XVI | DQ241188 | A, B |
| <i>A. velasci</i> | Locality 50 (A): Guanajuato, México | XVIII | DQ241189 | A, B |
| <i>A. velasci</i> | Locality 53 (A): Jalisco, México | LII | DQ241190 | A, B |
| <i>A. velasci</i> | Locality 57 (A): Michoacán, México | XIII | DQ241191 | A, B |
| <i>A. velasci</i> | Locality 66 (A): México, México | XXXIX | DQ241192 | A, B |
| <i>A. velasci</i> | Locality 67 (A): México, México | XI | DQ241193 | A, B |
| <i>A. velasci</i> | Locality 69 (A): Hidalgo, México | X | DQ241194 | A, B |
| <i>A. velasci</i> | Locality 70 (A): Puebla, México | XX | DQ241195 | A, B |
| <i>A. velasci</i> | Locality 77 (A): Veracruz, México | XXI | DQ241201 | A, B |

described in Templeton *et al.* (1992) and more accurately represents the genealogical relationships of haplotypes that are recently diverged. We ran the analysis under a 95% probability connection limit considering gaps as a fifth state. Our network included all *A. mexicanum* haplotypes and exemplar haplotypes of an additional seven closely associated species, based on the results of our phylogenetic analyses.

Malformations and parasites

All adult *Ambystoma* captured at Chapultepec were measured and visually inspected for deformities. We recorded the presence of *Lernaea*, an ectoparasitic copepod commonly found on amphibians (Green *et al.* 2002) and *Saprolegnia*, a pathogenic fungus that also infects amphibians (Blaustein *et al.* 1994). Three of the adult salamanders died in captivity, allowing for post mortem examination and detection of internal parasites.

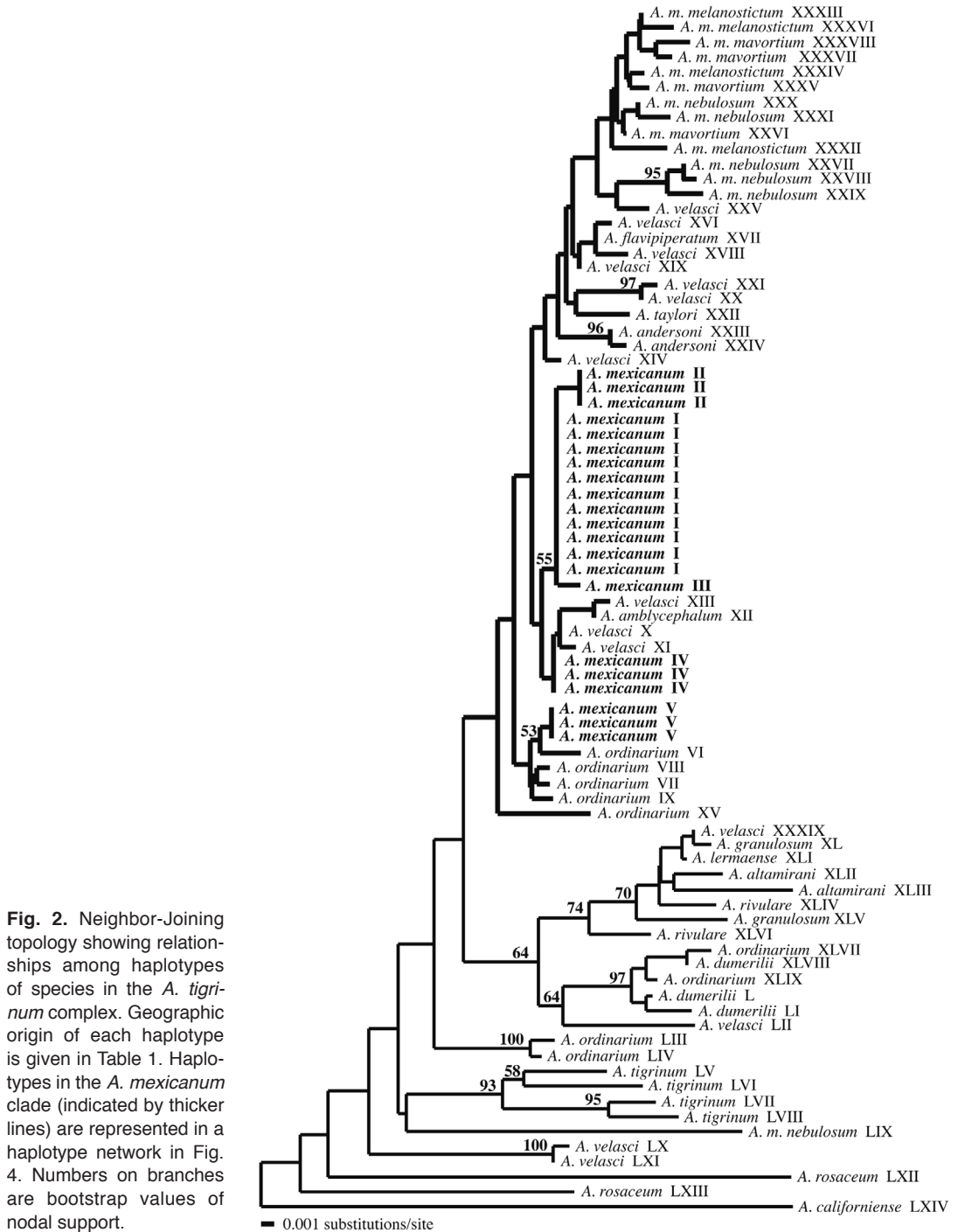
Results

A total of 26 adult *Ambystoma* and three egg masses were collected from the Lago Viejo.

All individuals and egg masses were removed from the lake and maintained in plastic containers at the facilities of the Chapultepec Zoological Garden while the lake was cleaned. Once the process was completed, the animals were released into the lake. One of the egg masses hatched, but two did not develop to hatching.

Genetic variation

Our results indicate low overall genetic variation among the sequences analyzed. Of the total of 1085 characters, only 86 were parsimony-informative (including the out-group). Among sequences of *Ambystoma mexicanum*, we found five haplotypes (Table 1, Figs. 2 and 3). Two of them were found both in Xochimilco and Chapultepec (Haplotypes I and IV); one was unique to Chapultepec (Haplotype V); the other two were found in the Chalco population (Haplotype II) or in the samples of unknown origin (Haplotypes II and III) (Figs. 2 and 3). Three of the GenBank sequences have a deletion at position 13 of the amplified fragment, in the threonine-proline intergenic spacer; all remaining individuals have cytosine at that position. The other variable positions among *A. mexicanum* sequences were located in the control region.



When all 80 sequences were analyzed together, we found low divergence among species. Both NJ (Fig. 2) and Bayesian (Fig. 3) reconstructions resulted in poorly supported

topologies, as expected in view of the low number of variable positions in the sequence data and previously published phylogenies (Shaffer & McKnight 1996). The Bayesian consensus tree

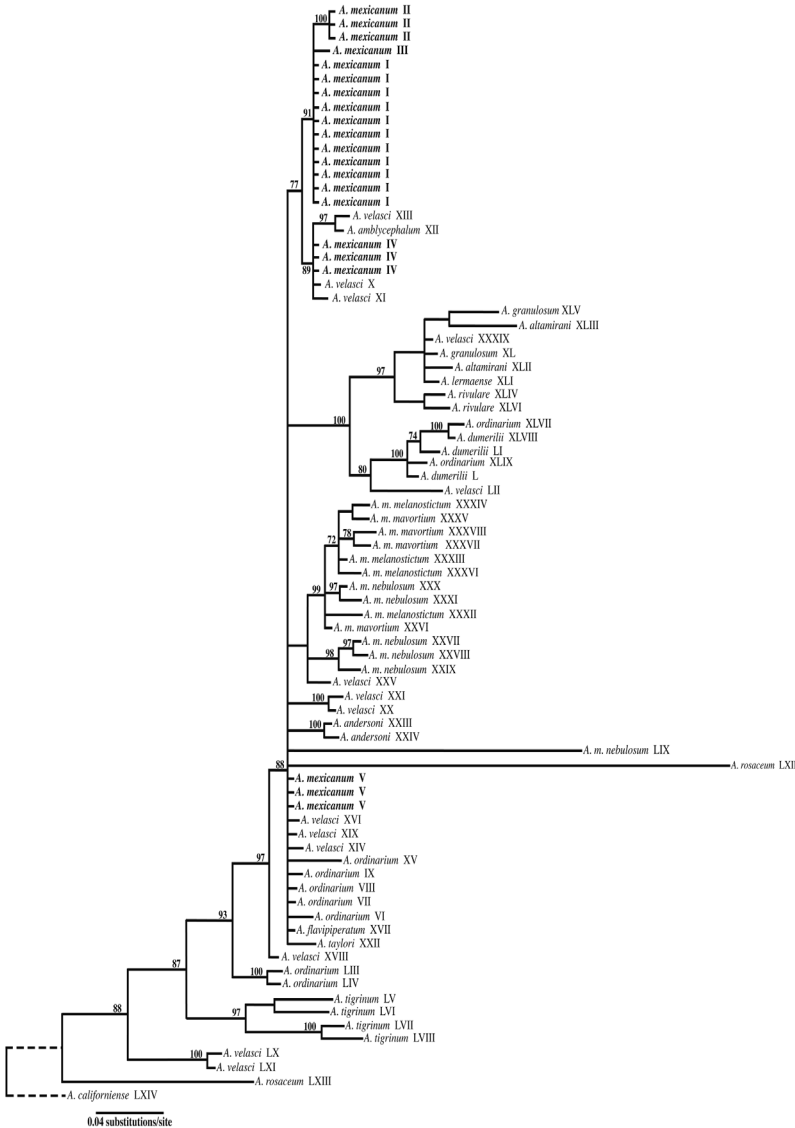


Fig. 3. Bayesian consensus tree showing relationships among haplotypes of species in the *A. tigrinum* complex. Number along branches are posterior probabilities values from the Bayesian analyses.

(Fig. 3) is characterized by polytomy with only a few distinct lineages, including an *A. tigrinum* clade, a second clade composed primarily of *A. mavortium* haplotypes, a third clade containing several Mexican species (*A. dumerilii*, *A. rivulare*, *A. lermaense* or *A. granulosum*), and a fourth clade composed primarily of *A. mexicanum* haplotypes. These groups are also found in the NJ tree (Fig. 2), with some changes. For example, the NJ topology has a clade formed by *A. mexicanum* haplotype V and haplotypes from *A. ordinarius*. The most remarkable result in both analyses is the lack of monophyly for

most taxa represented by more than one haplotype: *A. velasci* and *A. ordinarius* haplotypes are distributed in at least three different clades. *Ambystoma mexicanum* haplotypes I, II, III and IV are grouped in a single clade together with *A. velasci* and *A. amblycephalum* haplotypes. Haplotype V, as mentioned before, is grouped with *A. ordinarius* haplotypes in the NJ tree (Fig. 2), but forms part of a basal polytomy in the Bayesian topology (Fig. 3).

We reconstructed a haplotype network including all five *A. mexicanum* haplotypes plus 33 additional *Ambystoma* haplotypes, for a total

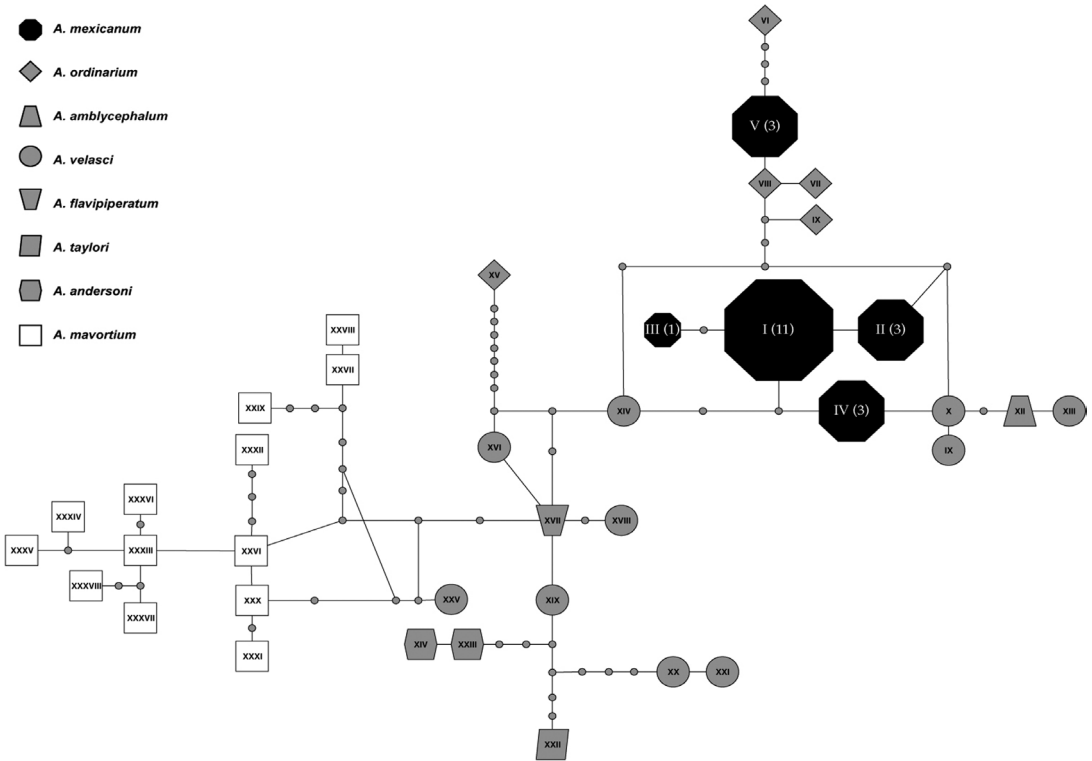


Fig. 4. Haplotype network showing position and distances among haplotypes in the *A. mexicanum* clade inferred in our phylogenetic analysis. Haplotype frequencies for *A. mexicanum* haplotypes are indicated in parentheses. For simplicity we included only unique haplotypes for the remaining species in the clade.

of 8 species (Fig. 4). This network includes representatives of all taxa in the *A. mexicanum* clade found in our NJ topology (Fig. 2). The resulting network reflects the low genetic differentiation among Mexican species of *Ambystoma* and corroborates results from the phylogenetic analyses. Despite the small number of mutations separating named species, our network does reveal some clustering among regional groups. For example, the Mexican species are generally more closely related to each other than to the North American samples (*A. mavortium*). Within the group of haplotypes from Mexican endemics, it is noteworthy that *A. velasci* haplotypes are spread throughout the network; this taxon is a metamorphic species widespread throughout the range of other *Ambystoma* species in southern México. The five haplotypes from *A. mexicanum* differ by only one or two mutational steps and are associated primarily with haplotypes of *A. velasci*, *A. amblycephalum*, and *A. ordinarium*.

Haplotype V, from the Chapultepec samples of *A. mexicanum*, is an exception. This haplotype differs by five mutational steps from the remaining *A. mexicanum* haplotypes, and may reflect the isolation of that population; however, this level of intraspecific differentiation is not unusual among other taxa in our sample.

Malformations and parasites

Twenty adult specimens were visually inspected for external deformities and parasites (Table 2). All observed deformities were present in the limbs. Eight specimens were polydactylous, four specimens had missing toes, two had fused toes and one had toes reduced in size. Three adults were dissected at the time of death and all hosted larval forms of the nematode *Eustrongylides* sp. The nematodes were found tightly coiled, encapsulated, and attached to the mesentery in the

coelomic cavity, liver, and subepidermal tissue; the latter formed bumps that were visible externally so the presence of this parasite was also detected in six additional specimens. The pathogenic fungus *Saprolegnia* spp. was found on two adult specimens. Approximately 60% of the individuals had the parasitic copepod *Lernaea*, which was found mostly in the cephalic area, but also on other parts of the body.

Discussion

Systematics of *Ambystoma* and species identification.

All Mexican species of *Ambystoma*, including *A. mexicanum*, belong to a taxonomic group defined as the *Ambystoma tigrinum* complex (Shaffer & McKnight 1996). Systematics of this species complex has been problematic due to the scarcity of reliable morphological characters (Shaffer 1984a, 1984b, Irschick & Shaffer 1997). Allozyme and mitochondrial DNA studies (Shaffer 1984a, Shaffer & McKnight 1996) revealed low genetic differentiation among species, and

poorly resolved phylogenetic trees with several apparently non-monophyletic taxa (Shaffer 1984a, Shaffer & McKnight 1996, Weisrock *et al.* 2006). This lack of monophyly could be the result of incomplete lineage sorting, which precludes the recovery of a resolved phylogenetic tree (Avice 2000). This is a common difficulty in studies of recently radiated species groups (Takahashi *et al.* 2001) and occurs due to large effective population sizes and short periods of time between speciation events that favor the retention of ancestral polymorphisms (Pamilo & Nei 1988). An alternate explanation for our results is that gene flow occurs among morphologically and ecologically differentiated *Ambystoma* species. Interbreeding among paedomorphic and metamorphic species produces fertile offspring under captive conditions (Brandon 1972, 1977, Voss 1995), thus hybridization in the wild among some species may be feasible.

Previous phylogenetic studies of this group included a maximum of two samples of *A. mexicanum* that showed no genetic differentiation (Shaffer 1984a, Shaffer & McKnight 1996, Weisrock *et al.* 2006). Our dataset included 19 individuals from three different populations

Table 2. Data on sex, snout-vent length (SVL), tail length (TL), toe abnormalities, and presence of *Eustrongylides* cysts for 20 specimens of *Ambystoma mexicanum* collected in Chapultepec Park.

| ID | Sex | SVL (mm) | TL (mm) | Polydactyly | Toe deformities | Nematode cysts |
|----|--------|----------|---------|-------------|-----------------|----------------|
| 1 | Female | 128.0 | 92.0 | Present | None | Present |
| 2 | Female | 130.9 | 86.7 | Absent | Missing | Present |
| 3 | Female | 154.9 | 104.0 | Absent | None | Present |
| 4 | Male | 139.4 | 120.0 | Absent | None | no data |
| 5 | Male | 131.1 | 88.3 | Absent | Missing | no data |
| 6 | Female | 127.1 | 94.6 | Absent | Reduced | no data |
| 7 | Male | 105.4 | 109.0 | Absent | None | no data |
| 8 | Female | 138.2 | 101.0 | Present | None | no data |
| 9 | Female | 127.2 | 78.1 | Absent | Fused | no data |
| 10 | Male | 131.3 | 111.0 | Absent | Missing | Present |
| 11 | Female | 130.0 | 94.0 | Present | None | no data |
| 12 | Male | 91.6 | 126.0 | Absent | None | no data |
| 13 | Male | 111.7 | 76.9 | Absent | None | no data |
| 14 | Female | 120.0 | 87.4 | Present | None | Present |
| 15 | Male | 105.9 | 146.0 | Present | None | Present |
| 16 | Female | 119.2 | 78.8 | Absent | None | no data |
| 17 | Female | 127.7 | 83.8 | Present | None | Present |
| 18 | Female | 132.6 | 83.4 | Absent | Fused | Present |
| 19 | Female | 119.0 | 79.0 | Present | None | no data |
| 20 | Female | 102.7 | 64.1 | Present | Missing | Present |

(plus two captive specimens of unknown origin) and we found five haplotypes with extremely low genetic divergences. However, the haplotypes did not form a well-supported clade. *Ambystoma mexicanum* haplotypes cluster with samples of *A. velasci*, *A. amblycephalum*, and *A. ordinarium*. Of these species, *A. amblycephalum* and *A. ordinarium* occur in regions that are relatively far from the current distribution of *A. mexicanum*, thus, incomplete lineage sorting might explain their association with *A. mexicanum* (Weisrock *et al.* 2006). In contrast, *A. velasci* is a widely distributed species, occurring across most of the Trans-Mexican Volcanic Belt of Central México (Frost 2009) and although this species has not been collected near Xochimilco, we cannot exclude the possibility that its presence may have gone unnoticed, and the possibility of hybridization with paedomorphic forms.

The most divergent haplotype of *A. mexicanum* collected at Chapultepec population clusters with *A. ordinarium*. This might lead us to infer the introduction of *A. ordinarium* in Chapultepec, however, that species is only narrowly distributed in the state of Michoacán, rarely metamorphoses, and is usually found in clean mountain streams (Weisrock *et al.* 2006, Stuart *et al.* 2008). It is unlikely that a species with such narrow ecological requirements could survive and reproduce in a highly eutrophic lake such as Chapultepec.

Clearly, the taxonomic issues in this clade will require additional studies, especially those focusing on species limits in the recently diverged Mexican *Ambystoma*. These studies should include measures of inter- and intra-population morphological diversity, autoecological and distributional data and in particular, an emphasis on population genetic approaches to better understand current and historical introgression among populations that might be species or incipient species.

Parasites and disease

We recorded the presence of different parasites affecting adult *Ambystoma* in Chapultepec. *Eustrongylides* is a nematode parasite with a complex life cycle that usually infects fish as

second intermediate hosts (Pérez-Ponce de León *et al.* 1996, Coyner *et al.* 2002), but it has also been reported in the amphibians *Ambystoma dumerilii* and *Rana dunni* (García-Altamirano *et al.* 1993). Outbreaks of this parasite often occur with anthropogenic alterations in aquatic systems that lead to high densities of first intermediate hosts. Thus, this parasite is considered an indicator of polluted aquatic environments (Measures 1988, Spalding *et al.* 1993, Franson & Custer 1994, Frederick *et al.* 1996). This nematode has been associated with high mortality and serious population declines in its final host, fish-eating birds (Spalding *et al.* 1993, 1994), but to date it has not been reported as a factor in amphibian declines.

We detected only two cases of *Saprolegnia* infection from the *Ambystoma* collected at Chapultepec. This fungus is a pathogen of fish and attacks eggs, larvae and adults. It has also an important pathogen of amphibian eggs in species such as *Ambystoma maculatum* (Bragg 1962), *Rana montezumae* (Frías 2005), *Bufo boreas*, and *Rana cascadae*, among others (Kiesecker & Blaustein 1995). Several studies have shown that a synergistic effect between *Saprolegnia* and other stressors, such as UV-B radiation and pollutants, can cause massive mortalities in wild amphibian populations (Kiesecker & Blaustein 1995, Blaustein *et al.* 1994, Lizana & Pedraza 1998). In our study, however, the fungus infected adult specimens that were probably already weakened by other diseases or by the polluted environment. The incidence of *Saprolegnia* in the lake must be monitored, as it can ultimately affect the viability of the population.

A very common parasite found in Chapultepec is the copepod *Lernaea*. Although it usually infects fish, it has been reported in aquatic amphibians, including several species of *Ambystoma* (Huacuz 2002) and anuran larvae (Martins & Souza 1996, Leong 2001). In a few cases, the presence of the copepod has been associated with amphibian limb abnormalities (Leong 2001), but this does not seem to be the case in Chapultepec axolotls. The high frequency of malformations axolotls from Chapultepec could again be a side effect result of water pollution. An abnormally high prevalence of malformed amphibians has been associated with outbreaks

of *Ribeiroia ondatrae*, a trematode parasite, due to the increased density of intermediate hosts in highly eutrophic lakes and ponds (Johnson & Chase 2004). This parasite was not found in the Chapultepec population, but Lago Viejo receives a large amount of nutrients from its water source (Alcocer *et al.* 1988, Alcocer-Durand & Escobar-Briones 1992, Lugo *et al.* 1998) and thus is at potential risk for parasites outbreaks.

Conservation implications for *A. mexicanum*

Ambystoma mexicanum is listed as Critically Endangered by the IUCN because its total area of occupancy is estimated to be less than 10 km². The aquatic habitat in which it evolved is highly reduced; of the original 180 km of channels in Xochimilco, less than 10 km still retain conditions required for survival of axolotls (e.g., high transparency, appropriate salinity and a lower density of exotic species) and within that area, the habitat is severely fragmented (Zambrano 2006).

Pollution, and introduced species are two of the main factors that have reduced habitat suitability in Xochimilco for *A. mexicanum*. The current and projected declines in the extent and quality of the habitat (Zambrano *et al.* 2006) paint a dire scenario for the two remaining wild populations, and in fact, rapid population reductions in axolotl densities have been observed in the last few years (Zambrano *et al.* 2004, Zambrano 2006). The major threats to remaining axolotl populations are clear (Sciences-Hernández *et al.* 2006, Zambrano *et al.* 2004, 2007, Zambrano 2006), but difficult to mitigate. In Chalco, a restoration program has increased the flooded surface in recent years (Zambrano 2006). In Xochimilco, natural water income has been adapted for human use, and the system is therefore now fed mainly with treated water and precipitation during the rainy season (Solís *et al.* 2006). Eutrophication levels are extremely elevated, chemical pollutants are present in excess, and infectious bacteria including *Pseudomonas* and *Aeromonas* are present in much higher concentrations than are recommended (Zambrano *et al.* 2004). In addition to habitat deterioration, illegal harvest of *A. mexicanum* for food and medicinal purposes has

also been a threat to the continued persistence of the species. Captive-breeding efforts and the inclusion of this species in appendix II of CITES have reduced the number of captures of wild specimens, but adult axolotls are still illegally captured today, both for international and local trade (Zambrano *et al.* 2004).

Given the threats to wild *A. mexicanum* populations, its confirmed presence in Chapultepec Park should be used as an opportunity to improve conservation of the species in its native range. The long-term persistence of this population is threatened by several factors similar to those in Xochimilco, but which may be easier to overcome because of the smaller size of the lake. If the lakes of Chapultepec are to serve a role in conservation of this species, it will be important to estimate the population size in Lago Viejo, explore other lakes in the park to detect the presence of axolotls, perform surveys to quantify population sizes and life stage distributions, and manage the lakes to promote restoration and survival of the native species living there.

In México, the coordination of researchers and institutions currently working on the conservation biology of *A. mexicanum* has yielded encouraging results in recent years, and culminated in the creation of several captive colonies in México and abroad, and the organization of a network of researchers (GIA-X, <http://ajolote.ibiologia.unam.mx/>) that promote multidisciplinary investigation of wild and captive populations, and conservation measures to preserve all paedomorphic *Ambystoma* species and their habitats (Graue *et al.* 1998, Griffiths *et al.* 2003, 2004, 2008, Griffiths & Bride 2005, Zambrano 2006, Zambrano *et al.* 2006, Bride *et al.* 2008). However, although the preservation of axolotl species may be guaranteed due to captive breeding colonies (Malacinski & Able 1989, Zambrano *et al.* 2006), wild populations and their habitats require urgent conservation measures to save them from extinction in their natural habitats (Sciences-Hernández *et al.* 2006, Zambrano *et al.* 2007). Aside from the challenges associated with restoration of habitat, other factors need to be considered before re-introduction of captive-bred animals into wild populations. The propagation of diseases in captivity has played a key role in spreading epidemics such as amphibian chytridiomycosis (Daszak

et al. 2003, Weldon *et al.* 2004). Most individuals in the captive colony of *Ambystoma mexicanum* at the Instituto de Biología (UNAM) were infected by *Batrachochytrium dendrobatidis*, the parasitic chytrid fungus that causes chytridiomycosis. However, none of the captive individuals show signs of disease (Frías-Álvarez *et al.* 2008), thus it is possible that this fungus is prevalent in other colonies without being detected. Researchers must ensure that all colonies are disease-free before reintroducing individuals to natural or restored populations. Our study also revealed population genetic diversity among local natural populations of *A. mexicanum* that was previously unknown. We identified five haplotypes among the 19 samples sequenced thus far. The spatial distribution of those haplotypes among localities is not yet clear, therefore, we recommend genetically characterizing all colonies, with emphasis on wild caught breeding adults, to maximize our potential of maintaining local variants and maximizing any potential natural genetic variability of the species.

Maintenance of the Chapultepec population is an alternative to *ex-situ* conservation in aquaria and terrariums, and may offer benefits because individuals experience natural seasonal dynamics and breed naturally in the lakes. This semi-natural setting might prevent some of the negative effects of captivity, such as loss of fitness due to small breeding groups and artificial selection for traits favored in captivity. A healthy breeding population in Chapultepec would also facilitate future reintroduction programs and serve as a source of acclimatized animals. Finally, the location of the population and the number of visitors to Chapultepec provide a suitable environment for the development of conservation and educational programs. Green areas such as large urban parks can be of great conservation value as reserves for local biodiversity and for education of the public about environmental challenges facing local flora and fauna.

Acknowledgments

We thank Claudia Colón, Arnaldo Cruzado, Martín Aguilar Montiel, Leonel Cruzado, Fernando Cea, Ing. Rosa María Gómez Sosa, Martín Aguilar Cervantes, Fideicomiso Pro-

Bosque de Chapultepec, and Grupo de Diseño Urbano, for their help in the capture and maintenance of the Chapultepec salamanders. Laura Marquez-Valdelamar for assistance in the laboratory. This study was funded by grants to GP-O from SEP-CONACyT (50563) and PAPIIT-UNAM (No. IN211808).

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