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RESEARCH ARTICLE

Considering alternative life history modes and genetic divergence in conservation: a case study of the Oklahoma salamander

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Abstract Alternative life history strategies can provide important variation for the long-term persistence of a lineage. However, conservation of such lineages can be complicated because each life history mode may have different habitat requirements and may be vulnerable to different environmental perturbations. The Oklahoma salamander (*Eurycea tynerensis*) is endemic to the Ozark Plateau of North America, and has two discrete life history modes, biphasic (metamorphic) and aquatic (paedomorphic). Until recently, these modes were considered separate species and conservation attention focused only on paedomorphic populations. We perform phylogenetic analyses of the mitochondrial gene *cytochrome b* (*Cytb*) and nuclear gene *proopiomelanocortin* (*POMC*) to assess patterns of historical isolation in *E. tynerensis*, and test whether life history mode is randomly distributed with respect to the phylogeny and geography. We find three divergent *Cytb* lineages and significant shifts in *POMC* allele frequencies between the eastern, western, and southwestern portions of the distribution. Life history mode varies extensively, but paedomorphosis is largely restricted to the widespread western clade. Therefore, the two most divergent and narrowly distributed clades (southwestern and eastern) were previously overlooked due to their metamorphic life history. Paedomorphosis has allowed *E. tynerensis* to drastically increase its niche breadth and distribution size. Nevertheless, metamorphosis is also an important attribute, and metamorphic populations are the ultimate source for paedomorphic evolution. Preservation of divergent genetic lineages, and regions that include adjacent habitat for both life history modes, may be the most effective way to maintain historical and adaptive variation and provide gateways for ongoing life history evolution.

Keywords Complex life cycles · Plethodontidae · Paedomorphosis · Metamorphosis · *Eurycea*

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Introduction

In species that exhibit complex life cycles, stage transitions typically coincide with distinct niche shifts that can alter an individual's habitat use and placement in the community (Hjelm et al. 2000; Olson 1996; Werner and Gilliam 1984; Wilbur 1980). Within some species, individuals exhibit alternative modes of development (life history) resulting in intraspecific niche variation at a particular life stage (e.g. adulthood; Moran 1992; Smith and Skúlason 1996; West-Eberhard 1986). Alternative life history modes can provide important evolutionary variation for the long-term persistence of a lineage, especially for organisms that inhabit dynamic environments (Beechie et al. 2006; Watters et al. 2003; see also "Discussion"). However, individuals of each life history mode may have different habitat requirements and may be vulnerable to different environmental perturbations, complicating the conservation management of such species.

The practice of defining Evolutionarily Significant Units (ESUs) was originally suggested as a method to conserve potentially important intraspecific genetic attributes that could aid in the long-term preservation of species (Ryder 1986). Subsequent authors suggested defining ESUs based primarily on the estimation of neutral markers to serve as a proxy for overall genomic diversity and history of isolated populations (Avise 1992; Dizon et al. 1995; Moritz 1994; Waples 1991). However, this method alone may not encompass and adequately protect important adaptive phenotypic variation, especially if phenotypic diversity does not correspond with the deepest genetic divergence within a species (Crandall et al. 2000). Consequently, integrative conservation approaches and diverse data types that consider both genetic divergence and phenotypic characteristics have been suggested (de Guia and Saitoh 2007; Fraser and Bernatchez 2001; Moritz 2002; Tymchuk et al. 2010). An integral part of these approaches is to understand the distribution of phenotypic traits on the phylogeny and/or the distribution of genetic divergence and phenotypic traits on the landscape. Conservation strategies for managing phenotypic diversity (e.g. life history variation) may need to be uniquely developed for each species (Moritz 2002; Watters et al. 2003).

Amphibians globally are in decline and multiple causes have been implicated in their disappearance (Collins and Storfer 2003; Stuart et al. 2004). The need to identify and conserve divergent intraspecific genetic lineages, as well as higher-level diversity, is well recognized (Bickford et al. 2007; Hanken 1999; Köhler et al. 2005; Rovito et al. 2009), but conservation of alternative life history modes has been underemphasized (Denoël et al. 2009; Whiteman and Howard 1998). Most amphibians exhibit a biphasic life history, with an aquatic larval stage followed by metamorphosis into a more terrestrial adult, and therefore require suitable aquatic and terrestrial habitats to complete their life cycle (Semlitsch 1998, 2000, 2003; Semlitsch and Bodie 2003). The developmental shift to paedomorphosis, where individuals retain the aquatic morphology and ecology throughout life (Duellman and Trueb 1986), has independently occurred in nine of the 10 families of salamanders, and is maintained as a variable trait (alternative life history modes) within and among species in five families (AmphibiaWeb 2011; Petranka 1998; Wiens et al. 2005).

The Oklahoma salamander, *Eurycea tynerensis*, is a small plethodontid species endemic to the Ozark Plateau of east-central North America. All individuals of this species have stream-dwelling larvae, while adults exhibit alternative life histories (metamorphic and paedomorphic), and most populations are exclusively metamorphic or paedomorphic (Bonett and Chippindale 2004, 2006). Until recently, the alternative life history modes of *E. tynerensis* were considered separate species (Moore and Hughes 1939, 1941; Petranka 1998; Tumlison et al. 1990a). Only paedomorphic individuals in the western Ozark Plateau were considered to be *E. tynerensis*, while metamorphic individuals were considered to be part of a more widespread species, *E. multiplicata* (specifically the Ozark subspecies *E. m. griseogaster*). Consequently, conservation attention focused only on the paedomorphic populations (Cline and Tumlison 1997, 2001; Tumlison et al. 1990b; Tumlison and Cline 2003), which were given special conservation status (Near Vulnerable on the IUCN Red List and a Species of Special Concern by the states of Arkansas and Oklahoma; Bonett 2005; IUCN 2008; ODWC 2009). However, recent phylogenetic analyses showed a well supported clade which includes *E. tynerensis* (paedomorphic individuals) and *E. m. griseogaster* (metamorphic individuals); yet neither taxon is itself monophyletic, and many adjacent populations of metamorphic and paedomorphic individuals are genetically identical based on mitochondrial DNA (Bonett and Chippindale 2004). Paedomorphosis occurs in multiple populations within this species and each life history mode is associated with distinctly different types of habitat (Tumlison and Cline 2003; Bonett and Chippindale 2004, 2006). The low dispersal abilities (resulting in regional genetic divergence), combined with evidence of repeated, adapted trait evolution, make this an ideal system for examining the conservation of genetic diversity and alternative life history modes.

In this study, we use both the mitochondrial gene *cytochrome b* (*Cytb*) and the nuclear gene *proopiomelanocortin* (*POMC*) to assess genetic divergence and diversity among metamorphic and paedomorphic populations of *E. tynerensis* at a fine scale. We then test whether life history variation is randomly distributed on the phylogeny and landscape, and determine the frequency of each life history mode with respect to the size of the distribution of each of the geographic clades of *E. tynerensis*. If life history mode is randomly distributed on the phylogeny, and genetic lineages and life history mode are randomly distributed on the landscape, then each life history mode or genetic lineage (i.e. population/region) could be granted equal priority. However, if traits and genetic lineages are non-randomly distributed, then the priority may need to be given based on uniqueness or potential long-term importance. Our study shows that previous conservation attention to only paedomorphic populations was misleading for several reasons, and highlights the importance of analyzing the evolution of life history and genetic divergence in a geographic context when developing conservation strategies for polymorphic species.

Materials and methods

Specimens

We collected tissue samples from 174 *E. tynerensis* from throughout their distribution across the Ozark Plateau (Fig. 1; Appendix Table 5; Table S1, Supplementary material). Tail tips were collected in the field or salamanders were returned alive to the University of Tulsa. These specimens were euthanized in a 10% solution of MS-222 and liver tissue was harvested for genetic analysis and vouchers were used for other morphological studies. All salamanders were handled according to IACUC procedures (TU-0029). Life history status was determined in the laboratory or the field by the presence of aquatic features such as external gills and caudal fin on salamanders that exhibited well-developed testicular lobes in males or mature oviductal eggs in females (paedomorphic); or salamanders that had entirely reabsorbed external gills and caudal fin (metamorphic).

DNA extraction, amplification, and sequencing

DNA was isolated from tissues using Qiagen DNeasy® Blood and Tissue extraction kits. 830 to 1118 base pairs (bp) of the mitochondrial gene *Cytb* were amplified from 174 *E. tynerensis*. As an independent test of genetic substructure, we amplified 433 bp of a more evolutionarily conserved nuclear gene, *POMC*, in 73 *E. tynerensis*. *Cytb* exhibits a high degree of variation in salamanders and is commonly used for studies of phylogeography and species diversity (e.g. Chippindale et al. 2000; Martínez-Solano et al. 2007; Moritz et al. 1992), including phylogeographic studies of *E. tynerensis* (Bonett and Chippindale 2004). *POMC* was selected because it showed one to two percent sequence divergence among populations of *E. tynerensis* and some phylogenetic structure in preliminary analyses. Amplification of both genes was performed by polymerase chain reaction (PCR) using a variety of primers and standard methods (Table S2, Supplementary material). Products were checked on 1% agarose gels, and unincorporated

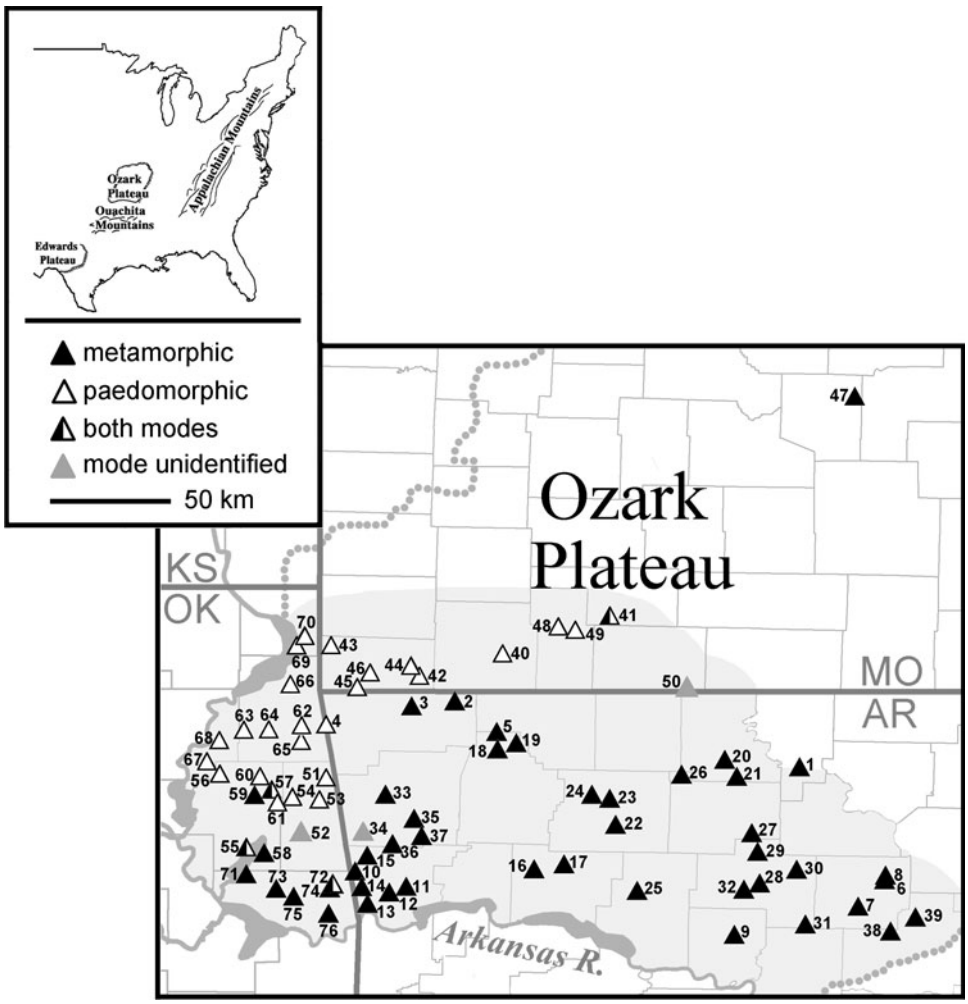

Fig. 1 Distribution of *E. tynerensis* and sampling localities on the Ozark Plateau. *Inset* map shows the location of the Ozark Plateau in eastern North America. *Grey dotted line* on the main map outlines the distribution of the Ozark Plateau, with the Arkansas River serving as the southern border of the Plateau. *Shaded grey area* indicates the known distribution of *E. tynerensis*, and triangles are sampling localities with adjacent locality numbers (Appendix Table 5; Table S1, Supplementary material). *Black triangles* are metamorphic locations, *white triangles* are paedomorphic locations, *black/white triangles* are locations where both life history modes occur, and *dark grey triangles* are locations where life history mode of the sample is unknown. The *black triangle* in the northeast corner of the map is a disjunct population of *E. tynerensis*. AR, KS, MO, and OK are the state abbreviations for Arkansas, Kansas, Missouri, and Oklahoma, respectively

dNTPs and primers were removed from PCR products using ExoSAP-IT (USB Corp). Sequencing reactions using Big Dye Version 3.1 (Applied Biosystems Inc.) were performed with either PCR primers or internal sequencing primers designed for *Eurycea*. Sequencing reactions were purified by centrifugation through columns of Sephadex G-50 (Invitrogen Corp.) in 96-well plates to remove unincorporated dye terminators, and sequenced on an ABI 3130xl capillary sequencer at The University of Tulsa. Direct sequencing of *POMC* (described above) revealed seven individuals that were heterozygous for divergent alleles. To determine the sequences of the distinct alleles, PCR products were ligated to a TA-cloning vector and transformed into *Escherichia coli* cells using an Invitrogen TA-Cloning® Kit. Transformed cells were plated on Ampicillin/X-gal plates for blue/white selection, and stored at 37°C overnight. White colonies were picked from each plate, amplified and sequenced with the original PCR primers, using the direct sequencing methods described above.

Genetic analyses

All sequences were edited and aligned using Sequencher™ v 4.8 (Gene Codes Corp). For *Cytb*, 68 additional sequences were collected from GenBank (41 sequences, Bonett and Chippindale 2004; 6 sequences, Bonett and Chippindale 2006; 21 sequences, McKnight and Nelson 2007). All of our *POMC* sequences were original. The alignments were trimmed to 678 bp (*Cytb*) and 416 bp (*POMC*) in order for all sequences in the alignment to be of the same length. *Cytb* and *POMC* were translated in Sequencher™ to verify open reading frames. The alignments were unambiguous and had no gaps/missing data. For both genes, *E. spelaea*, a metamorphic species closely related to *E. tynerensis*, was used as the outgroup (Bonett and Chippindale 2004).

Collapse v. 1.2 (Posada 2006) was used to consolidate each *Cytb* haplotype with four or fewer mutations of difference into a single operational taxonomic unit (OTU). Identical *POMC* alleles were collapsed manually into a single OTU. The haplotypes were collapsed for presentation purposes and also because we were primarily interested in the geographic distribution of the deeper tree structure. Analyses of the collapsed datasets did not differ in overall structure from analyses including the sequences of all individuals.

Since recombination can affect phylogenetic analyses (Schierup and Hein 2000; Hare 2001), we examined the relationship between linkage disequilibrium and the physical distance between sites in the program RecombiTEST (Piganeau et al. 2004) to test for evidence of recombination within our *Cytb* and *POMC* fragments (Lewontin 1964; Piganeau and Eyre-Walker 2004). We found no evidence of recombination in the *Cytb* (correlation coefficient 0.023; $P > 0.882$) or the *POMC* (correlation coefficient −0.053; $P > 0.210$) fragments. We also tested if our two markers were neutrally evolving using Tajima's D (Tajima, 1989) using the "Intrapop neutrality tests" calculator (Achaz, 2011). We found no evidence of significant deviations from neutrality in the *Cytb* ($D = -0.732$; $P > 0.261$) or the *POMC* ($D = -1.090$; $P > 0.131$) fragments.

MrModeltest v. 2.3 (Nylander 2004) was implemented to determine the appropriate model of sequence evolution for both genes (Table 1). Bayesian analyses were performed with MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), using four Markov chains (the default setting of three hot and one cold) and five million generations, sampling every 1000th generation. We conservatively discarded the first 1000 trees (one million generations) as burn-in, which was well beyond stationarity (reached in <100,000 generations in all analyses). The "sumt" command in MrBayes was used to calculate average branch lengths, and a 50% majority-rule consensus was used to calculate the posterior probability of each node in PAUP* v 4.0 (Swofford 2001), based on 4001 post-burnin trees. For both genes, PAUP* (Swofford 2001) was also used for maximum parsimony analysis and bootstrapping based on 1000 pseudoreplicates, with 10 random taxon replicates per pseudoreplicate.

Genetic divergence among populations was measured by calculating uncorrected pairwise sequence divergence (uncorrected *P*) in PAUP* (Swofford 2001). Measures of genetic diversity within the major lineages for both *Cytb* and *POMC* were estimated in Arlequin v 3.1 (Excoffier et al. 2005, 2006), including mean number of pairwise differences (π), nucleotide diversity or average genetic diversity over L loci (π_n), and θ_s, which is an estimate of the population parameter θ based on the number of segregating sites and the sample size. Arlequin v 3.1 (Excoffier et al. 2005, 2006) was also used to perform an analysis of molecular variance (AMOVA, Excoffier et al. 1992;

Table 1 Models of sequence evolution implemented based on MrModeltest and parameters used for Bayesian analysis in MrBayes

Lset	Model	Nst	Rates	Prset
Cytb				
Codon position 1	GTR	6	Invgamma	Fixed (equal)
Codon position 2	HKY	2	Equal	Dirichlet (1,1,1,1)
Codon position 3	GTR	6	Gamma	Dirichlet (1,1,1,1)
POMC				
Codon position 1	GTR	6	Equal	Dirichlet (1,1,1,1)
Codon position 2	HKY	2	Gamma	Dirichlet (1,1,1,1)
Codon position 3	GTR	6	Equal	Dirichlet (1,1,1,1)

Weir and Cockerham 1984; Weir 1996) to assess hierarchical genetic structure within and among populations and groups. For both *Cytb* and *POMC*, populations (sampling locations) were grouped by their placement in the three most divergent mitochondrial geographic groups (east, west, and southwest, see "Results"). Among group F_{ST} based on pairwise sequence divergence were also calculated in Arlequin. Exact tests were used to determine the level of population differentiation between our geographic groups compared to 10,100 permutations of randomly assigned *Cytb* haplotypes (or *POMC* alleles) to the groups. We used the *Cytb* groupings to test the significance of the phylogenetic structure at a population level, and also to test if the distribution of *POMC* alleles was significantly associated with the mtDNA. We also used Arlequin to calculate Harpending's Raggedness Index (HRI; Harpending 1994) for the mismatch distributions of *Cytb* haplotypes and *POMC* alleles for all populations and for each of the geographic clades. The mismatch distribution of a population that has undergone continuous expansion should be smooth and unimodal and have a low raggedness index. For each mismatch distribution we tested for significant deviations from a population expansion model by comparing the probability that simulated raggedness is greater than equal to the observed raggedness (Rogers and Harpending 1992).

Life history distribution analyses

To determine if life history mode is non-randomly distributed on the phylogeny of *E. tynerensis* we tested whether this characteristic shows significant phylogenetic signal on the Bayesian phylogeny of all unique *Cytb* haplotypes, which is largely congruent to the parsimony phylogeny (see "Results"). Of the 154 unique *E. tynerensis* *Cytb* haplotypes, four are found in both paedomorphs and metamorphs, so these four OTUs were duplicated in the Bayesian phylogenetic analysis so they could be coded for both life histories. The *Cytb* phylogeny was used for these analyses because this gene shows the most variation and yields the most structured tree. To test for phylogenetic signal of life history mode in a likelihood framework we used GEIGER (Harmon et al. 2008) in R to determine if the observed phylogeny explains the distribution of life history mode significantly better than when phylogenetic structure is removed by Pagel's Lambda (equaling zero; Pagel 1999). A likelihood ratio test was used to compare the difference in negative log likelihood ($-\ln L$) between these two scenarios (Freckleton et al. 2002). We note that this test assumes little gene flow among terminal nodes and is primarily designed for inter-species comparisons. Given that most plethodontid salamanders have very limited dispersal, and many *E. tynerensis* populations are somewhat isolated, gene flow is likely restricted among most populations. Using Mesquite v. 2.6 (Maddison and Maddison 2009) we also tested for phylogenetic signal of life history mode in a parsimony framework and examined rates of character evolution using likelihood. We calculated the most parsimonious number of steps needed to explain the observed distribution of life history on the *Cytb* phylogeny and compared it to the distribution of the numbers of steps calculated from 999 trees simulated under a uniform speciation process (Yule model). If the number of steps on the observed tree were to fall outside (below) the 95% confidence interval of the distribution of steps from the random trees, then life history mode shows significant phylogenetic signal and would be non-randomly distributed on the phylogeny of *E. tynerensis*. The rate of character change (between metamorphosis and paedomorphosis) was estimated across the *E. tynerensis* tree. The rate was examined using both the Markov k-state 1 model (Mk1, which assumes equal rates of change between states) and the Asymmetrical Markov k-state 2 model (AsymmMk, which assumes a greater rate of change in one direction than the reverse). A likelihood ratio test was used to determine which model (Mk1 or AsymmMk) is a better fit to the observed distribution of life history mode on the tree.

We determined whether the life history modes and a major habitat features (substrate type of streams containing *E. tynerensis*) are randomly distributed across the landscape by using Allelic Aggregation Index Analysis (AAIA) in the program Alleles in Space (AIS; Miller 2005). This method is a modification of the aggregation index used in spatial ecology (Clark and Evans 1954), but aggregation of non-genetic dichotomous data can also be analyzed with this program by treating them as dominant markers (M. P. Miller personal communication). The AAIA tests whether an allele (j; in our case the life history trait and stream substrate) is uniformly distributed ($R_j \geq 1$), randomly distributed ($R_j = 1$), or non-randomly distributed (aggregated, $R_j \leq 1$). Herein we will use j for metamorphic and paedomorphic (R_{meta} and R_{paedo}) in the life history analysis, and j for chert and non-chert gravel (R_{chert} and $R_{non\text{-}chert}$) in the stream substrate analysis. The significance of our observed R_{meta} and R_{paedo} or R_{chert} and $R_{non\text{-}chert}$ values were tested by comparison to the distribution of 10,000 Rs generated by randomly shuffling the life history mode or substrate data across sampled localities. Our life history analysis included 45 metamorphic and 35 paedomorphic observations. We only included one observation per locality for each life history mode because this characteristic is monomorphic in most locations (>95%). Since we do not know the Mendelian heritability of life history mode in these salamanders we treated this characteristic as a dominant marker (1 = metamorphic; 2 = paedomorphic), which effectively implements a standard aggregation

analysis. Our stream substrate analysis included 34 chert and 39 non-chert streams where we know *E. tynerensis* to occur. Non-chert streams were coded as 1 and chert streams were coded as 2. We converted all of our latitude and longitude coordinates to UTM prior to this analysis, and we also used AIS to generate interpolation plots of life history mode and stream substrate on the landscape. We used a 2×2 Chi-square test to determine if there is an association between paedomorphic life history and chert streams in 73 salamander locations where we have data on life history and streambed substrate.

Using Motic Images Software 2.0 we calculated the two-dimensional geographic area of the continuous distribution of *E. tynerensis* within each of the mitochondrial clades by measuring the minimum distance around our sampled localities. For the western clade, we did not include the disjunct northern Ozark locality (Pulaski Co., MO) in our area measurement because it is far from the rest of the distribution. To estimate the proportion of the geographic distribution of each mitochondrial clade that is paedomorphic versus metamorphic, we averaged the frequency of each life history mode at all localities (stream systems) for which we had life history information. We considered localities where we found only metamorphs as 100% metamorphic, those where we found only paedomorphic adults as 0% metamorphic, and localities that had both life histories as 50% metamorphic.

Results

Phylogenetic analyses based on *Cytb* reveal three divergent lineages corresponding to the eastern, western, and southwestern portions of the *E. tynerensis* distribution (Figs. 2, 3), with no overlap of divergent haplotypes at any location. Bayesian posterior probabilities (BAPP) and maximum parsimony bootstrap values (MPBS) show significant support for these geographic clades: eastern (BAPP = 1.0; MPBS = 87), western (BAPP = 1.0; MPBS = 100), and southwestern (BAPP = 1.0; MPBS = 100). Both BA and MP phylogenies show a sister relationship between the western and southwestern lineages, but this is not well supported (BAPP = 0.75; MPBS = 72). The uncorrected pairwise sequence divergence (P) averages $9.03 \pm 0.06\%$ between the western and eastern lineages, $9.97 \pm 0.05\%$ between the western and southwestern lineages, and $9.79 \pm 0.05\%$ between the eastern and southwestern lineages (Table 2). This is substantially higher than the uncorrected-P within each geographic clade (group), which is less than 4% (Table 2). AMOVA of *Cytb* shows 63.3% of the variation was among groups ($P < 0.00001$), while 26.8% of the variation is among populations within the groups ($P < 0.00001$, Table 3). Exact tests based on pairwise sequence divergence show significant differentiation of eastern, western, and southwestern groups ($P < 0.00001$ in all pairwise comparisons). For *Cytb*, π and π_n are both highest in the eastern lineage (27.04 ± 12.26 and 0.040 ± 0.020, respectively), lowest in the southwestern lineage (15.51 ± 7.58 and 0.022 ± 0.013, respectively), and at intermediate values in the western lineage (22.63 ± 10.04 and 0.033 ± 0.016; Table 4). θ_s estimates, however, are highest in the western lineage (42.05 ± 10.19), lowest in the southwestern lineage (13.08 ± 5.61), and with a value of 28.87 ± 9.55 for the eastern lineage (Table 4). There is also well-supported phylogenetic structure among most of the more terminal clades in the tree (Fig. 2) that is part of our ongoing research on this group.

The Bayesian phylogeny of *POMC* shows four distinct allele groups (Fig. 4). Support for the allele groups varies (group 1 BAPP = 0.94; group 2 BAPP = 0.68; group 3 BAPP = 0.93; group 4 is only one allele). The maximum parsimony analysis recovered a phylogeny congruent to the Bayesian tree, but with no bootstrap support. This is somewhat expected given the low degree of variation of most protein coding nuclear genes at this phylogenetic scale. This may also result from other phenomena such as retention of unsorted ancestral alleles. Average uncorrected P between these *POMC* allele groups range from 1.60 ± 0.06 to $2.15 \pm 0.03\%$, whereas within group divergence ranges from 0.0 to $0.82 \pm 0.05\%$ (Table 2). Given the distinct geographic structure of the divergent mitochondrial lineages, we mapped the frequency of the four *POMC* allele groups with respect to their geographic location and mitochondrial lineage (Fig. 5; eastern, western, southwestern). In the western mitochondrial clade, 80.2% of the alleles (69/86) are from the *POMC* haplotype group 1. The remainder of the alleles in the western mitochondrial clade are from groups 2 (1.25%), 3 (13.9%), and 4 (4.7%). In the eastern mitochondrial clade 73.5% of the alleles (25/34) correspond to *POMC* allele group 2, and the remainder of the alleles (26.5%) are from *POMC* allele group 1. 100% of the alleles (28/28) sampled in the southwestern mitochondrial group correspond to *POMC* allele group 3 (Fig. 5). AMOVA of *POMC* show 40.2% of the variation is among groups ($P < 0.00001$), while 41.3% of the variation is among populations within the groups ($P < 0.00001$, Table 3). Exact tests based on pairwise sequence divergence show significant differences in the *POMC* alleles present in the three groups (based on three mitochondrial clades, $P < 0.00001$ in all pairwise group comparisons). Estimates of π, π_n, and θ_s for *POMC* of the western, eastern and southwestern clades (grouped by *Cytb* divergence), show the western group has the highest degree of variation for all parameters (Table 4).

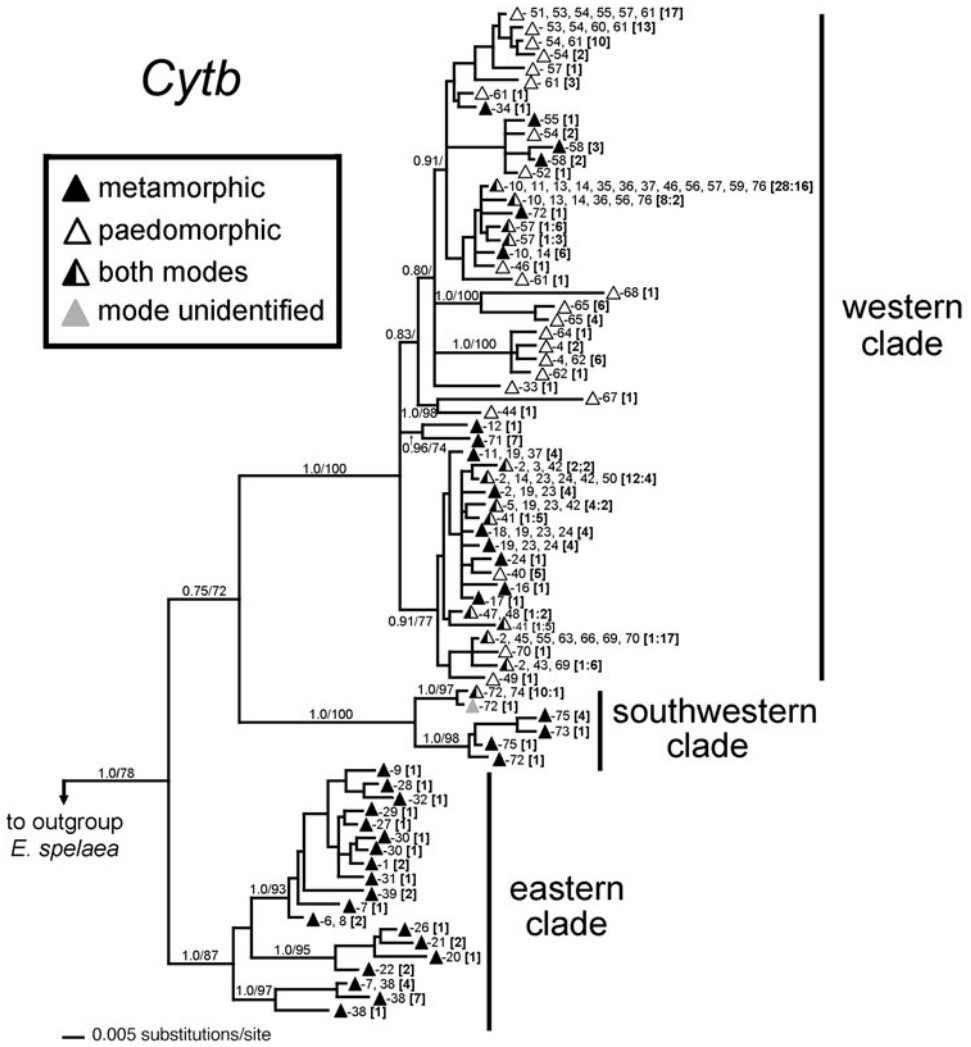

Fig. 2 Bayesian phylogram based on 678 bp of the mitochondrial gene *Cytb* for 243 *E. tynerensis*. Haplotypes with four or less mutations of difference were collapsed to a single OTU. Three major geographic clades (eastern, western, and southwestern) are indicated (see also Fig. 3). *Black triangles* indicate a haplotype group of metamorphic individuals, *white triangles* indicate a haplotype group of paedomorphic individuals, *black/white triangles* indicate a haplotype group containing both life history modes, and *dark grey triangles* are haplotype groups where the life history mode is unknown. Numbers to the right of triangles on the phylogram are the locality numbers for each collapsed haplotype and correspond to those in Fig. 1 and Appendix Table 5. The final number(s) in *brackets* and *bold* indicates the number of individuals represented by each collapsed haplotype. In haplotype groups that contain both life history modes (i.e. *black/white triangles*) the number of metamorphic individuals is listed first, followed by the number of paedomorphic individuals after the colon. Numbers subtending each major node are Bayesian posterior probabilities/maximum parsimony bootstrap values

The mismatch distribution of *Cytb* haplotypes from all populations appears multimodal, reflecting the three divergent genetic lineages; however, HRI for all populations is very low and does not differ significantly from a model of population expansion (Table 4). The low HRI of the distribution of all *Cytb* haplotypes is likely driven by our abundant sampling of haplotypes from the western clade, which has a very smooth unimodal mismatch distribution of haplotypes when analyzed alone. The HRI for most of the *Cytb* and *POMC* mismatch distributions for each region were very low, and we were unable to reject a model of population expansion for any of the groupings except for the *Cytb* haplotypes of the southwestern group (Table 4).

Life history mode shows significant phylogenetic signal on the *Cytb* phylogeny when analyzed by both likelihood and parsimony methods. Using the observed *Cytb* phylogeny, the trait tree parameter equals one indicating strong phylogenetic signal, and the –ln L of the distribution of life history is ($-\ln L = -72.32$). This is significantly better ($P < 0.0001$) than when phylogenetic structure is removed by setting Pagel's Lambda to zero ($-\ln L = -106.74$). The most parsimonious reconstruction of the observed distribution of life history states on the *Cytb* phylogeny is

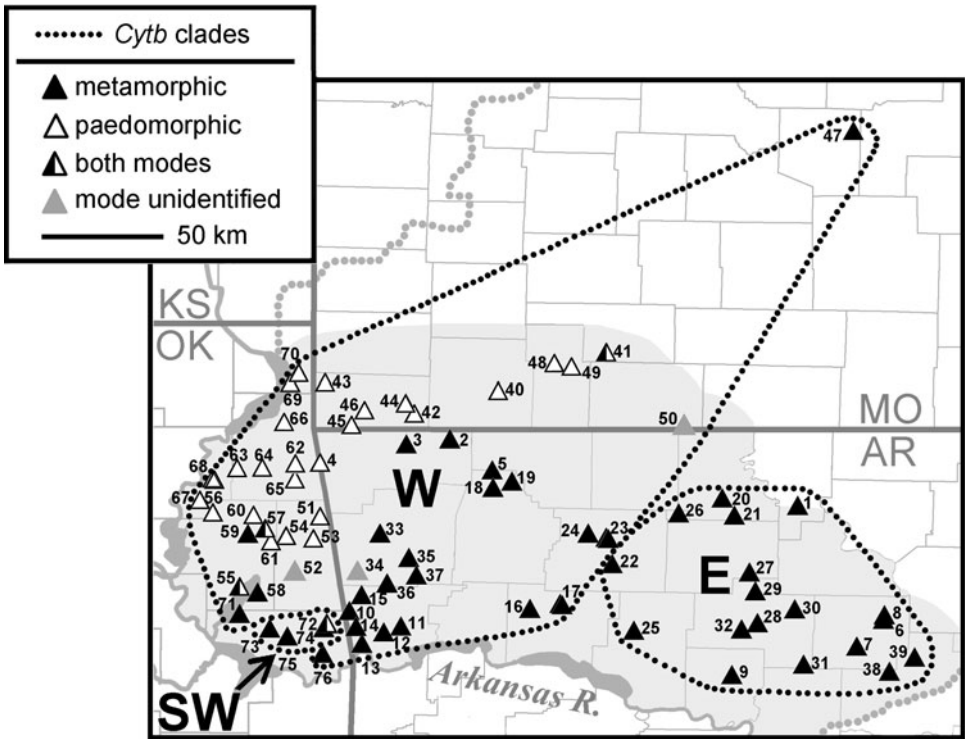

Fig. 3 Distribution of *E. tynerensis* and sampling localities with adjacent locality numbers for *Cytb* on the Ozark Plateau with *black dotted lines* surrounding the distribution of the three major mitochondrial lineages: eastern (E), western (W), and southwestern (SW). Additional symbols are described in the Fig. 1 legend

Table 2 Average uncorrected $P \pm$ standard deviation within and among *Cytb* and *POMC* clades

Clade/allele group	Average
Cytb	
Within eastern clade	3.98 ± 0.14%
Within western clade	3.29 ± 0.18%
Within southwestern clade	2.29 ± 0.14%
Between eastern and southwestern clades	9.79 ± 0.05%
Between eastern and western clades	9.03 ± 0.06%
Between western and southwestern clades	9.97 ± 0.05%
POMC	
Within allele group 1	0.79 ± 0.05%
Within allele group 2	0.72 ± 0.05%
Within allele group 3	0.82 ± 0.05%
Within allele group 4	0.00 ± 0.00%

22 steps. The numbers of steps calculated from 999 simulated trees averages 51.5 ± 3.4 steps (range: 39–62 steps). The 95% confidence interval for this distribution ranges from 44.8 to 58 steps, so the 22 steps for the observed tree falls well below this distribution and is highly significant ($P < 0.0001$). The AsymmMk model (forward rate = 28.71, reverse rate = 30.266, $-\ln L = -79.962$) is not a significantly better fit to our data than the Mk1 model (rate: 29.92, $-\ln L = -79.972$), indicating equal rates of change between metamorphosis and paedomorphosis on the phylogeny ($P < 0.887$), but the rate of evolution in the western clade is nevertheless high.

The entire continuous distribution of *E. tynerensis* is ~39,500 km^2 (not including the disjunct northern Ozark locality; Fig. 1). The three mitochondrial clades differ in the geographic area in which they are distributed. The western clade is by far the largest at over 28,500 km^2 (72.2% of the distribution) and is distributed across Arkansas, Missouri, and Oklahoma (Figs. 3, 6). The known distribution of the southwestern clade is very limited at about 480 km^2 (~1.2% of the distribution) in Sequoyah County, Oklahoma. The remainder of the distribution is occupied by the eastern clade which occurs across ~10,500 km^2 (26.6% of the distribution) in the eastern Ozark Plateau in Arkansas.

The geographic distribution of metamorphic individuals (by locality; n = 48) is aggregated ($R_{meta} = 0.80031$), but this pattern is not significant ($P < 0.59440$). However, paedomorphic individuals (by locality; n = 35) are significantly aggregated ($R_{paedo} = 0.46434$; $P < 0.00001$). The geographic distribution of chert bottom streams (n = 34) is also significantly aggregated ($R_{chert} = 0.46239$; $P < 0.00001$). Non-chert streams (n = 39) are also aggregated, but this pattern is not significant ($R_{non\text{-}chert} = 0.68047$; $P < 0.10400$). The landscape interpolation plots of life history mode and stream substrate, show that the aggregation of both paedomorphic and chert streams are aggregated in the western portion of the distribution (Fig. 6a, b). Chi-square analysis shows a strong association between life history and substrate ($\chi^2 = 61.5$; d.f. = 1; $P < 0.0001$). We know of only four locations

Table 3 AMOVA results for comparison of eastern, western, and southwestern groups for *Cytb* and *POMC*

Source of variation	d.f.	Sum of squares	Variance components	% Variation	*P*-value
Cytb					
Among groups	2	1741.4	19.386	63.3	<0.00001
Among populations	73	2095.7	8.205	26.8	<0.00001
Within populations	171	523.1	3.059	9.9	<0.00001
Total	246	4306.2	30.650		
POMC					
Among groups	2	106.3	1.072	40.20	<0.00001
Among populations	42	152.2	1.101	41.29	<0.00001
Within populations	95	46.9	0.494	18.51	<0.00001
Total	139	305.4	2.667		

Table 4 Estimates ± standard deviations of mean number of pairwise differences (π), nucleotide diversity (π_n), θ_s, for *Cytb* and *POMC* of all populations and the three regional mitochondrial clades (Western, Eastern, and Southwestern)

Clade	π	π_n	θ_s	HRI	*P* (sim ≥ obs)
Cytb					
All populations	35.45 ± 15.48	0.052 ± 0.025	43.22 ± 9.27	0.00095	0.86
Western	20.89 ± 9.26	0.031 ± 0.015	39.76 ± 8.87	0.00164	0.92
Eastern	28.47 ± 12.84	0.042 ± 0.021	28.01 ± 9.05	0.01158	0.19
Southwestern	17.84 ± 8.23	0.026 ± 0.014	23.59 ± 8.10	0.09709	0.02
POMC					
All populations	4.39 ± 2.18	0.011 ± 0.005	5.80 ± 1.65	0.01172	0.74
Western	3.06 ± 1.61	0.007 ± 0.004	4.87 ± 1.56	0.02519	0.81
Eastern	0.85 ± 0.62	0.002 ± 0.001	1.47 ± 0.72	0.05613	0.86
Southwestern	1.83 ± 1.08	0.004 ± 0.002	1.03 ± 0.58	0.13134	0.12

Tests of deviations from a population expansion model were assessed using Harpending's Raggedness Index (HRI) and the probability of simulated raggedness ≥ observed raggedness

where metamorphs occur on chert and in three of these locations they co-occur with paedomorphs. This is consistent with correlations between several stream substrate variables and life history mode in *E. tynerensis* (Bonett and Chippindale 2006). Mapping life history (and stream substrate) with respect to the *Cytb* clades shows that the proportion of metamorphic to paedomorphic populations varies among the western, southwestern, and eastern mitochondrial lineages (Figs. 2, 3, 6). The southwestern clade contains four known localities, three of which are metamorphic, while the other contains both paedomorphs and metamorphs. All known localities and individuals within the eastern lineage are metamorphic. Within the western lineage, paedomorphic and metamorphic localities are found in an approximately even ratio (52% metamorphic and 48% paedomorphic). There are two main subclades within the western lineage, both of which contain paedomorphs and metamorphs (Fig. 2). There are also several minor subclades that were exclusively paedomorphic, yet these are restricted to individual stream systems. In several cases, metamorphic and paedomorphic individuals are found to exist with identical haplotypes (Fig. 2), and the sharp peaks and valleys on the western edge of the interpolation plot show the close proximity of populations with alternative life histories (Fig. 6a).

Discussion

This system highlights the importance of understanding genetic relationships, neutral genetic divergence, and the distribution of key life history characteristics on both the phylogeny and the landscape when developing conservation strategies for polymorphic species. As shown here, this approach is especially important when the underlying geographic distribution of neutral genetic divergence is discordant with life history variation, and one or both of these parameters are unequally represented, or non-randomly distributed, on the landscape.

Distribution of genetic divergence

Neutral genetic divergence among currently or previously isolated populations may serve as a proxy for more cryptic

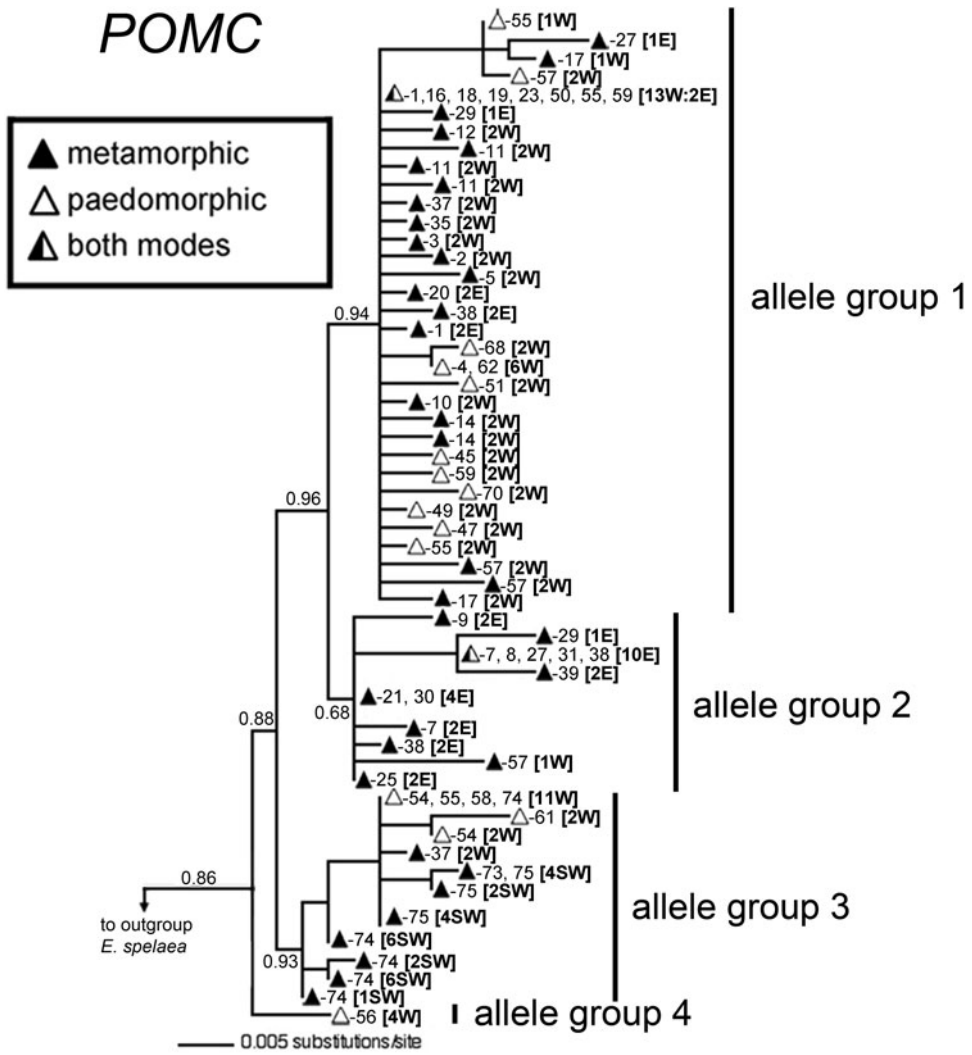

Fig. 4 Bayesian phylogram based on 416 bp of the nuclear gene *POMC* for 74 *E. tynerensis*. Alleles for seven individuals that were heterozygous for divergent alleles are represented independently in the tree. Redundant alleles are only included once in the tree. *Triangles* indicate life history mode of the OTU. Numbers to the right of *triangles* on the phylogram are the locality numbers for each OTU and correspond to those in Fig. 1 and Appendix Table 5. The final number(s) in *brackets and bold* indicates the number of individual alleles sampled from each of the three major mitochondrial clades (eastern, western, or southwestern). Numbers subtending each major node are Bayesian posterior probabilities. Additional *symbols* are described in the Fig. 2 legend

adaptive phenotypic diversity. Protection of divergent genetic lineages within a species may allow for evolution in the face of environmental change (Avise 1992; Dizon et al. 1995; Moritz 1994; Waples 1991), and furthermore divergent lineages are non-renewable in the short-term (Moritz 2002). Our population-level analyses of the mitochondrial gene *Cytb* show three divergent genetic lineages that correspond to the eastern, western, and southwestern portions of the distribution of *E. tynerensis* (Figs. 2, 3). Genetic variation in the nuclear gene *POMC* is also significantly different between the localities included in each of the three mitochondrial clades (Figs. 4, 5; Table 3). Therefore, the eastern, western, and southwestern portions of distribution of *E. tynerensis* fit Moritz's (1994) definition of genetic based ESUs (i.e. populations that are monophyletic for mitochondrial DNA haplotypes and show significant divergence of allele frequencies for nuclear loci). The mismatch distributions of *Cytb* haplotypes and *POMC* alleles follow models of population expansion for the eastern and western clades. Additionally, the three genetic lineages are drastically different from one another in terms of geographic size. The southwestern and eastern clades are the most restricted, comprising only about 1.2 and 26.6% of the species distribution respectively, whereas the western lineage makes up almost three quarters (72.2%) of the distribution (Figs. 3, 5, 6). If based only on genetic divergence and geographic distribution, the eastern lineage and especially the southwestern lineage would likely be given the highest conservation attention due to their limited occurrence. However, it is well recognized that delimitation of ESUs based on neutral genetic divergence alone may not adequately capture some evolutionarily important phenotypic characteristics (or quantitative genetic variation) that may be necessary for the long-term persistence of the species (Crandall et al. 2000; de Guia and Saitoh 2007; Fraser and Bernatchez 2001; Moritz 2002; Tymchuk et al. 2010). In the case of *E. tynerensis* the widespread western lineage actually exhibits the most extensive life history variation (detailed below).

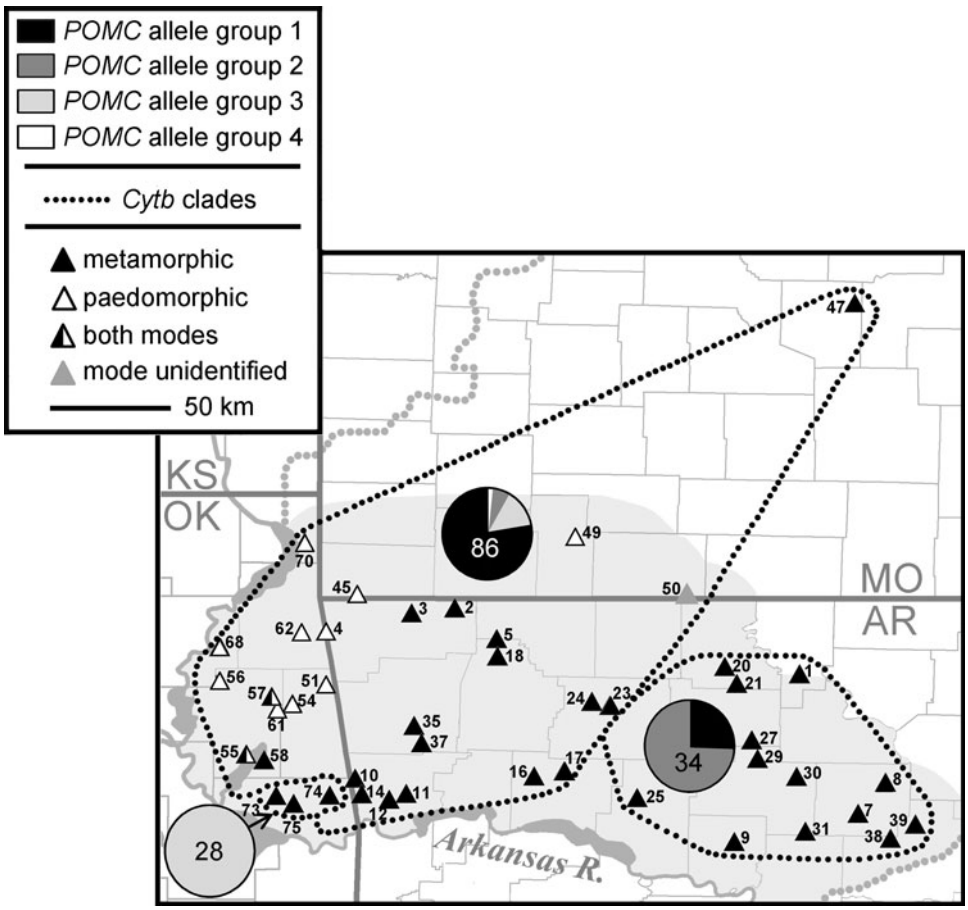

Fig. 5 Distribution of *E. tynerensis* and sampling localities with adjacent locality numbers for *POMC* on the Ozark Plateau with *black dotted lines* surrounding the distribution of the three major mitochondrial lineages: eastern (E), western (W), and southwestern (SW). *Pie charts* indicate the proportion of the four major *POMC* allele groups [*black* (1), *dark grey* (2), *light grey* (3), *white* (4)] that were found within each of the mitochondrial lineages (Fig. 4). Numbers on the *pie charts* indicate the total number of *POMC* alleles sampled for each mitochondrial group. *Shaded grey area* indicates the known distribution of *E. tynerensis*, and *triangles* are sampling localities for *POMC*. Additional *symbols* are described in the Fig. 1 legend

Distribution of life history variation

Phenotypic diversity is also an important attribute for withstanding environmental change, regardless of if a given phenotype is inherited or results from phenotypic plasticity (Watters et al. 2003). The evolution and maintenance of alternative life histories in a species (or lineage) can vastly broaden its ecological niche and distribution, including the colonization of distinctly different ecosystems or withstanding drastic environmental changes in situ. We find that the alternative life history modes of *E. tynerensis* (paedomorphic and metamorphic) are non-randomly distributed with respect to geography; paedomorphic populations are significantly aggregated in the western portion of the distribution (Fig. 6a). Similarly, the streambeds composed of chert gravel are also aggregated in the western portion of the distribution (Fig. 6b), and we find a strong association between life history and steam substrate. This is consistent with previous analyses of stream substrate parameters and alternative life history modes of *E. tynerensis* (Bonett and Chippindale 2006; Tumlison and Cline 2003). Paedomorphic *E. tynerensis* require a permanently aquatic environment and inhabit streams with gravel beds composed of coarse Ordovician/Silurian chert rock (Bonett and Chippindale 2006; Tumlison and Cline 2003). The interstitial spaces in the coarse streambed allow them access to subsurface water during dry summer months. Whereas, metamorphic individuals typically live in streams with compact streambeds where they do not necessarily have continuous access to water, yet require moist, forested habitat surrounding the stream (Bonett and Chippindale 2006). Therefore, in *E. tynerensis*, paedomorphosis and metamorphosis are strongly associated with distinct habitats, and this adaptive phenotypic variation may be the key characteristic that has allowed this species to exploit a diversity of stream systems across the Ozark Plateau (Bonett and Chippindale 2006).

The alternative life history modes of *E. tynerensis* (paedomorphic and metamorphic) are also non-randomly distributed with respect to both the divergence of neutral markers (Fig. 6c). Approximately half of the western lineage is composed of paedomorphic populations, the southwestern lineage is primarily metamorphic and the eastern lineage is exclusively metamorphic. Within the western lineage paedomorphosis appears in several seemingly unrelated lineages in different drainages. This life history variation may result from de novo evolution and selection for paedomorphosis in independent lineages.

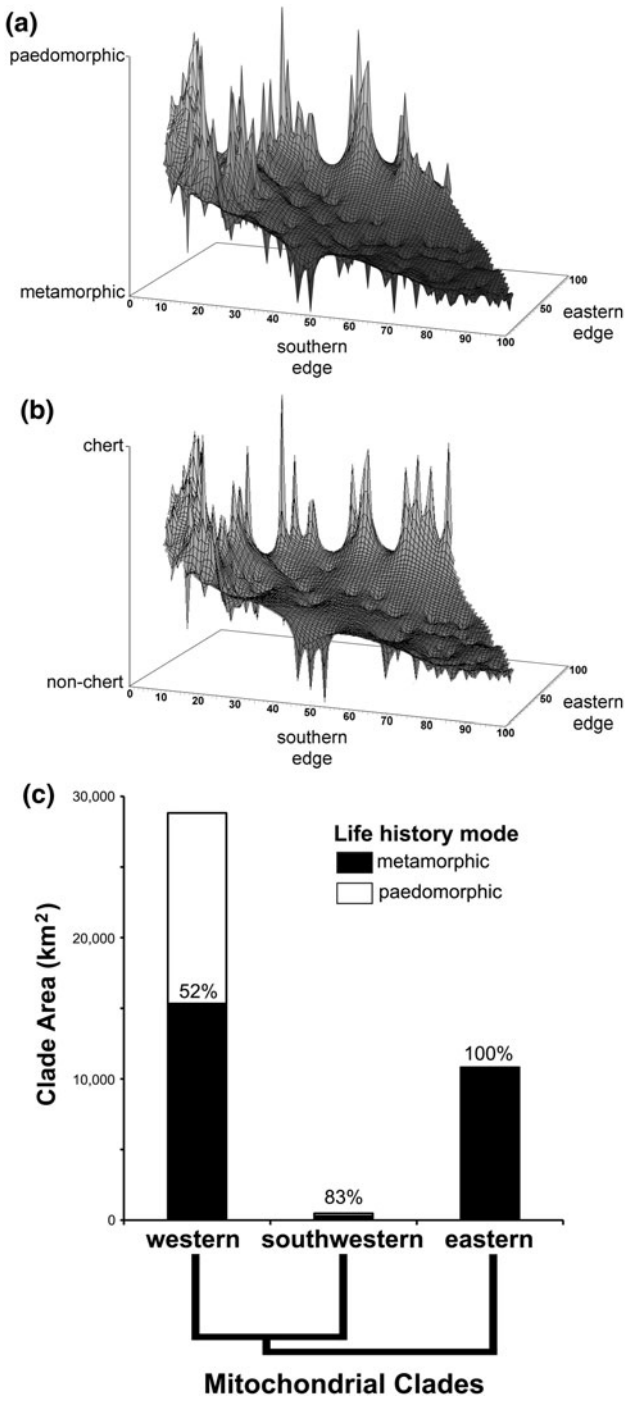

Fig. 6 The geographic distribution of alternative life history modes of *E. tynerensis* and steam substrates where they occur. Interpolation plots from the aggregation index analysis showing the distribution of **a** life history (metamorphic = 1; paedomorphic = 2), and **b** streambed substrate (non-chert = 1; chert = 2) plotted on the UTM coordinates of our collection localities (the distance weighting parameter a = 1; grid specified at X = 100; Y = 100). **c** Geographic area (km^2) of the three mitochondrial clades (eastern, southwestern, and western) and the proportion of metamorphic (*black*) and paedomorphic (*white*) localities within these clades. The number on each *bar* indicates the percentage of metamorphic localities for the clade

An alternative genetic basis of this phenomenon would be a single origin of "paedomorphic alleles" with subsequent gene flow and selection among populations, similar to patterns of allelic introgression and selection that have been recently observed in populations of *Ambystoma* (Fitzpatrick et al. 2010).

As paedomorphic *E. tynerensis* typically inhabit streams that metamorphs cannot exploit, the evolution of paedomorphosis allows the species to currently occur over an approximately one-third greater distribution (compared to the distribution size of strictly metamorphic populations; Fig. 6c). Conversely, the continued existence of the ancestral metamorphic life history allows *E. tynerensis* to inhabit ephemeral or unstable streams and currently occur across most of the other two-thirds of the distribution. The current distribution of the alternative life histories could have happened by the direct evolution of paedomorphosis or metamorphosis along habitat transition zones and subsequent expansion into new regions, or by shifting the frequency of alternative life histories within a given location due to changing environmental conditions.

The prior conservation attention and priority to only paedomorphic populations completely overlooked the most divergent and narrowly distributed southwestern and eastern genetic lineages, which would otherwise be given the highest priority based on neutral divergence. However, the widespread western lineage contains a high degree of life history variation (paedomorphosis and metamorphosis) and therefore, some populations of each life history mode should also carry high conservation value due to the long-term advantage of exhibiting alternative lifestyles (permanently aquatic versus semi-aquatic). We should also mention that if paedomorphic development in chert gravel streams were strictly plastic then it would be somewhat expected that this characteristic may be non-randomly distributed on the phylogeny and landscape (i.e. that the distribution of this trait would reflect the distribution of chert gravel streams). Nevertheless, plastic traits still have important conservation value, because it is the phenotypic variation of a lineage that may allow some individuals to survive environmental change (Boag and Grant 1981; Endler 1986).

Rate and direction of life history evolution

The direction and rate of phenotypic evolution are two other important parameters to consider when assigning priorities to alternative life history modes, and developing management strategies. Higher conservation priority should be afforded to rare phenotypes (Bernatchez 1995) and their habitats, especially if it is unlikely for another lineage to converge on the phenotype in a foreseeable

amount of time. Conversely, it may be possible to manipulate the frequency of some evolutionarily or developmentally labile phenotypes by altering or preserving particular microhabitats (Watters et al. 2003). Analyzing the direction of phenotypic change may provide further insight into whether or not a given phenotype is likely to diversify (or develop) into alternative phenotypes.

Even though paedomorphosis is largely restricted to the widespread western clade of *E. tynerensis*, it shows a high rate of evolution within the clade. Analysis of the direction of life history evolution on the tree suggests that the rate of change from metamorphosis to paedomorphosis is equal to evolution in the reverse direction (AsymmMk model does not explain the distribution of life history better on the phylogeny than an Mk1 model). However, our preliminary developmental endocrine data suggests that some populations of *E. tynerensis* may be incapable of metamorphosis. Amphibian metamorphosis is regulated at multiple levels by hormones from the hypothalamic–pituitary–thyroid axis and the hypothalamic–pituitary–adrenal axis (Bonett et al. 2010; Denver 2009; Kikuyama et al. 1993; Shi 2000). While studies have shown that paedomorphic populations of *E. tynerensis* will "metamorphose" in a weak solution of thyroxin (Kezer 1952), Myers and Bonett (unpublished) found that at the osteological-level metamorphosis is often incomplete and some populations of *E. tynerensis* are not very responsive to thyroid hormone. Therefore, some paedomorphic populations may not be able to reverse to the metamorphic life history if their permanently aquatic environment is lost. Metamorphosis is the likely ancestral condition for *E. tynerensis* (Bonett and Chippindale 2004) and the western clade. A previously overlooked aspect of the metamorphic life history is that it is in fact an important source for the ongoing colonization of paedomorphic populations.

Management of alternative life histories

"Phenotype management" strategies are used to promote the success of multiple phenotypes in a population/species through the restoration or development of habitat diversity or specific habitat features (Watters et al. 2003). For example, a management strategy for alternative male phenotypes of coho salmon (*Oncorhynchus kisutch*) involves restoring a balance between riffle and pool habitats in breeding streams to maintain both "jack" (maturity 1.5–2 years) and "hook jaw" (maturity 2.5–3 years) phenotypes, respectively (Groot and Margolis 1991; Gross 1985; Watters et al. 2003). Both coho phenotypes are oceanic migrants and are not restricted to spawning in the same habitat type in which they develop. In contrast, *E. tynerensis*, like most plethodontid salamanders, exhibits very low vagility (Larson et al. 1984). Furthermore, life history variation among populations of *E. tynerensis* is high in the western clade, but intra-population variation is low to nonexistent. The dichotomous nature of the alternative life history modes and broadly parapatric (or allopatric) distribution of their habitat, combined with low dispersal, may limit the ability of alternative life history modes to quickly re-colonize the habitats of extirpated populations. An alternative strategy for preserving phenotypic diversity, that does not necessarily involve habitat manipulation, is to conserve environmental gradients that allow for evolution and migration among populations (Moritz 2002). This strategy may be a very effective method for the conservation of *E. tynerensis* and other species that exhibit discrete habitat specific life history modes, but for species with low dispersal this may require conserving regions where distinct habitat types directly abut. Over the last few years we have surveyed transects between paedomorphic and metamorphic salamander populations (and their habitats) and we have identified several narrow locations in the western Ozarks where alternative life history modes come into very close contact and or are in sympatry (Bonett et al. unpublished). Regions containing developmentally or evolutionarily labile populations, as well as habitat for each life history mode and adjoining regions, could serve as reservoirs for *E. tynerensis* if either life history mode is threatened or extirpated. In general, this strategy could allow for continued life history evolution in the face of environmental change, as adjoining habitats could serve as a gateway for the re-colonization of alternative life history modes across the landscape.

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Appendix

See Table 5.

Table 5 Locality numbers, locality information, and life history for *E. tynerensis* populations

Loc #	State	County	Locality	Latitude	Longitude	Sample size *Cytb*	Sample size *POMC*	Life history
1	AR	Baxter	Farris spring, 3 mi S. of Lone Rock	36.1383	−92.3214	2	2	M
2	AR	Benton	Ashmore Cr, Garfield	36.4496	−93.9697	6	1	M
3	AR	Benton	Spring off of Spakner Cr. Rd.	36.4305	−94.1797	1	1	M
4	AR	Benton	Spavinaw Cr., Rt 43, 5 mi S. of Maysville	36.3426	−94.588	7	1	P
5	AR	Carroll	5 mi S. of Eureka Springs	36.3057	−93.7739	1	1	M
6	AR	Clerburne	1.3 mi E. of Ida	35.5953	−91.9125	1	0	M
7	AR	Clerburne	Bridal Veil Falls, Heber Springs	35.4703	−92.0398	2	2	M
8	AR	Clerburne	Tiger B Rd, 2.1 mi NE of Ida	35.6177	−91.9087	1	1	M
9	AR	Conway	Dutton Mt, Birdtown	35.3337	−92.6348	1	1	M
10	AR	Crawford	3 mi NE of Barcelona, Ozark N.F.	35.6401	−94.4223	6	1	M
11	AR	Crawford	5 mi S of Mountinburg	35.5653	−94.2046	3	3	M
12	AR	Crawford	Stream off of Hobbtown Rd.	35.5374	−94.2859	1	1	M
13	AR	Crawford	Pine Hollow Rd, 1.5 mi NW of Van Buren	35.4774	−94.3898	2	0	M
14	AR	Crawford	Rt 220, S of FR1716, Ozark N.F.	35.7062	−94.3178	2	2	M
15	AR	Crawford	Trib to Mt Fork Cr., 5 mi N of Natural Dam	35.6892	−94.4315	1	0	M
16	AR	Johnson	Washita Cr., N. of Yarbough Gap	35.6506	−93.5934	1	1	M
17	AR	Johnson	Ozone Campground	35.6708	−93.4487	1	1	M
18	AR	Madison	Spring off of CR42, 2 mi E. Clifty	36.2206	−93.7715	2	1	M
19	AR	Madison	Mr. Lang's, 2 mi S. of Rockhouse	36.2547	−93.6769	1	0	M
20	AR	Marion	Caney Cave, (Hartley's Cave)	36.0915	−92.6206	1	1	M
21	AR	Marion	Grey Springs, off of CR502, 0.5 mi E of Ralph	36.1718	−92.6809	2	1	M
22	AR	Newton	Norton Gap	35.8629	−93.1984	1	0	M
23	AR	Newton	Larry's Cave, 1.5 mi S. of Jasper	35.9881	−93.2330	6	1	M
24	AR	Newton	Low Gap Springs	36.0021	−93.3171	2	0	M
25	AR	Pope	Bates Knob	35.5444	−93.0994	1	1	M
26	AR	Searcy	Glenco Springs	36.1007	−92.8868	1	0	M
27	AR	Searcy	Spring off of Rt 65, 1.9 mi S of Leslie	35.8201	−92.5529	1	1	M
28	AR	Van Buren	Bradley Br., 2.8 mi W of Clinton	35.5798	−92.5118	1	0	M
29	AR	Van Buren	Rt. 65 Just S of Denard	35.7313	−92.5212	1	1	M
30	AR	Van Buren	Stream at Mt. Meadow Ests, Rt 16	35.6459	−92.3351	2	1	M
31	AR	Van Buren	Rt 124, 0.3 mi W of Cardon Cr.	35.3823	−92.2901	1	1	M
32	AR	Van Buren	Rt 95, 2.4 mi. NE of Scotland	35.5497	−92.5873	1	0	M
33	AR	Washington	Garrett Hollow Cave	35.8281	−94.3871	1	0	U
34	AR	Washington	Rt 62, 3 mi SW of Prairie Grove, vet clinic	35.9543	−94.3738	1	0	M
35	AR	Washington	Seeps along Rt 71, Woolsey	35.8866	−94.1683	2	1	M
36	AR	Washington	Trib. to Lee Cr, Rt 220, 2 mi S of Devel's Den S. P.	35.7671	−94.2732	2	0	M
37	AR	Washington	Winslow	35.8068	−94.1315	4	2	M
38	AR	White	Little Cr., 4.2 mi W of Letona	35.3487	−91.8832	8	3	M
39	AR	White	Seep off of Rt 305 between Clay and Dewey	35.4161	−91.7666	2	1	M
40	MO	Barry	Galena Spring	36.7076	−93.7633	5	0	P
41	MO	Christian	Camp Cr., Rt 65, 5 mi S of Spokane	36.8628	−93.2304	14	0	P/M
42	MO	McDonald	Big Sugar Cr., Rt 90, near intersection with KK	36.5754	−94.1416	3	0	P
43	MO	McDonald	Buffalo Cr., just N of Owens Bluff, 5 mi NE of Tiff City	36.7225	−94.5660	1	0	P
44	MO	McDonald	Mike's Cr., Rt E, NE of Powell	36.6271	−94.1806	1	0	P
45	MO	McDonald	Mill Cr., 4 mi SE of Noel	36.5217	−94.4400	3	1	P
46	MO	McDonald	Pineville, Dundee's site	36.5912	−94.3779	2	0	P
47	MO	Pulaski	Mudd Cave, Cliff Rd, 3 mi SW of Franks	37.9156	−92.0555	1	1	P

Table 5 continued

Loc #	State	County	Locality	Latitude	Longitude	Sample size *Cytb*	Sample size *POMC*	Life history
48	MO	Stone	Pine Run, Rt 13/Rt 265, Galena	36.8116	−93.4743	2	0	P
49	MO	Stone	Trib. to Railey Cr., KK 4 mi S of Abesville	36.7962	−93.3948	1	1	P
50	MO	Taney	Protem	36.5261	−92.8607	1	1	U
51	OK	Adair	Ballard Cr., Rt 59, Just S of Ballard	36.0867	−94.5881	1	1	P
52	OK	Adair	Spring 1 mi S of Cherry Tree, Rd to landfill	35.7158	−94.6393	1	0	U
53	OK	Adair	Trib. to Baron Fork River, 2 mi E of Strawberry Springs	35.9821	−94.6207	1	0	P
54	OK	Adair	Tyner Creek, off of Rt. 62	35.9955	−94.7502	13	3	P
55	OK	Cherokee	Balint Bridge	35.7562	−94.9711	3	3	P/M
56	OK	Cherokee	Cave Spring, 1.5 mi N of Peggs	36.1047	−95.0955	4	1	P
56	OK	Cherokee	Spring Creek, ~3 mi upstream from Cave Spring	36.0881	−95.0192	2	1	P
57	OK	Cherokee	Cedar Hollow, J. T. Nickel Preserve	36.0001	−94.8951	8	2	M
57	OK	Cherokee	Tulley Hollow, J. T. Nickel Preserve	35.9958	−94.8705	3	0	M
57	OK	Cherokee	Dog Hollow, J. T. Nickel Preserve	36.0581	−94.8509	8	0	P
57	OK	Cherokee	Sawmill Hollow, J. T. Nickel Preserve	36.0520	−94.8119	4	1	P
57	OK	Cherokee	Telamay Hollow, J. T. Nickel Preserve	36.0355	−94.8722	1	0	M
58	OK	Cherokee	Trib. of Elk Cr., 2 mi N of Cookson	35.7268	−94.9065	5	1	M
59	OK	Cherokee	McLeeland Spring, 2 mi S of Eagles Bluff	35.9786	−94.9276	2	1	M
59	OK	Cherokee	Spring off of Rt 10, 1 mi S of Eagles Bluff	35.9963	−94.9264	1	0	M
60	OK	Cherokee	Peavine Cr., at Hanging Rock	36.0700	−94.8843	2	0	P
61	OK	Cherokee	Rock Creek, Camp Egan	35.9605	−94.8172	9	1	P
62	OK	Delaware	Spavinaw Cr. at January/Stansbury Cave Stream	36.3219	−94.7097	2	1	P
62	OK	Delaware	January/Stansbury Cave	36.3219	−94.7097	0	1	P
63	OK	Delaware	Saline Cr., 0.5 mi E. of Kenwood	36.3154	−94.9833	1	0	P
64	OK	Delaware	Saline Cr., just E of Runaway Hollow	36.3170	−94.8357	1	0	P
65	OK	Delaware	Spring off of Rt 116, 2 mi W of Colcord	36.2642	−94.7228	7	0	P
66	OK	Delaware	White Water Cr., 1 mi N of Grand Lake O' the Cherokees	36.5349	−94.7606	1	0	P
67	OK	Mayes	Snake Cr., Rt 82, 5 mi S of Locust Grove	36.1646	−95.1571	1	0	P
68	OK	Mayes	Trib. of Saline Cr., just N of Chimney Rock Lake	36.2666	−95.0994	1	1	P
69	OK	Ottawa	Council Hollow, Stream off of Rt. 10	36.7212	−94.7301	7	0	P
70	OK	Ottawa	Sycamore Cr., Rt 10, 3 mi SE of Wyandotte	36.7681	−94.6926	6	1	P
71	OK	Sequoyah	Trib. of Lake Tenkiller, 10.5 mi NE of Gore	35.6287	−94.9789	7	0	M
72	OK	Sequoyah	Little Lee Cr., off of Rt 101	35.5786	−94.5574	5	0	P/M
73	OK	Sequoyah	Stream crossing Rt E1000, W of Brushy	35.5668	−94.7357	1	1	M
74	OK	Sequoyah	Polecat Cr., off of Rt 101	35.5582	−94.5674	10	9	M
75	OK	Sequoyah	Tin Cup Cr., 5 mi NE of Sallisaw	35.5068	−94.7434	6	4	M
76	OK	Sequoyah	Stream off of Weaver Loop, 2 mi NE of Muldrow	35.4156	−94.5856	3	0	M

P paedomorphic, *M* metamorphic, *U* unknown life history (larva)

Locality numbers 56, 57, 59, and 62 each contain multiple distinct collection sites that are lumped together for presentation purposes

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