Classification: Biological Sciences; Evolution

Geography is more important than life history in the recent diversification of the tiger salamander complex

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ABSTRACT

The North American tiger salamander species complex, including its flagship species the axolotl, has long been a source of biological fascination. The complex exhibits a wide range of variation in developmental life history strategies, including populations and individuals that undergo metamorphosis and those able to forego metamorphosis and retain a larval, aquatic lifestyle (i.e., paedomorphosis). Such disparate life history strategies are assumed to cause populations to become reproductively isolated, but the degree to which they have actually shaped population- and species-level boundaries is poorly understood. Using a large multi-locus dataset from hundreds of samples across North America, we identified genetic clusters with clear signs of admixture across the geographic range of the tiger salamander complex. Population clusters often contain a mixture of paedomorphic and metamorphic taxa, and we conclude that geography has played a large role in driving lineage divergence relative to obligate paedomorphosis in this system. This conclusion is bolstered by model-based analyses demonstrating gene flow between metamorphic and paedomorphic populations. Even the axolotl, a paedomorphic species with an isolated native range, apparently has a history of gene flow with its neighboring populations. This fine-scale genetic perspective on life-history variation establishes a framework for understanding how plasticity, local adaptation, and gene flow contribute to lineage divergence. The axolotl is currently used as the vertebrate model system in regenerative biology, and our findings chart a course for more informed use of these and other tiger salamander species in experimental and field research, including conservation priorities.

Keywords: *Ambystoma*, life history, phylogenetics, population genomics, salamanders

SIGNIFICANCE STATEMENT

Population structure and speciation are shaped by a variety of biotic and abiotic factors. In the tiger salamander complex, one factor that may influence diversification is life history: some taxa are obligately paedomorphic–a condition where adults maintain an aquatic, larval phenotype–while others are facultatively paedomorphic or entirely metamorphic. Using a large multi-locus dataset, we found evidence of gene flow and/or panmixia between obligately and facultatively paedomorphic taxa, suggesting that an obligately paedomorphic life history is not a strong driver of speciation in the tiger salamander complex. We also recovered a history of gene flow between the critically endangered axolotl and its neighboring populations, providing important information for its conservation and captive management.

INTRODUCTION

Life history—the complement of traits affecting survival and reproduction over an organism's lifetime—affects ecology and dispersal, and therefore plays a major role in shaping population structure and speciation (1, 2). The axolotl (*Ambystoma mexicanum*) and related salamander species of the North American *Ambystoma tigrinum* complex (Table 1) display enormous variation in life history (3). Some members of this group are considered obligate paedomorphs, where sexually mature adults retain a larval, aquatic body plan that includes external gills and an enlarged tail fin (4). Others are considered obligate metamorphs, transforming from aquatic larvae to terrestrial adults. Most populations are facultative in their developmental life history strategy, transforming under certain genetic and/or environmental conditions (5–8). This lability in life history is believed to have played an important role in the diversification of the *A. tigrinum* complex, particularly in the Trans-Mexican Volcanic Belt (TMVB) region, where several essentially paedomorphic species (including the axolotl) have been described (9–11). Obligate paedomorphosis is estimated to have evolved in this region multiple times (11–13) in association with relatively large, permanent bodies of water. Presumably, restricted gene flow between these isolated, paedomorphic populations led to speciation as well as morphological adaptations to the aquatic lifestyle (14).

The term "species complex," while not a formal taxonomic category, is often used to describe groups of closely related lineages early in the divergence process. The tiger salamander species complex has been highlighted as a potentially valuable example of a recent radiation (15) that could provide insight into the early mechanisms initiating and/or maintaining diversity (16–21). However, such species complexes poses a number of challenges: phenotypic differences among lineages may be subtle or absent (i.e., cryptic species), ancestral polymorphisms may be

Table 1. Currently recognized species in the *Ambystoma tigrinum* complex. Life histories, habitat preferences, and conservation status were compiled from AmphibiaWeb (2019) and the IUCN (2019).

Species	Description Reference	Life History	Habitat Preference	Conservation Status
Ambystoma altamirani	Dugès, 1895	Facultative paedomorph	Streams, occasional ponds	Endangered
Ambystoma amblycephalum	Taylor, 1940	Facultative paedomorph	Ponds	Critically endangered
Ambystoma andersoni	Krebs & Brandon, 1984	Paedomorph	Lake Zacapu	Critically endangered
Ambystoma bombypellum	Taylor, 1940	Facultative paedomorph	Lakes / Ponds, occasional streams	Data deficient
Ambystoma californiense	Gray 1853	Metamorph	Vernal pools	Vulnerable
Ambystoma dumerilii	(Dugès, 1870)	Paedomorph	Lake Patzcuaro	Critically endangered
Ambystoma flavipiperatum	Dixon, 1963	Facultative paedomorph	Lakes / Ponds, occasional streams	Endangered
Ambystoma granulosum	Taylor, 1944	Facultative paedomorph	Lakes / Ponds, occasional streams	Critically endangered
Ambystoma leorae	(Taylor, 1943)	Facultative paedomorph	Streams	Critically endangered
Ambystoma lermaense	(Taylor, 1940)	Facultative; often paedomorphic	Lakes / Ponds	Endangered
*Ambystoma mavortium**	Baird, 1850	Facultative paedomorph	Lakes / Ponds	Least concern
Ambystoma mexicanum	(Shaw and Nodder, 1798)	Paedomorph	Lakes Xochimilco & Chalco	Critically endangered
Ambystoma ordinarium	Taylor, 1940	Facultative paedomorph	Streams, ponds	Endangered
Ambystoma rivulare	(Taylor, 1940)	Facultative paedomorph	Streams	Endangered
Ambystoma rosaceum	Taylor, 1941	Facultative paedomorph	Streams, occasional ponds	Least concern
Ambystoma silvense	Webb, 2004	Facultative paedomorph	Lakes / Ponds	Data deficient
Ambystoma taylori	Brandon et al., 1982	Paedomorph	Lake Alchichica (saline)	Critically endangered
Ambystoma tigrinum	Green, 1825	Rare paedomorph	Lakes / Ponds	Least concern
Ambystoma velasci	(Dugès, 1888)	Facultative paedomorph	Lakes / Ponds, Streams	Least concern

**includes 5 recognized subspecies: A. m. mavortium Baird 1850, A. m. melanostictum (Cooper 1859), A. m. diaboli Dunn 1940, A. m. nebulosum Hallowell 1853, and A. m. stebbinsi Lowe 1954*

stochastically inherited (i.e., incomplete lineage sorting), and reproductive barriers may be porous (i.e., speciation with gene flow). Furthermore, phenotypic variation may not reflect lineage divergence or speciation processes; for example, trait variation may be the result of plasticity or balancing selection (22). The reliance on phenotype for species diagnosis in the *A. tigrinum* complex may require particular scrutiny, as several phenotypic traits, including developmental state and adult color pattern, can be plastic and highly variable (23–25). Given these challenges, fundamental questions remain concerning the evolutionary distinctiveness of component lineages within the tiger salamander complex, calling into question the current taxonomy as an accurate reflection of the underlying population biology.

Previous research has produced mixed results regarding levels of genetic differentiation among lineages in the *A. tigrinum* complex, as well as the relative importance of paedomorphosis as a driver of diversification. While population- and species-level structure is evident, an important theme emerging across multiple studies has been that reproductive barriers are porous or incomplete (11, 26, 27). Indeed, Shaffer and McKnight (11) noted "the striking lack of differentiation among the 14 species of the tiger salamander complex" (p. 425). Some studies have found an elevated degree of population structure among paedomorphic populations relative to metamorphic populations (12, 28), but others have demonstrated that paedomorphic taxa and neighboring metamorphic populations are not fully reproductively isolated (29) and can produce viable hybrid offspring under laboratory conditions (30, 31). Furthermore, there is evidence that some populations considered to be obligately paedomorphic are capable of transforming at low rates and may be more appropriately considered facultatively paedomorphic (10, 32, 33), although the absence of transformed individuals in nature is difficult to verify without targeted ecological studies (34).

To illuminate the processes underlying diversification in the tiger salamander complex, an important first step must be a range-wide assessment of population structure and the clarification of population- and species-level boundaries. With this groundwork, insights into the role of paedomorphosis and diversification can be addressed. For a study system of this geographic and taxonomic scale, robust inference of population structure requires information derived from throughout the genome combined with thorough range-wide sampling. To meet these criteria, we expand on a large multi-locus dataset containing 95 nuclear loci for 93 individuals (35), to produce a data matrix for 347 individuals across the full geographic range of tiger salamanders (Fig. S1). Given the complex and somewhat checkered history of the group's taxonomy, we take a naïve approach, performing population structure analyses without *a priori* identification of taxa to resolve geographic genetic clusters and characterize patterns of admixture. We couple these results with phylogenetic network analyses and model-based tests of migration between metamorphic and paedomorphic populations to provide an additional perspective on the degree to which populations are genetically connected, now or in the recent past. We then infer a species tree for identified genetic clusters in an attempt to provide an updated working hypothesis for the evolution of the group. Finally, we test the overarching hypothesis that life history evolution has driven speciation in the tiger salamander complex, particularly through the assumed isolation and independence of small-range endemics classified as paedomorphic species.

RESULTS AND DISCUSSION

Identification of genetic lineages and gene flow

A principal components analysis (PCA) and a discriminant analysis of principal components (DAPC; 36) both recovered *Ambystoma californiense*, the only confirmed obligate metamorph in the complex, as genetically distinct from all other tiger salamanders (Fig. 1A, S2, S3), which is consistent with previous work (e.g., 11, 37). After removing *A. californiense* from the dataset, DAPC analysis supported the recognition of three primary genetic clusters: U.S. (including samples from southern Canada), northwestern Mexico, and central Mexico (Fig. 1B, S4). All populations in the U.S. and northern Mexico clusters are considered facultatively paedomorphic or primarily metamorphic (38). The central Mexico cluster, however, contains a wealth of life history extremes: four currently recognized species are considered to be obligate paedomorphs and ten others are facultatively paedomorphic to varying degrees (13, 39). A PCA of these data produced similar clustering results, with ordination patterns largely mirroring geographic sampling (Fig. S5). For discussion of the taxonomic implications of our results, see the Supplementary Material.

Within central Mexico, both DAPC and STRUCTURE analyses identified four geographic clusters (hereafter referred to as CM1-CM4) with signatures of admixture within and among them (Figs. 2A, S6-S8). Network analysis (40) also recovered groups that generally correspond to CM1-CM4, but with a large number of reticulate branches (Fig. 3). This starburst-like pattern indicates the presence of conflicting topologies within the dataset (40), which can be caused by biological processes including incomplete lineage sorting (a historical legacy of recent diversification) and gene flow, either current or in the evolutionarily recent past. Phylogenetic

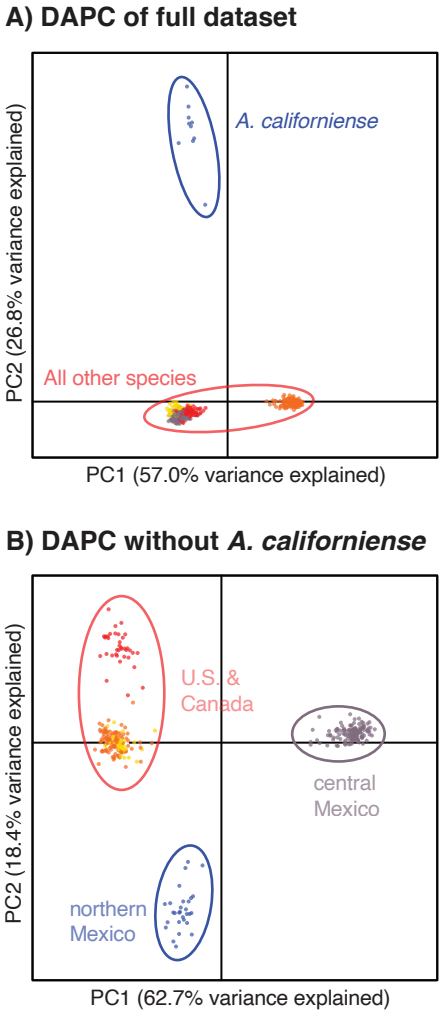

Figure 1. Results of discriminant analysis of principal components (DAPC) on (A) the entire genomic dataset (without *A. opacum* and *A. texanum* outgroups, and (B) the genomic dataset without the highly divergent *A. californiense*. In both plots, points represent individuals and ellipses show the groups identified by DAPC. The first and second principal components from the DAPC are on the x and y axes, respectively. Results from additional values of *K* are provided in Supplementary Figures 2 and 3.

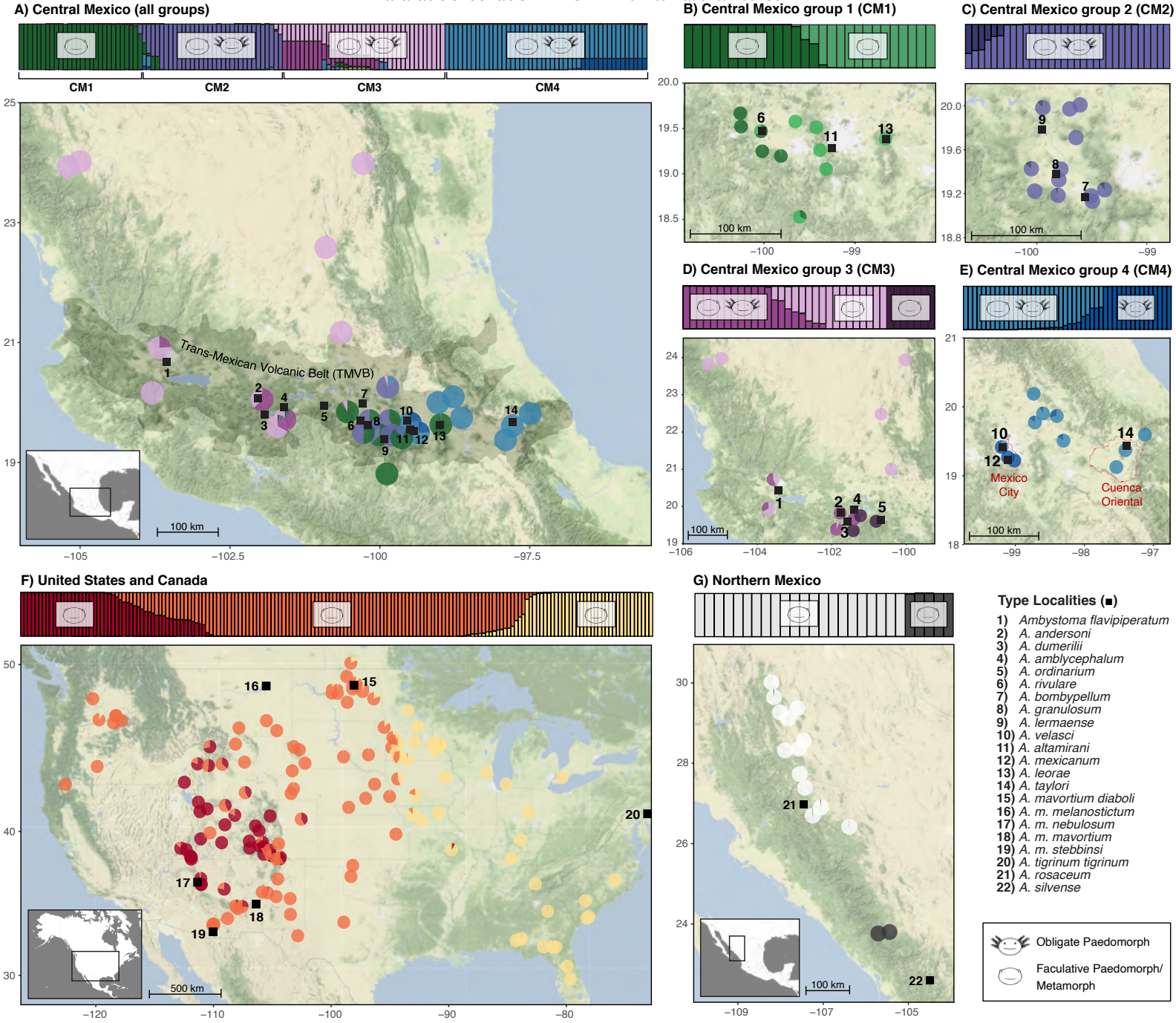

Figure 2. Results of population genomic analyses on the *Ambystoma tigrinum* species complex. (A) STRUCTURE analysis of the central Mexico cluster, which identified four major groups. Each vertical bar represents an individual, while the y-axis shows the membership probability for each group. Pie charts mapped below the assignment plot show the average group membership probability for that locality. Some localities were combined to a single pie chart to reduce visual clutter; see Table S1 for specific coordinates of each sample. The range of the Trans-Mexican Volcanic Belt (TMVB) is identified by shading and is modified from Ferrari (2004). (B-E) Detailed STRUCTURE analyses of each central Mexico subgroup CM1-CM4. A geographic outlier placed in CM2 is thought to represent a geographic range introduction and is not shown here, but is discussed in supplementary text and shown in Fig. S6. The range of the Cuenca Oriental is outlined in red and is modified from Percino-Daniel et al. (2016). (F,G) STRUCTURE results from analyses of the U.S. and northern Mexico groups, respectively. Detailed results are shown in Figs. S8-S9. Black squares on the maps denote the type localities of all species included in this study. On each STRUCTURE membership plot, salamander face symbols denote the presence of obligately or facultatively paedomorphic populations (with or without gills, respectively); groups labeled with both symbols contain a mixture of life history strategies.

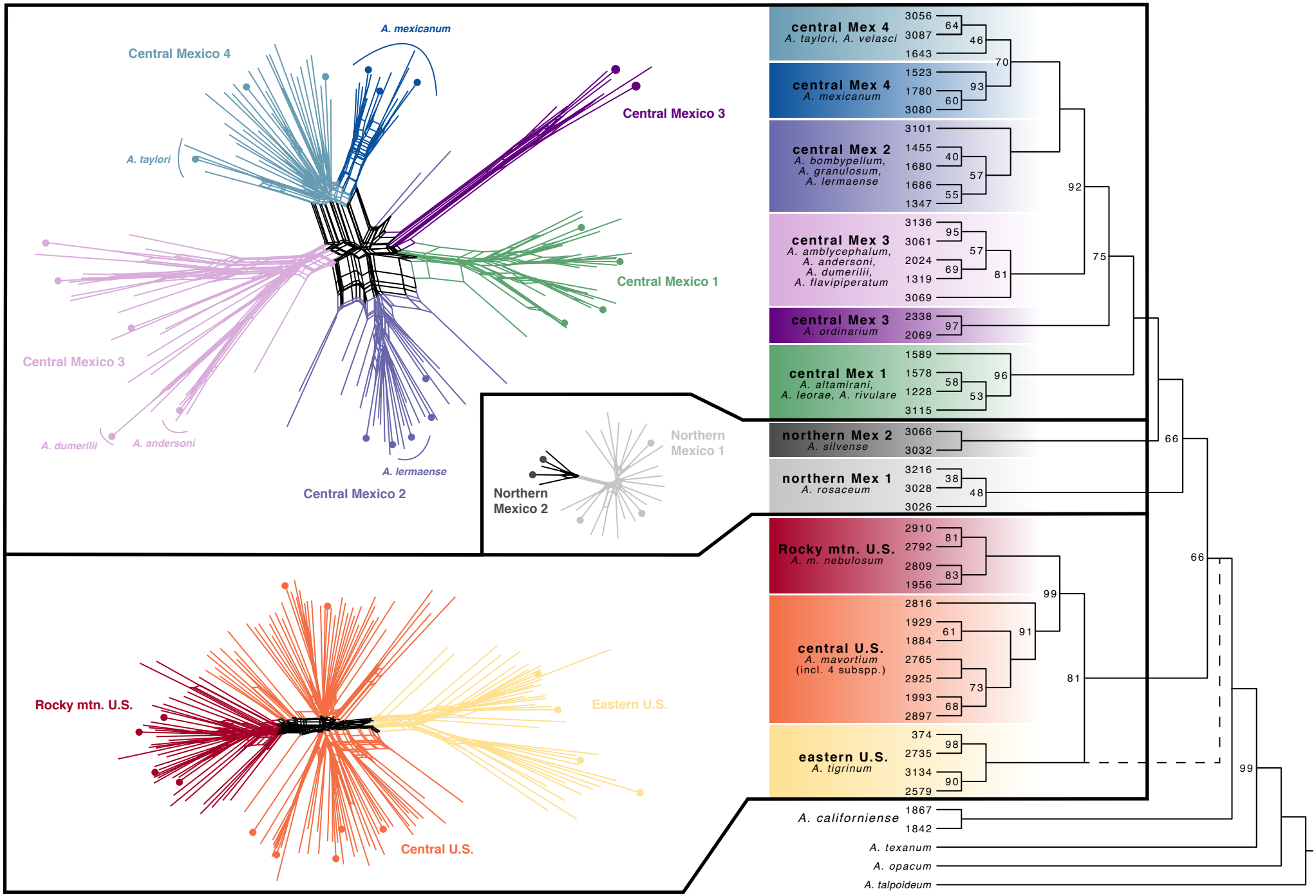

Figure 3. Results of phylogenetic network analyses (left) and the quartets-based phylogenetic analysis (right) of the *Ambystoma tigrinum* species complex. Clades are colored according to their cluster assignments in population genetic analyses, as shown in detail in Fig. 2. In phylogenetic networks, species names are only shown for taxa that are obligately or strongly paedomorphic; all other taxa (not shown) are considered to be facultatively paedomorphic or metamorphic. Note that the quartet tree analysis used a subset of individuals, indicated by solid circles on the phylogenetic networks. Phylogenetic results from Bayesian and maximum-likelihood concatenated analyses were largely concordant with the quartets tree (Fig. S10), although one important difference (the relationship of the eastern U.S. clade) is indicated by a dashed line. Bootstrap support (BS) values are indicated on each node of the quartets tree (BS values > 95 are not shown), while tips are labeled with individual IDs (Table S1). Branch lengths are not scaled to time or substitution rate.

analyses (41–43) recovered monophyletic CM1, CM2, and CM4 clusters (see below for discussion of CM3), but support values were often low within the central Mexico clade (Fig. 3).

Based on type locality and range information, the CM1 cluster includes three stream-breeding and facultatively paedomorphic taxa: *Ambystoma altamirani* Duges 1895 (44), *A. leorae* Taylor 1943 (45), and *A. rivulare* Taylor 1940 (9). We did not find evidence that these three taxa are reproductively isolated. Subsequent analyses of this cluster identified two admixed populations associated with eastern and western portions of its geographic distribution (Fig. 2B). Phylogenetically, CM1 was monophyletic and sister to all other central Mexican groups (Fig. 3), consistent with their original placement in a distinct genus, *Rhyacosiredon* Dunn 1928 (46).

Analyses of CM2 grouped *A. lermaense* Taylor 1940 (9) with samples corresponding to the type localities and range of *A. bombypellum* Taylor 1940 (9) and *A. granulosum* Taylor 1944 (47). There was no evidence for genetic isolation among any of these three taxa (Fig. 2C).

Within CM3, population-genetic analyses first separated samples of *A. ordinarium* Taylor 1940 (5) from the rest (with no hybridization detected; Fig 2D). Other samples were geographically structured according to the northern and southern portions of the distribution (Fig 1E). The more southern cluster included two obligately paedomorphic taxa, *A. andersoni* Krebs & Brandon 1984 (32) and *A. dumerilii* Duges 1870 (48), each endemic to single lake systems (Lakes Zacapú and Pátzcuaro, respectively). However, our results identified admixture between these lakes and other nearby localities. The samples from other CM3 localities likely represent *A. amblycephalum* Taylor 1940 (9) and *A. flavipiperatum* Dixon 1963 (49).

CM4 included the axolotl, *A. mexicanum* Shaw & Nodder 1798 (50), and *A. taylori* Brandon et al. 1981 (10)—both primarily considered to be near-obligate paedomorphs—as well as samples assigned to the polymorphic *A. velasci* Duges 1888 (51). Subsequent DAPC and

175 STRUCTURE analyses of CM4 placed all but one *A. mexicanum* individual sampled from Lakes
176 Xochimilco and Chapultepec (i.e., the remaining habitats of the axolotl) in a distinct cluster (Fig.
177 2E, S7-S8), with evidence of some admixture in samples to the northwest of Mexico City.
178 We used a Bayesian model-testing framework (Fig. 4A) to test for genetic isolation of
179 paedomorphic populations in three central Mexico groups. Specifically, we tested for the
180 presence or absence of gene flow between (near) obligate paedomorphs (*A. andersoni* and *A.*
181 *dumerilii* in CM3, and *A. mexicanum* and *A. taylori* in CM4) and their surrounding facultatively
182 paedomorphic populations. We also tested for gene flow between *A. lermaense* [which has been
183 described as "often neotenic [= paedomorphic]" (9, pg. 427)] and remaining populations in CM2.
184 In four out of the five groups, we recovered models of bidirectional gene flow or complete
185 panmixia between putatively obligate paedomorphs and facultative paedomorphs (Fig. 4B).
186 Alternative models of no migration or unidirectional migration were rejected with decisive Bayes
187 factors (Table S3). Migration rate estimates for full-migration scenarios consistently identified
188 higher levels of gene flow from facultative to obligate paedomorphic populations. We found
189 support for genetic isolation of one paedomorphic taxon, *A. andersoni* (Fig. 4B), a species
190 endemic to Lake Zacapú, Michoacán. As we are unable to account for landscape barriers or
191 sampling gaps in these analyses, we cannot be certain whether the lack of gene flow we
192 recovered for *A. andersoni* is due to its paedomorphic life history or to its geography. Another
193 important caveat to our migration analyses is that divergence time is not included as a model
194 parameter, which prevents distinguishing between historical and contemporary gene flow. Thus,
195 our ability to detect ongoing gene flow in these tests is limited.
196 Within the U.S. and Canada, DAPC and STRUCTURE identified three genetic clusters
197 (K = 3) excluding the allopatric, more differentiated *A. californiense*, with clear signs of

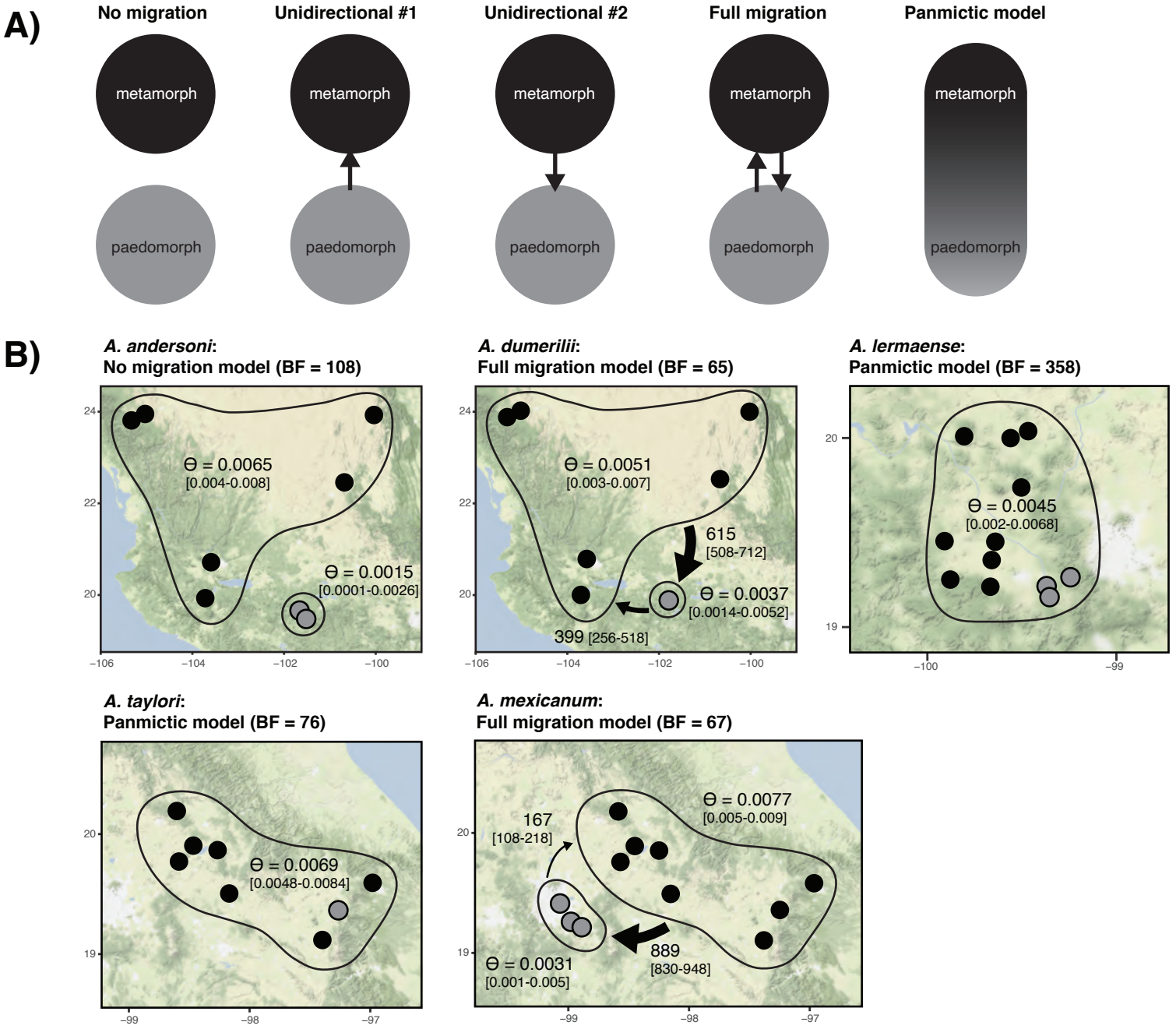

Figure 4. (A) Demographic models used to test hypotheses of gene flow. For each species considered to be obligately (*A. andersoni*, *A. dumerilii*, *A. taylori*, and *A. mexicanum*) or often (*A. lermaense*) paedomorphic, we evaluated five models of population structure and migration between that taxon and the facultatively paedomorphic taxa in the same genetic cluster (CM1-CM4). (B) Results of demographic model tests. Θ denotes the median estimated mutation-scaled effective size of each population. Values next to each arrow are median mutation-scaled migration rates ($M = m/\mu$, where m is the fraction of immigrants in each generation and μ is the mutation rate per generation per site). For both Θ and M, 95% confidence intervals are shown in brackets below the median value. Gray circles denote obligately paedomorphic lineages, while black circles denote facultatively paedomorphic lineages.

198 admixture along zones of contact (Figs. 2F, S9, S10). Geographically, these clusters are
199 associated with the eastern U.S., the central + western U.S., and the southwestern U.S. + Rocky
200 Mountains (hereafter, we refer to the latter two groups as U.S. central and U.S. Rocky
201 Mountains, respectively). Phylogenetic analyses corroborated these results, albeit with extensive
202 reticulations among clusters in the phylogenetic network (Fig. 3) and mixed support for species-
203 tree relationships among the three major groups (Fig. 3 vs. Fig. S12). Further exploration of
204 population structure within each of the three U.S. clusters recovered additional patterns of
205 differentiation (Figs. S9, S10), including northern and southern clusters in the eastern U.S. and
206 central U.S., and an allopatric cluster restricted to the Pacific Northwest. Overall, our U.S. results
207 are similar to mtDNA-based results from previous studies (11, 52), which identified haplotype
208 clades associated with the eastern U.S., the northern Great Plains, the southern Great Plains, and
209 the Pacific Northwest.
210 All analyses of the northern Mexico group identified two genetic clusters ($K = 2$; Figs.
211 2G, S11). The northern cluster corresponds to *A. rosaceum* Taylor 1941 (53), while the southern
212 cluster could represent either a second population of *A. rosaceum* or *A. silvense* Webb 2004 (54);
213 we did not include any samples from the type locality of *A. silvense* in this study. Phylogenetic
214 analyses recovered each of these two northern Mexico clusters as monophyletic, but not sister to
215 one another, and with few reticulate nodes in the phylogenetic network (Fig. 3, S12).
216
217 *The influence of life history on diversification*
218 A popular perspective on diversification in this species complex has been that the
219 evolution of obligate paedomorphosis promotes reproductive isolation and, ultimately, speciation
220 (10, 11). However, an important theme of our results is that taxa with different life history

strategies commonly cluster together by geography in population genetic and phylogeographic analyses (Figs. 2, 3). Moreover, our migration analyses support relatively recent gene flow and/or panmixia between facultative and paedomorphic populations (Fig. 4). Thus, the evolution of life history extremes does not appear to be the main driver of speciation in the tiger salamander complex. It is also worth noting that facultative paedomorphosis is the inferred ancestral condition (8) and nearly all extant lineages in the *Ambystoma tigrinum* complex are capable of metamorphosis, either in the wild or in the lab. Only *A. dumerilii* is not known to survive for long after metamorphosis (55). That only one described taxon in the complex is putatively fixed for paedomorphosis raises several questions: Are the rest of the primarily paedomorphic populations merely *en route* to fixation, rather than actually fixed? Do facultative populations fluctuate in paedomorphic frequency over time, dependent on prevailing environmental and/or demographic conditions (5)? Do transforming and non-transforming individuals readily interbreed outside of the laboratory setting? While these remain open questions, below we provide some relevant context for exploration and future research.

Theory predicts that paedomorphosis in salamanders is most likely to become fixed in a population when ecological conditions are favorable in the larval, aquatic environment but unfavorable on land (e.g., when terrestrial habitats are arid or resource-limited) (12, 56, 57). However, it is not known how long these fixations persist over evolutionary time; obligate paedomorphic lineages may have arisen and gone extinct multiple times over tiger salamander evolutionary history (58). One situation that would result in complete speciation is if paedomorphosis became fixed in the population and migration with surrounding facultatively paedomorphic populations ceased due to strong assortative mating and/or selection against hybrids (59–61). However, assortative mating within life history morphs has not been

244 demonstrated in other salamander groups (62–64) and there is evidence that different tiger
245 salamander morphs do interbreed (65). In fact, the only known instance of positive assortative
246 mating in *A. tigrinum* is associated with tail length (66, 67), which might favor metamorphosed
247 males as paedomorphic individuals can have relatively short tails (32). The evidence for
248 selection against intermorph offspring is also scarce. Captive breeding using artifical fertilization
249 has produced *A. mexicanum* x *A. tigrinum* hybrids (7, 31, 68) as well as *A. dumerilii* x *A.*
250 *tigrinum* and *A. dumerilii* x *A. rivularis* hybrids, although the fertility of offspring was not
251 investigated in *A. dumerilii* crosses (31). This suggests that postzygotic barriers are weak,
252 although the extent to which divergent lineages would interbreed in the wild remains a key
253 question in need of investigation.

254 If biological barriers to reproduction are limited or absent, there are several potential
255 mechanisms by which dispersal could mediate gene flow between paedomorphic and
256 metamorphic populations. First, metamorphosed individuals from facultatively paedomorphic
257 populations could move into bodies of water containing paedomorphic populations. Our
258 demographic analyses support this scenario, as they recovered higher rates of migration entering
259 rather than exiting obligately paedomorphic groups (Fig. 4). However, demographic analyses
260 also estimated non-zero emigration out of paedomorphic populations; thus, "obligate"
261 paedomorphs might also occasionally metamorphose and disperse. Occasional metamorphosis
262 has been documented in several putatively paedomorphic populations (9, 10, 33, 69, 70) and
263 metamorphosis can occur at higher frequencies in dispersing males in facultative populations
264 (71). Even a low frequency of transformed adults is likely sufficient to maintain evolutionary
265 cohesion with surrounding populations (10, 29, 72). Finally, gene flow may not necessarily be
266 mediated across a terrestrial environment; genetic connectivity could also be maintained if

267 populations have been forced into contact by water level fluctuations in the groundwater system
268 of a region. The dynamic lacustrine history of the Cuenca Oriental (73), for instance, suggests
269 that isolation of some paedomorphic populations has been punctuated by broader aquatic
270 connections over short geological time scales, potentially facilitating gene flow. Collectively,
271 these mechanisms are likely to explain the maintenance of geographic genetic groups, now or in
272 the evolutionarily recent past, containing a range of life histories.

273 The genetic cohesion we detected across wide swaths of the TMVB is particularly
274 striking given other evidence for local adaptation in this region. This scenario is perhaps best
275 exemplified in the Cuenca Oriental (Fig. 2E), where some lakes contain paedomorphic
276 salamanders adapted to saline conditions, a very rare trait among amphibians (74). At one site,
277 the saline Lake Alchichica, the population is currently considered a distinct species (*A. taylori*)
278 (10). However, our results indicate that *A. taylori* is not genetically distinct from surrounding,
279 putatively non-saline-adapted populations, a result consistent with a previous microsatellite-
280 based study that found significant gene flow to and from *A. taylori* (29). While we cannot rule
281 out the potential for localized signatures of selection and adaptation in the *A. taylori* genome, our
282 results highlight that even populations adapted to aquatic conditions intolerable to most other
283 *Ambystoma* have not reached a level of isolation consistent with an independent evolutionary
284 trajectory (75).

285

286 *Comparisons to previous work*

287 This study included extensive geographic sampling of the tiger salamander complex,
288 which provided a broad spatial context to more fully understand patterns of genetic variation.
289 However, it is important to consider the possibility that our data are simply not sensitive enough

290 to detect genetic differentiation associated with lineage divergence on an extremely recent time
291 scale. The common ancestor of *A. californiense* and the remainder of the species complex dates
292 to approximately five million years (11, 37), and phylogenomic data indicate that speciation
293 across the remaining tiger salamander lineages occurred within the last one million years (76).
294 Such timing could make lineage boundaries difficult to detect (15). In this context, our genome-
295 wide markers, developed from transcriptomic resources (35), may not have an overall
296 substitution rate sufficient to detect genetic differentiation. We recommend that future research
297 uses a larger genomic dataset or faster-evolving loci with particular focus on the sampling of
298 obligate paedomorphs in this system. To this point, perhaps the strongest evidence for the genetic
299 divergence of obligately paedomorphic populations comes from past microsatellite-based work
300 indicating the genetic distinctiveness of *A. andersoni* and *A. mexicanum* from a select set of
301 populations across central Mexico (77) and the genetic distinctiveness of *A. taylori* from
302 neighboring populations in the Cuenca Oriental (29). Both of these studies recovered signatures
303 of gene flow, but identified greater overall population structure compared to this study. These
304 previous results could be due to numerous factors, including a faster microsatellite mutation rate,
305 methodological differences, and more limited locality sampling.
306
307 **CONCLUSIONS**
308 The extent to which populations within the tiger salamander complex exhibit phenotypic
309 plasticity in life history traits is remarkable and is believed to have played a role in the rapid
310 accumulation of lineages observed in the highlands of central Mexico (11, 61). While our results
311 suggest there is less species-level diversity in that region than previously recognized, there is
312 clearly more diversity in central Mexico compared to the US and Canada where there is (a) more

geographic space and (b) less life history variation within and between lineages. While we cannot fully explain the greater diversity in Central Mexico, our results suggest that major patterns of diversification are related to a complex history of geographic isolation and secondary contact, in which life history strategy has played a less important role. We agree with previous work (12) that the complex geological history of the TMVB, including montane uplift and fluctuating drainage connectivity since the Miocene, has been the cornerstone of the evolutionary history of this species complex, and that the influences of geographic isolation and paedomorphosis may work synergistically to lead to the establishment of isolated populations. Lingering questions notwithstanding, this large-scale genetic and geographic study has established a framework for understanding the evolutionary history of the *Ambystoma tigrinum* species complex. The results presented here will facilitate comparative studies of the axolotl and its allies, provide direction for conservation prioritization and management, and strengthen the use of the tiger salamander species complex as a model system in biology.

MATERIALS & METHODS

Geographic sampling

We generated data from a total of 254 individuals sampled from across the range of the *A. tigrinum* complex (Fig. S1; Table S1). These individuals were combined with 93 individuals sampled in O'Neill et al. (35) to produce a data set comprising 347 individuals. To represent the large geographic range of this species complex, we chose to sample a large number of localities (188) with limited numbers of individuals sampled per locality (mean = 1.8, min. = 1, max. = 9). This sampling included a total of 166 individuals from the US, 2 individuals from Canada, and 178 individuals sampled from Mexico. Individuals of *A. californiense* were sampled from

336 localities with limited to no impacts of introgression from invasive western tiger salamanders
337 (52). Additional outgroup data were generated for two species outside of the *A. tigrinum*
338 complex: *A. opacum* and *A. texanum*. Full details regarding the generation of these data can be
339 found in the Supplementary Materials.
340
341 *Data collection and sequencing*
342 We generated DNA sequence data from a panel of 95 nuclear loci developed specifically
343 for the tiger salamander species complex. A more complete description of marker development
344 can be found in O'Neill et al. (35). Genomic DNA was extracted using a DNeasy Blood and
345 Tissue kit (Qiagen). For a small number of DNA extractions, we increased DNA quantities using
346 a Repli-g whole genome amplification kit (Qiagen). We used an initial round of PCR in 96-well
347 plate format to amplify all loci from an individual, followed by a smaller second round of PCR to
348 amplify loci that did not amplify in the initial PCR. See O'Neill et al. (35) for the details of PCR
349 conditions and primer sequences.
350 PCR products from all loci were pooled for each individual in roughly equal
351 concentrations based on the intensity of amplification as visualized on an agarose gel. Indexed
352 Illumina sequencing libraries were generated for each individual using an Illumina Nextera XT
353 DNA Library Preparation Kit (Illumina). Subsequent to library preparation, indexed libraries
354 were quantified using a Qubit Fluorometer, pooled in equimolar concentrations, and checked on
355 an Agilent Bioanalyzer to assure proper fragmentation.
356 Sequencing was performed in four rounds using an Illumina MiSeq. We performed an
357 initial round using a total of seven individuals to test the compatibility of the Illumina Nextera
358 XT library kit with our PCR amplicons. Three subsequent rounds of library preparation and

359 sequencing were performed on sets of 96 individuals each. Note that some individuals were
360 sequenced in multiple rounds due to initially low read counts. All sequencing was performed
361 with paired-end 150 bp reads. Overall, we generated a total of 29,426,894 PE reads across all
362 newly sequenced individuals, with an average of 309,757 PE reads, 1,148X coverage, and 7.6%
363 missing loci per individual (Table S2).
364
365 *Bioinformatics and dataset generation*
366 All sequence reads were processed using a newly developed bioinformatic pipeline
367 written for this project (https://doi.org/10.5281/zenodo.3585970) that produces multiple
368 sequence alignments for individual loci and genome-wide SNP matrices sampled from variable
369 sites. This pipeline was developed using the Snakemake workflow management system (78),
370 linking together multiple software tools to take sequence data from raw reads to phased sequence
371 alignments for each locus. Briefly, as used in this study, demultiplexed paired-end Illumina fastq
372 files were used as input, with separate forward and reverse read files for each individual.
373 Sequence data were trimmed and filtered in Trimmomatic (79) using a sliding window of 4 base-
374 pairs and a minimum average quality score of 15. Filtered sequence reads were then aligned to
375 reference sequences from the O'Neill et al. (2013) dataset (35); specifically, we used the clean
376 sequences of an *A. ordinarium* sample that had high coverage and low amounts of missing data.
377 The resulting aligned contigs were processed using SAMtools (80) to filter and prepare data for
378 FreeBayes (81), which was used to call variable sites. Variants were filtered with VCFTools (82)
379 by removing indels and setting a quality threshold of phred score > 20 and a minimum read
380 depth of 30. The program WhatsHap (83) was used to perform read-based phasing of the data for
381 each locus x individual contig. Finally, phased haplotypes from each individual (two copies,

382 regardless of homo- or heterozygosity) were combined into an alignment of all individuals using
383 MAFFT with the default auto parameter (84). We generated fasta files of SNPs using the SNP-
384 sites program (85) and created a SNP genotype matrix by sampling variable sites from a
385 concatenated sequence alignment of all loci. Through our informatic processing and manual
386 inspections of the data, we identified three loci (E12G1, E6A11, and E7G8) as potential paralogs
387 based on high alignment error and high levels of heterozygosity for all individuals. These three
388 loci were excluded from all analyses.

389 Across the remaining 92 loci, alignment lengths ranged from 123 to 630 bp (avg. = 270
390 bp) with a total concatenated alignment of 24,788 bp. For the full dataset, including *A. texanum*
391 and *A. opacum* outgroups, single-locus alignments contained an average of 55 variable sites
392 (min. = 15, max. = 121) and an average of 41 parsimony informative sites (min. = 11, max. =
393 102). Population genetic analyses restricted to the *A. tigrinum* complex including *A.*
394 *californiense*—which were further filtered for non-biallelic SNPs and a minor allele count ≥ 3—
395 contained a total of 2,360 SNPs.

396

397 *Population structure and lineage discovery*

398 We developed hypotheses of population-level lineages across the range of the *A. tigrinum*
399 complex using a bottom-up approach, starting with the identification of major geographic
400 patterns of differentiation, and then performing a recursive set of analyses on more restricted sets
401 of individuals, as identified in the previous analytical step. In our initial round of analyses we
402 used two non-parametric methods: principal components analysis (PCA) and discriminant
403 analysis of principal components (DAPC) (36). While both analyses provide a multivariate
404 summary of genetic data, DAPC is also used to assess the fit of data to varying numbers of

405 population clusters. These analyses were applied to our full genotypic dataset including *A.*
406 *californiense* and all remaining individuals from the *A. tigrinum* complex. The PCA was
407 calculated using the function 'prcomp' in the R package stats (86), while the DAPC was
408 calculated using the package adegenet (87). The optimal number of principal components to
409 retain for DAPC was identified using cross-validation via the xvalDapc function with default
410 parameter values. DAPC was performed without prior assignment of individuals to groups and
411 an exploratory approach was used to identify patterns of differentiation in ordination space
412 across a range of cluster levels (K = 1-20). We used two metrics to identify the best estimate of
413 the primary splits in our data. First, we used the Bayesian Information Criterion (BIC) calculated
414 in the DAPC analysis to assess the fit of the data to different levels of K. We note that the level
415 of K with the absolute lowest BIC may not be a better explanation of the data than a K with a
416 slightly higher BIC (88); therefore, we applied this measure for general guidance on a range of K
417 that may describe the data well. We paired this assessment with visualizations of the first and
418 second principal components (Fig. S2, S4) and DAPC ordination plots to identify the level of K
419 at which similar clustering patterns could be observed with minimal change at successively
420 higher levels of K. DAPC of the complete tiger salamander species complex identified a
421 consistent pattern beginning at K = 5 for high differentiation of all *A. californiense* samples (Fig.
422 S3). Further DAPC analysis with *A. californiense* removed identified a consistent pattern
423 beginning at K = 5 for differentiation between clusters of populations from northern and central
424 Mexico, and three clusters of U.S. populations (two from the Western U.S. and one from the
425 Eastern U.S.; Fig. S5).

426 Using the clusters identified in the DAPC analysis of the total data set, we then used both
427 DAPC and the program STRUCTURE v.2.3.4 (89) to analyze subsequent data sets comprising

428 smaller numbers of individuals. Recursive rounds of DAPC analyses were performed as
429 described above and were stopped when BIC scores showed little improvement (ΔBIC < 2) at
430 values of $K > 1$. STRUCTURE analyses were performed using an admixture model and a total of
431 500,000 generations following a burn-in of 100,000 generations. Analyses were performed for K
432 = 1-10 with 16 replicate analyses. We identified a "best" value of K using two approaches. First,
433 we calculated ΔK using the Evanno method (91) via the CLUMPAK web tool (90). A limitation
434 of the Evanno method is that it cannot identify the best value of $K = 1$ (91), thus, we also visually
435 inspected individual group assignments and concluded a value of $K = 1$ if the corresponding
436 DAPC cluster showed little improvement (ΔBIC < 2) at values of $K > 1$, or when visualization
437 showed extensive admixture without distinct clustering.
438
439 *Tests of migration and population structure*
440 We used the coalescent-based program Migrate-N v.3.2 (92) to explicitly test for
441 population structure and gene flow in each obligately paedomorphic species of *Ambystoma* (*A.*
442 *andersoni, A. dumerilii, A. taylori,* and *A. mexicanum,* plus the "often neotenic" species *A.*
443 *lermaense*). For each model we evaluated (Fig. 4A), we treated the obligately paedomorphic
444 species as one population, and the facultatively paedomorphic individuals from the same genetic
445 cluster (CM2-CM4) as the second population. The best-fitting model was determined via Bayes
446 factors (BF), which were calculated using Bézier-corrected marginal likelihoods (Table S2); the
447 highest-ranking models have a BF = 0. Each Migrate-N analyses ran for 50 million steps,
448 recording every 10 steps, with a burn-in of five million and the default heating scheme. Suitable
449 upper bounds for priors on population size (Θ) and migration rate (M) were determined from an

initial test run of 10 million steps for each analysis. Median values and 95% confidence intervals of Θ and *M* are reported (Fig. 4B).

Phylogenetic reconstruction

As our dataset included a large number of admixed individuals, we estimated phylogenetic networks to visualize reticulation events. We used SplitsTree v. 4.14.8 (40, 93) to generate four networks: one with all tiger salamander individuals, and one each for the U.S., central Mexico, and northern Mexico subgroups. Networks were constructed using uncorrected p-distances and the NeighborNet algorithm (94).

We also used three different analytical approaches to place hypothesized population lineages in a phylogenetic framework. For all analyses we used a reduced data set containing the concatenated data for 2-7 representative individuals from each population genetic cluster, which limited computation time and avoided violating the coalescent-model assumption of zero gene flow. We first inferred the phylogeny using Bayesian Inference in BEAST v.1.8.3 (42). Analyses were run for 5 million generations, sampling every 1,000 generations after the first 500,000 generations were removed as burn-in. Run convergence was assessed with Tracer v.1.6.0 (95). Next, we inferred a maximum-likelihood phylogeny using RAxML v.8 (43). Node support was assessed using a rapid bootstrap analysis with 1,000 replicates, which was summarized as a 95% rule consensus tree using the program SumTrees in the DendroPy python library (96). For both BEAST and RAxML, PartitionFinder (97) was used to identify the number of preferred gene partitions and their substitution models, and analyses were performed on the CIPRES Science Gateway server (98). Finally, we inferred phylogenetic relationships using SVDquartets (41) implemented in PAUP* version 4.0a164 (99), sampling all possible quartets and assessing node

support with 1,000 bootstrap replicates. For all phylogenetic analyses, trees were visualized using FigTree v.1.4.2 (100).

DATA AVAILABILITY

Supplementary figures and tables are provided with the online version of this manuscript. Input files for all population genetic and phylogenetic analyses are available via figshare (https://figshare.com/projects/Life_history_strategy_does_not_reflect_genetic_differentiation_in_the_tiger_salamander_species_complex/74115). Sequence data are available on the NCBI Sequence Read Archive (BioProject accession PRJNA594660).

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