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 best-known species, the Mexican 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, those able to forego metamorphosis and retain a larval, aquatic lifestyle (i.e., paedomorphosis), and those that do both. This life history variation has been assumed to lead to reproductive isolation and speciation, but the degree to which it has shaped population- and species-level divergence is poorly understood. Using a large multi-locus dataset from hundreds of samples across North America, we identified genetic clusters across the geographic range of the tiger salamander complex. These clusters often contain a mixture of paedomorphic and metamorphic taxa, indicating that geographic isolation has played a larger role in lineage divergence than paedomorphosis in this system. This conclusion is bolstered by geography-informed analyses indicating no effect of life history strategy on population genetic differentiation and by model-based analyses demonstrating gene flow between adjacent metamorphic and paedomorphic 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. Many members of the tiger salamander complex are endangered, and the Mexican axolotl is an important model system in regenerative and biomedical research. Our results chart a course for more informed use of these taxa in experimental, ecological, and conservation research.

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

51

52 **SIGNIFICANCE STATEMENT**

53 Population structure and speciation are shaped by a combination of biotic and abiotic
54 factors. The tiger salamander complex has been considered to be a key group where life history
55 variation has led to a rapid rate of speciation, driven in large part by the evolution of obligate
56 paedomorphosis–a condition where adults maintain an aquatic, larval phenotype. Using a large
57 multi-locus dataset, we present evidence of gene flow between taxa with different life history
58 strategies, suggesting that obligate paedomorphosis is not a strong driver of speciation in the
59 tiger salamander complex. Many of these nominal taxa are listed as critically endangered, and
60 our genetic results provide information and guidance that will be useful for their conservation.
61

62

63 **Introduction**

64 Life history—the complement of traits affecting survival and reproduction over an
65 organism's lifetime—affects ecology and dispersal, and therefore plays a potentially important
66 role in shaping population structure and speciation (1, 2). The Mexican axolotl (*Ambystoma*
67 *mexicanum*; hereafter, "axolotl") and related salamander species of the North American
68 *Ambystoma tigrinum* complex (Table 1) display enormous life history variation, particularly with
69 respect to the completion of metamorphosis (3). Some species are considered obligate
70 paedomorphs, where sexually mature adults retain a larval, aquatic body plan that includes
71 external gills and an enlarged tail fin (4). Others are considered obligate metamorphs,
72 transforming from aquatic larvae to terrestrial juveniles, eventually returning to the water to
73 breed as adults. Most populations are facultative, transforming under certain genetic and/or
74 environmental conditions (5–8). This lability in life history is believed to have played an
75 important role in the diversification of the *A. tigrinum* complex, particularly in the Trans-
76 Mexican Volcanic Belt (TMVB) region, where several paedomorphic species, including the
77 axolotl, are currently recognized (9–11). Obligate paedomorphosis is estimated to have evolved
78 in this region multiple times (11–13) in association with relatively large, permanent bodies of
79 water. Presumably, restricted gene flow between these isolated, paedomorphic populations led to
80 speciation as well as morphological adaptations to the aquatic lifestyle (14).

81 The term "species complex," while not a formal taxonomic category, is often used to
82 describe groups of closely related lineages early in the divergence process. The tiger salamander
83 species complex has been highlighted as a potentially valuable example of such a recent
84 radiation (15) that could provide insight into the early mechanisms initiating and/or maintaining
85 diversity (16–21). However, species complexes pose a number of challenges: phenotypic

86 differences among lineages may be subtle or absent (i.e., cryptic species), ancestral
87 polymorphisms may lead to incomplete lineage sorting that confounds molecular systematic
88 studies, and reproductive barriers may be incomplete. The reliance on phenotype for species
89 diagnosis in the *A. tigrinum* complex may require particular scrutiny, as several phenotypic traits,
90 including developmental state and adult color pattern, can be plastic and highly variable (22–24).
91 Given these challenges, fundamental questions remain concerning the reality of component
92 lineages within the tiger salamander complex, calling into question the current taxonomy as an
93 accurate reflection of its underlying evolutionary history.

94 Previous research has produced mixed results regarding levels of genetic differentiation
95 among lineages in the *A. tigrinum* complex, as well as the relative importance of paedomorphosis
96 as a driver of diversification. While population- and species-level structure is evident, an
97 important theme emerging across multiple studies has been that reproductive barriers are porous
98 (11, 25, 26). Shaffer and McKnight (11) noted "the striking lack of differentiation among the 14
99 species of the tiger salamander complex" (p. 425). Some studies have found an elevated degree
100 of population structure among paedomorphic populations relative to metamorphic populations
101 (12, 27). However, others have shown a lack of effect of paedomorphosis on population genetic
102 differentiation (28), and demonstrated that paedomorphic taxa and neighboring metamorphic
103 populations interbreed (29). Furthermore, crosses between metamorphic and paedomorphic taxa
104 can produce viable hybrid offspring under laboratory conditions (30, 31).

105 To understand the processes underlying diversification in the tiger salamander complex,
106 an important first step must be a range-wide assessment of population structure and the
107 clarification of population- and species-level boundaries. With this groundwork, insights into the
108 role of paedomorphosis and diversification can be addressed. For a study system of this

109 geographic and taxonomic scale, robust inference of population structure requires information
110 from multiple genes combined with thorough range-wide sampling. To meet these criteria, we
111 expand on a large multi-locus dataset containing 95 nuclear loci for 93 individuals (32), to
112 produce a data matrix for 347 individuals across the full geographic range of tiger salamanders
113 (Fig. S1). Given the complex and somewhat checkered history of the group's taxonomy, we
114 focus on a naïve approach, performing population structure analyses without *a priori*
115 identification of taxa to resolve geographic genetic clusters and characterize patterns of
116 admixture. We also overlay these results on the existing taxonomy to explore the correspondence
117 between current taxonomy and genetic differentiation. We then use these data for a phylogenetic
118 analysis to provide an updated working hypothesis for the evolution of the group. Finally, we test
119 the hypothesis that life history evolution has driven speciation in the tiger salamander complex,
120 particularly through the assumed isolation of paedomorphic species. In light of the life history
121 variation in this species complex, we use natural history information to place taxa into one of
122 five categories (Table 1) reflecting their propensity to metamorphose, then use model-based tests
123 of migration to ask whether paedomorphic populations (categories 4 and 5) exhibit greater levels
124 of genetic differentiation relative to those that regularly metamorphose (categories 1-3).
125

126 **RESULTS AND DISCUSSION**

127 *Identification of genetic lineages and phylogenetic relationships*

128 A principal components analysis (PCA) and a discriminant analysis of principal
129 components (DAPC; 33) both recovered *Ambystoma californiense* Gray 1853 as genetically
130 distinct from all other tiger salamanders (Fig. 1A, S2, S3), consistent with previous work (e.g.,
131 11, 34). Given that *A. californiense* is the only obligate metamorph in the complex (life history

132 category 1; Table 1) and is genetically divergent and geographically isolated from all other tiger
133 salamanders evaluated in this study, we focus the remaining analyses on the other members of
134 the complex. After removing *A. californiense* from the dataset, DAPC supported the recognition
135 of three primary genetic clusters: U.S. (including samples from southern Canada), northwestern
136 Mexico (primarily the Sierra Madre Occidental), and the central Mexican highlands (Fig. 1B,
137 S4). A PCA of these data produced similar clustering results, with ordination patterns largely
138 mirroring geographic sampling (Fig. S5). For a further discussion of the taxonomic implications
139 of our results, see the Supplementary Material.

140 Within central Mexico, we identified $K = 7$ as the best-fit number of clusters in both
141 DAPC and STRUCTURE analyses (Figs. 2A, S6). However, several of these clusters were only
142 represented as a small proportion of genetic assignments, rather than as meaningful groups of
143 individuals, and visual inspection at multiple levels of K revealed four clear geographic clusters
144 (hereafter referred to as CM1-CM4) that captured the primary patterns of genetic differentiation
145 and admixture across this region (Figs. 2A, S7-S8). Network analysis (35) also recovered groups
146 that generally correspond to CM1-CM4, but with evidence of many reticulations (Fig. 3). This
147 starburst-like pattern indicates the presence of conflicting topologies within the dataset (35),
148 which can be caused by biological processes including recent diversification with incomplete
149 lineage sorting, current or evolutionarily recent gene flow, or both. Phylogenetic analyses (36–
150 38) recovered monophyletic CM1, CM2, and CM4 clusters (see below for discussion of CM3),
151 but often with low statistical support (Fig. 3).

152 Based on type locality and range information, the CM1 cluster includes three stream-
153 breeding and facultatively paedomorphic taxa: *Ambystoma altamirani* Dugès 1895 (39), *A.*
154 *leorae* (Taylor 1943) (40), and *A. rivulare* (Taylor 1940) (9). Subsequent analyses of this cluster

155 identified two admixed populations associated with eastern and western portions of its
156 geographic distribution (Figs. 2B, S9). Phylogenetically, CM1 was monophyletic and sister to all
157 other central Mexican groups (Fig. 3).

158 CM2 grouped *A. lermaense* (Taylor 1940) (9) with samples corresponding to the type
159 localities and range of *A. bombypellum* Taylor 1940 (9) and *A. granulosum* Taylor 1944 (41).
160 There was no evidence for genetic isolation among any of these three taxa (Figs. 2C, S9).

161 Within CM3, population-genetic analyses first separated samples of *A. ordinarium* Taylor
162 1940 (5) from the rest (with no admixture detected; Figs. 2D, S9). Other samples clustered into
163 northern and southern groups with some admixture in intermediate localities. The more southern
164 cluster includes two obligately paedomorphic (category 5) taxa, *A. andersoni* Krebs & Brandon
165 1984 (42) and *A. dumerilii* (Dugès 1870) (43), endemic to Lakes Zacapú and Pátzcuaro,
166 respectively. The samples from other CM3 localities likely represent what are currently
167 classified as *A. amblycephalum* Taylor 1940 (9) and *A. flavipiperatum* Dixon 1963 (44).

168 CM4 included the axolotl, *A. mexicanum* (Shaw & Nodder 1798) (45), and *A. taylori*
169 Brandon et al. 1981 (10)—both category 4 paedomorphs—as well as samples assigned to the
170 facultatively paedomorphic (category 3) *A. velasci* (Duges 1888) (46). Subsequent DAPC and
171 STRUCTURE analyses of CM4 placed all but one *A. mexicanum* individual sampled from Lakes
172 Xochimilco and Chapultepec (i.e., the remaining habitats of the axolotl) in a distinct cluster (Fig.
173 2E, S9), with evidence of some admixture in samples to the northwest of Mexico City.

174 Within the U.S. and Canada (excluding *A. californiense)*, DAPC and STRUCTURE
175 identified three genetic clusters ($K = 3$), with clear signs of admixed contact zones (Figs. 2F,
176 S10, S11). Geographically, these clusters are associated with the eastern U.S., the central +
177 western U.S., and the southwestern U.S. + Rocky Mountains (hereafter, we refer to the latter two

178 groups as central U.S. and Rocky Mountain U.S., respectively). Populations in the eastern U.S.
179 are assignable to *A. tigrinum* Green 1825, while central U.S. and Rocky Mountain U.S.
180 populations to *A. mavortium* Baird 1850. Phylogenetic analyses corroborated these results, albeit
181 with extensive reticulations among clusters in the phylogenetic network (Fig. 3) and mixed
182 support for species-tree relationships among the three major groups (Fig. 3 vs. Fig. S13). Further
183 exploration of population structure within each of the three U.S. clusters recovered additional
184 patterns of differentiation (Figs. S10, S11), including northern and southern clusters in the
185 eastern U.S. and central U.S., and an allopatric cluster restricted to the Pacific Northwest.
186 Overall, our U.S. results are similar to mtDNA-based results from previous studies, which
187 identified haplotype clades associated with the eastern U.S., Great Plains and Rocky Mountains,
188 and the Pacific Northwest (11, 47).
189 All analyses of the northern Mexico group identified two genetic clusters ($K = 2$; Figs.
190 2G, S12). The northern cluster corresponds to *A. rosaceum* Taylor 1941 (48), while the southern
191 cluster is likely *A. silvense* Webb 2004 (49, see also 50). Phylogenetic analyses recovered each
192 of these two northern Mexico clusters as monophyletic, but not sister to one another, and with
193 few reticulate nodes in the phylogenetic network (Fig. 3, S13).
194
195 *Does paedomorphosis lead to increased genetic differentiation?*
196 As a first step in addressing this question, we used Bayesian modeling to test for genetic
197 isolation of paedomorphic taxa (i.e., species scored as life history category 4 or 5 in Table 1). We
198 tested for gene flow between paedomorphs–*A. andersoni* and *A. dumerilii* in CM3, and *A.*
199 *mexicanum* and *A. taylori* in CM4–and their surrounding facultatively paedomorphic
200 populations. For both CM3 and CM4, the top-ranking models included migration among all

201 populations, regardless of their degree of paedomorphosis (Fig. 4). Alternative models of no
202 migration or restricted migration (Fig. S14) were rejected with decisive Bayes factors (Table S3).
203 In CM3, migration rates entering the *A. dumerilii* population were higher than those leaving that
204 population, while migration rates entering and exiting *A. andersoni* were approximately equal.
205 In CM4, migration rates exiting the *A. mexicanum* population were much higher than those
206 entering the population while migration rates entering and exiting *A. taylori* were approximately
207 equal (Fig. 4, Table S4). An important caveat to our migration and population structure analyses
208 is that divergence time is not included as a model parameter, which prevents distinguishing
209 between historical and contemporary gene flow. Thus, our ability to differentiate ongoing from
210 historical gene flow in these tests is limited.

211 We also assessed whether a paedomorphic life history is associated with greater
212 population genetic differentiation (calculated here as Φ_{ST}), which would be expected if
213 paedomorphosis is an important reproductive barrier (12). Plots of Φ_{ST} against geographic
214 distance showed that comparisons involving paedomorphic populations were largely overlapping
215 with those based on facultative-facultative comparisons, with no difference in slope or y-
216 intercept (Fig. 4). In both CM3 and CM4, partial Mantel tests found no significant correlation
217 between life history and pairwise Φ_{ST} after correcting for geographic distance (Fig. 4).
218

219 *The influence of life history on diversification*

220 A popular perspective on diversification in this species complex is that paedomorphosis
221 promotes reproductive isolation and, ultimately, speciation (10, 11). An important theme of our
222 results is that taxa with different life history strategies commonly cluster together by geography
223 in population genetic and phylogeographic analyses (Figs. 2, 3). Furthermore, genetic

224 differentiation is not significantly greater for paedomorphic populations, relative to facultative
225 populations, and there has been substantial gene flow among populations with different life
226 history strategies (Fig. 4). Thus, the evolution of life history extremes does not appear to be the
227 main driver of speciation in the tiger salamander complex.

228 It is also worth noting that facultative paedomorphosis is the inferred ancestral condition
229 (8) and nearly all extant lineages in the *Ambystoma tigrinum* complex are capable of
230 metamorphosis, either in the wild or in the lab. Only *A. dumerilii* is not known to survive
231 metamorphosis, even under laboratory conditions (51). That only one described taxon in the
232 complex is demonstrably fixed for paedomorphosis raises several questions: Are the rest of the
233 primarily paedomorphic populations *en route* to fixation, rather than actually fixed? Have
234 lineages fixed for paedomorphosis gone extinct? Do facultative populations fluctuate in
235 paedomorph frequency over time, depending on environmental and/or demographic conditions
236 (5)? Do transforming and non-transforming individuals interbreed in nature? While these remain
237 open questions, below we provide some relevant context for exploration and future research.

238 Theory predicts that paedomorphosis in salamanders is most likely to become fixed in a
239 population when ecological conditions are favorable in the larval, aquatic environment but
240 unfavorable on land (e.g., when terrestrial habitats are arid or resource-limited) (12, 52, 53).
241 However, it is not known how long fixed populations persist over evolutionary time; obligate
242 paedomorphic lineages may have arisen and gone extinct multiple times over tiger salamander
243 evolutionary history (54). One situation that would result in complete speciation is if
244 paedomorphosis became fixed in the population and migration with surrounding facultatively
245 paedomorphic populations ceased due to strong assortative mating and/or selection against
246 hybrids (55–57). However, assortative mating within life history morphs has not been

247 demonstrated in other salamanders (58–60) and different tiger salamander morphs can interbreed
248 (61). In fact, the only known instance of positive assortative mating in *A. tigrinum* is associated
249 with tail length (62, 63), which might favor metamorphosed males as paedomorphic individuals
250 can have relatively short tails (42). The evidence for selection against intermorph offspring is
251 also scarce. Captive breeding using artificial fertilization has produced *A. mexicanum* x *A.*
252 *tigrinum*, *A. dumerilii* x *A. tigrinum*, *A. andersoni* x *A. tigrinum*, and *A. dumerilii* x *A. rivulare*
253 hybrids (7, 31, 64–66). This suggests that postzygotic barriers are weak, although the extent to
254 which divergent lineages would interbreed in the wild remains a key question in need of
255 investigation.

256 If biological barriers to reproduction are limited or absent, there are several potential
257 mechanisms by which dispersal could mediate gene flow between paedomorphic and
258 metamorphic populations. First, metamorphosed individuals from facultatively paedomorphic
259 populations could move into bodies of water containing paedomorphic populations. Our
260 demographic analyses support this scenario, as they recovered immigration from facultative into
261 paedomorphic populations (Fig. 4). However, demographic analyses also estimated emigration
262 out of paedomorphic populations; thus, "obligate" paedomorphs might also occasionally
263 metamorphose and disperse. Occasional metamorphosis has been documented in several
264 putatively paedomorphic populations (9, 10, 42, 67–69), and may be more prevalent than
265 currently recognized because rare transformed individuals in nature are virtually impossible to
266 verify (70). Importantly, even a low frequency of transformed adults is likely sufficient to
267 maintain evolutionary cohesion with surrounding populations (10, 29, 71). Alternatively, the
268 dispersal of paedomorphs might be human-mediated; this could partially explain the elevated
269 emigration rates we observed in the axolotl (Fig. 4). Axolotls are known to be sold in local

markets as food, bait, or aquarium pets, and are sometimes moved from one area of central Mexico to another (72). Finally, gene flow may not necessarily be mediated across a terrestrial environment; genetic connectivity could also be maintained if populations have been forced into contact by water level fluctuations in the groundwater system of a region. The dynamic lacustrine history of the Cuenca Oriental (73), for instance, suggests that isolation of some paedomorphic populations has been punctuated by broader aquatic connections over short geological time scales, potentially facilitating gene flow. Collectively, these mechanisms may explain the maintenance of geographic genetic groups, now or in the evolutionarily recent past, containing a range of life histories.

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

292 *Comparisons to previous work*

293 This study included extensive geographic sampling of the tiger salamander complex,
294 which provided a broad spatial context to more fully understand patterns of genetic variation.
295 However, it is important to consider the possibility that our data are simply not sensitive enough
296 to detect genetic differentiation associated with lineage divergence on an extremely recent time
297 scale. The common ancestor of *A. californiense* and the remainder of the species complex dates
298 to approximately five million years (11, 34), and phylogenomic data indicate that speciation
299 across the remaining tiger salamander lineages occurred within the last one million years (76).
300 Such recent timing renders lineage boundaries difficult to detect (15). Our markers, developed
301 from transcriptomic resources (32), may not have an overall substitution rate sufficient to detect
302 such subtle genetic differentiation. We recommend that future research uses a larger genomic
303 dataset or faster-evolving loci with particular focus on the sampling of paedomorphs in this
304 system. To this point, perhaps the strongest evidence for the genetic divergence of paedomorphic
305 populations comes from past microsatellite-based work indicating the genetic distinctiveness of
306 *A. andersoni* and *A. mexicanum* from a select set of populations across central Mexico (27) and
307 the genetic distinctiveness of *A. taylori* from neighboring populations in the Cuenca Oriental
308 (29). Both of these studies recovered signatures of gene flow, but identified greater overall
309 population structure compared to this study, which could be due to numerous factors, including a
310 faster microsatellite mutation rate and more limited locality sampling.

311

312 **CONCLUSIONS**

313 The extent to which populations within the tiger salamander complex exhibit phenotypic
314 plasticity in life history traits is remarkable and is believed to have played a role in the rapid

315 accumulation of lineages observed in the highlands of central Mexico (11, 57). While our results
316 suggest there is less species-level diversity in central Mexico than previously recognized, there is
317 clearly more diversity in this region compared to the US and Canada where there is (a) more
318 geographic space and (b) less life history variation within and between lineages. While we
319 cannot fully explain the greater diversity in central Mexico, our results suggest that major
320 patterns of diversification are related to a complex history of geographic isolation and secondary
321 contact, in which life history strategy has played a less important role, at least in the long
322 (evolutionary) run. We agree with previous work (12) that the complex geological history of the
323 TMVB, including montane uplift and fluctuating drainage connectivity since the Miocene, has
324 been the cornerstone of the evolutionary history of this species complex, and that the influences
325 of geographic isolation and paedomorphosis may work synergistically to lead to the
326 establishment of isolated populations. Lingering questions notwithstanding, this large-scale
327 genetic and geographic study establishes a framework for understanding the evolutionary history
328 of the *Ambystoma tigrinum* species complex. The results presented here will facilitate
329 comparative studies of the axolotl and its allies, provide direction for conservation prioritization
330 and management, and strengthen the use of the tiger salamander species complex as a model
331 system in biology.
332

333 **MATERIALS & METHODS**

334 *Geographic sampling*

335 We generated new data from 254 individuals sampled from across the range of the *A.*
336 *tigrinum* complex (Fig. S1; Table S1). These individuals were combined with 93 individuals
337 sampled in O'Neill et al. (32) to produce a data set comprising 347 individuals. We sampled a

338 large number of localities (188) with 1-9 individuals sampled per locality (mean = 1.8). This
339 sampling included 166 individuals from the US, 2 from Canada, and 178 from Mexico.
340 *Ambystoma californiense* samples were primarily included as a close outgroup to the remainder
341 of the species complex and were sampled from localities with limited to no impacts of
342 introgression from invasive *A. mavortium* (47). Additional outgroup data were generated for two
343 species outside of the *A. tigrinum* complex: *A. opacum* and *A. texanum*. Full details regarding the
344 generation of these data can be found in the Supplementary Materials. All *A. tigrinum* complex
345 species were assigned to a life history category based on review of the published literature and
346 private field records from authors of this study. These life history categories are as follow: 1 =
347 obligate metamorph (no paedomorphs have been documented in the field); 2 = strong bias
348 towards metamorphosis with rare paedomorphs found in the field; 3 = both metamorphosis and
349 paedomorphosis common in the field; 4 = strong bias towards paedomorphosis with rare
350 metamorphs found in the field; 5 = obligate paedomorph (no metamorphs have been documented
351 in the field). In the text, "paedomorph" describes taxa scored as 4 or 5, while "facultative
352 paedomorph" describes taxa scored as 2 or 3.
353
354 *Data collection and sequencing*
355 We generated DNA sequence data from a panel of 95 nuclear loci developed specifically
356 for the tiger salamander species complex. A more complete description of marker development
357 can be found in O'Neill et al. (32). Genomic DNA was extracted using a DNeasy Blood and
358 Tissue kit (Qiagen). For a small number of DNA extractions, we increased DNA quantities using
359 a Repli-g whole genome amplification kit (Qiagen). We used an initial round of PCR in 96-well
360 plate format to amplify all loci from an individual, followed by a smaller second round of PCR to

361 amplify loci that did not amplify in the initial PCR. See O'Neill et al. (32) for the details of PCR
362 conditions and primer sequences.
363 PCR products from all loci were pooled for each individual in roughly equal
364 concentrations based on the intensity of amplification as visualized on an agarose gel. Indexed
365 Illumina sequencing libraries were generated for each individual using an Illumina Nextera XT
366 DNA Library Preparation Kit (Illumina). Subsequent to library preparation, indexed libraries
367 were quantified using a Qubit Fluorometer, pooled in equimolar concentrations, and checked on
368 an Agilent Bioanalyzer to assure proper fragmentation.
369 Sequencing was performed in four rounds using an Illumina MiSeq. We performed an
370 initial round using a total of seven individuals to test the compatibility of the Illumina Nextera
371 XT library kit with our PCR amplicons. Three subsequent rounds of library preparation and
372 sequencing were performed on sets of 96 individuals each, and some individuals were sequenced
373 in multiple rounds due to initially low read counts. All sequencing was performed with paired-
374 end 150 bp reads. Overall, we generated a total of 29,426,894 PE reads across all newly
375 sequenced individuals, with an average of 309,757 PE reads, 1,148X coverage, and 7.6%
376 missing loci per individual (Table S2).
377
378 *Bioinformatics and dataset generation*
379 All sequence reads were processed using a newly developed bioinformatic pipeline
380 written for this project (https://doi.org/10.5281/zenodo.3585970) that produces multiple
381 sequence alignments for individual loci and genome-wide SNP matrices sampled from variable
382 sites. This pipeline was developed using the Snakemake workflow management system (77),
383 linking together multiple software tools to take sequence data from raw reads to phased sequence

384 alignments for each locus. Demultiplexed paired-end Illumina fastq files were used as input, with
385 separate forward and reverse read files for each individual. Sequence data were trimmed and
386 filtered in Trimmomatic (78) using a sliding window of 4 base-pairs and a minimum average
387 quality score of 15. Filtered sequence reads were then aligned to reference sequences from the
388 O'Neill et al. (2013) dataset (32); specifically, we used the clean sequences of an *A. ordinarium*
389 sample that had high coverage and low amounts of missing data. The resulting aligned contigs
390 were processed using SAMtools (79) to filter and prepare data for FreeBayes (80), which was
391 used to call variable sites. Variants were filtered with VCFTools (81) by removing indels and
392 setting a quality threshold of phred score > 20 and a minimum read depth of 30. The program
393 WhatsHap (82) was used to perform read-based phasing of the data for each locus x individual
394 contig. Finally, phased haplotypes from each individual (two copies, regardless of homo- or
395 heterozygosity) were combined into an alignment of all individuals using MAFFT with the
396 default auto parameter (83). We generated fasta files of SNPs using the SNP-sites program (84)
397 and created a SNP genotype matrix by sampling variable sites from a concatenated sequence
398 alignment of all loci. We identified three loci (E12G1, E6A11, and E7G8) as potential paralogs
399 based on high alignment error and high levels of heterozygosity for all individuals. These three
400 loci were excluded from all analyses.

401 Across the remaining 92 loci, alignment lengths ranged from 123 to 630 bp (avg. = 270
402 bp) with a total concatenated alignment of 24,788 bp. For the full dataset, including *A. texanum*
403 and *A. opacum* outgroups, single-locus alignments contained an average of 55 variable sites
404 (min. = 15, max. = 121) and an average of 41 parsimony informative sites (min. = 11, max. =
405 102). Population genetic analyses restricted to the *A. tigrinum* complex including *A.*

406 *californiense*—which were further filtered for non-biallelic SNPs and a minor allele count ≥ 3—
407 contained a total of 2,360 SNPs.
408
409 *Population structure and lineage discovery*
410 We developed hypotheses of population-level lineages across the range of the *A. tigrinum*
411 complex ignoring the existing taxonomy, starting with the identification of major geographic
412 patterns of differentiation, and then performing a recursive set of analyses on more
413 geographically restricted sets of individuals. In our initial round of analyses we used two non-
414 parametric methods: principal components analysis (PCA) and discriminant analysis of principal
415 components (DAPC) (33). While both analyses provide a multivariate summary of genetic data,
416 DAPC is also used to assess the fit of data to varying numbers of population clusters. These
417 analyses were applied to our full genotypic dataset including *A. californiense* and all remaining
418 individuals from the *A. tigrinum* complex. The PCA was calculated using the function 'prcomp'
419 in the R package stats (85), while the DAPC was calculated using the package adegenet (86).
420 The optimal number of principal components to retain for DAPC was identified using cross-
421 validation via the xvalDapc function with default parameter values. DAPC was performed
422 without prior assignment of individuals to groups across a range of cluster levels (K = 1-20). We
423 used two metrics to identify the best estimate of the primary splits in our data. First, we used the
424 Bayesian Information Criterion (BIC) calculated in the DAPC analysis to assess the fit of the
425 data to different levels of K. We note that the level of K with the absolute lowest BIC may not be
426 a better explanation of the data than a K with a slightly higher BIC (87); therefore, we applied
427 this measure for general guidance on a range of K that may describe the data well. We paired this
428 assessment with visualizations of the first and second principal components (Fig. S2, S5) and

429 DAPC ordination plots to identify the level of *K* at which similar clustering patterns could be
430 observed with minimal change at successively higher levels of *K*. DAPC of the complete tiger
431 salamander species complex identified a consistent pattern beginning at $K = 5$ for high
432 differentiation of all *A. californiense* samples (Fig. S3). Further DAPC analysis with *A.*
433 *californiense* removed identified a consistent pattern beginning at $K = 5$ for differentiation
434 between clusters of populations from northern and central Mexico, and three clusters of U.S.
435 populations (two from the Western U.S. and one from the Eastern U.S.; Fig. S4).
436 Using the clusters identified in the DAPC analysis of the total data set, we then used both
437 DAPC and STRUCTURE v.2.3.4 (88) to analyze subsequent data sets comprising smaller
438 numbers of individuals. Recursive rounds of DAPC analyses were stopped when BIC scores
439 showed little improvement ($\Delta BIC < 2$) at values of $K > 1$. STRUCTURE analyses used an
440 admixture model and 500,000 generations following a burn-in of 100,000 generations. Analyses
441 were performed for $K = 1$-10 with 16 replicate analyses for each *K*. To help identify an optimal
442 value for *K*, we calculated ΔK using the Evanno method (89) via the CLUMPAK web tool (90).
443 A limitation of the Evanno method is that it cannot estimate the likelihood of $K = 1$ (89), thus,
444 we also visually inspected individual group assignments and concluded a value of $K = 1$ if the
445 corresponding DAPC cluster showed little improvement ($\Delta BIC < 2$) at values of $K > 1$, or when
446 individuals were simply being split without any geographic or individual clustering. We also
447 evaluated models where *K* was equal to the number of currently recognized species in that
448 genetic cluster (e.g., $K = 3$ for CM1, which included the type localities for three described
449 species: *A. altamirani*, *A. leorae*, and *A. rivulare*). We mapped individual species assignments
450 onto these results to test the potential correspondence between naïve clustering and the existing
451 taxonomy (Fig. S9).

452

453 *Phylogenetic reconstruction*

454 Given the high levels of admixture among groups, we used SplitsTree v. 4.14.8 (35, 91)
455 to generate four phylogenetic networks: one with all tiger salamander individuals, and one each
456 for the U.S., central Mexico, and northern Mexico subgroups. Networks were constructed using
457 uncorrected p-distances and the NeighborNet algorithm (92).

458 We also used three different analytical approaches to place hypothesized population
459 lineages in a phylogenetic framework. For all analyses we used a reduced data set containing the
460 concatenated data for 2-7 representative individuals from each population genetic cluster, which
461 limited computation time and avoided violating the coalescent-model assumption of zero gene
462 flow. We first inferred the phylogeny using Bayesian Inference in BEAST v.1.8.3 (37). Analyses
463 were run for 5 million generations, sampling every 1,000 generations after the first 500,000
464 generations were removed as burn-in. Run convergence was assessed with Tracer v.1.6.0 (93).
465 Next, we inferred a maximum-likelihood phylogeny using RAxML v.8 (38). Node support was
466 assessed using a rapid bootstrap analysis with 1,000 replicates, which was summarized as a 95%
467 rule consensus tree using the program SumTrees in the DendroPy python library (94). For both
468 BEAST and RAxML, PartitionFinder (95) was used to identify the number of preferred gene
469 partitions and their substitution models, and analyses were performed on the CIPRES Science
470 Gateway server (96). Finally, we inferred phylogenetic relationships using SVDquartets (36)
471 implemented in PAUP* version 4.0a164 (97), sampling all possible quartets and assessing node
472 support with 1,000 bootstrap replicates. For all phylogenetic analyses, trees were visualized
473 using FigTree v.1.4.2 (98).

474

475 *Tests of migration and population differentiation of paedomorphic taxa*

476 We used the coalescent-based program Migrate-N v.3.2 (99) to explicitly test for
477 population structure and gene flow in each obligately paedomorphic species of *Ambystoma* (*A.*
478 *andersoni, A. dumerilii, A. taylori,* and *A. mexicanum*). For each model (Fig. S14), we treated
479 paedomorphic species as distinct populations, and the facultatively paedomorphic individuals
480 from the same genetic cluster (CM3 or CM4) as another population. The best-fitting model was
481 determined via Bayes factors (BF), which were calculated using Bézier-corrected marginal
482 likelihoods (Table S3); the highest-ranking models have a BF = 0. Each Migrate-N analyses ran
483 for 50 million steps, recording every 10 steps, with a burn-in of five million and the default
484 heating scheme. Suitable upper bounds for priors on population size (Θ) and migration rate (M)
485 were determined from an initial test run of 10 million steps for each analysis. Median values and
486 95% confidence intervals of Θ and M are in Fig. 4 and Table S4.

487 Within CM3 and CM4, we also calculated pairwise Φ_{ST} (a metric of population
488 differentiation) among all populations using the function 'pairPhiST' in the R package
489 haplotypes (100). These values were plotted against pairwise geographic distances (Fig. 4),
490 which were calculated from locality latitude/longitude data (Table S1) and converted to
491 kilometers using the function 'rdist.earth' in the R package fields (101). A life history matrix was
492 also generated by assigning a value of 0 for pairwise comparisons of populations with the same
493 life history and a value of 1 for populations with different life histories (i.e., facultatively
494 paedomorphic vs. obligately paedomorphic). Paedomorph-paedomorph comparisons were
495 omitted to avoid confounding them with facultative-facultative comparisons. Finally, we used
496 these matrices in a partial mantel test [function 'mantel.partial' package vegan (102)] to test for a

correlation between Φ_{ST} and life history while correcting for geographic distance. Significance of this test was determined using 999 permutations and an alpha level of 0.05.

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).

ACKNOWLEDGEMENTS

This project was supported by grants from the National Science Foundation [DEB-0949532, DEB-1355000, and DEB-1406876 (DDIG awarded to J. D. Kratovil)] and by a University Research Fellowship awarded to K. M. Everson from the University of Kentucky. We thank Chris Beachy, Jim Bogart, Ken Carbale, Sheri Church, Jeff LeClere, Carol Hall, Paul Moler, Nancy Staub, and Ken Wray for genetic samples, and Jose Bocanegra, Ben Browning, Mackenzie Humphrey, Mary Virginia Gibbs, Ricky Grewelle, Alan Lemmon, Emily Moriarty Lemmon, Deborah Lu, Stephanie Mitchell, Alex Noble, Tolu Odukoya, Ben Tuttle, and Josh Williams for assistance with data collection and analyses.

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745 **Figure Legends**

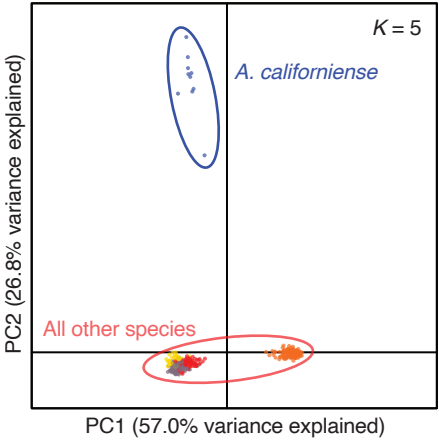
746 **Figure 1.** Discriminant analysis of principal components (DAPC) on (A) the entire genomic
747 dataset (without *A. opacum* and *A. texanum* outgroups), and (B) the genomic dataset without *A.*
748 *californiense*. In both analyses, patterns of genetic differentiation stabilized at values of $K \geq 5$ (K
749 = 5 is shown in both plots, with colors representing genetic clusters). Points represent individuals
750 and ellipses show the groups identified by DAPC. The first and second principal components
751 from the DAPC are on the x and y axes, respectively. Results from additional values of K are
752 provided in Supplementary Figures 3 and 4.
753
754 **Figure 2.** Population genomic analyses of the *Ambystoma tigrinum* species complex. (A)
755 STRUCTURE plot of central Mexico samples. While the Evanno et al. (2005) method identified
756 $K = 7$, four major geographic genetic groups are apparent and were analyzed separately in
757 subsequent analyses (B-E). In each plot, vertical bars represent individuals, while the y-axis
758 shows the membership probability for each group. Pie charts mapped below the assignment plot
759 show the average group membership probability for that locality. Some localities were combined
760 to a single pie chart to reduce visual clutter; see Table S1 for specific coordinates of each sample.
761 The range of the Trans-Mexican Volcanic Belt (TMVB) is identified by shading and is modified
762 from Ferrari (2004). The range of the Cuenca Oriental is outlined in red and is modified from
763 Percino-Daniel et al. (2016). Two geographic outliers placed in CM2 are thought to represent
764 range introductions and are not shown here, but are discussed in supplementary text and shown
765 in Fig. S9. (F,G) STRUCTURE results from analyses of the U.S. and northern Mexico groups,
766 respectively. Detailed results are shown in Figs. S11-S12. Black squares on the maps denote the
767 type localities of all species included in this study. On each STRUCTURE membership plot,
768 salamander face symbols denote the presence of salamanders with a life history category of 1-3
769 (the cartoon lacks gills) or 4-5 (paedomorphic, cartoon shows gills); groups labeled with both
770 symbols contain a mixture of these life history categories.
771
772 **Figure 3.** Results of phylogenetic network analyses (left) and the SVDquartets-based species
773 tree analysis (right) of the *Ambystoma tigrinum* species complex. Clades are colored according to
774 their cluster assignments in population genetic analyses, as in Fig. 2. In phylogenetic networks,
775 species names are only shown for paedomorphic taxa in categories 4 or 5 (Table 1). Life history
776 categories for all taxa are given in parentheses in the species tree. Note that SVDquartet analysis
777 used a subset of individuals, indicated by solid circles on the phylogenetic networks.
778 Phylogenetic results from Bayesian and maximum-likelihood concatenated analyses were largely
779 concordant with the quartets tree (Fig. S13), although two important differences are indicated by
780 dashed lines. Bootstrap support (BS) values are indicated on each node of the quartets tree (BS
781 values ≥ 95 are not shown), while tips are labeled with individual IDs (Table S1). Branch lengths
782 are not scaled to time or substitution rate.
783
784 **Figure 4.** Maps (left) show localities for CM3 and CM4, the two genetic clusters containing
785 paedomorphic taxa (life history categories 4 and 5). The top-ranked migration model is shown to
786 the right of each map, with median mutation-scaled effective population sizes (Θ) shown inside
787 the shapes denoting populations. Arrows indicate the direction of migration ($M = m/\mu$, where m
788 is the fraction of immigrants in each generation and μ is the mutation rate per generation per site)
789 and median migration rates are provided next to each arrow. Full model test results are provided

in Table S3 and full parameter estimates are provided in Table S4. Scatterplots (right) show pairwise geographic distances by pairwise ϕ_{ST} among all populations. Point colors and shapes denote the life history strategies of the pairwise comparison being made: black squares = both populations are facultatively paedomorphic; red circles = one population is facultatively paedomorphic and one population is obligately paedomorphic; black stars = both populations are obligately paedomorphic. Solid lines denote the line of best fit, calculated separately for each life history comparison. Regressions were not performed on paedomorph-paedomorph comparisons due to low sample sizes. Shaded areas denote the 95% confidence interval for the regression. P-values to the right of each graph were calculated using a partial mantel test, which tested for a correlation between ϕ_{ST} and life history while correcting for geographic distance (n.s. = not significant).

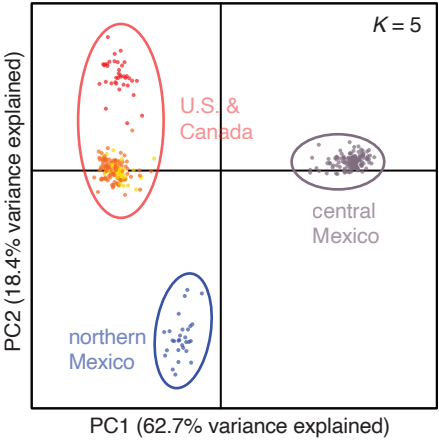
Table 1. Currently recognized species in the *Ambystoma tigrinum* complex. Habitat preference, range, conservation status ("Status"), and life history strategy were compiled from AmphibiaWeb (2019) and the IUCN (2019). Conservation status abbreviations: LC = least concern, V = vulnerable, E = endangered, CE = critically endangered, DD = data deficient. Life history codes: 1 = obligate metamorph (no paedomorphs documented in the field), 2 = Strong bias towards metamorphosis with rare paedomorphs found in the field, 3 = Both metamorphosis and paedomorphosis common in the field, 4 = Strong bias towards paedomorphosis with rare metamorphs found in the field, 5 = obligate paedomorph (no metamorphs documented in the field). In the text, "paedomorph" describes taxa scored as 4 or 5, while "facultative paedomorph" describes taxa scored as 2 or 3.

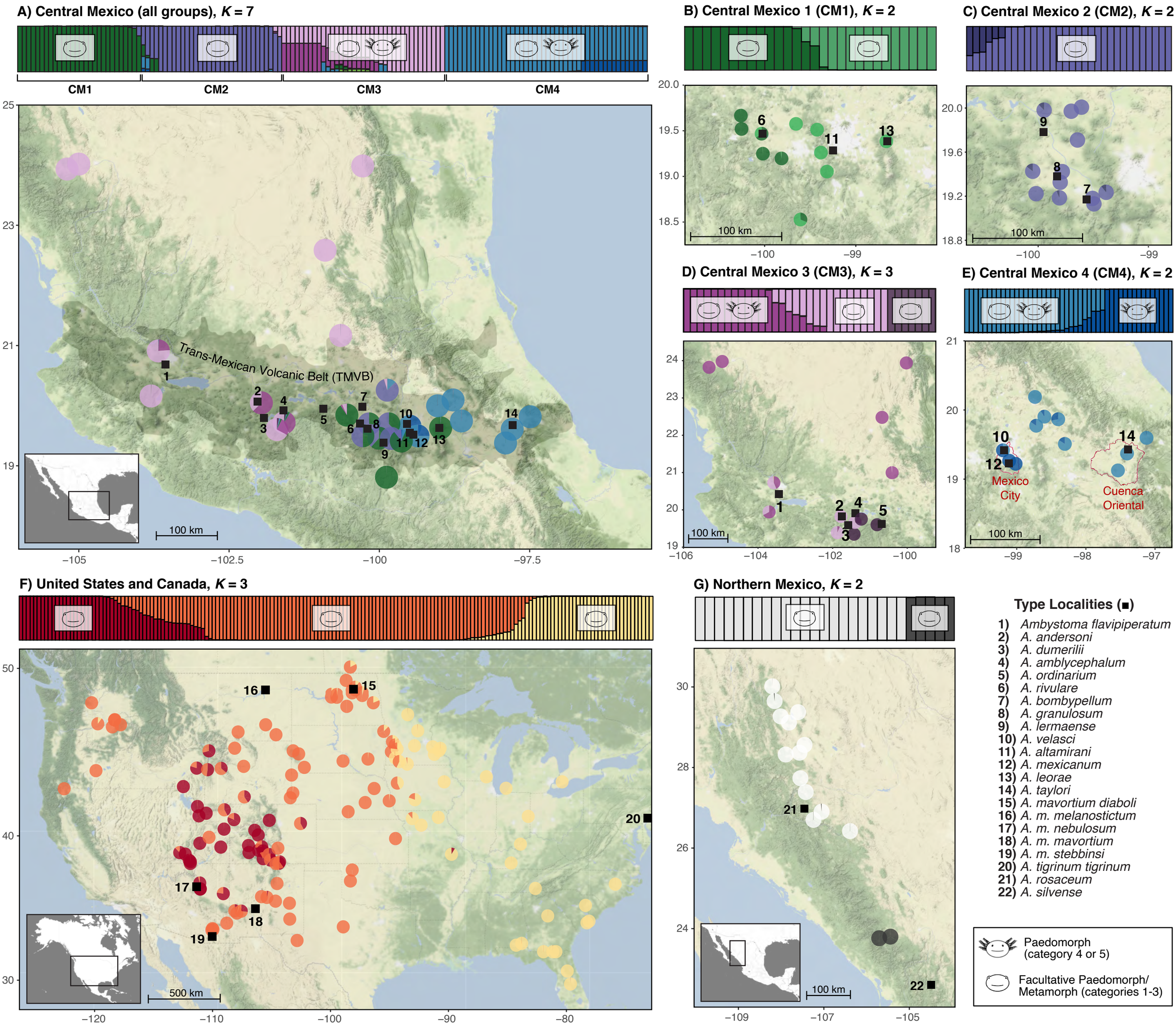
Species	Habitat Preference	Range	Status	Life History
Ambystoma altamirani Dugès, 1895	Streams, occasional ponds	High elevations in the west and south of the Valley of Mexico (Mexico State, Morelos, and Distrito Federal)	E	2
Ambystoma amblycephalum Taylor, 1940	Ponds	Small area of Tacicuaro (north and west Michoacan)	CE	2
Ambystoma andersoni Krebs & Brandon, 1984	Lakes / Ponds	Restricted to Lake Zacapu and its outflow streams (north-central Michoacan)	CE	5
Ambystoma bombypellum Taylor, 1940	Lakes / Ponds, occasional streams	Known only from type locality San Martin/Asunción, 15 km west of Morelia (Michoacan)	DD	2
Ambystoma californiense Gray 1853	Vernal pools	Great Central Valley and adjacent inner coast ranges, central California	V	1
Ambystoma dumerilii (Dugès, 1870)	Lakes / Ponds	Restricted to Lake Patzcauro (north-central Michoacan)	CE	5
Ambystoma flavipiperatum Dixon, 1963	Lakes / Ponds, occasional streams	Sierra de Quila (Jalisco). Historically known from the type locality in Santa Cruz, southwest of Guadalajara.	E	3
Ambystoma granulosum Taylor, 1944	Lakes / Ponds, occasional streams	Northwest of Toluca city (Mexico State and border of Michoacan)	CE	3
Ambystoma leorae (Taylor, 1943)	Streams	High elevations of Rio Frio in Iztaccihuatl -Popocatepetl Natl. Park (Mexico State and adjacent Puebla)	CE	2
Ambystoma lermaense (Taylor, 1940)	Lakes / Ponds	Highlands near Toluca, in Rio Lerma and Lake Lerma near Almoloya (Mexico State)	E	3
Ambystoma mavortium Baird, 1850*	Lakes / Ponds	Widespread distribution from west-central Canada through the USA to northern Mexico (Sonora, Chihuahua, Coahuila). Disjunct distribution in northwestern USA and Canada (Washington, British Columbia).	LC	3
Ambystoma mexicanum (Shaw and Nodder, 1798)	Lakes	Southern areas of Xochimilco (Mexico City). Historically distributed in Lakes of Xochimilco and Chalco.	CE	4
Ambystoma ordinarium Taylor, 1940	Streams	From south of Patzcuaro through Morelia City to eastern Michoacan	E	3
Ambystoma rivulare (Taylor, 1940)	Streams	Sierra Chinchua, Mariposa Monarca Biosphere Reserve, Michoacan, Valle de Bravo, Amanalco and Nevado de Toluca (Mexico State)	E	2
Ambystoma rosaceum Taylor, 1941	Streams	Sierra Madre Occidental (Sonora, Durango, Chihuahua, Sinaloa, Nayarit, Jalisco)	LC	3
Ambystoma silvense Webb, 2004	Lakes / Ponds	Sierra Madre Occidental (at least Chihuahua, Durango)	DD	3
Ambystoma taylori Brandon et al., 1982	Saline lake	Restricted to Lake Alchichica (Puebla)	CE	4
Ambystoma tigrinum Green, 1825	Lakes / Ponds	From southeast Canada south to east Texas and extending east of the Appalachian Mountains through the Atlantic Coastal Plain north to Long Island (New York)	LC	2
Ambystoma velasci (Dugès, 1888)	Lakes / Ponds, Streams	Sierra Madre Occidental (Chihuahua) and south across central Mexico (Durango, Jalisco, Nuevo León, Coahuila, Zacatecas, San Luis Potosi, Hidalgo, Queretaro, Tlaxcala, Puebla, Veracruz, Aguascalientes)	LC	3

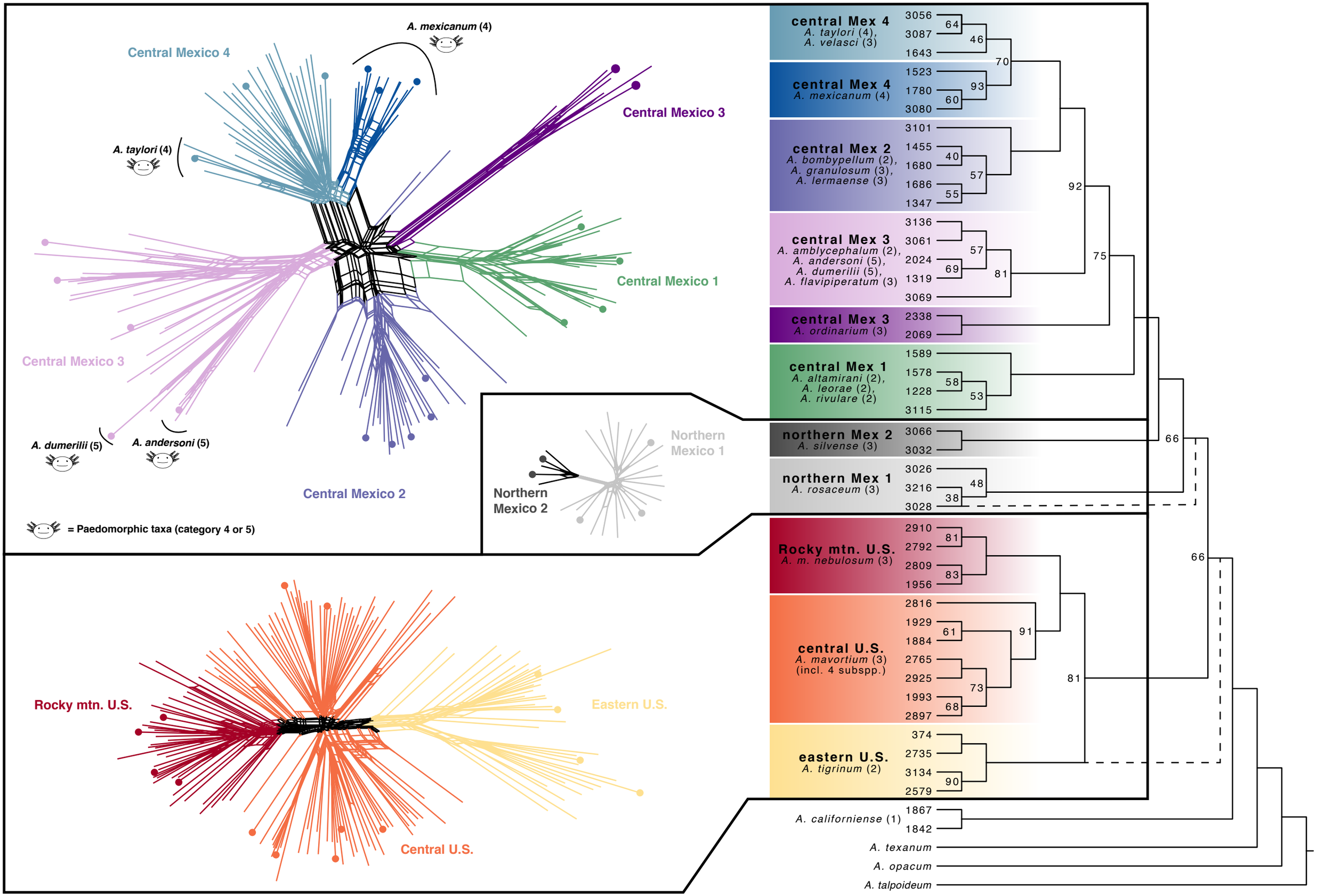
A) DAPC of full dataset



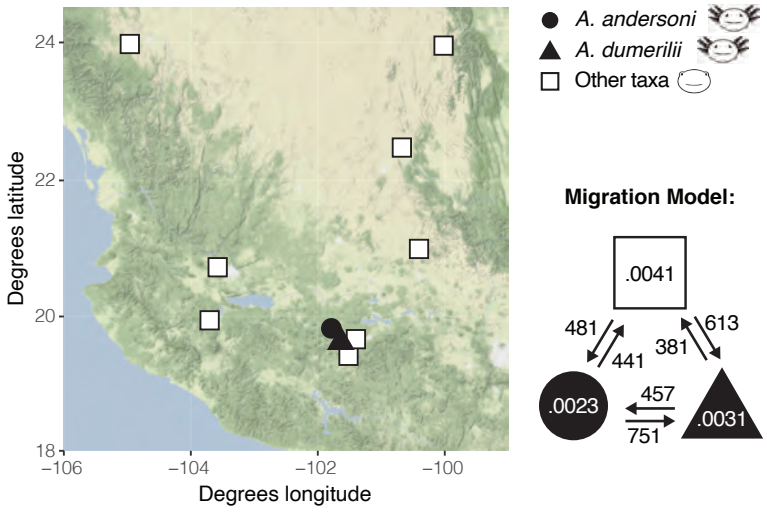
B) DAPC without *A. californiense*

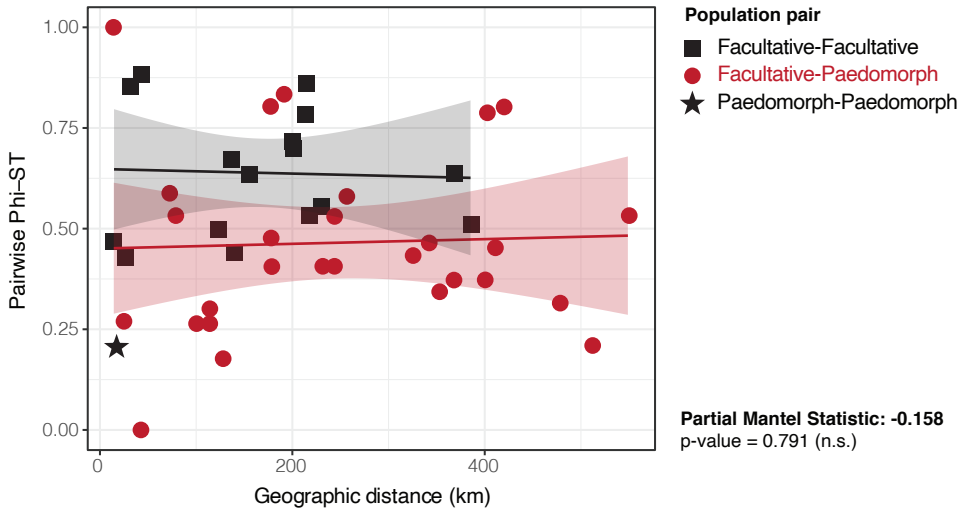
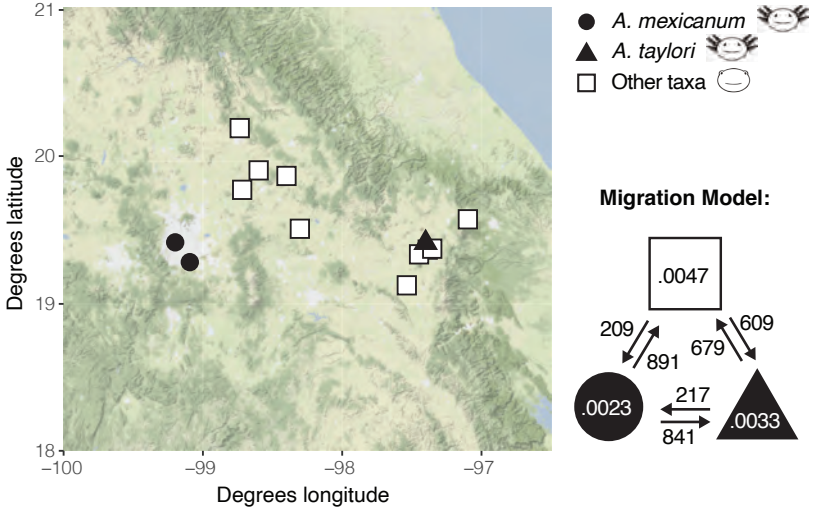
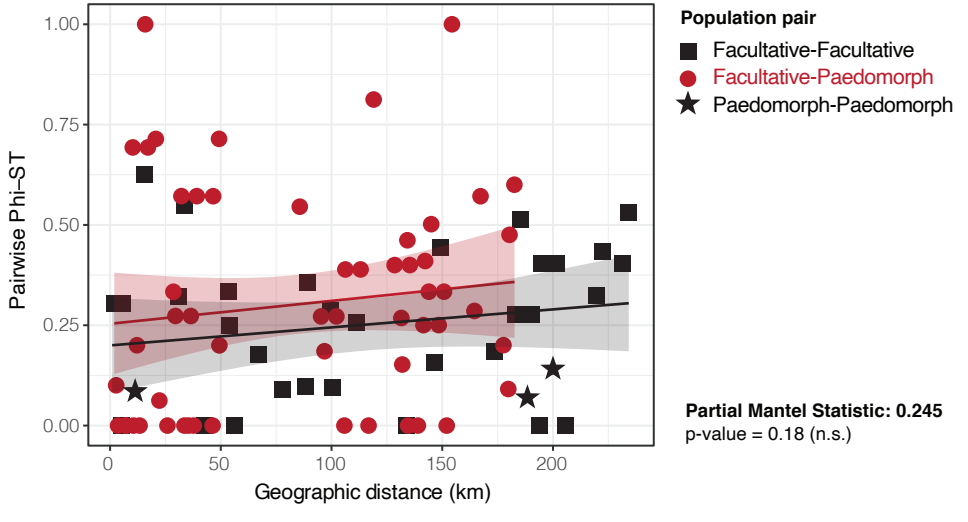



A) Central Mexico (all groups), K = 7
CM1
CM2
CM3
CM4
Trans-Mexican Volcanic Belt (TMVB)
100 km
B) Central Mexico 1 (CM1), K = 2
C) Central Mexico 2 (CM2), K = 2
D) Central Mexico 3 (CM3), K = 3
E) Central Mexico 4 (CM4), K = 2
Mexico City
Cuenca Oriental
F) United States and Canada, K = 3
500 km
G) Northern Mexico, K = 2
Type Localities (■)
1) Ambystoma flavipiperatum
2) A. andersoni
3) A. dumerilii
4) A. amblycephalum
5) A. ordinarium
6) A. rivulare
7) A. bombypellum
8) A. granulosum
9) A. lermaense
10) A. velasci
11) A. altamirani
12) A. mexicanum
13) A. leorae
14) A. taylori
15) A. mavortium diaboli
16) A. m. melanostictum
17) A. m. nebulosum
18) A. m. mavortium
19) A. m. stebbinsi
20) A. tigrinum tigrinum
21) A. rosaceum
22) A. silvense
Paedomorph (category 4 or 5)
Facultative Paedomorph/ Metamorph (categories 1-3)


A. mexicanum (4)
Central Mexico 4
Central Mexico 3
A. taylori (4)
Central Mexico 1
Central Mexico 3
A. dumerilii (5)
A. andersoni (5)
Central Mexico 2
= Paedomorphic taxa (category 4 or 5)
Northern Mexico 1
Northern Mexico 2
Rocky mtn. U.S.
Eastern U.S.
Central U.S.
central Mex 4
A. taylori (4), A. velasci (3)
3056
3087
1643
64
46
central Mex 4
A. mexicanum (4)
1523
1780
3080
93
60
70
central Mex 2
A. bombypellum (2), A. granulosum (3), A. lermaense (3)
3101
1455
1680
1686
1347
40
57
55
central Mex 3
A. amblycephalum (2), A. andersoni (5), A. dumerilii (5), A. flavipiperatum (3)
3136
3061
2024
1319
3069
57
69
81
92
75
central Mex 3
A. ordinarium (3)
2338
2069
central Mex 1
A. altamirani (2), A. leorae (2), A. rivulare (2)
1589
1578
1228
3115
58
53
northern Mex 2
A. silvense (3)
3066
3032
66
northern Mex 1
A. rosaceum (3)
3026
3216
3028
48
38
Rocky mtn. U.S.
A. m. nebulosum (3)
2910
2792
2809
1956
81
83
66
central U.S.
A. mavortium (3) (incl. 4 subspp.)
2816
1929
1884
2765
2925
1993
2897
61
91
73
68
81
eastern U.S.
A. tigrinum (2)
374
2735
3134
2579
90
A. californiense (1)
1867
1842
A. texanum
A. opacum
A. talpoideum

Central Mexico Group 3 (CM3)





Central Mexico Group 4 (CM4)