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The molecular phylogenetics of endangerment: cryptic variation and historical phylogeography of the California tiger salamander, *Ambystoma californiense*

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Abstract

A primary goal of conservation genetics is the discovery, delimitation and protection of phylogenetic lineages within sensitive or endangered taxa. Given the importance of lineage protection, a combination of phylogeography, historical geology and molecular clock analyses can provide an important historical context for overall species conservation. We present the results of a range-wide survey of genetic variation in the California tiger salamander, *Ambystoma californiense*, as well as a summary of the past several million years of inundation and isolation of the Great Central Valley and surrounding uplands that constitute its limited range. A combination of population genetic and phylogenetic analyses of mitochondrial DNA variation among 696 samples from 84 populations revealed six well-supported genetic units that are geographically discrete and characterized by nonoverlapping haplotype distributions. Populations from Santa Barbara and Sonoma Counties are particularly well differentiated and geographically isolated from all others. The remaining units in the Southern San Joaquin Valley, Central Coast Range, Central Valley and Bay Area are separated by geological features, ecological zone boundaries, or both. The geological history of the California landscape is consistent with molecular clock evidence suggesting that the Santa Barbara unit has been isolated for at least 0.74–0.92 Myr, and the Sonoma clade is equally ancient. Our work places patterns of genetic differentiation into both temporal- and landscape-level contexts, providing important insights into the conservation genetics of the California tiger salamander.

Keywords: *Ambystoma californiense*, California Great Central Valley, conservation genetics, declining amphibian, outgroup heteroduplex analysis, phylogeography

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Introduction

The interpretation of species boundaries among closely related lineages remains one of the primary challenges for systematists, historical biogeographers and conservation biologists alike (Avise *et al*. 1998; Sites & Marshall 2003). For well over 100 years, the tiger salamander complex has posed a fascinating set of challenges for amphibian systematists and biogeographers (Dunn 1940; Collins *et al*. 1980; Shaffer 1984a, 1984b; Templeton *et al*. 1995; Shaffer & McKnight 1996). Composed of ~15 species of closely related, largely allopatric taxa, the tiger salamander complex stands out as one of the primary groups in which life history evolution has led to rapid ecological and morphological diversification, particularly in central Mexico (Shaffer & Voss 1996; Voss & Shaffer 1997). Although full resolution of species boundaries remains incomplete (Shaffer & McKnight 1996; Highton 2000), one clear result is the deep divergence of the California tiger salamander, *Ambystoma californiense* from all remaining members of the complex. Current morphological and molecular evidence suggests that *A. californiense* is the sister group to the remaining species

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(Kraus 1988; Shaffer *et al*. 1991; Shaffer & McKnight 1996), implying that this narrowly distributed California endemic has maintained a separate and distinct evolutionary history since its separation from the rest of the complex. Given the antiquity of *A. californiense*, its current patchy distribution in the valleys of central California (Stebbins 2003), and the multiple speciation events that have taken place in its sister clade since their divergence, it seems plausible that considerable genetic structure may also exist within *A. californiense*.

Ambystoma californiense occupies a relatively narrow geographical and ecological range centred in the Great Central Valley of California from Tulare and San Luis Obispo Counties in the south, to Sacramento and Solano Counties in the north (Shaffer & Trenham in press). The species occurs from near sea level up to ~1200 m in the coast ranges and 500 m in the Sierra Nevada foothills. In addition to their primary range in the Great Central Valley and inner coast range, there are two disjunct, extralimital sets of populations; one is northwest of the main range near Santa Rosa, Sonoma County and the other is to the southwest in the Santa Maria region of northwestern Santa Barbara County (Storer 1925; Jennings & Hayes 1994; Fisher & Shaffer 1996). The entire range was historically dominated by grasslands ranging into open-canopy oak savannah, with salamanders breeding in vernal pools (Shaffer & Trenham in press). Ecologically, *A. californiense* has an obligate biphasic life cycle. Although larvae develop in the pools and ponds in which they were born, they are otherwise terrestrial salamanders that spend most of their postmetamorphic lives in widely dispersed, underground retreats (Trenham *et al*. 2001).

Recently, concern over the status of *A. californiense* has emerged as a major conservation issue. Stebbins (1989) was the first to pull together diverse lines of evidence documenting that the species was declining range wide, and he made strong recommendations for its immediate protection. Prompted by the Stebbins report and accumulating ecological and genetic data, the US Fish and Wildlife Service emergency listed the Santa Barbara (United States Fish and Wildlife Service 2000) and Sonoma (United States Fish and Wildlife Service 2003a) distinct population segments of *A. californiense* as endangered, and has proposed listing the remainder of the species as threatened (United States Fish and Wildlife Service 2003b). The Fish and Wildlife Service considers sets of populations to represent candidate 'distinct population segments' if they are both discrete and significant, based on ecological, genetic and/or distributional criteria (United States Fish and Wildlife Service 1996). Because of the strong reliance on genetics in the identification of distinct population segments, a key element in both the listing and recovery of *A. californiense* centres on the boundaries of genetically-defined units.

Our goal in this study was to explore the genealogical relationships of *A. californiense* populations across the range of the species, and if appropriate designate genetically-based distinct population segments. Our analyses were based on a comprehensive dataset of mitochondrial DNA (mtDNA) variation within and among populations. Given the high mutation rate and small effective population size of mtDNA, it remains an effective tool for identifying the major genetic units within a species (Hudson & Coyne 2002), even though the variance in coalescent times for any marker can be quite high (Hudson & Turelli 2003). We used both historical analysis of DNA sequences and population differentiation of haplotype frequencies to quantify genetic variation across the range of *A. californiense*, with a particular emphasis on the identification of deep, historically significant differentiation. We discuss our results in light of the historical geography of California, the systematics of *A. californiense* and the conservation genetics of the species.

Materials and methods

Sampling and geographical distribution

Our sampling is comprehensive, including sites from the main portion of the species range, as well as geographical outlier populations in Sonoma and Santa Barbara Counties (Stebbins 2003; Shaffer & Trenham in press). We also visited most of the historic sites beyond the current range limits, but these searches failed to locate specimens. We included specimens from all occupied sites reported in our recent field surveys (Shaffer *et al*. 1993; Fisher & Shaffer 1996), and new material from Alameda, Contra Costa, Merced, San Benito, San Luis Obispo, Santa Barbara and Sonoma Counties. Virtually all specimens were collected as larvae using seine nets. Specimens less than ~2 cm total length were generally frozen whole (–80 °C), and larger larvae were either sacrificed and tissued (vouchers retained in the University of California Davis Museum of Zoology), or tail-clipped and released within 1 h of capture. Alleles from nonnative *Ambystoma tigrinum* have been introduced into some native *A. californiense* populations (Riley *et al*. 2003; Fitzpatrick & Shaffer 2004), including several of our surveyed populations. We excluded all nonnative alleles from subsequent analyses, although we included native haplotypes from mixed populations.

Our 82 collection sites are mapped in Fig. 1 and described in Appendix 1; information on sample sizes for genetic analyses is given in Table 1. We have particularly dense sampling in Sonoma, Santa Barbara, the eastern Bay Area (Contra Costa and Alameda Counties) and northern Monterey/San Benito Counties, reflecting the density of historical localities and/or their importance as conservation targets. Although *A. californiense* occur in eastern Merced County (Vollmar 2002), we do not have specimens from this area (between sites 19 and 3, Fig. 1). Our total

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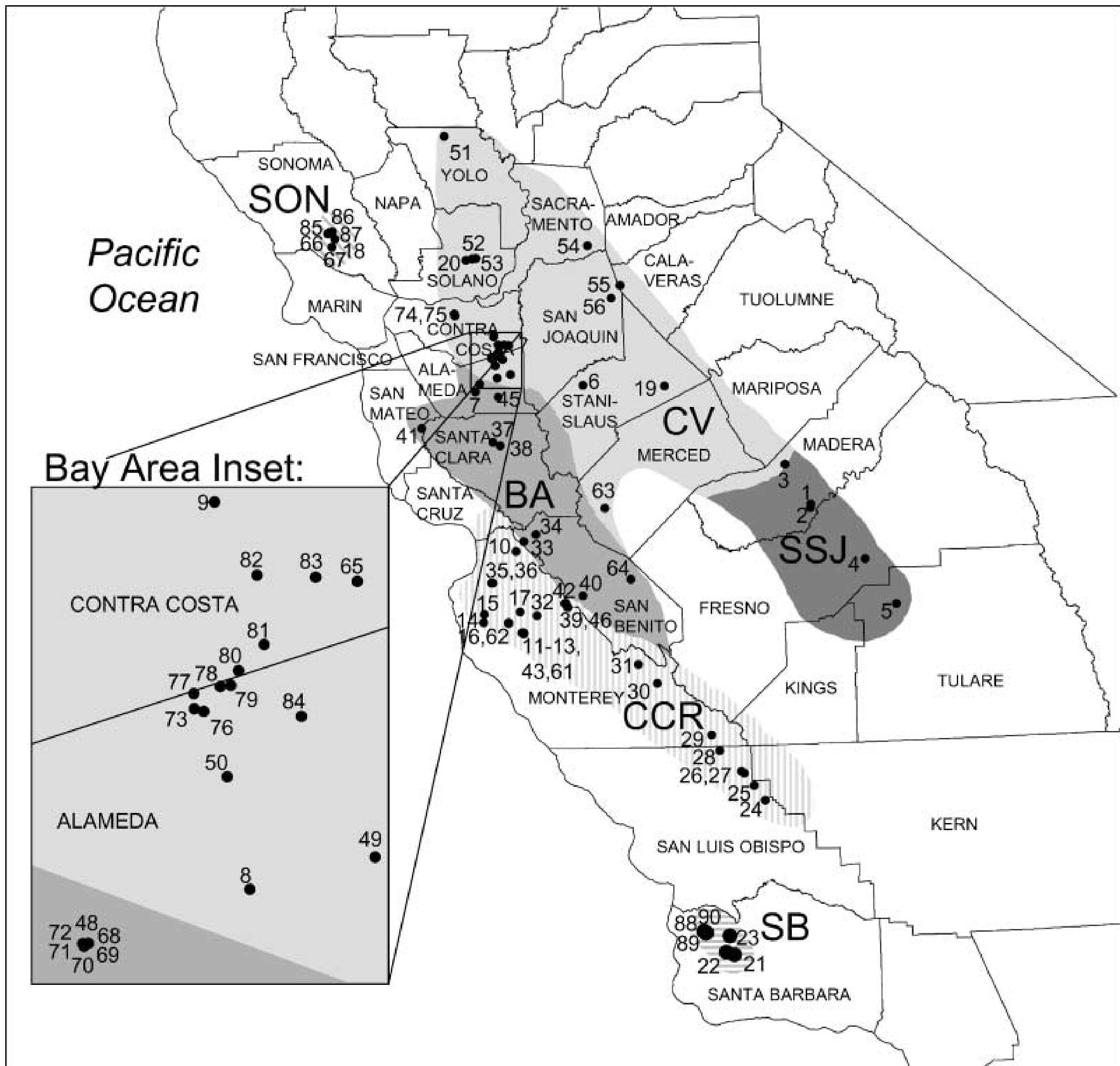

Fig. 1 Collection sites for our range-wide *Ambystoma californiense* genetic survey. Population numbers are the same as in Table 1 and Appendix 1. The approximate ranges of each of the six proposed distinct population segments are shaded. BA = Bay Area, CCR = Central Coast Range, CV = Central Valley, SB = Santa Barbara, SON = Sonoma, SSJ = Southern San Joaquin.

sample size for this study was 696 native specimens from 82 unique localities, for an average of 8.5 specimens per locality.

Molecular methods

mtDNA extraction and sequencing. We extracted DNA for polymerase chain reactions (PCR) using both phenol–chloroform and DNAzol (Chomczynski *et al.* 1997) methods. We used the primers THR (5′-AAACATCGATCTTGTA-AGTC-3′) and DL1 (5′-AATATTGATAATTCAAGCTCCG-3′) (Shaffer & McKnight 1996) to amplify an ~890 bp fragment of the mitochondrial control region that we used for direct sequence analysis. These primers were developed in our laboratory specifically for the tiger salamander complex, and were constructed from cloned *A. tigrinum* DNA. This DNA fragment has been shown to be informative regarding variation in several species of ambystomatid salamanders (Donovan *et al.* 2000; Zamudio & Savage 2003) including the tiger salamander complex (Shaffer

Table 1 Summary results of *Ambystoma californiense* mtDNA genetic survey

Popn	County	No. HD	No. sequenced	Haplotypes (Blitz–DL1/THR–DL1)	DPS†
1	Madera	10	1	A/A1	SSJ
2	Madera	5	1	B/B	SSJ
3	Madera	10	2	BB/BB, C/C	CV
4	Fresno	10	1	A/A2	SSJ
5	Tulare	10	1	A/A1	SSJ
6	Stanislaus	10	2	CC/CC, D/D1	CV
7	Alameda	10	1	E/E1	BA
8	Alameda	10	2	DD/DD1, F/F1	CV
9	Contra Costa	2	1	DD/DD1	CV
10	Monterey	7	3	G/G, H/H, Q/Q1	CCR
11	Monterey	10	1	H/H	CCR
12	Monterey	10	1	H/H	CCR
13	Monterey	10	1	H/H	CCR
14	Monterey	3	1	I/I	CCR
15	Monterey	8	2	J/J*	CCR
16	Monterey	9	2	H/H	CCR
17	Monterey	10	1	H/H	CCR
18	Sonoma	11	1	K/K	SON
19	Stanislaus	10	4	C/C, D/D1, D2, L/L	CV
20	Solano	9	1	D/D1	CV
21	Santa Barbara	5	2	M/M	SB
22	Santa Barbara	9	1	M/M	SB
23	Santa Barbara	11	1	M/M	SB
24	San Luis Obispo	10	2	N/N1	CCR
25	San Luis Obispo	10	1	N/N1	CCR
26	San Luis Obispo	6	1	N/N1	CCR
27	San Luis Obispo	4	1	N/N2	CCR
28	San Luis Obispo	12	1	N/N1	CCR
29	Monterey	9	1	N/N1	CCR
30	Monterey	4	1	N/N1	CCR
31	Monterey	2	1	N/N3	CCR
32	Monterey	4	1	O/O*	CCR
33	San Benito	10	1	P/P1	BA
34	San Benito	10	2	P/P2, Q/Q2	BA
35	Monterey	3	1	H/H	CCR
36	Monterey	10	1	Q/Q2	CCR
37	Santa Clara	10	2	R/R	BA
38	Santa Clara	10	2	E/E1, S/S	BA
39	San Benito	10	5	EE/EE, J/J*, Q/Q2, S/S, T/T1*, U/U*	BA
40	San Benito	10	2	P/P2, T/T1*	BA
41	Santa Clara	5	2	E/E2, S/S	BA
42	San Benito	9	1	T/T2*	BA
43	Monterey	9	2	H/H	CCR
45	Alameda	10	1	V/V	BA
46	San Benito	6	1	T/T1*	BA
48	Alameda	10	1	F/F1	BA
49	Alameda	10	1	F/F1	CV
50	Alameda	9	5	DD/DD1, DD2, D/D3, F/F1, HH/HH, JJ/JJ	CV
51	Yolo	8	1	W/W	CV
52	Solano	10	3	D/D1	CV
53	Solano	10	1	D/D1	CV
54	Sacramento	10	2	D/D4, DD/DD3	CV
55	San Joaquin	4	1	L/L	CV
56	Calaveras	10	1	D/D2	CV
61	Monterey	10	1	H/H	CCR
62	Monterey	10	1	H/H	CCR

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Table 1 *Continued*

Popn	County	No. HD	No. sequenced	Haplotypes (Blitz–DL1/THR–DL1)	DPS†
63	Merced	11	3	C/C, L/L, T/T1*	CV
64	San Benito	10	2	P/P2	BA
65	Contra Costa	11	2	D/D3, F/F1	CV
66	Sonoma	11	2	K/K	SON
67	Sonoma	10	1	K/K	SON
68	Alameda	10	1	E/E1	BA
69	Alameda	10	1	E/E3	BA
70	Alameda	10	1	F/F1	BA
71	Alameda	8	1	E/E1	BA
72	Alameda	10	1	E/E3	BA
73	Contra Costa	11	2	AA/AA, D/D?, F/F1	CV
74	Contra Costa	1	1	Z/Z1	CV
75	Contra Costa	10	1	D/D?, Z/Z1	CV
76	Alameda	11	3	AA/AA, D/D1, F/F2	CV
77	Contra Costa	10	2	D/D?, Z/Z2, DD/DD1	CV
78	Contra Costa	10	5	AA/AA, D/D1, D3, F/F1Z/Z?	CV
79	Alameda	2	1	D/D3	CV
80	Contra Costa	10	3	DD/DD1, D/D3, F/F1, AA/AA	CV
81	Contra Costa	10	4	F/F1, Z/Z2	CV
82	Contra Costa	10	1	D/D1	CV
83	Contra Costa	11	1	D/D3	CV
84	Alameda	8	3	DD/DD1, F/F2, Z/Z2	CV
85	Sonoma	10	1	FF/FF	SON
86	Sonoma	10	1	FF/FF	SON
87	Sonoma	8	2	GG/GG, K/K	SON
88	Santa Barbara	10	1	M/M	SB
89	Santa Barbara	10	1	M/M	SB
90	Santa Barbara	10	1	M/M	SB

Population numbers are the same as in Fig. 1 and Appendix 1. No. HD is the number of individuals genotyped using the outgroup heteroduplex analysis method for the fragment Blitz–DL1. No. sequenced is the number of individuals for which the fragment THR–DL1 was sequenced. Letter codes indicate the mtDNA haplotypes found in each population for the segments between primers Blitz–DL1, followed (/) by the equivalent haplotype for THR–DL1. Because the THR–DL1 fragment is more inclusive than the Blitz–DL1 fragment, we sometimes found two sequences that correspond to one Blitz–DL1 sequence, and we list those with the same letter(s) but sequential numbers (for example, population 19, THR–DL1 sequences D1 & D2) The last column indicated the proposed distinct population segment (DPS) for each population. Populations 13 and 43, and 18 and 67, are the same site sampled in different years; otherwise all populations are geographically unique.

*Nonnative *Ambystoma tigrinum* haplotypes.

†DPS codes: BA = Bay Area; CV = Central Valley; CCR = Central Coast Range; SSJ = Southern San Joaquin; SB = Santa Barbara; SON = Sonoma.

& McKnight 1996; Church *et al*. 2003). The temperature profile included an initial denaturation at 94 °C (3 min) followed by 35 cycles of denaturation at 94 °C (60 s) and annealing at 54.5 °C (90 s) with a final extension at 72 °C (5 min). Amplified fragments were sequenced with either an ABI 377 or ABI 3100 automated DNA sequencer in the University of California Davis Division of Biological Sciences DNA Sequencing Facility (http://dnaseq.ucdavis.edu/). All sequences will be deposited in GenBank, and the alignment is available from HBS.

mtDNA outgroup heteroduplex analysis. We also amplified a shorter, 734 bp fragment that is a subset of the THR–DL1 fragment, using primers Blitz (5′-GCCACTCCCTCCCTA-CTACC-3′) and DL1. For the Blitz–DL1 fragment, our goal was to genotype a much larger number of salamanders without sequencing every individual. We investigated two strategies, single-strand conformation polymorphism (SSCP) and outgroup heteroduplex analysis (OHA), for screening variation at the nucleotide level. Although SSCP (Chen *et al*. 1995) works well for short fragments (Shaffer *et al*. 2000), it is much less reliable for larger fragments (unpublished results). To accurately score 500–800 bp fragments, we developed a form of OHA that is similar to a method proposed by an independent group (Campbell *et al*. 1995).

We used OHA to screen variation among individuals within ponds only (i.e. the pond is the ingroup), and sequencing to confirm our OHA interpretations and haplotype identity among ponds. Equal amounts (3 μL) and approximately equal concentrations of amplified product of the ingroup individual was mixed with amplified product of a standard outgroup individual (*A. tigrinum*, HBS 24280) and 1 μL of heteroduplex annealing buffer (1 M NaCl, 100 mM Tris–HCl pH = 8.0, and 20 mM EDTA). Double-stranded ingroup and outgroup product mixture was then exposed to a denaturation/reannealing cycle (5 min denaturation at 95 °C followed by reannealing at 50 °C for 30 min and then 20 °C for 25 min) forcing some single-stranded product to reanneal to complementary strands. Homoduplexes are formed when the complementary strand is a perfect reverse complement (in this case from the same individual), whereas heteroduplexes form when partially complementary ingroup and outgroup strands pair. OHA products were mixed with 4 μL of loading buffer (20% ethylene glycol, 30% formamide, 0.25% bromophenol blue and 0.25% xylene cyanol) and subjected to standard electrophoresis (0.8 mm thick vertical gel) prepared with 10% polyacrylamide in a 49 : 1 acryl to bis-acryl ratio, 10% (by volume) ethylene glycol and 15% (by volume) formamide in 0.5× TTE buffer. We ran the gels for 18 h at 550 V and visualized DNA bands with ethidium bromide staining and UV illumination. Individuals were scored as being either identical or different to other members of the population. Questionable bands were reanalysed in additional electrophoresis runs.

We ran several tests to confirm the reliability of OHA at correctly identifying haplotypes. First, we constructed a test panel of 13 individual *A. californiense* sequences that varied by 1–14 bp for a 734 bp fragment of the control region based on direct sequencing. By running all 13 samples side-by-side on the same OHA gel we were able to determine whether these levels of differentiation were detectable for a large DNA fragment. Second, we sequenced 10 individuals from one population to confirm that OHA correctly assigned them to haplotype. Finally, we scored bands as liberally as possible with respect to differences within populations, even when we suspected that two bands might represent the same actual haplotype. We sequenced one example of every putative haplotype from each population, and collapsed together haplotypes that had been scored as different but were, in fact, the same based on sequencing. In following this strategy, we sequenced more individuals than necessary, but increased our confidence that we correctly scored variation within and between populations.

Data analysis

THR–DL1 sequences were aligned with CLUSTAL X (Thompson *et al*. 1997), and alignment was verified by eye and analysed in PAUP* (Swofford 1998). Phylogenetic trees were constructed using neighbour joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian analysis. All nucleotide positions were treated as unordered, equally weighted characters. Gaps (which were rare) were treated as missing data. For MP analyses, we conducted heuristic searches with 10 random addition sequence replicates, branch swapping using tree bisection–reconnection and the MULTREES option in PAUP*. NJ trees (Saitou & Nei 1987) were constructed using uncorrected 'p' distances in PAUP*. Nonparametric bootstrap probabilities based on 1000 replicates were used to determine nodal support for MP (MAXTREES = 100) and NJ trees. We used the likelihood ratio test in MODELTEST v. 3.0 (Posada & Crandall 2001) to identify the optimal model for ML analysis, constructed trees with tree bisection–reconnection branch swapping in PAUP*, and assessed nodal support using MRBAYES v. 3.0 (Huelsenbeck & Ronquist 2001) with the optimal model determined by the Bayesian search. For the Bayesian analysis we ran one cold and three heated chains for 10^6 generations, and sampled the chains every 100 generations. Stationarity was achieved after 38 000 generations. Sample points prior to stationarity were discarded as burn-in and the remaining values were used to generate a 50% majority-rule consensus tree. Posterior probabilities (PP) for a clade were then calculated as the proportion of samples recovering a particular clade. We ran all phylogenetic analyses on unique sequences with the following exception. In cases where an identical haplotype was found in more than one population, we included two copies of that haplotype to assess the support level for individuals or populations that are fixed for that haplotype. For example, all Santa Barbara haplotypes were identical in our study. By including two of them, we were able to test the statistical support for the Santa Barbara populations as a clade.

To analyse population variation we used AMOVA for sequence data (Excoffier *et al*. 1992) implemented in the computer package ARLEQUIN. We partitioned the total sequence variation into among- and within-subdivision components, using both sequence divergence and haplotype frequency information for the 734 bp OHA fragment.

We dated key nodes on the mtDNA tree by first checking for clock-like behaviour in the THR–DL1 sequences in PAUP*. We computed the log-likelihood score of our ML tree with and without an enforced molecular clock, and confirmed that we could reject a molecular clock (twice the difference in likelihood scores = 183.35, compared with χ^2 with 62 (# taxa–2) d.f., $P < 0.001$). Given nonclock-like rate heterogeneity, we used the penalized likelihood approach as implemented in the program R8S (Sanderson 2002) to estimate divergence times for the Santa Barbara and Sonoma isolates from the remainder of the range. We used our optimal ML tree, resolved the basal trichotomy among *A. californiense* lineages into all possible topologies, and

estimated divergence times on the three possible trees. We used the cross-validation procedure in R8S to obtain optimal smoothing levels. The date of the root node of the tiger salamander complex (that is, the outgroup to *A. californiense*) was set at 5 Ma based on current estimates of the uplift of the Sierra Nevada (see Discussion).

Results

Sequence variation

In total, 134 *Ambystoma californiense* were sequenced, including 10 individuals from population 3 to examine the effectiveness of OHA. This yielded 41 unique haplotypes varying by up to 3.07% uncorrected sequence divergence. Consistent with previous sequencing work on the tiger salamander complex (Shaffer & McKnight 1996), sequence divergence between ingroup samples and the two outgroup taxa ranged from 5.24 to 6.76%. The final alignment of the unique haplotypes plus the outgroup was 852 bp in length, and included 80 variable sites of which 64 were parsimony informative (40 among the ingroup haplotypes).

Heteroduplex results

The region used for OHA was a subset of the full region we sequenced, with the 3′ primer (DL1) being identical for both. As a result, sequencing often failed to provide data on potential variation in the nucleotide positions immediately internal to DL1. However, previous studies throughout the *A. tigrinum* complex (Shaffer & McKnight 1996) and unpublished sequence data from our laboratory suggest that there is little variation in this 25 bp region. Therefore, we consider it to be unlikely that the heteroduplex analysis detected variation that we were unable to account for in sequencing. Also, OHA seems to have reduced accuracy in detecting 1–2 bp differences if these occur in the ~25 bp region immediately internal to the primers (unpublished results).

Using OHA, 23 of the 82 population samples examined had multiple haplotypes. The numbers of base differences between detected haplotypes are shown in Table 2. Sixty-four comparisons can be made between the detected haplotypes; although some of these comparisons are between the same two haplotypes. For example, the difference between haplotypes F and DD was detected independently in populations 8, 50, 80 and 84 (Table 1). Because these haplotypes were scored without knowledge of the underlying sequence differences and generally on different gels, we consider them to be independent. In general, banding patterns are most similar between sequences with few differences. The large number of one and two base differences resolved suggests that OHA was effective at detecting variants (Table 2).

Table 2 Number of positions that differ among haplotypes detected by outgroup heteroduplex analysis

No. mismatches	Times detected	Unique times detected
1	15	7
2	15	12
4	8	3
5	8	3
6	6	3
8	1	1
9	1	1
11	1	1
31	1	1
33	3	3
35	1	1
36	1	1
37	1	1
38	2	2

These only include comparisons within populations. Values > 30 are comparisons between *Ambystoma californiense* and introduced *A. tigrinum*.

We also examined the effectiveness of OHA at identifying haplotypes by screening 13 individuals with sequences differing by 1–14 bp. In four of the seven cases in which individuals differed by one base, 11 of the 12 cases in which individuals differed by two bases, and in all other comparisons, individuals were scored as having different haplotypes. In one of the single-base differences and the two-base difference that went undetected, a mismatch existed three bases internal to the primer, a region for which we found decreased reliability of OHA. We also sequenced all members of population 3 following OHA. The analysis accurately assigned individuals to either of two haplotypes (BB, C; Table 1) that differed by 11 bp.

Phylogenetic results

Our phylogenetic results are summarized in Fig. 2. We consider bootstrap probabilities (BP) > 70, and Bayesian posterior probabilities (PP) > 95 to indicate strongly supported nodes (Hillis & Bull 1993; Wilcox *et al*. 2003). For the distribution of haplotypes across all populations, either for the full 852 bp THR–DL1 fragment or for the 734 bp Blitz–DL1 fragment, see Table 1.

In general, all analyses recovered a single set of statistically well-supported groups that correspond to geographically cohesive sets of populations. We identified four mtDNA lineages that are well-supported and correspond to distributional discontinuities or potential barriers to dispersal for the species. They are (i) Sonoma, (ii) Santa Barbara, (iii) the Southern San Joaquin Valley and (iv) the Central Coast Range. The remaining populations fell into two less well-defined clusters that we designate the

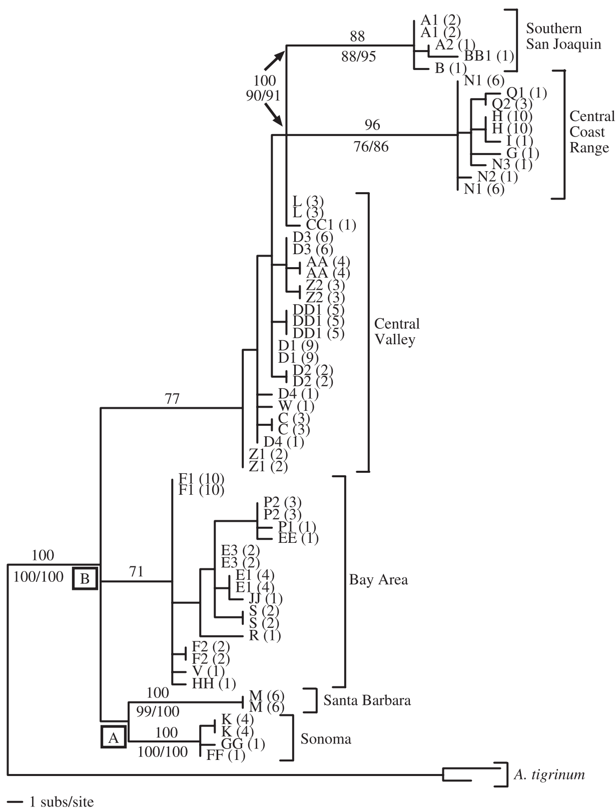

Fig. 2 Maximum likelihood phylogeny for all THR–DL1 haplotypes of native *Ambystoma californiense* plus *A. tigrinum* outgroups. The optimal model, derived from MODELTEST, was HKY + I + G (–ln L = 1917.4), with f(A) = 0.30, f(C) = 0.23, f(G) = 0.15, f(T) = 0.32, Ti/tv ratio = 5.0, the proportion of invariant sites I = 0.79, and the gamma distribution shape parameter G = 0.63. Numbers above branches (in bold) are Bayesian posterior probabilities; below branches are maximum parsimony/neighbour joining bootstrap probabilities. Bold letters 'A' and 'B' in boxes refer to dated nodes using the program R8S. Designated at the tree tips are haplotype, followed (in parenthesis) by the number of populations in which that haplotype was found. Haplotype nomenclature follows Table 1. The arrows indicate that although the sister group relationship of the Southern San Joaquin and Central Coast Range was not recovered under maximum likelihood, it was strongly supported by all other methods.

Central Valley (Madera County north to Yolo County) and the Bay Area (east of the San Andreas fault line, but west of the Great Central Valley).

Of these six units, Sonoma County and Santa Barbara County populations were each universally recovered with very strong bootstrap support and Bayesian posteriors. For Sonoma County, MP, NJ and Bayes all returned support values of 100, and Santa Barbara received essentially the same level of support (MP = 99, NJ = 100, PP = 100). The Santa Barbara County populations are further characterized by low mtDNA sequence variation, with only a single haplotype among 55 individuals from six widely-scattered localities.

The remaining four potential units were less strongly supported. The Central Coast Range was well-supported by all methods (MP = 76; NJ = 86; PP = 96), whereas the Southern San Joaquin received strong support in both MP (BP = 88) and NJ (BP = 95), but weaker Bayesian support (PP = 88). A sister group relationship between the Southern San Joaquin and Central Coast Range animals was strongly recovered (MP = 90; NJ = 91; PP = 100), although the ML analysis failed to recover this relationship with TBR branch swapping (but did recover it with NNI branch swapping). The Central Valley unit was recognized as monophyletic under NJ only (BP = 77), whereas the Bay Area unit was recovered under ML only (PP = 71).

In Fig. 1, we show the approximate geographical boundaries of these six groups. We drew range boundaries based on the results of our genetic analyses and the known distribution of *A. californiense* based on 596 population records downloaded from the California Natural Diversity Database (California Department of Fish and Game 2002). Where genetic units abut, we used biologically reasonable geological features to guide the precise placement of boundaries. Thus, the Fresno River forms the boundary between the Central Valley and the Southern San Joaquin units, and the San Andreas Rift Zone forms the boundary between the Bay Area and Central Coast Range. The boundary between the Central Valley and Bay Area does not coincide with any obvious geological feature, although it conforms very closely with an important ecological boundary (see below).

AMOVA *results*

We conducted four different AMOVA tests to examine the interpretation that these six regions represent well-differentiated sets of populations, and potential distinct population segments as defined by the US Fish and Wildlife Service. Because we used a much larger set of individuals and a completely different analytical approach, we view the AMOVA results as a reasonably independent test of our phylogenetic results, even though the Blitz–DL1 sequence is a subset of the longer THR–DL1 alignment used for phylogenetic analysis. Using the Blitz–DL1 OHA dataset, we asked whether there was a large and significant component of variation (i) among all six potential units, (ii) among the four units from the main core of the range of *A. californiense* but excluding Sonoma and Santa Barbara, (iii) between the contiguously distributed Central Coast Range and Bay Area units, and (iv) between the contiguously distributed Bay Area and Central Valley units (Table 3). In all cases the among-region component of variation was the largest, accounting for at least half of the total genetic variation. In contrast, when we divided the set of core populations (excluding Sonoma and Santa Barbara) into high (≥ 307 m), medium (153–306 m) and low (< 153 m)

Table 3 AMOVA results (Excoffier *et al*. 1992) for population variation in *Ambystoma californiense*

	Sources of variation		
	Among regions	Among ponds within regions	Within ponds
Six regions, including Sonoma and Santa Barbara	d.f. = 5 % var = 75.01 $\phi_{CT} = 0.75$***	d.f. = 65 % var = 17.86 $\phi_{SC} = 0.71$***	d.f. = 602 % var = 7.13 $\phi_{ST} = 0.92$***
Four regions, excluding Sonoma and Santa Barbara	d.f. = 3 % var = 73.26 $\phi_{CT} = 0.73$***	d.f. = 55 % var = 18.27 $\phi_{SC} = 0.68$***	d.f. = 499 % var = 8.47 $\phi_{ST} = 0.91$***
Two regions: Bay Area and Central Coast	d.f. = 1 % var = 74.01 $\phi_{CT} = 0.74$***	d.f. = 28 % var = 22.74 $\phi_{SC} = 0.87$***	d.f. = 255 % var = 3.25 $\phi_{ST} = 0.96$***
Two regions: Bay Area and Central Valley	d.f. = 1 % var = 52.90 $\phi_{CT} = 0.53$***	d.f. = 38 % var = 30.91 $\phi_{SC} = 0.83$***	d.f. = 341 % var = 16.19 $\phi_{ST} = 0.65$***
Three elevational regions: Low, medium and high	d.f. = 2 % var = 15.45 $\phi_{CT} = 0.15$***	d.f. = 56 % var = 74.30 $\phi_{SC} = 0.88$***	d.f. = 499 % var = 10.34 $\phi_{ST} = 0.89$***
Four randomly assigned regions	d.f. = 3 % var = 2.57 $\phi_{CT} = 0.02$	d.f. = 56 % var = 86.71 $\phi_{SC} = 0.89$***	d.f. = 503 % var = 10.72 $\phi_{ST} = 0.89$***

***$P < 0.001$.

elevation sets, the per cent of the total variation attributable to these ecologically defined regions dropped to 15%. When we randomly divided these populations into four sets of ~15 ponds, the among-set component of variation dropped to an insignificant 2.57%. In all analyses, the within- and among-pond components of variation were relatively large and significant, suggesting that there is considerable genetic differentiation among breeding ponds as well as between regions.

Divergence times

We used the penalized likelihood procedure as implemented in R8S (Sanderson 2002) to estimate the divergence times of the highly divergent Sonoma and Santa Barbara clades by estimating the age of either the node subtending the separation of Sonoma and Santa Barbara from each other (node A, Fig. 2) or the node subtending the divergence of node A from the remainder of the *A. californiense* (node B, Fig. 2). Because the latter is represented as a trichotomy, we resolved the base of the *A. californiense* tree in all three possible ways, and estimated the age of each; we refer to these as tree 1 (Sonoma–Santa Barbara clade sister to remaining *A. californiense*), tree 2 (Bay Area plus Sonoma–Santa Barbara sister to remaining *A. californiense*) and tree 3 (Bay Area sister to remaining *A. californiense*). Depending on the tree used, smoothing rates varied from 0.32 to 10.0, and ages varied from 0.74 to 2.49 Myr (Table 4). However,

Table 4 Absolute divergence time estimates for the Sonoma and Santa Barbara County clades from the remainder of *Ambystoma californiense*

	Smoothing rate	Node A date (Ma)	Node B date (Ma)
Tree 1	10.0	0.76	0.92
Tree 2	0.32	2.19	2.49
Tree 3	1.0	0.74	0.86

tree 2 had a large number of zero branch lengths and divergence times that were about a factor of three larger than trees 1 or 3, making us tend to feel that the values for trees 1 and 3 are more reasonable. Thus, the Santa Barbara and Sonoma clades are estimated to have diverged from the remainder of *A. californiense* ~0.74–0.92 Ma, although they could be considerably older.

Discussion

Distinct population segments and cryptic species within Ambystoma californiense

Although geographically restricted in range, our genetic analyses have uncovered considerable cryptic variation within *Ambystoma californiense* that suggests a long history of restricted gene flow and independent evolutionary

Table 5 Distribution of private and shared haplotypes across potential distinct population segments (DPS) of *Ambystoma californiense*

DPS	Private OHA haplotypes (N1, N2)	Private sequence haplotypes (N1, N2)	Shared haplotypes or ambiguous populations
BA	E, P, R, S, V, EE (168, 18)	E1, E2, E3, P1, P2, Q, R, S, V, Z, EE (28, 18)	Haplotype F (OHA) found in: CV: 8, 49, 50, 65, 73, 76, 78, 80, 81, 84 BA: 48, 70 Haplotype F1 (seq.) found in: CV: 8, 49, 50, 65, 73, 78, 80, 81 BA: 48, 70 Haplotype Q (OHA) found in: BA: 34, 39 CCR: 10, 36
CV	C, D, L, W, Z, AA, CC, DD, HH, JJ (248, 28)	C, D1, D2, D3, D4, F2L, W, Z1, Z2, AA, BB1, CC1, DD1, DD2, DD3, HH, JJ (59, 28)	
CCR	G, H, I, N (180, 22)	G, H, I, N1, N2, N3 (28, 22)	
SB	M (55, 6)	M (7, 6)	
SON	K, FF, GG (60, 6)	K, FF, GG (8, 6)	
SSJ	A, B (35, 4)	A1, A2, B (4, 4)	Pop. 3 contains both SSJ and CV haplotypes

DPS abbreviations and haplotype designations as in Table 1. N1 is the number of individuals surveyed, and N2 is the number of populations. N1 and N2 include native and nonnative alleles and populations, although only native alleles are listed in the body of the table.

lineages. Theoretically, deep gene tree breaks can occur among linear, continuously distributed populations purely as a by-product of coalescent processes (Irwin 2002). In such cases, interpreting a deep break in a gene tree as representing the history of a population or species would be incorrect. We take the geographical concordance of genetically identified groups with distributional gaps, ecological zone transitions or potential barriers to gene flow as supporting evidence that our gene trees represent real population history (Irwin 2002). However, we also refrain from suggesting taxonomic changes until additional genetic and morphological data become available.

Six genetically and geographically coherent sets of populations emerged from our analysis that we consider to be strong candidates for 'distinct population segment' status. We recognize that gaps in our sampling still exist, making it difficult to place precise boundaries around some of these units. We proceeded by mapping our known sites based on genetic data, and then looking for reasonable geographical barriers or ecological transitions that coincide with our genetically determined groupings. Thus, the precise placement of a boundary (e.g. the Fresno River) becomes a hypothesis that can be tested with additional detailed sampling, although the general placement of boundaries is empirically determined by the mtDNA data. Each of our proposed distinct population segments is characterized by a number of unique or private alleles that are diagnostic even when very large sample sizes are available (Table 5). We summarize the key attributes of each distinct population segment in Table 6.

Sonoma and Santa Barbara are geographically isolated and genetically distinct. Sonoma has an 85 km distributional gap from the nearest population and is ~2.25% divergent (average uncorrected 'p' distance) from all other *A. californiense*. Santa Barbara has a 90 km distributional gap and is ~1.67% divergent from all other *A. californiense*. The remaining four distinct population segments (DPS) abut, and some show intermixing. However, these mixed sites are limited to boundary populations and have a negligible impact on the levels of differentiation among groups (Table 3). The boundary between the Central Valley and Bay Area unit is the least well defined genetically and geographically, and the monophyly of each is only marginally supported (Table 6). Although no obvious barriers to gene flow exist between them, when we mapped their occurrence on the widely accepted Jepson ecological zonation of California (Hickman 1993), we found that all Bay Area populations fall cleanly in the Western California Ecological Zone and all Central Valley populations fall in the Great Central Valley Ecological Zone. This coincidence with an important ecological boundary suggests to us that the Central Valley and Bay Area

Table 6 Important features of six distinct population segments (DPS) of *Ambystoma californiense*

DPS	No. of popns	Location	Isolated?	Monophyletic? (ML, MP, NJ)	No. of private alleles
Sonoma	5	South-central Sonoma County	Yes	Yes, Yes, Yes	3
Santa Barbara	6	Northwest Santa Barbara County	Yes	Yes, Yes, Yes	1
Southern San Joaquin Valley	4	Central Fresno, southern Madera, and northwest Tulare Counties	Yes?	Yes, Yes, Yes	2
Central Valley	28	Yolo County south to northern Madera County	No	No, Yes, Yes	10
Bay Area	18	Diablo Range	No	Yes, No, Yes	6
Central Coast Range	21	Central Coast Range	No	Yes, Yes, Yes	4

Isolated means that a DPS is geographically isolated from all others. Monophyletic indicates that the group was recovered under (ML, MP [strict consensus of the first 2000 equally parsimonious trees], NJ). Private alleles are those found only in a single DPS.

population segments probably are real historical entities, perhaps with adaptive differentiation maintaining their current distribution.

Thus, the mtDNA analyses provide strong support for independent evolutionary lineages corresponding to six geographical regions based on the following evidence: (i) all are monophyletic in at least some analysis (Table 6), (ii) most genetic variation was accounted for in among-region comparisons in our AMOVA analyses (Table 3), (iii) very few alleles were shared among regions (Table 5), and (iv) those few populations possessing haplotypes of multiple regions were always found on regional borders (Table 5). Do these data argue for the recognition of cryptic species within *A. californiense*? The populations from Sonoma and Santa Barbara are the strongest candidates for recognition as separate species, because they are demonstrably monophyletic and geographically isolated from the remainder of the species. Others have suggested that cryptic species within the tiger salamander complex, at this level of differentiation, are recognizable based on mtDNA divergence alone (Highton 2000). Although Sonoma and Santa Barbara may well represent diagnosable phylogenetic species, we prefer to wait until our ongoing studies on unlinked nuclear genes and adult colour pattern are complete before making formal taxonomic recommendations. We therefore propose to maintain the current taxonomy of *A. californiense* as a single variable species pending the completion of these additional analyses.

Historical biogeography

Several recent studies have examined the phylogeography of species that ring the Great Central Valley of California (Tan & Wake 1995; Rodriguez-Robles *et al*. 1999; Feldman & Spicer 2002; Jockusch & Wake 2002; Matocq 2002). Although high levels of endemicity and species richness in crustaceans have been documented (King *et al*. 1996), we are unaware of any published genetic analyses of taxa that are restricted to the Great Central Valley itself. Our historical reconstructions rely on understanding both the uplift of the Sierra Nevada (to date the separation of *A. californiense* from the remaining members of the *A. tigrinum* complex) and the geological history of the Great Central Valley.

Based on current distribution and ecological requirements, we assume that the uplift of the Sierra Nevada and subsequent drying of the Great Basin was the vicariant event that separated *A. californiense* from the remaining members of the tiger salamander complex (Shaffer & McKnight 1996). The history of the Sierra Nevada is complex, but current reconstructions indicate two periods of uplift; an ancient uplift at ~50 Ma, followed by a second period of uplift at ~5 Ma (Unruh 1991; Wakabayashi & Sawyer 2001). The Basin and Range began extending at ~35 Ma, and late Cenozoic deformations may have led to a lowering of mean elevation in this region as the Sierra Nevada was rising at 5 Ma (Wolfe *et al*. 1997; Wakabayashi & Sawyer 2001). Based on both the rapid uplift and potential synchronous slumping of the intervening Basin and Range, we date the vicariant split between *A. californiense* and its *A. tigrinum* outgroup at 5 Ma, and use that date as a calibration point for the R8S program (Sanderson 2002).

The geological history of the Great Central Valley itself is somewhat difficult to interpret because of the massive levels of natural erosion that obscure past historical events (Dupré *et al*. 1991). The reconstruction described below is based primarily on Dupré. By the early Pliocene a southern marine connection between the San Joaquin Valley and the Pacific had closed, and nonmarine sediments were accumulating in the then-existing Santa Maria Plain (current range of the Santa Barbara tiger salamander) as well as the Salinas–Paso Robles Valleys. Although some estuarine intrusions were present 5 to 2.5 Ma (Jacobs *et al*. 2004), these areas have largely remained free of marine sediments until the present. The Santa Maria Plain, in particular, was an isolated region of lowland alluvial fill surrounded by

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upland mountainous regions that are currently not appropriate *A. californiense* habitat. Thus, the current range of the Santa Barbara clade has existed as continuous, isolated lowland habitat for the last several million years. By the late Pliocene (~2–2.5 Ma), the Great Central Valley was again inundated with a large marine embayment with its outlet near present-day Santa Cruz in Monterey County (Dupré *et al*. 1991; Jacobs *et al*. 2004), restricting California tiger salamanders and other vernal pool endemics to the alluvium deposits ringing the Valley. Sometime in the late Pliocene or early Pleistocene, ~1 Ma, this marine outlet of the Great Central Valley was sealed off, and the resulting interior drainage of streams and rivers led to the formation of a large, north–south orientated lake from ~0.6–0.72 Ma that covered most of the San Joaquin Valley (Dupré *et al*. 1991). Thus, in the San Joaquin Valley, vernal pool species including *A. californiense* would have been restricted to the narrow fringe of low-lying alluvial fill habitat between the lake and the surrounding mountains. However, more extensive habitat was available in the northern and northeastern portions of the current range, from roughly the position of current-day San Jose north. By ~0.6 Ma the Great Valley re-established a marine connection to the Pacific through San Francisco Bay, and the configuration of the Sacramento and San Joaquin drainages of the northern and southern arms of the Great Central Valley, respectively, have remained intact to the present.

There are at least three key elements of this brief history of the Great Central Valley and associated alluvial fill habitats that are relevant to the phylogeographical history of *A. californiense*. First, the history of inundation of the Valley has led to a persistent separation of the terrestrial habitat into a narrow ring with a predominantly north–south orientation. Second, both the Salinas Valley and the Santa Maria Plain have been separate, low-lying entities for several million years. Third, much of the Great Central Valley has been available habitat for a relatively short time, in the order of one or a few hundred thousand years.

Patterns of genetic differentiation in *A. californiense* are generally consistent with the interpretation of long-term isolation and fragmentation during the last several million years. The long branches and deep divergences of the Southern San Joaquin, Central Coast Range and Santa Barbara clades, and the shallow differentiation of the populations in the Central Valley itself (Fig. 2) are consistent with isolation in northern Santa Barbara and the Salinas Valley, but recent expansion into the floor of the Great Central Valley proper. Recent expansion from formerly more isolated ranges, as opposed to the persistence of ancestral polymorphisms in a more widely distributed taxon, is also suggested by the deep among-region differentiation (Table 3) characterized by many private alleles (Table 5) and minimal introgression of haplotypes among regions (Table 5). Finally, the timing of separation of the Santa Barbara clade (Table 4) is consistent with this scenario in that it dates from ~0.7–2.5 Ma (Table 4), and the Santa Maria Plain has been both available habitat and isolated from the Great Central Valley for at least that long. We have not been able to find similarly detailed geological information for the Santa Rosa region in Sonoma County, but our prediction is that it has been similarly intact, and isolated from the Great Central Valley for most of the Pleistocene.

Conservation genetics

There are two similar goals in conservation genetics: (i) conserve genetically distinct population/lineages, and (ii) conserve genetic variation that may be important for the long-term evolutionary success of a species/population/lineage. Mitochondrial markers likely have greater bearing on the former. Our detailed measurements of regional/population differentiation have identified areas of 'genetic endemism' that merit independent conservation status as distinct population segments in *A. californiense*. When these genetic data are linked to historical geological events and analysed in a molecular clock framework, we can go a long way toward inferring both the mechanism and time course of genetic isolation and differentiation. In this study, this approach highlighted the antiquity of the Sonoma and Santa Barbara clades, further emphasizing their importance in the management and recovery of *A. californiense*. For the second goal, additional studies of nuclear markers, including QTLs for important traits, are crucial in identifying 'hotspots' of functional and genomic diversity. Because the tiger salamander complex is becoming increasingly well characterized as a genomic model system (http://salamander.uky.edu/; Voss *et al*. 2001), we look forward to applying QTL (Voss & Shaffer 1997) and functional genomic approaches to future conservation prioritization in this system.

Finally, as populations continue to become fragmented and demographically unstable (the most striking examples are currently in Sonoma County), reintroductions of recently extirpated populations may become a necessary tool for species survival. As we have noted in another declining California amphibian (Shaffer *et al*. in press), such reintroductions should be based on both ecological and genetic similarity with the source population. If such aggressive conservation strategies become necessary for *A. californiense*, our mtDNA-based distinct population segments, in combination with comparative ecological studies, should help guide future reintroduction efforts.

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Brad Shaffer's lab group has research interests in amphibian and reptilian genetics, conservation and systematics, and the molecular work for this project was conducted while Pauly and Oliver were part of that lab group. Pete Trenham's primary research interests are in amphibian ecology and conservation, particularly the California tiger salamander. Jeff Oliver and Greg Pauly are pursuing their Ph.D. research on insects and amphibians, respectively.

Appendix 1

Population location for 84 sampling sites of Ambystoma californiense

For each locality, we provide the population number, HBS catalogue numbers for genotyped specimens, site number, and exact geographical localities including latitude/longitude coordinates (decimal degrees N, decimal degrees W). Site # refers to sites listed in Shaffer *et al*. (1993). Population numbers are the same as in Table 1 and Fig. 1. The two sites listed for population 54 are within a few hundred metres of each other, and were combined in all analyses. We excluded sites with only introduced *Ambystoma tigrinum* haplotypes (57–60). Populations 13 and 43, and 18 and 67, are the same site sampled in different years; otherwise all populations are geographically unique.

1 11149–11155, 11157–11159 (site 107) Along Road 204, 2 km S of Hwy 145, on Urrutina Ranch, Madera Co., CA. 36.9995 N, 119.7490 W

2 11171–11175 (site 111) Along Road 204, 3 km S of Hwy 145, on Urrutina Ranch, Madera Co., CA. 36.9822 N, 119.7528 W

3 11183–11191, 11204 (site 117) Just NE of intersection of Roads 606 and 600, Madera Co., CA. 37.2044 N, 119.9015 W

4 11207–11216 (site 124) Cattle pond, 150 m N of Hwy 180 at intersection with Alta Ave. 12.3 km W of Hwy 63, Fresno Co., CA. 36.7182 N, 119.4212 W

5 11243–11252 (site 126) Along N side of Hwy 201, 9.6 km W of Road 153, Tulare Co., CA. 36.4872 N, 119.2410 W

6 11268–11277 (site 136) 16 km SW of Paradise Road, 1.6 km S of Hwy 132, 0.8 km N of California Ave., Stanislaus Co., CA. 37.6270 N, 121.1602 W

7 11332, 11333, 11335–11342 (site 154) 75 m S of Hwy 84, 4.3 km E of Hwy 680, Alameda Co., CA. 37.6043 N, 121.8369 W

8 11371, 11372, 11374, 11376–11382 (sites 103 and 158) 10 m W of Greenville Road, 0.6 km N of Tesla Road, Alameda Co., CA. 37.6707 N, 121.6972 W

9 11400, 11401 (site 159) S side of Camino Diablo Road, 0.3 km E of Marsh Creek Road, Contra Costa Co., CA. 37.8772 N, 121.7175 W

10 11415–11420, 12854 (site 162) 0.8 km NW of San Juan Grade Road, 2 km N of Crazy Horse Canyon Road, Monterey Co., CA. 36.7978 N, 121.5945 W

11 11422–11437 (site 163) Laguna Conejo, Hastings Natural History Reservation, Carmel Valley, Monterey Co., CA. 36.3898 N, 121.5546 W

12 11475–11484 (site 165) Just N of the large pond at Laguna Conejo, Hastings Natural History Reservation, Carmel Valley, Monterey Co., CA. 36.3834 N, 121.5571 W

13 11507–11516 (site 166) Blomquist Pond, Hastings Natural History Reservation, Carmel Valley, Monterey Co., CA. 36.3873 N, 121.5558 W

14 11537–11539 (site 170) Along dirt road leading to Pinyon Peak, 4 km NW of Robinson Canyon Road, Rancho San Carlos, Monterey Co., CA. 36.4852 N, 121.7967 W

15 11548–11553, 11555a, 11555b (site 172) Along dirt road 2.3 km SW of Robinson Canyon Road, Rancho San Carlos, Monterey Co., CA. 36.4441 N, 121.8000 W

16 11556–11564 (site 174) Along road adjacent to Rana Creek Road, 1.3 km S of Carmel Valley Road, Carmel Valley, Monterey Co., CA. 36.4358 N, 121.6458 W

17 11569–11574, 11576–11578, 11581 (site 175) Along Rana Creek Road, N of Carmel Valley Road, Carmel Valley, Monterey Co., CA. 36.4936 N, 121.5768 W

18 11618–11620, 11623, 11624, 11626–11628, 11630, 11631 (site 186) S side of Stony Point Road, 15 m S of intersection with Hwy 116, Sonoma Co., CA. 38.334 N, 122.7380 W

19 11666–11675 (site 275) Hickman Vernal Pool, ~2.3 km SE of intersection of Lake Road and Hawkins Road, Stanislaus Co., CA. 37.6155 N, 120.6435 W

20 11718–11720, 11722–11724, 11726–11728 4.8 km W of Olcott Lake at Jepson Prairie, Solano Co., CA. 38.2632 N, 121.8868 W

21 11761, 11762, 11771, 11773, 11777 (site 200) 5.6 km NW of Los Alamos, Los Flores Ranch, Careaga Divide, Santa Barbara Co., CA. 34.7788 N, 120.3217 W

22 11800–11808 (site 202) 9.6 km NW of Los Alamos, Los Flores Ranch, Careaga Divide, Santa Barbara Co., CA. 34.7934 N, 120.3698 W

23 11914–11922, 11926 (site 210) Gill's Pond, 3.0 km SW of Garey, Santa Barbara Co., CA. 34.8742 N, 120.3445 W

24 11997–12006 (site 223) Vernal pond 0.4 km W of Bitterwater Road, 4.3 km S of Bitterwater Valley Road, San Luis Obispo Co., CA. 35.5236 N, 120.0905 W

25 12051–12060 (site 225) Grant's Lake, on Bitterwater Road, 6.4 km N of Bitterwater Valley Road, San Luis Obispo Co., CA. 35.5983 N, 120.1529 W

26 12080, 12081, 12083, 12091, 12092, 12095 (site 226) Twisselman Lake, 0.3 km NE of Bitterwater Road, 9.6 km SE of Cholame, San Luis Obispo Co., CA. 35.6617 N, 120.2142 W

27 12109, 12112, 12118, 12119 (site 227) Obrien's Lake, 1.0 km NE of Bitterwater Road, 8 km SE of Cholame, San Luis Obispo Co., CA. 35.6725 N, 120.2275 W

28 12150–12159 (site 230) Vernal pond adjacent to Kerr Lake, 1.6 km S of Monterey/San Luis Obispo Co. line, 2.8 km W of Cholame Valley Road, San Luis Obispo Co., CA. 35.7770 N, 120.3613 W

29 12223–12225, 12227, 12230, 12232, 12234, 12236, 12237 (site 233) Covington Lake, 1.6 km W of Cholame Road, 7.2 km N of Monterey/San Luis Obispo Co. line., Monterey Co., CA. 35.8565 N, 120.4067 W

30 12244, 12248, 12252, 12253 (site 235) Berm pond just E of Peach Tree Road, ~11.2 km S of Hwy 198, Monterey Co., CA. 36.1217 N, 120.7325 W

31 12268, 12269 (site 236) Berm pond 1.6 km NE of Hwy 25, 6.4 km N of Hwy 198, Monterey Co., CA. 36.2197 N, 120.8477 W

32 12364, 12365, 12367, 12368 (site 242) Berm pond along River Road, 0.8 km S of Gonzales River Road, Monterey Co., CA. 36.4748 N, 121.4698 W

33 12402–12406, 12408, 12410, 12412, 12413, 12418 (site 248) Berm pond 50 m N of Hwy 156 just W of Monterey St., San Benito Co., CA. 36.8473 N, 121.5478 W

34 12429–12438 (site 250) Vernal pool 0.3 km S of the end of Hudner Lane, 7.2 km NW of Hollister in the Flint Hills, San Benito Co., CA. 36.8825 N, 121.4685 W

35 12467–12469 (site 252) Approx. 2.8 km SE of Marina on Fort Ord, Monterey Co., CA. 36.6432 N, 121.7512 W

36 12477–12485 (site 253) Approx. 2.9 km SE of Marina on Fort Ord, Monterey Co., CA. 36.6420 N, 121.7415 W

37 12499–12508 (site 258) Just N of Mt. Hamilton Road, 11.4 km E of Alum Rock Road, Santa Clara Co., CA. 37.3493 N, 121.7308 W

38 12530–12539 (site 259) Berm pond along Dairy Trail, Grant Ranch County Park, Santa Clara Co., CA. 37.3308 N, 121.6850 W

39 12553, 12554, 12556–12563 (site 239) Gloria Lake, 4.8 km SSE of La Gloria Road, 5.1 km W of Pinnacles National Monument, San Benito Co., CA. 36.5125 N, 121.2785 W

40 12605–12614 (site 261) Berm pond just W of Hwy 25, 0.4 km N of La Gloria Canyon Road, San Benito Co., CA. 36.5720 N, 121. 1845 W

41 12619–12622, 12626 (site 268) Lake Lagunita, SW end of Stanford Campus, Palo Alto, Santa Clara Co., CA. 37.4218 N, 122. 1767 W

42 12651–12660 (site 260) Berm pond NW of Gloria Lake, 0.5 km S of La Gloria Canyon Road, San Benito/Monterey Co. line, CA. 36.5347 N, 121.2983 W

43 12661–12669 (site 166) Second visit to Blomquist Pond, Hastings Natural History Reservation, Carmel Valley, Monterey Co., CA. 36.3873 N, 121.5558 W

45 12709, 12711–12713, 12715–12718, 12721, 12723 (site 267) Ohlone Regional Park, Alameda Co., CA. 37.5752 N, 121.6938 W

46 12855, 12857–12860, 12864, 12866, 12868, 12871 (site 272) Gloria Lake, bern pond, 4.6 km W of Pinnacles National Monument, San Benito Co., CA. 36.5125 N, 121.2783 W

48 8745–8754 (site 328) S of Route 84 extension, Livermore Valley, Alameda Co., CA. 37.6415 N, 121.8088 W

49 8857–8866 (site 333) Just N of Patterson Pass Road, 6.6 km E of intersection of Patterson Pass Road and Greenville Road, Alameda Co., CA. 37.6868 N, 121.6130 W

50 6718–6726 Frick Lake, along Laughlin Road, 1.8 km N of Hwy 580, Alameda Co., CA. 37.7308 N, 121.7113 W

51 9739, 9740, 9742–9747 Along Road E4, 3.8 km W of Dunnigan, Yolo Co., CA. 38.8872 N, 122.0172 W

52 8840–8849 (site 333) Jepson Prairie Reserve, Solano Co., CA. 38.2727 N, 121.8245 W

53 9827–9836 (site 57) 2.2 km W of Cook Road along dirt road that intersects Cook Road, 1.6 km S of Hwy 113, Solano Co., CA. 38.2710 N, 121.8475 W

54a 9800–9802 (site 53) Approx. 0.1 km S of Twin Cities Road, 4.2 km E of Clay Station Road, Sacramento Co., CA. 38.3267 N, 121.1117 W

54b 9803–9809 (site 50) Pond on W side of dirt road leading to Rancho Seco Power Plant, 2.9 km N of the end of Borden Road, Sacramento Co., CA. 38.3267 N, 121.1117 W

55 9844, 9845, 9847, 9848 (site 63) N of Shelton Road, 4.2 km E of intersection of Shelton Road and Hwy 26, San Joaquin Co., CA. 38.0608 N, 120.9657 W

56 9855–9864 (site 64) N of Sheri's Road, 0.2 km W of intersection of Sheri's Road and Burson Road, Calaveras Co., CA. 38.1235 N, 120.9095 W

61 14047–14056 (site 164) Just SE of intersection of Carmel Valley Road and Old County Road, 0.5 km NW of Laguna Conejo, Oak Ridge Ranch, Hastings Natural History Reservation, Carmel Valley, Monterey Co., CA. 36.3885 N, 121.5648 W

62 14076–14085 (site 176/276) Near end of main driveway into Rana Creek Ranch, Carmel Valley, Monterey Co., CA. 36.4420 N, 121.6445 W

63 14154–14160, 14162–14164 Pond along Jasper-Sears Road, 1.3 km W of intersection of Jasper-Sears Road and Billy Wright Road, 8.8 km S of Hwy 152, Merced Co., CA. 37.0082 N, 121.0368 W

64 14256–14264, 14266 Approx. 0.8 km W of Panoche Road, 5.6 km N of intersection of Panoche Road and Little Panoche Road, San Benito Co., CA. 36.6490 N, 120.8820 W

65 14304–14313, 14315 Cattle pond at intersection of Byron Hot Springs Road and Holey Road, Contra Costa Co., CA. 37.8338 N, 121.6223 W

66 14338–14347, 14351 Natural stream bed under Ludwig Road, between Wright Road and Daniels Road, Sonoma Co., CA. 38.4033 N, 122.7663 W

67 14359–14368 (site 186) Second visit to the S side of Stony Point Road, 15 m S of intersection with Hwy 116, Sonoma Co., CA. 38.3340 N, 122.7380 W

68 8755–8764 (site 329) Hills S of Route 84 extension, Livermore Valley, Alameda Co., CA. 37.6415 N, 121.8088 W

69 8766–8775 (site 330) Hills S of Route 84 extension, Livermore Valley, Alameda Co., CA. 37.6415 N, 121.8088 W

70 8797–8806 (site 327) Hills S of Route 84 extension, Livermore Valley, Alameda Co., CA. 37.6415 N, 121.8088 W

71 8809–8816 (site 332) Hills S of Route 84 extension, Livermore Valley, Alameda Co., CA. 37.6415 N, 121.8088 W

72 8817–8826 (site 334) Hills S of Route 84 extension, Livermore Valley, Alameda Co., CA. 37.6415 N, 121.8088 W

73 14390, 14392–14400 (site 298) Along dirt road that intersects Vasco Road, 3.6 km N of the Contra Costa/Alameda Co. line, Contra Costa Co., CA. 37.7673 N, 121.7330 W

74 14405 (site 295) 30 m N of dirt road to a spring ~2.4 km from Wilden Road, Concord Naval Weapons Station, Contra Costa Co., CA. 37.9948 N, 121.9649 W

75 14407–14416 (site 296) 100 m behind bunker 4AT35 off of T St., Concord Naval Weapons Station, Contra Costa Co., CA. 37.9857 N, 121.9604 W

76 14424–14433 (site 299) 0.6 km E of Vasco Road on a dirt road that intersects Vasco Road, 0.4 km S of the Contra Costa/Alameda Co. line, Alameda Co., CA. 37.7657 N, 121.7265 W

77 14434–14443 (site 300) Approx. 0.6 km E of Vasco Road on a dirt road that intersects Vasco Road, 0.4 km N of the Contra Costa/Alameda Co. line, Contra Costa Co., CA. 37.7753 N, 121.7332 W

78 14456–14465 (site 301) Approx. 2.6 km E of Vasco Road on a dirt road that intersects Vasco Road. 0.4 km S of the Contra Costa/Alameda Co. line, Contra Costa Co., CA. 37.7788 N, 121.7153 W

79 14475, 14476 (site 302) Approx. 3.2 km E of Vasco Road on a dirt road that intersects Vasco Road, 0.4 km S of the Contra Costa/Alameda Co. line, Alameda Co., CA. 37.7795 N, 121.7080 W

80 14478–14487 (site 303) Approx. 3.8 km E of Vasco Road on a dirt road that intersects Vasco Road, 0.4 km S of the Contra Costa/Alameda Co. line, Contra Costa Co., CA. 37.7872 N, 121.7027 W

81 14497–14506 (site 304) Approx. 6.1 km NE of Vasco Road on a dirt road that intersects Vasco Road, 3.6 km S of the Contra Costa/Alameda Co. line, Contra Costa Co., CA. 37.8008 N, 121.6855 W

82 14517–14526 (site 305) Approx. 2.6 km S of intersection of Vasco Road and Camino Diablo Road, Contra Costa Co., CA. 37.8377 N, 121.6895 W

83 14537–14546 (site 306) Approx. 3.2 km W of intersection of Armstrong Road and Byron Hot Springs Road, Contra Costa Co., CA. 37.8362 N, 121.6503 W

84 14575–14582 (site 325) Altamont Pass Land Fill, Alameda Co., CA. 37.7622 N, 121.6614 W

85 26398–26407 N end of Ash Dr., 0.3 km N of intersection of Ash Dr. and Yuba Dr., 0.8 km W of Stony Point Road, Santa Rosa, Sonoma Co., CA. 38.4090 N, 122.7510 W

86 26364–26373 Southwest Community Park, just S of Hearn Ave. between Stony Point Road and Dutton Ave., Santa Rosa, Sonoma Co., CA. 38.4122 N, 122.7367 W

87 27739–27746 W of railroad crossing at Scenic Ave. near Hwy 101, Sonoma Co., CA. 38.3758 N, 122.7193 W

88 28365–28374 2.2 km NW of the intersection of Black Road and Hwy 1, Santa Maria Valley, Santa Barbara Co., CA. 34.9005 N, 120.5048 W

89 28390–28399 Along W side of Black Road, 1.0 km N of Hwy 1, Santa Maria Valley, Santa Barbara Co., CA. 34.8887 N, 120.4917 W

90 28420–28429 1.6 km NE of the intersection of Black Road and Hwy 1, Santa Maria Valley, Santa Barbara Co., CA. 34.8922 N, 120.4842 W