HYBRIDIZATION BETWEEN A RARE, NATIVE TIGER SALAMANDER (AMBYSTOMA CALIFORNIENSE) AND ITS INTRODUCED CONGENER

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Abstract. Exotic species threaten native biodiversity through predation, competition, and habitat alteration, but also by hybridizing with native species. A lack of reproductive isolation between exotic and native species can lead to genetic swamping, loss of native genetic diversity, and, in rare or endangered species, extirpation or extinction. We examined hybridization between a declining native salamander, the California tiger salamander, Ambystoma californiense, and an introduced congener, A. tigrinum. Ambystoma californiense is restricted to central California where A. tigrinum has been deliberately introduced as fish bait. In the Salinas Valley, we sampled salamanders from four artificial ponds and two natural vernal pools. Based on mitochondrial DNA and two nuclear loci, we found that hybrids were present in all six ponds, and that these hybrids were viable and fertile. No potentially pure A. californiense were present in three of the six ponds, and only one pond had more than 8% possibly pure native animals. Despite a relatively ancient split and wide genetic divergence between these taxa, they are interbreeding and threatening the genetic purity of the native species.

Our data also suggest that the extent of the genetic mixing depends on the breeding habitat. There is little evidence of barriers to gene exchange in the four artificial breeding ponds. However in the two vernal pools, we found significantly fewer larvae with hybrid genotypes and significantly more with pure parental genotypes than expected. Linkage disequilibria revealed positive associations between native alleles and genotypes, and negative associations between native and introduced alleles and genotypes in these two ponds. Despite rampant hybridization, these data provide evidence of some constraints on hybridization in the native breeding habitats. Our results suggest that habitat characteristics of native species should be exploited in management strategies to limit hybridization with exotics.

Key words: Ambystoma californiense; Ambystoma tigrinum; amphibian decline; conservation biology; endangered and rare species; genetic swamping; Great Central Valley, California, USA; hybridization; introduced species; reproductive isolation; tiger salamander.

INTRODUCTION

Introduced species are a major cause of ecological breakdown and the loss of biological diversity worldwide (Elton 1958, Lodge 1993). Exotic species can significantly alter ecosystem properties such as nutrient levels and vegetation structure (e.g., Vitousek 1989), and they can eliminate populations, species, and entire faunas through predation (e.g., Savidge 1987). These ecological impacts pose significant threats to the recovery of many endangered species (Foin et al. 1998, Czech et al. 2000). Threats to biological diversity from introduced species can also occur through more subtle mechanisms such as hybridization with native species (Rhymer and Simberloff 1996). Hybridization with exotic species reduces the distinctness of native species and, if native species are rare or endangered, can virtually eliminate them by genetic swamping. Worldwide many rare species are threatened by hybridization with exotics, ranging from mammals (Cervus elaphus, Goodman et al. 1999; Mustela putorius, Davison et al. 1999), to fishes (Hypomesus transpacificus, Trenham et al. 1998), to plants (Spartina foliosa, Ayres et al. 1999; see Rhymer and Simberloff [1996] for more examples). In this paper we investigate the potential for hybridization between the California tiger salamander (Ambystoma californiense), a rare and declining amphibian (Fisher and Shaffer 1996), and an introduced congener, the tiger salamander (Ambystoma tigrinum).

While human activities threaten diversity in many groups of plants and animals, alarming declines in amphibian populations worldwide have focused particular attention on frogs, toads, and salamanders (Blaustein and Wake 1990, Phillips 1990, Reaser 1996). In the western United States including California, amphibian declines have been associated with a number of potential causes including UV-B radiation (Blaustein et al. 1997, 1998), disease (Kiesecker and Blaustein 1997,
Kiesecker et al. 2001), pesticides (Davidson et al. 2001, Relyea and Mills 2001), habitat destruction (Davidson et al. 2002), and introduced predators and competitors (Kupferberg 1997, Knapp and Matthews 2000). Field correlative data (Fisher and Shaffer 1996) and experimental evidence (K. Leyse, unpublished data) have demonstrated the impact of introduced bullfrogs and fishes, including mosquitofish (Gambusia affinis), on California tiger salamanders.

While these introduced species all reduce the distribution and abundance of native amphibians through competition or predation, no attention has been given to the additional threat of genetic introgression through hybridization. Although this threat may be less obvious than predation and competitive exclusion, its long-term impacts may be at least as severe. If an introduced species eats or displaces a native one, the introduced taxon can in principle be removed and the native species allowed to recover to its former population size. However if the introduced species invades genetically, the native species may be disappearing before our eyes without our knowledge, especially if genotype is not easily determined from phenotype. No efforts of eradication or captive rearing and breeding, no matter how heroic, can reproduce the original biological entity from a hybrid population, since the original genetic sample no longer exists.

Fortunately, a rare combination of circumstances must occur in order for exotic species introductions to result in hybridization. The exotic species must be sympatric with a native species that is evolutionary similar enough that interbreeding is possible, and reproductive isolating mechanisms must never have evolved, or must have been disrupted, perhaps through human disturbance. This threat may be important in amphibian conservation, since hybridization can be common among amphibians (Arntzen and Wallis 1991, Green 1996, Werner and Watson 1996). When species do hybridize, ecological characteristics often play an important role. The importance of ecology in the dynamics of hybridization and reproductive isolation has been repeatedly demonstrated in animals as diverse as sticklebacks (Schluter 1996, Vamosi and Schluter 1999), Darwin’s finches (Grant and Grant 1992, 1996), aphids (Via 1999, Via et al. 2000), crickets (Rand and Harrison 1989), and fire-bellied toads (Szymura 1993, 1996). When species do hybridize, it often occurs in environments that are intermediate between those to which each taxon is specifically adapted (Anderson 1948). Consequently when environmental conditions are altered by human disturbance, reproductive isolating mechanisms may break down (Anderson 1948, Seehausen et al. 1997). The suggestion that human disturbance can cause a breakdown in reproductive isolation between naturally sympatric amphibians has been made repeatedly (e.g., Schlefer et al. 1986, Sullivan 1986, Gollmann 1996). However all of these cases involve two native species. Here we examine a situation in which an exotic species has been deliberately introduced into the range of A. californiense where breeding habitats have been highly modified.

Our aim is to investigate the nature and extent of hybridization between A. californiense and nonnative tiger salamanders. Though there may be cases where hybridization between native and nonnative taxa benefits conservation goals (e.g., Hedrick 1995, see Rhymler and Simberloff [1996] for discussion), in this case we take the position that hybridization threatens the purity and identity of a declining native species and is unequivocally negative. We demonstrate first that native and nonnative salamanders co-occur. Tiger salamander taxa can be difficult to distinguish morphologically (Petranka 1998), particularly in the most easily obtained larval form. However our large database of mitochondrial DNA (mtDNA) information from across the range of the tiger salamander complex (Shaffer and McKnight 1996, H. B. Shaffer, unpublished data) allows us to overcome this identification problem with mtDNA typing. Given that animals with native and nonnative mtDNA are now sympatric in central California, we determined the potential origin of the introduced animals using phylogenetic analysis of mtDNA sequences. Since mtDNA is inherited only from the mother, we used nuclear DNA to explore the extent of hybridization in these ponds, and to show that hybrids are breeding successfully. We demonstrate that allele and genotype frequencies in some ponds are significantly different from Hardy-Weinberg expectations and that significant linkage disequilibrium is present, indicating the presence of partial reproductive isolation or sustained immigration of pure genotypes. Finally, we present evidence that altered environmental conditions may affect the dynamics of hybridization, and specifically that hybridization is less constrained in more-disturbed breeding ponds. This is important given that almost all of the natural breeding habitat of A. californiense has been extensively modified (Fisher and Shaffer 1996). This hybridization threatens the conservation and ultimate persistence of A. californiense, and presents significant management challenges throughout its range. Ultimately, if we can understand the extent and nature of this threat, we can develop techniques for reducing further genetic introgression and decreasing the range and abundance of nonnative salamanders in California.

**Study Species and Area**

**Study Species**

The phylogeny of the tiger salamander complex (Ambystoma tigrinum) has been extensively studied with mitochondrial DNA (mtDNA), allozymes, and morphology (Shaffer et al. 1991, Shaffer 1993, Shaffer and McKnight 1996). The deepest split, estimated at 3–5 million years ago, is between A. californiense and all other lineages, which includes 15 Mexican species and
FIG. 1. Neighbor-joining tree (redrawn from Shaffer and McKnight, [1996]) based on 840 base pairs of mitochondrial DNA control-region sequence data showing one depiction of the phylogenetic relationships among the major clades within the tiger salamander complex (Ambystoma tigrinum). The two hybridizing species are shown as black triangles; sequence divergences among the hybridizing populations range from 6.03% through 6.99%.

all other U.S. tiger salamanders (Shaffer and McKnight 1996; Fig. 1). While parts of the phylogeny remain unresolved, the geographically widespread A. tigrinum from the Rocky Mountains/Great Plains (USA) area is clearly a recently derived taxon that is distantly related to A. californiense (Shaffer and McKnight 1996). It is also geographically separated from A. californiense by about 800 km of Great Basin desert (Fig. 2).

Along with the large phylogenetic divergence between these species, there are major differences in life history. Coastal and central California is the only region of North America with a Mediterranean climate, characterized by cool, wet winters and hot, dry summers when it rarely rains. Ambystoma californiense breeds during winter rains in November–March, and larvae metamorphose and leave drying vernal pools by April–June (Trenham et al. 2000, Shaffer and Trenham, in press). However, in the Great Plains part of their native range, A. tigrinum hibernate during cold winters and generally breed during spring/summer rains from March–August (Degenhardt et al. 1996). Throughout the Great Plains and Rocky Mountains, they either metamorphose in late summer or become paedomorphic (reproductively mature in the larval form) gilled adults in permanent water bodies (Collins 1981). Thus, allopatric A. californiense and A. tigrinum differ both in timing of reproduction and expression of life-history mode (metamorphs or paedomorphs). However, in sympatry in California, A. tigrinum apparently shift their breeding phenotype to match the local environment, and this shift creates the opportunity for hybridization with A. californiense.

Study area
At our study site near Gonzales, Monterey County, California, A. tigrinum from the southwestern United State were introduced as bass bait into ponds within the breeding range of native A. californiense. Introductions of nonnative Ambystoma may be related to bass (Mycropterus spp.) fishing in two ways. Ambystoma larvae are popular bass bait throughout North America, and because A. californiense larvae are available only between April and June in the Salinas area, animals were brought from Texas, Arizona, and Colorado to stock local ponds for bait throughout the summer. We learned from interviews with longtime residents of the Salinas valley, including one of the bait dealers who performed the introductions, that “water-dogs” had been introduced throughout the area since at least 1950 (Don Green, personal communication [1999]). Some introductions may also have been “bait bucket” releases by fisherman who purchased salamander larvae from local bait dealers and discarded leftover animals in nearby ponds. The introductions by local bait dealers occurred approximately 50 years or 10–25 salamander generations ago (Trenham et al. 2000), allowing ample time for interbreeding.

We studied salamander populations in six ponds, labeled A, C, D, F, 2, and 2a, all within a 1-km² area near the Johnson Canyon landfill, just east of Gonzales, California. Ponds F and A are vernal pools, typical of natural breeding habitat for A. californiense (Storer 1925, Barry and Shaffer 1994, Shaffer and Trenham, in press). Pond F is unmodified, and while there is an old dam below Pond A, it is broken and no longer functional. Ponds 2 and 2a are permanent landfill ponds in the same drainage system, and Ponds C and D are ephemeral, but greatly modified, cattle ponds. These four ponds are artificial and have all been modified to varying degrees, primarily by deepening to prolong their water-holding capacity. As a result, they hold water almost every year regardless of rainfall, they hold water for longer into the summer and fall than natural vernal pools, and in the case of Ponds 2 and 2a, they generally do not dry even in the summer months.

METHODS
We collected salamanders in winter and spring of 1997–1998. Paedomorphic larvae and adults were collected from Pond 2 in November 1997, adults were collected from Ponds 2 and 2a between January and April 1998, and larvae from all six ponds were collected in early April 1998. Metamorphosed adults were collected in pitfall traps along a drift fence, and larvae were collected by seining.
Molecular techniques

We extracted DNA for polymerase chain reactions (PCR) by standard methods (Kocher et al. 1989, Palumbi 1996). We used the primers THR and DL1 (Shaffer and McKnight 1996) to amplify an approximately 840 base-pair (bp) fragment of the mitochondrial control region. Temperature profiles of 94°C (45 s), 54.5°C (90 s) for 35 cycles were used for these reactions. We used an extensive library of sequences from tiger salamanders collected throughout North America (Shaffer and McKnight 1996; H. B. Shaffer, unpublished data) to determine sequence differences between *A. californiense* and *A. tigrinum* mitochondrial DNA (mtDNA) sequences. A restriction site for enzyme *Ssp*-1 was present in all *A. californiense* sequences but not in any of the *A. tigrinum* samples. PCR products were digested with *Ssp*-1 for 4h at 37°C (Dowling et al. 1996), producing two fragments of 280 bp and 560 bp for animals with the *A. californiense* haplotype. To verify that our RFLP (restriction fragment length polymorphism) results correctly identified each species, we bidirectionally sequenced four presumed *A. californiense* and four presumed *A. tigrinum* alleles using an ABI 377 automated sequencer (Applied Biosystems, Foster City, California, USA), added these new sequences to our existing database, and confirmed that haplotypes identified as *A. californiense* or *A. tigrinum* associated phylogenetically with their inferred species.

For nuclear DNA, we amplified an approximately 220-bp fragment of the sodium bicarbonate co-transporter gene, *Slc4a*, and an approximately 220-bp fragment of the homeobox-containing *Dlx3* gene using primers reported by Voss et al. (2001). These genes are unlinked in the ambystomatid genome (Voss et al. 2001). Temperature profiles of 94°C (30 s), 54°C (60 s), 72°C (120 s) for 30 cycles were used for PCR amplification. We bidirectionally sequenced one *A. tigrinum* and one *A. californiense* individual for both of these nuclear markers. In the *A. tigrinum* sequence we found restriction sites for enzyme *Sau*-96 in *Slc4a*, producing fragments of 50 bp and 170 bp, and for enzyme *Alu*-1 in *Dlx3*, producing fragments of 70 bp and 150 bp. Both of these restriction sites were absent in *A. californiense*, and we confirmed that they were diagnostic differences for the two taxa by digesting 20 individuals each from a pure *A. californiense* (Solano County, California) and an introduced *A. tigrinum* (Lake County, California) population. Enzyme digestions were incubated for 4 h at 37°C, and digestion products were separated on 1.5% agarose gels with pure *A. californiense* (Monterey County, California) and pure *A. tigrinum* (Lincoln County, Colorado, USA).
controls and a low-DNA mass ladder to aid interpretation. Based on our initial work on pure populations, we predicted that upon restriction–digestion of each nuclear gene, pure *A. californiense* animals would show single undigested fragments, pure *A. tigrinum* animals would show the smaller restriction fragments, and hybrid individuals would show all fragments, but of lesser intensity (see also Evans et al. 1998). Our predicted fragment patterns were observed, although a faint undigested fragment was sometimes seen for homozygous *A. tigrinum* individuals, and for heterozygotes the (undigested) *A. californiense* allele was often darker than the *A. tigrinum* allele. To confirm that our interpretation of these patterns correctly reflects the underlying alleles, we bidirectionally sequenced two individuals of all three genotypes for both genes. Homozygous genotypes indicated by RFLP were confirmed by sequencing, but putative heterozygotes did not produce readable sequences. Therefore, we cloned alleles for each gene from three heterozygote individuals (for *Slc4a*, individuals HBS 21729, 21817, 21857; for *Dlx3* HBS 21510, 21823, 21847 ["HBS" stands for H. Bradley Shaffer; these are catalog numbers in the second author’s specimen collection]), and confirmed that both alleles were present using RFLP. We then sequenced a representative *A. californiense* and *A. tigrinum* clone from one heterozygote for each gene (*Slc4a*, HBS 21817; *Dlx3*, HBS 21823) to confirm our RFLP identifications. Representative sequences of mitochondrial and nuclear genes are available from GenBank (accession numbers AY185773–AY185795).

**Data analysis**

We scored both the mtDNA marker and the nuclear markers for at least 30 animals per pond. For each individual we computed a hybrid index, \( I \), consisting of the number of introduced alleles summed across the three loci. This index ranges from 0 for animals with all *A. californiense* alleles to 5 for animals with all *A. tigrinum* alleles (see Appendix A). F1 hybrids should be heterozygotes at both nuclear markers and have either mtDNA marker, yielding a hybrid index score of 2 or 3. Individuals with hybrid index scores of 1 or 4, or of 2 or 3 by other combinations, are necessarily F2 or backcross hybrids, and their identification as such is unequivocal. Given that we have information for only five alleles for each animal, there is a chance that additional molecular markers would yield a different conclusion about the status of a "pure" animal with a hybrid index of 0 or 5, or of an F1 animal. For example, consider an animal that is in fact a later generation hybrid and has 50% native and 50% nonnative alleles. We would score such an individual as having a hybrid index score of 0, thus appearing to be a pure native animal, 3.1% (0.5\(^2\)) of the time if all alleles sort independently.

We tested whether there was evidence of reproductive isolation, selection, or migration in the ponds by performing an overall test for three-locus disequilibrium. We computed expected genotype frequencies for each of the 18 possible genotypes—nine combinations of the two diploid nuclear markers, each with either *A. californiense* or *A. tigrinum* mtDNA—under the assumption that each pond’s population was in multilocus Hardy-Weinberg equilibrium (HWE). Monte Carlo simulation was used to evaluate the correspondence of expected genotypic frequencies under HWE to genotypic frequencies calculated from observed allele frequencies. We calculated \( P \) values as the fraction of 1000 Monte Carlo replicates that deviated from multilocus HWE expectations as much or more than the observed data.

For each pond we also tested for significant deviation from single-locus HWE for each nuclear gene separately, using \( \chi^2 \) tests. To further quantify the nature of genetic associations in these hybrid populations, we tested for linkage disequilibria (\( D \)) between all pairs of loci. These disequilibria quantify nonrandom associations between genes or genotypes within a pond. Significant disequilibrium indicates nonrandom mate choice (which may or may not be symmetrical for the two species), nonrandom survival of different genotypic combinations, or significant migration into the population from more pure populations (Barton 1979, Asmussen et al. 1989, Arnold 1993). For cytonuclear disequilibria, a significantly positive \( D \) indicates a positive association between mitochondrial and nuclear alleles derived from the same parental species, while \( D_2 \), \( D_3 \), and \( D_4 \) represent associations between native *A. californiense* mitochondria and native homozygote, heterozygote, and introduced homozygote genotypes, respectively (see Appendix B, Table B1). \( D_2 \) is particularly interesting because a significant value shows that heterozygotes are more likely to have one species’ mitochondria than the other. This indicates an inequality of heterozygote fitness in different cytoplasmic back- grounds, or an asymmetry of mate discrimination between the species (Asmussen et al. 1987, 1989). Disequilibria, \( D_1 \)’s, were converted to standardized disequilibria, \( R \)’s, for comparisons between ponds (Asmussen and Basten 1994; see Table 1). We tested for the significance of cytonuclear disequilibria with exact tests using the program CND (Asmussen and Basten 1994).

We tested for significance of allelic (gametic) nuclear–nuclear disequilibria with the chi-square methods of Weir (1979) using the program Popgene (F. C. Yeh, R.-C. Yang, and T. Boyle [version 1.21]). We used the ANOVA method of Langley et al. (1978) to test whether nuclear disequilibria in the vernal pools were the same as in the artificial ponds. We used the Monte Carlo \( R \times C \) contingency table simulation (B. Engels, [University of Wisconsin], *unpublished program* [version 2.1]) to test for genotypic (zygotic) disequilibrium between nuclear genes using 3 \( \times \) 3 tables and at least 10 000 Monte Carlo replicates. Significance of indi-
Table 1. Cytocnuclear disequilibria between mitochondrial DNA (mtDNA) and two nuclear loci (Slc4a and Dlx3) in tiger salamanders across five of the six study ponds near Gonzales, California, USA.

<table>
<thead>
<tr>
<th>Pond‡</th>
<th>n§</th>
<th>R</th>
<th>Slec4a</th>
<th>Dlx3</th>
<th>Slec4a</th>
<th>Dlx3</th>
<th>Slec4a</th>
<th>Dlx3</th>
<th>Slec4a</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>55</td>
<td>0.983***</td>
<td>0.691***</td>
<td>0.671***</td>
<td>0.535***</td>
<td>0.225</td>
<td>-0.021</td>
<td>-0.850**</td>
<td>-0.522***</td>
</tr>
<tr>
<td>A</td>
<td>37</td>
<td>0.422*</td>
<td>0.125</td>
<td>0.289*</td>
<td>0.138</td>
<td>0.150</td>
<td>-0.041</td>
<td>-0.385**</td>
<td>-0.071</td>
</tr>
<tr>
<td>D</td>
<td>36</td>
<td>-0.166</td>
<td>0.086</td>
<td>-0.372**</td>
<td>-0.070</td>
<td>0.401**</td>
<td>0.221</td>
<td>-0.104</td>
<td>-0.200</td>
</tr>
<tr>
<td>C</td>
<td>43</td>
<td>0.412**</td>
<td>0.240</td>
<td>0.183</td>
<td>-0.079</td>
<td>0.336**</td>
<td>0.366**</td>
<td>-0.422***</td>
<td>-0.334**</td>
</tr>
<tr>
<td>2</td>
<td>61</td>
<td>0.290*</td>
<td>0.058</td>
<td>0.094</td>
<td>0.037</td>
<td>0.274**</td>
<td>0.039</td>
<td>-0.316**</td>
<td>-0.054</td>
</tr>
<tr>
<td>Pooled χ²</td>
<td>42.67***</td>
<td>15.33***</td>
<td>10.04***</td>
<td>4.15*</td>
<td>17.21***</td>
<td>2.32</td>
<td>45.18***</td>
<td>13.58***</td>
<td></td>
</tr>
</tbody>
</table>

Notes: Standardized cytonuclear disequilibria (R) are equivalent to correlation coefficients between mtDNA haplotypes and nuclear alleles (R = D]^½x(1 - x)p(1 - p) + D]u(1 - x) + D](1 - 2x) [^½)] and nuclear genotypes (e.g., R_i = D]i(x(1 - x)u(1 - u)] [^½]), where D is the coefficient of linkage disequilibrium (Asmussen and Basten 1994). D]p is the deviation from single-locus Hardy-Weinberg equilibrium, and other variables are as defined in Appendix B, Table B1.

Pooled tests (1 df) indicate that most disequilibria are significantly different from zero on average across ponds, and heterogeneity tests (4 df) show that disequilibria tend to vary significantly among ponds (Manly 1985, Weir 1996). Individual coefficients were tested using χ² approximations described by Asmussen and Basten (1994).

* P < 0.05; ** P < 0.01; *** P < 0.001.
‡ Pond 2a had no mtDNA variation (no individuals with the native haplotype) and therefore is not included.
§ Number of individuals in each pond, scored for all three genes.

Results

We genotyped 264 salamanders from the six ponds for all three markers. From mitochondrial DNA (mtDNA) typing we determined that salamanders with both native and nonnative haplotypes were present in five of the six ponds, confirming the potential for past and present hybridization. We sequenced mtDNA fragments from four individuals with presumptive A. californiense alleles and four individuals with A. tigrinum alleles. We then constructed neighbor-joining trees with PAUP* software (Swofford 2000) using the three Salinas sequences, sequences from known pure A. californiense from throughout its range, and other A. tigrinum from throughout the United States. All four presumptive A. californiense alleles were identical in nucleotide sequence and clustered with known A. californiense alleles, while the four presumptive A. tigrinum alleles yielded two similar nucleotide sequences. The two introduced haplotypes from Salinas were most closely related to animals from the Great Plains of Nebraska, New Mexico, and Texas (Figs. 1 and 2), consistent with information provided by the bait dealer who performed the introductions.

The presence of putative F1 and F2/backcross genotypes in all six ponds (Fig. 3, Appendix A) demonstrates that these two distantly related phylogenetic species (Fig. 1) produce viable, fertile hybrids under field conditions. Salamanders with a hybrid index I = 0, the only potential examples of pure A. californiense, were present only in Ponds F, A, and D (Fig. 3, Appendix A). Of these animals with I = 0, only one (2.8%) occurred in pond D and three (8.1%) in pond A. Pond F, with 15 animals (27%), was the only pond with an appreciable number of I = 0.94, I = 0.13). However, for the most part of our study ponds, Pond F, there was a highly significant deficit of heterozygotes for Dlx3 (Dlx3, χ² = 22.25, P = 0.00001; Slc4a, χ² = 4.08, P = 0.13). Because we only examined progeny (i.e., larvae) rather than breeding adults in all ponds (except Ponds 2 and 2a), departures from single-locus HWE expectations cannot result from immigration. Rather, they must be due to nonrandom mating or differential survival of larvae.

There were strong, symmetrical genotypic cytonuclear disequilibria, for Dlx3 in the two vernal pools, Ponds F and A, and for Slc4a in Pond F (Table 1, R, and R significant and of opposite sign). In the modified ponds, there were weaker allelic disequilibria between

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FIG. 3. Observed (black bars) and expected (open bars) frequency distributions of the additive hybrid index of tiger salamanders in six ponds in the Salinas Valley, Monterey County, California, USA. Ponds F and A are vernal pools, Ponds D and C are cattle ponds, and Ponds 2 and 2a are permanent landfill ponds (see Study species...: Study area). The distributions in vernal pools are bimodal (Pond F) or relatively flat (Pond A), while in artificial ponds the distributions are unimodal as expected under three-locus Hardy-Weinberg equilibrium. Goodness of fits to three-locus Hardy-Weinberg expectations were tested with Monte Carlo simulations of random union of gametes given the allele frequencies and sample sizes in each pond. Bonferoni-corrected P values based on 1000 replicates are given along with goodness-of-fit statistics for each pond. Salamanders with hybrid index \( I = 0 \), the only potentially pure native \( Ambystoma californiense \) animals, are present primarily only in Ponds F and A, with one animal in Pond D.

There were significant linkage disequilibria between the two nuclear genes in four of the six ponds (Table 2). Pond F had by far the highest linkage disequilibrium, followed by Pond A, and nuclear disequilibria in these two vernal pools were significantly greater than

**Table 2.** Nuclear linkage disequilibria between two unlinked loci.

<table>
<thead>
<tr>
<th>Pond</th>
<th>n</th>
<th>Allelic R</th>
<th>Dlx3\textsuperscript{R}</th>
<th>Slc4a\textsuperscript{R}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Genotypic ( \chi^2 )</td>
<td>( R_{\text{HW}} )</td>
<td>( R_{\text{HW}} )</td>
</tr>
<tr>
<td>F</td>
<td>55</td>
<td>0.472***</td>
<td>29.87***</td>
<td>0.636***</td>
</tr>
<tr>
<td>A</td>
<td>37</td>
<td>0.251**</td>
<td>13.44**</td>
<td>0.103</td>
</tr>
<tr>
<td>D</td>
<td>36</td>
<td>0.198***</td>
<td>18.97**</td>
<td>-0.111</td>
</tr>
<tr>
<td>C</td>
<td>43</td>
<td>0.054</td>
<td>4.34</td>
<td>0.055</td>
</tr>
<tr>
<td>2</td>
<td>61</td>
<td>0.165*</td>
<td>6.89</td>
<td>0.092</td>
</tr>
<tr>
<td>2a</td>
<td>33</td>
<td>0.121</td>
<td>4.43</td>
<td>-0.364*</td>
</tr>
<tr>
<td>Pooled</td>
<td>1 df</td>
<td>( \chi^2 )</td>
<td>51.95***</td>
<td>68.99***</td>
</tr>
<tr>
<td>Heterogeneity</td>
<td>5 df</td>
<td>( \chi^2 )</td>
<td>20.980**</td>
<td>8.94</td>
</tr>
</tbody>
</table>

Notes: Standardized allelic disequilibria are correlation coefficients between \( Ambystoma californiense \) Dlx3 and Slc4a alleles: \( R = D[p_i q_i p_j q_j] \)^\(1/2 \) (Langley et al. 1978; variables defined in Appendix B). Statistical significance (\( \alpha = 0.05 \)) was assessed using Weir’s (1979) methods in the Popgene program (F. C. Yeh, R.-C. Yang, and T. Boyle).

* \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \).

\( \dagger \) Genotypic \( \chi^2 \) values were computed from 3 x 3 contingency tables of the form shown in Appendix B, Table B2, and statistical significance was assessed with Engel’s Monte Carlo \( R \times C \) program. Standardized deviations from single-locus Hardy-Weinberg equilibrium (HWE), \( R_{\text{HW}} = D_{\text{HW}}/[p(1-p)] \), were evaluated with traditional \( \chi^2 \) tests.
in the four modified ponds ($F_{1,4} = 17.47, P = 0.014$). In three of the six ponds, Ponds A, D, and F, there were also significant genotypic associations (Tables 2 and 3). Specifically, double heterozygotes and both “pure species” double homozygotes were significantly more common than expected ($D_{13}, D_{22}, D_{33} > 0$; $P < 0.05$), and the other six combinations were all less common (Table 3).

### Discussion

We found clear genetic evidence of extensive hybridization between the declining native salamander *Ambystoma californiense* and its invasive relative *A. tigrinum*. Nonrandom genetic associations provide some evidence of partial barriers to genetic exchange, particularly in natural breeding ponds. However, these genetic barriers alone are not likely to prevent eventual merger of the two taxa, especially since most available breeding ponds in California are artificial or highly modified. The dynamics of hybridization between these divergent salamanders provide some important lessons about the evolution of reproductive isolation and the conservation consequences of species introductions.

The first and most important conclusion from our study is that *A. californiense* and introduced *A. tigrinum* are interbreeding in the wild and producing viable and fertile hybrid offspring. This hybridization was occurring in all six of the ponds that we examined within a small, 1-km² geographic area, and the known range of hybridization extends for over 160 km in a north-south direction at least from Santa Clara to San Luis Obispo counties (California, USA) (H. B. Shaffer and B. M. Fitzpatrick, *unpublished data*). For the California tiger salamander, which has declined over the vast majority of its range and is impacted by other threats such as past and continuing habitat loss and predation from other exotic species (Fisher and Shaffer 1996, Davidson et al. 2002), this genetic mixing is a serious threat. Its declining status may make *A. californiense* particularly vulnerable to genetic swamping. This problem will be exacerbated if nonnative traits provide any kind of selective advantage, particularly in altered habitats (e.g., facultative paedomorphosis in perennial ponds).

Given the ancient split and considerable geographic distance between these two taxa, (Figs. 1 and 2; Shaffer and Mc Knight 1996), it is noteworthy that these animals are successfully interbreeding. It is particularly remarkable to document this interbreeding in the field, as it is rare to examine reproductive isolation between allopatric species under natural conditions. Most tests of reproductive isolation between allopatric taxa, both in amphibians (Tilley et al. 1990, Voss and Shaffer 1996, Sasa et al. 1998), and in non-amphibian taxa (Coyne and Orr 1989, 1997, Colbourne and Hebert 1996, Edmands 1999, Lee 2000, Fitzpatrick 2002), have been conducted in the laboratory. The extent of this hybridization between genetically, geographically, and phylogenetically distant species is interesting from an evolutionary perspective, and it is important for conservation biologists. Apparently, even a distant relationship between a rare species and other sympatric taxa (introduced or native) may not indicate strong enough reproductive isolation to prevent genetic introgression and the loss of genetic purity in the rare species.

Despite the presence of F2/backcross animals in all six ponds, significant departures from Hardy-Weinberg expectations and significant linkage and cytonuclear disequilibria indicate that these pools are not randomly interbreeding hybrid swarms in which survival and mating are unrelated to genetic identity. We do not know the specific mechanisms behind these results, but assortative mating, differential survival of larvae, and migration could all be involved. The fact that the strongest departures from expectation also occur in the most ecologically intact breeding habitats is encouraging from a conservation point of view. The symmetrical cytonuclear disequilibria in Ponds A and F—natural vernal pools—suggest that positive assortative mating
or decreased survival of hybrid larvae occur in these habitats to a greater degree than in human-modified ponds. The disequilibria coupled with the positive genotypic associations at nuclear loci could also indicate that the hybridization events in these ponds are more recent, or that pure native and nonnative animals continue to enter these ponds for breeding, although the close proximity of all the ponds in this study argues against these interpretations.

The strong association in modified ponds between native mitochondrial DNA (mtDNA) and Dlx3 heterozygotes suggests either increased mating between females with A. californiense mtDNA and hybrid males with nonnative Dlx3 alleles relative to the reciprocal cross, or asymmetrical epistatic interactions between mitochondrial proteins and alleles of Dlx3 or nearby genes (Orr 1995, Edmands and Burton 1999). In permanent ponds, if many of the nonnative and hybrid males are paedomorphic (and preliminary results suggest that this is the case), they may have opportunities to mate with early or late-arriving native females, since native males come to ponds only to breed before returning to upland areas (Trenham et al. 2000). Paedomorphic males in productive aquatic environments may also be bigger than native males, and able to out-compete them for matings. However this would not explain the results for ponds C and D—ephemeral but modified for cattle use—because they are not permanent and do not support paedomorphs. Current and future work will refine our knowledge of these mechanisms.

The fact that there is any evidence of potential genetic separation between the native and introduced species in this case is also encouraging. In other cases of hybridization with nonnative species there is a range of results regarding genetic introgression and interchange. In the endemic pupfish Cyprinodon pecosensis and the introduced Cyprinodon variegatus, a small introduction at one location generated a complete hybrid swarm covering half the range of the native species in less than five years with no evidence of deviations from Hardy-Weinberg equilibrium (HWE) (Echelle and Connor 1989). Hybridization between the federally threatened, native Apache trout and the introduced cutthroat trout (Oncorhynchus clarki) similarly showed no evidence of deviation from HWE based on allozyme frequencies (Dowling and Childs 1992). In other situations there is more separation between the native and introduced taxa. Hybridization appears to be quite rare between the threatened delta smelt and the introduced wakasagi (Hypomesus nipponensis; Trenham et al. 1998). Where red deer and exotic sika deer (Cervus nippon) interbreed in Scotland, recent evidence (Goodman et al. 1999) indicates that the extent of hybridization is less than once thought (Abernethy 1994), and that the degree of introgression is less than would be predicted by hybridization rates. While European polecats and introduced ferrets (Mustela furo) maintain distinct mtDNA haplotype distributions and the native mustelid may be better adapted to wild habitats, more detailed genetic work involving nuclear markers is necessary to determine the extent of hybridization and introgression (Davison et al. 1999). There was evidence of F1 inviability or sterility in hybrids between bull trout and nonnative brook trout (Salvelinus fontinalis) in a Montana stream, where 73 of 75 naturally occurring hybrid individuals appeared to be F1’s (Leary et al. 1993). However even where there is evidence of hybrid breakdown, if the introduced species is numerically dominant, as is the case in many situations, hybridization will lead to significant wasted reproductive effort for the native species and a potentially rapid decline in the native population (Leary et al. 1993).

The long-term prospects for the genetic legacy of A. californiense depend also on the geographic range of introduced and hybrid tiger salamanders. We know already of other hybrid populations sprinkled throughout the range of the California tiger salamander, and we are currently investigating the geographic extent of this problem. In the long run, biologists and managers will have to develop criteria to define hybrid populations and establish strategies for their management. In an early discussion of these issues, Allendorf and Leary (1988) suggest eliminating populations of cutthroat trout with introgression in >1% of individuals. Dowling and Childs (1992) argue, however, that population elimination may be a risky alternative, especially in very rare species and in cases where local adaptation may be important. In the case of these tiger salamanders, elimination of all, or perhaps even most, of the impure populations is probably not feasible due to the geographic extent of the invasion, the predominance of private land in the Salinas Valley, the longevity of adult salamanders, and their habit of spending most of their lives in secluded underground retreats (Shaffer and Trenham, in press). Thus, it may be difficult to define a management goal that is both feasible and satisfactory.

It has long been postulated that habitat disturbance, including human habitat alteration, can break down reproductive barriers and lead to increased hybridization (Anderson 1948, Seehausen et al. 1997), particularly in pond-breeding amphibians. At constructed ponds in Alabama (USA) without surrounding vegetation, there is less differentiation between calling sites and therefore increased hybridization between the treefrogs Hyla gratiosa and Hyla cinerea (Schlefer et al. 1986). Fire-bellied toads (Bombina spp.) in Austria hybridize in areas of transition between lowlands and hills, but many of these hybrid populations occur in disturbed habitats (Gollmann 1996). In Arizona, the toad Bufo microscaphus, which prefers shallow, fast-moving streams, may be increasingly hybridizing with Bufo woodhousei in lentic habitats created by stream impoundments and urbanization (Sullivan 1986). Certainly not all cases of disturbance produce increased
interbreeding. Among South African clawed frogs, the rare and threatened *Xenopus gilli* occupies only native, blackwater, fynbos ponds, while the widespread *X. laevis* occurs also in highly disturbed ponds of lighter color and higher pH (Picker 1985). *Xenopus gilli* is threatened by loss of the native fynbos ponds, and potentially by competition with *X. laevis*. However while hybridization was originally feared to be a serious threat to *X. gilli* (Picker 1985, Picker and de Villiers 1989), more recent molecular work with both mtDNA and nuclear DNA reported very low levels of hybridization and introgression (Evans et al. 1998).

Given a goal of preserving *A. californiense* in its native form, we are thrice unlucky. An exotic species has been introduced into the range of a declining native salamander, reproductive isolation is not strong enough to have prevented extensive genetic mixing, and widespread disturbance of native breeding habitats may have eliminated or dampened what reproductive isolation exists. At this point, a combination of geographical containment and further exploiting habitat-specific variation in hybrid fitness may be the best solutions to managing these populations.

**ACKNOWLEDGMENTS**

We thank the Salinas Valley Solid Waste Authority for funding, for access to their property, and for arranging access to nearby properties. Dana Bland collected and transported the salamanders for this study and provided invaluable site information and consultation. We thank Greg Pauly and especially Jeff Oliver for assistance and guidance with the molecular work, the Shaffer laboratory for discussion, John Gillespie, Chuck Langley, Peter Chesson, and Jason Hoeksema for statistical consultation, and Michael Turelli for comments on an earlier draft of the manuscript. This material is based upon work supported by the EPA under grant number R-82889601-0 (to H. B. Shaffer), and U-91572401-3 (to B. M. Fitzpatrick), the NSF (9727161) to H. B. Shaffer, and the University of California Agricultural Experiment Station to H. B. Shaffer.

**LITERATURE CITED**


APPENDIX A

Observed (Obs.) and expected (Exp.) counts of each salamander (Ambystoma) genotype in each of the six study ponds in Monterey County, California, USA; expected counts are based on Hardy-Weinberg equilibrium assumptions and observed allele frequencies.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Genotype†</th>
<th>Hybrid index, I</th>
<th>Pond F</th>
<th>Pond A</th>
<th>Pond D</th>
<th>Pond C</th>
<th>Pond 2</th>
<th>Pond 2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>All A. tigrinum</td>
<td>T/T/T/TT</td>
<td>5</td>
<td>14</td>
<td>1.5</td>
<td>7</td>
<td>2.9</td>
<td>3</td>
<td>1.3</td>
</tr>
<tr>
<td>F2 or Backcross</td>
<td>C/T/T/TT</td>
<td>4</td>
<td>0</td>
<td>1.9</td>
<td>1</td>
<td>1.3</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>F2 or Backcross</td>
<td>T/T/T/CT</td>
<td>4</td>
<td>0</td>
<td>3.0</td>
<td>2</td>
<td>4.1</td>
<td>1</td>
<td>2.4</td>
</tr>
<tr>
<td>F2 or Backcross</td>
<td>C/T/T/CT</td>
<td>3</td>
<td>1</td>
<td>3.9</td>
<td>1</td>
<td>1.7</td>
<td>1</td>
<td>2.1</td>
</tr>
<tr>
<td>F2 or Backcross</td>
<td>T/T/CC/CC</td>
<td>3</td>
<td>0</td>
<td>1.5</td>
<td>0</td>
<td>1.4</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>F2 or Backcross</td>
<td>C/T/CC/CT</td>
<td>2</td>
<td>2</td>
<td>1.9</td>
<td>1</td>
<td>0.6</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>F2 or Backcross</td>
<td>T/CC/T/TT</td>
<td>4</td>
<td>6</td>
<td>3.0</td>
<td>5</td>
<td>4.4</td>
<td>2</td>
<td>2.4</td>
</tr>
<tr>
<td>F2 or Backcross</td>
<td>C/CT/TT</td>
<td>3</td>
<td>1</td>
<td>3.9</td>
<td>0</td>
<td>1.9</td>
<td>2</td>
<td>2.1</td>
</tr>
<tr>
<td>F1</td>
<td>T/CT/CT</td>
<td>3</td>
<td>2</td>
<td>6.1</td>
<td>6</td>
<td>6.1</td>
<td>6</td>
<td>4.6</td>
</tr>
<tr>
<td>F1</td>
<td>C/CT/CT</td>
<td>2</td>
<td>5</td>
<td>7.7</td>
<td>5</td>
<td>2.6</td>
<td>11</td>
<td>4.1</td>
</tr>
<tr>
<td>F2 or Backcross</td>
<td>T/CT/CC</td>
<td>2</td>
<td>1</td>
<td>3.0</td>
<td>2</td>
<td>2.1</td>
<td>2</td>
<td>2.2</td>
</tr>
<tr>
<td>F2 or Backcross</td>
<td>C/CT/CC</td>
<td>1</td>
<td>5</td>
<td>3.9</td>
<td>0</td>
<td>0.9</td>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td>F2 or Backcross</td>
<td>T/CC/TT</td>
<td>3</td>
<td>1</td>
<td>1.5</td>
<td>1</td>
<td>1.7</td>
<td>0</td>
<td>1.2</td>
</tr>
<tr>
<td>F2 or Backcross</td>
<td>C/CC/TT</td>
<td>2</td>
<td>0</td>
<td>1.9</td>
<td>0</td>
<td>0.7</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>F2 or Backcross</td>
<td>T/CC/CT</td>
<td>2</td>
<td>0</td>
<td>3.0</td>
<td>2</td>
<td>2.3</td>
<td>0</td>
<td>2.2</td>
</tr>
<tr>
<td>F2 or Backcross</td>
<td>C/CC/CT</td>
<td>1</td>
<td>2</td>
<td>3.9</td>
<td>0</td>
<td>1.0</td>
<td>1</td>
<td>2.0</td>
</tr>
<tr>
<td>F2 or Backcross</td>
<td>T/CC/CC</td>
<td>1</td>
<td>0</td>
<td>1.5</td>
<td>1</td>
<td>0.8</td>
<td>3</td>
<td>1.1</td>
</tr>
<tr>
<td>All A. californiense</td>
<td>C/CC/CC</td>
<td>0</td>
<td>15</td>
<td>1.9</td>
<td>3</td>
<td>0.3</td>
<td>1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Notes: Ponds A and F are natural vernal pools; Ponds C and D are ephemeral, but greatly modified, cattle ponds; and Ponds 2 and 2a are artificial, permanent landfill ponds.

† CC means A. californiense (i.e., native) homozygote; CT means californiense/tigrinum (i.e., native/introduced) heterozygote; and TT means tigrinum (introduced) homozygote.
APPENDIX B

LINKAGE DISEQUILIBRIUM DEFINITIONS

Table B1. Contingency table showing how cytonuclear disequilibria were described by Asmussen et al. (1987) and Arnold (1993). In our case, C represents a native Ambystoma californiense allele, and T represents an A. tigrinum allele.

<table>
<thead>
<tr>
<th>mtDNA</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>$f_{11} = ux + D_1$</td>
<td>$f_{12} = vx + D_2$</td>
<td>$f_{13} = wx + D_3$</td>
<td>$x$</td>
</tr>
<tr>
<td>T</td>
<td>$f_{21} = uy - D_1$</td>
<td>$f_{22} = vy - D_2$</td>
<td>$f_{23} = wy - D_3$</td>
<td>$y$</td>
</tr>
<tr>
<td>Totals</td>
<td>$u$</td>
<td>$v$</td>
<td>$w$</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Notes: The following identities illustrate interdependencies and constraints. $D$ is the allelic (or gametic) cytonuclear disequilibrium:

$p = u + \frac{1}{2}v; \quad q = 1 - p$

$D = f_{11} + \frac{1}{2}f_{12} - px = D_1 + \frac{1}{2}D_2 = -D_3 - \frac{1}{2}D_2 = \frac{1}{2}D_1 - \frac{1}{2}D_3$

$D_1 + D_2 + D_3 = 0$

$-px, -qy \leq D \leq py, qx$

† Mitochondrial DNA.

‡ $u$, $v$, and $w$ are the respective frequencies of CC, CT, and TT.

Table B2. Contingency table defining nuclear–nuclear disequilibria for a pair of diploid loci, $i$ and $j$.

<table>
<thead>
<tr>
<th>Locus $j$</th>
<th>$i_1 i_1$</th>
<th>$i_1 i_2$</th>
<th>$i_2 i_2$</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>$j_1 j_1$</td>
<td>$f_{11} = R_1 C_1 + D_{11}$</td>
<td>$f_{12} = R_1 C_2 + D_{12}$</td>
<td>$f_{13} = R_1 C_3 + D_{13}$</td>
<td>$R_1$</td>
</tr>
<tr>
<td>$j_2 j_2$</td>
<td>$f_{21} = R_1 C_1 + D_{21}$</td>
<td>$f_{22} = R_2 C_1 + D_{22}$</td>
<td>$f_{23} = R_2 C_2 + D_{23}$</td>
<td>$R_2$</td>
</tr>
<tr>
<td>Totals</td>
<td>$C_1$</td>
<td>$C_2$</td>
<td>$C_3$</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Notes: The alleles $i_1$ and $j_1$ are derived from species 1, and alleles $i_2$ and $j_2$ from species 2. $D$ is the allelic (or gametic) disequilibrium; $R_i$ are row totals, and $C_i$ are column totals; $D_{ij}$ are genotypic associations. The following expressions illustrate the interdependencies and constraints on these associations (Weir 1996, Yang 2000):

$p_1 = C_1 + \frac{1}{2}C_2; \quad p_2 = R_1 + \frac{1}{2}R_2; \quad q_1 = 1 - p_1; \quad q_2 = 1 - p_2$

$D = 2f_{11} + f_{12} + f_{21} + \frac{1}{2}f_{22} - 2p_1 p_2 = 2D_{11} + D_{12} + D_{21} + \frac{1}{2}D_{22}$

$D_{12} = -(D_{11} + D_{13}); \quad D_{21} = -(D_{11} + D_{13}); \quad D_{23} = -(D_{13} + D_{23}); \quad D_{32} = -(D_{31} + D_{33})$

$D_{22} = D_{11} + D_{13} + D_{23}$

$-p_1 q_1, -p_2 q_2 \leq D \leq p_2 q_2, p_1 q_1$