

INTRODUCTION HISTORY AND HABITAT VARIATION EXPLAIN THE LANDSCAPE GENETICS OF HYBRID TIGER SALAMANDERS

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Abstract. Genetic introgression from introduced species into native populations is a growing challenge for biological conservation, and one that raises unique practical and ethical issues. Here, we describe the extent of introgression between native California tiger salamanders (*Ambystoma californiense*) and introduced barred tiger salamanders (*A. tigrinum mavortium*) relative to habitat, distance from introduction sites, and watershed boundaries. We used ancestry informative markers (AIMs) to characterize the degree of introgression at 85 sites within the range of *A. californiense*. Eight unlinked markers showed concordant patterns, indicating that different chromosomal segments are introgressing at similar rates. The current distribution of introduced alleles is largely contained in the Salinas Valley, California. Within it, the distribution of nonnative alleles was best explained at a broad geographic scale by the history of introductions, with limited introgression beyond 12 km from multiple independent release sites. The spatial transition from highly admixed to nearly pure native populations was abrupt, suggesting either cryptic barriers to dispersal or locally rapid displacement of natives by an advancing hybrid swarm. At a more ecological level, highly modified perennial breeding ponds had higher introduced allele frequencies than more natural seasonal ponds, suggesting greater invasion success in perennial breeding ponds. Management favoring natural habitat characteristics may substantially decrease the rate of spread of introduced alleles.

Key words: *Ambystoma* spp.; biological invasion; conservation genetics; DNA; Endangered Species Act; hybridization; intercross; molecular markers; tiger salamanders.

INTRODUCTION

Invasive species constitute a growing economic nuisance and threat to endangered species conservation (e.g., Pimentel et al. 2001). One of the most difficult issues presented by biological invasions is hybridization between native species and introduced or domesticated forms (Rhymer and Simberloff 1996, Allendorf et al. 2001, Daniels and Corbett 2003). Hybridization is a particularly complex type of invasion, where the unit of invasion is the unit of heredity (i.e., a nucleotide, gene, or chromosomal segment) rather than the individual organism (Petit 2004, Mallet 2005). A primary concern of many conservation biologists is that hybridization may lead to rapid replacement of native species by hybrid swarms or largely nonnative admixtures. Such genetic change has been characterized as a kind of extinction (Rhymer and Simberloff 1996), and may result in a population with fundamentally altered ecological function (Ellstrand and Schierenbeck 2000). A key element in understanding the consequences of hybridization between introduced and native species is quantifying the importance of natural dispersal, human-

mediated dispersal, and habitat in determining the geographic distribution of introduced alleles.

Barred tiger salamanders (*Ambystoma tigrinum mavortium*) have been translocated extensively in the western United States by bait dealers and fishermen, who use the larvae (“waterdogs”) as live bait for trophy bass (Espinosa et al. 1970, Collins 1981, Riley et al. 2003). Tiger salamander larvae are dominant predators in fishless ponds, lakes, and vernal pools, and can have substantial effects on the distribution and abundance of aquatic arthropods, frogs, and other salamanders (Wilbur 1972, Holomuzki et al. 1994, Werner and McPeck 1994, Benjoy 2005). Introduced *A. t. mavortium* hybridize with threatened California tiger salamanders, *A. californiense* (Riley et al. 2003), and endangered Sonora tiger salamanders, *A. tigrinum stebbinsi* (Storfer et al. 2004). While population genetic consequences of this invasion have been examined at the local pond level (Fitzpatrick and Shaffer 2004), factors influencing the spatial spread of introduced populations and gene flow into native species are not well understood.

California tiger salamanders are listed as “threatened” (“endangered” in Santa Barbara and Sonoma Counties) under the U.S. Endangered Species Act due to range-wide declines primarily associated with habitat destruction (Fisher and Shaffer 1996, USFWS 2004). Hybridization with barred tiger salamanders is also recognized

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as a major threat, yet the geographic distribution, ecological effects, and legal status of hybrid/introgressed populations are not yet well characterized. Here, we focus on the range-wide pattern of admixture and introgression in relation to the spatial distribution of both introductions and aquatic habitat modification. In particular, we ask: (1) what is the spatial extent of introgression, based on multiple unlinked molecular markers; (2) how far have introduced alleles moved from known sites of introduction; (3) is the invasion marked by a sharp front or a diffusion gradient; and (4) do different breeding habitats contribute differentially to the invasion at the landscape scale? We end with a discussion of some of the inherent difficulties in managing this invasion, and outline some important legal and ethical points requiring resolution.

METHODS

Introduction sites and habitat types

In the 1940s and 1950s, bait dealers from the Salinas Valley (Monterey County, California, USA) imported thousands of tiger salamander larvae from Texas and other parts of the southwestern United States (Riley et al. 2003). Many were released in the hope of establishing harvestable populations in central California because they attain larger size prior to metamorphosis and can be available further into the summer than native *A. californiense*. These introductions have resulted in a series of hybridization foci scattered across central California.

An important component of this study is separating human-mediated animal movement (bait dealers relocating salamanders) from naturally occurring spread of nonnative genotypes. We identified sites as known or suspected introduction sites based on interviews with long-time residents and the one surviving member of the group of bait dealers responsible for importing and releasing *A. t. mavortium* in California. The introduction effort was well-known and involved the cooperation of several individuals; however, it was not coordinated or documented. Therefore the quality of information about specific release sites varies. We classified potential release sites according to the information used: (A) specific pond mentioned by bait dealer or landowner in our interviews as a release site; (B) specific pond strongly suspected based on known bait dealer harvest, but not specifically on known release of nonnative salamanders; and (C) strong candidate based on access and releases known to have occurred in the vicinity (i.e., on the same property), but with no specific release or harvest documented. The sites are identified in Appendix A.

We also classified each pond as seasonal (does not hold water throughout the year) or perennial (does not usually dry up) based on personal observation, statements of ranchers or local biologists, and presence/absence of specialized organisms. Specialized "indicator" species included fairy shrimp (*Branchinecta* and *Linderiella*) and clam shrimp (*Cyzicus*) for seasonal ponds, and

paedomorphic tiger salamanders and bullfrog tadpoles (*Rana catesbeiana*) for perennial ponds, since both require at least 12 months for development.

Sampling and molecular methods

All salamander DNA samples used in this study were taken from young-of-the-year larvae. We used seines or dip nets, drawn haphazardly through each breeding pond, to capture larvae. Focusing on larvae allowed us to obtain large samples from populations with well-understood population genetic properties. In particular, fully aquatic larvae in landlocked ponds comprise discrete populations consisting of a single generation with no immigrants.

Using genetic resources derived from the tiger salamander genome project (Voss et al. 2001), we developed eight ancestry-informative markers (AIMs). We identified candidate single nucleotide differences between *A. californiense* and *A. t. mavortium* by sequencing DNA of 2–4 individuals of each pure species with primers described by Voss et al. (2001). We chose to use nucleotide differences that cause restriction enzymes to cut the PCR-amplified DNA of one species but not the other. This yields markers that are easy to score in large numbers and that are informative with respect to individual genotype (homozygous native, heterozygous, or homozygous introduced) for each genomic region. We accepted a marker as diagnostic if the difference was fixed between samples of ~50 pure native and 50 pure nonnative animals; details on marker development can be found in Fitzpatrick and Shaffer (2004). Table 1 provides genomic and PCR primer information on the final set of markers used in this study. The marker *GNAT1*, used in Fitzpatrick and Shaffer (2004), was not used here because it is not 100% diagnostic.

Multivariate analysis of eight markers

To accomplish a geographically thorough analysis while minimizing costs and laboratory time, we sought to identify a subset of the eight markers for use on a larger number of individuals that would identify the current limits of the hybrid zone with a smaller number of loci. According to our conversations with Mr. Don Green, one of the original bait dealers who brought *A. t. mavortium* to California, most animals were released in the Gonzales area of the Salinas Valley, whereas none were released to the north in Santa Clara and Alameda Counties. Therefore, we chose an initial transect of 28 breeding ponds starting at Gonzales (Monterey County) and extending north to the Ohlone Wilderness just east of Fremont (East Bay Regional Parks, Alameda County; Fig. 1 and Appendix A). This rough transect extended from the major center of the introduction into a relatively natural area that we predicted was still pure *A. californiense*. We scored all eight markers (seven nuclear, one mitochondrial, Table 1) for all individuals in these 28 samples.

TABLE 1. The eight diagnostic molecular markers that were used to characterize hybrid tiger salamanders (modified from Fitzpatrick and Shaffer [2004]).

Marker	Linkage	Detection†	Primers	Primer source
<i>D-loop</i>	mtDNA	<i>SspI</i> cuts cal	THR-AAACATCGATCTTGTAAGTC DL1-AATATTGATAATTCAAGCTCCG	Shaffer and McKnight (1996)
<i>GNAT2</i>	17	<i>AluI</i> cuts mav	F6-AACCACATCCGCTTCTCAAC DR-CAGCCCTAG (C/T) (C/T) (A/G) AGGCCTTC	Designed from GenBank AF050654
<i>SLC4A4</i>	2	<i>Sau96I</i> cuts mav	F-TCCGCTTGACAGCAGTCTCCTTGCTCTC R-TAACGGCTGATTGATGACCAGCGAAG	Voss et al. (2001)
<i>HOXD8</i>	9	<i>Fnu4HI</i> cuts mav	F-CGGACTGTAATCGTCCAGTGGTCC R-GCTGGGAACCTGTCTTTGTTGTTCTC	Voss et al. (2001)
<i>DLX3</i>	23	<i>AluI</i> cuts mav	F-GGCGAGGCGCACCTCTCCAAGTGGTGA R-AGGCTCCACCTTCTGAGTTGGGAAGG	Voss et al. (2001)
<i>HOXB13</i>	23	<i>HpyCH4V</i> cuts mav	F-GTCTCCTTTGCTTGATTTCGG R-TCACAAAATCACAGAACACCTGC	Voss et al. (2001)
<i>FOXG1B</i>	16	<i>BspCNI</i> cuts cal	F-GATGGACTGCTAATCTTTGAGACCC R-AACAAGTGGGGCAAACAACCAACCAGC	Voss et al. (2001)
<i>WNT1</i>	7	<i>RsaI</i> cuts cal	F-CAGCGCAAATCACACCTCA R-TCCATAAACGCTGCCAAAATAAAG	Voss et al. (2001)

† "Cuts cal" and "cuts mav" indicate which species allele (*Ambystoma californiense* and *A. tigrinum mavortium*, respectively) is cut by the restriction enzyme. Linkage groups are according to Voss et al. (2001).

We used principal components analysis (PCA) to describe allele frequency variation among ponds and assess concordance in the information provided by different molecular markers. Each pond was treated as a single observation with eight variables (introduced allele frequency for each marker) and PCA was performed on the correlation matrix (using the covariance matrix produced equivalent results). PCA finds linear combinations (principal components, or PCs) of variables that best describe the variation among observations in the data set. Thus, if one or a few PCs describe most of the variance among ponds, and if most marker frequencies are highly correlated with that PC, then we can choose a smaller number of markers for more thorough geographic analysis without losing much information compared to the eight-gene analysis. That is, a single dominant PC indicates that the information obtained from multiple markers is largely redundant. Alternatively, if different markers are correlated with different (independent) PCs, then there are multiple dimensions of variation among breeding ponds that would be missed by only a few molecular markers. Statistical analyses were performed using R Version 2.3.0 unless otherwise noted (*available online*).⁴

Allele frequencies were highly concordant among markers (Fig. 2). The first principal component accounted for 94.2% of the variance among ponds and was highly associated with all eight markers (Appendix B). The second component, accounting for 3% of the variance, suggested that mtDNA was somewhat deviant from the other markers (Appendix B); this can be seen visually as greater dispersion in the mtDNA panel in Fig. 2. Based on these multivariate results, we chose

three markers for further analysis: mtDNA, *SLC4A4*, and *DLX3*. These three markers were the first to be used in describing hybrid tiger salamander populations (Riley et al. 2003) and include the marker most closely associated with PC1 (*SLC4A4*) and the one least closely associated (mtDNA).

Geographic analysis of three markers

We used our subset of three markers in an expanded set of breeding pond samples that included 85 ponds and

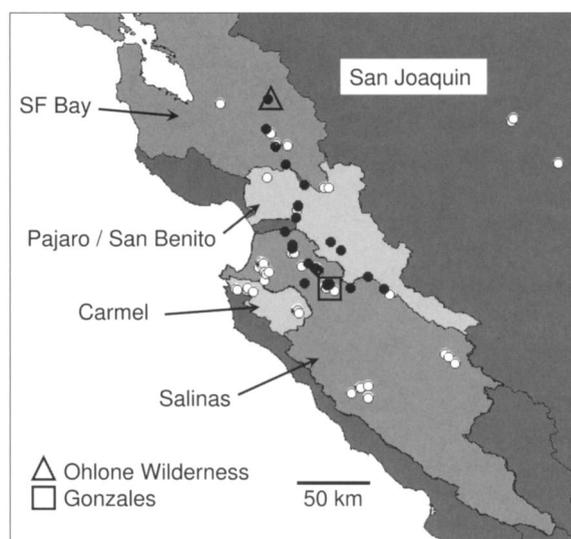


FIG. 1. Sample locations in California, USA: solid circles represent sites assayed for all eight DNA markers, and open circles represent sites assayed for only three markers. Boundaries of watersheds (shown in different shades of gray) were adapted from the CalWater GIS database (<http://www.ca.nrcs.usda.gov/features/calwater/>).

⁴ (<http://www.r-project.org>)

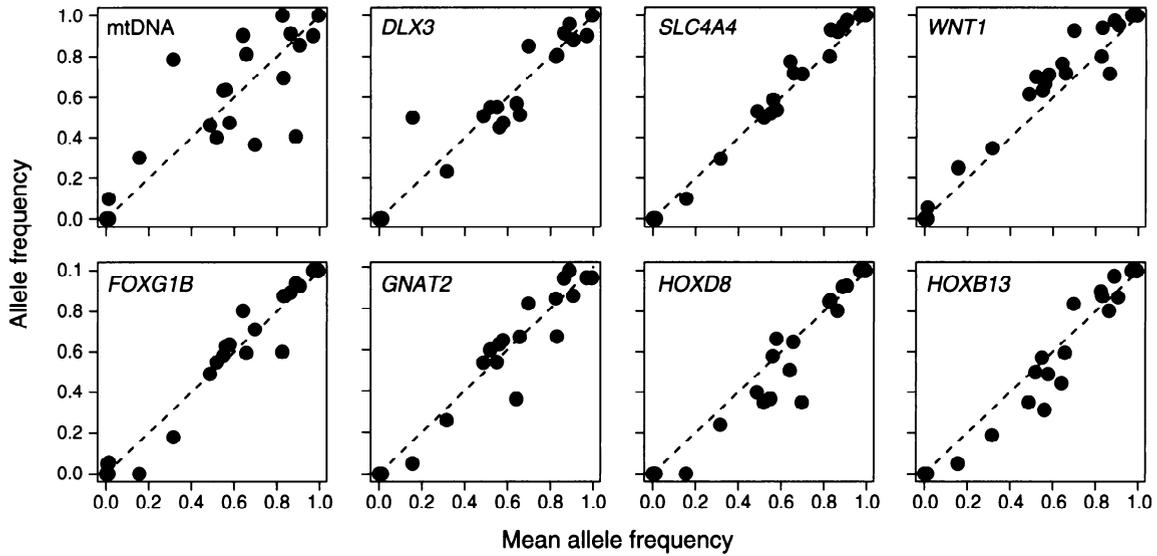


FIG. 2. Associations between introduced allele frequencies at individual markers (y-axes) and the mean introduced allele frequency (x-axes) at California ponds. The dashed line represents one-to-one correspondence.

1671 salamander larvae (Appendix A). To obtain a snapshot of contemporary introgression, we included all ponds in which we found tiger salamander larvae in the years 2000–2005. We did not generally include older tissue samples (which span the last three decades and generally include only pure native salamanders from outside the hybrid swarm; Shaffer et al. 2004) because we wanted to avoid confounding sample year and sample locality. Given the low vagility and long generation time of California tiger salamanders (Trenham et al. 2000), we assumed that no dramatic changes in geographic distribution occurred over this six-year sampling interval. We included a few sites where evidence of introduced alleles was found in samples taken prior to 2000 and no contemporary samples were available (sites 54, 58–60, 63 in Appendix A). Excluding these sites does not substantially change our results as they are centrally located in the Salinas Valley (see *Results*).

Statistical analyses

To assess the importance of dispersal, habitat, and the history of introductions in determining the contemporary pattern of introduced allele frequencies, we performed three sets of statistical analyses. First, we used a multiple Mantel test (Smouse et al. 1986, Legendre et al. 1994) to evaluate geographic distance (great circle distances from latitude/longitude coordinates derived from topographic maps or hand-held GPS units) and pond type (seasonal vs. perennial) as predictors of similarity in allele frequencies between ponds. The number of years between samples was included as a potentially confounding variable. There is some controversy about the accuracy of type I error rates estimated from multiple Mantel tests (Raufaste and Rousset 2001, Castellano and Balletto 2002,

Rousset 2002). Therefore, we also performed a parametric ANCOVA testing watershed, pond type, and sampling year as predictors of introduced allele frequency, taking spatial covariation into account using an autoregression term and/or a spatial trend surface (Lichstein et al. 2002). That is, the expected mean introduced allele frequency at pond *i* was modeled as

$$E(Y_i) = f(\text{watershed, pond type, year}) + \rho \sum_{j \neq i} w_{ij} Y_j + [b_1x + b_2y + b_3x^2 + b_4xy + b_5y^2 + b_6x^3 + b_7x^2y + b_8xy^2 + b_9y^3] + \text{error.} \quad (1)$$

The function *f* is a standard linear model. The autoregression term, $\rho \sum_{j \neq i} w_{ij} Y_j$, consists of a fitted coefficient, ρ , and a weighted average of the allele frequencies at all other ponds (we used weights w_{ij} of $1/\text{distance}_{ij}$ and $1/\text{distance}_{ij}^2$). The trend surface (in square brackets) is a third-order polynomial function of standardized UTM coordinates (*x* and *y*) with fitted coefficients b_k . Alternative weighting schemes and simplifications of this full model were compared using Akaike’s information criterion (AIC).

Finally, we tested two alternative models for the relationship between introduced allele frequency and distance to the nearest known or suspected introduction site. We used likelihood ratio tests to compare the fit of a simple step cline vs. a smooth sigmoidal curve. The step cline model for the mean frequency of introduced alleles P_i at distance x_i from a known introduction site,

$$P_i = \begin{cases} y_1 & \text{if } x_i \leq x_{\text{mid}} \\ y_2 & \text{if } x_i > x_{\text{mid}} \end{cases} \quad (2)$$

was fitted by searching for the value x_{mid} with maximum likelihood. The sigmoidal model (Richards 1959),

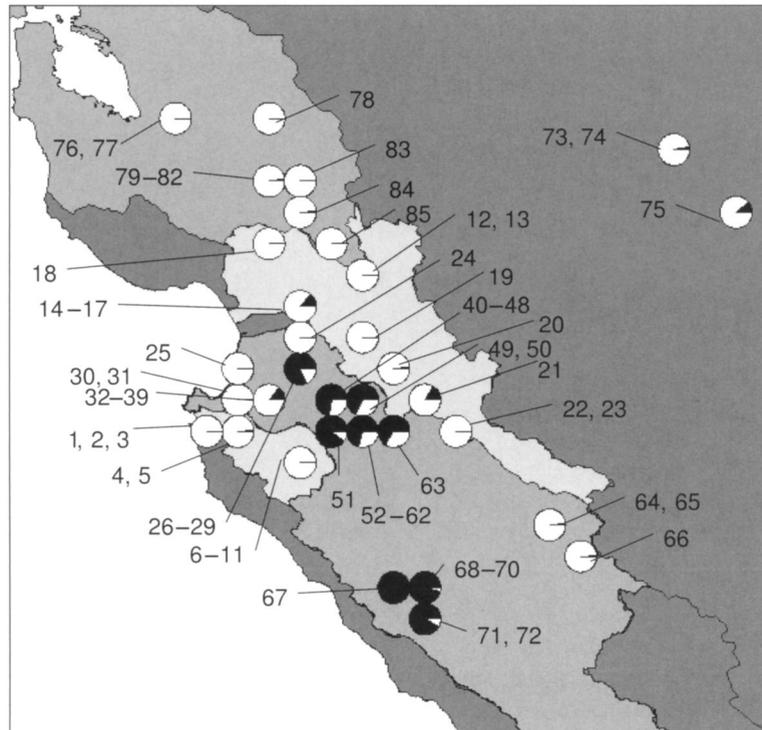


FIG. 3. Introduced allele frequencies (solid portions of pie diagrams) across 85 breeding ponds in California. For display, site coordinates were rounded to the nearest 0.1 degree latitude and longitude, and sites rounding to the same location were averaged. Different watersheds are represented by different shades of gray, as in Fig. 1. Site numbers correspond to Appendix A.

$$P_i = y_1 + \frac{y_2 - y_1}{1 + e^{(x_{\text{mid}} - x_i)/w}} \quad (3)$$

where w describes the width of a cline (defined as the reciprocal of the slope at the cline center x_{mid} [Barton and Gale 1993]), was fitted using the nonlinear regression algorithm nls in R. As the width w becomes very small, the sigmoidal model approaches the step cline model, so the step cline can be considered nested within the more general model with one fewer degrees of freedom (the parameter w is fixed at $1/\infty$). Therefore, the models can be compared with a likelihood ratio test with one degree of freedom (Hilborn and Mangel 1997). In an advancing wave moving across discrete populations, cline width increases with dispersal probability and decreases with local selection (Tufto 1999: Eq. 3). That is, a step cline suggests that an allele rises to a high frequency within one population before moving to the next population. By contrast, a gradual cline suggests that dispersal tends to equalize allele frequencies in nearby populations almost as fast as selection increases the local frequency.

As a complementary analysis, we fitted models 2 and 3 to the relationship between introduced allele frequency and distance from the Ohlone Wilderness (site 78, a pure native site far removed from any introductions) along a transect south through part of the Pajaro watershed into the Salinas Valley. The 42 ponds included in the transect are indicated in Appendix A. With these transect data,

we tested for correspondence between the breakpoint x_{mid} and the Pajaro River, because it is a recognized biogeographic boundary for many taxa (associated with an ancient trans-California seaway; Yanev 1980, Wake 1997, Lapointe and Rissler 2005), and the only natural barrier to dispersal between the introduction sites in the Salinas Valley and pure native sites in the San Francisco Bay area.

RESULTS

Our expanded geographic analysis indicated that the Salinas Valley (sites 24–72) is largely a hybrid swarm with most samples containing high frequencies of introduced alleles (Fig. 3). The only ostensibly pure native samples from the Salinas Valley were from an isolated vernal pool complex on Old Fort Ord Public Lands (sites 25 and 30–35). A very low level of introgression was detected in the Peachtree Valley near the San Andreas Fault in the southeast part of the Salinas watershed (sites 64–66). Gaps between samples in the central and southern Salinas Valley represent areas where we could not find suitable breeding ponds, even after intensive, multiyear searching. According to local ranchers, the soil in these areas is too porous to hold water, and neither natural nor artificial ponds exist in the region. There may be no suitable habitat between Fort Hunter-Liggett (sites 67–72) and Peachtree Valley (sites 64–66).

TABLE 2. Multiple Mantel test results for difference in introduced allele frequencies between ponds.

Prediction variable	Coefficient	<i>P</i> †
Intercept	0.497	0.036
Distance (km)	0.0003067	0.600
Time difference (yr)	0.043	0.646
Same watershed	-0.112	0.010
Same pond type	-0.220	<0.001

† *P* values are based on 1000 matrix randomizations.

With a few exceptions, nonnative genotypes were largely restricted to the Salinas Valley watershed (Fig. 3). The San Benito and Pajaro River watersheds were mostly native (the San Benito is a tributary of the Pajaro and they were treated as a single watershed in our analyses). The highest nonnative allele frequencies in the San Benito Valley occur at site 21, where hybrids from the Salinas Valley were released by Don Green over 20 years ago. The highest nonnative allele frequencies in the San Benito-Pajaro watershed occur at site 14, which was used as a source of salamanders by bait dealers as recently as 1998, and presumably represents another introduction site. Sites in the hills east of the Santa Clara Valley (San Francisco Bay watershed) were nearly pure native; introduced mtDNA was found in single individuals from each of three samples (sites 79, 80, 84). The Carmel River watershed contained predominantly pure native ponds with evidence of a low level of introgression on the Santa Lucia Preserve near the mouth of the Carmel River. Even sites in the vicinity of the Hastings Reservation (within 5 km of the Carmel-Salinas watershed boundary) showed no introgression of nonnative alleles (sites 6-11). Other outlying occurrences of nonnative alleles were detected in Merced County on the east side of the San Joaquin Valley (sites 74, 75).

To statistically evaluate the proposition that introduced alleles are largely restricted to individual watersheds (primarily the Salinas), we added an indicator variable for same vs. different watershed (as defined in Fig. 1) to our multiple Mantel test. The *Y* matrix was the matrix of absolute pairwise differences in mean allele frequency between ponds. The predictor matrices were (1) geographic distances in km, (2) differences in sample year, (3) indicator variable for same vs. different pond type (perennial vs. seasonal), and (4) indicator variable for same vs. different watershed. The multiple Mantel test (Table 2) indicated that differences in introduced allele frequencies between ponds were not predicted by geographic distance or difference in sampling year, but were strongly predicted by differences in watershed location and habitat type. Based on the multiple Mantel test, there was no support for a geographical gradient in allele frequencies. However, a simple Mantel test fitted to seasonal ponds within the Salinas watershed did show a significant relationship between genetic and geographic distances (seasonal ponds: $r = 0.311$, $P = 0.0003$;

TABLE 3. Akaike's Information Criteria (AIC) for alternative ANCOVA models for introduced allele frequencies.

Model	AIC
1) Watershed + pond type + year	77.79
2) Model 1 + second order trend surface	55.65
3) Model 1 + third-order trend surface	36.84
4) Model 1 + linear autoregression	77.59
5) Model 1 + quadratic autoregression	79.76
6) Model 1 + linear and quadratic autoregression	77.51
7) Model 3 + linear autoregression	38.80
8) Model 3 + quadratic autoregression	38.54
9) Model 3 + linear and quadratic autoregression	39.60

perennial ponds: $r = 0.080$, $P = 0.8280$, 10 000 Monte Carlo replicates each). This is probably due to regional differences (e.g., Fort Ord, sites 25, 30-35, and Peachtree Valley, sites 64-66, have uniformly low frequencies of introduced alleles, while Fort Hunter-Liggett, sites 67-72, has uniformly high frequencies), rather than fine-scale gradients (simple Mantel test of genetic vs. geographic distance for seasonal ponds within the Salinas Valley but excluding the above mentioned regions: $r = 0.135$, $P = 0.769$; 10 000 Monte Carlo replicates).

The best ANCOVA included a third-order trend surface, but not an autoregression term (Tables 3 and 4). This analysis also supported watershed and pond type as predictors of introduced allele frequency. Lichstein et al. (2002) indicated that trend surfaces tend to describe large-scale spatial covariation, while autoregression better captures fine-scale autocorrelation. Thus, our ANCOVA and Mantel test results seem to reflect predictability at the landscape scale. However, neither the analyses nor visual inspection of the data suggest evidence of fine-scale gradients consistent with isolation by distance (Fig. 3; also see Fitzpatrick and Shaffer 2004).

The cline analyses did not favor a sigmoidal pattern over a simpler step function (Table 5). In fact, the algorithm could not fit a specific cline width to the

TABLE 4. ANCOVA of introduced allele frequencies with third-order trend surface describing spatial covariation.

Source	df	<i>F</i> ratio	<i>P</i>	Coefficient
Watershed	4, 81	11.031	<0.0001	
Pond type	1, 84	18.652	<0.0001	
Year	1, 84	0.0279	0.868	-0.0023
<i>x</i>	1, 84	11.103	0.001	0.403
<i>x</i> ²	1, 84	0.828	0.366	-0.073
<i>x</i> ³	1, 84	2.776	0.100	-0.041
<i>y</i>	1, 84	1.635	0.205	0.193
<i>y</i> ²	1, 84	8.899	0.0039	0.178
<i>y</i> ³	1, 84	7.163	0.0093	-0.125
<i>xy</i>	1, 84	2.919	0.0921	0.200
<i>x</i> ² <i>y</i>	1, 84	1.381	0.244	-0.137
<i>xy</i> ²	1, 84	5.532	0.0215	-0.314

Notes: Whole-model $r^2 = 0.799$, $F_{15,69} = 18.247$, $P < 0.0001$. *F* ratios and associated *P* values are based on Type III sums of squares and test the effect of omitting each variable on the whole-model fit.

TABLE 5. Comparison of cline models for (a) distance from nearest known or suspected introduction site and (b) distance from Ohlone Wilderness along a transect south to Gonzales.

Model	Allele frequencies†	Midpoint (km)‡	Width (km)	Log likelihood	LRT§
a) Distance from nearest introduction site					
Sigmoid	0.008/0.70	8.0	2.27	-10.037 (df = 4)	0.171
Step	0.08/0.64	7.4–8.1	NA	-10.975 (df = 3)	
b) Transect from Ohlone to Gonzales					
Sigmoid	0.03/0.73	81.9	0.5	18.125 (df = 4)	>0.999
Step	0.03/0.71	78–86	NA	18.126 (df = 3)	

† For the sigmoid model, the values are the estimated asymptotic frequencies of introduced alleles on each side of the cline. For the step model, the values are average frequencies of introduced alleles on each side of the step cline.

‡ The midpoint for the step clines could not be localized any more precisely because there were no sites within the range given.

§ *P* values from likelihood ratio tests of the sigmoid vs. step models.

transect data (Table 5b) because the likelihood surface approached an asymptote as the cline width decreased lower than 0.5 km, indicating that a width of 0.5 km fits no better than a width of $1/\infty$. Ponds within 12 km of a known or suspected introduction site often had high frequencies of introduced alleles, but more distant ponds had very low introduced allele frequencies, with no evidence of a gradient on either side of the step (Fig. 4). Along the Ohlone–Gonzales transect (Fig. 5), the fitted position of the step (between 78 km and 86 km) was significantly better than constraining the step to coincide with the Pajaro River at 70.6 km (log likelihood ratio 27.88, $P_{df=1} < 0.00001$). Limiting these analyses to seasonal ponds did not change the results other than lowering the average frequency of introduced alleles (not shown). Our sample of perennial ponds outside the Salinas Valley was too sparse to support an analysis limited to perennial ponds.

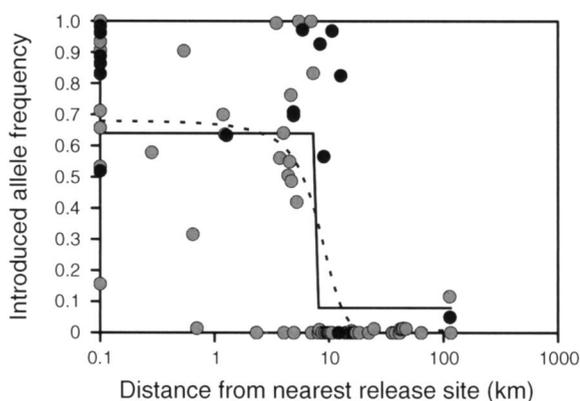


FIG. 4. Introduced allele frequency as a function of minimum distance (log scale) between a pond and a known or suspected *Ambystoma t. mavortium* release site (based on first-hand conversations with bait salesmen and landowners). Allele frequencies for release sites are shown at 0 km. The sites past 100 km are in eastern Merced County, where we are not aware of any specific release activities (although they presumably occurred). Cline models are described in Table 5a; the solid line is the step cline, and the dashed line is the sigmoid cline. Gray circles represent seasonal ponds, and solid circles represent perennial ponds.

DISCUSSION

Biological invasions of introduced genotypes via hybridization are among the most difficult situations to measure and manage on natural landscapes. Even detecting the initial stages of invasion, when the first few genotypes introgress into pure native populations, is a challenge; understanding the factors influencing the success or failure of invasions is both a daunting task and critical to developing management strategies and predicting outcomes across a range of cases. Here, we describe a broad hybrid swarm in the Salinas Valley of central California where native *Ambystoma californiense* has been replaced by admixed tiger salamander populations, often consisting of predominantly introduced genotypes (Figs. 3–5). We used methods from landscape genetics and hybrid zone analysis to show that the distribution of introduced tiger salamander genotypes in California populations is largely confined to within 12

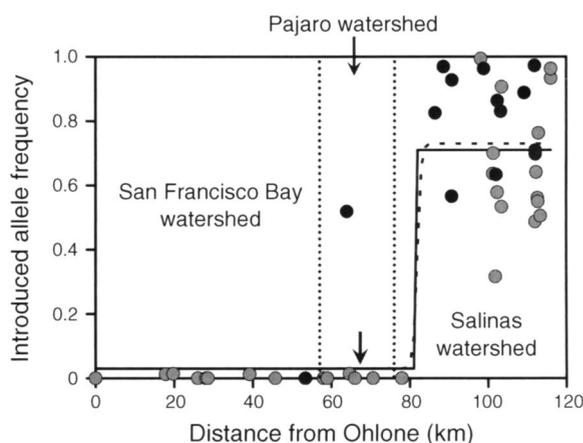


FIG. 5. Mean frequency of introduced alleles as a function of distance from the Ohlone Wilderness (pure *Ambystoma californiense*) along a rough transect south through the Pajaro watershed to Gonzales in the Salinas Valley, California. The arrow illustrates the position of the Pajaro River. Cline models are described in Table 5a; the solid line is the step cline, and the dashed line is the sigmoid cline. Gray circles represent seasonal ponds, and solid circles represent perennial ponds.

km of introduction sites, where the transition between the hybrid swarm and native populations is abrupt. This is consistent with other studies of hybridization between native and introduced species in which hybrid swarms were initiated by a series of dispersed introductions (Weigel et al. 2003, Lambrinos 2004). In addition, human modifications of natural breeding habitat from vernal pools to perennial ponds appears to favor high frequencies of introduced genotypes (Table 4; Fitzpatrick and Shaffer 2004), supporting the view that interactions between human land use and species introductions facilitate and intensify biological invasions (Vitousek et al. 1997).

The broad-scale distribution of allele frequencies is best described by an abrupt step from high (but variable) to extremely low frequency of introduced alleles in samples >12 km from known or suspected release sites (Figs. 4 and 5, Table 5). Even the best fit sigmoidal models indicate cline widths of <3 km, indicating that the rate at which gene flow tends to homogenize allele frequencies among ponds is far slower than the rate at which allele frequencies change within ponds. This is inconsistent with a simple neutral diffusion hypothesis. Four other scenarios are possible. (1) Extrinsic barriers such as highways, waterways, and unsuitable habitat block dispersal very far from introduction sites. This is certainly not the case in two instances (Fort Ord, sites 37, 38; north of the Pajaro River, sites 14, 15), and it appears unlikely in many other regions. (2) Natural selection against dispersers or certain recombinant genotypes inhibits gene flow between invaded and pure native populations. (3) Strong selection causes introduced allele frequencies to rise within local populations much faster than they are dispersed to new populations. (4) The invasion is contracting and strong selection causes introduced alleles to go extinct much faster at the edge of the hybrid swarm than in its interior. (Given that introduced alleles predominate in most admixed populations, it seems unlikely that they are decreasing in frequency.) Whichever is true (scenarios 2 and 3 appear most likely), an interaction between selection and dispersal most likely explains the steep invasion front.

Although we have been fortunate in identifying many of the actual release sites in the Salinas Valley, there is still a potential bias due to incomplete information on release sites. It seems likely that some ponds with high allele frequencies, particularly near the city of Salinas, may be closer to undocumented release sites than we currently assume, although we cannot test this empirically. If so, the true pattern may be more of a gradient than our analyses suggest, particularly if many of the sites with high introduced allele frequencies around 10 km in Fig. 4 are actually much closer to sites of introduction. While this bias may reduce the step-cline nature of fall-off from introduction sites, it only strengthens the important inference that high frequencies of introduced alleles are extremely unlikely beyond ~12 km from introduction sites. This short distance

after 50–60 years may indicate that the invasion is slow, or that a stable hybrid zone is developing, or even that the hybrid swarm is contracting. However, many biological invasions are characterized by a lag time between establishment and accelerated spread (Sakai et al. 2001, Crooks 2005).

While the pattern of restricted distribution of nonnative alleles is generally true, introduced alleles are not completely absent from more distant sites. The presence of introduced alleles in eastern Merced County, ~114 km from the nearest known release site in the Salinas Valley, is better explained by separate introductions or translocations than by natural movement, given the substantial barriers to natural dispersal across the San Joaquin Valley (interstate highways, the California aqueduct, and ~100 km of unsuitable habitat in the intensively farmed valley). In the San Francisco Bay area, low frequencies of introduced alleles (1.25% at site 79) may be found as far away as 47 km from the nearest suspected release site (site 14). This may reflect a small but detectable amount of natural gene flow on the scale of 40–50 km over 60 years or less, at least for a limited fraction of the genome. The implied rate of expansion of ≤ 1 km/yr for the extreme tail of the distribution of introduced alleles is consistent with direct estimates of California tiger salamander movement on natural landscapes, where observed interpond migrations and seasonal terrestrial movements of up to 650–1000 m/yr are both relatively common (Trenham et al. 2001, Trenham and Shaffer 2005; H. B. Shaffer, unpublished data).

The history of introduction and translocation appears to explain much of the distribution of introduced alleles at the broad landscape level (Fig. 4, Table 5a). At a smaller scale, ponds only a few hundred meters apart sometimes harbor very different allele frequencies (Appendix A; Fitzpatrick and Shaffer 2004). This variation may be explained by the influence of ecological heterogeneity on natural selection among genotypes. Our analyses confirm and strengthen the earlier interpretation (Fitzpatrick and Shaffer 2004) that introduced alleles are significantly more common in perennial than in seasonal breeding ponds. This difference is maintained between ponds in very close proximity (e.g., site 14 vs. 15, 704 m; 46 vs. 47, 280 m; 49 vs. 50, 10 m). Even if introductions were strongly biased toward perennial ponds as release sites, differences in allele frequency on this fine scale must be maintained by selection if they persist for more than a few generations in the face of known terrestrial movement abilities of *A. californiense* (Fitzpatrick and Shaffer 2004).

An advantage for introduced *A. t. mavortium* in perennial ponds is consistent with several life history adaptations, including facultative paedomorphosis, that allow them to exploit the extended hydroperiods provided by perennial ponds. Thus, landscapes with high densities of fishless, perennial ponds are likely the most conducive to continued increases in the frequency

and geographic range of introduced alleles. In the Salinas watershed, 13 of 49 breeding ponds that we sampled were perennial, whereas in all other watersheds combined only three of 32 occupied ponds were perennial ($P = 0.0479$; Monte Carlo contingency table test with 10 000 replicates). This unequal distribution of perennial breeding ponds may help explain why the hybrid swarm remains contained within the Salinas Valley (particularly considering that the two areas of the Salinas watershed with pure or nearly pure native tiger salamanders, Fort Ord and Peachtree Valley, have high concentrations of natural seasonal pools).

Management implications

Our analysis of the geographic distribution of introduced *A. tigrinum mavortium* alleles in California tiger salamander populations demonstrates that most tiger salamanders in the Salinas Valley are part of a hybrid swarm but that the invasion has not proceeded extensively in other parts of central California. This indicates that the tiger salamander populations in the Salinas Valley are fundamentally altered from their original condition, and require the development of a different set of management goals than for other California tiger salamander populations. This hybrid swarm occurs over most of the geographic range of the "central coast range population segment" of California tiger salamanders described by Shaffer et al. (2004) based on genetic variation in native mtDNA. Areas peripheral to the Salinas Valley currently have low levels of introgression, and may require different management from both pure *A. californiense* and the highly introgressed Salinas Valley populations. Range wide, there is a surprisingly clear distinction between admixed populations (mostly above 40% introduced ancestry) and near-native populations (mostly well below 10% introduced ancestry; Fig. 4), suggesting that it is feasible to develop separate management schemes for each. Whether the frequency of introduced alleles will increase, decrease, or remain stable is an empirical question, and our data provide a baseline for monitoring the potential spread or retreat of introduced alleles.

Wildlife managers have two decisions to make with regard to hybrids between introduced and endangered species. First, what individuals and populations qualify for legal protection under state and federal law? Second, what, if any, genotypes should be eradicated? Despite some attempts to formulate a standard policy (USFWS 1996), legal treatment of hybrids under the Endangered Species Act is currently argued and decided on a case-by-case basis (e.g., Allendorf et al. 2004, 2005, Campton and Kaeding 2005). Specific information on the ecological consequences of hybridization and introgression is required to provide scientific and ethical guidance, particularly if eradication is an option. If introduced genes are not detrimental to tiger salamander population viability and do not cause problems for other native species, then management can only be based on

aesthetics or some concept of "naturalness" (Simberloff 2003). In such a case, a biologically arbitrary but aesthetically compelling legal decision could reasonably be made as to the level of genetic impurity that disqualifies an individual or population from protection.

Eradicating animals simply because they are genetically impure is ethically more problematic than merely denying them protection. However, whether or not it is justified or desirable, eradication of introduced tiger salamander alleles is probably not feasible (short of outright habitat destruction) because adults may live for 11 years in underground burrows (Trenham et al. 2000), there is low but detectable gene flow over large distances (Figs. 4 and 5), and most of the hybrid swarm occupies private ranch land where long-term access and management are difficult. Some level of management could be accomplished by converting perennial breeding ponds to more naturalistic seasonal ponds, and we encourage this as a concrete practice that private and public land managers can facilitate. This would remove an ecological advantage for introduced genotypes, and generally select for tiger salamander genotypes that function best in more natural habitat.

A further practical issue is the uncertainty of genetic classification of individuals. Given stochasticity and variance in invasion success of different parts of the genome at the local pond level (Fitzpatrick and Shaffer 2004) and our ability to sample only a very small fraction of the genome, there will always be substantial uncertainty about declaring individuals or populations pure native after more than a few generations of potential admixture (Boecklen and Howard 1997, Reiseberg and Linder 1999, Anderson and Thompson 2002). Operational criteria for protection must at least recognize this uncertainty. For genetically unknown populations, we feel that the discussion of Haig et al. (2004) of the "similarity of appearance" provision (section 4e) in the Endangered Species Act provides a sound strategy for field management. This provision allows nonlisted species to be protected if they are difficult to distinguish from listed species. That is, mistaking an animal for a nonnative or hybrid should not be considered a valid excuse for destroying a California tiger salamander, and if there are doubts about the status of a population or individual, they should be treated as protected under the Act. While this may end up protecting nonnative individuals in some cases, it uses best available evidence in the field to make decisions. By similar logic, if a population has previously been demonstrated to be introgressed beyond a management threshold, it is probably reasonable to declare the entire population "not protected," rather than verifying the genotype of each individual larva in a pond. Again, this may mean that a few individuals that would fall below the threshold are eliminated, but on average it is a rational approach under field conditions. These population-based recommendations depart from the normal focus of the Endangered Species Act on

individuals, but recognize the well-documented population biology of this system; it thus uses "best available science" in a defensible, practical way.

While introduced DNA certainly lowers the genealogical distinctiveness and historical authenticity of California tiger salamander populations, we emphasize that the ecological consequences of hybridization are largely unknown. Research into potential effects on tiger salamander population viability and impacts on other native species is necessary to establish a management plan with both strong scientific and ethical bases. Given the widespread influence of introduced tiger salamander genes and the legal protection of California tiger salamanders under the U.S. Endangered Species Act, decisions will have to be made as to the conservation value and legal status of genetically impure tiger salamander individuals and populations in California.

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APPENDIX A

A table showing tiger salamander breeding pond sites assayed for introduced alleles at three or eight molecular markers (*Ecological Archives* A017-023-A1).

APPENDIX B

Principal components analysis of introduced allele frequencies at eight DNA markers among 28 tiger salamander breeding ponds (*Ecological Archives* A017-023-A2).