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Conservation genetics of evolutionary lineages of the endangered mountain yellow-legged frog, *Rana muscosa* (Amphibia: Ranidae), in southern California

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ABSTRACT

Severe population declines led to the listing of southern California *Rana muscosa* (Ranidae) as endangered in 2002. Nine small populations inhabit watersheds in three isolated mountain ranges, the San Gabriel, San Bernardino and San Jacinto. One population from the Dark Canyon tributary in the San Jacinto Mountains has been used to establish a captive breeding population at the San Diego Zoo Institute for Conservation Research. Because these populations may still be declining, it is critical to gather information on how genetic variation is structured in these populations and what historical inter-population connectivity existed between populations. Additionally, it is not clear whether these populations are rapidly losing genetic diversity due to population bottlenecks. Using mitochondrial and microsatellite data, we examine patterns of genetic variation in southern California and one of the last remaining populations of *R. muscosa* in the southern Sierra Nevada. We find low levels of genetic variation within each population and evidence of genetic bottlenecks. Additionally, substantial population structure is evident, suggesting a high degree of historical isolation within and between mountain ranges. Based on estimates from a multi-population isolation with migration analysis, these populations diversified during glacial episodes of the Pleistocene, with little gene flow during population divergence. Our data demonstrate that unique evolutionary lineages of *R. muscosa* occupy each mountain range in southern California and should be managed separately. The captive breeding program at Dark Canyon is promising, although mitigating the loss of neutral genetic diversity relative to the natural population might require additional breeding frogs.

1. Introduction

Over the last few decades, dramatic and persistent population declines have been documented in amphibian species throughout the world (Wake and Vredenburg, 2008) resulting in an unprecedented crisis with over 32.5% of known species considered "globally threatened". Tremendous effort has been expended to collect information and document declines (Global Amphibian Assessment, 2004), but in many cases adequate information is lacking for prioritizing major threats or management actions. In those few cases where restoration and recovery efforts have been undertaken for amphibians (Griffiths and Pavajeau, 2008; Semlitsch, 2002), basic information on population demography and connectivity have been essential in setting management guidelines. However, this information can be difficult to obtain using field ecological methods, especially if populations have declined to very low numbers. Genetic data provide an alternative method to reconstruct the demography of populations and can be used to estimate historical migration rates between populations, even after substantial fragmentation has taken place (Delaney et al., 2010; Frankham, 1995).

In the United States, the number of endangered frog species or candidates for listing is disproportionally represented in the west (US Fish and Wildlife Service), where population declines have been particularly notable in *Rana* species for several decades (Fisher and Shaffer, 1996; Hayes and Jennings, 1986). The southern mountain yellow-legged frog, *Rana muscosa*, along with its sister species, the Sierra mountain yellow-legged frog *Rana sierrae*, is a prominent example of an enigmatic range-wide population crash.

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Although these species were once considered abundant in montane aquatic habitats of California (Schoenherr, 1976; Stebbins and Cohen, 1995), they have declined dramatically (>93%) across their range, even in well-protected habitats of the National Parks and National Forests (Vredenburg et al., 2007). The decline of *R. muscosa* in southern California has been even more severe, with extinction at >99% of historical sites (Backlin et al., 2004), leading to its listing as an endangered distinct population segment (US Fish and Wildlife Service, 2002). A number of factors have been linked to the decline of *R. muscosa* in the Sierra Nevada, including chytridiomycosis (Briggs et al., 2005) and predation by invasive trout (Vredenburg, 2004). In southern California, additional threats include habitat degradation, stream channelization, fire, post-fire debris flows, and pollution, and remaining populations mostly persist in headwater sites that do not experience these issues.

Previously, mitochondrial data were used to characterize the phylogenetic relationships, split *R. muscosa* and *R. sierrae*, and identify three major geographically isolated clades within each lineage (Macey et al., 2001; Vredenburg et al., 2007). While this information has been critical in the conservation of this species, there is a pressing need to examine genetic diversity within the remaining populations and connectivity among populations. In southern California, ongoing monitoring efforts have located nine extant populations in three mountain ranges (Backlin et al., 2004; Lewis, 2009), some separated by only a few kilometers. Efforts to restore habitat are ongoing and a program for captive breeding and reintroduction has been set up by the San Diego Zoo Institute for Conservation Research. In order to facilitate the management and recovery of endangered populations of southern California *R. muscosa*, we analyze patterns of mitochondrial and microsatellite genetic diversity. Our analysis focuses on answering three central questions, utilizing data from over 600 individuals and including the closest remaining population in the Sierra Nevada. First, what are the levels of genetic variation in southern California *R. muscosa* and is there a reduction of variation in the captive population relative to its source population? Second, how is genetic variation structured among populations at a local and regional scale? And third, what is the history of population divergence and gene flow in southern *R. muscosa*? Based on our results, we discuss how management efforts and captive breeding programs can maintain evolutionary diversity in populations of southern California *R. mucosa.*

2. Material and methods

2.1. Field sampling

U.S. Geological Survey (USGS) personnel collected tissue samples of *Rana muscosa* during surveys in 2003–2009 from nine unique locations: South Fork Big Rock Creek, Little Rock Creek, Bear Gulch, Vincent Gulch, Devils Canyon, East Fork City Creek, Fuller-Mill Creek, Dark Canyon, and Tahquitz Creek (Fig. 1). Additionally, we obtained samples in 2004 from Milestone Basin, the nearest extant population in the southern Sierra Nevada. Other populations in the southern Sierra Nevada are considered extinct (Vredenburg et al., 2007). Tissue samples consisted of toe clips of post-metamorphic frogs and tail clips of tadpoles, preserved in 95% ethanol, taken from 614 individuals across all populations (Supplementary Table 1S), representing the majority of animals alive and present in southern California for the last decade and are currently maintained by USGS.

2.2. Genetic data collection

Genomic DNA was extracted using PrepMan Ultra™ reagent (Applied Biosystems), with the following modifications to the manufacturer's protocol: tissue was combined with 45 μl of PrepMan and 40 mg of silica beads, shaken on a beadbeater for 90 s, digested and centrifuged for 30 s. We amplified each individual at nine microsatellite loci (Supplementary Table 2S) by polymerase chain reaction (PCR) using primers designed by Genetic Information Services (GIS). PCR products were run on a 3730 capillary sequencer with the GeneScan® Liz 500 size standard (Applied Biosystems).

Sequence data encompassing the mitochondrial ND1–ND2 genes, including tRNAs Ile, Gln and Met (~1500 bp), was targeted to match sequences available from previous studies (Macey et al., 2001; Vredenburg et al., 2007). In a subset of 57 individuals sampled across populations of southern California *R. muscosa* (Supplementary Table 1S), we PCR amplified four overlapping segments and sequenced in both directions using BigDye v3.1® chemistry. We edited and aligned chromatograms in Sequencher® v.4.8 (Gene Codes Corporation). Sequences from previous analyses include populations of *R. muscosa* and its sister taxon, *R. sierrae*, as well as the out-groups *Rana catesbeiana*, *Rana aurora*, and *Rana cascadae*. All unique haplotypes have been submitted to GenBank at the National Center for Biotechnology Information (NCBI, JF27239-JF27259).

2.3. Population genetic analysis

Variability at each microsatellite locus was tested for deviation from Hardy–Weinberg equilibrium (HWE) using chi-square and Fisher's Exact tests, across all available samples and for a subset of the data including only adult frogs. HWE tests were conducted for each locus within each population, and due to the large number of tests ($n = 90$), the level of statistical significance ($\alpha = 0.05$) was adjusted by Dunn-Šidák correction ($1 - (1 - \alpha)^{1/n}$). We also calculated the mean number of alleles (allelic richness), observed and expected heterozygosity, and the fixation index averaged across loci for each population. All statistical tests were conducted within GENALEX v6.4 (Peakall and Smouse, 2006) and ARLEQUIN v3.5.1.2 (Excoffier and Lischer, 2010).

To test for genetic bottlenecks in each population, we calculated the sign-test (Cornuet and Luikart, 1996) and *M-ratio* statistic (Garza and Williamson, 2001). The sign-test examines whether there is an excess of heterozygosity across loci, which occurs when the effective population size is sharply reduced during a population bottleneck. This was calculated in the program BOTTLENECK (Piry et al., 1999) assuming the two-phase model (T.P.M.) with variance set to 30 and probability set to 70%, and significance assessed over 10,000 replicates. The *M-ratio* statistic examines the ratio of the number of alleles and the allele range, with the expectation that the number of alleles declines faster than the allele range in a bottlenecked population. The *M-ratio* was calculated in ARLEQUIN and tested for significance by comparison to simulated data. We simulated genetic diversity in a population with constant size at a microsatellite locus evolving under a single step mutation model, with a mean size of multistep mutation set to 3.5 and the proportion of single steps set at 0.89 (as suggested by Garza and Williamson, 2001, based on their survey of the literature). From each simulated dataset, a sample size of 20 diploid individuals was drawn, from a total of 10,000 datasets. Critical values ($\alpha = 0.05$) of the *M-ratio*, below which an *M-ratio* value would be likely to indicate a bottleneck, were determined for two levels of ancestral theta, $\theta = 1$ and 10.

In order to compare the captive population of *R. muscosa* from Dark Canyon to the resident population, a principal coordinate analysis (PCoA) was used to summarize microsatellite genetic variation in these two groups. The eigenvectors of the PCoA were calculated from a covariance matrix with data standardization using the program GENALEX.

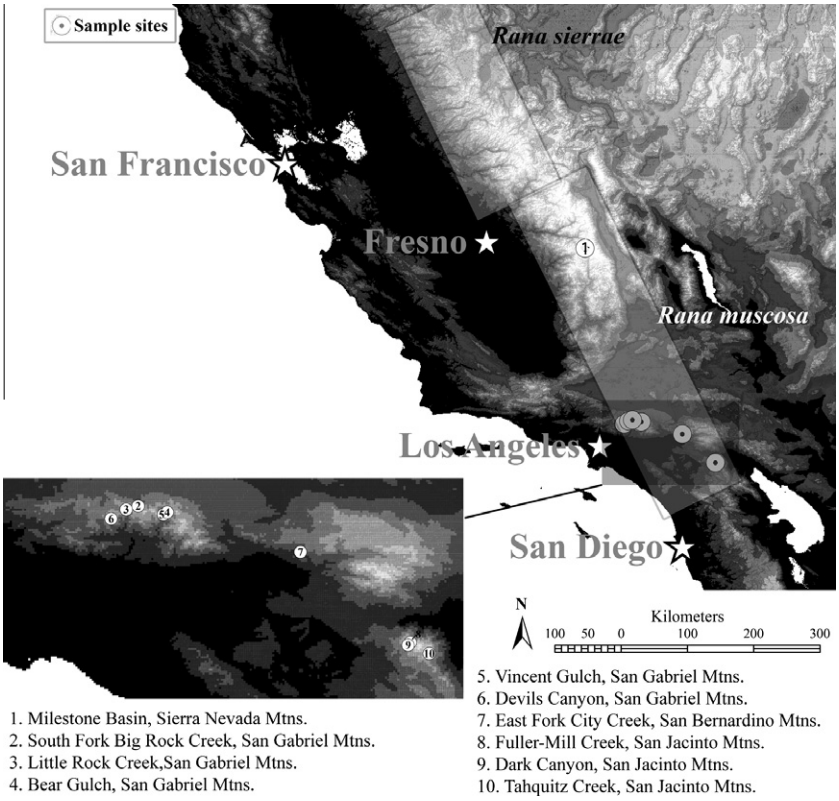

Fig. 1. Approximate geographical distribution of *Rana muscosa* and *R. sierrae* in California (boxes), with genetic sampling locations (circles) superimposed on a digital elevation map.

2.4. Geographic pattern of genetic variation

A mitochondrial gene tree was estimated using an unrooted Bayesian method in the program MRBAYES v3.1.2 (Ronquist and Huelsenbeck, 2003). The data were partitioned by gene (ND1, t-Ile, t-Met, t-Gln, and ND2) and the software MRMODELTEST2 v2.3 (Nylander, 2004) was used to estimate models of molecular evolution for each partition using Akaike Information Criteria (AIC, Akaike, 1973). The best fitting models were: ND1 GTR + G, t-Ile K80, t-Met K80, t-Gln HKY, and ND2 GTR + G. Two independent runs of MRBAYES were used to build a 50% majority-rule consensus tree. The following conditions were used: 30 million steps, four chains, genealogies sampled every 1000 steps. Performance of the Markov Chain Monte Carlo (MCMC) sampler was assessed for convergence using TRACER v1.5 (Rambaut and Drummond, 2009), where a 25% burn-in was selected before summarizing genealogies. To visualize evolutionary relationships using the microsatellite data, average Nei's genetic distance was calculated for pairwise population comparisons and used to plot an unrooted neighbor-joining tree.

Population structure in the microsatellite data was assessed using a Bayesian clustering algorithm implemented in the program STRUCTURE v2.3.3, with population identifiers used as prior information (Hubisz et al., 2009; Pritchard et al., 2000). We used the admixture model with correlated allele frequencies to account for any migrants in the dataset, following recommendations of François and Durand (2010). STRUCTURE was run for the adult frog dataset by setting the cluster ("*k*") value incrementally from 1 to11 with eleven independent runs at each *k* value. A burn-in period of 100,000 steps was followed by MCMC sampling for 1 million steps. After determination of the *k* value with the lowest log-likelihood score ($k = 9$), the 11 independent runs at $k = 9$ were summarized using the program CLUMPP (Jakobsson and Rosenberg, 2007) with the LargeKGreedy algorithm and 10,000 permutations. The program DISTRUCT (Rosenberg, 2004) was used to graphically display the output. The STRUCTURE analysis was also run using the entire dataset, with a three iterations at each *k* and an additional 10 iterations at $k = 9$.

In addition, population differentiation based on microsatellite genetic variation was measured using pairwise *F*-statistics (F_{ST}), an analysis of molecular variance (AMOVA), and principle coordinates analysis. F_{ST} was measured in the adult frogs using two metrics of genetic variation, the number of alleles and the corrected pairwise difference based on the sum of squared differences in the number of repeats. Geographical partitioning of microsatellite genetic variation between the San Gabriel, San Bernardino and San Jacinto Mountains was assessed using an AMOVA with *F* statistics (Excoffier et al., 1992). Genetic variation was partitioned hierarchically at four levels: within individuals, among individuals, among populations, and among regions. Differentiation at these hierarchical levels was assessed for statistical significance by permuting the data 1000 times in ARLEQUIN. PCoA was used to summarize microsatellite genetic variation of all adult frogs, among all populations. The eigenvectors of the PCoA were calculated from a covariance matrix with data standardization using the program GENALEX.

Finally, both mitochondrial and microsatellite genetic data were examined for genetic isolation by distance (IBD). Matrices of genetic distance were compared to two different measures of geographic distance. First, a matrix of log-transformed Euclidean distance was calculated between all sites. However, amphibians often have strong preferences and ecological restrictions for habitat type during dispersal, which can alter the observed relationship between geographic distance and genetic distance of population pairs (Wang et al., 2009). Therefore, a second matrix of least-cost distance was calculated using python scripts (Etherington, 2010) in ArcGIS v9.3 (ESRI), with cost penalties assigned for changes in elevation and movement away from freshwater habitat. Maps of digital

elevation (30 arcsecond resolution) and water boundaries were downloaded from the California Spatial Information Library (CaSIL, www.atlas.ca.gov). Mitochondrial genetic variation (available from eight sites) was used to calculate Phi_{ST} assuming a Jukes-Cantor substitution model, and a mantel test of genetic and geographic distance was conducted using the program IBD (Jensen et al., 2005), with statistical significance assessed with 1000 permutations. Similarly, microsatellite genetic distance (available from nine sites) based on the estimator $\hat{a}$ was tested against geographical distance in the program GENEPOP'007 (Rousset, 2008), with statistical significance assessed with 1000 permutations.

2.5. Biogeographic history

We examined the fit of an isolation with migration model (Nielsen and Wakeley, 2001) to our data in order to reconstruct the population history of southern California populations of *R. muscosa*. This model parameterizes the divergence time, population size and migration rate of diverging populations using multi-locus coalescent analysis. Recently, the method has been extended in the program IMa2 (Hey, 2010) to infer the divergence time and demography of up to four populations assuming a known phylogeny. We use this model to examine the history of four regions in our study (Sierra Nevada, San Gabriel, San Bernardino, and San Jacinto Mountains). Populations within each mountain range were combined and relationships based on the mitochondrial gene tree were used to define the four-population phylogeny. The analysis was run using both mitochondrial and microsatellite data (the Vincent Gulch population was excluded due to missing mitochondrial data), with 40 heated chains (geometric heating with nonlinearity = 0.975 and $\beta = 0.75$). After an initial run was monitored for convergence to determine an appropriate burn-in (350,000 steps), the state of the Markov chain was saved and used to seed five separate runs (1 million steps, with 10,000 genealogies saved from each). These runs were combined to estimate demographic and divergence time parameters. Because no mitochondrial mutation rate is available based on fossil studies of *Rana*, we set the substitution rate to 0.65%/million years (with a range of 0.57–0.69%) following Macey et al. (2001) and the generation time was set to 7 years. The generation time is based on unpublished estimates from field ecological data (A. Backlin and V. Vredenburg). Likelihood ratio tests were used to assess the significance of migration rate estimates ($\alpha = 0.001$).

3. Results

3.1. Genetic variation

Several populations exhibit statistically significant deviations from HWE after Dunn-Šidák correction (Supplementary Table 3S) and the number of significant tests is higher using the chi-square method versus Fisher's Exact method. When only adult frogs are considered, the number of significant tests declines, irrespective of the statistical method. There is no clear trend of deviation from HWE at specific loci across all populations and there is no clear predominance of deviations in specific populations, which suggests that HWE deviations are not a result of null alleles or admixture. For measures of population differentiation (STRUCTURE, F_{ST}, and AMOVA) that might be sensitive to deviations from HWE, we conducted analyses using adult samples and all individuals. The results are qualitatively similar, so all individuals were used in other analyses.

Comparison of microsatellite variability averaged across loci (Table 1) shows that the mean number of alleles and observed heterozygosity are similar across populations, except that the captive population has lower values. Differences in the observed and expected heterozygosity, as measured in the fixation index, show that most populations do not have a deficit of heterozygous individuals. However, East Fork City Creek and Little Rock Creek do have deficits that differ significantly from zero, but the level of inbreeding is not high. Several other populations, including Vincent Gulch, Bear Gulch, Fuller-Mill Creek and the captive population, have an excess of observed heterozygosity resulting in negative fixation indices, but these are not significantly different from zero. Signatures of population bottlenecks are evident across southern California *R. muscosa* populations (Table 2). Based on the sign-test and Wilcoxin sign-rank test, Dark Canyon shows a significant bottleneck, while East Fork City Creek is significant under the standardized difference test. Using the *M*-ratio statistic, all populations have values below critical thresholds of a sample drawn from a moderate ($\theta = 1$) to large ($\theta = 10$) population. This includes the Milestone Basin population in the Sierra Nevada. Differences in genetic variability in the resident Dark Canyon population and captive population are evident in the principal coordinates analysis (Fig. 2). A plot of the first two components (representing 27.1% and 24.4% of the variation, respectively) indicates less dispersion (or total genetic variation) along the two axes in the captive population.

3.2. Geographic structure of population genetic variation

Mitochondrial haplotypes from the San Gabriel, San Bernardino and San Jacinto Mountains differ by 0.1–2.0% in an uncorrected pairwise comparison and unique mitochondrial haplotypes are found in almost every population. Shared haplotypes occur only between Little Rock Creek and Devils Canyon, and Fuller-Mill Creek and Dark Canyon. The Bayesian gene tree (Fig. 3A) places southern California *R. muscosa* in two distinct clades, one exclusive to the southern mountain ranges and the other shared between the southern mountain ranges and the Sierra Nevada. Samples from Little Rock Creek and Devils Canyon in the San Gabriel Mountains occur in both clades. The neighbor-joining tree estimated from average Nei's genetic distance of the microsatellite data (Supplementary Fig. 1S) is topologically similar to the mitochondrial gene tree, except that populations in the San Gabriel Mountains are not split into two clades and the Milestone Basin population is estimated to be more closely related to the San Bernardino and San Joaquin Mountain populations.

The analysis of microsatellite variation using the admixture model of STRUCTURE shows a distinct plateau in the log likelihood of the data at nine clusters (Fig. 3B). The improvement in the log-likelihood from $k = 8$ to $k = 9$ is slight, and results in subdividing individuals with incomplete assignment to a 9th cluster in the Dark Canyon population. Clusters represent every distinct sampling site, except Vincent Gulch and Bear Gulch are indistinguishable, and the sample from Tahquitz Creek and the captive population are not distinguished from the resident Dark Canyon population. Two individuals from South Fork Big Rock Creek appear to be admixed or migrants from Devils Canyon. When the data include all samples, the peak in the log-likelihood occurs at $k = 9$ and the results are qualitatively the same (Supplementary Fig. 2S).

Population differentiation based on pairwise F_{ST} of the microsatellite data (Table 3), using the number of alleles or corrected pairwise divergence, provides evidence of significant population differentiation in all populations comparisons, except in contrasts of Vincent Gulch and Bear Gulch, and the Dark Canyon resident and captive populations. Similarly, the AMOVA of populations from San Gabriel, San Bernardino and San Jacinto (Supplementary Table 4S) shows evidence of significant genetic structure among populations within each mountain range (24% of the total variation) and among mountain ranges (25% of the total variation).

Table 1
Genetic variability of microsatellite loci in populations of southern California *Rana muscosa*.

	All Individuals					Adults				
	N	*AR*	*Ho*	*He*	*F*	*N*	*AR*	*Ho*	*He*	*F*
Milestone Basin										
Mean	56.000	4.444	0.358	0.400	0.108	56.000	4.667	0.358	0.400	0.109
SE	14.056	1.271	0.107	0.112	0.068	14.056	1.333	0.107	0.112	0.068
Devils Canyon										
Mean	53.667	4.222	0.480	0.504	0.082	23.667	3.889	0.512	0.480	−0.052
SE	3.543	0.364	0.079	0.059	0.070	1.795	0.309	0.089	0.069	0.071
Little Rock Creek										
Mean	61.778	4.333	0.475	0.523	0.106	40.667	4.111	0.434	0.521	0.178
SE	2.414	0.289	0.063	0.052	0.049	4.298	0.611	0.060	0.053	0.053
South Fork Big Rock Creek										
Mean	71.889	4.556	0.411	0.433	0.116	9.111	2.889	0.446	0.454	0.023
SE	6.617	0.648	0.080	0.079	0.076	0.772	0.512	0.089	0.077	0.090
Vincent Gulch										
Mean	12.667	3.222	0.556	0.454	−0.254	12.444	3.333	0.511	0.426	−0.218
SE	1.213	0.683	0.118	0.094	0.119	1.435	0.553	0.115	0.088	0.129
Bear Gulch										
Mean	20.556	3.778	0.539	0.466	−0.129	40.333	2.556	0.514	0.431	−0.170
SE	2.205	0.521	0.125	0.104	0.037	4.055	0.338	0.127	0.104	0.104
East Fork City Creek										
Mean	80.556	3.333	0.263	0.305	0.236	18.333	4.778	0.253	0.301	0.210
SE	8.774	0.333	0.072	0.068	0.127	1.312	0.364	0.076	0.067	0.129
Fuller-Mill Creek										
Mean	42.667	5.556	0.647	0.583	−0.060	29.889	4.111	0.651	0.577	−0.115
SE	3.371	0.242	0.100	0.077	0.062	2.632	0.389	0.101	0.086	0.027
Dark Canyon										
Mean	76.778	4.667	0.467	0.468	0.013	56.000	4.667	0.358	0.400	0.109
SE	5.387	0.289	0.046	0.026	0.066	14.056	1.333	0.107	0.112	0.068
Captive population										
Mean	36.556	1.667	0.248	0.241	−0.028	36.556	1.778	0.250	0.242	−0.032
SE	7.848	0.373	0.066	0.064	0.035	7.848	0.401	0.067	0.064	0.036
Total over Loci and populations										
Mean	46.737	3.737	0.434	0.413	−0.012	27.424	3.323	0.432	0.411	−0.035
SE	3.144	0.201	0.031	0.025	0.032	2.183	0.194	0.031	0.026	0.033

N sample size; *AR* mean number of alleles; *Ho* observed heterozygosity; *He* expected heterozygosity; *F* fixation index ((*Ho* − *He*)/*He*).

Table 2
Statistical tests of genetic bottlenecks in *Rana muscosa* populations.

	Sign test *p*-value	Standardized difference test	Wilcoxin sign-rank deficiency test	*M-ratio*[*]	Standard deviation
Milestone Basin	0.53257	0.25193	0.5	**0.16001**	0.08982
Devils Canyon	0.58308	0.46852	0.78711	**0.19986**	0.08104
Little Rock Creek	0.55832	0.4858	0.82031	**0.20755**	0.06429
South Fork Big Rock Creek	0.43969	0.0566	0.45508	**0.21913**	0.08367
Vincent Gulch	0.12511	0.05119	0.99609	**0.27455**	0.11485
Bear Gulch	0.62448	0.44211	0.54492	**0.2175**	0.09429
East Fork City Creek	0.38881	**0.04406**	0.15039	**0.17473**	0.11237
Fuller-Mill Creek	0.5487	0.18393	0.5	**0.19571**	0.0658
Dark Canyon	**0.00512**	0.05855	**0.00977**	**0.17857**	0.03724
Captive population	0.08826	0.08043	0.98438	**0.20969**	0.12407

[*] Statistically significant tests shown in bold type. Based on simulations, *M-ratio* values less than 0.42 ($\theta = 10$) or 0.43 ($\theta = 1$) are significant and indicate a bottleneck.

Within-individual variation is also significant and accounts for 51% of the total variation. PCoA analysis of all adult frogs (Supplementary Fig. 3S) shows clear separation of populations in the three mountain ranges along the first two axes (~54% of the total variation). Populations within each mountain range occupy unique coordinate space in the PCoA, with the exception of Vincent Gulch and Bear Gulch and the captive and resident Dark Canyon population. Finally, a weak trend of isolation by distance is supported in the mitochondrial dataset, with log-transformed Euclidean distance significantly correlated to genetic distance ($r = 0.393$, $p = 0.018$) as well as least-cost geographic distance ($r = 0.395$, $p = 0.022$). However, isolation by distance is not evident in the microsatellite dataset with either log-transformed Euclidean distance ($\hat{a} = 0.329$, $p = 0.123$) or least-cost distance ($\hat{a} = 0.730$, $p = 0.116$).

3.3. Biogeographic history

The multi-population isolation with migration model (Fig. 4) estimates the split of San Bernardino and San Jacinto populations at 47,000 years before present (95% range: 33,654–127,885 YBP), the split of San Gabriel and the combined San Bernardino-San Jacinto lineage at 289,423 YBP (95% range: 275,962–3641,346 YBP), and the split of southern California lineages from the Sierra Nevada

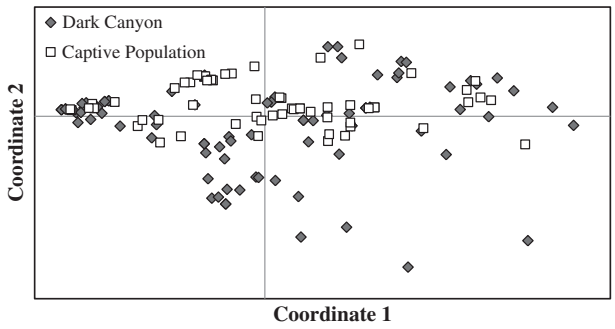

Fig. 2. Principal coordinates analysis of microsatellite variation in the Dark Canyon resident population and captive breeding population. The first two components account for 52% of the total genetic variation.

(Milestone Basin) at 1.42 MYBP (95% range: 1299,038–4745,192 YBP). San Jacinto and San Bernardino have smaller effective populations (*Ne* = 10,817, 95% range: 10,817–39,664) compared to either the San Gabriel (*Ne* = 284,856, 95% range: 191,106–782,452) or Milestone Basin populations (*Ne* = 118,991, 95% range: 104,567–400,241; Fig. 4). Going backwards in time, effective population size grows considerably in the combined San Jacinto and San Bernardino lineage (*Ne* = 616,586, 95% range: 219,952–1914,663), the combined southern California mountains lineage (*Ne* = 659,856, 95% range: 501,202–7020,432), and the ancestral lineage of the entire genealogy (*Ne* = 4546,875, 95% range: 2635,817–6991,579). Migration between populations is only observed in the most recent time period, from San Gabriel into San Jacinto (*2Nm* = 0.157, 95% range: 0.093–0.412), San Bernardino into San Gabriel (*2Nm* = 1.079, 95% range: 0.592–1.983), and San Gabriel into San Bernardino (*2Nm* = 0.098, 95% range: 0.024–0.219).

4. Discussion

4.1. Conservation genetics of southern california rana muscosa

The precipitous decline of populations of *R. muscosa* in southern California led to their listing as a federally endangered distinct population segment in 2002 (US Fish and Wildlife Service, 2002). Based on mark-recapture surveys, the nine remaining populations are small and have not been observed exchanging migrants (Backlin et al., 2004). These populations have continued to decline and

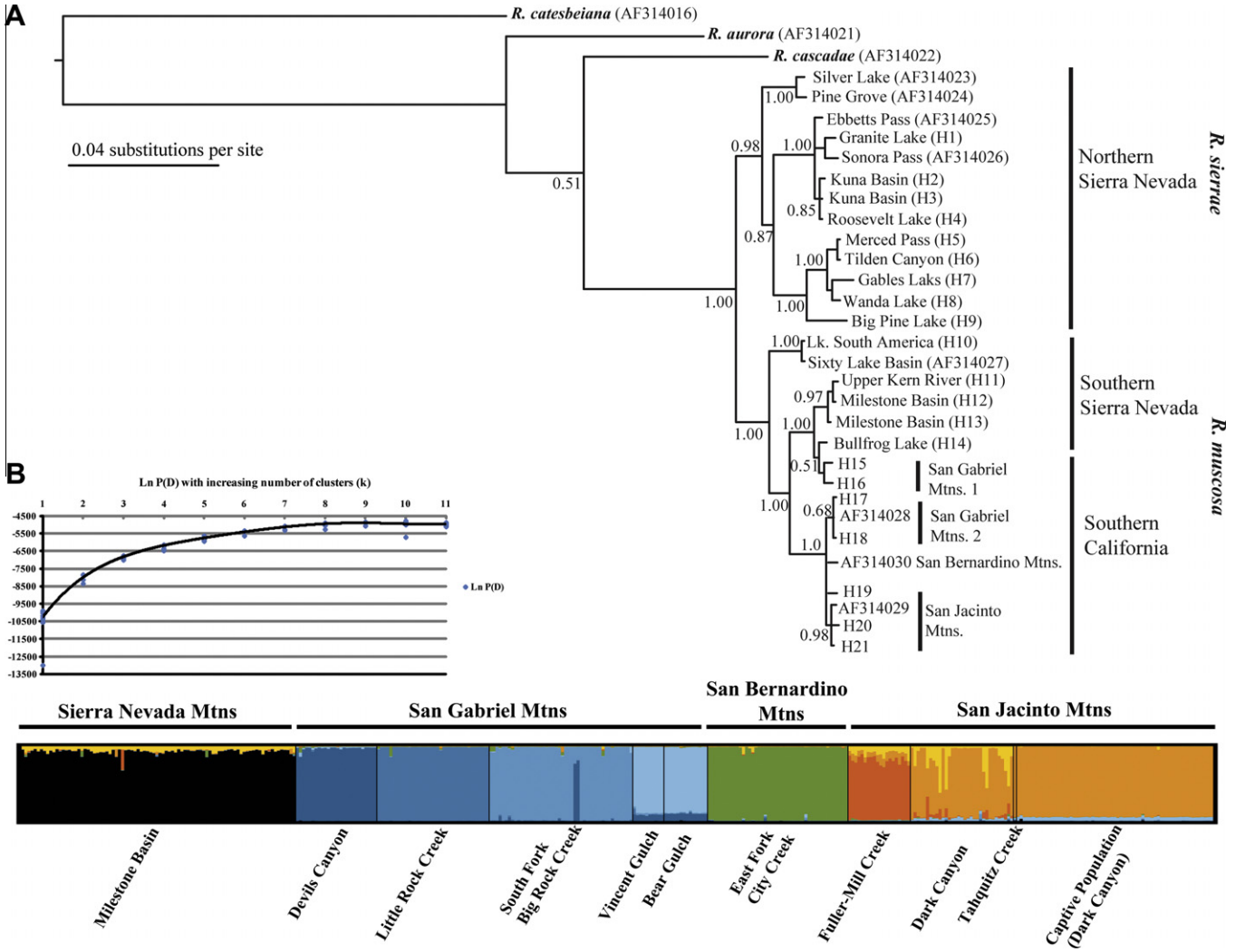

Fig. 3. Population genetic structure in southern California *Rana muscosa* indicated by the (A) Bayesian mitochondrial gene tree of *R. muscosa*, *R. sierrae*, and related out-groups (posterior probability support values shown at the nodes) and (B) STRUCTURE analysis of microsatellite variation of adult frogs (plot of the posterior likelihood of the data with increasing number of clusters (*k*), ten replicates at each *k*, showing a distinct plateau at *k* = 9), with corresponding plot of individual posterior probabilities for cluster membership shown at *k* = 9.

Table 3
Pairwise F_{ST} of microsatellite data of adult frogs among populations of southern California *Rana muscosa*.[a].

	Milestone Basin	Devils Canyon	Little Rock Creek	South Fork Big Rock Creek	Vincent Gulch	Bear Gulch	East Fork City Creek	Fuller-Mill Creek	Dark Canyon	Captive population
Milestone Basin	–	0.43211	0.37263	0.38728	0.47317	0.4888	0.40869	0.24811	0.31381	0.25051
Devils Canyon	1.97977	–	0.21116	0.25398	0.27119	0.27058	0.56467	0.50622	0.48135	0.60699
Little Rock Creek	1.58778	0.68509	–	0.31194	0.375	0.38896	0.42986	0.43625	0.40476	0.51249
South Fork Big Rock Creek	1.69041	0.87793	1.21968	–	0.15325	0.17079	0.54702	0.43363	0.36759	0.43348
Vincent Gulch	2.34484	0.84183	1.52919	0.47783	–	0.01539	0.63916	0.56367	0.50939	0.7051
Bear Gulch	2.45418	0.81284	1.56958	0.52398	0.02839	–	0.64924	0.57639	0.52823	0.70437
East Fork City Creek	1.6044	2.46094	1.58259	2.61614	2.96868	3.09761	–	0.50452	0.40384	0.51485
Fuller-Mill Creek	0.8571	2.3608	1.95433	1.95402	2.73468	2.79654	1.83779	–	0.28326	0.36367
Dark Canyon	1.21494	2.3342	1.80956	1.54897	2.57231	2.68621	1.38804	0.97975	–	−0.11439
Captive population	0.62301	1.83288	1.49937	1.18266	2.04476	2.14287	1.15927	0.59796	−0.14751	–

[a] F_{ST} calculated based on the number of different alleles (above diagonal) and the corrected average pairwise difference (below diagonal). Bold type indicates statistical significance corrected for multiple tests.

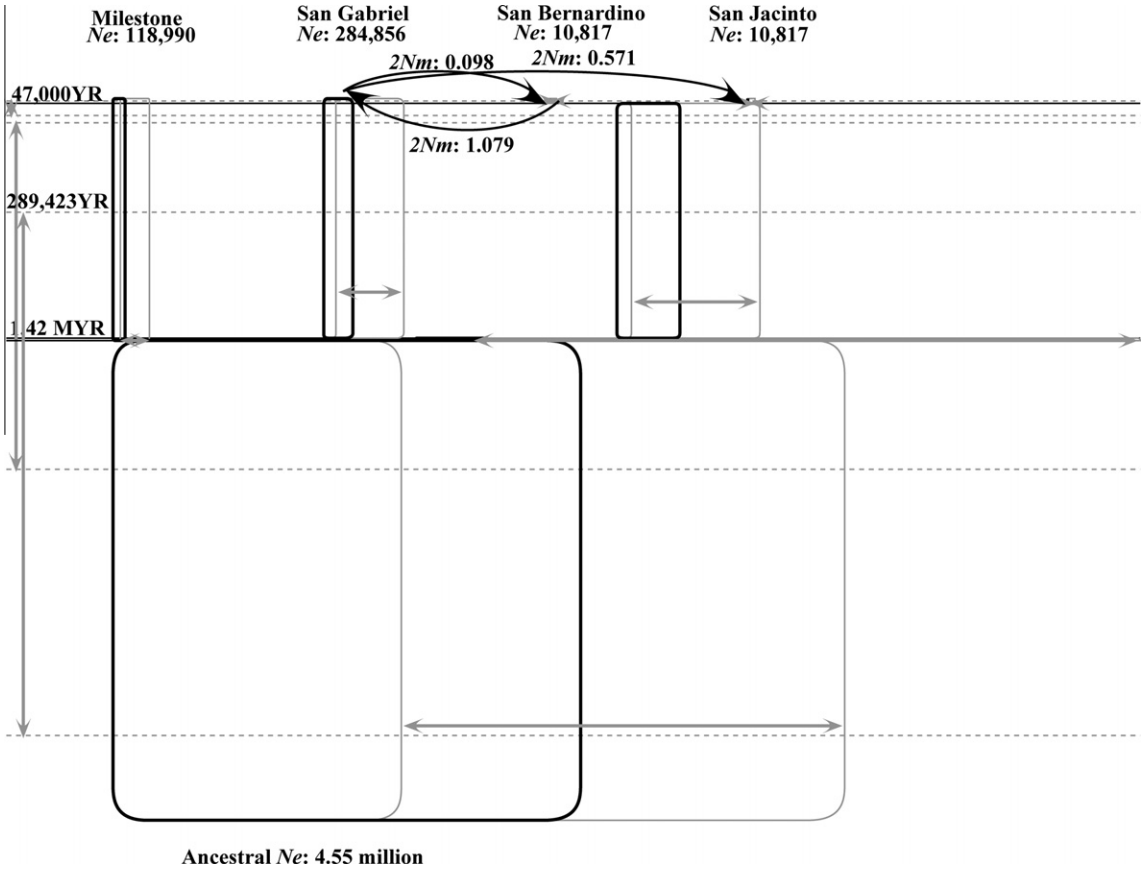

Fig. 4. Estimated split times and demographic parameters from the isolation with migration model of southern California *Rana muscosa*. Only statistically significant migration estimates shown as horizontal black arrows. Confidence intervals of split times and population sizes are indicated by gray shaded lines and arrows.

have been threatened by natural disasters, including wildfires and flooding, as well as predation by invasive trout (Compton et al., 2005a,b). We examined both mitochondrial and microsatellite loci to assess levels of genetic variation remaining in these populations, and in one of the last known populations inhabiting the southern Sierra Nevada.

Genetic diversity within these populations, as measured by the mean number of alleles per microsatellite locus and expected heterozygosity, is quite low compared to other montane *Rana* (Monsen and Blouin, 2004; Zhan et al., 2009; Zhao et al., 2009). While these studies were conducted with different microsatellite loci, it is noteworthy that only a few threatened or endangered ranids show similar low levels of microsatellite variation, including *Rana luteiventris* (Funk et al., 2005), *Rana latastei* (Ficetola et al., 2007), and *Rana pipiens* (Wilson et al., 2008). It is not known whether reduced levels of genetic variability will affect fitness, and, currently, inbreeding in *R. muscosa* is not strong, with the highest inbreeding (F ranges from 0.11 to 0.21 in adults) found in the East Fork City Creek, Little Rock Creek, Dark Canyon and Milestone Basin populations. However, there is evidence that genetic bottlenecks have recently occurred. The *M-ratio* test shows significant population size reduction in all populations. The sign-test, standardized difference

test, and Wilcoxin sign-rank test show a significant result in two different populations. The negative fixation indices in several additional populations (Table 1) show a trend towards an excess of observed heterozygosity. However, it is well known that there is less statistical power to detect a bottleneck with the sign-test or Wilcoxin sign-rank test (Williamson-Natesan, 2005), unless a strong bottleneck is very recent and/or ongoing, because heterozygosity rapidly returns to equilibrium values.

Population structure is evident among populations in southern California in both mitochondrial and microsatellite datasets (Fig. 3). With the exception of Vincent and Bear Gulch (which are not distinguishable in our data), every population appears to be genetically isolated with very little inter-population gene flow. This is not entirely surprising, as closely related ranids show a high degree of population structure among drainage basins (Funk et al., 2005; Lind et al., 2011). While the observed bottlenecks could explain allele frequency differences between our populations of *R. muscosa*, the analysis of the Dark Canyon wild and captive population suggests that recent bottlenecks are not inflating population structure. Isolation by distance is also known to exaggerate population structure (Frantz et al., 2009) and to positively bias detection of population bottlenecks (Leblois et al., 2006), but it does not appear to be a factor in the observed genetic structure of the microsatellite data. While a weak signature of isolation by distance is evident in the mitochondrial dataset, it is likely to reflect older demographic events that are retained in the coalescent history of the mitochondrial loci. The pattern of IBD in the mitochondrial data is consistent with our biogeographic model, where IBD is an expected outcome of the initial spatial expansion and stepwise diversification of *R. muscosa* into southern California.

4.2. Biogeographic history of Southern California mountains

R. muscosa occupies streams in montane forests of the San Jacinto, San Bernardino and San Gabriel Mountains, with a surrounding landscape of unsuitable habitat. While dispersal between these populations and to/from the southern Sierra Nevada is unlikely to occur in the current climate, paleoclimate records suggest a greater degree of habitat connectivity in the past (Hall, 2007). Fossil records indicate the presence of mixed conifer forest almost 900 m lower than present during the last glacial period (~41 KYBP; Anderson et al., 2002) and a significantly cooler and wetter climate lasting into the Holocene period (Bird and Kirby, 2006). During periods of wetter climate, stream drainages and lacustrine environments expanded around the southern California mountains and the western Mojave Basin (Orme, 2008). It is likely that pluvio-glacial cycles of the Pleistocene epoch increased the amount of suitable habitat for *R. muscosa*, facilitating spatial movement and population expansion, while warm and dry interglacial climates led to periods of genetic isolation.

Estimates of divergence time from the isolation with migration model are a plausible fit to this scenario (Fig. 4). Our analysis suggests that population divergence between San Jacinto and San Bernardino populations began near the end of last glacial stage (~47 KYBP), as gene flow was sufficiently reduced ($2Nm \leqslant 1$) to facilitate divergence due to genetic drift. The San Gabriel lineage is estimated to have diverged from the combined San Jacinto-San Bernardino lineage near the end of an earlier glacial stage (~289 KYBP). Finally, the divergence of southern California populations from the Sierra Nevada is estimated at 1.42 MYBP, occurring near the end of an early Pleistocene glacial stage (Lisiecki and Raymo, 2005). While estimates of effective population size have large confidence intervals, the relative increases going backwards in time may be indicative of substantial population declines during the present interglacial period. Estimates of effective population size suggest that the San Jacinto and San Bernardino lineages are smaller than the other lineages. However, these estimates may be inflated by underlying population structure in each mountain range (Wakeley, 2001), which is not accounted for in the isolation with migration model (Hey, 2010). While the effect of recent bottlenecks might decrease estimated rates of gene flow, it is not likely to have an effect on estimates of divergence time (Johnson et al., 2007).

4.3. Captive breeding, reintroduction and conserving evolutionary lineages

Efforts to maintain viable populations during severe population decline typically benefit from programs that facilitate recruitment, including captive breeding and reintroduction (Ballou and Foose, 2010). Amphibians are considered good candidates for captive breeding programs because they have high fecundity, breed multiple times as adults, and have low maintenance requirements (Griffiths and Pavajeau, 2008). Estimates of population size in southern California *R. muscosa*, based on field surveys and mark-recapture results, projected that the eight main extant populations were already in the range of a few hundred individuals or less in 2003 (Backlin et al., 2004). Theoretical research suggests that a genetic meltdown can occur when a small number of individuals (i.e. less than 100) comprise the entire breeding population (Lynch et al., 1995), although in practice high inbreeding rates have not always resulted in the demographic collapse of small populations (Keller and Waller, 2002). Irrespective of the effects of genetic inbreeding, it is essential to maintain larger populations in order to avoid stochastic changes in population size due to unpredicted environmental events. During an extreme drought event in August 2006, 82 tadpoles were salvaged from drying pools in Dark Canyon tributary under federal and state permit, and used to initiate a captive breeding program at the San Diego Zoo. The captive population cannot be distinguished genetically from the natural population based on microsatellite variation, a result which is supported by both the Bayesian clustering analysis and measurements of pairwise F_{ST}. However, there is evidence that the captive population has less neutral genetic variation, with fewer alleles across loci, lower heterozygosity and less total genetic variation in the principle components analysis.

While conservation efforts of southern California *R. muscosa* should be directed at maintaining populations in as many sites as possible, more recent surveys have found very few frogs at sites in the San Gabriel and San Bernardino Mountains (Compton et al., 2005a,b). Due to recent catastrophic environmental events (fires and subsequent flooding), remaining populations of southern California *R. muscosa* continue to decline and genetic diversity loss is likely to be rapid in these populations. At some point, a translocation program may be necessary to bring breeding frogs into contact or to avoid severe inbreeding depression. Translocation methods have improved substantially and become an increasingly successful strategy for restoring viable amphibian populations (Germano and Bishop, 2009). However, it is also important to maintain the unique characteristics of natural populations and to avoid out-breeding depression by mixing evolutionary independent lineages (Moritz, 1999). Based on the observed genetic structure and biogeographic history, the remaining populations of southern California *R. muscosa* represent seven distinct populations, with separate lineages represented in each mountain range. Efforts should be taken quickly to ensure that the integrity of these lineages is not sacrificed to maintain a viable gene pool.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocon.2011.04.025.

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