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Analysis of long-term river regulation effects on genetic connectivity of foothill yellow-legged frogs (*Rana boylii*) in the Alameda Creek watershed

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Cover photos: Alameda Creek at Camp Ohlone, Arroyo Hondo, Alameda Creek downstream of Calaveras Creek, and *Rana boylii* adult female (Clockwise from upper left).

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1 BACKGROUND AND PURPOSE

Amphibians are uniquely suited as indicators of ecological stability; the typical life cycle of a frog includes aquatic development of eggs and larvae, and terrestrial activity as adults. In addition, frog larvae are generally herbivorous, and adults are carnivorous, linking a single individual to a wide range of environments and trophic connections (Duellman and Trueb 1986; Wake and Vredenburg 2008). The foothill yellow-legged frog (*Rana boylii*), a California Species of Special Concern, provides a suitable cornerstone for assessing stream health. *Rana boylii* occupies a critical niche in the ecosystem, functioning as periphyton grazers and prey for aquatic macroinvertebrates and snakes when in the tadpole form, and as predators of arthropods post-metamorphosis. As an obligate river-breeding frog, *R. boylii* are particularly sensitive to changes in the ecosystem due to their physiology and life histories, and has disappeared from over 50 percent of their known historical range (Davidson et al. 2002; Lind 2005).

Amphibians are useful indicators of landscape fragmentation and genetic connectivity. Sensitive amphibian species have shown strong landscape patterns of extinction due to habitat degradation and fragmentation (Ficetola and De Bernardi 2004). Vos et al. (2001) found that barriers like roads and railways had a greater influence in restricting gene flow compared with geographic distances in moor frog (*Rana arvalis*) populations. For the California native river breeding foothill yellow-legged frog (*Rana boylii*), river regulation may significantly alter landscapes through flow modification, landscape structures such as reservoirs and dams, which limit movement, and by altering connectivity between populations.

Among multiple ecosystem stressors, river regulation has a great potential impact on *R. boylii* because of the impairment of important fluvial processes necessary for creating and sustaining important frog habitat (e.g., bar formation), as well as alteration of flow and temperature regimes (Kupferberg 1996; Poff et al. 1997; Yarnell et al. 2012). Lind (2005) observed that *R. boylii* population absences were positively correlated with proximity to large dams. Flow modification has pervasive effects on river ecosystems (Renofalt et al. 2010), and ultimately riverine processes are driven by flow variables (Poff et al. 1997). However, little is known about the impact of river regulation on genetic connectivity in non-fisheries aquatic vertebrates. Most research on *R. boylii* has focused on the short-term effects of river regulation on breeding, survival, and fluctuations in population size. There has been little long-term research on the effects of river regulation on gene flow, genetic diversity, and connectivity.

The maintenance of genetic diversity and gene flow are driving forces behind the process leading to speciation or extinction (Avise 1994). Gene flow is the successful migration of alleles between populations, and is dependent upon population size and migration rates (Frankham et al. 2002). Aseasonal high flows that restrict connectivity within a watershed (i.e., prevent movement from one tributary to another, alter habitat due to regulation of flow, allow dispersal or introduction of non-native species into a region) may have impacts on local population dynamics (Kupferberg et al. 2008; Lind et al. 1996; Reese and Welsh 1998). Patterns of genetic divergence have been observed to correlate with landscape features (Slatkin 1985; Vignieri 2005; Vos et al. 2001; Wang et al. 2008), and discerning the connections between *R. boylii* population genetic structure and the landscape that these frogs inhabit is critical for developing effective conservation measures for the species (Moritz 2002).

Both water regulation and landscape features like dams or reservoirs can fragment the riverine landscape and increase genetic divergence among frog populations (Peek 2010). Lind et al.

(2011) observed that genetic variation in *R. boylii* was highly structured along hydrologic boundaries, and suggested that future conservation efforts, particularly genetically-based management strategies, should strive to preserve these patterns as they represent historical lineages that could not be recovered if lost. From the perspectives of conservation and ecosystem services, populations with more genetic variation may have greater resilience in the face of environmental change (Luck et al. 2003). Genetically isolated populations have a much higher risk of extirpation, and conservation management should ultimately balance the preservation of genetic diversity associated with subpopulations—critical to potential adaptive plasticity required for long term survival in stochastic river systems—against the risks associated with isolation when managing connectivity through habitat manipulations or assisted migration to provide sensitive species with the greatest chance for survival.

Connectivity of populations that facilitates movement of individuals is critical for the persistence of ecological and evolutionary processes and occurs at many scales. In rivers, connectivity links organisms at various scales (Power and Dietrich 2002; Puth and Wilson 2001; Wiens 2002), reduces genetic divergence, increases gene flow (Raeymaekers et al. 2008; Slatkin 1987; Vignieri 2005), and protects biodiversity (Fahrig 2003). Restoring riparian connectivity may be a key factor in promoting adaptation and resilience to climate change (Seavy et al. 2009). Interactions between landscape structure and organism movement behavior may ultimately influence dispersal and gene flow (the successful migration of alleles between populations), which both depend upon population size and migration rates (Frankham et al. 2002).

Rana boylii has been extant as a native species in the rivers and streams of California and Oregon for approximately 8 million years (Macey et al. 2001), yet it has only taken the last 150 years for human influence to drastically transform their habitat (Karr and Chu 2000; Mount 1995). This frog species is important because it links with multiple trophic levels in dynamic and complex ecosystems, and because it is a current California Species of Special Concern (Jennings et al. 1994). This particular species currently lacks the large-scale conservation efforts implemented for federally threatened or endangered species. Studies of the effects of river regulation on *R. boylii* have largely focused on the negative impacts of pulse flows (or aseasonal flow fluctuations) on *R. boylii* breeding and breeding habitat (Kupferberg et al. 2008; Lind et al. 1996; Lind 2005). Large changes in flow can scour egg masses from breeding locations, and because *R. boylii* females only lay one egg mass per year, loss of any egg mass can severely reduce population fecundity (Kupferberg et al. 2009; Kupferberg et al. 2008).

This species has adapted to inhabit a dynamic ecosystem, and flow regulation has altered patterns of natural hydrologic variation. Aseasonal high flows that restrict connectivity within a watershed (i.e., prevent movement from one tributary to another, alter habitat due to regulation of flow, allow dispersal or introduction of non-native species into a region) counter the natural flow regime, which provides consistent ecological and hydrological signals that local populations have adapted life history strategies around (Kupferberg et al. 2008; Lind et al. 1996; Reese and Welsh 1998; Yarnell et al. 2010). Lind et al. (1996) found that reduced peak flows and increased summer flows permitted the encroachment of riparian vegetation, which limited *R. boylii* breeding habitat by increasing shade, and caused incision and armoring of the streambed. Research has shown that *R. boylii* occur in stream reaches with greater habitat heterogeneity in the foothills of the Sierra Nevada (Van Wagner 2006; Yarnell 2005), and changes in the geomorphic composition of a river can influence ecosystem function and productivity (Beisel et al. 2000; Poff et al. 2006). The impacts of river regulation have been documented extensively in recent literature for a wide range of species and ecosystems; however, genetic diversity and gene flow have rarely been assessed in regulated systems, particularly for river-breeding amphibians. As a result, conservation management strategies rarely consider connectivity at both genetic and

spatial scales. Instead, most research has focused on the short-term effects of river regulation on *R. boylii* breeding and there has been little research on the effects of river regulation on genetic connectivity. Genetic structuring may occur naturally due to small population sizes and potentially strong philopatric associations with breeding habitat. However, given sufficient time over generations, isolated subpopulations (i.e., above a reservoir or separated by river regulation) may exhibit lower levels of genetic diversity and higher levels of genetic drift, particularly if existing population sizes are small. This study attempted to address the following questions:

1. Does the presence of Calaveras Dam (built in 1913), reservoir, and associated river regulation limit gene flow and connectivity within *R. boylii* populations in the Alameda watershed?
2. Are *R. boylii* populations within the Alameda Creek watershed genetically divergent from extant *R. boylii* populations in adjacent or proximal watersheds (Lind et al. 2011)?

2 METHODS

2.1 Study Area

Calaveras Reservoir, located in Alameda and Santa Clara Counties, California, was formed when SFPUC began constructing Calaveras Dam in 1913, and the project was completed in 1925. Calaveras Dam is located on Calaveras Creek about 1.3 km (0.8 miles) upstream (south) of the confluence with Alameda Creek. Calaveras Reservoir captures the flow from Calaveras Creek and the large Arroyo Hondo tributary watershed. In addition, flows from upper Alameda Creek are diverted about 5 km (3 miles) upstream of the Calaveras Creek confluence through the Alameda Creek Diversion Dam (ACDD) tunnel into Calaveras Reservoir.

Seismic safety restrictions imposed by the Division of Safety of Dams (DSOD) in 2001 and later refined in 2003 require that Calaveras Reservoir be kept just under 40% of the original design capacity. These DSOD restrictions have led to increased releases to Calaveras Creek since 2001. The Calaveras Dam Replacement Project (CDRP), scheduled for completion in 2015–2016, will allow the SFPUC to return storage in the reservoir to its original capacity.

Before 2002, the ACDD tunnel gates were kept open throughout the winter, diverting up to 650 cfs even at times when Calaveras Reservoir was full, resulting in spills into Calaveras Creek. Since 2002, the ACDD has operated infrequently because SFPUC has had to maintain lower storage levels in Calaveras Reservoir in compliance with DSOD restrictions. As part of the CDRP, the ACDD will be re-operated with a 370-cfs maximum diversion capacity. Diversions will be limited to January–March and minimum flows will be bypassed to provide water below the ACDD as long as there is flow in Alameda Creek above ACDD.

Extant populations of *R. boylii* occur in Arroyo Hondo upstream of the reservoir, and in Alameda Creek, both upstream and downstream of the diversion (see Figure 2-1). Three study sites were identified (Table 2-1, Figure 2-1):

- Arroyo Hondo upstream of Calaveras Dam (Arroyo Hondo) – The creek drains into the southeast arm of Calaveras Reservoir, and Calaveras Reservoir and Calaveras Dam have blocked connectivity via stream pathways between Arroyo Hondo and Alameda Creek (via Calaveras Creek) for over 85 years.
- Alameda Creek upstream at Camp Ohlone (Camp Ohlone) – Alameda Creek upstream of the diversion is unimpaired (no large dam or reservoir is present upstream). A diversion dam and diversion tunnel, 3.5 km downstream of Camp Ohlone, divert water from Alameda Creek into Calaveras Reservoir. There are no screens on the diversion tunnel opening in Alameda Creek, and although water is diverted into Calaveras Reservoir when possible, there are stretches of Alameda Creek that can be intermittent in all but the wettest years throughout this reach.
- Alameda Creek at Calaveras Creek confluence (Calaveras Creek Confluence) – Approximately 7 km downstream of Camp Ohlone, and 9 km downstream from the Arroyo Hondo site Calaveras Creek flows into Alameda Creek. This site was centered on the confluence between these two creeks. Flow is highly regulated below Calaveras Dam, and therefore Calaveras Creek and Alameda Creek downstream of the confluence with Calaveras Creek are influenced heavily by flow diversion above the dam and releases downstream of the dam.

Study reaches were selected to maximize survey data on frog populations, and therefore information on accessibility and current population presence was compiled before choosing the final stream segments. Surveys have been conducted in Alameda Creek for nearly 10 years as part

of ongoing amphibian monitoring by East Bay Regional Park District, and these field data helped inform selection of sites near known breeding sites. Each site was surveyed in 2011 between April and May to maximize the probability of encountering *R. boylii* breeding. Genetic comparisons within populations were from sampling locations less than 10 km apart, to avoid confounding patterns associated with geographic distance [for gene flow see, (Dever 2007; Monsen and Blouin 2003); for movement see (Bourque 2008; Gonsolin 2010)].

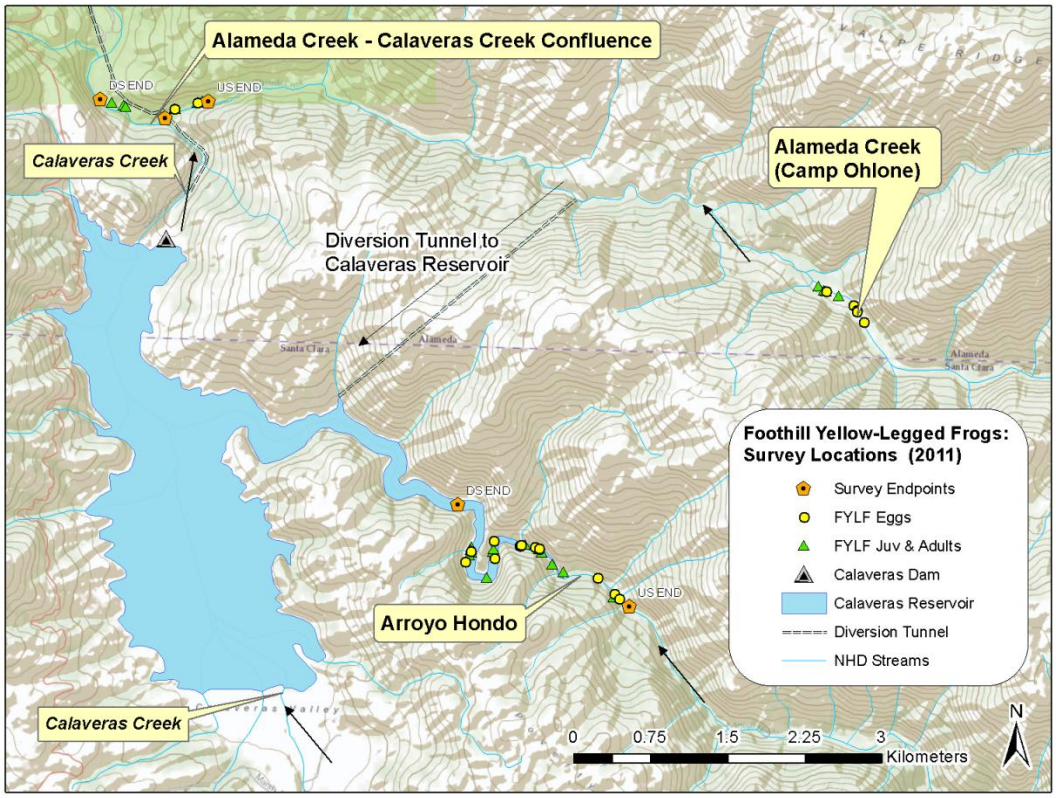

Figure 2-1. *Rana boylii* sampling locations within the Study Area.

Table 2-1. Study sites for 2011.

Site Name	Site Characterization	Site Length (km)
Arroyo Hondo	Unregulated—Upstream of Calaveras Dam	3.25
Camp Ohlone	Unregulated—Upstream of the Diversion Tunnel in Alameda Creek	1.25
Calaveras Creek Confluence	Regulated—Downstream of Calaveras Dam and Alameda Creek Diversion Tunnel	1.15

2.2 Field Surveys

Timed visual surveys were conducted at each site. For each *R. boylii* observed, data collected included frog location, life history stage, sex, morphometric measurements, survey time, and habitat use. Individuals exhibiting secondary sexual characteristics (i.e., enlarged nuptial pad on the inner-most digit of the forelimb) were categorized as males, and individuals greater than 40

mm (SUL) that did not exhibit nuptial pads were categorized as females. Waypoints for each frog location were logged in decimal degrees, with a hand-held GPS receiver using NAD83 datum, and averaged for approximately 30 seconds per location to increase accuracy (generally 3–5 m per point).

Tissue was collected from captured post-metamorphic frogs following USGS (2001) toe-clipping protocols. All individuals were released at the same location they were captured after a brief observation period. The longest toe on the left foot was clipped at the most distal joint and immediately placed in 70% ethanol in a cryogenic tube. Because it is often difficult to capture a sufficient number of post-metamorphic individuals for genetic analysis, a unique method was tested using egg masses. Female *R. boylii* oviposit one egg mass per breeding season, and each egg mass may contain as many as 2,000 eggs. Where possible, 1–2 eggs from each *R. boylii* egg mass were collected and reared until hatched, after which tadpole tissue was utilized for DNA extraction. Egg masses were not moved and were minimally disturbed during the sampling process. DNA from tissue was isolated using the Qiagen DNeasy kit. DNA concentrations were quantified using a Nanodrop. Samples were stored at -80°C, and aliquots of 50 µL were stored at -20°C for polymerase chain reaction (PCR) reactions.

2.3 Molecular Methods

DNA was isolated for two distinct types of genetic markers: randomly amplified polymorphic DNA (RAPDs) and a region of mitochondrial DNA (mtDNA) from the NADH-Dehydrogenase gene (ND2). RAPDs were used to assess the level of genetic diversity, and ND2 mtDNA were used to assess gene flow. These markers have already been successfully employed as a means for estimating genetic structure within and among subpopulations (Dever 2007). Additionally, *R. boylii* sequences collected in 2011 were compared with additional samples from Lind et al. (2011) to assess genetic divergence from other regional populations. Choice of molecular marker for the examination of genetic structure is important as different markers can produce different results (Mila et al. 2010) due to differences in mutation rate and inheritance pattern. Therefore, both mtDNA and RAPDs, were used to determine genetic haplotype variability, gene flow, and genetic structuring within and among *R. boylii* populations.

2.3.1 Mitochondrial DNA

The mtDNA has a much higher rate of nucleotide substitution relative to nuclear DNA, because it does not contain the proofreading machinery that nuclear DNA does, and it has several mutational hot spots (DeWoody 2005; Galtier and Boursot 2000). In addition, mtDNA does not deviate significantly from expectations of neutral evolution (Bos et al. 2008). Thus mtDNA is a fairly stable marker not influenced by environmental selection that can be used to determine gene flow among populations (Avise 1994; DeWoody 2005; Moritz 2002). Since mtDNA is maternally inherited and is haploid, it provides a robust indicator of genetic drift and has been used extensively in determining lineage and population structure (Shaffer et al. 2004). For this study, a region of the NADH dehydrogenase subunit-2 gene (ND2) mtDNA was amplified and sequenced. This region was chosen because substitution rates are elevated at ND2 relative to other regions of the mtDNA genome (Bos et al. 2008). Following methods described in Dever (2007), PCR primers yielded a 1000 base pair (bp) amplicon, and final PCR conditions included 5.0 µL of each primer (tRNAtrp_R: 5'-TTA AAGGGC CTG AGT TGC ATT-3' and tRNAmet_F: 5'-AAG CTT TCG GGC CCA TAC C-3', Lind 2005), 5.0 µL of PCR buffer (Tris-Cl), 4.0 µL of dNTP, and 0.24 µL of Taq polymerase in a final volume of 50 µL. The reaction consisted of 30 cycles of

30s at 94°C, annealing for 30s at 56°C, and elongation for 1 min at 72°C. The amplified product was separated and examined on an agarose gel stained with ethidium bromide. Samples were then cleaned with a Millipore kit and sent to Davis Sequencing Lab for sequencing. Sampled mtDNA sequences were aligned using Sequencher v. 5.0. To determine haplotype diversity, aligned sequences were analyzed using DNASP.

2.3.2 Random Amplified Polymorphic DNA

RAPD markers were used to estimate genetic diversity within and among populations. These markers are suitable for estimating genetic diversity in a population, and are a useful and cost-effective marker in population genetic studies (Bagley et al. 2001; D'Surney et al. 2001; Gibbs et al. 1994; Karuppudurai et al. 2007; Lynch and Milligan 1994). Microdifferentiation between genetically similar subpopulations can be detected using RAPDs (by examining loci with high number of alleles) (Nei 1973), and have been used to quantify levels of diversity in *R. boylii* (Dever 2007). Compared with mtDNA, RAPDs have higher mutation rates, which may reflect more recent changes in the genetic structure of a population (Dever 2007; DeWoody 2005). In addition, RAPDs are much easier to develop, generate greater numbers of loci for genetic analysis, require less DNA than other markers, and are an inexpensive PCR-based technique for generating a DNA banding profile for each individual (Kimberling et al. 1996; Lynch and Milligan 1994). This makes RAPDs a good alternative for studying rare species, or populations that have very small population sizes, as minimal tissue is required (Kimberling et al. 1996).

Nonetheless, RAPDs have certain limitations as they are not locus-specific, and are dominant markers. Estimation of diversity cannot be as accurate as other locus specific and co-dominant markers (because it is only possible to estimate a single dominant parameter when in actuality there may be two, the recessive and dominant) (Lynch and Milligan 1994; Wang 2004). Co-dominant markers are generally preferred over dominant markers, because all alleles in a genotype are observable phenotypically. Therefore allele frequencies and relatedness are more accurately estimated (Lynch and Milligan 1994; Wang 2004).

In order to avoid biased diversity estimates, Lynch and Milligan (1994) and Wang (2004) outlined several options, including sampling more individuals, equalizing sampling sizes in different populations, using a similarity estimator, and standardizing the amplification protocol. Nei and Tajima (1987) observed that small sample sizes do not have as adverse an effect in estimating genetic diversity and genetic distance when large numbers of loci are examined. Assumptions that were required for RAPD analysis include: (1) each marker must represent a Mendelian locus in which the amplified marker allele is in Hardy-Weinberg equilibrium with a null recessive allele; and (2) marker alleles from different loci do not co-migrate to the same position, or no linkage disequilibrium (Kimberling et al. 1996; Lynch and Milligan 1994). RAPDs provide useful estimates of genetic variation if the limitations and assumptions are taken into account (D'Surney et al. 2001; Kimberling et al. 1996; Lynch and Milligan 1994).

PCR reactions for each sample utilized the Ready-To-Go RAPD Analysis Beads Kit (Biosciences) since it included all the necessary PCR components in a dried bead optimized for RAPD amplification. Following the methodology described in Dever (2007), RAPD primers #2, #4, and #5 were used as they produced identifiable polymorphic bands. Banding patterns were created for each individual using a stratified sampling system, and each band was identified using a binary code. Three different technicians conducted separate band counts to provide an accurate estimate of band diversity. Allele frequencies for RAPDs were estimated following Lynch and Milligan (1994), as described in Dever (2007). Genetic differentiation was calculated using the program TFPGA (Miller 1998) to calculate θ (Weir and Cockerham 1984), which is a measure of

genetic distance that has little bias and can be used across many genetic data, and a bootstrap procedure consisting of 1000 replicates over loci to determine 95% confidence limits. F_{ST} and pair-wise genetic distances between subpopulations were estimated using Reynolds et al. (1983) coancestry distance in ARLEQUIN (Excoffier and Lischer 2009). Coancestry distance is an inbreeding coefficient, used as the basis for measurement of genetic distance in short-term evolution, when divergence between populations with a common ancestral population is considered solely due to drift (Reynolds et al. 1983). Mantel's tests were used to analyze isolation-by-distance with 10,000 randomizations in the web program IBD (Jensen et al. 2005) following methods outlined in Dever (2007).

3 RESULTS

Surveys were conducted on April 9, April 20, and April 30, 2011 at Camp Ohlone, Arroyo Hondo, and Calaveras Creek Confluence respectively. Incidental observations of additional herpetofauna documented a total of 8 different aquatic species (*Rana draytonii, Thamnophis couchii, Pseudacris sierra, Anaxyrus boreas, Emys marmorata, Taricha torosa,* and the invasive American bullfrog *Lithobates catesbeiana*). All species except for American bullfrogs were observed at Camp Ohlone. Additional species may be present at these sites which were not observed during surveys; therefore this list should not be considered a comprehensive list of species diversity without conducting additional surveys. Invasive American bullfrogs (*Lithobates catesbeiana*) were observed only at the Calaveras Creek Confluence site.

Survey effort was comparable between sites in 2011, but distances surveyed were different and therefore calculations of *R. boylii* egg mass density varied; the highest density was observed at Camp Ohlone (20 eggs/km), followed by Arroyo Hondo (5.2 eggs/km) and Calaveras Creek Confluence (1.6 eggs/km). The confluence site had the fewest number of *R. boylii* egg masses and very few post-metamorphic individuals were observed compared with Camp Ohlone and Arroyo Hondo.

Genetic samples were collected from a subset of observations. A total of 42 unique *R. boylii* DNA samples were collected, mainly from eggs (Table 3-1).

Table 3-1. *Rana boylii* genetic samples from 2011.

Site	Adult Female	Adult Male	Egg Masses	Sequenced ND2[a]	RAPD
Arroyo Hondo	0	0	17	8	18
Camp Ohlone	0	0	22	12	28
Calaveras Creek Confluence	1	4	2	5	6

[a] Not all samples were successfully sequenced.

3.1 Mitochondrial DNA (ND2) Results

Mitochondrial DNA (mtDNA) sequenced from the ND2 loop yielded an alignment of 609 base pairs and only 2 unique haplotypes from 28 samples from three sites (Table 3-2). Several samples could not be sequenced successfully, and combined with the single polymorphism, haplotype diversity could not be calculated and comparative analysis was limited. The polymorphism identified at a single base pair was only shared by five total samples (four at Alameda Upstream and one at Arroyo Hondo).

Table 3-2. *Rana boylii* mtDNA summary.

Site	Calaveras Creek Confluence	Arroyo Hondo	Camp Ohlone	All
Sample size	6	7	15	28
Samples with polymorphism[a]	0	1	4	5

[a] Only one polymorphic site identified.

A comparison of the above samples with *R. boylii* mtDNA sequences from clades B and D (San Francisco and Central Coast region) identified by Lind et al. (2011) revealed an overlapping region of 179 bp. Analysis of aligned mtDNA sequences yielded two shared haplotypes among samples from Camp Ohlone, Arroyo Hondo, and neighboring watershed, Arroyo Mocho (Locality 26 in Table 5, Lind et al. 2011). Although this result is limited by the small sample size and short comparison sequence, it does indicate that populations in Arroyo Hondo and Alameda Creek are most likely phylogeographically similar to Clade D (Lind et al. 2011). Euclidean distances from samples in Arroyo Mocho collected by Lind et al. (2011) and samples in Arroyo Hondo and Camp Ohlone were approximately 15 km. Although the alignment provided a short sequence for comparison, genetic similarities with samples from Arroyo Mocho lends support to observations of shared ancestry within hydrologic regions (Lind et al. 2011).

3.2 Random Amplified Polymorphic DNA Results

A total of 115 loci were identified across all three sites using three different RAPD primers. Estimates of average band heterozygosity (H_z) were based on Lynch and Milligan's (1994) Taylor expansion estimates, and allele frequencies were rounded to exactly match the observed sample (Table 3-3). Camp Ohlone had the highest average band heterozygosity and Arroyo Hondo had the lowest. Despite the limited RAPD sample size for the Calaveras Creek Confluence site (six samples); average H_z was greater than at Arroyo Hondo. Percent polymorphic loci per site (using 99% criterion) indicate that the Camp Ohlone site had the greatest proportion of band diversity, followed by Arroyo Hondo and Calaveras Creek Confluence. The low proportion of polymorphic loci in Calaveras Creek Confluence compared with the other two sites may be attributed to the small sample size.

Table 3-3. Descriptive statistics of analysis of RAPDs.

Site	Calaveras Creek Confluence	Arroyo Hondo	Camp Ohlone	All
Sample size	6	18	28	52
Number of polymorphic loci	45	72	99	97
Percent polymorphic loci	39.10	62.61	86.09	84.35
Expected H_z	0.16	0.18	0.22	n/a
Average H_z	0.13	0.11	0.14	0.14

3.2.1 Genetic divergence

Genetic divergence within and among populations was calculated using several methods. Estimates of θ (Weir and Cockerham 1984) and coancestry identity (Reynolds et al. 1983) are both approximate equivalents of Wright's F_{ST} metric(Wright 1943), and describe the proportion of genetic diversity from differences in allele frequencies between populations. Generally, F_{ST} estimates range from 0 to 1, with estimates nearing 0 indicating very little divergence between

subpopulations and estimates nearing 1 indicating complete genetic divergence. Values of 0.1 or greater often indicate significant levels of genetic divergence.

An estimate of θ from TFPGA using 10,000 replicates and bootstrapping across loci showed significant divergence between all sites (θ = 0.128 [sd=0.020]). Estimates of pairwise F_{ST} calculated with Arlequin (Excoffier and Lischer 2009) and coancestry identities calculated with TFPGA also showed high levels of subpopulation divergence, although divergence was the lowest [0.008 and 0.05] between the two sites that occurred in separate drainages (Camp Ohlone and Arroyo Hondo) (Table 3-4). Divergence was highest between the Calaveras Creek Confluence site and Arroyo Hondo using either F_{ST} or coancestry identity. Furthermore, divergence was high (~0.3) between the Calaveras Creek Confluence site and Camp Ohlone, which both occur on Alameda Creek.

Table 3-4. Pairwise F_{ST} estimates among sites (on the lower diagonal) and coancestry identities (Reynolds et al. 1983) (on the upper diagonal).

Site	Calaveras Creek Confluence	Arroyo Hondo	Camp Ohlone
Calaveras Creek Confluence	--	0.399	0.309
Arroyo Hondo	0.092	--	0.048
Camp Ohlone	0.076	0.008	--

Statistical tests based on the mutation (segregating site) frequency, mismatch distribution, or haplotype distribution provide a way to distinguish population growth from constant population sizes (tests of selective neutrality) (Fu 1997; Slatkin and Hudson 1991; Tajima 1989). Tests of selective neutrality infer whether a population is experiencing neutral selection, or evolving under a variety of non-random processes including directional selection, genetic hitchhiking, or demographic expansion or contraction. For example, population growth can generate an excess number of mutations which translate as an excess of singletons (substitutions present in only one sampled sequence) which can be quantified using DNA sequences (Ramos-Onsins and Rozas 2002). Therefore, changes in population size may be detected in DNA sequence data. Tests of selective neutrality gave conflicting results. Tajima's D ((Tajima 1989, 1996) was not significant within any of the three populations. Fu's F_S statistic (Fu 1997) was significant for populations at Arroyo Hondo and Camp Ohlone (Table 3-5). Ramos-Onsins and Rozas (2002) found Fu's F_S was most statistically informative when comparing multiple tests of neutrality, particularly with larger sample sizes, while Tajima's D had comparatively less power to reject they neutral selection hypothesis in a population. Large negative Fu's F_S values may indicate a recent population demographic expansion (as an excess number of low frequency polymorphisms within a population) or genetic hitchhiking. Arroyo Hondo and Camp Ohlone had the highest F_S value of the three sites and the Calaveras Creek Confluence had the lowest, although the limited sample size from the Calaveras Creek Confluence site may be an important factor for this value (see Ramos-Onsins and Rozas 2002).

Table 3-5. Tests of selective neutrality: Tajima's D and Fu's F within populations. (p-values: * < 0.01).

Site	Tajima's D (pairwise difference)	Fu's F
Calaveras Creek Confluence	-0.554 (0.366)	-0.128 (0.302)
Arroyo Hondo	-0.106 (0.491)	-5.969* (0.007)
Camp Ohlone	-0.062 (0.503)	-11.436* (0.001)

3.3 Migration Rates and Isolation by Distance

Migration rates (M) can be defined as movement of alleles from one population to another. Migration rates varied among study sites, and did not correlate with geographic distances between sites (Euclidean). Migration rates can be defined as the number of migrants per generation carrying a copy of a gene (allele). Migration rates between Arroyo Hondo and Camp Ohlone were very high compared with rates between the Calaveras Creek Confluence and Arroyo Hondo or Camp Ohlone (Table 3-6). Geographically, Euclidean distances between Camp Ohlone and Arroyo Hondo were shortest (based on straight line measurements between the mid-point of each survey site).

Table 3-6. M-values (migration rates) between sites (on the lower diagonal) and geographic distances (km), Euclidean [Stream network] (on the upper diagonal).

Site	Calaveras Creek Confluence	Arroyo Hondo	Camp Ohlone
Calaveras Creek Confluence	--	6.7 [8.9]	5.4 [7.9]
Arroyo Hondo	0.904	--	3.8 [16.6]
Camp Ohlone	1.441	5.721	--

The M-value between the Calaveras Creek Confluence and Arroyo Hondo was 0.904, compared with 5.7 between Arroyo Hondo and Camp Ohlone, despite similar Euclidean and stream network geographic distances separating these sites (Calaveras Creek Confluence to Arroyo Hondo, and Calaveras Creek Confluence to Camp Ohlone).

Mantel tests of isolation by distance (IBD) were not significant (Figure 3-1, Figure 3-2). Two different distance matrices were tested, a Euclidean distance (measured from the centroid or midpoint of each survey site) and a stream-network distance (measured from the centroid of each survey site along a stream network). Genetic distances between sites (based on Nei 1978 model)

were positively correlated with Euclidean distances (r = 0.966, p = 0.155) and negatively correlated with the stream-network distances (r = -0.997, p = 0.162). For example, the sites separated by the largest stream network and shortest Euclidean distance (Sites Camp Ohlone and Arroyo Hondo) had the highest migration rate and lowest F_{ST} of all three sites. The two sites separated by the shortest Euclidean and stream network distance (Calaveras Creek Confluence to Camp Ohlone) had one of the lowest migration rates and highest F_{ST} values. Therefore, geographic distance between sites did not explain differing levels of genetic variation and divergence among study sites.

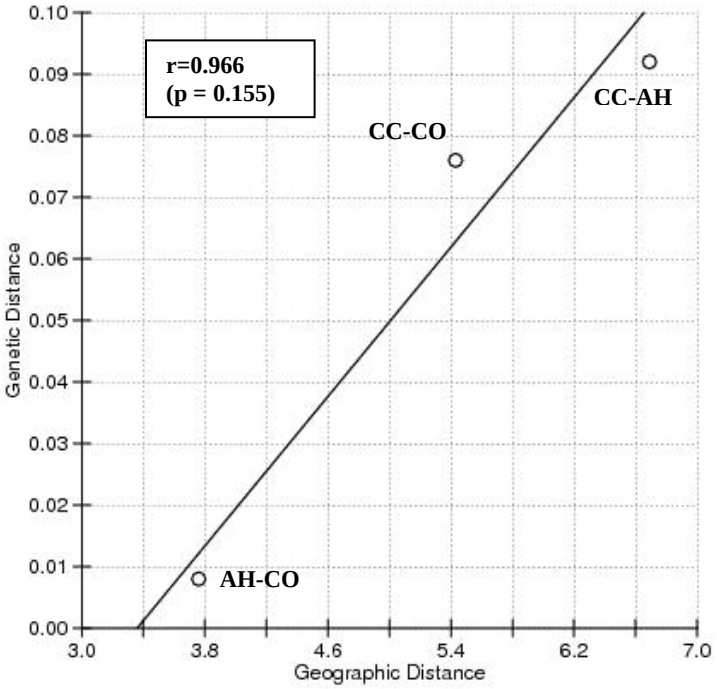

Figure 3-1. Test of isolation by distance using Euclidean distances (AH=Arroyo Hondo, CO=Camp Ohlone, CC=Calaveras Creek Confluence).

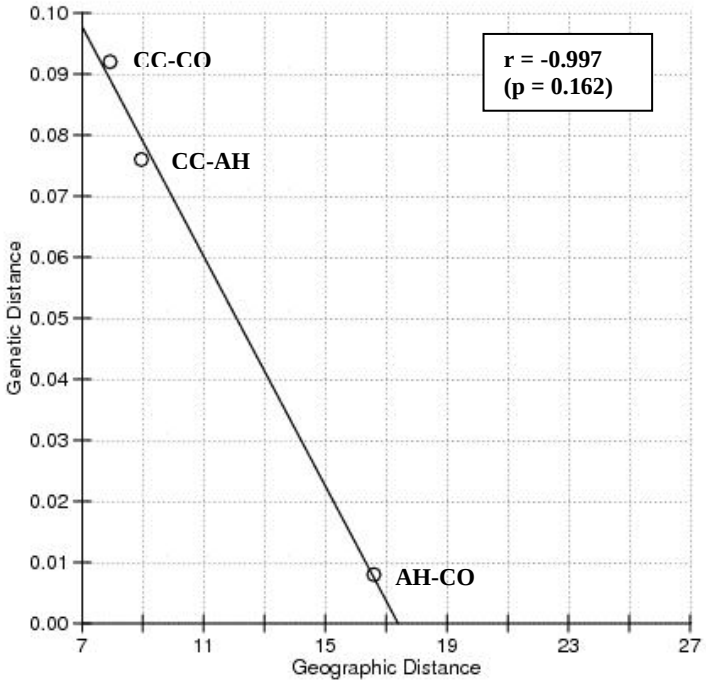

Figure 3-2. Test of isolation by distance using stream network distances (km) (AH=Arroyo Hondo, CO=Camp Ohlone, CC=Calaveras Creek Confluence).

4 DISCUSSION

Calaveras Reservoir and the associated dam and diversion facilities have altered the landscape in the Alameda Creek watershed for over 85 years. For *R. boylii* in Arroyo Hondo, both geographic and anthropogenic factors may limit genetic connectivity with adjacent populations at Calaveras Creek Confluence and Arroyo Hondo. Based on RAPD results, genetic diversity was lowest in the Arroyo Hondo population compared with the Calaveras Creek Confluence and Camp Ohlone. Despite the small sample size (n=6) for the Calaveras Creek Confluence site, genetic diversity was higher than site Arroyo Hondo, which may indicate less genetic connectivity between Arroyo Hondo and adjacent populations. Genetic assessments of *R. boylii* at the Calaveras Creek Confluence site should be treated cautiously because of the small sample size, which may not include rare haplotypes, and therefore could underestimate diversity and gene flow.

The mtDNA data are limited due to small sample sizes, and ultimately would require additional samples for a more robust test of gene flow in the watershed and comparison with RAPDs. Based on 609 bp for 28 individuals in the southern Alameda Creek watershed, only one polymorphism was identified, compared with a 511 bp ND2 sequence for 51 individuals from the Eel River, CA, that identified nine polymorphisms and a haplotype diversity of 0.824 (Dever 2007). Lind et al. (2011) sequenced 1525 bp ND2 fragments for 77 individuals and found that haplotype diversity ranged from 0.679 to 0.914 based on hydrologic region, and samples from the San Francisco Bay (inclusive of Alameda Creek watershed) had a haplotype diversity of 0.700. Peek (2010) sequenced 583 bp for 62 individuals in regulated and unregulated rivers in the Sierra Nevada and identified 20 haplotypes and a haplotype diversity of 0.625.

Geographic distances between sites were not significant indicators of genetic isolation using RAPDs data. Dever (2007) observed significant genetic divergence in *R. boylii* populations separated by more than 9 km in the Eel River. In addition, Bourque (2008) observed that *R. boylii* remained within 12 meters of the stream channel and the greatest distance moved was approximately 7 km (by a female), indicating a preference for movement within stream corridors. All of the study sites in the Alameda Creek watershed were separated by less than 9 km using Euclidean distances, and isolation by distance was not significant. However, stream network distance was 16.6 km between Arroyo Hondo and Camp Ohlone, and yet isolation by distance was not significant; in fact coancestry identity and F_{ST} values were lowest for this pair of sites and highest in the sites separated by much smaller geographic distances. Euclidean distance between Camp Ohlone at Camp Ohlone and Arroyo Hondo was only 3.8 km, although this distance spans across a ridge that separates the watersheds. Based on genetic distance data, patterns of *R. boylii* connectivity between the study sites do not support previous movement observations. Although radio telemetry data and genetic data cover different time scales, dispersal is determined by successful movements (by way of introduction of new alleles) among populations, and generation times for *R. boylii* are short enough that RAPDs would be expected to show recent isolation events.

In addition to significant results showing high levels of connectivity and low divergence between Arroyo Hondo and Camp Ohlone (based on coancestry identity and F_{ST}), Fu's F_S statistical test of selective neutrality was significant for both Arroyo Hondo and Camp Ohlone, and values were negative. Fu's F_S statistic is very sensitive to population demographic expansion or contraction, and generally large negative values for F_S indicate an excess number of alleles in the population. Based on RAPD results, this would imply recent expansions or contractions. Whether potential expansion is a result of recent connectivity between Arroyo Hondo and Camp Ohlone (through the diversion tunnel, over the ridge, or successful migration of individuals from farther upstream

in the watershed), or because populations have declined due to isolation is unclear. A combination of factors may be plausible, and without further detailed information from mtDNA or more detailed markers such as microsatellites, it is not possible to determine at this time.

When comparing genetic divergence between the Calaveras Creek Confluence and Arroyo Hondo, the combination of lower genetic diversities, high F_{ST} values, and coancestry identities indicate the presence of significant barriers to gene flow. The relationship between geographic distance and genetic distance was not significant; therefore landscape distance is not attributable to the genetic divergences observed in RAPDs between these populations. A combination of flow regulation, a large dam and reservoir, and the presence of invasive predators of *R. boylii* all lend support to anthropogenic alteration limiting genetic connectivity between these populations. The pattern of larger F_{ST} values (greater than 0.1) indicate that genetic connectivity was also limited between the Calaveras Creek Confluence site and Camp Ohlone, despite the fact these sites occur within the same drainage. In addition, potential dispersal in the downstream direction (towards the Calaveras Creek Confluence site) from Camp Ohlone seems probable, and well within the range of *R. boylii* dispersal ability, although Twitty et al. (1967) observed newly metamorphosed *R. boylii* moving in an upstream direction. Dispersal between Camp Ohlone and the Calaveras Creek Confluence may be limited because of the intermittent nature of the stream in this reach. Bourque (2008) found that recently metamorphosed frogs (young-of-the-year) moved with the first fall rains; however, during the early fall in Alameda Creek there are long dry stretches upstream of the diversion. Therefore, connectivity between these two sites may be limited during dry periods, as the channel is probably not an effective corridor when dry (see Gonsolin 2010). Based on F_{ST} estimates and coancestry identities, there is significant genetic structuring occurring between the Calaveras Creek Confluence site and Camp Ohlone, as well as between the Calaveras Creek Confluence and Arroyo Hondo (separated by a dam and reservoir). Geographic distance was not significantly correlated with genetic distances, and distances between Calaveras Creek Confluence and Camp Ohlone are comparable to distances between the Calaveras Creek Confluence site and Arroyo Hondo.

There are several factors that may limit *R. boylii* dispersal between Camp Ohlone and the Calaveras Creek Confluence on Alameda Creek, including the presence of invasive bullfrogs, flow regulation at the confluence, and the presence of a large diversion dam and tunnel on Alameda Creek midway between the sites. Kupferberg (1997) found that larval competition with introduced American bullfrogs caused a reduction in *R. boylii* size at metamorphosis, which could reduce the survivorship of young-of-year and reduce overall population fecundity. In particular, the diversion tunnel on Alameda Creek may be a critical determinant of *R. boylii* connectivity within the watershed, not only as a barrier that frogs must circumvent when moving along Alameda Creek, but also providing a separate movement pathway out of Alameda Creek and into Calaveras Reservoir and Arroyo Hondo. This is potentially more probable for individuals dispersing in a downstream direction (towards Calaveras Creek Confluence from Camp Ohlone). Results for the genetic distance F_{ST} between site Camp Ohlone and Arroyo Hondo provide interesting information regarding the research question of whether or not Calaveras Reservoir and Dam limit genetic connectivity. Although genetic diversity was lowest at the Arroyo Hondo site, the F_{ST} value was the smallest between Camp Ohlone and Arroyo Hondo, which indicates these two populations were most similar and not genetically isolated based on current data.

Two possible explanations for this lack of genetic divergence include successful dispersal events occurring between the two populations via a fairly short distance (less than 4 km) directly over the ridge separating Arroyo Hondo and Camp Ohlone, or potential successful dispersal via the diversion tunnel downstream of the Camp Ohlone site. For the former, individual frogs could travel along small ephemeral and intermittent drainages for much of the distance between Arroyo

Hondo and Camp Ohlone before making a short (less than 1 km) traverse over the ridge to descend into the adjacent canyon. Measurements between upstream (headwater) endpoints of small ephemeral and intermittent drainages occurring on the west slope of Alameda Creek and the east slope of Arroyo Hondo ranged from 0.5 km to 1.5 km, with an average of approximately 1 km. Other ranids have been documented moving overland between adjacent habitats; *R. cascadae* have been observed successfully moving over a kilometer through rugged mountainous terrain (with slopes >70 degrees), often at night and across dry land, and *R. muscosa* have also been documented moving overland, although movement varied seasonally and by sex (Garwood and Welsh Jr. 2007; Pope and Matthews 2001). Little is known about *R. boylii* migration and dispersal behavior. Gonsolin (2010) observed movements over 2 km by individuals in Coyote Creek, and these movements were greatly restricted by channels drying early in the summer.

Dispersal overland may occur as frequently as dispersal along stream corridors, but it is difficult to extrapolate from telemetry data collected in other regions. Movement depends largely on local conditions and habitat characteristics, particularly in a species adapted to stochastic environments such as *R. boylii*.

It is also probable that frogs may move downstream from the Camp Ohlone site, before reaching the diversion tunnel and diverting into Calaveras Reservoir. The tunnel is approximately 3 km long and remains open year-round. Post-metamorphic frogs would likely be dispersing in late summer or early fall, which correlates with the drier periods (and greater discontinuity of wetted upslope stream corridors) during which little or no water may be diverted from Alameda Creek into Calaveras Reservoir. In addition, the tunnel is likely cool and moist relative to the surrounding dry channel, and may provide suitable refugia for frogs. The distance from the outlet of the tunnel in the reservoir to the nearest egg mass location in Arroyo Hondo was approximately 3 km, and therefore the total distance for frogs moving from Camp Ohlone to the Arroyo Hondo site via the diversion tunnel would be approximately 9 km. It is unknown if a single individual *R. boylii* would successfully move this distance, but *R. boylii* has been observed moving distances greater than 6–7 km (Bourque 2008).

5 CONCLUSIONS

Because *R. boylii* have been shown to generally remain within and near stream channels, populations in Arroyo Hondo and Camp Ohlone were more genetically similar than would be expected based on landscape structure and stream network distances. Connectivity between these two populations cannot preclude dispersal overland or via hydroregulation structures (diversion tunnel and reservoir), neither of which have been documented previously for this species using traditional movement or mark-recapture studies.

The mtDNA data are limited, and ultimately would require additional samples for a more robust test of gene flow in the watershed. From the observed data, limited haplotype diversity suggests that this is a small, fragmented population. However, RAPDs illustrate more recent patterns of genetic drift, and genetic diversity was the lowest in Arroyo Hondo, indicating stronger influences of isolation on *R. boylii* in Arroyo Hondo. Preserving natural processes that maintain genetic diversity and gene flow should be a primary focus for conservation management, as genetic diversity is a major factor in the process leading to species survival or extinction, particularly in the face of climate warming.

Isolation by distance in natural populations is expected, but genetic drift is not expected to occur among populations from nearby geographic regions unless gene flow is limited. In addition, small population sizes are at greater risk of genetic drift, particularly if connectivity between subpopulations is restricted. Although the sample size for the Calaveras Creek Confluence site was small, F_{ST} estimates between the Calaveras Creek Confluence and Camp Ohlone, as well as Calaveras Creek Confluence and Arroyo Hondo, showed significant divergence, despite similar geographic distances between each of these site pairs. Physical structures associated with river regulation such as dams, reservoirs, and powerhouses are generally permanent landscape components. Although *R. boylii* may be fairly motile aquatic vertebrates (see Bourque 2008, Gonsolin 2010), results from this study show that genetic connectivity is limited among these three sites.

Long-term conservation goals must focus on restoring and enhancing genetic connectivity within *R. boylii* populations, in addition to limiting flow fluctuations during the breeding and rearing periods in regulated rivers. Flow regulation will continue given California's growing population and the need for renewable energy, drinking water, and agricultural irrigation. Therefore, preserving genetic diversity within *R. boylii* populations is paramount to conservation efforts for this species. Protection of the river environments that *R. boylii* inhabit alone will not be sufficient for long-term sustainability of populations. Conservation must also include managing entire river networks to preserve genetic diversity and promote gene flow.

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