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Fine-Scale Genetic Structure in the Threatened Foothill Yellow-Legged Frog (*Rana boylii*)

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ABSTRACT.—**The Foothill Yellow-Legged Frog (*Rana boylii*) is a threatened river-dwelling amphibian endemic to California and Oregon. Determining the genetic structure of populations that have not yet declined is an important tool for their conservation. In this study, molecular markers were used to asses the genetic structure of *R. boylii*. The ND2 region of the mitochondrial genome and Random Amplified Polymorphic DNA (RAPD) markers were examined amongst 51 individuals collected from seven relatively pristine tributaries branching off the Eel River in Northern California. Both markers exhibited significant genetic differentiation among tributaries; however, the RAPD markers revealed a positive correlation between geographic distance and genetic distance. Cluster analysis illustrated a distinct separation between northern and southern tributaries within the study site. In contrast, relatively little geographic structure was apparent when mtDNA haplotypes were examined. Discordance may be caused by the number of loci examined in the mitochondrial and nuclear genomes, recent divergence, and sex-biased dispersal.**

The Foothill Yellow-Legged Frog (*Rana boylii*), endemic to the foothill and mountain streams of California and Oregon, was once highly abundant (Fitch, 1936). However, *R. boylii* populations have declined precipitously in the last 30 years (M. R. Jennings and M. P. Hayes, Amphibian and Reptile Species of Special Concern in California, A Report to the California Department of Fish and Game, Rancho Cordova, CA, 1994; Lind, 2004) to the point that it is now listed as a California State Species of Special Concern. Unfortunately, from a conservation management perspective, very little is known about this species (M. R. Jennings and M. P. Hayes, Amphibian and Reptile Species of Special Concern in California, A Report to the California Department of Fish and Game, Rancho Cordova, CA, 1994). However, this frog has been shown to be unlike other members of the *Rana* species group, which are ordinarily found in ponds, lakes, and marshes. By contrast, *R. boylii* is a riparian species restricted to creeks and rivers (Zweifel, 1955; Stebbins, 2003). Although dispersal patterns are undocumented for this species (M. R. Jennings and M. P. Hayes, Amphibian and Reptile Species of Special Concern in California, A Report to the California Department of Fish and Game, Rancho Cordova, CA, 1994), it has been suggested that adults may spend the majority of their time in the smaller tributaries and migrate to the confluence of larger rivers during the breeding season (Twitty et al., 1967; Kupferberg, 1996). Because their egg masses and tadpoles have not been observed in the smaller tributaries, combined with evidence of breeding at the confluences of tributaries and rivers (Kupferberg, 1996), it was predicted that breeding *R. boylii* return to a particular confluence, resulting in a genetically heterogenous population.

In this study, mitochondrial and nuclear genetic data were examined from a population that is relatively free of anthropogenic impacts, unlike the majority of populations that are limited to only a few regions and considered under ''concern'' or ''threat'' of extinction (M. R. Jennings and M. P. Hayes, Amphibian and Reptile Species of Special Concern in California, A Report to the California Department of Fish and Game, Rancho Cordova, CA, 1994; Lind, 2004). The findings demonstrate that tributaries are important confluences for the preservation of genetic diversity in this species and that geographic distance may affect gene flow. These factors reveal implications for both conservation management of the species and for understanding evolutionary processes such as genetic drift. The detection of the amount of genetic variation within and among natural populations is vital to an understanding of evolutionary processes (Moritz, 2002); in turn, broadening our understanding of these processes is an integral part of any successful conservation management program, as such information suggests methods of conservation specific to the species. With the connection between evolutionary processes and management programs for species of special concern in mind, this study is the first attempt to document the fine-scale genetic structure of *R. boylii*.

MATERIALS AND METHODS

During the summer of 2004, *R. boylii* were sampled from seven different tributaries: Corbin (CO), Horse (HO), Rattlesnake (RS), Skeleton (SK), Berry (BE), Hummingbird (HU), and Thistleglade (TG) Creeks along a section of the Eel River in Mendocino National Forest (Fig. 1). Distances between tributaries were calculated by directly measuring the route of the Eel River on a 1:24,000 topographic map. Frogs were hand captured, and sample tissue was obtained by clipping the end of the fourth toe on the left foot (as described in the USGS toe-clipping Standard Operating Procedure, 2003). The tissue was placed immediately in 70% ethanol in a cryogenic tube and DNA was extracted from the tissue.

For this study, DNA from 51 individuals was examined using two molecular markers: the NADH Dehydrogenase subunit 2 gene (ND2) from the mitochondrial genome (mtDNA) and randomly amplified polymorphic DNA (RAPD). The ND2 region

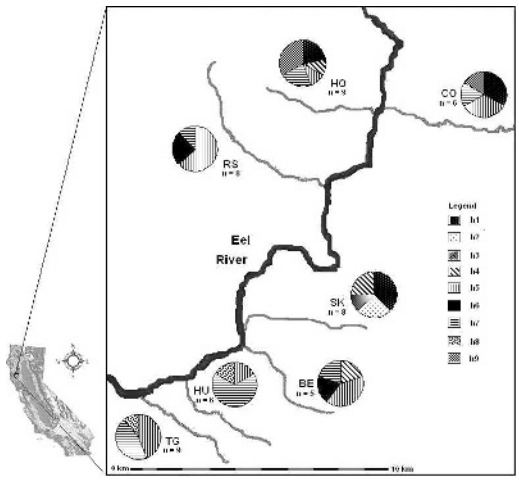

FIG. 1. Location of study tributaries branching off Eel River in Northern California. The distribution and frequency of the nine haplotypes found for the mitochondrial DNA locus ND2 for *Rana boylii* from tributaries is indicated as pie charts.

was amplified using PCR with primers tRNAmet_F and tRNAtrp_R (Lind, 2004). PCR products were purified and using the tRNAmet_F primer, automated sequencing was performed at the UCSF Biomolecular Resources Center and the Davis Sequencing Center on ABI 7900HT and ABI3730 instruments. Sequences were aligned using SEQUENCHER™ 4.1. DnaSP 4.01 (Rozas et al., 2003) was used to obtain simple descriptive statistics, calculate Nei's (1987) haplotype diversity (*h*), and test neutrality using Tajima's D (Tajima, 1989). To determine intertributary variation, pairwise genetic distances were computed using Nei's genetic distance, G_{ST} (Nei, 1973), which, similar to Wright's *F*-statistic, F_{ST}, is a measure of differentiation among subpopulations (Wright, 1943). The level of dispersal and gene flow between subpopulations was estimated using a variance-partitioning algorithm (AMOVA, Excoffier et al., 1992) calculated in ARLEQUIN (S. Schneider, D. Roessli, and L. Excoffier, vers. 2.0., A software for population genetic data analysis. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland, 2000). Genetic and geographic distance matrices were compared for correlations using a Mantel test (Mantel, 1967) with 10,000 randomizations to test for isolation-by-distance using the IBD program web version 2.6 (J. L. Jensen, A. J. Bohonak, and S. T. Kelley, Isolation by distance, web service. BMC Genetics 6:13, http://phage.sdsu.edu/~jensen/, 2005).

In addition to mtDNA, RAPD (Williams et al., 1990) was also used because it enables several nuclear biparentally inherited loci to be analyzed (Hadrys et al., 1992). This method is a relatively inexpensive PCR-based technique that generates a DNA banding profile for each individual, and has been used for a variety of species (Mockford et al., 1999; Jaeger et al., 2001; Zeisset and Beebee, 2003; Wagner et al., 2005). For this study, the Ready-To-Go™ RAPD Analysis Beads Kit (Biosciences) was used as it includes all the necessary PCR components in a dried bead optimized for RAPD amplification. Using the kit procedure, PCR was conducted with primers #2, #4, and #5 to produce identifiable polymorphic bands that were scored as present (1) or absent (0). Reproducibility was tested on three random DNA samples by repeating PCR reactions three times and directly comparing profiles.

Lynch and Milligan's (1994) allele frequency estimate for RAPDs was calculated using the TFPGA computer program (M. P. Miller, Tools for Population Genetic Analyses [TFPGA], http://www.marksgeneticsoftware.net/, 1998). Genetic differentiation was then calculated using θ (Weir and Cockerham, 1984) with a bootstrap procedure consisting of 1000 replicates over loci to determine 95% confidence limits. Pairwise genetic distances between subpopulations were estimated using Reynolds et al. (1983) coancestry distance. Using ARLEQUIN both overall and between F_{ST} subdivision estimates were calculated (Weir and Cockerham, 1984). Additionally, F_{ST} estimates between two subgroups, northern region tributaries (CO, HO, and RS) and southern region tributaries (SK, BE, HU, and TG) were calculated.

Coancestry genetic distances were used for the Mantel test and isolation-by-distance as described in the mtDNA analyses section. Additionally, isolation-by-distance within the northern and southern region tributaries was tested using the binary indicator variable pairwise comparison option in the IBD program (0 for within the northern region or within the southern region comparison and 1 for between the northern region and southern region comparison). A UPGMA analysis (unweighted pair-group method using an arithmetic average; Tamura, 1992) was conducted using both coancestry distances (Reynolds et al., 1983) and Nei's distances (Nei, 1972). From these distances, dendrograms were constructed with a bootstrap procedure over loci based on 1000 replicates using TFPGA.

RESULTS

A total of 511 bp of the ND2 gene was sequenced from 51 individuals revealing nine mitochondrial haplotypes (Table 1). Unique haplotype sequences were deposited in GenBank (accession numbers DQ088653–DQ088661, DQ973814). Among the nine polymorphic sites observed, two were singleton variable sites, and seven were parsimony informative sites. For the overall diversity of individuals, an *h* estimate of 0.824 was calculated. Two nucleotide substitutions were observed at the first codon position, one at the second and six at the third, producing three aminoacid replacements. A Tajima's *D*-statistic was not significantly different from 0 ($D = -0.694$, $P > 0.1$), suggesting that this variation was neutral. The genetic variation within tributaries accounted for 90.82% of the total variation. There were three tributary-specific haplotypes—(h2 and h3) for SK, (h8) for TG—yet no haplotypes were shared by all tributaries. Furthermore, two haplotypes were found in over half (55%) of all individuals across tributaries (frequencies shown in Fig. 1).

High levels of intratributary diversity were exhibited by the RAPD profiles with the three primers generating 93 polymorphic bands. The phenotypic

TABLE 1. Variable positions in the 511 bp segment of the NADH 2 gene defining nine different haplotypes and their distributions across *Rana boylii* collected from seven different tributaries of the Eel River.

Haplotypes	Nucleotide position									Locality						
				3	*4*	*4*	4	5	5							
	1	5	9	7	*1*	*5*	8	0	0							
	2	9	8	7	*4*	*2*	8	2	3	CO	HO	RS	SK	BE	HU	TG
h1	C	C	C	A	A	G	G	G	G	2	2	-	3	-	-	-
h2	.	.	.	G	.	.	A	.	A	-	-	-	2	-	-	-
h3	.	.	.	.	.	.	.	A	A	-	-	-	1	-	-	-
h4	T	.	.	.	.	A	.	.	A	-	1	-	2	1	1	-
h5	.	T	.	.	.	.	.	.	A	2	1	5	-	2	1	4
h6	.	.	T	.	.	.	.	.	A	-	-	2	-	1	-	-
h7	.	.	.	.	.	.	.	.	A	1	2	1	-	1	4	4
h8	.	T	.	.	G	.	.	.	A	-	-	-	-	-	-	1
h9	.	.	.	.	.	A	.	.	A	1	3	-	-	-	-	-

Dots indicate the same nucleotide is present as in haplotype 1.
Italic characters indicate amino acid replacement substitutions.

band frequencies of loci ranged from 0.0132–0.4114. No tributary-specific bands were found.

Subpopulations along the Eel River exhibited mtDNA differentiation (G_{ST} = 0.084, $P < 0.018$). By comparison, the RAPD analyses of differentiation exhibited a slightly higher degree among tributaries. An average θ-value of 0.101 (upper confidence limit = 0.145, lower confidence limit = 0.062) was observed. This value was similar to Weir and Cockerham's overall estimate of F_{ST} obtained using AMOVA (F_{ST} = 0.123, $P < 0$).

Results from the RAPD Mantel test reflected a positive correlation between the geographic distance and genetic distance (Z = 2.73, r = 0.68, $P < 0.015$; Fig. 2A). UPGMA analyses conducted on both the coancestry distance and Nei's distance matrices resulted in dendrograms with the same topology (Fig. 3), which indicated a separation between the northern and southern regions. The RAPD pairwise comparison of the two regions also revealed a significant difference (F_{ST} = 0.195, $P < 0$) between the two. To determine whether the isolation by distance pattern was evident within and between the northern and southern region tributaries, a second Mantel test was performed using a third indicator pairwise comparison. The results of this test were significant (r = 0.677, $P < 0.0182$) for a partial correlation between genetic distance and geographic distance within each subgroup.

Conversely, the mtDNA sequence analyses did not support such a pattern. The Mantel test statistic (Z = 0.541, r = −0.052, $P < 0.5$) was not significantly different from 0, and a graph plotting mtDNA genetic distance versus log geographical distance indicated a lack of correlation between these two factors (Fig. 2B). When the northern and southern regions were compared, absolutely no differentiation was observed (F_{ST} = 0, $P < 0$).

DISCUSSION

Like so many other amphibians, *R. boylii* are experiencing a significant reduction in their range, yet little information exists in regards to their basic biology (Lind, 2004; Stuart et al., 2004). In this study, both mtDNA and RAPD markers suggest that *R. boylii* inhabiting this region comprise a genetically diverse population. The number of mtDNA haplotypes observed on this small geographic scale, accompanied by the high amount of within-population molecular variance for both markers, implies the presence of a large population.

Because these frogs are strictly associated with riparian corridors and have been observed breeding at the confluence of tributaries the hypothesis was that their behavior would lead to the genetic subdivision of the population, with subgroups clustering around specific tributaries. The RAPD pairwise comparison supported this hypothesis, results of both Mantel tests suggested an isolation-by-distance pattern (Fig. 2A). UPGMA analyses of pairwise genetic distances from RAPD data among tributaries produced a topology indicative of geographic clustering with a distinct north-south division of subpopulations along the Eel River (Fig. 3). Although no physical barriers exist between the two subgroups the two regions are separated by a distance of 10.45 km. In a study of the cascades frog (*R. cascadae*), reduced gene flow was observed between populations separated by a distance greater than 10 km (Monsen and Blouin, 2003); indicating the physical distance between the north-south subgroups in this study may be prohibitive to high levels of gene flow.

The pattern of structuring described here was not exhibited by the mitochondrial sequence data. Instead, lower levels of overall among-tributary differentiation were exhibited, the Mantel test was unsubstantiated (Fig. 2B), and no difference was observed between the north-south subgroups, suggesting that gene flow between the two regions was high enough to offset genetic drift. Small sample size could be a factor, because additional sampling could produce rare haplotypes that may change the level of differentiation and pattern exhibited. However, it is likely that the discrepancy between the two markers may be caused by the following distinctions regarding RAPD markers. First, RAPDs have a higher mutation rate, and thus, the more variable RAPD markers compared to the ND2 gene reflect a more recent

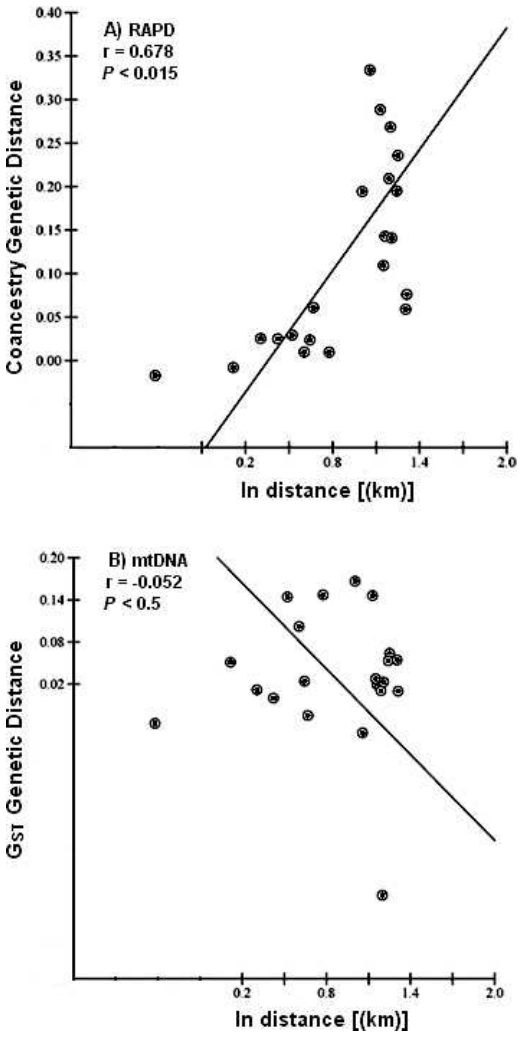

FIG. 2. The influence of isolation by geographic distance log-transformed (km) on genetic distance (A) from Reynolds coancestry in RAPD variation and (B) from mitochondrial DNA genetic distance from Nei's G_{ST}. Significant relationships indicated by trend line (solid line) based on 10,000 replicates.

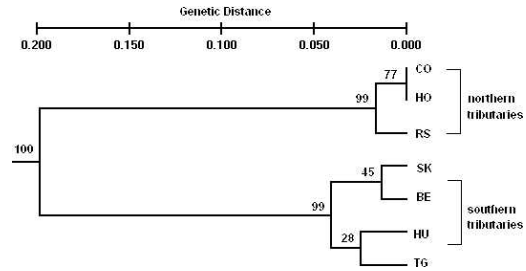

FIG. 3. UPGMA dendrogram produced from Reynolds et al., (1983) coancestry distance statistics for RAPD allele frequencies measured from 93 loci between all possible pairs from seven subpopulations along the Eel River. Numbers on branches reflect the percent of 1000 bootstrap replicates that supported the given topology.

pattern of genetic structure (Vandewoestijne and Baguette, 2002). Second, RAPDs provide an increased number of loci, and it has been noted that the ability to detect microdifferentiation between genetically similar subpopulations may be augmented by examining loci with a high number of alleles (Nei, 1973). Third, the RAPD technique provides a greater ability to screen a wider region of the genome. All these distinctions may result in RAPD yielding higher resolution of more recent genetic structure and greater detection of genetic drift (Vandewoestijne and Baguette, 2002). Alternately, one cannot discount the possibility of female-biased dispersal acting in this species based on the distinct mode of inheritance for the two types of markers, (mtDNA is maternally inherited and RAPD is biparentally inherited). If female dispersal is greater than male dispersal, nuclear markers (i.e., RAPDs) would potentially become fixed via genetic drift at a faster rate than mtDNA markers (i.e., ND2) because mtDNA is maternally inherited and haploid (Ballard and Whitlock, 2004).

This particular region of the Eel River can be considered relatively pristine, lacking any immediate source of anthropogenic contamination from either agricultural or urban runoff. Nor are there any dams spanning the study site that would result in habitat degradation and act as a barrier to dispersal. One could hypothesize that for this reason, the region supports a relatively large population with a high level of genetic diversity: Nine ND2 haplotypes were observed with a within-locality variation of 91%. This is not the case for the vast majority of *R. boylii*'s habitat and likely why in their historic range numbers appear to be declining rapidly (M. R. Jennings and M. P. Hayes, Amphibian and Reptile Species of Special Concern in California, A Report to the California Department of Fish and Game, Rancho Cordova, CA, 1994; Kupferberg, 1996; Lind and Wilson, 1996). In a previous study, Lind (2004) reported mtDNA within-locality variation of this species over its historical range (which included six distinct hydrologic regions) from 40–73%, values much lower than was observed for the *R. boylii* in the Eel River tributaries of this study.

Implications of these observations on management strategies for *R. boylii* are that tributaries are important confluences for the preservation of genetic diversity and populations separated by more than 10 km may be subject to the effects of genetic drift. Either physical barriers to dispersal (dams) or habitat fragmentation will presumably result in a reduction of genetic diversity within populations and perhaps the fixation of deleterious alleles. Thus, the overall connectivity between subpopulations must be considered for any successful management planning rather than simply focusing upon just the river or specific tributary. Conservation management is hampered, however, by the low level of information on this species. In particular, comparative studies of riverine watersheds on the same local scale and the testing of female-

biased dispersal through the study of additional nuclear markers are warranted.

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Literature Cited

Ballard, J. W., and M. C. Whitlock. 2004. The incomplete natural history of mitochondria. Molecular Ecology 13:729–744.

Cockerham, C. C. 1973. Analysis of gene frequencies. Genetics 74:679–700.

Excoffier, L., P. E. Smouse, and J. M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction sites. Genetics 131:479–491.

Fitch, H. S. 1936. Amphibians and reptiles of the Rogue River Basin, Oregon. American Midland Naturalist 17:634–652.

Hadrys, H., M. Balick, and B. Schierwater. 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Molecular Ecology 1:55–63.

Jaeger, J. R., Riddle, B. R., Jennings, R. D., and D. F. Bradford. 2001. Rediscovering *Rana onca*: evidence for phylogenetically distinct Leopard Frogs from the border region of Nevada, Utah, and Arizona. Copeia 2001:339–354.

Kupferberg, S. J. 1996. Hydrologic and geomorphic factors affecting conservation of a River-Breeding Frog (*Rana boylii*). Ecological Applications 6: 1332–1344.

Lind, A. J. 2004. Reintroduction of a Declining Amphibian: Determining an Ecologically Feasible Approach for the Foothill Yellow Legged Frog (*Rana boylii*). Unpbubl. Ph.D. diss., University of California, Davis.

Lind, A. J., and R. A. Wilson. 1996. The effects of a dam on breeding habitat and egg survival of the Foothill Yellow-Legged Frog (*Rana boylii*) in Northwestern California. Herpetological Review 27:62–67.

Lynch, M., and B. G. Milligan. 1994. Analysis of population genetic structure with RAPD markers. Molecular Ecology 3:91–99.

Mantel, N. 1967. The detection of disease clustering and generalized regression approach. Cancer Research 27:209–220.

Mockford, S. W., M. Snyder, and T. B. Herman. 1999. A preliminary examination of genetic variation in a peripheral population of Blanding's Turtle, *Emydoidea blandingii*. Molecular Ecology 8:323–327.

Monsen, K. J., and M. S. Blouin. 2003. Genetic structure in a montane ranid frog: restricted gene flow and nuclear-mitochondrial discordance. Molecular Ecology 12:3275–3286.

Moritz, C. 2002. Strategies to protect biological diversity and the evolutionary processes that sustain it. Systematic Biology 51:238–254.

Nei, M. 1972. Genetic distance between populations. American Naturalist 106:283–292.

———. 1973. Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Science 70:3321–3323.

———. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Reynolds, J., B. S. Weir, and C. C. Cockerham. 1983. Estimation of the coancestry coefficient: basis for a short-term genetic distance. Genetics 105: 767–779.

Rozas, J. J. C., Sanchez-Delbarrio, X. Messeguer, and R. Rozas. 2003. DnaSP, DNA polymorphism analices by the coalescent and other methods. Bioinformatics 19:2496–2497.

Stebbins, R. C. 2003. A Field Guide to Western Reptiles and Amphibians. 3rd ed. Houghton Mifflin Co., Boston, MA.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786.

Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595.

Tamura, K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. Molecular Biology and Evolution 9:678–687.

Twitty, V. C., D. Grant, and O. Anderson. 1967. Amphibian orientation: an unexpected observation. Science 155:352–353.

Vandewoestijne, S., and M. Baguette. 2002. Genetic structure of endangered populations in the butterfly *Boloria aquilonaris*: different evolution of allozymes and RAPDs depict population structure at two different time scales. Heredity 89:436–445.

Wagner, R. S., M. P. Miller, C. M. Crisafulli, and S. M. Haig. 2005. Geographic variation, genetic structure, and conservation unit designation in the Larch Mountain Salamander (*Plethodon larselli*). Canadian Journal of Zoology 83:396–406.

Weir, B. S., and C. C. Cockerham. 1984. Estimating *F*-statistics for the analysis of population structure. Evolution 38:1358–1370.

Williams, J. G., A. R. Kubelik, J. Livak, J. A. Rafalski, and S. V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18:6531–6535.

Wilson, A. J., J. A. Hutchings, and M. M. Ferguson. 2004. Dispersal in a stream dwelling salmonid: inferences from tagging and microsatellite studies. Conservation Genetics 5:25–37.

WRIGHT, S. 1943. Isolation by distance. Genetics 28:114–138.

ZEISSET, I., AND T. J. C. BEEBEE. 2003. Population genetics of a successful invader: the Marsh Frog *Rana ridibunda* in Britain. Molecular Ecology 12:639–646.

ZWEIFEL, R. G. 1955. Ecology, distribution and systematics of frogs of the *Rana boylii* group. University of California Publications in Zoology 54:207–292.

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