



Extreme isolation by distance in a montane frog *Rana cascadae*

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Abstract

Given the recent interest in declining amphibian populations, it is surprising that there are so few data on genetic drift and gene flow in anuran species. We used seven microsatellite loci to investigate genetic structure and diversity at both large and small geographic scales, and to estimate gene flow in the Cascades frog, *Rana cascadae*. We sampled 18 sites in a hierarchical design (inter-population distances ranging from 1–670 km) to test for isolation by distance and to determine the geographic scale over which substantial gene flow occurs. Eleven of these sites were sampled as three fine-scale clusters of three, three, and five sites separated by pairwise distances of 1–23 km to estimate number of migrants exchanged per generation via F_{ST} and by a coalescent approach. We found *R. cascadae* exhibits a strong pattern of isolation by distance over the entire species range, and that there is a sharp drop in migrants exchanged between sites separated by greater than 10 km. These data, in conjunction with results of other recent studies, suggest that montane habitats promote unusually strong genetic isolation among frog populations. We discuss our results in light of future management and conservation of *R. cascadae*.

Introduction

Assessing the geographic scale over which substantial gene flow occurs is critical for the successful management and conservation of threatened and endangered species. Gene flow patterns can affect genetic structure and diversity, population size, and long-term population survival (Whitlock and McCauley 1999). Understanding current patterns of gene flow between populations is crucial for effective conservation because species survival may depend on dispersal between patches of suitable habitat (Hanski and Gilpin 1991; Driscoll 1997 and references within). This is especially important considering the dramatic increase in habitat fragmentation facing many threatened and endangered species.

Among vertebrates, amphibians generally have low vagility and high genetic differentiation across

small geographic scales (Driscoll 1998a, b; but see Burrowes and Joglar 1999; Storfer 1999; James and Moritz 2000; Shaffer et al. 2000). Additionally, dispersal has been shown to be an important factor in amphibian local population survival by increasing population size or re-establishing populations following extinction (Gill 1978; Gulve 1994).

Given the recent interest in conservation and management of declining amphibian populations, it is surprising that there are so few molecular data on gene flow across a fine geographic scale in anuran species. Most molecular genetic studies on anuran populations were phylogeographic studies (large-scale patterns of subdivision within a species), not population genetic structure studies, and therefore often have low sample sizes per site and widely spaced populations (e.g. Wilkinson et al. 1996; Evans et al. 1997; Macey et al. 1998; Sumida

et al. 1998; James and Moritz 2000; Monsen and Blouin 2003). A hierarchical sampling design that includes populations sampled at different spatial scales is necessary to infer patterns of gene flow and to estimate the geographic scale over which gene flow occurs. Additionally, in order to reasonably estimate the number of migrants exchanged per generation using molecular genetic data, it is necessary to sample more than a few individuals per population. Furthermore, many molecular genetic studies on anurans used eggs, tadpoles, and juvenile animals in addition to or instead of adults (Reh and Seitz 1990; Hitchings and Beebee 1997; Rowe et al. 1998; Shaffer et al. 2000; Newman and Squire 2001). The use of non-adult animals complicates the estimation of allele frequencies because juveniles may be related (Allendorf and Phelps 1981), especially given the small effective number of breeders per generation that may characterize many frog species (Waldman and McKinnon 1993; Scribner et al. 1997). Therefore, there is a need for more studies designed to estimate basic parameters such as migration rates and the geographic scale of gene flow.

Rana cascadae is an anuran endemic to the Pacific Northwest that appears healthy in its Northern range in Oregon and Washington, but has recently experienced severe population declines in the southern part of its range in Northern California (Fellers and Drost 1993). This animal occurs at elevations between 800–2740 m from the Olympic and Cascade Mountains in Washington state to the Klamath- Siskiyou Mountains in Northern California (Figure 1; Stebbins 1985). Although severe population declines have only been documented in Northern California, *R. cascadae* is still a conservation concern owing to its restricted distribution. During a recent phylogeography study of *R. cascadae*, we observed strong genetic differentiation for both mitochondrial and nuclear loci between populations separated by approximately 50 km (the smallest scale at which we sampled; Monsen and Blouin 2003). Therefore, an understanding of the scale at which populations are substantially connected is important for management of the species.

In the current study, we used seven tetranucleotide microsatellite loci from adult *R. cascadae* to investigate isolation by distance at both large and small geographic scales. We then determined the geographic scale over which substantial

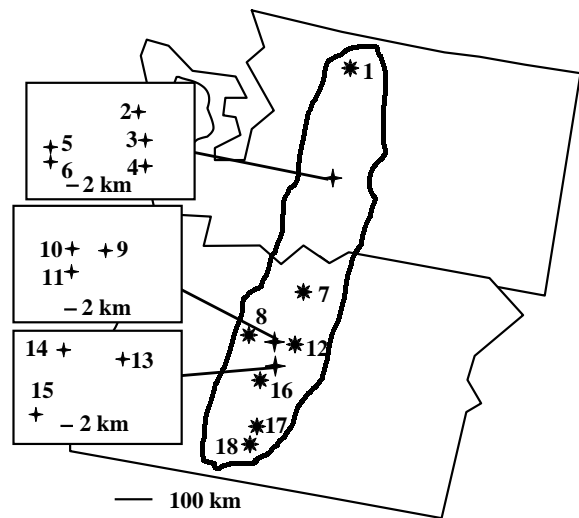


Figure 1. *Rana cascadae* sample sites. Populations marked with + were used for gene flow estimates, and are illustrated as insets on a finer geographic scale. The species range in Washington and Oregon is outlined (R. Nauman and D. Olson pers.com.). See Table 1 for location names and sample sizes.

gene flow occurs by estimating the number of migrants exchanged between populations ($N_e m$) using an F_{ST} -based method as well as a coalescent approach. We discuss the implications of our results for *R. cascadae* conservation.

Methods

Sampling

Tissue was collected from adult *Rana cascadae* by toe clipping during the summers of 1997–1999. Samples were collected from 18 populations throughout Oregon and Washington (Figure 1, Table 1). It is important to note that we refer to a population in this study as a single collection site. It is likely that individual sampling sites do not represent discrete populations, but are in fact part of a larger set of inter-breeding ponds that compose a larger neighborhood. Sample sizes ranged from 13 to 30 individuals per population (Table 1). Eleven of these populations were sampled in three clusters of three, three, and five sites where pairs were separated by a distance of 1–23 km (Figure 1, Table 1). These three “fine-scale” sets were used to estimate the number of

Table 1. *Rana cascadae* sampling site information. Populations marked with * were used in pairwise estimates of the number of migrants exchanged per generation

Population (Abbreviation) ^a	Latitude, Longitude	Average Sample Size ^b
Illabot Creek, WA (1)	48.4402W, -121.3876N	21
Elysian Fields, WA (2)*	46.9435W, -121.7554N	29
Berkeley Park, WA (3)*	46.9131W, -121.6872N	19
Mt. Rainier, WA (4)*	46.9160W, -121.6531N	29
Paradise River, WA (5)*	46.7786W, -121.7368N	19
Reflection Lakes, WA (6)*	46.7680W, -121.7264N	23
Breitenbush, OR (7)	44.7716W, -121.9495N	26
Grassy Mountain, OR (8)	44.2750W, -122.7755N	13
McKenzie Pass, OR (9)*	44.2448W, -121.8414N	28
Benson Lake Trail, OR (10)*	44.2322W, -121.9157N	23
Melakwa Lake, OR (11)*	44.1973W, -121.9089N	15
Todd Lake, OR (12)	44.0250W, -121.6821N	22
Many Lakes Trail, OR (13)*	43.8155W, -121.9068N	22
Waldo Lake, OR (14)*	43.7623W, -122.0131N	27
Gold Lake, OR (15)*	43.6332W, -122.0464N	30
Crystal Springs, OR (16)	43.3123W, -122.1404N	21
Seven-Mile Creek, OR (17)	42.7161W, -122.1278N	13
Big Frank Swamp, OR (18)	42.4422W, -122.2416N	16

^aPopulation numbers correspond to those in Figure 1.

^bSample sizes are averaged across seven microsatellite loci.

migrants exchanged between populations per generation.

Molecular methods

Total genomic DNA was extracted from each toe using a standard phenol–chloroform protocol (Hillis et al. 1996). We used 7 microsatellite loci that were originally developed for *R. pretiosa* and *R. luteiventris* (SFC120, RC287, RP193, SFC134, SFC128, RP17, and SFC139; Table 2; Blouin, unpublished data; Monsen and Blouin 2003). PCR amplifications were carried out in 25 μ l reactions using the following components and conditions: 100–200 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl pH9, 0.1% Triton X-100, 1.5 mM MgCl₂, 0.8 μ M of both the forward (fluorescently-labeled) and reverse primers, 0.2 mM dNTPs, 2.5 units *taq* polymerase, and water to a final volume of 25 μ l. Amplification was carried out in a Perkin–Elmer 9600 thermocycler under the following conditions: 94 °C 3 min, followed by 30 cycles of 94°C 45 s, locus-specific annealing temperature (Table 2) 30 s, 72°C 30 s, and a final extension at 72°C for 7 min. Microsatellite PCR product was run on an

ABI 377 automated sequencer, and allele sizes were scored using the program GENOTYPER v. 2.0 (Applied Biosystems, Inc.).

Data analysis

The program GENEPOP v. 3.2a (Raymond and Rousset 1995) was used to calculate general measures of genetic diversity including number of alleles per locus per population, and observed and expected heterozygosities. Departure from Hardy–Weinberg equilibrium was tested using an exact test based on the procedure described by Guo and Thompson (1992) using a Markov chain method as implemented in GENEPOP v. 3.2a. Sequential Bonferroni corrections were used to account for simultaneous statistical tests (Rice 1989). Pairwise F_{ST} estimates among all populations were made using GENEPOP v. 3.2a after the method of Weir and Cockerham (1984).

Isolation by distance for the entire set of 18 populations was tested using Mantel's test (Mantel 1967) from the comparison of all pairwise $F_{ST}/(1-F_{ST})$ values with pairwise geographic distances using the program ISOLDE in GENEPOP v. 3.2a.

Table 3. Comparison of anuran microsatellite studies.

Species	Reference	Range N	# of Loci	# of Populations	Measures of differentiation	Average # alleles/locus/pop	H_o , H_e Range	Geographic range (km)
<i>Bufo bufo</i>	Scribner et al. (1994)	40–40 (40) ^a	1	3	$F_{IS} = -0.07$	6	not reported	5.5–14.5
<i>Rana luteiventris</i>	Call (1997)	8–28 (15) ^a	3	8	$F_{ST} = 0.016$ $\hat{F} = 0.066$	4.4	0.625–0.750 0.00–1.0	– 49–165
<i>Hyla regilla</i>	Call (1997)	15–22 (16) ^a	3	3	$\hat{\phi}_{ST} = 0.056$ $\hat{F} = 0.056$	7.8	0.16–0.91 0.37–0.87	3–34
<i>Bufo calamita</i>	Rowe et al. (1998)	25–40 (39) ^l	8	40 (4 regions)	$\hat{\phi}_{ST} = 0.07$ $F_{ST} = 0.068-0.531$	3.3	0.42–0.8 0.245–0.350	100–300
<i>Rana sylvatica</i>	Newman and Squire (2001)	32–102 (50) ^{a,m}	5	12	$D_N = 0.38-0.807$ $D_C = 0.208-0.652$ $\delta\mu^2 = 0.127-1.752$ $R_{ST} = 0.099-0.568$ $F_{ST} = 0.00-0.03$	1.7	0.16–0.60	0.05–20
<i>Physalaemus pustulosus</i>	Lampert et al. (2003)	24–40 (39) ^a	5	17	$F_{ST} = 0-0.039$	14	0.24–0.73 0.45–0.95	0.26–11.8
<i>Rana luteiventris</i>	Funk et al. (in review)	19–55 (28) ^{a,m,l}	6	28	$F_{ST} = -0.009-0.345$	4	0.617–0.875 not reported	0.1–90
<i>Rana cascadae</i>	Monsen and Blouin (this study)	13–30 (22) ^a	7	18	$F_{ST} = 0.01-0.26$ (1–23 km)	4.5	0.29–0.70 0.45–0.73	1–670
					$F_{ST} = 0.04-0.52$ (26–670 km)		0.45–0.78	

Measures of differentiation: F_{IS} and F_{ST} (Wright 1951); D_N (Nei 1972); \hat{F} (Lynch 1991); $\hat{\phi}_{ST}$ (Michalakis and Excoffier 1996); D_C (Cavalli-Sforza and Edwards 1967); $\delta\mu^2$ (Goldstein et al. 1995); and R_{ST} (Slatkin 1995). H_o – observed heterozygosity, H_e – expected heterozygosity. N is the range of sample sizes used in each study with the average N in parentheses. Superscript lower case letters indicate life stage source of tissue: a – adults; m – metamorphs; l – larvae

geographic scale of 1–23 km F_{ST} was high with pairwise estimates from 0.01 to 0.24.

Isolation by distance

Strong isolation by distance was apparent for the entire set of 18 populations from the graph of $F_{ST} / (1 - F_{ST})$ versus geographic distance (Mantel test P -value < 0.0001, Figure 2). This pattern of isolation by distance was apparent at both small and large geographic scales across the entire 670 km study area (Figure 2).

Estimates of N_m

Populations of *R. cascadae* showed substantial genetic differentiation and low migration at very small geographic distances (Figure 3a; Table 3). For N_m estimated from F_{ST} , there was a marked decrease in migration between populations separated by 10 km or more. Although the coalescent estimates of N_m were more variable, the graph of coalescent N_m versus distance shows the same pattern, with a marked inflection in the number of migrants at approximately 10 km (Figure 3b).

Discussion

Variation within populations

Although there have been relatively few microsatellite studies on anurans, it appears that levels of genetic variation (average heterozygosities and number of alleles per locus per population) at microsatellite loci in anurans are similar to those in

other vertebrates (Newman and Squire 2001). *R. cascadae* populations appear to have levels of variation that are typical for anurans (Table 3).

Differentiation among populations

Although *R. cascadae* has levels of genetic variation within populations typical of anurans, the species shows greater genetic differentiation across small geographic scales than other anurans for which population differentiation at microsatellite loci has been investigated (Table 3). Indeed, F_{ST} estimates at the smallest geographic scale (1–23 km) ranged from 0.01 to 0.26. Moreover, *R. cascadae* populations show clear isolation by distance over the entire study area that constitutes most of the species range (Figure 2) and a striking pattern of reduced gene flow between populations separated by more than 10 km (Figure 3). These estimates of low migration agree with previous research on migration distances in anurans that suggest adult frogs rarely move distances more than a few km (Dole 1971; Breden 1987, 1988; Berven and Grudzien 1990; Gulve 1994). Therefore, gene flow is most likely occurring in a stepping stone fashion where nearest neighbors exchange migrants in a set of closely spaced

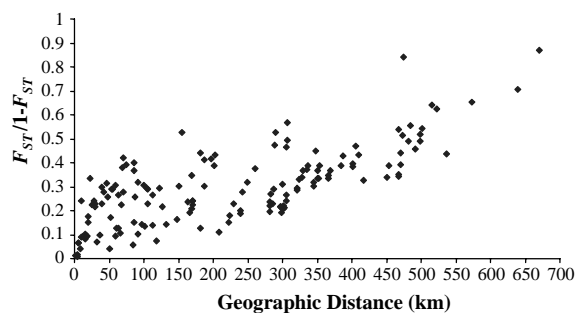


Figure 2. Isolation by distance in 18 *R. cascadae* Oregon and Washington populations. Comparisons are pairwise between all populations.

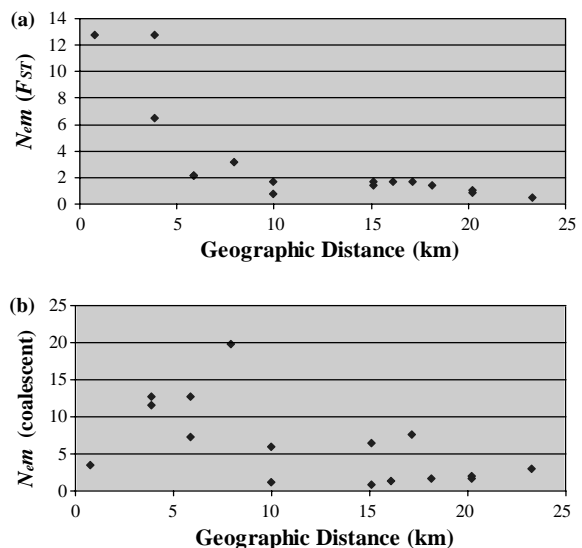


Figure 3. Number of migrants exchanged per generation between 3 fine-scale sets of populations of *R. cascadae* estimated from F_{ST} (a) and the coalescent (b). Estimates from the coalescent are averages of pairwise estimates within each fine-scale cluster.

populations that cover the species range in the Cascades mountains. The strong and significant pattern of isolation by distance is consistent with the hypothesis that *R. cascadae* populations are at equilibrium (Slatkin 1993; Hutchison and Templeton 1999; Castric and Bernatchez 2003).

A growing literature suggests that genetic structure in frogs is very sensitive to the habitat that separates populations (Reh and Seitz 1990; Hitchings and Beebee 1997; James and Moritz 2000; Rowe et al. 2000; Lampert et al. 2003; Funk et al. in review). Montane habitats, in particular, appear to promote very strong genetic isolation among frog populations (Waldman and Tocher 1998; Shaffer et al. 2000; Mosen and Blouin 2003; Funk et al. in review). For example, Funk et al. (in review) found lower diversity within, and higher differentiation among, high elevation populations of *R. luteiventris* than among nearby populations from lower elevations. Our data are consistent with a hypothesis that montane frog species are prone to strong genetic subdivision.

Conservation implications for R. cascadae

The data presented here suggest a substantial reduction in gene flow among *R. cascadae* populations separated by geographic distances greater than 10 km. Consequently, future conservation efforts that may involve transplanting *R. cascadae* between populations should use animals from populations in very close proximity to avoid the admixture of genetically distinct groups. Additionally, the extreme genetic differentiation at such small geographic distances suggests metapopulation structure is weak within *R. cascadae*. Populations that go extinct may be unlikely to be re-colonized quickly, especially if they are greater than 10 km from the nearest population. Consistent with this conclusion is the observation that recolonization of one historic *R. cascadae* site was reported to have taken 12 years despite the presence of a *R. cascadae* population within 2 km (Blaustein et al. 1994). This species spends over half the year in hibernation. When not in hibernation, *R. cascadae* breed and feed in ephemeral creeks and ponds filled by snowmelt. Given the limited amount of time *R. cascadae* are active, combined with their ephemeral habitat, it is not surprising long distance gene flow is rare in this species. Stable metapopulations of *R. cascadae*

may exist in a precarious balance between extirpation and re-colonization of sites that could easily be disrupted by environmental change. Indeed, it is possible that a subtle shift towards increased extinction rates or reduced migration caused the surprisingly quick collapse of the species in Northern California. Future studies should focus on investigating the geographic and habitat features that are likely to promote or reduce gene flow in order to maintain current levels of connectedness between *R. cascadae* populations.

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