AN ABSTRACT OF THE DISSERTATION OF

Kirsten J. Monsen for the degree of Doctor of Philosophy in Zoology presented on October 25, 2002.
Title: Population and Conservation Genetic Structure of the Cascades Frog, *Rana cascadae* Throughout the Species' Range.

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Abstract approved:

Michael S. Blouin

A major goal of conservation biology is to elucidate the population genetic structure in threatened species and assess the relative importance of the evolutionary forces that shape that population genetic structure. I conducted three studies in the declining amphibian *Rana cascadae* to assess levels of population genetic differentiation and the relative importance of gene flow versus random genetic drift throughout the species' range. In the first study, I examined phylogeographic structure on a species-wide geographic scale with both mitochondrial and nuclear molecular markers. I found three mitochondrial groups within *R. cascadae* that are as divergent at the mitochondrial DNA as sister species. However, I only found two nuclear groups within *R. cascadae*, suggesting there are two Distinct Population Segments and three Management Units within the species' range.

In the second study, I compared sequence data from mtDNA and nuclear DNA of the three *R. cascadae* mtDNA groups to several closely related Pacific

Northwestern ranid species. I found the surprising result that the mtDNA of *R. aurora aurora* is more closely related to the mtDNA of *R. cascadae* than to the mtDNA of its own subspecies *R. aurora draytoni*. The nuclear data support the sub-specific relationship between *R. aurora aurora* and *R. aurora draytoni*. This result is most likely due to incomplete lineage sorting of ancestral mtDNA alleles.

Finally, in the third study, I examined the relative importance of gene flow versus random genetic drift on a fine geographic scale using microsatellite loci. Additionally, I estimated the long-term effective population sizes and genetic neighborhood size for 11 *R. cascadae* populations. *Rana cascadae* shows extreme isolation by distance with very little gene flow occurring past a distance of 10 km. Long-term effective population sizes were unrealistically large for current effective population sizes, but the estimates of genetic neighborhood size are consistent with those expected based on current census population size and genetic neighborhood size in other amphibians.

My research suggests *Rana cascadae* should be managed as three separate groups corresponding to the Olympic Peninsula, the Cascades of Washington and Oregon, and Northern California. Additionally, *R. cascadae* exhibits extreme isolation by distance with reduced gene flow at distances greater than 10 km, suggesting metapopulation structure is weak, and populations that go extinct are unlikely to be re-colonized quickly despite the presence of nearby *R. cascadae* populations.

Population and Conservation Genetic Structure of the Cascades Frog,
Rana cascadae Throughout the Species' Range

by

Kirsten J. Monsen

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APPROVED:

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Chair of the Department of Zoology

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Kirsten J. Monsen, Author

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I would like to thank the other members of my cohort for your friendship: Glenn Almany (sorry I suspected you of being an axe murderer when we first met in stats class), Michael Webster (I apologize for all of the times I made Tess late to meet you), and Tess Freidenburg. I am especially thankful to Tess for her friendship, laughter, and empathy. I miss you terribly!

My family has been a constant source of emotional support and encouragement. Although I am the black sheep of the family, my parents Ron and Joan, my brother Pete, and my Aunt Jannie and Uncle Boris, have always encouraged me in my pursuits. I am especially thankful to my mother Joan for teaching me to appreciate and respect all living things, and for giving me the sensitivity to do so.

Words will never be able to express the love and gratitude I have for Todd Lagestee. Without his emotional (and let's face it financial) support, and without his help during my last two field seasons, I'm not sure if I would have made it through this. Although I frequently complained when a simple day hike to look for frogs turned into a death-defying National Geographic expedition, Todd always found frogs, and he always caught them. I want to apologize to Todd for all of my Jekyll and Hyde, psychotic, neurotic fits, and thank him for loving me in spite of them.

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CONTRIBUTION OF AUTHORS

Dr. Michael S. Blouin was directly involved in the design and data analysis of the studies reported in Chapters 2, 3, and 4. Because of his contribution to the research presented in this dissertation, he is a co-author on the three manuscripts submitted for publication resulting from this dissertation.

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DEDICATION

I would like to dedicate my dissertation to my Grandfather, Curtis E. Williams. He was the first person to get me interested in biology by teaching me the life cycle of a may fly (he was showing me how to raise them for fish bait). Grampa encouraged me to look for and appreciate the beauty that connects all living things and it worked a little too well. I eventually quit fishing because I actually felt sorry for the bait. In addition to influencing me directly, he had a profound influence on my Mom, Joan, by raising her to respect all living things, and she in turn had a profound influence on me. I miss him more than words can describe and I am thankful to him for everything he has taught me.

Population and Conservation Genetic Structure of the Cascades Frog, *Rana cascadae* Throughout the Species' Range

CHAPTER 1: GENERAL INTRODUCTION

A fundamental tenet of conservation biology is to identify and protect the evolutionary heritage and future evolutionary potential in threatened species. Although there is still substantial debate over the best way to identify populations that are an important part of a species' evolutionary past or potential evolutionary future, there is no doubt that elucidating a species' population genetic structure can be instrumental in management and conservation of threatened species. Species-wide studies of population genetic differentiation can provide valuable insight into the evolutionary forces, and their relative importance, that shape current and future population genetic structure. Additionally, studies of population genetic differentiation can also provide details of past evolutionary events that have shaped current population genetic structure. For example, one can determine historical patterns of gene flow. It is important to identify gene flow patterns in order to focus on protecting environmental or landscape features necessary to maintain current patterns of connectedness between populations, and to effectively group populations into cohesive conservation units. If populations show substantial genetic differentiation, gene flow is limited between them, creating the opportunity for local adaptation, and necessitating separate management of these populations. In contrast, populations that show limited genetic differentiation are likely connected by gene flow, and should be grouped together as a conservation unit in

order to ensure current levels of connectedness between populations, and prevent population isolation. Additionally, one can identify populations that because of their genetic uniqueness represent an important component to the evolution of the species. In order to maximize variation maintained in a threatened species, it is essential to recognize genetically unique populations.

How to group populations into cohesive conservation units that significantly contribute to the evolution of a species has been the focus of debate for many years (Pennock and Dimmick 1997; Waples 1998; Paetkau 1999). Conservation units have been defined several ways. Evolutionarily Significant Units (ESUs) have been described as populations that possess genetic attributes significant for present and future generations of a species (Ryder 1986), and as reproductively isolated populations that represent an important component to the evolutionary legacy of a species (Waples 1991, 1995). Moritz (1994) suggested ESUs can be recognized as populations that show reciprocal monophyly at mitochondrial DNA loci and significant divergence of allele frequencies at nuclear loci, and Management Units (MUs) can be recognized as populations with significant divergence of allele frequencies at nuclear or mtDNA loci, regardless of the phylogenetic distinctness of the alleles. Taylor and Dizon (1999) defined MUs as geographical areas with little or no exchange of the individuals of interest with adjacent areas, and stress that the amount of interchange defining an MU depends on the management objectives . Geminate Evolutionary Units (GEUs) have been defined as

populations that may be progenitors of future biodiversity and evolution (Bowen 1998). Currently, in order to be considered for protection under the Endangered Species Act of the United States, a vertebrate population must be considered a Distinct Population Segment (DPS). A DPS is defined as a population that is discreet, biologically or ecologically significant, and threatened or endangered regardless of the status of other populations in the same species (US Dept. of the Interior, Federal Register 1996). Although most authors emphasize it is important to avoid making these divisions solely on the basis of information from genetic markers, (Ryder 1986; Paetkau 1999; Taylor and Dizon 1999), in practice, molecular genetic differentiation is often the only or main criterion used to identify threatened or endangered populations that conform to these classifications.

Most genetic studies attempting to define conservation groups in animals use a single type of molecular marker, and most often it is mitochondrial. For example, of 35 papers published over the last eight years specifically attempting to classify vertebrate populations into conservation units, 29 used a single marker; 22 of those 29 used mtDNA. Although there are numerous benefits to using mtDNA for intraspecific studies (Avise et al. 1987), there can be discordance between genetic patterns seen in mtDNA and nuclear loci (Buonaccorsi et al. 1999; Nyakaana and Arctander 1999; Franck et al. 2001). Owing to the expected fourfold reduction in effective population size (Birky et al. 1983), one would expect mitochondrial DNA to reach reciprocal monophyly faster than nuclear DNA, but

given enough time after separation of two populations, both markers should produce the same signal (Avise et al. 1987; Moore 1995). However, differences in sex ratio and mating system can significantly alter this relationship (Hoelzer 1997). Additionally, fluctuating effective population size (N_e) can have profound and different effects on the population genetic patterns at nuclear and mitochondrial genomes (Hoelzer 1997), and stochastic events play a more significant role in shaping the genetic pattern of mtDNA than nuclear DNA (Birky et al. 1983; Avise et al. 1984).

Of most vertebrates studied, amphibians generally show the highest genetic differentiation across small geographic scales (Driscoll 1998a, 1998b; Storfer 1999; James and Moritz 2000; Shaffer et al. 2000; but see Burrowes and Joglar 1999). However, most studies showing high genetic differentiation among amphibian populations are phylogeographic studies, not population genetic structure studies, and therefore often have low sample sizes (Wilkinson et al. 1996; Evans et al. 1997; Macey et al. 1998; Sumida et al. 1998; James and Moritz 2000). Low sample sizes make estimation of population genetic parameters, such as number of migrants exchanged per generation, population differentiation, and effective population size unreliable. Some studies have reasonable sample sizes that could be used to estimate these parameters between populations. However, many of these studies 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 population genetic parameters because juveniles may be related, and because they do not represent reproductively contributing members of the population.

Many studies of migration, reproduction, and survival in amphibian populations suggest there is high variance in reproductive success and cohort survival leading to small effective population sizes (Samollow 1980; Waldman and McKinnon 1993 and references within). In fact, several studies of skeletochronology used to assess age structure in frog populations have found a dominance of a single age class, suggesting large fluctuations in cohort survival (Friedl and Klump 1997; Driscoll 1999a; Reaser 2000; Measey 2001). In populations of small N_e, random genetic drift will play a major role in population differentiation. Migration will reverse the effects of random genetic drift, but low N_e will exacerbate the effects of random genetic drift, especially in the absence of migration. Additionally, populations with low *Ne* may be subject to loss of genetic diversity and an increase in inbreeding depression (Frankham 1995). Given the recent interest in conservation and management of declining amphibian populations, it is important to understand population genetic structure within a species range and the relative importance of random genetic drift and migration in shaping that population genetic structure.

One geographic area that has experienced severe amphibian declines is the Pacific Northwest of the USA (Blaustein et al. 1994; Blaustein and Wake 1995). Owing to the recent decline of many Pacific Northwestern ranid frogs, studies of intraspecific phylogeny have become prevalent (e.g. *Rana pretiosa*, *R. luteiventris* Green et al. 1996, 1997; Blouin, unpublished data; *R. boylii*, Macey et al. 2001; *R. aurora aurora*, *R. aurora draytoni* Schaffer and Fellers, pers. comm.). Additionally, the phylogeny of Pacific Northwestern ranid species has been the focus of many molecular studies using allozymes (Case 1978; Green 1986b), immunology (Case 1978; Post and Uzzell 1981; Farris et al.1982), rDNA (Hillis and Davis 1986), mtDNA (Macey et al. 2001), and karyotypes (Green 1986a).

Rana cascadae is an anuran endemic to the Pacific Northwest that has recently experienced population declines in the southern part of its range (Fellers and Drost 1993). This animal occurs at elevations between 800-2740 m from the Olympic and Cascade Mountains in the state of Washington to the Cascade Mountains in Northern California (Fig. 2.1; Stebbins 1985). Although severe population declines have only been observed in Northern California (Fellers and Drost 1993), *R. cascadae* is still a conservation concern owing to its restricted distribution.

Because of the isolated nature of *R. cascadae*'s range, and because of its recent decline in the southern part of its range, I examined population genetic structure in this species with the aim of identifying genetically unique populations

and estimating the geographic scale over which gene flow occurs. In Chapter two, I used mitochondrial DNA sequence data and microsatellite allele frequency data to: 1) assess population genetic differentiation across the entire species range of *R. cascadae*, 2) determine if any populations of *R. cascadae* warrant separate conservation status because of genetic uniqueness, and 3) compare patterns of genetic differentiation between mtDNA and nuclear markers. In Chapter three I use mtDNA and nuclear sequence data to compare unique *R. cascadae* genetic groups to other Pacific Northwestern ranid species. In Chapter four I use microsatellite allele frequency data to determine the geographic scale over which substantial gene flow occurs, and to estimate the effective population size in *R. cascadae* populations in order to determine the relative importance of gene flow and random genetic drift in shaping population genetic structure.

CHAPTER TWO: DISCORDANCE BETWEEN MITOCHONDRIAL AND NUCLEAR DATA IN A DECLINING AMPHIBIAN: THE POTENTIAL FOR MISLEADING CONSERVATION RECOMMENDATIONS

ABSTRACT

There is substantial debate over the criteria that should be used to group populations of a species into distinct units for conservation (e.g. Evolutionarily Significant Units, Management Units, Distinct Populations Segments). However, in practice, molecular genetic differentiation is often the only or main criterion used to identify such units. Most genetic studies attempting to define conservation units in animals use a single molecular marker, most often mitochondrial, and use samples from a limited number of populations throughout the species' range. Although there are many benefits to using mtDNA, certain features (haploid, maternal, non-recombining) can cause it to show patterns of differentiation among populations that do not reflect the history of differentiation at the nuclear genome (where loci controlling traits of adaptive significance presumably occur). Here I illustrate an example of such mitochondrial-nuclear discordance in a ranid frog, and show how using mtDNA alone could have led to erroneous conservation recommendations. I used mtDNA sequence data and allele frequency data from five microsatellite loci and two randomly cloned single copy nuclear loci to explore

patterns of genetic differentiation throughout the species range of a declining amphibian, *Rana cascadae*. I also used these molecular data to estimate levels of gene flow between six populations of *R. cascadae* on a smaller geographic scale in Oregon. Genetic differentiation was high and gene flow was low for mtDNA and nuclear markers on species-wide and local scales. I discovered three major mtDNA haplotype groups within *R. cascadae*'s range, but found only two major groups at the nuclear loci. I suggest hypotheses to explain the discordant results and discuss the erroneous conservation recommendations I would have made if I had used only mtDNA. This study illustrates the dangers of basing conservation recommendations on a limited sample of populations and a single molecular marker.

INTRODUCTION

A fundamental tenet of conservation biology is to identify and protect the evolutionary heritage and future evolutionary potential in threatened species. How to group populations into cohesive conservation units that significantly contribute to the evolution of a species has been the focus of debate for many years (Pennock and Dimmick 1997; Waples 1998; Paetkau 1999). Conservation units have been defined several ways. Evolutionarily Significant Units (ESUs) have been described

as populations that possess genetic attributes significant for present and future generations of a species (Ryder 1986), and as reproductively isolated populations that represent an important component to the evolutionary legacy of a species (Waples 1991, 1995). Moritz (1994) suggested ESUs can be recognized as populations that show reciprocal monophyly at mitochondrial DNA loci and significant divergence of allele frequencies at nuclear loci, and Management Units (MUs) can be recognized as populations with significant divergence of allele frequencies at nuclear or mtDNA loci, regardless of the phylogenetic distinctness of the alleles. Taylor and Dizon (1999) defined MUs as geographical areas with little or no exchange of the individuals of interest with adjacent areas, and stress that the amount of interchange defining a MU depends on the management objectives. Geminate Evolutionary Units (GEUs) have been defined as populations that may be progenitors of future biodiversity and evolution (Bowen 1998). Currently, in order to be considered for protection under the United States Endangered Species Act, a vertebrate population must be considered a Distinct Population Segment (DPS). A DPS is defined as a population that is discreet, biologically or ecologically significant, and threatened or endangered regardless of the status of other populations in the same species (US Dept. of the Interior, Federal Register 1996). Although most authors emphasize it is important to avoid making these divisions solely on the basis of information from genetic markers, (Ryder 1986; Paetkau 1999; Taylor and Dizon 1999), in practice, molecular genetic

differentiation is often the only or main criterion used to identify threatened or endangered populations that conform to these classifications.

Most genetic studies attempting to define conservation groups in animals use a single molecular marker, and most often it is mitochondrial. For example, of 35 papers published over the last eight years specifically attempting to classify vertebrate populations into conservation units, 29 used a single type of marker; 22 of those 29 used mtDNA. Although there are numerous benefits to using mtDNA for intraspecific studies (Avise et al. 1987), there can be discordance between genetic patterns seen in mtDNA and nuclear loci (Nyakaana and Arctander 1999; Buonaccorsi et al. 1999; Franck et al. 2001). Owing to the expected fourfold reduction in effective population size (N_e) (Birky et al. 1983), one would expect mitochondrial DNA to reach reciprocal monophyly faster than nuclear DNA, but given enough time after separation of two populations, both markers should produce the same signal (Avise et al. 1987; Moore 1995). However, differences in sex ratio and mating system can significantly alter this relationship (Hoelzer 1997). Additionally, fluctuating effective population size can have profound and different effects on the population genetic patterns at nuclear and mitochondrial genomes (Hoelzer 1997), and stochastic events play a more significant role in shaping the genetic pattern of mtDNA than nuclear DNA (Birky et al. 1983; Avise et al. 1984). In this study, I compare mitochondrial and microsatellite data on genetic structure

in a declining amphibian, *Rana cascadae*, to illustrate the drawbacks of using a single molecular marker to define conservation units.

Rana cascadae is an anuran endemic to the Pacific Northwest that has recently experienced population declines in the southern part of its range (Fellers and Drost 1993; Drost and Fellers 1996). This animal occurs at elevations between 800-2740 m from the Olympic and Cascade Mountains in the state of Washington to the Cascade Mountains in Northern California (Fig. 2.1; Stebbins 1985).

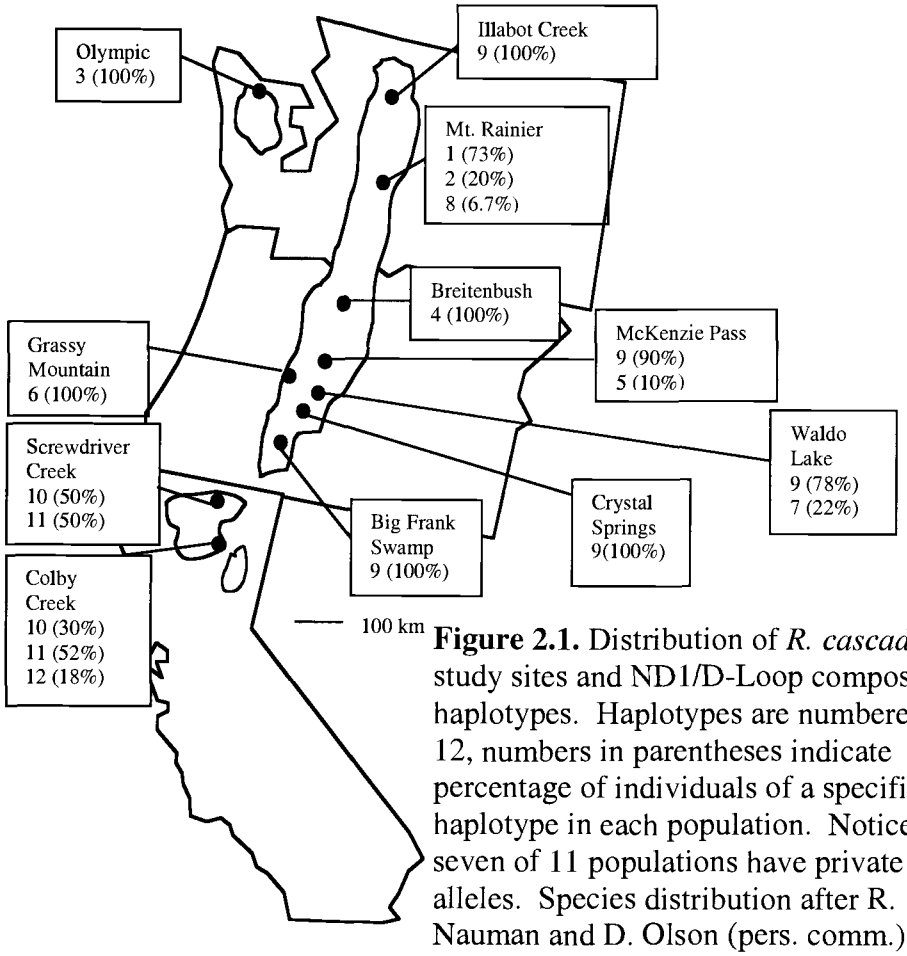


Figure 2.1. Distribution of *R. cascadae* study sites and ND1/D-Loop composite haplotypes. Haplotypes are numbered 1-12, numbers in parentheses indicate percentage of individuals of a specific haplotype in each population. Notice seven of 11 populations have private alleles. Species distribution after R. Nauman and D. Olson (pers. comm.).

Although severe population declines have only been observed in Northern California (Fellers and Drost 1993; Drost and Fellers 1996), *R. cascadae* is still a conservation concern owing to its restricted distribution.

Because of the isolated nature of *R. cascadae*'s range, and because of its recent decline in the southern part of its range, I examined population genetic structure in this species with the aim of providing management recommendations for the remaining populations. I used mitochondrial DNA sequence data and microsatellite allele frequency data to: 1) assess population genetic differentiation across the entire species range of *R. cascadae*, 2) determine if any populations of *R. cascadae* warrant separate conservation status because of genetic uniqueness, 3) determine the geographic scale over which substantial gene flow occurs, and 4) compare patterns of genetic differentiation between mtDNA and nuclear markers. I found a major discrepancy between the patterns of phylogeographic subdivision suggested by nuclear and mtDNA markers and show how decisions on the ESU/DPS status of disjunct populations of *R. cascadae* would depend heavily on which marker was examined.

METHODS

Sampling

Tissue was collected from adult *Rana cascadae* by toe clipping during the summers of 1997 and 1998. Samples were collected from 11 populations throughout the species range (Fig. 2.1, Table 2.1). Sample sizes ranged from 18 to 73 individuals per population, with the exception of one site in California from which I could only obtain 11 individuals (Fig. 2.1, Table 2.1, Appendix 2.1). Populations for collection were chosen in order to cover the entire range for a species-wide analysis. Additionally, six of these 11 populations were sampled at a scale of approximately 30-50 km apart in central Oregon to examine gene flow on a smaller scale.

Table 2.1. Site location and sample size (N) information for *R. cascadae*. Nuclear N is an average of sample sizes across all seven nuclear loci.

Site Name	Latitude	Longitude	mtDNA N	Nuclear N
Olympic, WA	47.9163 N	-123.7814W	72	28
Illabot Creek, WA	48.4402 N	-121.3876 W	18	21
Mt. Rainier, WA*	46.9160 N	-121.6351 W	46	30
Breitenbush, OR	44.7716 N	-121.9495 W	27	26
McKenzie Pass, OR	44.2448 N	-121.8414 W	21	29
Grassy Mountain, OR *	44.2750 N	-122.7755 W	21	14
Waldo Lake, OR	43.7623 N	-122.0131 W	60	28
Crystal Springs, OR	43.3123 N	-122.1404 W	24	22
Big Frank Swamp, OR	42.4422 N	-122.2416 W	20	18
Screwdriver Creek, CA*	40.9981 N	-121.7493 W	11	11
Colby Creek, CA*	40.1113 N	-121.4846 W	33	30

*Tissue collection done by others: Mt. Rainier, WA, Robert Hoffman; Grassy Mt., OR, Marc Hayes; Both CA sites, Gary Fellers

Molecular Methods

Total genomic DNA was extracted from each toe using a standard phenol-chloroform protocol (Hillis et al. 1996). Three-hundred-forty-eight *R. cascadae* individuals were genotyped at a 335 bp fragment of the mitochondrial D-loop, and at a 347 bp fragment that includes 281 bp of the 5' end of the mitochondrial ND1 gene and 66 bp of the flanking tRNA leucine gene. For each fragment I initially sequenced 1 to 10 individuals from each population, and then screened the other individuals for the presence of new alleles by running PCR product on single strand conformation polymorphism gels (SSCP, Orita et al. 1989).

D-loop Fragment Amplification

Primers MB75 and MB76 (D. Call, pers. comm. Table 2.2) were used to amplify a 335 bp fragment in *R. cascadae* individuals from 100-200 ng of genomic DNA in a 50 μL polymerase chain reaction. PCR components were: 50 mM KCl, 10 mM Tris-HCl pH9, 0.1% Triton X-100, 1.5 mM MgCl2, 0.8 μM of both the forward and reverse primers, 0.2 mM dNTPs, 2.5 units taq polymerase, and water to a final volume of 50 μL. Amplification was carried out in a Perkin-Elmer 9600 thermocycler under the following conditions: 94° C 3 minutes, followed by 30

cycles of 94° C 45 seconds, 50° C 30 seconds, 72° C 30 seconds, and a final extension at 72° C for 7 minutes.

ND1/tRNA Fragment Amplification

Primers MB77 and MB129 (Table 2.2) were used to amplify a 347 bp fragment in *R. cascadae* individuals from 100-200 ng of genomic DNA in a 25 μL polymerase chain reaction. PCR components and conditions were the same as the D-loop fragment with a locus-specific annealing temperature (Table 2.2).

Table 2.2. Primer and product information for mtDNA D-Loop and ND1/tRNA fragments. Primer positions relative to published sequences of the bullfrog, *R. catesbeiana* are also given.

Locus	F Primer R Primer	Size (bp)	Anneal (°C)	Position in *R. catesbeiana* mitochondrial genome
D-Loop	MB75 5'gacgcccatacatcagcc3'	335	50	1359 Yoneyama 1987
	MB76 5'acctgcaccgttagtccaa3'			1689 Yoneyama 1987
ND1/tRNA small fragment	MB77 5'tggcagagcttggttatgcaaaaga3'	349	52	2669 Nagae 1988
	MB129 5'ggaaattggggttcatatrattattrg3'			
ND1/tRNA large fragment	MB74 5'ggtatgagcccgatagctta3'	1204	46	3853 Nagae 1988
	MB77 5' tggcagagcttggttatgcaaaaga3'			2669 Nagae 1988
	internal sequencing			
	MB130 5'gaaatggyraargaagagagggt3'			3227 Nagae 1988
	MB143 5'ggattcaccctctcttc3'			3221 Nagae 1988

SSCP

Six μL of PCR product was mixed with 4 μL of loading buffer (95% formamide, 0.1 mg/ml xylene cyanol, 0.1 mg/ml bromophenol blue), heated at 94° C for 3 minutes, and placed immediately on ice. Three μL of this mixture was loaded on a 0.5X MDE (BioWhittaker Molecular Applications), 0.6X TBE gel at 4° C. Samples were run at 8 W for 16-18 hours at 4° C in 0.6X TBE buffer. Bands were visualized by Sybr Gold staining (Molecular Probes), and photographed using a Polaroid camera. Unique banding patterns were scored as unique haplotypes. I found that D-loop reaction products were easier to visualize on SSCP gels if I first concentrated the product two-fold by ethanol precipitation. This step was not necessary with the ND1/tRNA fragment. Two individuals of each putative new haplotype were sequenced in both directions to verify unique sequence.

Sequencing

Products to be sequenced were first purified with Ultrafree-MC 30,000 NMWL spin columns (Millipore Corp.). Purified product was then sequenced on an automated ABI 377 sequencer (Applied Biosystems, Inc.). All sequences were aligned by eye in the program SeqEd v. 1.0.3 (Applied Biosystems, Inc.).

Microsatellite Isolation and Amplification

I used five microsatellite loci that were originally developed for *R. pretiosa* and *R. luteiventris* (RP17, RP193, SCF120, SCF128, and SCF134, Table 2.3; Blouin, unpublished data). In addition, I used two putative microsatellite loci that do not show microsatellite variation in *R. cascadae* (RP123 and RC174; Table 2.3). They each showed size variation owing to a single indel, and so were treated as bi-allelic loci and kept in the study. PCR amplifications were carried out in 25 μL reactions using the same components and conditions as the D-Loop fragment with locus-specific annealing temperatures (Table 2.3). Microsatellite PCR product was run on an ABI 377 automated sequencer, and allele sizes were scored using the program GENOTYPER v. 2.0.

Table 2.3. Primer and product information for five microsatellite loci and 2 size variant nuclear loci.

Locus	F Primer R Primer	Clone Size (bp)	Repeat #	Allele Size Range (bp)	Total # Alleles	Ave.# Alleles Per Population	Anneal (°C)
SCF120	5'aaccctggtagtatgaccaac3' 5'gtggaactccagttatgatcc3'	180	16	137-185	13	5.2	56
RP193	5'ccattttctctctgatgtgtgt3' 5'tgaagcagatcactggcaaagc3'	183	21	143-203	16	6.5	49
SCF134	5'tgggaaaagactctgtggt3' 5'aggaaatgtgtggaagcat3'	240	11	225-273	20	6.0	57
SCF128	5'agaaaagcggacttctgaaat3' 5'agccataatccctgttaaacc3'	236	7	221-261	10	4.5	57
RP17	5'gtgtagacaaacaaatgaaagtcag3' 5'gtctctacttccatccaaccattcc3'	118	7	111-119	3	1.8	50
RP123	5'atgaaacaataaaatctccagagacc3' 5'caaaataaagttggggaaggatgcg3'	135	na	145 and 146	2	1	52
RC174	5'ggtcacacacacccgctgccgag3' 5'accatacatcacagtttttcccacc3'	140	na	86 and 140	2	1	51

mtDNA Data Analysis

Because mtDNA is a single non-recombining molecule, the D-loop sequence and ND1/tRNA sequence were combined to create a single composite haplotype for each individual. F_{st} estimates among the six Oregon populations were made using FSTAT v. 2.9.3 (Goudet 1995, 2001) after the method of Weir and Cockerham (1984). Arlequin v. 2.0 (Schneider et al. 2000) was used to obtain AMOVA estimates of F-statistics (ϕ_{st}) from the uncorrected number of nucleotide differences between haplotypes (Excoffier et al. 1992) among Oregon populations. Because the six Oregon populations are in a continuous habitat and in close proximity (pairs separated by 30-50 km; Fig. 2.1) it is probably reasonable to assume they are in approximate drift-migration equilibrium. Therefore, the number of migrants per generation (N_em) among the six Oregon populations was estimated from $F_{st}=1/(N_em+1)$ (Wright 1951, 1965) and via the private allele method (Slatkin 1985) using GENEPOP v. 3.2a (Raymond and Rousset 1995). A statistical parsimony network was created of all *R. cascadae* mtDNA alleles using the TCS software (Templeton et al. 1992; Clement et al. 2000).

Nuclear DNA Data Analysis

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). A neighbor-joining tree based on Nei's standard genetic distance *D* (Nei 1972) was constructed in the program POPULATIONS v. 1.2.26 (© Olivier Langella 2000), and viewed in the program TREEVIEW v. 1.6.6 (Page 1996). I was unable to obtain allele frequency data for the Screwdriver Creek, CA population for size variant locus RP123, most likely due to poor DNA quality. Consequently, this population was eliminated from the neighbor-joining tree.

F_{st} estimates among the six Oregon populations were made using GENEPOP v. 3.2a after the method of Weir and Cockerham (1984) for the five microsatellite loci. The number of migrants per generation (N_em) among Oregon populations was estimated under an assumption of approximate drift-migration equilibrium in an island model from $F_{st}=1/(4N_em+1)$ (Wright 1951, 1965), and via the private allele method (Slatkin 1985) using GENEPOP v. 3.2a.

Estimated Times of Divergence Between mtDNA Alleles

Sequence analysis of the D-Loop and ND1/tRNA variants suggested the presence of three very divergent groups of mtDNA alleles. In order to more accurately date the split between these three groups, I sequenced a 1204 bp mtDNA fragment that included the entire ND1 gene and four flanking tRNA genes (tRNA Leu, tRNA Ile, tRNA Gln, tRNA Met) using the primers MB74, MB77, MB130, and MB143 (Table 2.2) from the most common haplotype found in each of the three groups. Times of divergence between mtDNA alleles were based on a molecular clock of 1.4% sequence divergence per million years for anuran mtDNA based on the ND1 gene (Macey et al. 1998a). The clock estimates sequence divergence of 1.4% per million years between taxa (Macey et al. 1998a). Similar rates of divergence have been shown in other anurans (A. Crawford, pers. comm.), salamanders (Spolsky et al. 1992), lizards (Macey et al. 1998b), and snakes (Zamudio and Greene 1997). I have confidence in the accuracy of this clock because it yields dates of separation among Pacific Northwestern ranid species (Monsen and Blouin, in review; Macey et al. 2001) that are very similar to dates based on allozyme distances (Green 1986b).

RESULTS

mtDNA

There were 12 mtDNA haplotypes observed throughout the range of *R. cascadae* (Fig. 2.1 and 2.2). The statistical parsimony network of mtDNA haplotypes showed three distinct groups: the Olympic Peninsula, the Cascades of Oregon and Washington, and California. It was not possible to connect these three groups with 95% confidence in TCS, and 14 steps were needed to force connections between them (Fig. 2.2). When forced, TCS originally joined Olympic allele 3 to Waldo Lake allele 7 (a rare private allele) based on a single ambiguous reversal. I changed this connection in Fig. 2.2 based on the geographic separation of these two populations and the other basic assumptions described by Crandall and Templeton (1993).

Of the 11 populations sampled, seven contained private alleles (Fig. 2.1). Three of these populations are fixed for their private allele. Three of the remaining four populations without private alleles are fixed for the most common central Cascades allele 9. The final population lacking a private allele, Screwdriver Creek, shared its two alleles (10 and 11) with the neighboring Colby Creek, CA population. Although there are multiple alleles within the Cascades and California groups, there are few genetic differences between alleles within each group (Fig.

2.2). There was, however, substantial divergence between the three groups. The sequence divergence at the 1204 bp mtDNA ND1/tRNA fragment between the Cascades and California is 3.4%, between Cascades and Olympic is 3.2%, and between California and Olympic is 4%. The anuran ND1 clock dates the split of these three groups at 2.3 to 2.9 MYA.

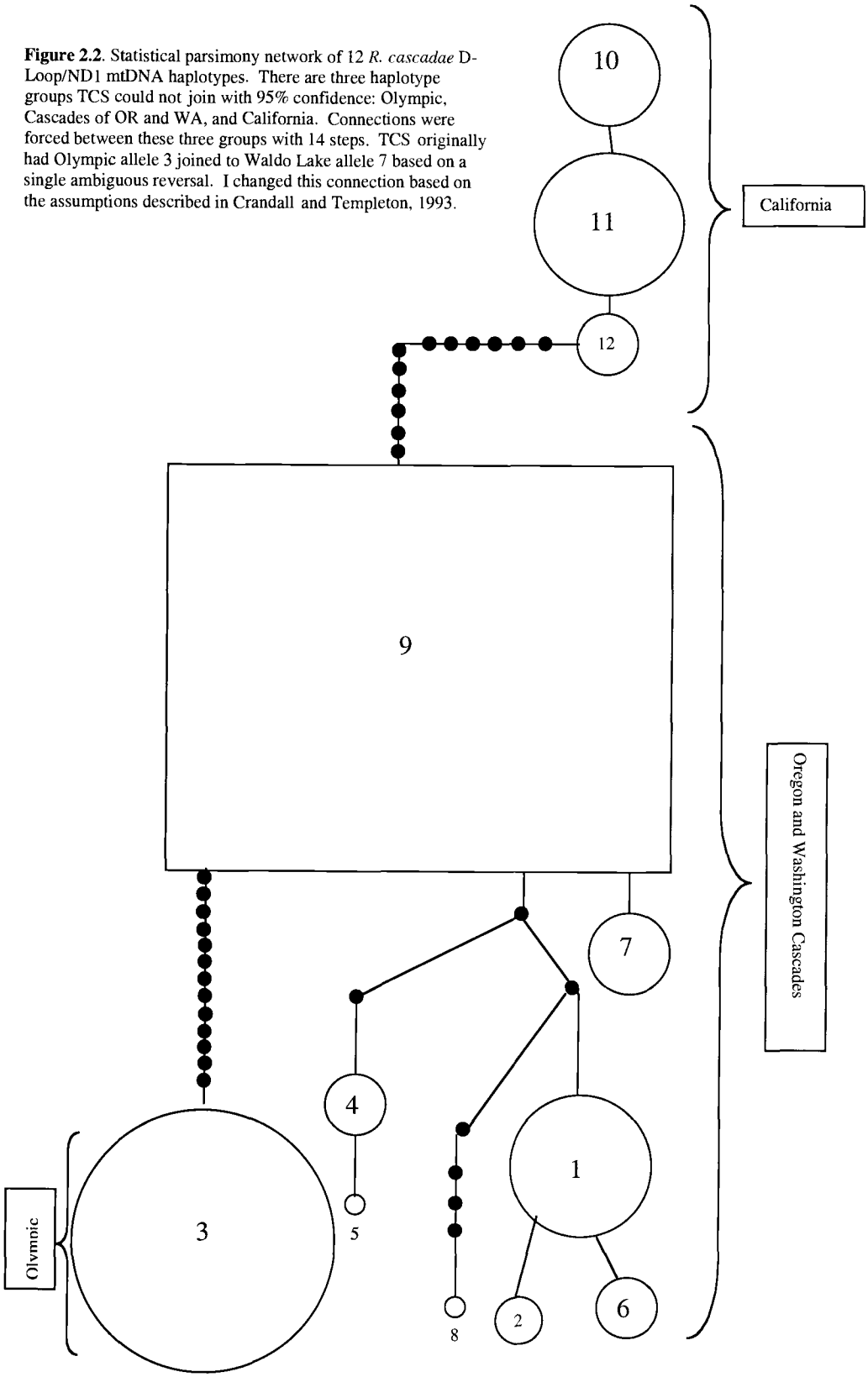


Figure 2.2. Statistical parsimony network of 12 *R. cascadae* D-Loop/ND1 mtDNA haplotypes. There are three haplotype groups TCS could not join with 95% confidence: Olympic, Cascades of OR and WA, and California. Connections were forced between these three groups with 14 steps. TCS originally had Olympic allele 3 joined to Waldo Lake allele 7 based on a single ambiguous reversal. I changed this connection based on the assumptions described in Crandall and Templeton, 1993.

Populations of *R. cascadae* in Oregon showed strong genetic differentiation and limited gene flow on a small scale (nearest neighbors separated by 30-50 km). F_{st} is estimated as 0.78, and ϕ_{st} is estimated as 0.93, giving estimates of gene flow (N_em) that range from 0.28 to 0.07. The private allele method placed this estimate at 0.024 (Table 2.4).

Nuclear Loci

The total number of alleles per microsatellite locus ranged from 3 to 20 and expected heterozygosities within populations ranged from 0.25 to 0.87 (Appendix 1). After a Bonferroni correction for multiple statistical tests, three of eleven populations were not in Hardy-Weinberg equilibrium at single loci (Mt. Rainier-SCF120, Olympic-SCF134, and Waldo Lake-RP17), and one population was not in Hardy-Weinberg equilibrium at three loci (Colby Creek-SCF120, RP193, and SCF128). Deviations from Hardy-Weinberg equilibrium could result from the presence of a null allele or unsuspected subdivision creating a Wahlund effect. There were few or no failed PCR reactions at these loci in these populations, suggesting heterozygote deficits are not due to the presence of high-frequency null alleles. It is possible that deviations from HWE in the Colby Creek population

were due to the sampling of multiple genetic groups mistakenly considered one population.

The neighbor-joining tree of Nei's *D* for nuclear data among *R. cascadae* populations gave results incongruent with the mtDNA data in two ways (Fig. 2.3). First, the nuclear data suggest a break between Oregon and Washington populations, but there is no evidence of reciprocal monophyly for mtDNA across the same break (Fig. 2.2). Second, the nuclear data do not show evidence of a break between the Olympic Peninsula and the Washington Cascades, even though the mtDNA data suggest that the two groups have been separated for two to three million years. On the other hand, both data sets identified the California populations as being distinct (Fig. 2.2 and 2.3). Note that the distinctness of California populations at the nuclear loci results from unique alleles as well as distinct frequencies of shared alleles (Appendix 2.1).

Populations of *R. cascadae* showed less genetic differentiation on a small scale for microsatellites than for mtDNA. Microsatellite F_{st} among the six Oregon populations is 0.16, which suggests an N_em of 1.31. The private allele method placed this estimate at 0.54. N_em estimated from mtDNA ranged from 4 to 15 times lower than N_em estimated from nuclear DNA when the same method of estimation was used (F_{st} or private allele; Table 2.4). This range is consistent with the expected fourfold difference in N_e between mtDNA and nuclear loci, especially given the stochastic variance in F_{st} expected from sampling a single locus (Nei

1987). So there is no reason to believe that nuclear and mtDNA are giving very different pictures of small scale gene flow in *R. cascadae*. The important point is, taken together, these data suggest that gene flow in the species is quite restricted on a scale of 30-50 km between populations.

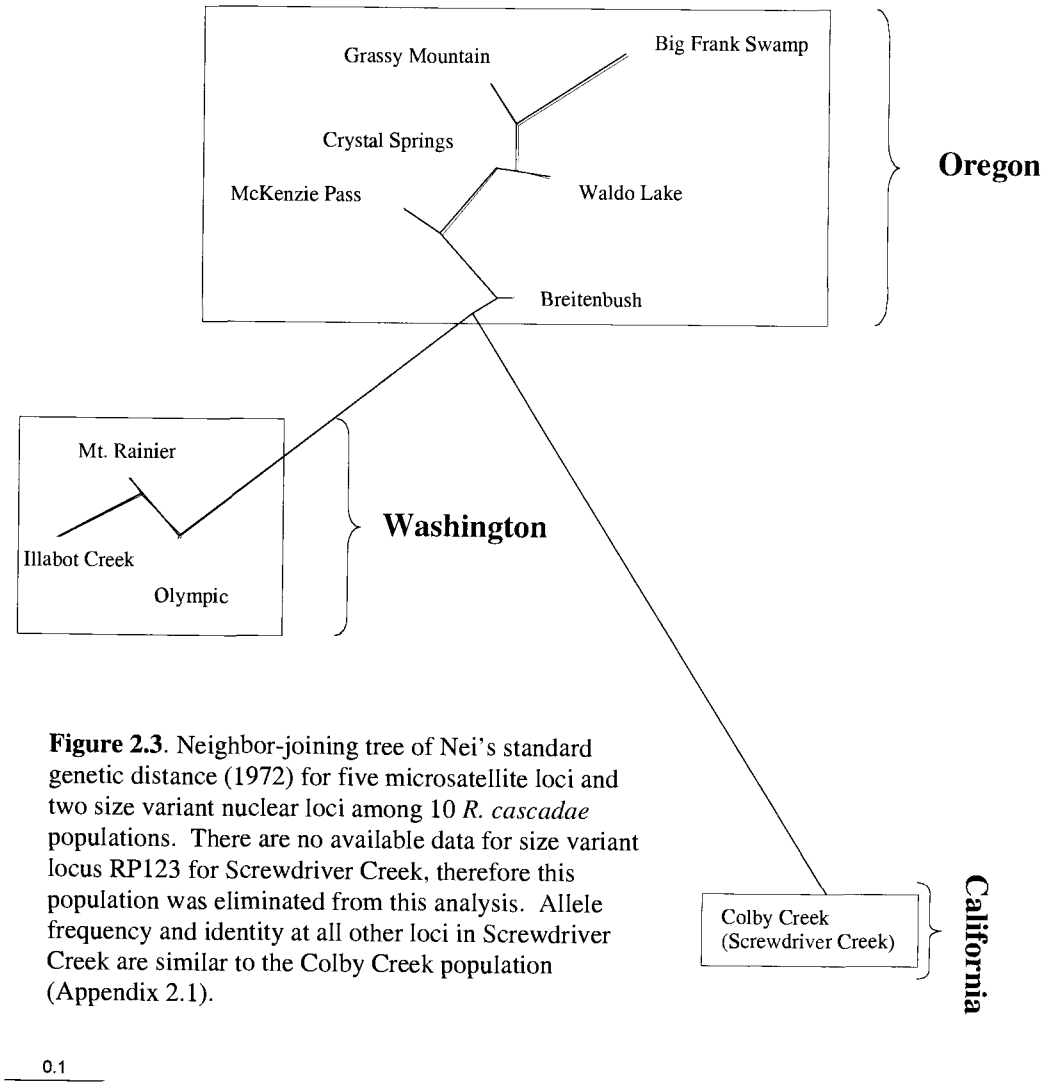


Figure 2.3. Neighbor-joining tree of Nei's standard genetic distance (1972) for five microsatellite loci and two size variant nuclear loci among 10 *R. cascadae* populations. There are no available data for size variant locus RP123 for Screwdriver Creek, therefore this population was eliminated from this analysis. Allele frequency and identity at all other loci in Screwdriver Creek are similar to the Colby Creek population (Appendix 2.1).

Table 2.4. N_em estimates between six Oregon populations of *R. cascadae* for mtDNA and nuclear data.

mtDNA	F_{st}	ϕ_{st}	N_em (F_{st})	N_em (ϕ_{st})	N_em (private alleles)
	0.78	0.93	0.28	0.08	0.024

nuclear	F_{st}	N_em (F_{st})	N_em (private alleles)
	0.16	1.31	0.54

DISCUSSION

There are two obvious discrepancies between the mtDNA and nuclear data throughout the species range of *R. cascadae*. First, there is no apparent genetic break between Oregon and Washington populations according to mtDNA data, but there is a break between Oregon and Washington populations at the nuclear loci (Figs. 2.1, 2.2, and 2.3). Second, the mtDNA data suggest there are three major genetic groups (Olympics, Cascades, and California) that diverged 2 to 3 MYA, while the nuclear data suggest there are only two (Oregon/Washington, and California) (Figs. 2.1, 2.2, and 2.3).

It is possible there is no obvious split between Oregon and Washington populations for the mtDNA data because there simply has not been enough time since separation of the populations to achieve reciprocal monophyly among

mtDNA alleles. The presence and fixation of private alleles in many populations suggest the N_e of individual populations at the mitochondrial genome is very small. However, across the entire species range N_e is most likely quite high, as subdivision into many small populations is expected to increase the overall N_e (Kimura and Crow 1963; Roberston 1964; Nei and Takahata 1993; Lande 1995). Having a high species-wide N_e will increase the time necessary for mtDNA alleles to achieve reciprocal monophyly (Neigel and Avise 1986).

The discrepancy between mtDNA and microsatellites over the distinctness of the Olympic Peninsula population is more difficult to explain. Although the mtDNA data suggest the Olympic and Cascades populations have been separated for two to three million years, the microsatellite data refute this conclusion. Given a typical vertebrate microsatellite mutation rate of $5x10^{-5}$, after 2000 generations of isolation between two populations every ancestral allele will have mutated once on average (Estoup and Angers 1998). *R. cascadae* has a generation time of approximately three years (Briggs and Storm 1970), so after 6000 years of complete separation there should have developed substantial differentiation between the two groups owing to mutation. Clearly this is not so at the microsatellite loci. One might argue that mutational constraints on allele size have put a cap on the total microsatellite differentiation possible between the two groups, as appears to have been the case with other vertebrates separated for millions of years (Ostrander et al. 1993; Bowcock et al. 1994; Garza et al. 1995; Paetkau et al.

1997). However, the Olympic and central Washington populations share the same small and size-restricted subset of the total alleles observed in this species, and they have those alleles in similar frequencies (Appendix 2.1; Fig.2. 4). In order to estimate the effect of mutation, I calculated R_{st} between Washington and Olympic populations and compared it to F_{st} between these populations. Pairwise R_{st} values between the Olympic population and the other Washington populations range from 0.13 to 0.28, which is not substantially larger than F_{st} (0.17 to 0.25), as would be expected if there was a large mutational component to the differentiation between these groups (Slatkin 1995). Thus, it appears implausible that the two groups have been completely isolated for thousands of years, much less two million.

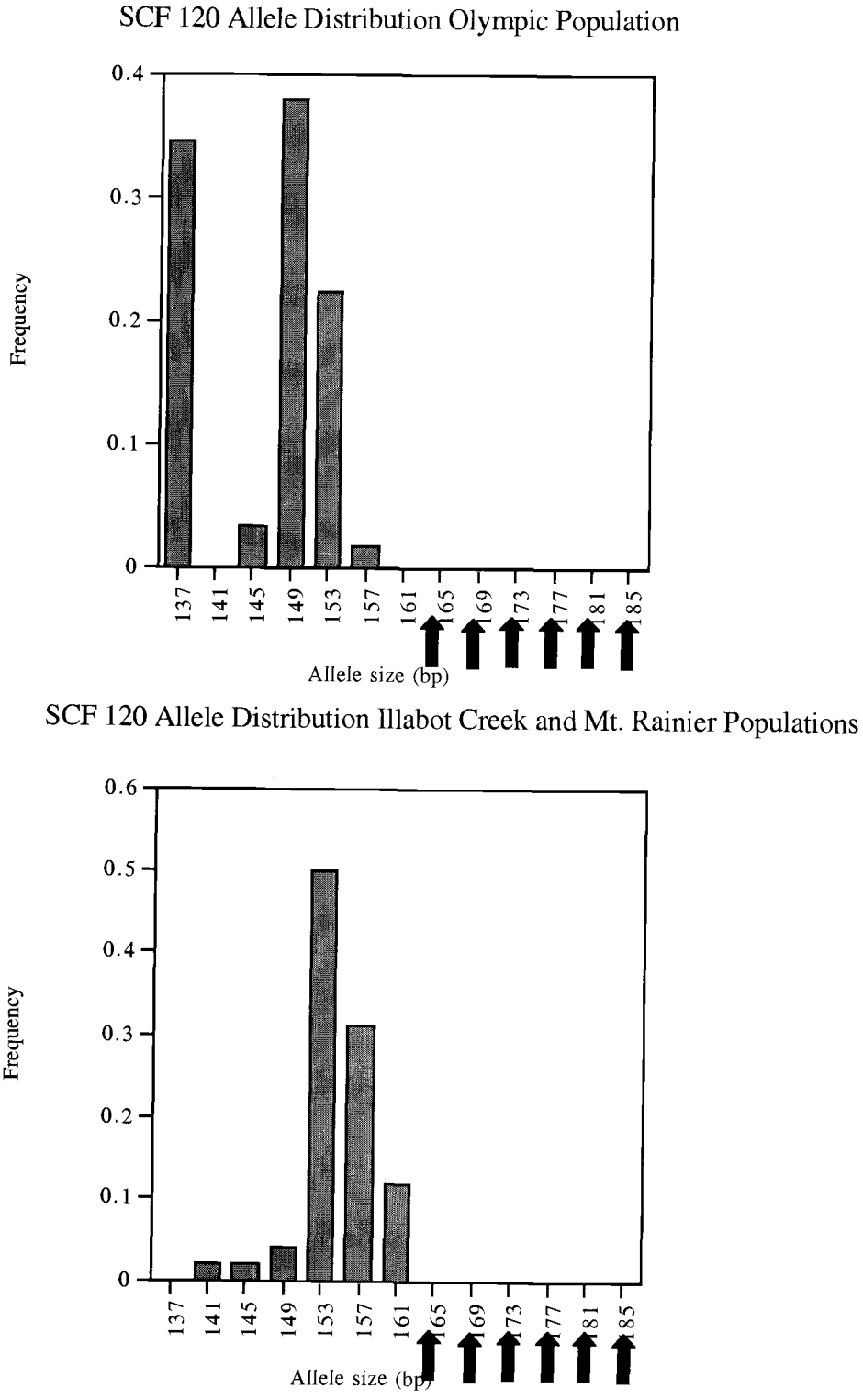
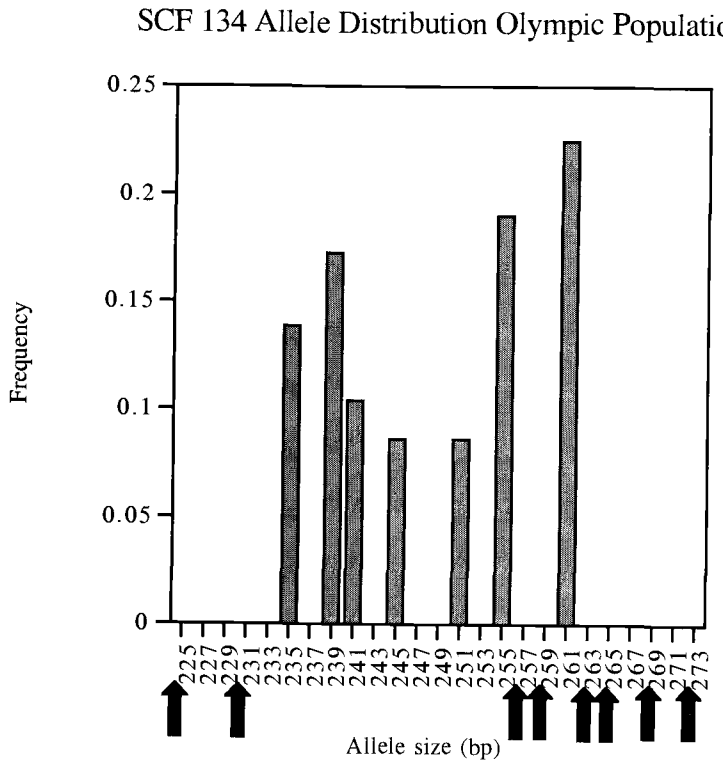


Figure 2.4. Allele distributions for SCF120 and SCF134 in *R. cascadae* Washington populations.



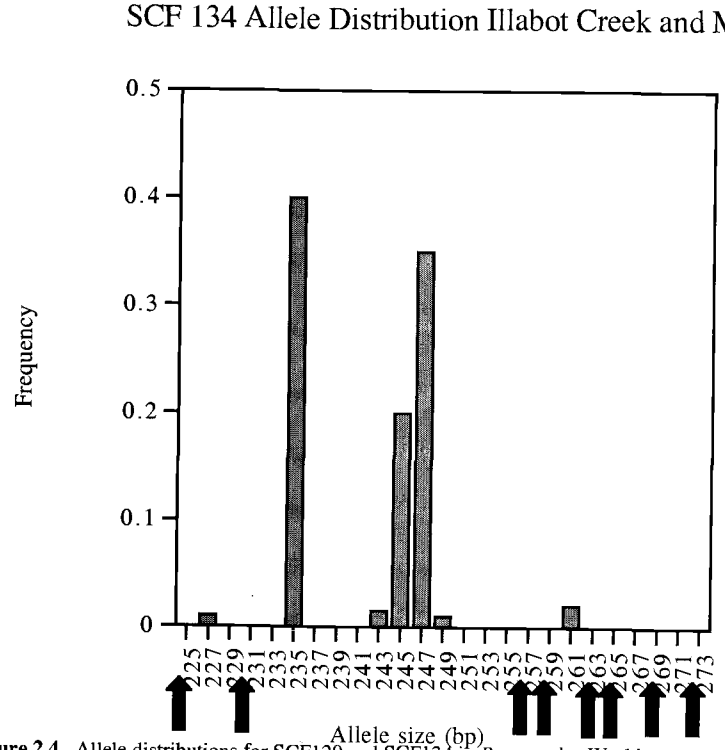


Figure 2.4. Allele distributions for SCF120 and SCF134 in *R. cascadae* Washington populations. Alleles marked with an arrow are present in Oregon or California populations but not Washington. If there were a constraint on allele size at these loci, one would not expect to see other alleles present in other populations.

Sex-biased dispersal can produce discordant patterns between mtDNA and microsatellites, but this does not seem a plausible explanation for the Olympic-Cascades discrepancy. There is currently no evidence of sex-biased dispersal among Oregon populations based on the estimates of N_e from both mitochondrial and nuclear data (see results). Additionally, adult anurans are very philopatric, and most gene flow probably occurs during juvenile dispersal away from breeding ponds (Waldman and McKinnon 1993, and references within). Also, *R. cascadae* does not appear prone to long-distance gene flow. So migration between Cascades and Olympics must have proceeded in a bi-directional stepping-stone fashion, which would have reduced the opportunity for male-biased movement to generate such a discordant pattern between mtDNA and microsatellites.

So how did the Olympic population come to be fixed for such a divergent mtDNA allele if microsatellites show that the Olympic population could not have been isolated nearly as long as the age of that allele? The Olympic Peninsula was isolated from the Cascades during the last glacial maximum which ended approximately 13,600 years ago (Waitt and Thorson 1983). It is plausible that a population of *R. cascadae* was isolated on the peninsula for several million years during which time the distinct Olympic and Cascades mtDNA lineages evolved in isolation. Following secondary contact, there would have been a mixing of Olympic and Cascades alleles as animals colonized from the East. An Olympic allele may have by chance drifted to high frequency on the peninsula even though

the nuclear genomes were homogenized. This scenario is plausible given the small and fluctuating effective sizes that probably typify anuran populations (Waldman and McKinnon 1993, and references within), and the expected fourfold smaller N_e of mtDNA relative to nuclear loci. Furthermore, I only sampled one site on the peninsula. Even though that site appears fixed for the Olympic allele (n = 72), if the above scenario is correct, then there may be Cascades-lineage alleles still present elsewhere on the Olympic Peninsula.

I sampled a few populations throughout the species range as is typically done for conservation studies. This sampling scheme often means a single population is sampled from a unique genetic group when strong genetic subdivision is observed in a species. For example, I only have one population from the Olympic Peninsula, and this population appears fixed for the Olympic mtDNA allele. If I sampled additional populations within the Olympic Peninsula, I may observe other mtDNA alleles more closely related to Cascades mtDNA alleles, yielding results more concordant with the nuclear data. Therefore, I suggest a better approach to these types of conservation genetics studies is to sample in two stages. First, one should sample a few populations throughout a species range and identify a general pattern of subdivision. Then, one should sample multiple populations within each region of genetic subdivision to verify the pattern of subdivision and describe the boundaries of genetic differentiation. This sampling

strategy has been proposed by other authors (Baverstock and Moritz 1996). Additionally, one should use both mitochondrial and nuclear markers.

If I had based this study, and subsequent conservation recommendations, on the mtDNA data alone, I would have made erroneous conservation recommendations. Based on the geographical separation and the large mtDNA genetic distance between the Olympic population and other Washington populations, I would have suggested there were three DPSs in the species range of *R. cascadae*. Indeed, the three *R. cascadae* mtDNA groups are as divergent at the ND1/tRNA fragment as the sister species *R. pretiosa* and *R. luteiventris* (Monsen and Blouin, in review). One might have even suggested the Olympic *R. cascadae* are approaching the status of subspecies or even cryptic species based on the mtDNA divergence. Clearly the microsatellite data do not support such a division between the Olympic and other populations. This problem of discordance between molecular markers may be especially pronounced in organisms with demographically unstable populations (Hoelzer 1997). Owing to fluctuations in population size and large variance in reproductive success, amphibians are very likely to have unstable demographic structure (Waldman and McKinnon 1993, and references within). In fact, several studies of skeletochronology used to assess age structure in frog populations have found a dominance of a single age class, suggesting large fluctuations in cohort survival (Friedl and Klump 1997; Driscoll

1999a; Reaser 2000; Measey 2001). Amphibians may therefore be more prone to show discordance between mtDNA and nuclear markers than other taxa.

Conservation Implications for *R. cascadae*

Based on the mitochondrial and microsatellite data presented here, I suggest there are two Distinct Population Segments within the species range of *R. cascadae*: California, and Oregon/Washington populations. These two groups show reciprocal monophyly at mitochondrial DNA loci, and significant divergence of allele frequencies at nuclear loci. They therefore meet the definition of "discreet and significant". Additionally, these groups are physically separated by a known faunal break across southern Oregon and northern California (Bury and Pearl 1999). Based on the mtDNA data, California populations were most likely separated at the beginning of the last glacial maximum (approximately 2 MYA), but never experienced secondary contact after glacial retreat. This glacial history has been invoked to explain species breaks in other herpetofauna (Bury and Pearl 1999) and plants (Soltis et al. 1997) in the same geographic region.

The status of the Olympic populations as a DPS is debatable. Many authors have criticized the designation of populations as unique based solely on molecular genetic data, citing other characteristics such as unique habitat use or physical isolation as good indicators of distinctness even when genetic data do not show a

difference between populations (Pennock and Dimmick 1997; Paetkau 1999; Taylor and Dizon 1999). Because of habitat restrictions, it is unlikely there is current gene flow between the Olympic Peninsula and the Cascades, even though there has clearly been gene flow in recent evolutionary time. Therefore, the physical isolation and unique habitat of the Olympic populations suggest they should be managed separately, regardless of their status as a Distinct Population Segment. Further sampling of other Olympic populations is necessary to determine if mtDNA alleles more closely related to Cascades mtDNA alleles are present.

Finally, estimates of gene flow on a small geographic scale based on mtDNA were less than one, suggesting the opportunity for substantial genetic drift (Mills and Allendorf 1996). Remarkably, five of six populations sampled within 50 km of each other contain private mtDNA alleles, with two of these populations fixed for a private allele (Fig. 2.1). The widespread presence and fixation of mtDNA private alleles has not been observed in most other anurans (Yang et al. 1994; Wilkinson et al. 1996; Evans et al. 1997; Shaffer et al. 2000), but has been observed in other Pacific Northwestern ranid populations on a similar geographic scale (*R. pretiosa*, Blouin, unpublished data). Gene flow estimates based on the microsatellite data are larger than those based on mtDNA data, but the difference is consistent with the expected difference in N_e between the two markers. The low gene flow observed among Oregon populations suggests metapopulation structure is weak with low connectivity between populations. Consistent with this

conclusion is the observation that re-colonization 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). Thus, effective conservation of this species will require further studies on the scale of gene flow, and on the habitat features that enhance or reduce connectivity between populations.

CHAPTER THREE: DISCORDANCE BETWEEN MITOCHONDRIAL AND NUCLEAR DATA IN PACIFIC NORTHWESTERN RANID PHYLOGENY

ABSTRACT

The phylogeny of Pacific Northwestern ranid species has been the focus of many molecular studies, often with conflicting results. Additionally, many new studies are exploring intraspecific molecular differentiation within Pacific Northwestern ranid species. During a recent intraspecific study of the Cascades frog, *Rana cascadae*, I discovered three mtDNA haplotype groups within the species' range. In order to elucidate the phylogenetic relationships among these three groups, I compared sequence data from the mitochondrial ND1 gene and flanking tRNA genes and two single copy nuclear loci from the three *R. cascadae* groups and six other ranid species. I found the surprising result that the mtDNA of the Northern red-legged frog, *R. aurora aurora*, is more closely related to the mtDNA of *R. cascadae* than to the mtDNA of its own subspecies, *R. aurora draytoni* (California red-legged frog). This result conflicts with several independent studies, including the nuclear data reported here. I discuss two possible explanations for the discordance between mtDNA and nuclear data in

Pacific Northwestern ranid species: past hybridization and incomplete lineage sorting of mtDNA alleles.

INTRODUCTION

Phylogeny of Pacific Northwestern ranid species has been the focus of many molecular studies using allozymes (Case 1978; Green 1986b), immunology (Wallace et al. 1973; Case 1978; Farris et al. 1979, 1982; Post and Uzzell 1981), rDNA (Hillis and Davis 1986), mtDNA (Macey et al. 2001), and karyotypes (Green 1986a). Owing to the recent decline of many Pacific Northwestern amphibians, studies of intraspecific phylogeny have also become prevalent (e.g. *Rana pretiosa*, *R. luteiventris* Green et al. 1986, 1987; Blouin unpublished data; *R. boylii*, Macey et al. 2001; *R. aurora aurora*, *R. aurora draytoni* Schaffer and Fellers, pers. comm.). During a recent study of gene flow among populations of the Pacific Northwestern frog *R. cascadae*, I discovered three major mtDNA groups that are as divergent as the mtDNA of the sister species *R. pretiosa* and *R. luteiventris* (Monsen and Blouin, in review). Each *R. cascadae* mtDNA group is restricted to one of the following regions: the Olympic Peninsula, the Cascades of Washington and Oregon, and California.

To compare the phylogenetic relationships among these three *R. cascadae* mtDNA groups relative to other Pacific Northwestern ranid species, I compared

mitochondrial DNA sequence data of all three *R. cascadae* groups to the related species *R. aurora aurora*, *R. aurora draytoni*, *R. boylii*, *R. luteiventris*, and *R. pretiosa*. I found the surprising result that *R. aurora aurora* mtDNA appears more closely related to *R. cascadae* mtDNA than to the mtDNA of its own subspecies *R. aurora draytoni*. I examined the relationships among these taxa further by comparing sequence data from two randomly cloned single copy nuclear loci for the three *R. cascadae* mtDNA groups and the other related Pacific Northwestern ranid species. I estimated the times of divergence between the three *R. cascadae* mtDNA groups and other Pacific Northwestern ranid species with a molecular clock specific to anuran mtDNA. Additionally, I compared the dates of divergence estimated from mtDNA to dates estimated from previously published allozyme data and a molecular clock specific to anuran allozymes.

METHODS

Sampling

Tissue was collected from adult ranid frogs by toe clipping. Samples were collected from six Pacific Northwestern species (*R. aurora aurora*, *R. aurora draytoni*, *R. boylii*, *R. cascadae*, *R. luteiventris*, and *R. pretiosa*) and two outgroup species (*R. catesbeiana* and *R. pipiens*) (Table 3.1). Previous research has

identified three distinct and geographically separated mtDNA groups in the species range of *R. cascadae* (Monsen and Blouin, in review). One individual representing the most common haplotype from each of these three regions was used.

Table 3.1. Specimen location information.

Species	Collection location	Latitude	Longitude
R. aurora aurora	Benton Co., OR	48.4718 N	-123.2050W
R. aurora draytoni	Santa Cruz Co., CA	37.0667N	-122.0500W
R. boylii	Coos Co., OR	43.1667N	-124.0003W
R. cascadae – Olympic	Clallam Co., WA	47.9163N	-123.7814W
R. cascadae – Cascades	Skagit Co., WA	48.4402N	-121.3876W
R. cascadae – California	Butte Co., CA	40.1113N	-121.4846W
R. catesbeiana	Benton Co., OR	44.7011N	-123.2096W
R. luteiventris	Chelan Co., WA	48.4674N	-120.6533W
R. pretiosa	Deschutes Co., OR	43.8842N	-121.4375W
R. pipiens	Bear Lake Co., ID	42.1385N	-111.2623W

ND1/tRNA fragment

Total genomic DNA was extracted from each toe using a standard phenol-chloroform protocol (Hillis et al. 1996). A 1204 bp fragment containing the ND1 gene and four flanking tRNA genes (tRNA Leu, tRNA Ile, tRNA Gln, tRNA Met) was amplified in each species using forward primer MB77 and reverse primer MB74 (Table 3.2) in 50 µL reactions with the following components: 50 mM KCl, 10 mM Tris-HCl pH9, 0.1% Triton X-100, 1.5 mM MgCl2, 0.8 µM of both the forward and reverse primers, 0.2 mM dNTPs, 2.5 units taq polymerase, and water to a final volume of 50 µL. Amplification was carried out in a Perkin-Elmer 9600 thermocycler under the following conditions: 94° C 3 minutes, followed by 30

cycles of 94° C 45 seconds, 46° C 30 seconds, 72° C 30 seconds, and a final extension at 72° C for 7 minutes. Double-stranded sequence was then obtained using MB74, MB77, and internal primers MB130 and MB 143 (Table 3.2).

Table 3.2. Primer and product information for mtDNA ND1/tRNA fragment and single copy nuclear loci. Primer positions relative to published sequences of the bullfrog, *R. catesbeiana* are also given for mtDNA.

Locus *catesbeiana*	F Primer R Primer	Size (bp)	Anneal (°C)	Position in *R.* mitochondrial genome
ND1/tRNA fragment	MB74 5'ggtatgagcccgatagctta3' MB77 5' tggcagagcttggttatgcaaaaga3'	1204	46	3853 Nagae 1988 2669 Nagae 1988
	internal sequencing MB130 5'gaaatggyraargaagagagggt3'			3227 Nagae 1988
	MB143 5'ggattcaccctctcttc3'			3221 Nagae 1988
RP304	5'gatccccgttacaaggacaa3' 5'gtggacacatactgctcaac3'	336	55	
B22	5'taatccattcttcttgtattgtgac3' 5'cgcagtccaacccaggcactatctg3'	346	53	

Single copy nuclear loci

Randomly cloned single copy nuclear loci were isolated from a *R. pretiosa* genomic library. Two loci, RP304 and B22 (Table 3.2), successfully amplify in *R. cascadae*, *R. aurora aurora*, *R. aurora draytoni*, *R. luteiventris*, and *R. pretiosa*. RP304 successfully amplifies in *R. boylii* as well. PCR amplifications were carried out in 50 μL reactions using the same components and conditions as the ND1/tRNA fragment and locus-specific annealing temperatures (Table 3.2).

Amplified single copy nuclear loci were sequenced in both directions with their respective PCR primers (Table 3.2).

Sequencing

Products to be sequenced were first purified with Ultrafree-MC 30,000 NMWL spin columns (Millipore Corp.). Purified product was then sequenced on an automated ABI 377 sequencer (Applied Biosystems, Inc.). All sequences were aligned by eye in the program SeqEd v.1.0.3 (Applied Biosystems, Inc.).

Phylogenetic analysis

All mitochondrial phylogenetic analyses were conducted using PAUP version 4.0b8 (Swofford 1998). The ND1/tRNA sequences were used in both maximum likelihood and maximum parsimony analyses. All phylogenetic analyses for mtDNA were done with the two-parameter HKY'85 model (Hasegawa et al. 1985). All searches were done using the heuristic option, and the tree bisection-reconstruction (TBR) method was used. Confidence for each analysis was determined through a bootstrap analysis of 1000 replications. All trees were

viewed in the program TREEVIEW v. 1.6.6 (Page 1996). Statistical parsimony networks were drawn from the sequence data of single copy nuclear loci using the program TCS (Clement et al. 2000) following the algorithm of Templeton et al. (1992).

Estimated times of divergence between species

Times of divergence between taxa were estimated by use of a molecular clock for anuran mtDNA that includes the ND1 gene (Macey et al. 1998a). The clock estimates sequence divergence of 1.4% per million years between taxa (Macey et al. 1998a). Similar rates of divergence have been shown in other anurans (A. Crawford, pers. comm.), salamanders (Spolsky et al. 1992), lizards (Macey et al. 1998b), and snakes (Zamudio and Greene 1997). In order to assess the accuracy of the mtDNA clock, divergence times between species calculated from the mtDNA data were compared to divergence times among the same species calculated from allozyme data (Green 1986b) using a protein molecular clock specific to anurans (0.1 units of Nei's *D* per million years (Beerli et al. 1996; Nei 1972).

RESULTS

mtDNA

There were no differences between trees constructed from the parsimony and likelihood analyses, therefore, only results of the likelihood analysis are reported here. The topology of the tree produced by the likelihood analysis is the same as the tree produced from Green's allozyme data (1986b; Fig. 3.1 and 3.2), with the exception of the relationship between *R. cascadae* and *R. aurora aurora.* Interestingly, the mtDNA data suggest that *R. aurora aurora* mtDNA is more closely related to *R. cascadae* mtDNA than to the mtDNA of its own subspecies *R. aurora draytoni* (Fig. 3.1).

The three *R. cascadae* mtDNA haplotype groups are monophyletic and are estimated to have split from each other between 2-3 MYA (Fig. 3.1; Table 3.3). Although there were only allozyme data available for *R. cascadae* from the Washington and Oregon Cascades, allozyme (Green, 1986b) and mtDNA data yield similar estimates of divergence times among the other Pacific Northwestern ranids (Table 3.3; Fig. 3.3). However, the mtDNA data give slightly larger times of divergence, especially at higher levels of differentiation (Table 3.3; Fig.3.3). Similarity between clocks suggests both allozyme data and mtDNA data can be

used to accurately estimate times of divergence between Pacific Northwestern ranids.

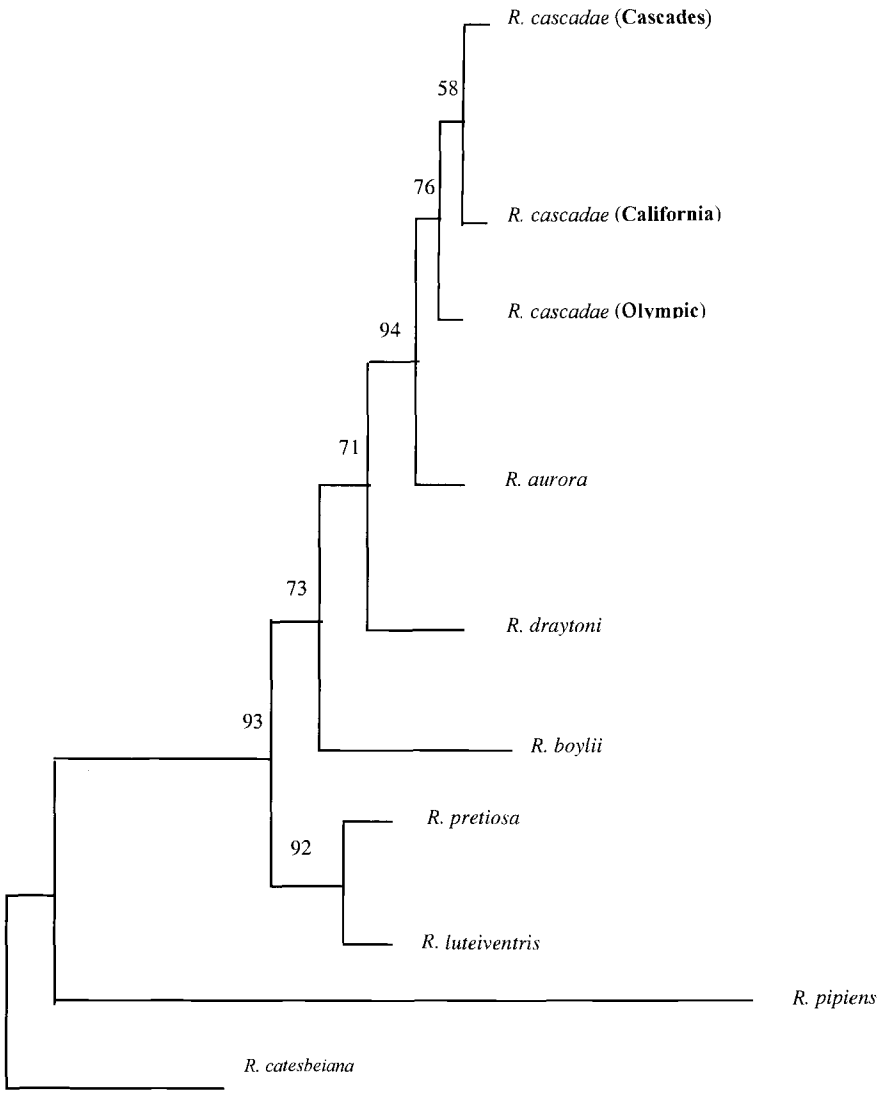


Figure 3.1 Maximum likelihood tree of 1204 bp ND1/tRNA mtDNA fragment from Pacific Northwestern ranid species. Bullfrog (*R. catesbeiana*) and Northern leopard frog (*R. pipiens*) are outgroups. Numbers are bootsrap values after 1000 replications. Only bootstrap values greater than 50 are reported.

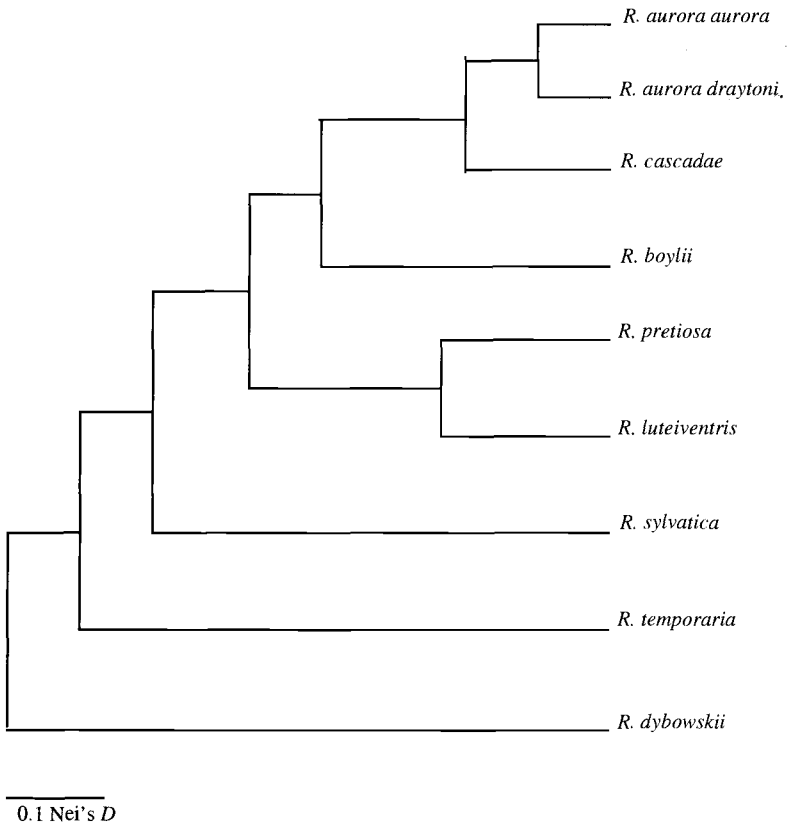


Figure 3.2. UPGMA tree of allozyme data after Green, 1986b.

Table 3.3. Estimated times of divergence among Pacific Northwestern ranid species for allozyme data (above diagonal), and mtDNA data (below diagonal). The allozyme clock is 0.1 Nei's *D* (1972)/Myr after Beerli et al., 1996, using Green's 1986a allozyme data. The mtDNA clock is 1.4% sequence divergence /Myr after Macey et al., 1998a.

	R. boylii	*R. luteiventris*	*R. pretiosa*	*R. aurora draytoni*	*R. aurora aurora*	*R cascadae* (Cascades)	*R. cascadae* (California)	*R. cascadae*(Olympic)
R. boylii	----------	5.9	6.6	5.3	5.6	4.6	?	?
R. luteiventriss	9.6	----------	2	6.9	6.8	4.4	?	?
R. pretiosa	9.6	2.3	----------	5.9	5.8	4.5	?	?
R. aurora draytoni	9.9	8.2	7.7	----------	2	2.3	?	?
R. aurora aurora	9.1	7.9	7.9	6.9	----------	2.2	?	?
R cascadae (Cascades)	8.6	7.5	7.3	6.4	3.7	----------	?	?
R. cascadae (California)	9.1	7.6	7.9	7.1	3.8	2.3	----------	?
R. cascadae(Olympic)	9.2	7.3	7.3	6.5	3.5	2.1	2.6	-------

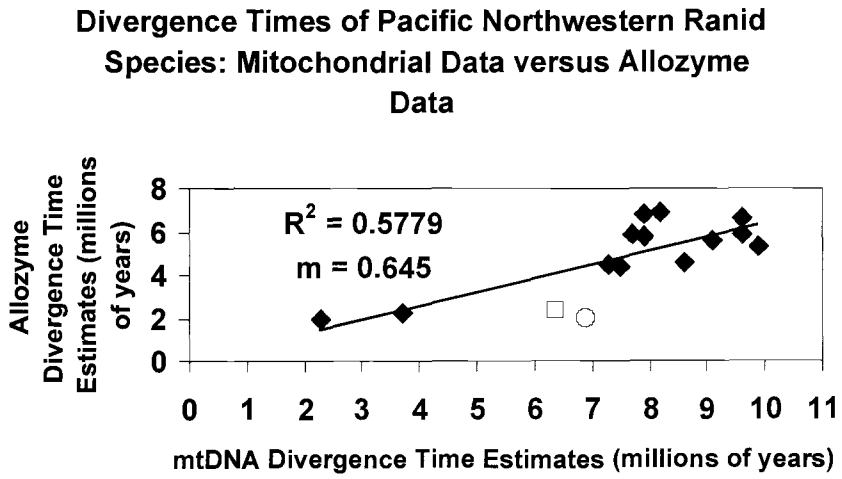


Figure 3.3. Plot of divergence time estimates among Pacific Northwestern ranid species for mtDNA using a molecular clock of 1.4% sequence divergence per million years (Macey et al. 1998a), versus allozyme data from Green (1986b) using a molecular clock of 0.1 Nei's *D* (1972) per million years (Beerli et al. 1996). The white square is the comparison between *R. aurora draytoni* and *R. cascadae*. The white circle is the comparison between *R. aurora aurora* and *R. aurora draytoni*. The comparisons among *R. aurora aurora*, *R. cascadae*, and *R. aurora draytoni* are very different at the mtDNA and allozyme data owing to the close evolutionary relationship between *R. aurora aurora* and *R. cascadae* mtDNA.

Single copy nuclear DNA

Both single copy nuclear loci show *R. aurora aurora* and *R. aurora draytoni* to be each other's closest relative, conflicting with the mtDNA data (Fig.3.4). All taxa had a unique sequence for B22, except for the three *R. cascadae* mtDNA groups (Fig. 3.4).

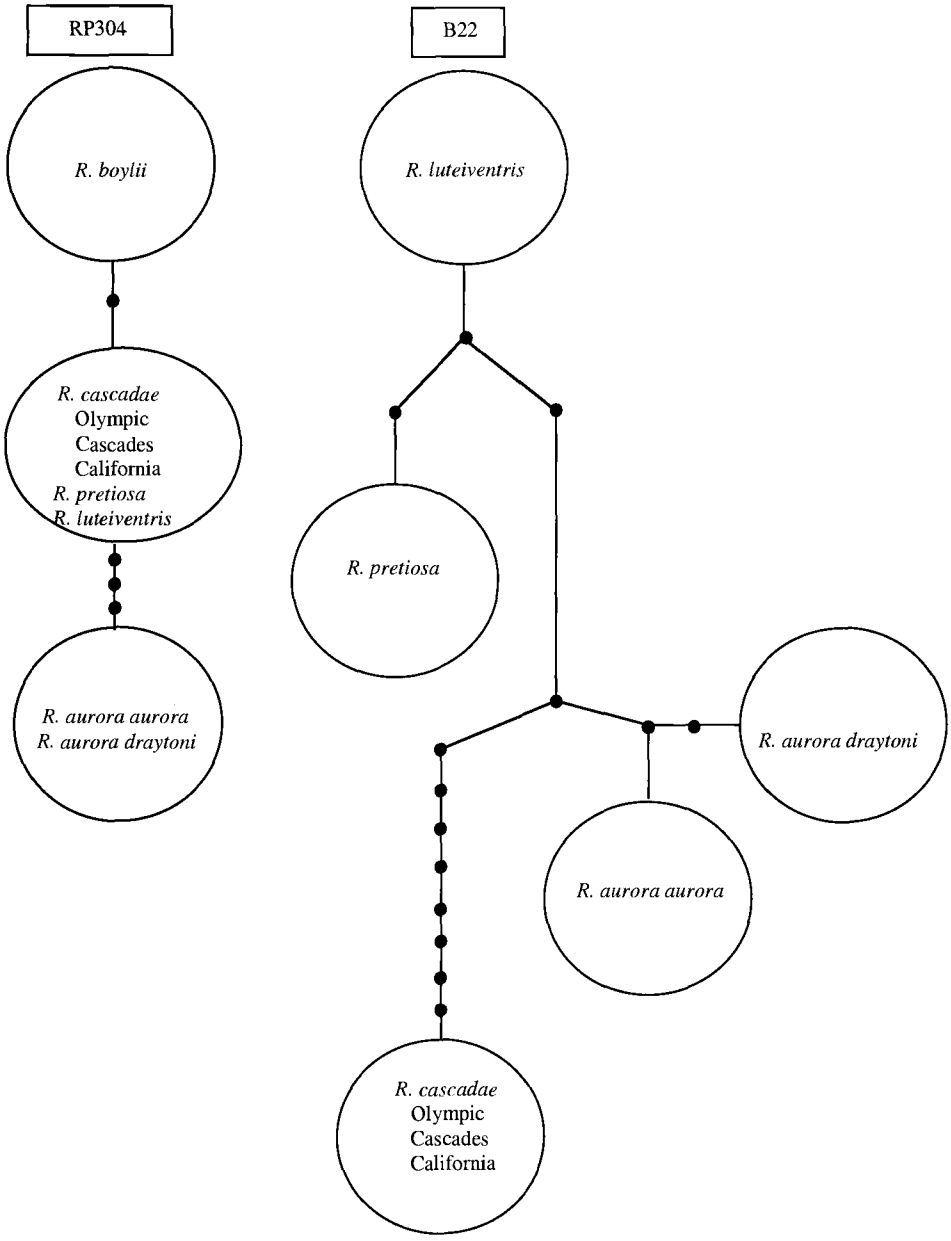


Figure 3.4. Statistical parsimony networks of single copy nuclear loci B22 and RP304. For B22, TCS joined *R. aurora aurora* and *R. aurora draytoni* with 95% confidence in seven steps suggesting they are each other's closest relative. Connections between the remaining taxa were forced with 16 steps. For RP304, TCS joined all taxa with 95% confidence in six steps.

TCS was only able to join *R. aurora aurora* and *R. aurora draytoni* with 95% confidence for locus B22. Connections were forced between the remaining taxa with 16 steps. Taxa-specific differences were not present between some taxa at RP304, and shared sequences are grouped as follows: *R. aurora aurora* and *R. aurora draytoni*, *R. pretiosa*, *R. luteiventris*, and *R. cascadae* (all three mtDNA groups), and *R. boylii* (Fig 3.4). TCS was able to join all taxa with 95% confidence in six steps (Fig. 3.4).

DISCUSSION

The three *R. cascadae* mtDNA groups are monophyletic and appear to have split from each other 2-3 MYA. Additionally, they are as genetically divergent at the ND1/tRNA fragment as the sister species *R. pretiosa* and *R. luteiventris* (between 3-4% sequence divergence). The three *R. cascadae* mtDNA groups are genetically identical at the randomly cloned single copy nuclear loci suggesting that although the mitochondrial genomes of these three groups are substantially differentiated, their nuclear genomes have not yet diverged.

The mtDNA and allozyme molecular clocks give similar dates of divergence among Pacific Northwestern ranids. However, the mtDNA data consistently yield higher dates. It is possible that the allozyme data underestimate the dates of divergence, or have reached a plateau in divergence owing to the

constraints of differentiation of protein versus DNA sequence. For example, there may be sequence divergence at the allozyme loci that is not detected in the allozyme assays due to synonymous substitutions. Because I analyzed mtDNA sequence divergence directly, even synonymous substitutions contributed to the overall estimate of divergence from a common ancestor.

The mtDNA data very clearly indicate *R. aurora aurora* mtDNA is more closely related to *R. cascadae* mtDNA than to *R. aurora draytoni* mtDNA. This result conflicts with previous studies of allozymes (Green 1986b) and karyotypes (Green 1986a), in addition to the nuclear data reported here. There are two likely explanations for the disparity between the mtDNA data and other nuclear molecular markers. First, there may have been hybridization between *R. aurora aurora* and a small population of *R. cascadae* followed by rapid range expansion of the hybrid population, during which time the *R. aurora aurora* mtDNA allele drifted to fixation. Hybridization has been shown in natural populations of Pacific Northwestern ranids (*R. pretiosa* and *R. cascadae*, Green 1985; *R. aurora aurora* and *R. pretiosa*, Blouin, unpublished data). Although there is evidence that hybrids are sterile (Green 1985), hybrid sterility may not have been present when these species first diverged. In order for the hybridization scenario to be true, at some point the effective population size (N_e) of *R. cascadae* would have to have been small. While I have previously shown the N_e of individual *R. cascadae* populations is likely to be quite small (Monsen and Blouin, in review), the N_e across the entire species range is likely to be quite high, as subdivision into many small populations

is expected to increase the overall Ne (Kimura and Crow 1963; Roberston 1964; Nei and Takahata 1993; Lande 1995). Because it is unknown if the genetic structure of *R. cascadae* populations immediately following species radiation was similar to the structure observed today, I can not reject the hybridization hypothesis.

A second explanation for the discrepancy between the mtDNA and nuclear data is that there may have been incomplete lineage sorting of ancestral mtDNA alleles among *R. aurora* and *R. cascadae* complexes. The retention of ancestral polymorphisms has been documented in other species (Helm-Bychowski and Cracraft 1993; Fehrer 1996; Schneider-Broussard et al. 1998; Wilding et al. 2000). In order for this scenario to be true, the speciation events giving rise to the two *R. aurora* species and the *R. cascadae* complexes would have to have occurred in relatively close succession, and there would have to have been a mtDNA polymorphism in the ancestral species at the time of radiation (Fig. 3.5).

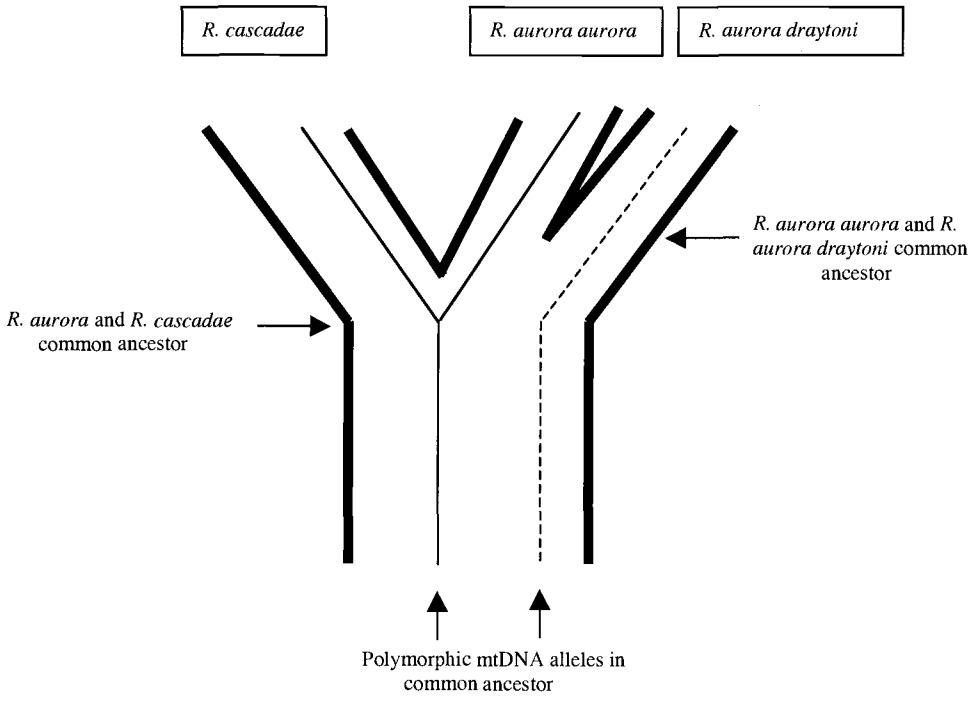


Figure 3.5. Incomplete lineage sorting in the *R. aurora* and *R. cascadae* complexes. *R. cascadae* and *R. aurora aurora* inherited the same mtDNA allele from a common ancestor, while *R. aurora draytoni* inherited a different mtDNA allele. Even though *R. aurora aurora*'s closest relative is *R. aurora draytoni*, *R. aurora aurora*'s mtDNA is most closely related to that of *R. cascadae*.

Both the allozyme and mtDNA molecular clocks date the split of *R. aurora aurora* and *R. cascadae* between 2-3 MYA, and the allozyme clock dates the split between *R. aurora aurora*, *R. aurora draytoni*, and *R. cascadae* between 2-3 MYA. Additionally, Savage (1960) has estimated these species colonized North America at the beginning of the Pleistocene (approximately 2 MYA). Given these species split from a common ancestor in close succession, it is quite plausible that incomplete lineage sorting of ancestral mtDNA alleles has occurred among these species. Based on the data presented here, both the hybridization and incomplete lineage sorting hypotheses are plausible explanations for the discordance between mtDNA and nuclear data. However, given the evidence for rapid radiation of the *R. aurora* and *R. cascadae* groups from a common ancestor, the incomplete lineage sorting hypothesis seems to be the simplest explanation.

CHAPTER FOUR: EXTREME ISLATION BY DISTANCE: THE RELATIVE IMPORTANCE OF GENE FLOW VERSUS RANDOM GENETIC DRIFT IN A DECLINING AMPHIBIAN

ABSTRACT

Given the recent interest in declining amphibian populations, it is surprising that there are so few data on effective population size, genetic drift, and gene flow in anuran species. I used data from seven microsatellite loci to compare the relative importance of gene flow versus random genetic drift in the Cascades frog, *Rana cascadae*. I sampled 18 sites in a hierarchical design (inter-population distances ranging from1-670 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. I also used these 11 fine-scale sites to estimate long-term N_e from expected heterozygosity and from the coalescent, and to estimate a genetic neighborhood size from F_{st}. I 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. The estimate of average genetic neighborhood size (43) agrees with expectations based on the current census sizes of single sites (tens to hundreds of

frogs). Estimates of long-term N_e ranged from hundreds to tens of thousands, depending on the assumed mutation rate. Estimates in the hundreds are consistent with census sizes and with the neighborhood size estimate, but require mutation rates on the order of 10^{-3}. Effective sizes in the thousands are unrealistic for single isolated sites, but could represent the long-term diversity maintained by migration in a metapopulation. Given the large estimates of long-term effective size, the clear pattern of isolation by distance at all spatial scales, and the sharp drop in migration between sites separated by greater than 10 km, I conclude that strong genetic structuring in *R. cascadae* results mainly from low gene flow rather than high genetic drift.

INTRODUCTION

Assessing the scale over which substantial gene flow occurs, and determining the relative importance of gene flow versus random genetic drift are critical for successful management and conservation of threatened and endangered species. Given the recent interest in conservation and management of declining amphibian populations, it is surprising that there are so few data on effective population size and gene flow in anuran species. Among vertebrates, amphibians generally show the highest genetic differentiation across small geographic scales

(Driscoll 1998a, 1998b; Storfer 1999; James and Moritz 2000; Shaffer et al. 2000; but see Burrowes and Joglar 1999). However, 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 (Wilkinson et al. 1996; Evans et al. 1997; Macey et al. 1998; Sumida et al. 1998; James and Moritz 2000). A hierarchical sampling design that includes populations sampled at different spatial scales is necessary to infer patterns of gene flow, and larger sample sizes are needed for accurate estimation of parameters such as effective size. Furthermore, many 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 (Phelps and Allendorf 1983), especially given the small effective number of breeders per generation that may characterize many frog species (Scribner et al. 1997; Waldman and McKinnon 1993). Therefore, there is a need for more studies designed to estimate basic population genetic parameters such as the geographic scale of gene flow, effective sizes and migration rates in anurans.

During a recent phylogeography study of the Cascades frog, *Rana cascadae*, I observed strong genetic differentiation for both mitochondrial and nuclear loci between populations separated by 30-50 km (in review). *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 the state of Washington to the Cascade Mountains in Northern California (Fig. 4.1; Stebbins 1985). Although severe population declines have only been observed in Northern California, *R. cascadae* is still a conservation concern owing to its restricted distribution. Therefore, an understanding of the scale at which populations are substantially connected is important for management of the species.

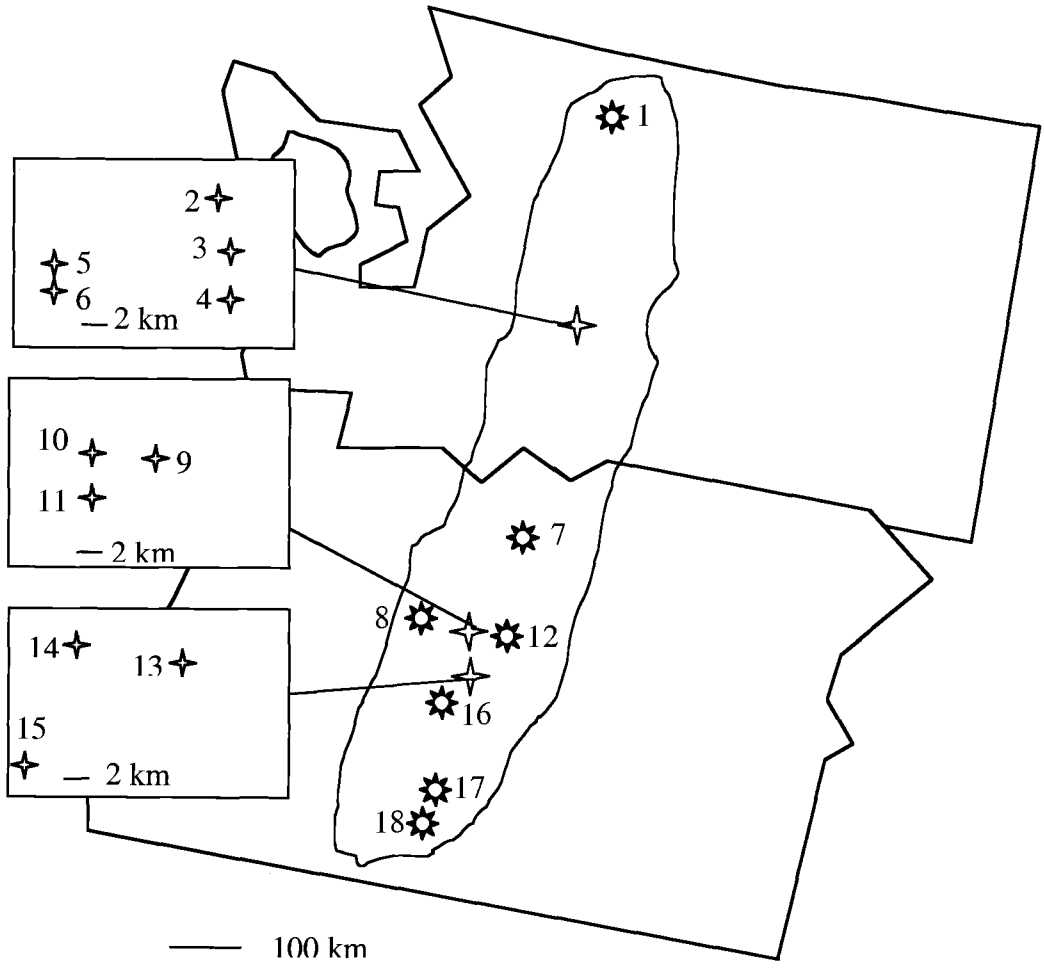


Figure 4.1. *Rana cascadae* sample sites. Populations marked with ✦ were used for gene flow and effective population size estimates, and are illustrated as insets on a finer geographic scale. The species' range in Washington and Oregon is outlined (R. Nauman and D. Olson pers.com.). See Table 4.1 for location names and sample sizes.

In the current study, I used microsatellite loci from adult frogs to determine the geographic scale over which substantial gene flow occurs in *R. cascadae*, and to estimate the long-term effective population size in several small scale populations. Additionally, I investigated if isolation by distance is present among populations throughout most of the species' range. From the data I infer the relative importance of gene flow and random genetic drift in shaping the population genetic structure of *R. cascadae*.

METHODS

Sampling

Tissue was collected from adult *Rana cascadae* by toe clipping during the summers of 1997, 1998, and 1999. Samples were collected from 18 populations throughout Oregon and Washington (Fig. 4.1, Table 4.1). It is important to note that I 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 interbreeding ponds that compose a larger neighborhood. Eight populations were used in the previous study. Sample sizes ranged from 18 to 30 individuals per population (Fig. 4.1, Table 4.1). Populations were chosen to cover

the majority of the species' range where gene flow is most likely to occur. 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 (Fig. 4.1, Table 4.1). These three "fine-scale" sets were used to estimate long term effective population sizes and the number of migrants exchanged between populations per generation.

Table 4.1. *Rana cascadae* sampling site information. Sample Sizes are averaged across seven microsatellite loci. Populations marked with * were used to estimate number of migrants exchanged per generation and effective populations size.

Population (Abbreviation)	Latitude, Longitude	Average Sample Size
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

Molecular methods

Total genomic DNA was extracted from each toe using a standard phenol-chloroform protocol (Hillis et al. 1996). I used seven microsatellite loci that were

originally developed for *R. pretiosa* and *R. luteiventris* (SCF120, RC287, RP193, SCF134, SCF128, RP17, and SCF139; Table 4.2; Blouin, unpublished data). PCR amplifications were carried out in 25 μL 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 μM 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 μL. Amplification was carried out in a Perkin-Elmer 9600 thermocycler under the following conditions: 94° C 3 minutes, followed by 30 cycles of 94° C 45 seconds, locus-specific annealing temperature (Table 2) 30 seconds, 72° C 30 seconds, and a final extension at 72° C for 7 minutes. 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.).

Table 4.2. Primer and product information for seven microsatellite loci.

Locus	F Primer R Primer	Clone Size (bp)	Repeat #	Allele Size Range (bp)	Total # Alleles	Ave.# Alleles Per Population	Anneal (°C)
SCF120	5'aaccctggtagtatgaccaac3' 5'gtggaactccagttatgatcc3'	180	16	137-185	13	5.2	56
SCF134	5'tgggaaaagactctgtggt3' 5'aggaaatgtgtggaagcat3'	240	11	225-273	20	6.0	57
SCF128	5'agaaaagcggacttctgaaat3' 5'agccataatccctgttaaacc3'	236	7	221-261	10	4.5	57
SCF139	5'ggcatggttaaagtggaactc3' 5'tgcatgtctgtaatggacctc3'	277	17	230-278	13	5.4	58
RP17	5'gtgtagacaaacaaatgaaagtcag3' 5'gtctctacttccatccaaccattcc3'	118	7	111-119	3	1.8	50
RP193	5'ccattttctctctgatgtgtgt3' 5'tgaagcagatcactggcaaagc3'	183	21	143-203	16	6.5	49
RC287	5'atagggatgtgggcgggaagattga3' 5'caaggcactgtagatagatagaat3'	279	8	268-284	4	1.7	51

Data analysis

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 (Raymond and Rousset 1995). 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). All populations are in a continuous habitat with nearest neighbors separated by 1-50 km (Fig. 4.1). For the three fine-scale clusters, it is probably reasonable to assume they are in approximate drift-migration equilibrium (Whitlock and McCauley 1999). Therefore, in the three fine-scale clusters (Fig. 4.1) I estimated the number of migrants exchanged per generation between populations (N_em) from $F_{st}=1/(4N_em+1)$ (Wright 1951, 1965). Pairwise migration in the three fine-scale clusters was also estimated using the MIGRATE program v. 1.5.1 (Beerli 1997-2001; Beerli and Felsenstein 1999, 2001). MIGRATE estimates number of migrants exchanged between populations per generation using an expansion of the coalescent theory (Kingman 1982 a and b) which includes migration (Hudson 1990; Nath and Griffiths 1993; Notohara 1994). MIGRATE yields pairwise migration estimates that may be asymmetric between populations. Therefore, migration estimates between pairs of populations within a cluster were averaged to obtain a value comparable to that obtained from F_{st}. Isolation by distance for the

entire set of 18 populations was tested with a Spearman rank correlation coefficient from the comparison of all pairwise $F_{st}/1\text{-}F_{st}$ values with pairwise geographic distances using the program ISOLDE in GENEPOP v. 3.2a. Comparing $F_{st}/1\text{-}F_{st}$ versus geographic distance has been shown to be a more accurate indicator of isolation by distance than F_{st} versus geographic distance (Rousset 1997).

Estimation of N_e

For the three groups of fine-scale populations (Fig. 4.1, Table 4.1), microsatellite allele frequency and repeat number data were used to generate maximum likelihood estimates of the parameter θ using the program MIGRATE v. 1.5.1. It is possible to estimate the long-term N_e of a population using the relationship $\theta = 4N_e\mu$ for nuclear loci where μ is the mutation rate per site per generation. I used a microsatellite mutation rate of 10^{-3} to 10^{-4} per generation to estimate a range of N_e. This rate is typical of other poikilotherms (Banks et al. 1999; Balloux and Lugon-Moulin 2002; Steinberg et al. 2002), including anurans (Call 1997).

I also estimated the long term N_e from expected heterozygosity at microsatellite loci for the three small scale clusters. Because there is considerable debate over whether microsatellite loci follow the infinite alleles model (IAM) or stepwise mutation model (SMM) of mutation, it has been suggested that both

models should be considered when estimating effective population sizes (Lehmann et al. 1998). I used average expected heterozygosity across all loci with both 10^{-3} and 10^{-4} as a mutation rate to estimate a range of N_e for both the SMM and the IAM using the following formulae (SMM: Nei 1987; IAM: Maruyama and Nei 1981):

$$\text{IAM:} \quad N_e = \frac{H}{4\mu(1-H)}$$

$$\text{SMM:} \quad N_e = \left[\left[\frac{1}{1-H}\right]^2 - 1\right] \Big/ 8\mu$$

I estimated the genetic neighborhood size from the y intercept of the regression of log of geographic distance versus log of $F_{st}/1\text{-}F_{st}$ (Rousset 1997).

RESULTS

Genetic variation

The number of alleles per locus ranged from 1-11 per population with an average of 4.5 alleles per population per locus (Appendix 4.1; Table 4.5). Average observed heterozygosities per population ranged from 0.44 to 0.80 (Appendix 4.1). After a Bonferroni correction for multiple statistical tests, almost all populations

were in HWE for all loci. Five populations were not in Hardy-Weinberg equilibrium at single loci (Gold Lake-RP193, 7 Mile Creek-SCF139, Reflection Lakes-SCF120, Mt. Rainier-SCF120, and Waldo Lake-RP17). Deviations from Hardy-Weinberg equilibrium could result from the presence of a null allele or unsuspected subdivision creating a Wahlund effect. A Wahlund effect would influence all loci, so null alleles are more likely. However, there were few or no failed PCR reactions at these loci in these populations, suggesting null alleles were not in high frequency.

Strong isolation by distance is apparent for the entire set of 18 populations from the graph of $F_{st}/1\text{-}F_{st}$ versus geographic distance (Spearman rank correlation coefficient p-value = 0.00, Fig. 4.2; Table 4.3). At the finest geographic scale, there is substantial genetic differentiation and low migration at very small geographic distances (Fig. 4.3A; Table 4.3). For N_em estimated from F_{st}, there is a marked decrease in migration around 10 km, with populations separated by greater distances exchanging many fewer migrants per generation. Although the coalescent estimates of N_em are more variable, the graph of coalescent N_em versus distance shows the same trend (Fig. 4.3B).

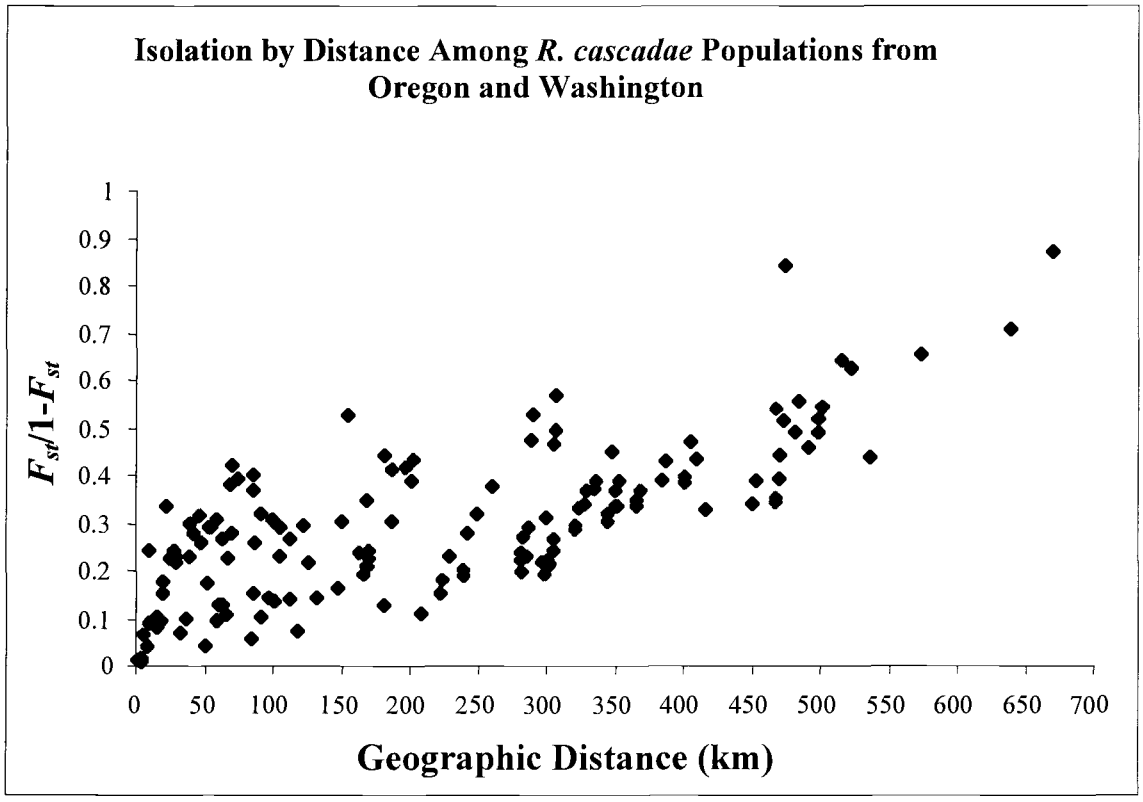


Figure 4.2. Isolation by distance in 18 *R. cascadae* Oregon and Washington populations. Comparisons are pairwise between all populations.

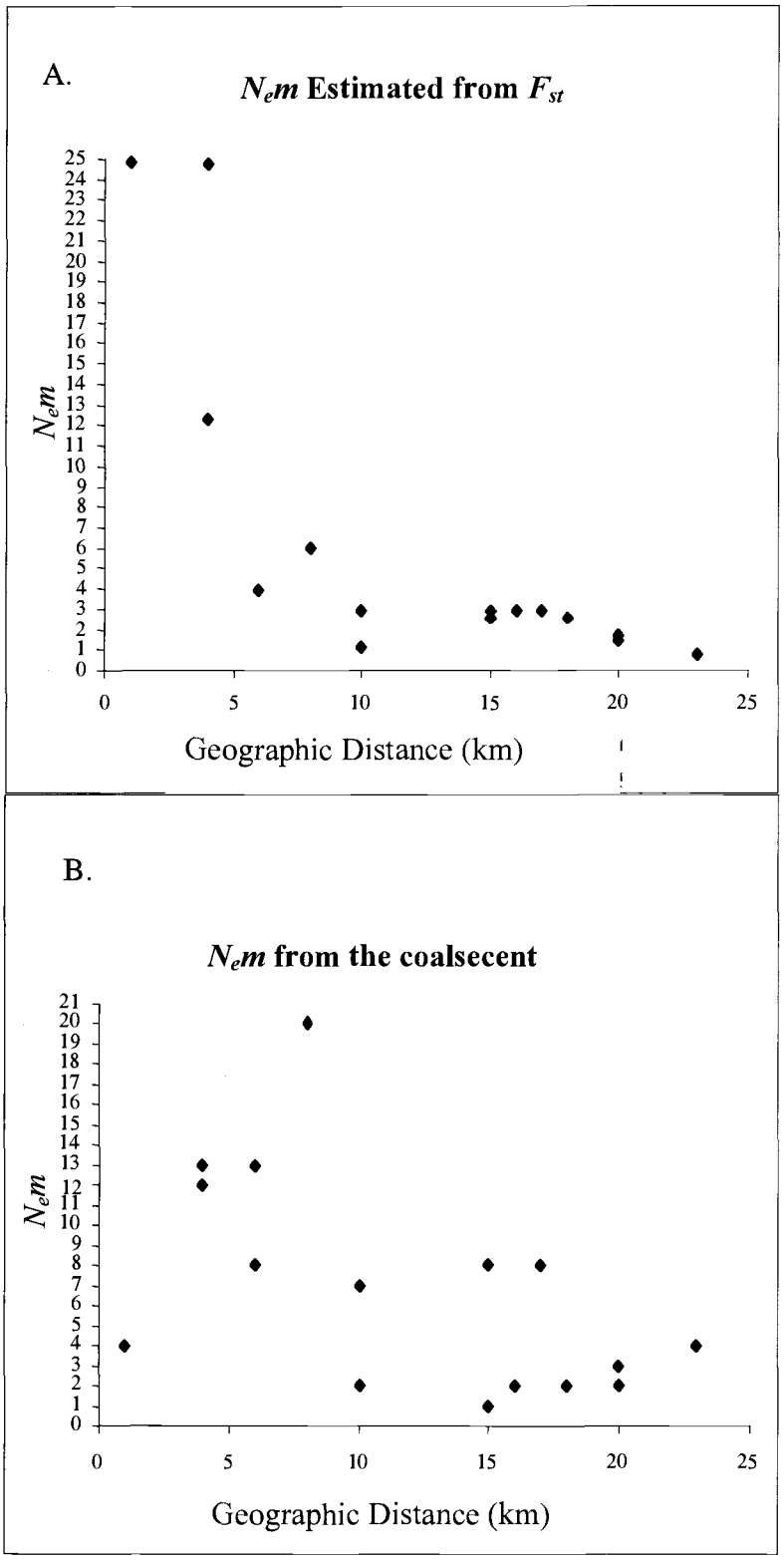


Figure 4.3. Number of migrants exchanged per generation between 3 fine-scale sets of populations of *R. cascadae* estimated from F_{st} (A) and the coalescent (B). Estimates from the coalescent are averages of pairwise estimates within each fine-scale cluster.

Table 4.3. The upper diagonal is estimated N_em from F_{st} and the coalescent approach (in parentheses) for three fine-scale population comparisons in *R. cascadae*. The lower diagonal is geographic distance in km.

Population	9	10	11
9	---	4 (13)	6 (20)
10	6	---	12.3 (12)
11	8	4	---

Population	13	14	15
13	---	1.1 (1.8)	0.75 (3.5)
14	10	---	2.5 (7)
15	23	15	---

Population	2	3	4	5	6
2	---	3.9 (7.8)	2.9 (6.5)	1.4 (2.2)	1.7 (2.6)
3	6	---	24.8 (13.1)	2.9 (1.4)	2.9 (2)
4	10	4	---	2.9 (8)	2.5 (2.2)
5	20	15	17	---	24.8 (4)
6	20	16	18	1	---

Effective population size and genetic neighborhood

Estimates of long-term N_e from the coalescent estimate of Θ among the 11 fine-scale populations range from 50 to 178 when $\mu = 10^{-3}$ and 500 to 1 780 when $\mu = 10^{-4}$ (Table 4.4). Estimates of long-term N_e from expected average heterozygosities range from 205 to 750 for the IAM and 288 to 1 875 for the SMM when $\mu = 10^{-3}$. When $\mu = 10^{-4}$, the corresponding N_e estimates range from 2 050 to 7 500 for the IAM and 2 880 to 18 750 for the SMM (Table 4.4). The estimate of genetic neighborhood size was 43 based on a y intercept of 1.63.

Table 4.4. Estimates of N_e for 11 fine-scale *R. cascadae* populations at seven microsatellite loci. N_e estimates from the parameter θ are over all loci with 95% confidence limits in parentheses. N_e estimates from average expected heterozygosity were calculated for both the infinite alleles model (IAM) and the stepwise mutation model (SMM). For all estimates, the top set of numbers is calculated using a mutation rate of 10^{-3} per generation and the bottom set of numbers is calculated using a mutation rate of 10^{-4} per generation.

Population	N_e (from θ)	Average *He*	N_e (IAM)	N_e (SMM)
Mt. Rainier, WA	105 (97-115)	0.75	750	1 875
	1 050 (970-1 150)		7 500	18 750
Berkeley Park, WA	82 (75-92)	0.73	730	1 590
	820 (750-920)		7 300	15 900
Elysian Fields, WA	50 (47-54)	0.64	640	840
	500 (470-540)		6 400	8 400
Paradise River, WA	164 (146-185)	0.61	305	697
	1 640 (1 460-1 850)		3 050	6 970
Reflection Lakes, WA	89 (82-98)	0.64	640	697
	890 (820-980)		6 400	6 970
Benson Lake Trail, OR	84 (77-92)	0.54	270	466
	840 (770-920)		2 700	4 660
Melakwa Lake, OR	134 (119-152)	0.73	730	1 590
	1 340 (1 190-1 520)		7 300	15 900
McKenzie Pass, OR	130 (120-142)	0.67	670	1 023
	1 300 (1 200-1 420)		6 700	10 230
Many Lakes Trail, OR	76 (69-84)	0.54	270	466
	760 (690-840)		2 700	4 660
Gold Lake, OR	178 (162-196)	0.73	730	1 590
	1 780 (1 620-1 960)		7 300	15 900
Waldo Lake, OR	160 (147-175)	0.69	690	1 176
	1 600 (1 470-1 750)		6 900	11 760

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 4.5).

Differentiation among populations

Although *R. cascadae* has typical levels of genetic variation within populations, the species shows greater genetic differentiation across small geographic scales than other anurans for which population differentiation at microsatellite loci has been investigated (Table 4.5).

R. cascadae populations show clear isolation by distance over their entire range (Fig. 4.2) and a striking pattern of reduced gene flow between populations separated by more than 10 km (Fig. 4.3). Previous research on migration distances in anurans suggests adult frogs rarely move distances more than a few km (Dole 1971; Breden 1987,1988; Berven and Grudzien 1990). Therefore, gene flow is most likely occurring in a stepping stone fashion where nearest neighbors exchange migrants in a set of closely-spaced populations that cover the species' range in the Cascades Mountains (Fig. 4.1).

This study is one of the few that allows an estimate of neighborhood size for anurans. I estimated the genetic neighborhood size in *R. cascadae* to be 43 frogs. Census sizes per site are typically tens to at most a few hundred frogs (pers. obs.), so this estimate of neighborhood size is consistent with expectations of the current N_e for individual ponds (Nunney and Elam 1994). Additionally, this neighborhood size estimate is consistent with estimates for other amphibians (Driscoll 1999b; Funk et al. 1999).

Table 4.5. Comparison of anuran microsatellite studies.

Species	Authors	Range N	# of Loci	# of Populations (average)	Measures of Differentiation	Average # Alleles/Locus/Pop.	H_o H_e Range	Geographic Range (km)
Bufo bufo	Scribner et al., 1994	40-40 (40) adults	1	3	Fis=-0.07 Fst=0.016	6	not reported 0.625-0.750	5.5-14.5
Rana luteiventris	Call, 1997	8-28 (15) adults	3	8	$\hat{F}$ = 0.066 $\hat{\phi}$st = 0.056	4.4	0.00-1.0 0.16-0.91	49-165
Hyla regilla	Call, 1997	15-22 (16) adults	3	3	$\hat{F}$ = 0.056 $\hat{\phi}$st = 0.07	7.8	0.37-0.87 0.42-0.83	3-34
Bufo calamita	Rowe et al., 1998	25-40 (39) larvae	8	40 (4 regions)	Fst=0.068-0.531 DNei=0.38-0.807 Dcsc=0.208-0.652 $\delta\mu^2$=0.127-1.752 Rst=0.099-0.568	3.25	0.245-0.350 0.242-0.376	100-300
Rana sylvatica	Newman and Squire, 2001	32-102 (50) adults and metamorphs	5	12	Fst=0.00-0.03	1.67	0.16-0.60	0.05-20 0.24-0.73
Rana cascadae	Monsen and Blouin (this study)	13-30 (22) adults	7	18	Fst=0.01-0.52	4.5	0.04-0.93	1-670 0.04-0.88

Measures of differentiation: Fis and Fst (Wright, 1951), DNei (Nei, 1972), $\hat{F}$ (Lynch, 1991), $\hat{\phi}$st (Michalakis and Excoffier, 1996), Dcsc (Cavalli-Sforza and Edwards, 1967), $\delta\mu^2$ (Goldstein et al., 1995), and Rst (Slatkin, 1995).

Ho= observed heterozygosity, He= expected heterozygosity.

Drift vs. migration

Although small effective size and low migration can both explain high F_{st} among populations, there are two reasons that low migration may be the dominant factor controlling genetic structure in *R. cascadae*. First, there is a clear signal of isolation by distance, even at the smallest geographic scale. High dispersal in combination with small and/or fluctuating population sizes could produce a high overall F_{st}, but would produce a more random pattern of differentiation among populations. Second, the species shows normal levels of genetic variation within populations, and the long-term effective size estimates were surprisingly large.

Effective population size

I observed N_e values ranging from tens to a few thousands depending on what mutation rate was used, and whether the estimate was based on coalescent theory or average expected heterozygosity (Table 4.4). Long-term estimates based on the IAM were consistently larger than those based on the SMM, but the differences are not substantial. Additionally, the alleles present in *R. cascadae* at the seven microsatellite loci used in this study do not obviously appear to deviate from the SMM (there are no major gaps in allele sizes, Appendix 4.1). Therefore,

there is no reason to discount the SMM as a mutational model for microsatellite loci in *R. cascadae*.

Given census population sizes are in the tens to hundreds of adults for these sites, N_e values that are in the hundreds to thousands seem biologically unrealistic for current N_e of individual populations. Despite the high genetic differentiation and low gene flow observed between *R. cascadae* populations, enough gene flow is probably occurring to maintain moderate levels of genetic diversity over time (Vuchetich and Waite 1999). The genetic neighborhood size estimates are more consistent with the expectation for current N_e per site. Estimates of N_e based on the coalescent approach calculated from $\mu = 10^{-3}$ are consistent with other studies of neighborhood size and current effective population size of amphibian populations (Merrell 1968; Gill 1978; Berven and Grudzien 1990; Scribner et al. 1997; Driscoll 1999b; Funk et al. 1999; Carpenter et al. 2001; Jehle et al. 2001; but see Eastseal 1985 and Crawford 2000). However, given the lack of data on microsatellite mutation rate in amphibians, future studies estimating this mutation rate will be instrumental in testing the accuracy of the N_e estimates reported here.

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.

The extreme genetic differentiation at such small geographic distances suggests metapopulation structure is weak within *R. cascadae*, and populations that go extinct are 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 re-colonization 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 *Rana 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.

CHAPTER FIVE: GENERAL CONCLUSIONS

The research presented in Chapters 2-4 describes the population genetic structure of a declining amphibian, *Rana cascadae*, throughout the entire species' range for both mitochondrial and nuclear DNA markers. My results indicate mtDNA and nuclear DNA can show different patterns of evolutionary genetic history on both interspecific and intraspecific levels, and that *R. cascadae* exhibits a classic isolation by distance pattern of genetic differentiation that is likely shaped by reduced gene flow between populations across a very small geographic scale.

In Chapter 2 I described the phylogeographic structure of *R. cascadae* across the entire species' range for both mtDNA and nuclear markers in order to assess population genetic differentiation, to determine if any populations of *R. cascadae* warrant separate conservation status because of genetic uniqueness, and compare patterns of genetic differentiation between mtDNA and nuclear markers. I found three major mtDNA haplotype groups corresponding to three geographic locations: the Olympic Peninsula, the Cascades of Washington and Oregon, and Northern California. A different pattern emerged with the nuclear markers. Specifically, there was no break at the Olympic Peninsula. This discordance between mtDNA and nuclear markers is most likely explained by gene flow between the Olympic and Washington populations in recent evolutionary time that

has homogenized the nuclear genome. This homogenization is not apparent in the mtDNA owing to the expected fourfold reduction in N_e for the mitochondrial genome, and potential fixation of the Olympic mtDNA allele in the single Olympic population I examined. Taken together, the results from both markers suggest the presence of two DPSs in *R. cascadae*: Washington/Oregon, and California populations. Although the status of the Olympic populations as a DPS is questionable, they should still be managed as a separate group owing to the strong mtDNA divergence and physical separation of the Olympic population used in this study. The results from Chapter 2 underscore the importance of using multiple molecular markers when using molecular genetic data to group populations for management purposes.

In Chapter 3 I examined the phylogenetic relationship between the three *R. cascadae* mtDNA haplotype groups and compared them to other closely related ranid taxa for both mtDNA and nuclear markers. I found that the mtDNA of the three *R. cascadae* haplotype groups is as divergent as the sister species *R. pretiosa* and *R. luteiventris*. I observed no differences between the three *R. cascadae* groups at the two randomly-cloned single copy nuclear loci used in this study. Additionally, the mtDNA of *R. aurora aurora* is more closely related to the mtDNA of *R. cascadae* than to the mtDNA of its own subspecies *R. aurora draytoni*, despite several independent studies of nuclear DNA including data presented in Chapter 3. There are two hypotheses to explain the discordance between the mitochondrial and nuclear DNA. First, there may have been a past

extensive hybridization between *R. aurora aurora* and *R. cascadae*. Second, there may have been incomplete lineage sorting of ancestral mtDNA alleles during the radiation of the *R. aurora* and *R. cascadae* complexes. I used an mtDNA molecular clock to date the split of the three *R. cascadae* mtDNA haplotype groups and *R. aurora aurora* and found they all diverged from each other approximately 2-3 million years ago. Although both hypotheses are possible, the rapid radiation of the *R. aurora* and *R. cascadae* complexes suggests incomplete lineage sorting is the most plausible explanation for the discordance between mtDNA and nuclear markers at the interspecific level.

In Chapter 4 I used microsatellite allele frequency data to determine the geographic scale over which gene flow occurs in *R. cascadae*, and to estimate long-term effective population and genetic neighborhood sizes. *R. cascadae* displays a classic isolation by distance pattern of geographic differentiation with very low gene flow occurring between populations separated by 10 km or more. Long-term estimates of effective population sizes are high, but genetic neighborhood size estimates are low and in agreement with current census sizes. Such low gene flow across a small geographic scale suggests re-colonization of *R. cascadae* populations is unlikely to occur once they have gone extinct.

Several general patterns are apparent when looking at the results of all Chapters presented here. Mitochondrial and nuclear markers can show very different patterns of genetic structure on both interspecific and intraspecific levels. Most studies that use molecular genetic markers to group populations for

management and conservation generally only use a single type of marker, most often mtDNA. The results presented in this dissertation illustrate the need to use multiple molecular markers when identifying groups for management purposes. Additionally, most studies of interspecific phylogeny based on molecular genetic markers often only use a single type of molecular marker. The differences presented here among mtDNA and nuclear markers in ranid phylogeny also illustrate the need to use multiple molecular markers when describing interspecific relationships. In addition to accurately grouping populations for management and more accurately describing interspecific phylogenies, exploring the differences in mitochondrial and nuclear markers can shed light on past evolutionary events that have shaped the current population genetic structure and interspecific relationships.

Finally, the data presented here illustrate gene flow and genetic neighborhood size are limited in *R. cascadae*, suggesting re-colonization of extinct populations may be unlikely to occur. It is possible that rapid extinction with no re-colonization is the cause of the recent and sudden decline of *R. cascadae* in Northern California.

BIBLIOGRAPHY

Avise, J., J. Neigel, and J. Arnold. 1984. Demographic influences on mitochondrial DNA lineage survivorship in animal populations. Journal of Molecular Evolution **20:** 99-105

Avise, J., J. Arnold, R. Ball, E. Bermingham, T. Lamb, J. Neigel, C. Reeb, and N. Saunders. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annual Review of Ecology and Systematics. **18:** 489-522

Balloux, F. and N. Lugon-Moulin. 2002. The estimation of population differentiation with microsatellite markers. Molecular Ecology **11:**155-165

Banks, M., M. Blouin, B. Baldwin, V. Rashbrook, H. Fitzgerald, S. Blankenship, and D. Hedgecock. Isolation and inheritance of novel microsatellites in chinook salmon (*Onchorhynchus tschawytscha*). Journal of Heredity **90(2):**281-288

Baverstock P, Moritz C. 1996. Project design. In: Molecular Systematics (eds. Hillis DM, Moritz C, Mable BK), pp. 17-27. Sinauer Associates, Inc. Publishers

Beerli, P., H. Hotz, and T. Uzzell. 1996. Geologically dated sea barriers calibrate a protein clock for Aegean water frogs. Evolution **50(4):**1676-1687

Beerli, P. 1997-2001. MIGRATE: documentation and program, part of LAMARC. Version 1.5.1

Beerli, P. and J. Felsenstein. 1999. Maximum likelihood estimation of migration rates and population numbers of two populations using a coalescent approach. Genetics **152(2):**763-773

Beerli, P and J. Felsenstein. 2001. Maximum likelihood estimation of a migration matrix and effective population sizes in n subpopulations using a coalescent approach. Proceedings of the National Academy of Sciences USA **98(8):**4563-4568

Berven, K and T. Grudzien. 1990. Dispersal in the wood frog (*Rana sylvatica*): implications for genetic population structure. Evolution **44:**2047-2056

Birky, C., T. Maruyama, and P. Fuerst. 1983. An approach to population and evolutionary genetic theory for genes in mitochondria and chloroplasts, and some results. Genetics 1983 **103:**513-527.

Blaustein, A., D. Wake, and W. Sousa. 1994. Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. Conservation Biology **8(1):**60-71

Blaustein, A., and D. Wake. 1995. The puzzle of declining amphibian populations. Scientific American. **1995:**52-57

Bowcock, A., A. Ruiz-Linares, J. Tomfohrde, E. Minch, and J. Kidd. 1994. High resolution of human evolutionary trees with polymorphic microsatellites. Nature **368:**455-457

Bowen, B. 1998. What is wrong with ESUs?: the gap between evolutionary theory and conservation principles. Journal of Shellfish Research **17(5):**1355-1358

Breden, F. 1987. The effect of post-metamorphic dispersal on the population genetic structure of Fowler's Toad, *Bufo woodhousei fowleri*. Copeia **1987:**386-395

Breden, F. 1988. Natural history and ecology of Fowler's toad, *Bufo woodhousei fowleri* (Amphibia: Bufonidae), in the Indiana Dunes National Lakeshore. Fieldiana Zoology **49:**1-16

Briggs, J., and R. Storm. 1970. Growth and population structure of the cascades frog, *Rana cascadae* Slater. Herpetologica **26(3):**283-300

Buonaccorsi, V., K. Reece, L. Morgan, J. Graves. 1999. Geographic distribution of molecular variance within the blue marlin (*Makaira nigricans*): a hierarchical analysis of allozyme, single-copy nuclear, and mitochondrial DNA markers. Evolution **53(2):**568-579

Bury, R., and C. Pearl. 1999. Klamath-Siskiyou herpetofauna: biogeographic patterns and conservation strategies. Natural Areas Journal **19(4):**341-350

Burrowes, P., and R. Joglar. 1999. Population genetics of the Puerto Rican cave-dwelling frog, *Eleutherodactylus cooki*. Journal of Herpetology **33(4):**706-711

Call, DR. 1997. Microsatellite characteristics and population structure for two

anurans *Rana luteiventris* and *Hyla regilla*. PhD Thesis, Washington Sate University, Pullman, WA

Carpenter, D, R. Jung, and J. Sites. 2001. Conservation genetics of the endangered Shenandoah salamander (*Plethodon shenandoah*, Plethodontidae). Animal Conservation **4:**111-119

Case, S. 1978. Biochemical systematics of members of the genus Rana native to Western North America. Systematic Zoology. **27**:299-311

Cavalli-Sforza, L. and A. Edwards. 1967. Phylogenetic analysis: models and estimation procedures. Evolution **21:**550-570

Clement, M., D. Posada, and K. Crandall. 2000. TCS: a computer program to estimate gene genealogies. Molecular Ecology **9:**1657-1659

Crandall, K., and A. Templeton. 1993. Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. Genetics **134:**959-969

Crawford, A. 2000. Evolution and maintenance of a color pattern polymorphism in neotropical frogs. PhD Thesis, University of Chicago, Chicago, IL

Dole, J. 1971. Dispersal of recently metamorphosed leopard frogs, *Rana pipiens*. Copeia **1971:**221-228

Driscoll, D. 1998a. Genetic structure, metapopulation processes and evolution influence the conservation strategies for two endangered frog species. Biological Conservation **83(1):**43-54

Driscoll, D. 1998b. Genetic structure of the frogs *Geocrinia luteas* and *Geocrinia rosea* reflects extreme population divergence and range changes, not dispersal barriers. Evolution **52(4):**1147-1157

Driscoll, D. 1999a. Skeletochronological assessment of age structure and population stability for two threatened frog species. Australian Journal of Ecology **24(2):**182-189

Driscoll, D. 1999b. Genetic neighborhood and effective population size for two endangered frogs. Biological Conservation **88:**221-229

Drost, C., and G. Fellers. 1996. Collapse of a regional frog fauna in the Yosemite area of the California Sierra Nevada, USA. Conservation Biology **10 (2):** 414-425

Eastseal, S. 1985. The ecological genetics of introduced populations of the giant toad *Bufo marinus*. II. effective population size. Genetics **110:**107-122

Estoup, A., and B. Angers. 1998. Microsatellites and minisatellites for molecular ecology: theoretical and empirical considerations *in* Advances in molecular ecology, ed. G. Carvalho. IOS Press

Evans, B., J. Morales, M. Picker, D. Kelley, and J. Melnick. 1997. Comparative molecular phylogeography of two *Xenopus* species, *X. gilli* and *X. laevis*, in the South-Western Cape Province, South Africa. Molecular Ecology **6:**333-343

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

Farris, J., A. Kluge, and M. Mickevich. 1982. Immunological distance and the phylogenetic relationships of the *Rana boylii* species group. Systematic Zoology **31:**479-491

Fellers, G., and C. Drost. 1993. Disappearance of the cascades frog *Rana-cascadae* at the southern end of its range California USA. Biological Conservation **65(2):**177-181

Fehrer, J. 1996. Conflicting character distribution within different data sets on cardueline finches: artifact or history? Molecular Biology and Evolution **13(1):**7-20

Frankham, R. 1995. Conservation Genetics. Annual reviews in genetics **29:**305-327

Franck, P., L. Garnery, A. Loiseau, B. Oldroyd, H. Hepburn, M. Solignac, and J. Cornuet. 2001. Genetic diversity of the honeybee in Africa: microsatellite and mitochondrial data. Heredity **86(2001):**420-430

Friedl, T., and G. Klump. 1997. Some aspects of population biology in the European treefrog, *Hyla arborea*. Herpetologica **31(1):**52-59

Funk, C., D. Tallmon, and F. Allendorf. 1999. Small effective population size in the long-toed salamander. Molecular Ecology **8:**1633-1640

Garza, J., M. Slatkin, and N. Freimer. 1995. Microsatellite allele frequencies in humans and chimpanzees, with implications for constraints on allele size. Molecular Biology and Evolution **12:**594-603

Gill, D. 1978. Effective population size and interdemic migration rates in a meta-population of the red-spotted newt, *Notophthalmus viridescens*. Evolution **32:**839-849

Goldstein, D., A. Linares, L. Cavalli-Sforza, and M. Feldman. 1995. Genetic absolute dating based on microsatellites and the origin of modern humans. Proceedings of the National Academies of Sciences USA **92:**6723-6727

Goudet, J. 1995. FSTAT (vers. 1.2): a computer program to calculate F-statistics. Journal of Heredity **86:**485-486

Goudet, J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3)

Green, D. 1985. Natural hybrids between the frogs *Rana cascadae* and *Rana pretiosa* (Anura: Ranidae). Herpetologica **41(3):**262-267

Green, D. 1986a.Systematics and evolution of Western North American frogs allied to *Rana aurora* and *Rana boylii*: karyological evidence. Systematic Zoology **35(3):**273-282

Green, D. 1986b.Systematics and evolution of Western North American frogs allied to *Rana aurora* and *Rana boylii*: electrophoretic evidence. Systematic Zoology **35(3):**283-296

Green, D., T. Sharbel, J. Kearsley, and H. Kaiser. 1996. Postglacial range fluctuation, genetic subdivision and speciation in the Western North American spotted frog complex, *Rana pretiosa*. Evolution **50(1):**374-390

Green, D., H. Kaiser, T. Sharbel, J. Kearsley, and K. McAllister. 1997. Cryptic species of spotted frogs, *Rana pretiosa* complex, in Western North America. Copeia **1997(1):**1-8

Guo, S., and E. Thompson. 1992. Performing the exact test of Hardy-Weinberg proportions for multiple alleles. Biometrics **48:**361-372

Hasegawa, M., H. Kishino, and T. Yano.1985. Dating of the human ape splitting by a molecular clock of mitochondrial-DNA Journal of Molecular Evolution **22 (2):**160-174

Helm-Bychowski, K., and J. Cracraft. 1993. Recovering phylogenetic signal from DNA sequences: relationships within the corvine assemblage (Class Aves) as inferred from complete sequences of the mitochondrial DNA cytochrome-b gene. Molecular Biology and Evolution **10(6):**1196-1214

Hillis, D., and S. Davis. 1986. Evolution of ribosomal DNA: fifty million years of recorded history in the Frog *Rana*. Evolution **40:**1275-1288

Hillis, D., B. Mable, A. Larson, S. Davis, E. Zimmer. 1996. Nucleic acids IV: sequencing and cloning pg. 342-343 *in* Molecular systematics. Editors D.M. Hillis, C. Moritz, B.K. Mable. Sinauer Associates, Inc. Publishers

Hitchings, S. and T. Beebee. 1997. Genetic substructuring as a result of barriers to gene flow in urban *Rana temporaria* (common frog) populations: implications for biodiversity conservation. Heredity **19 (1997):**117-127

Hoelzer, G. 1997. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees revisited. Evolution **51(2):**622-626

Hudson, R. 1990. Gene genealogies and the coalescent process. Oxford Surveys in Evolutionary Biology **7:**1-44

James, C., and C. Moritz. 2000. Intraspecific phylogeography in the sedge frog *Litoria fallax* (Hylidae) indicates pre-Pleistocene vicariance of an open forest species from Eastern Australia. Molecular Ecology **9:**349-358

Jehle, R., J. Arntzen, T. Burke, P. Krupa, and W. Hodl. 2001. The annual number of breeding adults and the effective population size of syntopic newts (*Triturus cristatus*, *T. marmoratus*). Molecular Ecology **10:**839-850

Kingman, J. 1982a. On the genealogy of large populations. Pg. 27-43 *in* Essays in statistical science. eds J. Gani and E. Hannan. Applied Probability Trust, London

Kingman, J. 1982b. The coalescent. Stochastic Processes and Their Applications **13:**235-248

Kimura, M., and J. Crow. 1963. The measurement of effective population number. Evolution **17:**279-288

Lande, R. 1995. Breeding plans for small populations based on the dynamics of quantitative genetic variance. Pg. 318-340 *in* Population management for survival and recovery: analytical methods and strategies in small population conservation. eds. J. Ballou, M. Gilpin, and T. Foose. Columbia University Press, New York

Lehman, T., W. Hawley, H. Grebert, F. Collins. 1998. The Effective Population

Size of *Anopholes gambiae* in Kenya: Implications for Population Structure. Molecular Biology and Evolution **15(3):**264-276

Lynch, M. 1991. Analysis of population genetic structure by DNA fingerprinting. Pg. 113-126 *in* DNA fingerprinting: approaches and applications. eds, T. Burke, G. Dolf, A. Jeffreys, and R. Wolff. Birkhauser, Basel, Switzerland

Macey, J., J. Schulte II, A. Larson, Z. Fang, Y. Wang, B. Tuniyev, and T. Papenfuss. 1998a. Phylogenetic relationships of toads in the *Bufo bufo* species group from the Eastern escarpment of the Tibetan Plateau: a case of vicariance and dispersal. Molecular Phylogenetics and Evolution **9(1):**80-87

Macey, J., J. Schulte II, N. Ananjeva, A. Larson, N. Rastegar-Pouyani, S. Shammakov, and T. Papenfuss. 1998b. Phylogenetic relationships among agamid lizards of the *Laudakia caucasia* species group: testing hypotheses of biogeographic fragmentation and an area cladogram for the Iranian Plateau. . Molecular Phylogenetics and Evolution **10(1):**118-131

Macey J., J. Strasburg, J. Brisson, V. Vredenburg, M. Jennings, and A. Larson. 2001. Molecular phylogenetics of Western North American frogs of the *Rana boylii* species group. Molecular Phylogenetics and Evolution **19 (1):**131-143

Maruyama, T. and M. Nei. 1981. Genetic variability maintained by mutation and overdominant selection in finite populations. Genetics **98:**441-459

Measey, G. 2001. Growth and ageing of feral *Xenopus laevis* (Daudin) in South Wales, UK. Journal of Zoology **254:**547-555

Merrell, D. 1968. A comparison of the estimated size and the "effective size" of breeding populations of the leopard frog, *Rana pipiens*. Evolution **(22):**274-283

Michalakis, Y. and L. Excoffier. 1996. A generic estimation of population subdivision using distances between alleles with special reference for microsatellite loci. Genetics **142:**1061-1064

Mills, L., and F. Allendorf. 1996. The one-migrant-per-generation rule in conservation and management. Conservation Biology **10 (6):**1509-1518

Moore, W. 1995. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. Evolution **49(4):**718-726

Moritz, C. 1994. Defining "Evolutionarily significant units" for conservation. Trends in Ecology and Evolution **9(10):**373-375

. US Geological Survey Bulletin 1194-0

Naggae, Y. 1988. The primary and secondary structures of the ribosomal RNAs of *Rana catesbeiana* mitochondrion. Journal of the Nippon Medical School **55:**417-430

Nath, H. and R. Griffiths. 1993. The coalescent in two colonies with symmetric migration. Journal of Mathematical Biology **31:**841-851

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

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York

Nei, M., and N. Takahata. 1993. Effective population size, genetic diversity, and coalescence time in subdivided populations. Journal of Molecular Evolution **37:**240-244

Neigel, J., and J. Avise. 1986. Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation, *in* Evolutionary processes and theory, eds. E. Nevo and S. Karlin. Academic Press, New York

Newman, R., and T. Squire. 2001. Microsatellite variation and fine-scale population structure in the wood frog (*Rana sylvatica*). Molecular Ecology **10:**1087-1100

Notohara, M. 1994. The coalescent and the genealogical process in geographically structured populations. Journal of Mathematical Biology **29:**59-75

Nunney, L., and D. Elam. 1994. Estimating the effective population size of conserved populations. Conservation Biology **8:**175-184

Nyakaana, S., and P. Arctander. 1999. Population genetic structure of the African elephant in Uganda based on variation at mitochondrial and nuclear loci: evidence for male-biased gene flow. Molecular Ecology **8:**1105-1115

Orita, M., H. Iwahana, H. Kanazawa, K. Hayashi, and T. Sekiya.1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proceedings of the National Academy of Sciences **86:**2766-2770

Ostrander, E., G. Sprague, J. Rine. 1993. Identification and characterization of dinucleotide repeat (CA)n markers for genetic mapping in dog. Genomics **16:**207-213

Paetkau, D., L. Waits, P. Clarkson, L. Craighead, C. Strobeck. 1997. An empirical evaluation of genetic distance statistics using microsatellite data from bear (Ursidae) populations. Genetics **147:**1943-1957

Paetkau, D. 1999. Using genetics to identify intraspecific conservation units: A critique of current methods. Conservation Biology **13(6):**1507-1509

Page, R. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences **12:**357-358

Pennock, D., and W. Dimmick. 1997. Critique of the evolutionarily significant unit as a definition for "distinct population segments" under the US Endangered Species Act. Conservation Biology **11(3):**611-619

Phelps, S., and Allendorf, F. 1983. Genetic identity of pallid and shovenose sturgeon

Post, T., and T. Uzzell. 1981. The relationships of *Rana sylvatica* and the monophyly of the *Rana boylii* group. Systematic Zoology **30:**170-180

Raymond, M., F. Rousset. 1995. GENEPOP (Version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity **86(3):**248-249

Reaser, J. 2000. Demographic analysis of the Columbia spotted frog (*Rana luteiventris*): case study in spatiotemporal variation. Canadian Journal of Zoology **78 (7):**1158-1167

Reh, W., and A. Seitz. 1990. The Influence of land use on the genetic structure of populations in the common frog *Rana temporaria*. Biological Conservation **54:**239-250

Rice, W. 1989. Analyzing tables of statistical tests. Evolution. **43:**223-225.

Robertson, A. 1964. The effect of non-random mating within inbred lines on the rate of inbreeding. Genetic Research **5:**164-167

Rousset, F. 1997. Genetic differentiation and estimation of gene flow from F-Statistics under isolation by distance. Genetics **145:**1219-1228

Rowe, G., T. Beebee, and T. Burke. 1998. Phylogeography of the natterjack toad *Bufo calamita* in Britain: genetic differentiation of native and translocated populations. Molecular Ecology **7:**751-760

Ryder, O. 1986. Species conservation and systematics: the dilemma of subspecies. Trends in Ecology and Evolution **1(1):** 9-10

Samollow, P. 1980. Selective mortality and reproduction in a natural population of *Bufo boreas*. Evolution **34 (1):** 18-39

Savage, J. 1960. Evolution of a peninsular herpetofauna. Systematic Zoology **9:**184-212

Schneider, S., D. Roessli, and L. Excoffier. 2000. Arlequin ver. 2.000: a software for population genetic data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland

Schneider-Broussard, R. D. Felder, C. Chlan, J. Neigel. 1998. Tests of phylogeographic models with nuclear and mitochondrial DNA sequence variation in the stone crabs, *Menippe adina* and *Menippe mercenaria.* Evolution **52(6):** 1671-1678

Scribner K., J. Arntzen, and T. Burke. 1997. Effective number of breeding adults in *Bufo bufo* estimated from age-specific variation at minisatellite loci. Molecular Ecology **6:**701-712

Shaffer, H., G. Fellers, A. Magee, and R. Voss. 2000. The genetics of amphibian declines: population substructure and molecular differentiation in the Yosemite toad, *Bufo canorus* (Anura, Bufonidae) based on single-strand conformation polymorphism analysis (SSCP) and mitochondrial DNA Sequence Data. Molecular Ecology **9:**245-257

Slatkin, M. 1985. Rare alleles as indicators of gene flow. Evolution **39:**53-65

Slatkin, M. 1995. A measure of population subdivision based on microsatellite allele frequencies. Genetics **139:**457-462

Soltis D, Gitzendanner M, Strenge D, Soltis P. 1997. Chloroplast DNA intraspecific phylogeography of plants from the Pacific Northwest of North America. Plant Systematics and Evolution **206:**353-373

Spolsky, C., C. Phillips, and T. Uzzell. 1992. Antiquity of clonal salamander lineages revealed by mitochondrial DNA. Nature **356:**706-708

Stebbins, R. 1985. A field guide to Western reptiles and amphibians. Houghton Mifflin, Boston

Steinberg, E., K. Lidner, J. Gallea, A. Maxwell, J. Meng, and F. Allendorf. 2002. Rates and patterns of microsatellite mutations in pink salmon. Molecular Biology and Evolution **19(7):**1198-1202

Storfer, A. 1999. Gene flow and population subdivision in the streamside salamander, *Ambystoma barbouri*. Copeia **1999(1):**174-181

Sumida, M., A. Allison, and M. Nishioka. 1998. Genetic relationships and phylogeny of Papua New Guinean hylid frogs elucidated by allozyme analysis. Japanese Journal of Herpetology **17(4):**164-174

Swofford, D. 1998. PAUP. Phylogenetic analysis using parsimony (and other methods). Version 4. Sinauer Associates, Sunderland, Mass.

Taylor, B., and E. Dizon. 1999. First policy then science: why a management unit based solely on genetic criteria cannot work. Molecular Ecology **8:**S11-S16

Templeton, A., K. Crandall, C. Sing. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram Estimation. Genetics **132:** 619-633

US Department of the Interior and US Department of Commerce. 1996. Policy regarding the recognition of distinct vertebrate population segments under the Endangered Species Act. Federal Register **61:**4722-4725

Vuchetich, J.A., and T.A. Waite. 1999. Erosion of heterozygosity in fluctuating populations. Conservation Biology **13:**860-868

Waitt, R., and R. Thorson. 1983. The Cordilleran ice sheet in Washington, Idaho, and Montana *in* late Quaternary environments of the United States Volume 1 The late Pleistocene, eds. H. Wright, Jr. and S. Porter. University of Minnesota Press, Minneapolis, Minn.

Waldman, B., and J. McKinnon. 1993. Inbreeding and outbreeding in fishes, amphibians, and reptiles, *in* A natural history of inbreeding and outbreeding , theoretical and empirical perspectives, ed. N. Wilmsen Thornhill. University of Chicago Press, Chicago and London

Waples, R.1991. Pacific Salmon, Oncorhynchus spp., and the definition of

"species" under the Endangered Species Act. US National Marine Fisheries Service Marine Fisheries Review **53(3):**11-22

Waples, R.1995. Evolutionarily significant units and the conservation of biological diversity under the Endangered Species Act. American Fisheries Society Symposium **17:** 8-27

Waples, R. 1998. Evolutionarily significant units, distinct population segments, and the endangered species act: reply to Pennock and Dimmick. Conservation Biology **12(3):** 718-721

Weir, B. and C. Cockerham. 1984. Estimating F-Statistics for the analysis of population structure. Evolution **38(6):**1358-1370

Whitlock, M, and D. McCauley. 1999. Indirect measures of gene flow and migration: Fst does not equal 1/4Nm+1. Heredity **82:**117-125

Wilding, C, J. Grahame, and P. Mill. 2000. Mitochondrial DNA col haplotype variation in sibling species of rough periwinkles. Heredity **85:**62-74

Wilkinson, J., M. Matsui, and T. Terachi. 1996. Geographic variation in a Japanese tree frog (*Rhacophorus arboreus*) revealed by PCR-aided restriction site analysis of mtDNA. Journal of Herpetology **30(3):**418-423

Wright, S. 1951. The genetical structure of populations. Annals of Eugenetics **15:** 323-354

Wright, S. 1965. The interpretation of population structure by F-statistics with special regard to systems of mating. Evolution **19:**395-420

Yang, Y., L. Yao-Sung, J. Wu, and C. Hui. 1994. Variation in mitochondrial DNA and population structure of the Taipei treefrog *Rhacophorus taipeinus* in Taiwan. Molecular Ecology **3:**219-228

Yoneyama, Y. 1987. The nucleotide sequences of the heavy and light strand replication origins of the *Rana catesbeiana* mitochondrial genome. Nippon Ika Daigaku Zasshi **54(4):** 429-440

Zamudio, K. and H. Greene. 1997. Phylogeography of the bushmaster (*Lachesis muta*: Viperidae): implications for neotropical biogeography, systematics, and conservation. Biological Journal of the Linnean Society **62:**421-442

APPENDICES

Appendix 2.1. Allele frequency data for 11 populations of *R. cascadae* for five microsatellite loci and one size variant nuclear locus.

Locus SCF120

Population	Sample Size	Expected Het.	Alleles (bp)												
			137	141	145	149	153	157	161	165	169	173	177	181	185
Olympic, WA	29	0.70	0.345	-----	0.034	0.379	0.224	0.017	-----	------	-----	-----	-----	-----	-----
Illabot Creek, WA	20	0.33	-----	-----	-----	-----	0.800	0.200	-----	------	-----	-----	-----	-----	-----
Mt. Rainier, WA	30	0.74	-----	0.033	0.033	0.083	0.200	0.417	0.233	------	-----	-----	-----	-----	-----
Breitenbush, OR	27	0.76	-----	-----	0.204	0.352	0.241	-----	0.167	-----	0.037	-----	-----	-----	-----
McKenzie Pass, OR	29	0.80	-----	-----	0.138	0.293	0.103	0.276	0.017	0.034	0.138	-----	-----	-----	-----
Waldo Lake, OR	27	0.78	-----	0.296	-----	-----	0.093	0.352	0.074	-----	-----	0.093	0.037	0.037	0.019
Grassy Mountain, OR	14	0.76	-----	0.393	-----	0.071	0.143	0.286	-----	0.071	0.036	-----	-----	-----	-----
Crystal Springs, OR	22	0.87	-----	0.068	0.159	0.068	0.182	0.159	0.205	0.023	0.023	0.114	-----	-----	-----
Big Frank Swamp, OR	18	0.67	-----	-----	-----	-----	0.222	0.361	0.417	-----	-----	-----	-----	-----	-----
Screwdriver Creek, CA	11	na	-----	1.000	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
Colby Creek, CA	28	0.60	0.036	0.607	-----	-----	0.107	0.143	-----	-----	0.107	-----	-----	-----	-----

Locus RP193

Population	Sample Size	Expected Het.	Alleles (bp)												
			143	147	151	155	159	163	167	171	175	179	183	187	191
Olympic, WA	29	0.79	-----	-----	-----	-----	-----	0.069	0.207	0.328	0.103	0.034	0.241	0.017	-----
Illabot Creek, WA	22	0.58	-----	-----	-----	0.364	-----	-----	-----	0.023	0.045	0.023	0.545	-----	-----
Mt. Rainier, WA	28	0.82	-----	-----	-----	-----	0.018	-----	0.071	0.214	-----	0.143	0.321	-----	0.107
Breitenbush, OR	26	0.83	-----	-----	0.038	-----	0.231	0.269	0.019	0.115	0.058	0.096	0.173	-----	-----
McKenzie Pass, OR	30	0.80	-----	-----	0.133	-----	0.033	0.333	-----	-----	0.117	0.233	0.033	0.017	0.100
Waldo Lake, OR	30	0.72	-----	-----	-----	0.017	0.133	0.133	0.450	0.233	0.033	-----	-----	-----	-----
Grassy Mountain, OR	15	0.54	-----	-----	-----	0.033	-----	0.433	0.533	-----	-----	-----	-----	-----	-----
Crystal Springs, OR	21	0.66	-----	-----	0.071	-----	-----	0.119	0.548	0.119	-----	0.143	-----	-----	-----
Big Frank Swamp, OR	18	0.66	-----	-----	-----	-----	0.028	0.528	0.056	0.167	-----	0.222	-----	-----	-----
Screwdriver Creek, CA	10	0.70	0.350	-----	0.300	0.350	-----	-----	-----	-----	-----	-----	-----	-----	-----
Colby Creek, CA	32	0.90	0.172	0.047	0.047	0.141	-----	-----	0.016	0.031	0.109	0.047	0.031	0.094	0.141

Locus RP193, continued

Population	Sample Size	Expected Het.	Alleles (bp)		
			195	199	203
Olympic, WA	29	0.79	-----	-----	-----
Illabot Creek, WA	22	0.58	-----	-----	-----
Mt. Rainier, WA	28	0.82	0.071	0.018	0.036
Breitenbush, OR	26	0.83	-----	-----	-----
McKenzie Pass, OR	30	0.80	-----	-----	-----
Waldo Lake, OR	30	0.72	-----	-----	-----
Grassy Mountain, OR	15	0.54	-----	-----	-----
Crystal Springs, OR	21	0.66	-----	-----	-----
Big Frank Swamp, OR	18	0.66	-----	-----	-----
Screwdriver Creek, CA	9	0.70	-----	-----	-----
Colby Creek, CA	32	0.98	0.125	-----	-----

Locus SCF134

Population	Sample Size	Expected Het.	Alleles (bp)												
			225	227	231	235	239	241	243	245	247	249	251	253	255
Olympic, WA	29	0.85	-----	-----	-----	0.138	0.172	0.103	-----	0.086	-----	-----	0.086	-----	0.190
Illabot Creek, WA	20	0.54	-----	-----	-----	0.500	-----	-----	-----	-----	0.475	-----	-----	-----	-----
Mt. Rainier, WA	29	0.71	-----	0.017	-----	0.310	-----	-----	0.034	0.397	0.207	0.017	-----	-----	-----
Breitenbush, OR	26	0.84	-----	-----	-----	-----	-----	-----	-----	0.192	0.058	-----	-----	-----	0.019
McKenzie Pass, OR	29	0.75	-----	-----	-----	-----	0.431	-----	-----	-----	0.034	-----	0.017	0.086	-----
Waldo Lake, OR	27	0.77	-----	-----	-----	-----	-----	-----	-----	-----	0.111	0.278	-----	-----	-----
Grassy Mountain, OR	12	0.79	-----	-----	-----	-----	-----	-----	-----	-----	0.250	0.125	-----	0.083	-----
Crystal Springs, OR	21	0.79	-----	-----	-----	-----	-----	-----	-----	-----	0.119	0.238	-----	-----	-----
Big Frank Swamp, OR	18	0.39	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
Screwdriver Creek, CA	11	0.57	-----	0.500	0.455	0.045	-----	-----	-----	-----	-----	-----	-----	-----	-----
Colby Creek, CA	27	0.86	0.093	0.093	0.278	0.148	0.074	-----	0.037	-----	-----	-----	0.037	0.019	0.056

Locus SCF134, continued

Population	Sample Size	Expected Het.	Alleles (bp)						
			257	259	261	263	265	269	273
Olympic, WA	29	0.85	-----	-----	0.224	-----	-----	-----	-----
Illabot Creek, WA	20	0.54	-----	-----	0.025	-----	-----	-----	-----
Mt. Rainier, WA	29	0.71	-----	-----	0.017	-----	-----	-----	-----
Breitenbush, OR	26	0.84	0.173	0.058	0.269	0.115	0.115	-----	-----
McKenzie Pass, OR	29	0.75	0.121	-----	0.069	-----	0.207	0.034	-----
Waldo Lake, OR	27	0.77	0.370	0.111	0.074	-----	0.019	-----	0.037
Grassy Mountain, OR	12	0.79	0.042	-----	0.375	-----	0.125	-----	-----
Crystal Springs, OR	21	0.79	0.333	-----	0.190	-----	0.095	0.024	-----
Big Frank Swamp, OR	18	0.39	-----	-----	0.750	-----	0.250	-----	-----
Screwdriver Creek, CA	11	0.57	-----	-----	-----	-----	-----	-----	-----
Colby Creek, CA	27	0.86	0.167	-----	-----	-----	-----	-----	-----

Locus SCF128

Population	Sample Size	Expected Het.	Alleles (bp)									
			221	225	229	233	237	241	245	249	253	261
Olympic, WA	29	0.72	0.034	0.017	0.414	-----	0.310	0.017	0.155	0.052	-----	-----
Illabot Creek, WA	20	0.51	-----	-----	0.475	-----	-----	0.525	-----	-----	-----	-----
Mt. Rainier, WA	30	0.67	-----	-----	0.517	0.200	-----	0.167	0.033	0.050	-----	0.033
Breitenbush, OR	27	0.62	-----	0.500	-----	0.148	0.352	-----	-----	-----	-----	-----
McKenzie Pass, OR	27	0.51	-----	0.574	-----	0.407	-----	-----	0.019	-----	-----	-----
Waldo Lake, OR	27	0.79	-----	0.259	0.056	0.185	0.222	0.019	0.259	-----	-----	-----
Grassy Mountain, OR	11	0.69	-----	0.273	-----	-----	-----	0.318	0.409	-----	-----	-----
Crystal Springs, OR	22	0.68	-----	0.523	-----	0.068	0.068	0.091	0.205	0.045	-----	-----
Big Frank Swamp, OR	18	0.60	-----	0.139	-----	0.056	-----	0.139	0.611	0.056	-----	-----
Screwdriver Creek, CA	30	069	-----	-----	-----	-----	0.250	-----	-----	0.400	0.350	-----
Colby Creek, CA	31	0.76	-----	0.081	-----	0.097	0.226	0.016	-----	0.371	0.210	-----

Locus RP17

Population	Sample Size	Expected Het.	Alleles (bp)		
			111	115	119
Olympic, WA	28	na	-----	1.000	-----
Illabot Creek, WA	22	na	-----	1.000	-----
Mt. Rainier, WA	30	na	-----	1.000	-----
Breitenbush, OR	25	0.25	0.120	0.860	0.020
McKenzie Pass, OR	27	0.33	0.204	0.796	-----
Waldo Lake, OR	28	0.57	0.464	0.464	0.071
Grassy Mountain, OR	14	0.48	0.643	0.357	-----
Crystal Springs, OR	22	0.50	0.432	0.568	-----
Big Frank Swamp, OR	18	0.60	0.528	0.361	0.111
Screwdriver Creek, CA	11	na	-----	1.000	-----
Colby Creek, CA	27	na	-----	1.000	-----

Locus RC174

Population	Sample Size	Expected Het.	Alleles (bp)	
			86	140
Olympic, WA	29	na	-----	1.000
Illabot Creek, WA	22	na	-----	1.000
Mt. Rainier, WA	30	na	-----	1.000
Breitenbush, OR	27	na	-----	1.000
McKenzie Pass, OR	30	na	-----	1.000
Waldo Lake, OR	27	na	-----	1.000
Grassy Mountain, OR	14	na	-----	1.000
Crystal Springs, OR	22	na	-----	1.000
Big Frank Swamp, OR	18	na	-----	1.000
Screwdriver Creek, CA	11	na	1.000	-----
Colby Creek, CA	32	na	1.000	-----

Locus RP123

Population	Sample Size	Expected Het.	Alleles (bp)	
			145	146
Olympic, WA	27	na	-----	1.000
Illabot Creek, WA	19	na	-----	1.000
Mt. Rainier, WA	29	na	-----	1.000
Breitenbush, OR	16	na	1.000	-----
McKenzie Pass, OR	30	na	1.000	-----
Waldo Lake, OR	26	na	1.000	-----
Grassy Mountain, OR	13	na	1.000	-----
Crystal Springs, OR	20	na	1.000	-----
Big Frank Swamp, OR	18	na	1.000	-----
Screwdriver Creek, CA	na	na	no data	
Colby Creek, CA	17	na	1.000	-----

Appendix 4.1. Microsatellite allele frequency data for 18 *R. cascadae* populations at seven microsatellite loci.

Locus : SCF120

Population	Alleles 137	141	145	149	153	157	161	165	169	173	177	181	185	N	He	Ho
1 Illabot Creek, WA	.	.	.	.	0.800	0.200	.	.	.	.	.	.	.	20	0.33	0.30
2 Mt. Rainier, WA	.	0.033	0.033	0.083	0.200	0.417	0.233	.	.	.	.	.	.	30	0.73	0.36
3 Berkely Park, WA	.	0.048	.	0.119	0.310	0.476	0.048	.	.	.	.	.	.	21	0.68	0.29
4 Elysian Fields, WA	0.019	.	.	0.056	0.389	0.537	.	.	.	.	.	.	.	27	0.57	0.33
5 Reflection Lakes, WA	0.042	0.021	.	0.438	0.271	0.188	0.042	.	.	.	.	.	.	24	0.71	0.29
6 Paradise River, WA	.	0.050	.	0.400	0.200	0.275	0.075	.	.	.	.	.	.	20	0.74	0.45
7 Breitenbush, OR	.	.	0.204	0.352	0.241	.	0.167	.	0.037	.	.	.	.	27	0.74	0.78
8 Grassy Mountain, OR	.	0.393	.	0.071	0.143	0.286	.	0.071	0.036	.	.	.	.	14	0.78	0.93
9 Melakwa Lake, OR	.	.	0.200	.	0.267	0.367	.	0.033	0.133	.	.	.	.	15	0.76	0.80
10 McKenzie Pass, OR	.	.	0.138	0.293	0.103	0.276	0.017	0.034	0.138	.	.	.	.	29	0.80	0.72
11 Benson Lake Trail, OR	.	.	0.083	0.083	0.354	0.438	0.021	.	0.021	.	.	.	.	24	0.68	0.71
12 Todd Lake, OR	.	0.370	0.109	0.043	0.413	.	0.022	.	0.043	.	.	.	.	23	0.69	0.65
13 Many Lakes Trail, OR	.	0.458	0.438	.	.	0.104	.	.	.	.	.	.	.	24	0.60	0.54
14 Waldo Lake, OR	.	0.296	.	.	0.093	0.352	0.074	.	.	0.093	0.037	0.037	0.019	27	0.78	0.78
15 Gold Lake, OR	0.161	0.129	.	0.032	0.516	0.097	.	0.016	.	0.032	0.016	.	.	31	0.69	0.61
16 Crystal Springs, OR	.	0.068	0.159	0.068	0.182	0.159	0.205	0.023	0.023	0.114	.	.	.	22	0.87	0.91
17 Seven-Mile Creek, OR	.	0.038	0.346	.	0.115	0.385	0.115	.	.	.	.	.	.	13	0.73	0.46
18 Big Frank Swamp, OR	.	.	.	.	0.222	0.361	0.417	.	.	.	.	.	.	18	0.67	0.67

Locus : RC287

Population	Alleles 268	276	280	284	N	He	Ho
1 Illabot Creek, WA	.	.	1.000	.	21	na	na
2 Mt. Rainier, WA	.	.	1.000	.	30	na	na
3 Berkely Park, WA	.	.	1.000	.	20	na	na
4 Elysian Fields, WA	.	.	1.000	.	28	na	na
5 Reflection Lakes, WA	0.021	.	0.979	.	24	0.04	0.04
6 Paradise River, WA	0.028	.	0.972	.	18	0.06	0.06
7 Breitenbush, OR	.	0.077	0.923	.	26	0.15	0.15
8 Grassy Mountain, OR	.	0.846	0.154	.	13	0.31	0.27
9 Melakwa Lake, OR	.	.	1.000	.	14	na	na
10 McKenzie Pass, OR	.	.	1.000	.	29	na	na
11 Benson Lake Trail, OR	.	.	1.000	.	24	na	na
12 Todd Lake, OR	.	.	1.000	.	22	na	na
13 Many Lakes Trail, OR	.	0.152	0.848	.	23	0.22	0.27
14 Waldo Lake, OR	.	0.722	0.278	.	27	0.41	0.41
15 Gold Lake, OR	.	0.552	0.328	0.121	29	0.59	0.45
16 Crystal Springs, OR	.	0.727	0.068	0.205	22	0.44	0.55
17 Seven-Mile Creek, OR	.	0.375	0.208	0.417	12	0.67	0.42
18 Big Frank Swamp, OR	.	1.000	.	.	5	na	na

Locus : RP193

Population	Alleles 143	147	151	155	159	163	167	171	175	179	183	187
1 Illabot Creek, WA	.	.	.	0.364	.	.	.	0.023	0.045	0.023	0.545	.
2 Mt. Rainier, WA	.	.	.	.	0.018	.	0.071	0.214	.	0.143	0.321	.
3 Berkely Park, WA	.	.	.	.	.	.	.	0.132	0.105	0.105	0.368	0.026
4 Elysian Fields, WA	.	.	.	.	.	.	0.036	0.321	0.036	0.089	0.518	.
5 Reflection Lakes, WA	.	.	0.043	.	.	0.065	.	0.130	0.130	0.261	0.283	0.043
6 Paradise River, WA	.	.	.	.	.	.	.	0.111	0.056	0.111	0.333	0.306
7 Breitenbush, OR	.	.	0.038	.	0.231	0.269	0.019	0.115	0.058	0.096	0.173	.
8 Grassy Mountain, OR	.	.	.	0.033	.	0.433	0.533	.	.	.	.	.
9 Melakwa Lake, OR	.	.	0.100	.	0.033	0.567	.	0.033	0.033	0.067	0.100	.
10 McKenzie Pass, OR	.	.	0.133	.	0.033	0.333	.	.	0.117	0.233	0.033	0.017
11 Benson Lake Trail, OR	.	.	0.065	.	0.043	0.435	.	.	.	0.065	0.174	0.087
12 Todd Lake, OR	.	.	0.239	.	0.022	0.022	0.500	0.196	0.022	.	.	.
13 Many Lakes Trail, OR	.	.	.	0.021	0.229	.	0.167	0.146	0.104	.	0.042	0.292
14 Waldo Lake, OR	.	.	.	0.017	0.133	0.133	0.450	0.233	0.033	.	.	.
15 Gold Lake, OR	.	.	0.081	0.032	0.065	0.306	0.226	0.065	.	.	0.194	0.032
16 Crystal Springs, OR	.	.	0.071	.	.	0.119	0.548	0.119	.	0.143	.	.
17 Seven-Mile Creek, OR	.	.	.	0.038	0.077	0.615	0.038	0.192	.	0.038	.	.
18 Big Frank Swamp, OR	.	.	.	.	0.028	0.528	0.056	0.167	.	0.222	.	.

Locus : RP193 continued

Population	Alleles 191	195	199	203	N	He	Ho
1 Illabot Creek, WA	.	.	.	.	22	0.58	0.77
2 Mt. Rainier, WA	0.107	0.071	0.018	0.036	28	0.82	0.71
3 Berkely Park, WA	0.105	0.158	.	.	19	0.81	0.79
4 Elysian Fields, WA	.	.	.	.	28	0.63	0.68
5 Reflection Lakes, WA	0.043	.	.	.	23	0.83	0.83
6 Paradise River, WA	0.056	0.028	.	.	18	0.78	0.83
7 Breitenbush, OR	.	.	.	.	26	0.83	0.77
8 Grassy Mountain, OR	.	.	.	.	15	0.55	0.47
9 Melakwa Lake, OR	0.067	.	.	.	15	0.67	0.67
10 McKenzie Pass, OR	0.100	.	.	.	30	0.80	0.87
11 Benson Lake Trail, OR	0.130	.	.	.	23	0.76	0.83
12 Todd Lake, OR	.	.	.	.	23	0.67	0.70
13 Many Lakes Trail, OR	.	.	.	.	24	0.82	0.67
14 Waldo Lake, OR	.	.	.	.	30	0.72	0.60
15 Gold Lake, OR	.	.	.	.	31	0.81	0.58
16 Crystal Springs, OR	.	.	.	.	21	0.67	0.67
17 Seven-Mile Creek, OR	.	.	.	.	13	0.60	0.38
18 Big Frank Swamp, OR	.	.	.	.	18	0.63	0.44

Locus : SCF134

Population	Alleles 225	227	231	235	237	239	241	243	245	247	249	251	253	255
1 Illabot Creek, WA	.	.	.	0.500	.	.	.	.	.	0.475	.	.	.	.
2 Mt. Rainier, WA	.	0.017	.	0.310	.	.	.	0.034	0.397	0.207	0.017	.	.	.
3 Berkely Park, WA	.	.	.	0.300	.	.	.	0.033	0.433	0.233	.	.	.	.
4 Elysian Fields, WA	.	0.121	.	0.103	0.052	.	.	0.103	0.500	0.103	0.017	.	.	.
5 Reflection Lakes, WA	.	.	.	0.413	.	.	.	0.326	0.196	.	.	.	.	.
6 Paradise River, WA	.	0.028	.	0.417	.	.	.	0.222	0.306	.	.	.	.	.
7 Breitenbush, OR	.	.	.	.	.	.	.	.	0.192	0.058	.	.	.	0.019
8 Grassy Mountain, OR	.	.	.	.	.	.	.	.	.	0.250	0.125	.	0.083	.
9 Melakwa Lake, OR	.	.	0.071	.	.	0.321	.	.	.	.	.	0.071	0.036	.
10 McKenzie Pass, OR	.	.	.	.	.	0.431	.	.	.	0.034	.	0.017	0.086	.
11 Benson Lake Trail, OR	.	.	0.130	.	.	0.130	.	.	.	.	.	0.022	0.022	.
12 Todd Lake, OR	.	.	.	.	.	0.065	.	.	.	0.043	.	.	.	.
13 Many Lakes Trail, OR	.	.	.	.	.	.	.	.	.	0.048	0.095	.	.	.
14 Waldo Lake, OR	.	.	.	.	.	.	.	.	.	0.111	0.278	.	.	.
15 Gold Lake, OR	.	.	.	.	0.016	.	0.097	.	0.032	.	0.129	.	.	.
16 Crystal Springs, OR	.	.	.	.	.	.	.	.	.	0.119	0.238	.	.	.
17 Seven-Mile Creek, OR	.	.	.	.	.	.	.	.	.	.	0.115	0.038	.	.
18 Big Frank Swamp, OR	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Locus : SCF134 continued

Population	Alleles 256	257	259	261	263	265	269	272	273	277	281	N	He	Ho
1 Illabot Creek, WA	.	.	.	0.025	.	.	.	.	.	.	.	20	0.51	0.50
2 Mt. Rainier, WA	.	.	.	0.017	.	.	.	.	.	.	.	29	0.72	0.72
3 Berkely Park, WA	.	.	.	.	.	.	.	.	.	.	.	15	0.69	0.67
4 Elysian Fields, WA	.	.	.	.	.	.	.	.	.	.	.	29	0.71	0.72
5 Reflection Lakes, WA	.	.	.	0.065	.	.	.	.	.	.	.	23	0.70	0.74
6 Paradise River, WA	.	.	.	0.028	.	.	.	.	.	.	.	18	0.70	0.83
7 Breitenbush, OR	.	0.173	0.058	0.269	0.115	0.115	.	.	.	.	.	26	0.84	0.62
8 Grassy Mountain, OR	.	0.042	.	0.375	.	0.125	.	.	.	.	.	12	0.79	0.75
9 Melakwa Lake, OR	.	0.107	0.036	0.071	.	0.286	.	.	.	.	.	14	0.81	0.93
10 McKenzie Pass, OR	.	0.121	.	0.069	.	0.207	0.034	.	.	.	.	29	0.76	0.66
11 Benson Lake Trail, OR	.	0.043	.	0.043	.	0.370	0.174	.	0.065	.	.	23	0.81	0.91
12 Todd Lake, OR	.	0.087	.	0.326	.	0.130	.	.	0.348	.	.	23	0.76	0.70
13 Many Lakes Trail, OR	.	0.405	.	.	.	0.452	.	.	.	.	.	21	0.63	0.45
14 Waldo Lake, OR	.	0.370	0.111	0.074	.	0.019	.	.	0.037	.	.	27	0.77	0.70
15 Gold Lake, OR	.	0.081	.	0.242	.	0.048	0.048	.	0.145	0.097	0.065	31	0.88	0.87
16 Crystal Springs, OR	.	0.333	.	0.190	.	0.095	0.024	.	.	.	.	21	0.79	0.62
17 Seven-Mile Creek, OR	.	0.038	.	0.500	.	0.308	.	.	.	.	.	13	0.66	0.62
18 Big Frank Swamp, OR	.	.	.	0.750	.	0.250	.	.	.	.	.	18	0.38	0.39

Locus : SCF128

Population	Alleles 221	225	229	233	237	241	245	249	253	261	N	He	Ho
1 Illabot Creek, WA	.	.	0.475	.	.	0.525	.	.	.	.	20	0.51	0.45
2 Mt. Rainier, WA	.	.	0.517	0.200	.	0.167	0.033	0.050	.	0.033	30	0.67	0.70
3 Berkely Park, WA	.	0.075	0.350	0.025	.	0.350	0.050	.	0.125	0.025	20	0.75	0.50
4 Elysian Fields, WA	.	0.017	0.167	.	.	0.067	0.483	.	0.017	0.250	30	0.68	0.67
5 Reflection Lakes, WA	.	.	0.136	0.068	.	0.205	0.205	0.364	.	0.023	22	0.78	0.68
6 Paradise River, WA	.	.	0.325	.	0.050	0.050	0.200	0.375	.	.	20	0.73	0.80
7 Breitenbush, OR	.	0.500	.	0.148	0.352	.	.	.	.	.	27	0.61	0.67
8 Grassy Mountain, OR	.	0.273	.	.	.	0.318	0.409	.	.	.	11	0.71	0.64
9 Melakwa Lake, OR	.	0.533	.	0.233	.	.	0.233	.	.	.	15	0.63	0.80
10 McKenzie Pass, OR	.	0.574	.	0.407	.	.	0.019	.	.	.	27	0.51	0.52
11 Benson Lake Trail, OR	.	0.659	.	0.136	.	.	0.182	.	0.023	.	22	0.53	0.59
12 Todd Lake, OR	.	0.304	.	.	0.391	0.043	0.261	.	.	.	23	0.70	0.65
13 Many Lakes Trail, OR	.	.	.	0.396	0.188	0.417	.	.	.	.	24	0.65	0.79
14 Waldo Lake, OR	.	0.259	0.056	0.185	0.222	0.019	0.259	.	.	.	27	0.79	0.67
15 Gold Lake, OR	.	0.339	0.065	0.161	0.113	0.065	0.258	.	.	.	31	0.78	0.52
16 Crystal Springs, OR	.	0.523	.	0.068	0.068	0.091	0.205	0.045	.	.	22	0.68	0.64
17 Seven-Mile Creek, OR	.	0.192	.	0.154	.	0.077	0.385	0.192	.	.	13	0.77	0.46
18 Big Frank Swamp, OR	.	0.139	.	0.056	.	0.139	0.611	0.056	.	.	18	0.60	0.44

Locus : RP17

Population	Alleles 111	115	119	N	He	Ho
1 Illabot Creek, WA	.	1.000	.	22	na	na
2 Mt. Rainier, WA	.	1.000	.	30	na	na
3 Berkely Park, WA	.	1.000	.	21	na	na
4 Elysian Fields, WA	.	1.000	.	30	na	na
5 Reflection Lakes, WA	.	1.000	.	22	na	na
6 Paradise River, WA	.	1.000	.	19	na	na
7 Breitenbush, OR	0.120	0.860	0.020	25	0.25	0.28
8 Grassy Mountain, OR	0.643	0.357	.	14	0.48	0.57
9 Melakwa Lake, OR	.	1.000	.	15	na	na
10 McKenzie Pass, OR	0.204	0.796	.	27	0.33	0.26
11 Benson Lake Trail, OR	0.182	0.818	.	22	0.30	0.36
12 Todd Lake, OR	0.136	0.682	0.182	22	0.50	0.55
13 Many Lakes Trail, OR	0.143	0.857	.	14	0.26	0.14
14 Waldo Lake, OR	0.464	0.464	0.071	28	0.58	0.07
15 Gold Lake, OR	0.403	0.194	0.403	31	0.65	0.48
16 Crystal Springs, OR	0.432	0.568	.	22	0.50	0.32
17 Seven-Mile Creek, OR	0.385	0.615	.	13	0.49	0.46
18 Big Frank Swamp, OR	0.528	0.361	0.111	18	0.59	0.50

Locus : SCF139

Population	Alleles 230	234	238	242	246	250	254	258	262	266	270	274	278	N	He	Ho
1 Illabot Creek, WA	.	.	.	.	0.825	0.175	.	.	.	.	.	.	.	20	0.30	0.25
2 Mt. Rainier, WA	.	0.052	0.086	0.138	0.224	0.345	0.155	.	.	.	.	.	.	29	0.79	0.66
3 Berkely Park, WA	0.033	0.100	.	0.200	0.200	0.467	.	.	.	.	.	.	.	15	0.71	0.47
4 Elysian Fields, WA	0.033	.	0.033	0.067	0.350	0.517	.	.	.	.	.	.	.	30	0.61	0.53
5 Reflection Lakes, WA	0.114	0.023	.	0.386	0.273	0.182	0.023	.	.	.	.	.	.	22	0.75	0.55
6 Paradise River, WA	0.056	0.028	.	0.583	0.083	0.194	0.056	.	.	.	.	.	.	18	0.63	0.50
7 Breitenbush, OR	.	.	0.204	0.352	0.241	.	0.167	.	0.037	.	.	.	.	27	0.76	0.78
8 Grassy Mountain, OR	.	0.278	.	0.222	0.222	0.278	.	.	.	.	.	.	.	9	0.79	0.67
9 Melakwa Lake, OR	.	.	0.200	.	0.267	0.367	.	0.033	0.133	.	.	.	.	15	0.76	0.80
10 McKenzie Pass, OR	.	.	0.146	0.271	0.104	0.271	0.021	0.021	0.167	.	.	.	.	24	0.81	0.75
11 Benson Lake Trail, OR	.	.	0.100	0.075	0.375	0.425	.	.	0.025	.	.	.	.	20	0.68	0.65
12 Todd Lake, OR	.	0.361	0.111	0.028	0.444	.	.	.	0.028	.	0.028	.	.	18	0.68	0.61
13 Many Lakes Trail, OR	.	0.457	0.457	.	.	0.087	.	.	.	.	.	.	.	23	0.59	0.52
14 Waldo Lake, OR	.	0.308	.	.	0.096	0.346	0.077	.	.	0.077	0.038	0.038	0.019	26	0.78	0.77
15 Gold Lake, OR	0.185	0.130	.	0.037	0.500	0.074	.	0.019	.	0.037	0.019	.	.	27	0.70	0.59
16 Crystal Springs, OR	.	0.075	0.175	0.050	0.225	0.150	0.200	0.025	0.025	0.075	.	.	.	20	0.87	0.85
17 Seven-Mile Creek, OR	.	0.038	0.269	.	0.192	0.385	0.115	.	.	.	.	.	.	13	0.75	0.31
18 Big Frank Swamp, OR	.	.	.	.	0.222	0.361	0.417	.	.	.	.	.	.	18	0.67	0.67