Type N Experimental Buffer Treatment Study: Baseline Measures of Genetic Diversity and Gene Flow of Three Stream-Associated Amphibians

Stephen Spear, Jason Baumsteiger, and Andrew Storfer

WASHINGTON STATE DEPARTMENT OF
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Washington State Forest Practices Adaptive Management Program
Cooperative Monitoring, Evaluation, and Research Committee (CMER)
Report

Type N Experimental Buffer Treatment Study: Baseline Measures of Genetic Diversity and Gene Flow of Three Stream-Associated Amphibians

Prepared by:

Stephen Spear, Jason Baumsteiger, and Andrew Storfer

Prepared for the

The Cooperative Monitoring, Evaluation, and Research (CMER) Committee
Washington State Forest Practices Board
Adaptive Management Program
Washington State Department of Natural Resources
Olympia, Washington

May 2011 **CMER 06-605**

Washington State Forest Practices Adaptive Management Program

The Washington State Forest Practices Board (FPB) has established an Adaptive Management Program (AMP) by rule in accordance with the Forests & Fish Report (FFR) and subsequent legislation. The purpose of this program is to:

> *Provide science-based recommendations and technical information to assist the FPB in determining if and when it is necessary or advisable to adjust rules and guidance for aquatic resources to achieve resource goals and objectives. The board may also use this program to adjust other rules and guidance.* (Forest Practices Rules, WAC 222-12-045(1)).

To provide the science needed to support adaptive management, the FPB established the Cooperative Monitoring, Evaluation and Research (CMER) committee as a participant in the program. The FPB empowered CMER to conduct research, effectiveness monitoring, and validation monitoring in accordance with WAC 222-12-045 and Board Manual Section 22.

Report Type and Disclaimer

This technical report contains scientific information from research or monitoring studies that are designed to evaluate the effectiveness of the forest practices rules in achieving one or more of the Forest and Fish performance goals, resource objectives, and/or performance targets. The document was prepared for the Cooperative Monitoring, Evaluation and Research Committee (CMER) and was intended to inform and support the Forest and Fish Adaptive Management program. The project is part of the Type N Amphibian Response Program, and was conducted under the oversight of the Landscape and Wildlife Advisory Group.

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Full Reference

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Author Contact Information

Stephen Spear
University of Idaho, Department of Fish and Wildlife Resources,
P.O. Box 442282
Moscow, ID 83844-2282
sfspear2@yahoo.com

Jason Baumsteiger
University of California, Department of Natural Sciences
5200 N. Lake Rd.
Merced, CA 95343
cutthroat99@hotmail.com

Andrew Storfer
Washington State University, School of Biological Sciences
423 Neill Hall
Pullman, WA 99164-3140
astorfer@wsu.edu

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Executive Summary

One of the major objectives of the Type N Experimental Buffer Treatment Study is to investigate how different forest buffers surrounding non-fish-bearing streams may influence stream-associated amphibian populations. Measures of genetic diversity and genetic differentiation within and among amphibian populations can allow for species identification, provide insight into trends in population size and identify the level of migration among sites. Herein, we report baseline, pre-treatment measures of genetic structure for three species of stream-associated amphibians: Coastal tailed frog (*Ascaphus truei*), Cope's giant salamander (*Dicamptodon copei*) and Coastal giant salamander (*Dicamptodon tenebrosus*). We addressed four main objectives:

1) Classify giant salamander individuals to the correct species and identify any hybrids
2) Estimate indices of genetic diversity for the three species at each sampled basin
3) Test for significant differences by region, block and anticipated treatment for any genetic diversity parameter
4) Determine genetic clusters for each species

We identified giant salamander individuals to species with high probabilities, and found 31 hybrids out of 1504 total sampled *Dicamptodon* individuals. However, no hybrid individuals represented the current F_1 generation, suggesting hybridization is not currently ongoing. Levels of genetic diversity (based on allelic richness and heterozygosity) were high in Coastal tailed frogs and intermediate for Cope's giant salamander and Coastal giant salamander. Fourteen sites (out of 47 possible) had high levels of inbreeding and/or evidence of declines, but no consistent patterns were found among the three species. Effective population sizes varied greatly among species, with very large sizes for Coastal tailed frogs (3000-7000), intermediate sizes for Cope's giant salamander (150-3150) and low sizes for Coastal giant salamanders (50-750). Genetic parameters revealed no differences that would suggest biased results based on site selection for any species, demonstrating that the approach for assigning treatments was successful. Generally, no differences existed by region or block, with the notable exception of Cope's giant salamander, which had lower values of allelic richness, heterozygosity, inbreeding coefficient and effective population size in the Olympics. Finally, we detected geographically large genetic clusters (at a regional scale) for Coastal tailed frogs and Coastal giant salamanders, consistent with their broad species range and increased ability to disperse terrestrially. In contrast, Cope's giant salamander had geographically restricted genetic clusters, whereby individual sites often were genetically distinct from nearby sites. However, degree of divergence varied by region, with a larger cluster in the Olympics and the most restricted gene flow in the South Cascades.

1. Introduction and Objectives

The Type N Experimental Buffer Treatment Study (hereafter the Type N Study) has the primary objective of identifying how different harvest treatments influence the biotic and physical resources and processes in non-fish-bearing streams, with the ultimate goal of informing forest management about which buffer prescription(s) is(are) the most effective in maintaining those processes and biotic resources. The Type N Study is investigating four main categories of response variables including amphibian abundance and genetic diversity, downstream fish abundance, export of nutrients, detritus and macroinvertebrates and water temperature. The inclusion of amphibian variables is due to several factors. Nearly 40% of all amphibian species are thought to be in decline and are thus of conservation concern. Due to their apparent greater sensitivity to environmental degradation when contrasted with other vertebrate groups, amphibians are recognized as important indicators of ecosystem health (Stuart et al. 2004). Especially pertinent to the goals of the Type N Study, amphibians are important trophic components in stream ecosystems, comprising a large percentage of the vertebrate biomass in Pacific Northwest streams (Bury et al. 1991). Therefore, forest practices that may influence amphibian populations could subsequently alter entire stream assemblages. Studies generally demonstrate a reduction in numbers of stream amphibians in managed stands relative to old-growth (Corn and Bury 1989, Welsh 1990). However, other studies have shown little effect of harvest on stream amphibians in second-growth forests (Diller and Wallace 1999, Wilkins and Peterson 2000. Although factors such as sedimentation, gradient, geology, and stream temperature have influenced abundance in the previous studies, there is little consensus on the reasons for the differences in harvest effects on stream amphibians. Therefore, there is need for carefully designed studies to test different mechanisms that moderate amphibian response to harvest. Additionally, previous studies have primarily been focused on assessing abundance of amphibians in streams. However, stream amphibians are most easily detected as larvae (Spear and Storfer 2008; Kroll et al. 2010), and larval numbers may not accurately represent adult population status (Goldberg and Waits 2009). A high number of larval individuals could be produced by only a few adults, and if this was the case, demographic studies would conclude a large population size, when in reality the breeding population was low. However, genetic data can assess levels of effective population size or reductions in population size that are not immediately obvious demographically (Luikart et al. 1998, Garza and Williamson 2001). As a result, we proposed molecular population genetic studies to assess pre-treatment amphibian population status, as well as post-treatment responses (Beebee 2005; Jehle and Arntzen 2005; Storfer et al. 2009). Coastal tailed frogs (*Ascaphus truei*) and giant salamanders (Cope's giant salamanders [*Dicamptodon copei*] and Coastal giant salamanders [*Dicamptodon tenebrosus*]) were chosen as the stream-associated amphibians for the focus of the baseline (pre-harvest) population genetics portion of the Type N Study. Coastal tailed frogs and Cope's giant salamanders (hereafter the two focal species) were the two species originally designated in the Type N Study design to be the focus of the genetic portion of this study as they were the only

stream-associated amphibian species occurring over a geographic range encompassing all study basins. However, identification and hybridization issues between the two species of giant salamanders, as later explained, resulted in the default incorporation of Coastal giant salamanders into the genetic analysis portion of the Type N Study.

Herein, we present the results of the amphibian genetic portion of the Type N Study, to provide baseline data for comparisons with future post-treatment analyses. Occurrence and abundance data are important to this study to provide an index of immediate population response and numbers, but the inclusion of genetic data is complementary because it provides a longer-term picture of population trends as well as insight into future population viability. Specifically, genetic data are pertinent to this study for the following reasons: (1) Both species of giant salamander are nearly impossible to identify using exclusively morphology during their larval stages, hence genetic markers are the best means of ensuring unambiguous identifications. (2) Amphibian population samples are usually larval-biased, which can lead to errant conclusions if one depends exclusively on abundance data. Multiple amphibian larvae can be the offspring of one parent, and larvae typically suffer high mortality rates prior to metamorphosis. Therefore, larval abundance estimates are unlikely to fairly represent adult population size. (3) Measures of genetic diversity provide insight into the long-term viability of a population. A population with low genetic diversity and a high level of inbreeding may not be sustainable at the scale of tens to hundreds of generations. Additionally, genetic diversity statistics can be used to detect changes in population size. (4) Finally, genetic data can estimate the degree of gene flow across a study area. The Type N Study design examines the effect of a treatment on a single basin, presumably reflected by the amphibians resident in that basin. In this design is the implicit assumption that individuals sampled post-treatment at that site were exposed to that treatment and thus are not immigrants from another site. Measures of genetic differentiation can be used to test this assumption. In addition, streams connected to Type N treatment basins can be affected by harvest in those basins; analyzing gene flow (and hence connectivity) among nearby streams can provide information of how the *local network* of streams may be affected.

The population genetics portion of the Type N Study had four main objectives:

1) Correctly identify individual giant salamanders to species (Cope's or Coastal) as well as identify any hybrids between the two species that might confound results.

2) Generate measures of genetic diversity for each species for each Type N treatment basin that would provide the baselines for comparisons to post-treatment data. Changes in measures of genetic diversity such as allelic richness, heterozygosity and effective population size can provide evidence for treatment effects that either would not be immediately apparent or would be undetectable through demographic measures.

3) Examine whether significant *a priori* differences exist among regions, blocks and treatments using measures of genetic diversity. As this study occurred before any harvest treatments were applied, we expect no initial differences to exist. However, it is critical that we determine that no initial differences exist (especially by treatment), so we can ensure that any potential differences seen in post-treatment analysis are not the result of any pre-existing conditions.

4) Identify the genetic cluster (spatial extent of gene flow) for each species using population clustering techniques. The genetic cluster will not only be useful for identifying the correct spatial or geographic scale of management for each species (i.e. stream, watershed, region etc.), but will also indicate whether treatments and blocks are independent or connected by gene flow.

2. Materials and Methods

2.1 General Methods

2.1.1 Sample collection and study sites

In collaboration with the Washington Department of Fish and Wildlife (WDFW), we collected tissues for genetic samples from three regions in western Washington: the Olympics, Willapa Hills and South Cascades (Fig. 1). These were the physiographic regions in the WDFW-developed Type N Study design; treatment basins were identified within each region. Each treatment is being applied to an entire non-fish-bearing basin, and four different treatments, grouped as study blocks, are being applied in the Type N Study: an unharvested reference (or control), and three prescriptions that vary in the length of the two-sided 50-foot wide stream buffer applied to each (one prescription has buffer along 100% of the stream length, one has no buffer, and one has the current Forest Practices Rules prescribed buffer (FF HCP, 2005), which approximates at least 50% of the stream length). For the purposes of this study, basins were 1st, 2nd, or 3rd order non-fish-bearing basins defined as the area from the non-fish-bearing point of the stream network upstream and including all headwaters of the stream network. One block (of the four aforementioned treatments) was sampled in the Olympics (sites 363, 1099, 1197, 1236; Fig. 2), 2.5 blocks were sampled in the Willapa Hills (sites 2260, 2468, 3074, 3098, 3110, 3111, 3437, 3576, 3914, 5785; Fig. 3) and 1 block was sampled in the South Cascades (sites 5378, 5595N, 5595S, 6000; Fig. 4). Table 1 lists all basins and their respective treatments. An extensive site selection process was conducted over two years to identify all non-fish-bearing basins meeting *a priori* selection criteria. Once all basins meeting these criteria were identified researchers worked with landowners to determine which basins were available for inclusion in the study as either a reference (not harvested) or a treatment (harvested according to a specified prescription) basin. For a detailed description of selection criteria and the site selection process see McIntyre et al. (2009). After determining a list of all suitable non-fish-bearing basins, sites were blocked

based on geographic location (location within the Olympic, Willapa Hill or South Cascade physiographic regions). The exception was in the Willapa Hills, where selection of basins for blocks was restricted by unavoidable logistical issues (land ownership and willingness of landowners to enact particular harvest treatments). As a result, inconsistencies exist among blocks with regard to spatial proximity. However, genetic cluster analyses will give insight into whether genetic differences exist within blocks that need to be accounted for. Within each block, treatments were randomized unless prevented by these same logistical constraints. We selected additional sites within the Olympic and South Cascade regions to serve as sites for the genetic cluster analysis to estimate genetic connectivity across the entire study area and within individual regions (see Objective 4 for details). We did not need to sample additional cluster sites in the Willapa Hills because we already had ten sites chosen for this region for the Type N Study.

Up to 50 individuals each of Coastal tailed frog and Cope's giant salamander were sampled from each treatment basin. We chose this sample size to attain the level of power required to confidently describe trends in genetic diversity, and in particular, to provide a robust baseline with which to compare to future post-harvest sampling. At nearby sites likely to be connected genetically ("cluster sites"), our desired sample size was 30 individuals, as these sites would not be directly impacted by application of the treatments to the study basins. Rather, we sampled cluster sites primarily to detect any changes in extent or directionality of gene flow that might be altered by forest practice harvest treatments applied to the study basins. Sampling occurred from 2006-2008. WDFW crews sampled each basin consistently each year, utilizing two sampling methods designed to detect both tailed frogs and giant salamanders. Sampling was conducted diurnally between 0700 and 1900 h, from 7 July – 1 November. Light-touch sampling was conducted along systematically selected stream reaches from the fish-end-point and upstream along every tributary to the headwall. A minimum of 25% of the stream length in each basin was sampled each year. Light-touch sampling involves turning all surface cover objects within the stream channel that are small cobble-sized (64 mm) and larger and visually searching for amphibians. Rubble-rouse sampling was conducted in 20 1-m sample plots randomly placed in each of the first 20 10-m intervals above the fish end point. Plots were restricted with block nets and intensively sampled by removing all cover objects larger than small gravel, (≥32 mm) and excavating down to 30 cm or bedrock. For both methods, substrates were returned to their original positions. Every effort was made to collect tissues from individuals distributed throughout the entire stream network from the fish-end-point upstream and including all tributaries. Where more than adequate numbers of amphibians were encountered, tissues were collected from the first individual of each genus encountered within each 10-m stream reach. This was important in the event that the two giant salamander species were differentially distributed throughout the stream network. This was also done in an attempt to minimize sampling full siblings. For example, when multiple tailed frogs were encountered in close proximity, only the first individual encountered in the area was sampled. WSU crews

also visited treatment basins that had low sample sizes in an attempt to increase sample size. WSU crews used a protocol that used kick sampling and visual surveys to locate individuals. WSU surveys started at the fish end point and continued upstream to the end of the basin or until sufficient sample size was reached. Therefore, sites with low sample size are likely to have actual low abundance as multiple sampling visits were conducted at each site. While inter-annual differences may influence the data it is unlikely to result in misleading genetic conclusions, as the pre-harvest sampling period (3 years) for the two focal species is considerably shorter than the generation time of either species (7-8 years). We obtained tissue from larval tailed frogs and giant salamanders using tail clips and we used mouth swabs (Goldberg et al. 2003) and toe clips to collect tissue from metamorphosed tailed frogs. Finally, because Cope's giant salamanders are difficult to distinguish from Coastal giant salamanders in the field (Nussbaum 1976, Good 1989), giant salamander samples included both Coastal and Cope's giant salamanders, as indicated by downstream genetic analyses. In fact, as previously stated, one of the four main objectives of the genetics portion of the study was to correctly identify individual giant salamanders to species. As a result, we included analyses for Coastal giant salamanders.

2.1.2. Laboratory Methods

We extracted DNA from all tissue samples using the Qiagen DNEasy Tissue Kit (Qiagen, Inc.). To develop microsatellite primers to use for genetic analyses, we sent extracted DNA from tailed frogs collected from the Olympic region and from Cope's giant salamander collected from the South Cascades region to Ecogenics GmbH molecular marker services. Ecogenics developed microsatellite primer sequences for 15 loci in each species. For the tailed frog samples, 13 loci could be easily amplified; the remaining 2 could not be amplified consistently (Spear et al. 2008). For Cope's giant salamanders, we used 11 of the loci in analyses; the other 4 loci contained a number of non-specific alleles that overlapped with alleles specific to Cope's giant salamanders, and thus led to unreliable scoring of genotypes. Additionally, nine of the Cope's giant salamander loci cross-amplified successfully in the Coastal giant salamander (Steele et al. 2008). We used multiplex PCR (polymerase chain reaction) amplification for both species using the Qiagen Multiplex PCR kit (Qiagen Inc.). Multiplex PCR allowed us to run PCR reactions for several microsatellite loci in the same mix, thereby reducing the overall number of PCR reactions required to conduct the analyses. We describe the specific PCR conditions and multiplex panels for both tailed frog and giant salamander loci in Appendices 1-2. All PCR products were run on an ABI 3730 sequencer at the Washington State University LBB1 core facility and then genotyped using GeneMapper 3.7 software (Applied Biosystems, Inc.).

2.1.3. Genetic Analyses

We formatted all genotypic data for the program CONVERT (Glaubitz 2004), which in turn reformats data for a number of popular genetic programs, including those used in

this study. We first tested that each locus conformed to Hardy-Weinberg expectations at each sampling site. One primary assumption of Hardy-Weinberg equilibrium is random mating across a population. For sites such as the individual streams for each Type N study basin, this is a reasonable assumption and any deviations from Hardy-Weinberg equilibrium might be due to processes such as inbreeding or substructure within streams, either of which would be important to identify. We tested for Hardy-Weinberg equilibrium using an exact test in GENEPOP v 3.4 (Raymond and Rousset 1995). Second, we tested for linkage disequilibrium among loci. Linkage disequilibrium is an estimate of statistical association among loci, which can be caused via physical linkage due to close proximity on a chromosome or population processes related to drift or selection. Tests of whether pairs of loci are in linkage disequilibrium are needed to ensure that each microsatellite locus can be considered statistically independent. If alleles at any two of the tested loci are significantly correlated with each other, then overall genetic results may be biased. We also used GENEPOP to perform tests of linkage disequilibrium.

Further, we tested for presence of null alleles, which can occur with microsatellite loci and produce misleading results. A null allele is an instance when a certain allele fails to amplify, and as a result, leads to individuals with missing data (when null alleles are homozygous) or an excess of apparent homozygotes that occur when the null allele is part of a heterozygous pair. The most common reason for a null allele is when a mutation occurs at the site that the microsatellite primer anneals to during PCR reactions. Thus, the annealing site is "unrecognizable" to the primer and replication of that fragment cannot occur. Such mutations are most likely in populations that have diverged from the population that was used to develop the microsatellite markers. In our case, because microsatellite development used individuals from only one region for each species, we may encounter null alleles if the populations in the other regions have been genetically isolated for some time. We estimated the occurrence of null alleles at each locus for each site using the software FreeNA (Chapuis and Estoup 2007).

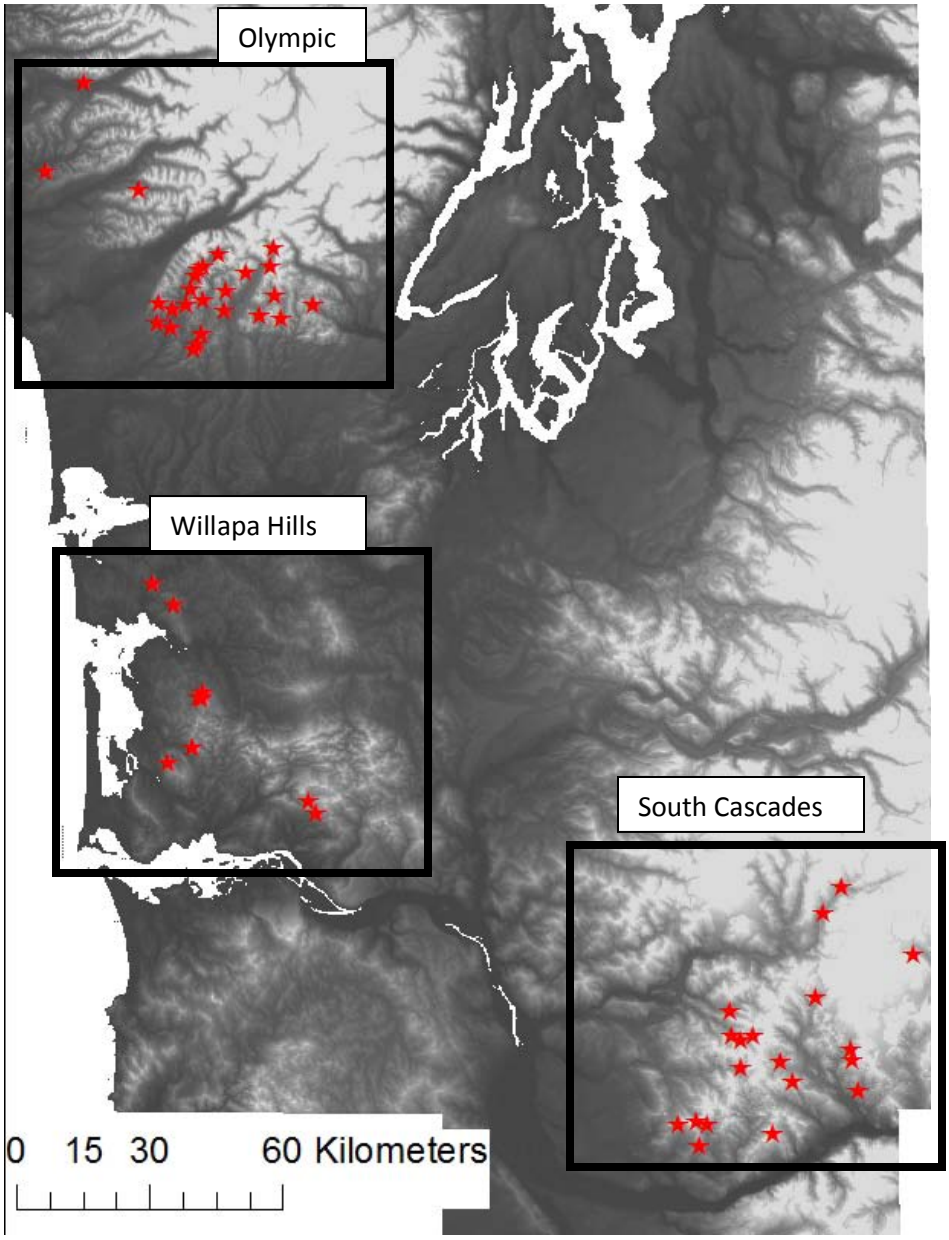

Figure 1. Overview map of Type N study area with each region designated by a black box. Background is a USGS Digital Elevation Model (DEM) with lighter colors as higher elevations. White patches indicate background areas with zero elevation.

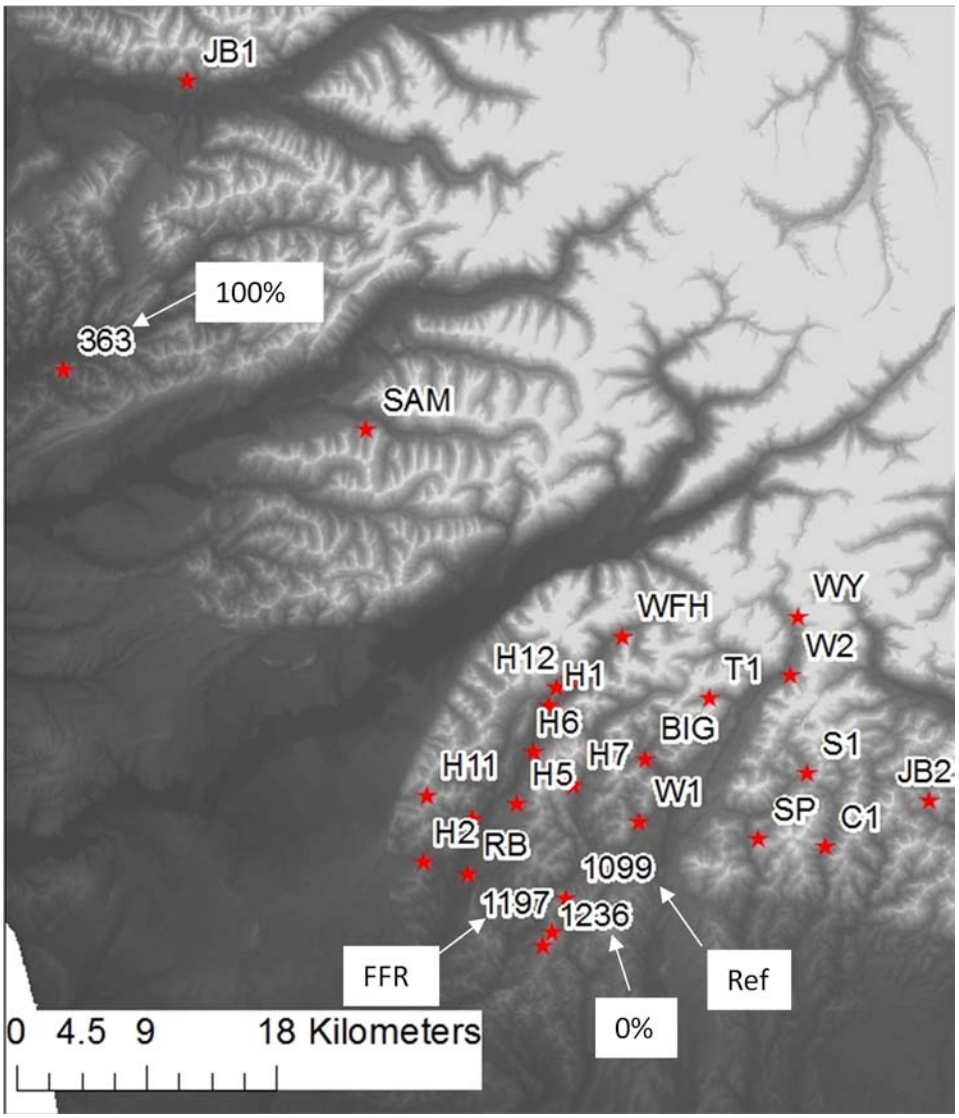

Figure 2. Map of both Type N basin (sites 363, 1099, 1197 and 1236) and cluster sites across the Olympic region. Text box and arrow represents assigned treatment for each basin.

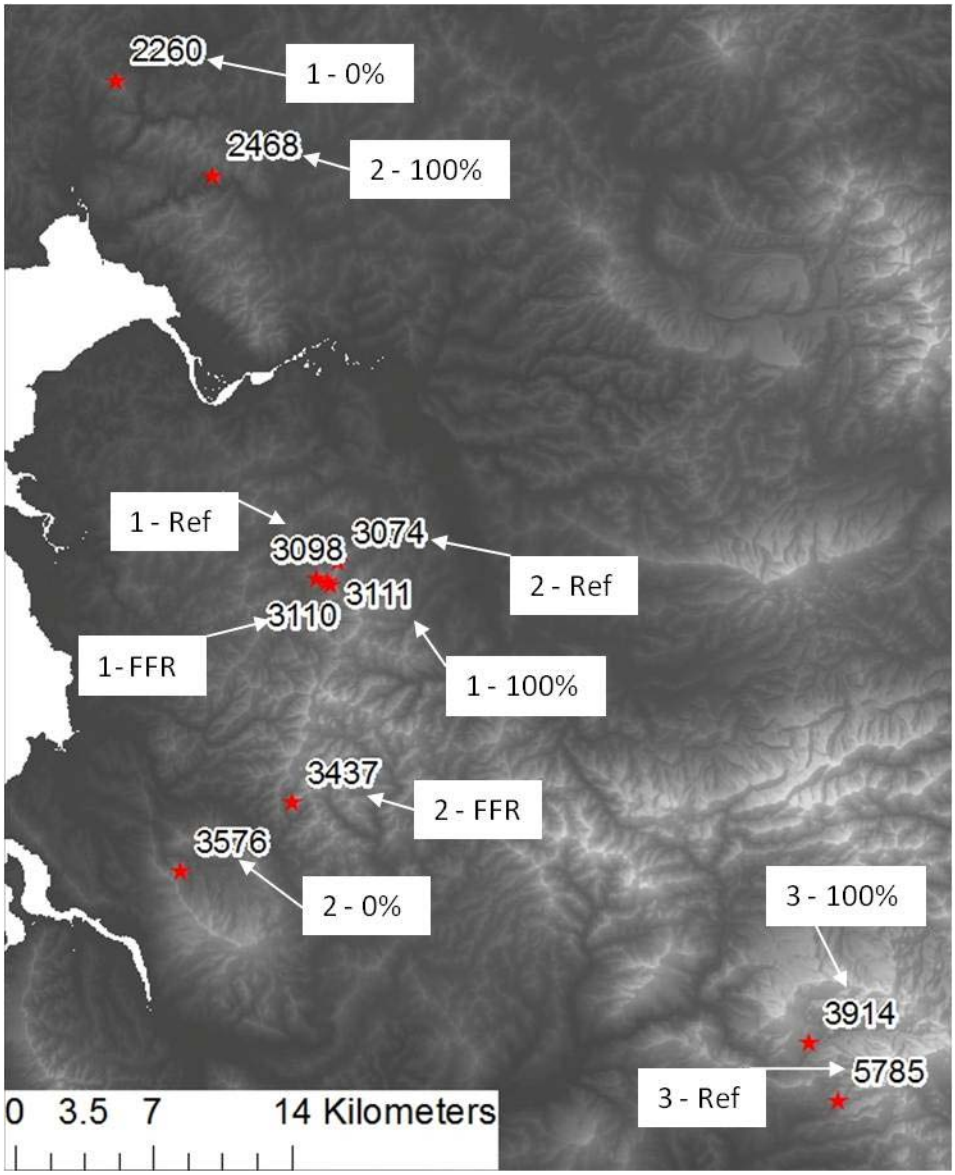

Figure 3. Map of Type N basin sites across the Willapa Hills region. Text boxes with areas indicate block and treatment types (number before dash is block number, followed by treatment type).

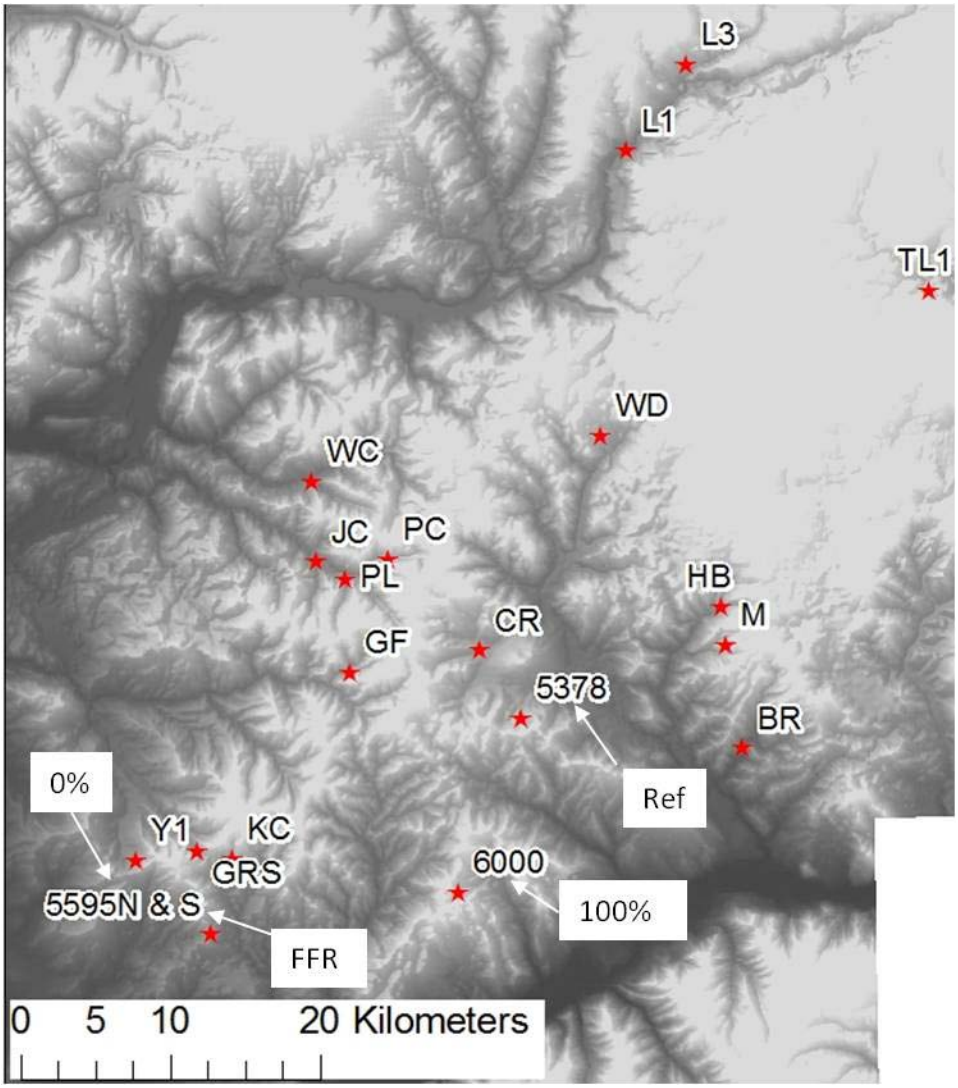

Figure 4. Map of Type N basins (sites 5378, 5595 N, 5595S, and 6000) and cluster sites across the South Cascades region. Text box and arrow represents assigned treatment for each basin.

For both tailed frogs and giant salamanders, most sampled individuals were larvae; this increases the likelihood that full siblings were sampled. If a sample has a proportionally large number of full siblings by chance, then sites may appear as inbred or as subdivided from other populations, even if the population as a whole is randomly mating and/or not subdivided. We identified full sibling pairs using a maximum likelihood approach implemented in the software COLONY (Wang 2004). This method was demonstrated to be appropriate for larval amphibians by Goldberg and Waits (2009). We identified the number of full sibling pairs per site, and in such cases we only included one family member for our analyses. Therefore, this method might create unequal

sample sizes among basins, and as a result, we account for unequal sample sizes in our estimates of genetic diversity (see Objective 2 methods).

2.2. Objective 1 Methods: Distinguishing giant salamander species

The first objective, determining the species identity of giant salamander samples, used a method that did not require us to identify *a priori* "pure" individuals belonging to each species. The program NEWHYBRIDS (Anderson and Thompson 2002) can accomplish this using a Bayesian method that assigns a posterior probability of an individual belonging to each species. With 10 or more microsatellites, probabilities can usually be assigned with very high probability (typically above 99%) if individuals are pure bred. Therefore, this program allowed us to confidently distinguish Cope's giant salamander individuals from the Coastal giant salamander individuals. In addition, if hybridization has occurred between the two species, the method can determine the probability of different types of hybrids (F_1, F_2, backcross Cope's [BC_{Dc}], or backcross Coastal [BC_{Dt}]). If any hybrids were observed, they were removed from the dataset and were not analyzed further.

Table 1. List of Type N basins by region, experimental block and buffer treatment. UTM coordinates are in NAD 83, Zone 10 and represent field verified fish end points for each basin. Numbers are used to identify sites in figures of clustering analysis results presented later in this report. FFR represents the current Forest Practices prescription buffer.

#	Basin/Site	Region	Block	Treatment	UTME	UTMN
1	363	Olympic	Olympic	100%	409832	5277868
2	1099	Olympic	Olympic	Reference	444753	5241629
3	1197	Olympic	Olympic	FFR	443819	5238402
4	1236	Olympic	Olympic	0%	442850	5237549
14	2260	Willapa Hills	Willapa 1	0%	433824	5184969
15	2468	Willapa Hills	Willapa 2	100%	438413	5179481
16	3074	Willapa Hills	Willapa 2	Reference	445225	5160174
17	3098	Willapa Hills	Willapa 1	Reference	443721	5159250
18	3110	Willapa Hills	Willapa 1	FFR	443750	5159054
19	3111	Willapa Hills	Willapa 1	100%	444223	5158712
20	3437	Willapa Hills	Willapa 2	FFR	442298	5148062
21	3576	Willapa Hills	Willapa 2	0%	442297	5148060
22	3914	Willapa Hills	Willapa 3	100%	469149	5135507
23	5785	Willapa Hills	Willapa 3	Reference	470128	5132348
30	5378	South Cascades	South Cascades	Reference	577925	5073227
25	5595N	South Cascades	South Cascades	0%	557451	5058323
26	5595S	South Cascades	South Cascades	FFR	557388	5058295
24	6000	South Cascades	South Cascades	100%	574450	5061390

2.3. Objective 2 Methods: Providing baseline levels of genetic diversity

2.3.1. Summary indices of genetic diversity

We estimated summary measures of genetic diversity for each sampled site for each species. Specifically, we estimated allelic richness, observed and expected heterozygosity and the inbreeding coefficient (F_{IS}). Allelic richness is a measure of the number of alleles present per locus per site corrected for the variance in sample sizes among sites. Observed and expected heterozygosity are additional measures of diversity. Expected heterozygosity is the percentage of heterozygotes that are expected to occur based on Hardy-Weinberg equilibrium and the number of alleles present. Observed heterozygosity is the percentage of heterozygotes that are actually found at the sampling site. F_{IS} is estimated based on the difference between observed and expected heterozygosity in a population, and value significantly different from 0 suggests a population is out of equilibrium. This metric ranges from -1 to 1, with positive values indicative of some degree of inbreeding and negative values suggestive of outbreeding. Values not different from zero suggest random mating. Finally, we estimated F_{ST} as a measure of genetic diversity among sites, and assessed significance of pairwise F_{ST} values. F_{ST} (range: 0-1) is positively correlated with differentiation, and thus low values indicate sites that are similar genetically. All genetic diversity parameters were estimated using either the software GDA (Lewis and Zaykin 2001) or FSTAT (Goudet 2001).

2.3.2. Estimate of effective population size

Effective population size (N_e) is a more meaningful metric of population viability than census population size. Effective population size is a measure of the number of breeding individuals in a population. Thus, a measure of effective population size can provide insight into its long-term viability, as well as predict the rate of inbreeding and loss of genetic diversity. We used a Brownian motion microsatellite model in MIGRATE 3.0.3 (Beerli 2008), which uses a coalescent approach (essentially following lineages back in time) to estimate the parameter θ, which is equal to $4N_e\mu$, with μ representing the microsatellite mutation rate. While the exact microsatellite mutation rate for each locus is unknown, a generally accepted estimate for microsatellites is 5×10^{-3} (Busch et al. 2007). However, it should be noted that mutation rate is only an estimate and likely subject to error. This would influence the exact value of N_e estimated, but would not change the relative differences among sites. Therefore, from θ we can estimate the differences in effective population size among sites, as well as a 95% confidence interval around the maximum likelihood estimate. Due to time limitations and memory limitations we could not estimate migration rates among basins, so we ran each Type N basin individually for each species to estimate effective population sizes. Each run consisted of 10 short chains of 10,000 generations each (sampled every 20 generations) and three long chains of 100,000 generations each (sampled every 20 generations). For each chain, a burn-in length of 10,000 generations was discarded.

The coalescent model used above represents historic effective population size. To provide more recent estimate of effective population size, we used a technique based on approximate Bayesian computation summarizing eight different metrics known as OneSamp (Tallmon 2004, 2008). This method generates 50,000 simulations with the same number of individuals and loci as the real population and with an initial effective size chosen from a range specified by the user. Effective population sizes from simulated populations that have similar summary statistics as the real population are accepted and the final estimated N_e is inferred from a weighted local regression of simulated values. We always chose 2 as the lower limit of N_e for simulations and the upper limit was chosen based on the upper confidence limit of MIGRATE results.

2.3.3. Evidence for recent population declines

We used three tests to assess whether any sites had undergone recent declines in effective population size. Specifically, we tested for: 1) significant heterozygosity excess relative to equilibrium expectations (Cornuet and Luikart 1996); 2) shifted distribution of allele frequencies (Luikart et al. 1998); and 3) M-ratios lower than those expected under random mating (Garza and Williamson 2001). Heterozygosity excess is an ephemeral pattern that can only be detected for the first several generations after a decline. While a heterozygosity excess may seem counterintuitive, it initially occurs because allelic diversity is lost faster than heterozygosity; this results in the appearance of greater heterozygosity than expected based on the number of alleles in the population (Cornuet and Luikart 1996). This method typically has low power unless declines are 90% or greater (Cornuet and Luikart 1996) and thus detected declines are likely to be severe. We assumed a two-phase mutation model with 10% multistep mutations. The choice of mutation model can influence the result of heterozygosity excess test in a predictable fashion (Cornuet and Luikart 1996). The two-phase model we use is the most conservative test that is realistic for our microsatellite loci. We used a Wilcoxon sign-rank test to detect significant excess in the software BOTTLENECK (Piry et al. 1999).

We also used BOTTLENECK to detect shifts in allelic distributions. Specifically, we tested for deviations from the expected distribution of microsatellite allele frequency of many rare alleles and few common alleles. As rare alleles are more likely to be lost during a bottleneck event, a population that has been through a recent decline will have an allele frequency distribution that is shifted so that common alleles occur at an even greater frequency.

Finally, the M-ratio is the ratio of number of alleles (*k*) to allelic size range (*r*). Allelic size range is simply the range in size of the microsatellite alleles that are determined by an automated DNA sequencer. To illustrate, if the microsatellite repeat is GATA and an individual has 8 repeats, their allele size would be 32. The allele size range (*r*) thus reflects the variation in number of repeats across all individuals in a population. The loss of alleles during a decline should be independent of allelic size, and thus *k* should decline faster than *r*. M-ratios are best suited for detecting more severe

declines over a longer period of time. Garza and Williamson (2001) suggested an overall critical M-ratio of 0.68, below which populations can be considered bottlenecked. However, sample size and effective population size can influence the critical value of the M-ratio. Therefore we used the Critical M software to determine the correct critical value for populations associated with Type N Study sites.

2.4. Objective 3 Methods: Testing for differences in genetic diversity among sites or blocks

To identify any pre-existing significant differences in genetic parameters, we conducted three comparisons of allelic richness, observed heterozygosity, F_{IS}, and effective population size (estimated through MIGRATE). We compared basins at three levels: among regions, blocks and treatment types. We used FSTAT v 2.9.3 (Goudet 2001) to run comparisons of allelic richness, heterozygosity, and F_{IS} using a randomization procedure with 10,000 permutations to assess significance. If we detected a significant difference by group for a variable, we tested all pairwise comparisons using the same randomization procedure to identify the specific comparisons that were significant after Bonferroni correction. We compared values for both estimates of effective population size using an ANOVA. To meet ANOVA assumptions, we conducted tests for normality using the R package normtest. If normality assumptions were violated, we log-transformed the variables and tested whether this corrected deviations from normality. For this test, if we detected a significant effect with the ANOVA, we used a t-test with a Bonferroni correction to assess which pairwise comparisons were different from each other. Second, we used simulations and population genetic expectations to predict the magnitude of decline necessary to detect changes in genetic diversity. To model changes in allelic richness, we used a population genetic simulator based on the current allelic diversity and number of loci of our data for each species. The simulation consisted of one population for one generation, with the number of loci and maximum number of alleles set to the specific values generated for each species in this study. We assumed equal sex ratio and ran 999 simulations for a number of population sizes. All simulations were run in EASYPOP 1.7 (Balloux 2001). To predict change in heterozygosity, we used Wright's equation for genetic drift (Wright 1969):

$$H_t = H_0\left[1-1/\left(2N_e\right)\right]^t$$

Where H_t is the final heterozygosity, H_o is the average heterozygosity of the study species from this study, N_e is the effective population size, and *t* is the generation time (in this case, 1). We used this equation for a range of possible effective population sizes. For both allelic richness and heterozygosity, we estimated effect size and identified the population size necessary to get a statistically significant result at the 0.05 level.

2.5. Objective 4 Methods: Estimating genetic clusters

We collected samples at an additional number of sites proximal to treatment basins to estimate the number and size of genetic clusters for each species. The genetic cluster size represents the extent of gene flow across the study areas and elucidates how dispersal may influence experimental results, thereby providing insight for spatial scale of management. Based on preliminary data from the Olympics and South Cascades, we predicted a maximum cluster size of 10-20 km for tailed frogs (Spear and Storfer 2008) and less than 5 km for Cope's giant salamander (Steele et al. 2009). The most important variable for the cluster analysis was spatial proximity to previously selected treatment basins. Therefore, we attempted to sample all accessible streams that were within 20 km of all treatment basins, although this was exceeded if suitable sites were difficult to find. We identified sites primarily by driving roads within the designated radius of Type N treatment sites and stopping at all streams that appeared permanent and contained suitable amphibian habitat (flowing water, riffles, pools, etc.). To maximize efficiency of sampling, we attempted to obtain sufficient sample size in one survey, and thus sites were only chosen if the focal species were found within the first 10-15 minutes. As our primary goal for this objective was obtaining sufficient number of samples, we did not standardize survey times or stream length, although most surveys lasted 1-2 hours and covered 50-100 meters of stream length. We only conducted cluster site sampling for the Olympics and South Cascades; however, the number of treatment basins sampled in the Willapa Hills allowed for a cluster analysis of this region as well.

We used a Bayesian population clustering algorithm, STRUCTURE 2.3.1 (Pritchard et al. 2000) as our primary tool to determine genetic cluster size. This analysis allows us to deduce which sites are exchanging migrants and thus estimate the spatial scale of gene flow. Additionally, conducting this analysis for each study region will allow us to infer whether the extent of gene flow varies across the entire Type N Study area. In STRUCTURE, log likelihood values for each run at a hypothesized K (number of clusters) are used to determine the most likely number of population groupings. However, estimating K by raw likelihood values can be problematic because as K increases, likelihood values tend to plateau after the true K is reached with slight improvements in value (Evanno et al. 2005). Therefore, choosing a clustering group based solely on the highest likelihood value may not make biological sense. To address this, Evanno et al. (2005) developed a method that estimated ΔK, the second order rate of change, to identify the value of K that had the greatest increase in likelihood with the lowest variance. The one problem with this method is that it does not allow for the evaluation of $K = 1$, since ΔK cannot be estimated for this value. However, if K truly equals one, then iterations assuming $K = 2$ will assign individuals to each cluster equally.

Finally, STRUCTURE often creates hierarchical subdivisions; clusters identified in one run of the program can often be further subdivided in a subsequent run that only includes sites that belong to the initial cluster. As a result, we ran additional iterations of STRUCTURE for all identified clusters until the program indicated that a group of sites

equaled 1 cluster, or when *K* equaled the number of sites in the run. All STRUCTURE results will be presented as bar plots by sampling site. Different clusters are denoted in the bar plots by different colors and the probability that individuals at a site assign to a particular cluster is determined by the amount of that color at each site in the bar plot. For each value of *K* we ran five replicates. Each replicate consisted of 100,000 iterations with 10,000 burn-in runs, which was sufficient for convergence in our dataset. For each hierarchical analysis, we used a range of *K* ranging from 1-10. We used an Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992) to verify the hierarchical clusters indicated by STRUCTURE. An AMOVA is simply an ANOVA based on genetic data. In particular, the analysis partitions the amount of genetic variation due to between groups, individual sites within groups, and individuals within sites. Additionally, the analysis tests the significance of each component. If we have clustered sites into the proper genetic groups, then variation among groups should be a greater percentage than variation among sites within groups. AMOVA analyses were implemented in the software ARLEQUIN 3.1.1 (Excoffier et al. 2005).

3. Results and Conclusions

3.1. Objective 1 – *Dicamptodon* species identification and hybrids

We are able to assign individuals to either pure species group with high probability (98% of individuals that were assigned to either species had a probability greater than 0.90). As expected, we identified both species of giant salamanders at sites in the South Cascades and Willapa Hills (Table 2). We did not detect Coastal giant salamanders in the Olympics, which is consistent with previous observations from that region and the documented range limit for Coastal giant salamanders (Welsh 2005). Interestingly, the northern Willapa Hills site numbers 2260 and 2468 (Fig. 3) contained only Cope's giant salamanders, which suggests the species boundary of Coastal giant salamanders is at the Willapa River. A geographic trend in species composition also seems to occur in the Willapas (Fig. 3). Sites centrally located (3098/3110/3111) and in the southeast (3914/5785) were predominantly Coastal giant salamanders, whereas the southwest pair of sites (3437/3576) were dominated by Cope's giant salamanders.

We also detected 31 hybrid individuals, almost exclusively in the Willapa Hills. Four hybrid categories were possible: F_1 (offspring of pure Cope's and Coastal), F_2 (offspring of two hybrid individuals), backcross to Cope's (offspring of hybrid individual and pure Cope's) and backcross to Coastal (offspring of hybrid individual and pure Coastal). We detected 31 hybrid individuals belonging to only two of these hybrid types: F_2 (26) and backcross to Cope's (5), with the majority in the F_2 type. This means that we have no evidence for hybridization between the two species in the current generation. The posterior probabilities of the assigned F_2 hybrids ranged between 0.48-0.99. Eighteen of the 26 putative hybrids had posterior probabilities 0.75 or greater. Therefore, some of our assigned hybrids may be misidentified, but the majority had high posterior

probabilities for the F_2 classification. Although we cannot say when in the past this hybridization occurred, it is possible that it resulted from recent droughts that may have forced increased contact between the two species in the Willapa Hills. Consistent with this hypothesis is the fact that WDFW personnel have observed over 50 post-metamorphic *D. copei* during this time period. Nonetheless, this analysis clearly suggests that hybrid offspring are viable with a possible differential in mating success among hybrid types.

The presence of the two species in several treatment sites may complicate our ability to interpret post-treatment results, but it also provides opportunities for additional insight. Potentially the biggest challenge is the disparity in numbers between the two species, and how some of the Willapa sites are arranged into blocks. The Willipa 2 block is completely composed of sites that are dominated by Cope's giant salamanders and thus this block should provide inference for this species. However, the Willapa 1 block is composed of one site (2260) with exclusively Cope's giant salamanders, while the other 3 basins in this block have a greater abundance of Coastal giant salamanders. This could cause a problem after experimental treatment, particularly if a significant change occurs in 2260 but not the other 3 sites; that is, treatment effects may be confounded if the two giant salamander species respond differently to disturbance. We have little reason to believe that hybridization will greatly affect experimental results, as low numbers of hybrids were present, and introgression appears to be historic and not ongoing. It will be interesting, however, to see whether the relative abundance of species or number and type of hybrids changes from the observed baseline levels in post-treatment samples.

Table 2. Numbers of giant salamander individuals belonging to four classes: pure Cope's giant salamanders, pure Coastal giant salamanders, F_2 hybrids, and backcross to Cope's hybrids (Bc_{cope}).

Region	Site	Cope's	Coastal	F_2	Bc_{cope}	Total
Olympics	363	31	0	0	0	31
	1099	62	0	0	0	62
	1197	28	0	0	0	28
	1236	44	0	0	0	44
	Total	**165**	**0**	**0**	**0**	**165**
Willapa Hills	2260	96	0	0	0	96
	2468	59	0	0	0	59
	3074	21	4	0	0	25
	3098	12	41	0	0	53
	3110	22	78	2	2	104
	3111	22	137	5	0	164
	3437	67	5	1	3	76

Region	Site	Cope's	Coastal	F_2	Bc_{cope}	Total
	3576	37	16	6	0	59
	3914	6	71	6	0	83
	5785	28	40	5	0	73
	Total	**370**	**392**	**25**	**5**	**792**
South Cascades	5595N	19	66	0	0	85
	5595S	69	102	1	0	172
	5378	52	31	0	0	83
	6000	100	107	0	0	207
	Total	**240**	**306**	**1**	**0**	**547**
	Grand Total	**775**	**698**	**26**	**5**	**1504**

3.2. Equilibrium tests, presence of null alleles and sibling estimation

While these results do not clearly fit one of the study objectives, the analyses are necessary to ensure the data do not violate assumptions of analyses that are contained in the following sections.

3.2.1. Coastal tailed frogs

Tailed frogs were collected at 15 of the 18 total Type N basins, with no frogs found at 363, 5595N and 5595S despite multiple collecting visits. We found that despite a large number of larval individuals, relatively few full siblings were detected (Table 3). Eighty to 95% of all sampled individuals were from different families, which likely partly reflects the WDFW approach of non-proximate sampling of sequential individuals. Little evidence exists of a relationship between sample size and number of siblings, as the total number of individuals explained only 13% of variation in the number of sibling individuals (Fig. 5). This suggests that the difference in the number of samples collected at sites is representative of the actual differences in census size. In other words, large sample sizes at some sites (such as 5785, 3110, etc.) are not simply due to sampling many related individuals.

We detected deviations from Hardy-Weinberg equilibrium at several sites for 4 loci: A14, A2, A29 and A3. Sites out of Hardy-Weinberg equilibrium at these loci displayed a clear geographic pattern, with A14 and A29 out of equilibrium at South Cascades sites and A2 and A3 out of equilibrium at the Willapa Hills sites. For each of these loci, the Hardy-Weinberg deviations are most likely due to the presence of null alleles at each locus from individuals in the respective regions. Specifically, across the South Cascades, A14 had an estimated 18% of alleles characterized as null, with 27% for locus A29. Similarly, within the Willapa Hills region, 18% of alleles were null for A2

and 10% for A3. Other than these locus/region pairs, we found no other evidence of null alleles. Because the presence of null alleles can provide misleading estimates of genetic diversity parameters, we eliminated A14 and A29 for South Cascades sites and A2 and A3 for Willapa Hills sites from further analyses. In addition, because we conducted statistical tests among all 3 regions for genetic diversity parameters, we only used the 9 loci the three regions had in common for estimation of allelic richness, heterozygosity and inbreeding. However, this reduced number of loci should still be sufficient for detecting significant changes in genetic diversity.

The fact that no evidence existed for null alleles in the Olympic region (the area used to sample individuals for marker development) strongly suggests that null alleles are due to mutation as described in the methods. This result also implies that tailed frogs on the Olympic Peninsula are genetically divergent from other tailed frog populations and have not regularly exchanged migrants in the recent past. This is consistent with findings from Nielson et al. (2006) who examined genetic variation in tailed frogs with allozyme and mitochondrial DNA markers (less variable genetic markers best used for historical inference) and found that Olympic sites were significantly divergent from Cascade sites. In fact, Nielson et al. (2006) argue that the Olympic population should be considered an Evolutionarily Significant Unit (ESU), which designates a lineage that contains genetic diversity unique to that population. In particular, they demonstrated that Olympic tailed frogs formed a monophyletic mitochondrial lineage (i.e. Olympic sites share a common ancestor that is not shared with any other site) and had high allozyme differences from other regions, including a unique allozyme allele only found in the Olympics.

Table 3. Sibling results and genetic diversity measures for Coastal tailed frogs at Type N Study basins. N is total sample size, Family # represents the number of unique families sampled at the site, A_r is allelic richness, H_e is expected heterozygosity, H_o is observed heterozygosity, F_{IS} is the inbreeding coefficient (* indicates significant after Bonferroni correction; adjusted *p*-value = 0.003), and N_e is effective population size (derived from θ, assuming a mutation rate of 0.005), along with the 95% confidence interval. N_e was estimated through either a coalescent approach (MIGRATE) or approximate Bayesian computation (ONESAMP).

Basin	N	Family #	A_r	H_e	H_o	F_{IS}	MIGRATE N_e	ONESAMP N_e
1099	42	41	11.21	0.9	0.86	0.05	4750 (4300-5050)	24 (16-36)
1197	24	19	9.96	0.88	0.92	-0.04	3550 (3250-3900)	214 (122-552)
1236	15	12	9.89	0.9	0.91	-0.01	3500 (3100-4000)	44 (31-110)
2260	50	46	10.44	0.86	0.84	0.02	2700 (2500-2850)	28 (21-38)
2468	33	27	10.52	0.87	0.85	0.03	4600 (4250-4950)	7572 (2182-76162)
3074	26	23	11.99	0.9	0.86	0.04	7500 (6850-8250)	29676 (4653-1794483)
3098	26	22	11.79	0.91	0.86	0.06	5700 (5100-6350)	3729 (1209-31533)
3110	54	51	12.5	0.91	0.84	0.08*	6750 (6350-7150)	5175 (1256-38934)
3111	54	52	11.76	0.9	0.86	0.06*	6350 (6000-6750)	29292 (5318-1220356)
3437	39	34	12.22	0.9	0.88	0.03	9450 (8750-10150)	4721 (1195-35933)
3576	48	42	11.91	0.9	0.89	0.01	7300 (6850-7750)	57 (34-94)
3914	25	24	11.95	0.9	0.89	0.01	8450 (7800-9150)	15971 (2849-345476)
5785	61	52	12.37	0.91	0.89	0.03	7700 (7250-8100)	0.19 (0.18-0.20)
5378	28	24	11.98	0.85	0.82	0.02	6100 (5600-6700)	1254285 (0-infinity)
6000	48	41	11.47	0.85	0.84	0.02	4300 (4000-4600)	0.38 (0.36-0.4)

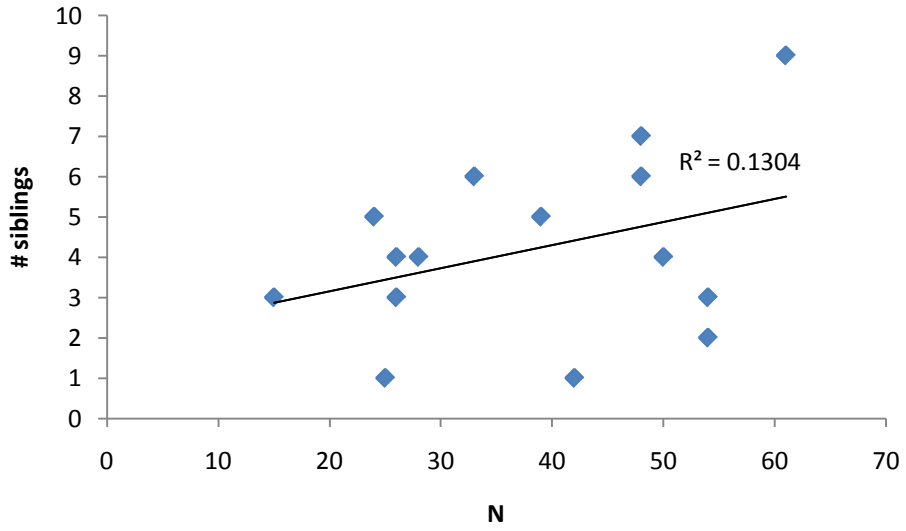

Figure 5. Relationship between number of individuals eliminated from data set as siblings and total sample size for Coastal tailed frogs.

Tests for linkage disequilibrium revealed that loci can be considered independent. No locus pair was significantly out of equilibrium at more than 2 or 3 sites (out of 47 total sampled sites). If two loci were truly statistically linked, we would expect to see disequilibrium for that pair at a majority of the 47 sites. As a result, once we accounted for null alleles, all loci used for tailed frogs are consistent with Hardy-Weinberg expectations and can be considered statistically independent, and thus are useful for further analyses.

3.2.2. Cope's giant salamanders

Cope's giant salamanders were collected at all Type N Study basins. In contrast to tailed frogs, full siblings often comprised a large portion of the total individuals of Cope's giant salamanders sampled (Table 4). Though broad variation existed in the number of siblings per site, the number of unique families at a basin averaged only 76% (compared to 88% for tailed frogs). The higher number of siblings may reflect fundamentally different microhabitat use than what was observed in tailed frog; we often found giant salamanders in low-flow areas including pools and under large debris, and their occurrence in such areas may reflect more sedentary behavior. Additionally, the number of full sibling groups is dependent on sample size for Cope's giant salamander. Extra siblings range from 0-34 per site, and total sample size explains 67% of the variation in sibling individuals (Fig. 6). Thus, the more total individuals that are sampled, the greater the likelihood that additional siblings will be sampled.

In general, all 11 loci conform to Hardy-Weinberg expectations, as each locus was in equilibrium at most sampling sites. However, 5 loci at basin 6000 violated equilibrium assumptions. This suggests that non-random mating is occurring, though the specific mechanism is unknown and could range from populations substructure to assortative mating Similarly, evidence of linkage disequilibrium among several locus pairs was found only at site 6000, again likely due to non-random mating. Finally, we found no evidence of null alleles at any loci for any region, and as such, all loci are included for further analyses.

Table 4. Sibling results and genetic diversity measures for Cope's giant salamanders at Type N Study basins. N is total sample size, Family # represents the number of unique families at the site, A_r is allelic richness, H_e is expected heterozygosity, H_o is observed heterozygosity, F_{IS} is the inbreeding coefficient (* indicates significant after Bonferroni correction; adjusted *p*-value = 0.003), and N_e is effective population size (derived from θ, assuming a mutation rate of 0.005), along with the 95% confidence intervals. N_e was estimated through either a coalescent approach (MIGRATE) or approximate Bayesian computation (ONESAMP).

Basin	N	Family #	A_r	H_e	H_o	F_{IS}	MIGRATE N_e	ONESAMP N_e
363	31	17	3.03	0.54	0.48	0.12	150 (120-155)	48 (30-159)
1099	62	46	4.01	0.65	0.65	0	450 (440-500)	157 (104-425)
1197	28	17	3.92	0.66	0.65	0.01	300 (275-350)	17 (14-32)
1236	44	29	3.92	0.64	0.65	-0.01	300 (265-315)	26 (18-55)
2260	96	62	4.75	0.74	0.71	0.05	600 (585-650)	188 (118-529)
2468	59	47	4.94	0.75	0.66	0.12*	600 (575-650)	363 (207-1304)
3074	21	17	6.14	0.85	0.8	0.06	2250 (2000-2550)	44 (29-93)
3098	12	11	6.13	0.83	0.77	0.08	3150 (2750-3600)	26 (18-61)
3110	22	14	5.93	0.82	0.81	0.01	2400 (2100-2700)	36 (26-69)
3111	22	18	6.39	0.86	0.78	0.09*	3200 (2900-3600)	67 (40-155)
3437	67	41	6.56	0.88	0.84	0.04	1800 (1700-1950)	592 (276-2381)
3576	37	33	6.18	0.85	0.75	0.12*	1250 (1150-1350)	126 (71-355)
3914	6	6	5.84	0.8	0.74	0.09	1150 (950-1450)	10 (7-18)
5785	28	23	6.23	0.85	0.75	0.11*	2750 (2500-3000)	742 (365-2947)
5595N	19	16	5.58	0.83	0.75	0.09*	1950 (1750-2200)	36 (25-80)
5595S	69	62	5.66	0.83	0.76	0.09*	700 (650-750)	170 (88-524)
5378	52	37	4.14	0.69	0.66	0.04	400 (350-415)	122 (60-370)
6000	100	81	5.44	0.8	0.74	0.09*	1050 (1000-1100)	2798 (883-24298)

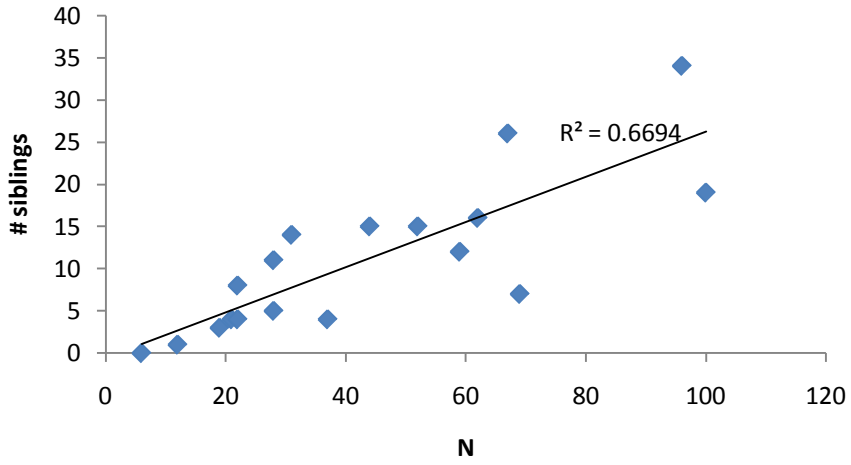

Figure 6. Relationship between number of individuals eliminated from data set as siblings and total sample size for Cope's giant salamanders.

3.2.3. Coastal giant salamanders

We detected high proportions of siblings across most sites for Coastal giant salamanders (Table 5). The average proportion of unique families was only 55%, and site 3110 had only 25% unrelated individuals. Furthermore, the number of siblings was strongly related to sample size, as $r^2 = 0.91$ (Figure 7), suggesting that high individual sample sizes will include many sibling pairs. The high number of sibling individuals may be related to moderately large clutch sizes, up to 200 eggs (Welsh 2005) which are larger than those laid by Cope's giant salamanders (average of 60; Jones and Bury 2005).

Three loci were consistently out of Hardy-Weinberg equilibrium at multiple sites. These loci were D05, D07, and D23. Additionally, basins 3914 and 6000 were out of Hardy-Weinberg equilibrium at several other loci besides the three above. As with basin 6000 for the Cope's giant salamander, the likely reason for equilibrium deviations is non-random mating, although the mechanism is unclear. Estimation of null alleles indicated that loci D05, D07 and D23 had high (>0.10) proportions of null alleles, likely resulting in deviations from equilibrium of those loci. Thus, these three loci were excluded from further analyses, leading to the use of 6 total loci for Coastal giant salamanders. Therefore, we probably have less power to detect changes in Coastal giant salamanders as compared to the other two species. Finally, no evidence existed of linkage disequilibrium among locus pairs.

Table 5. Sibling results and genetic diversity measures for Coastal giant salamanders at Type N Study basins. N is total sample size, Family # represents the number of unique families at the site, A_r is allelic richness, H_e is expected heterozygosity, H_o is observed heterozygosity, F_{IS} is the inbreeding coefficient (* indicates significant after Bonferroni correction; adjusted *p*-value = 0.004), and N_e is effective population size (derived from θ, assuming a mutation rate of 0.005), with the 95% confidence intervals. N_e was estimated through either a coalescent approach (MIGRATE) or approximate Bayesian computation (ONESAMP).

Basin	N	Family #	A_r	H_e	H_o	F_{IS}	MIGRATE N_e	ONESAMP N_e
3074	4	4	3.83	0.67	0.79	-0.21	600 (400-1050)	5 (4-7)
3098	41	14	3.82	0.68	0.67	0.01	550 (450-650)	31 (23-72)
3110	78	20	3.38	0.61	0.62	-0.02	350 (300-400)	56 (35-156)
3111	137	53	3.96	0.69	0.65	0.07	500 (450-550)	347 (154-1085)
3437	5	4	2.33	0.4	0.54	-0.42	50 (40-75)	7 (5-13)
3576	16	10	3.68	0.7	0.56	0.19	550 (450-650)	18 (12-39)
3914	71	43	3.79	0.67	0.51	0.25*	650 (600-700)	455 (188-1996)
5785	40	23	3.75	0.67	0.61	0.1	400 (350-450)	106 (59-388)
5595N	66	35	3.26	0.57	0.49	0.14*	350 (300-400)	120 (72-572)
5595S	102	53	3.42	0.63	0.63	0	450 (400-465)	99 (62-332)
5378	31	14	2.67	0.43	0.42	0.02	200 (190-255)	17 (12-44)
6000	107	56	4.43	0.74	0.69	0.07	750 (700-800)	2260 (739-1582)

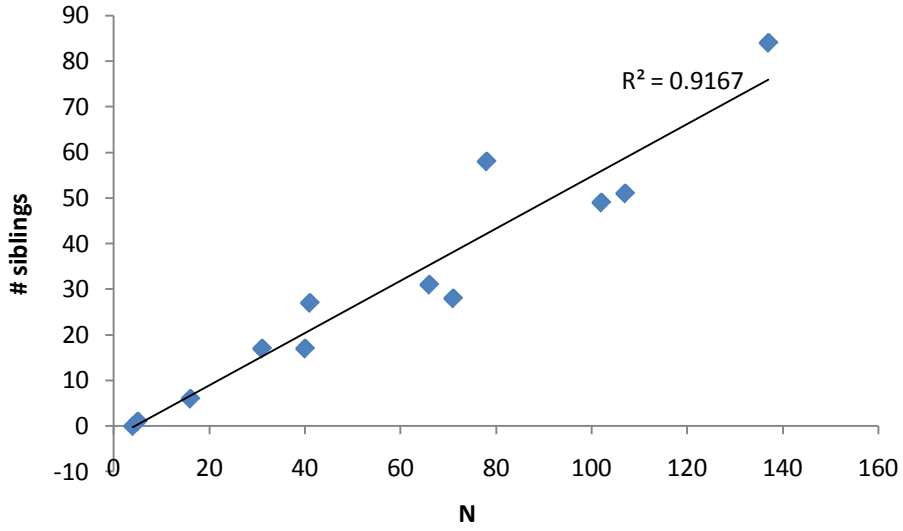

Figure 7. Relationship between number of individuals eliminated from data set as siblings and total sample size for the Coastal giant salamander.

3.3. Objective 2: Measures of genetic diversity and population size

3.3.1. Coastal tailed frogs

A. Summary measures of genetic diversity

Levels of genetic diversity in all Coastal tailed frog populations sampled were high (Table 3). The mean number of alleles per locus per site (once corrected for sample size) was greater than 11, and average expected and observed heterozygosity of 0.89 and 0.87, respectively. Since the observed heterozygosity was lower than the expected value, a positive, but low F_{IS}, averaging 0.03, was estimated. However, only two basins had F_{IS} values significantly greater than zero: 3110 and 3111. These two basins are adjacent, suggesting an unknown common cause. These high levels of F_{IS} must be considered in analysis of post-treatment genetic data, such that if significant levels of inbreeding are observed post-harvest at 3110 and 3111, it may be due to a prexisting condition. Significant F_{ST} values primarily corresponded to regional differences (Appendix 3), although there were some significant differences between sites within the Olympics and Willapa Hills, suggesting that genetic diversity is primarily apportioned by region.

Tailed frogs are an ancient lineage that has no closely related extant frog lineage to which to compare as to whether these levels of genetic diversity are typical. Similar high levels of heterozygosity and allelic richness are seen in the Rocky Mountain tailed frog (Spear 2008), so our results are at least consistent between the two tailed frog species. However, comparison with microsatellite studies among other frog species

suggests that these observed levels of diversity are much higher than expected for frogs in general. Ficetola et al. (2007) summarized 16 studies of frog microsatellite genetic diversity, and only one had levels of genetic diversity similar to that documented here. Most studies reported a mean allelic richness of 4-7 and heterozygosity of 0.4-0.6 (although differing sample sizes could lead to some differences in allelic richness). The high diversity detected in tailed frogs may reflect their long evolutionary history (a long time has been available to accumulate new alleles).

B. Effective population size

Based on coalescent MIGRATE analyses, tailed frogs appear to have very high effective population sizes across the entire study area, numbering between 3000-9000 (Table 3). Comparison with recent estimates of N_e (based on ONESAMP) demonstrates that many sites have similar or greater values as compared to the coalescent analysis. This is particularly true in the Willapa Hills region, where most sites have very high sizes. The Olympic region has significantly lower recent population sizes (based on non-overlap of confidence intervals), as well as sites 2260 and 3576 in the Willapas. Finally, sites 5785 and 6000 had extremely low sizes (less than 1). However, despite the differences in the two methods, it is clear that tailed frogs generally have a high effective population sizes Effective population size is often much smaller than the census population size, so the census population sizes associated with these N_e estimates are probably much larger. High effective population sizes undoubtedly play a large role in the high genetic diversity observed in these populations. High effective population sizes serve to buffer populations against loss of alleles due to inbreeding and genetic drift. Therefore, because such populations are less likely to undergo genetic changes due to drift, even small detected changes in post-treatment estimates of genetic diversity could reflect a strong treatment effect. However, smaller sizes in the Olympics suggest this area might respond to treatment effects of smaller magnitude, and sites 5785 and 6000 should be evaluated carefully due to the apparent current loss of N_e.

Table 6. Population bottleneck results for Coastal tailed frogs. H_e is the actual expected heterozygosity, H_{eq} is the expected heterozygosity under the assumed two-phase mutation model, and p-value is for the significance test of heterzygosity excess. Italics represent significant values.

Basin	H_e	H_{eq}	p-value	Shifted?	M-ratio	M-crit
1099	0.91	0.89	*0.0026*	normal	0.841	0.612
1197	0.90	0.88	0.0199	normal	0.712	0.520
1236	0.91	0.89	*0.0003*	normal	0.709	0.441
2260	0.88	0.89	0.4155	normal	0.738	0.656
2468	0.89	0.89	0.7935	normal	0.676	0.547
3074	0.91	0.90	0.3501	normal	0.679	0.473
3098	0.91	0.91	0.2886	normal	0.728	0.493

Basin	H_e	H_{eq}	p-value	Shifted?	M-ratio	M-crit
3110	0.92	0.91	*0.0034*	normal	0.812	0.615
3111	0.92	0.91	*0.0024*	normal	0.821	0.621
3437	0.91	0.91	0.4492	normal	0.769	0.522
3576	0.91	0.91	0.1602	normal	0.774	0.580
3914	0.91	0.90	0.0615	normal	0.714	0.469
5785	0.92	0.92	0.0415	normal	0.840	0.610
5378	0.86	0.90	0.1602	normal	0.689	0.502
6000	0.86	0.87	0.2597	normal	0.879	0.617

C. Declines in effective population size

We detected evidence of significant heterozygosity excess at 4 basins, but no evidence of declines due to shifted allele distributions or M-ratios (Table 6). The four basins with significant heterozygosity excess were 1099, 1236, 3110 and 3111. However, it is puzzling why these sites should not also have shifted allele distributions or low M-ratios. A likely confounding factor is migration. Bottleneck analyses assume closed populations, which is clearly not the case with tailed frogs, which have high levels of gene flow. Busch et al. (2007) conducted a study of kangaroo rats that had recently undergone a known demographic decline, but did not display signatures of bottlenecks using these three tests. They attributed this result to migration among their study sites, which would replenish rare alleles and obscure a bottleneck signature. Pope et al. (2000) used a simulation, based on a stepping stone migration model (which assumes that individuals only move to the closest suitable patch), to show that high migration could lead to heterozygosity excess, and lead to a false positive of population bottleneck. However, this explanation is somewhat unsatisfactory. This is because all basins have high migration rates and if high migration alone was contributing to false positives, we would expect all sites to display heterozygosity excess. Furthermore, the sites with significant heterozygosity excess also had some independent evidence of reduced size. Specifically, the significant inbreeding coefficient detected at 3110 and 3111 could be consistent with a very recent decline, and 1099 and 1236 have lower effective population sizes than the average. Obviously, none of these possibilities are compelling, and as such we cannot conclusively determine whether recent declines have occurred. Including the post-treatment samples in the future will be much more insightful, as temporal comparisons should be more sensitive to demographic changes (Luikart et al. 1998).

3.3.2. Cope's giant salamanders

A. Summary measures of genetic diversity

Overall, levels of genetic diversity in Cope's giant salamanders were lower than those documented for tailed frogs. Average allelic richness across all basins was 5. Expected heterozygosity and observed heterozygosity were 0.77 and 0.72, respectively.

Due to the deficiency of heterozygotes, a positive F_{IS} with an average value of 0.07 was found. Several individual basins had significantly high F_{IS} values, including: 2468, 3111, 3576, 5785, 5595N, 5595S and 6000. No strong geographic pattern appears to exist among sites with significant inbreeding, with the potential exception of 3 sites in the South Cascades. However, Steele et al. (2009) sampled 11 sites in the South Cascades in an area between sites 5595N, 5595S and 6000, and these sites displayed a low F_{IS}, so inbreeding does not seem uniformly distributed across the South Cascades region. Based on available data, Cope's giant salamanders appear to be primarily restricted to streams even as adults (Jones and Corn 1989), and the higher numbers of siblings detected suggests that individuals may not move frequently. Such limited mobility and observed proximity of full siblings could lead to an intrinsically higher prevalence of inbreeding than a species with a life history that promotes greater mobility. Pairwise measures of genetic differentiation (Appendix 4) indicate that almost all Cope's giant salamanders sampled at Type N basins are differentiated from one another. This suggests that if population sizes become small, Cope's giant salamanders are especially vulnerable to erosion of genetic diversity.

Overall, the genetic diversity of Cope's giant salamander is similar to that found in a number of other population genetic studies done primarily with ambystomatid salamanders (the salamander family thought to be most closely related to *Dicamptodon*). Such studies have found levels of heterozygosity ranging from 0.3-0.7 and a pattern of significant levels of F_{IS} in some, but not all, sites sampled (Spear et al. 2006, Eastman et al. 2007, Giordano et al. 2007, Chandler and Zamudio 2008, Purrenhage et al. 2009). The level of heterozygosity is much higher than observed values (0-0.244) in a population of Coastal giant salamanders in British Columbia (Curtis and Taylor 2003), although this population likely underwent a historic population bottleneck due to recent colonization after glaciations (Steele et al. 2006). Thus, Cope's giant salamander seems to have maintained a higher level of diversity relative to founder populations of *D. tenebrosus*.

B. Effective population sizes

The estimated effective population sizes based on coalescent analyses for Cope's giant salamander per site are much lower than those documented for tailed frogs (on average about 1/5 of tailed frog population sizes) and highly variable across sites (150-3150). In general, current effective sizes are significantly lower than the historic sizes, and most sites have N_e less than 200. There is also much more consistency among sites for Cope's giant salamander as compared to tailed frogs. Interestingly, sites 5785 and 6000 have the highest population size for Cope's giant salamanders, but extremely low for tailed frogs. Overall, we should expect smaller effective population sizes for Cope's giant salamander than for tailed frogs due to the former's more restricted habitat requirements (i.e. the post-metamorphic stages of tailed frogs can use terrestrial habitats, whereas most Cope's giant salamander adults are restricted to the stream environment). A geographic pattern seems to exist to the magnitude of effective population size.

Cope's giant salamanders reach their highest population size in the Willapa Hills and South Cascades and the lowest in the Olympics. This is contrary to expectations because Cope's giant salamanders are sympatric with Coastal giant salamanders in the Willapa Hills and South Cascades and may experience competition. This result also suggests that Olympic populations are more susceptible to genetic and demographic changes than in other areas, although the Olympic sites have a lower average F_{IS}, which could be due to behavioral avoidance of inbreeding due to prolonged small population sizes, a hypothesis in need of further testing. Finally, in comparison to tailed frogs, it appears that Cope's giant salamanders have been more susceptible to recent declines across their range, although it is unclear whether this is due to population subdivision or declines at each site.

Table 7. Population bottleneck results for Cope's giant salamanders. H_e is the actual expected heterozygosity, H_{eq} is the expected heterozygosity under the assumed two-phase mutation model, and p-value is for the significance test of heterzygosity excess. Italics represent significant values.

Basin	**H_e**	**H_{eq}**	**p-value**	**Shifted?**	**M-ratio**	**M-crit**
363	0.543	0.573	0.6812	normal	0.955	0.718
1099	0.66	0.71	0.9966	normal	0.833	0.706
1197	0.66	0.69	0.5508	normal	0.846	0.669
1236	0.64	0.68	0.5508	normal	0.870	0.700
2260	0.74	0.74	0.1826	normal	0.868	0.714
2468	0.75	0.75	0.2065	normal	0.817	0.701
3074	0.85	0.84	0.1602	normal	0.723	0.536
3098	0.83	0.83	0.1826	normal	0.692	0.432
3110	0.82	0.82	0.4155	normal	0.699	0.501
3111	0.86	0.87	0.6177	normal	0.764	0.514
3437	0.88	0.86	*0.0002*	normal	0.894	0.659
3576	0.85	0.83	0.2065	normal	0.807	0.657
3914	0.88	0.88	0.2783	normal	0.583	0.448
5785	0.85	0.87	0.3823	normal	0.718	0.568
5595N	0.83	0.83	0.2324	normal	0.718	0.538
5595S	0.83	0.84	0.6499	normal	0.876	0.712
5378	0.69	0.76	0.9939	normal	*0.656*	0.699
6000	0.80	0.87	0.9919	normal	0.724	0.723

C. Declines in effective population size

No widespread evidence exists for recent population declines across the Type N basins for Cope's giant salamanders (Table 7), in contrast to expectations based on effective population size estimates. Only two sites had a significant result for any of the

three bottleneck tests: 3437 had significant heterozygosity excess and 5378 had a low M-ratio. Basin 3437 had a high M-ratio and a normal allelic distribution, in contrast to the heterozygosity excess results. Due to their reliance on the stream environment, Cope's giant salamanders are more likely to follow a stepping-stone dispersal strategy, and thus stepping-stone migration to 3437 could potentially have led to the observed false positive (Pope et al. 2000). However, the explanation for the low M-ratio at 5378 is much more straight-forward. This site does not have a heterozygosity excess; in fact it has a strong heterozygote deficit. Low M-ratios without a corresponding heterozygosity excess is evidence of a more historic decline (Garza and Williamson 2001). Basin 6000 has a M-ratio that is barely above the critical level, and also has a heterozygote deficit. Finally, the sites of the Olympic peninsula all have strong heterozygote deficits (although higher M-ratios), suggesting historically low population sizes in this area as well. This is also consistent with the lower effective population sizes estimated across the Olympics and South Cascades.

3.3.3. Coastal giant salamanders

A. Summary measures of genetic diversity

Average values of genetic diversity in Coastal giant salamanders were lower than Cope's giant salamanders (average allelic richness = 3.5, average expected heterozygosity = 0.62 and average observed heterozygosity = 0.60) (Table 5). However, the levels of heterozygosity described here are much higher than a previous study of Coastal giant salamanders in British Columbia, which ranged from 0-0.244 (Curtis and Taylor 2003), although the BC population is a recent colonization, as described previously. Levels of F_{IS} were variable among sites, with 3 sites actually showing negative values (which indicates outbreeding, or mating among individuals less related than random), but two sites had significantly positive inbreeding (3914 and 5595N). Based on F_{ST} values, sites 3110 and 3111 were among the most differentiated, but there were several site comparisons between the Willapa Hills and South Cascades that have low F_{ST} values and thus low differentiation (Appendix 5). Note that sites 3074 and 3437 often had high F_{ST}, but values were not significant due to very low sample sizes and thus low power. It is somewhat surprising that Coastal giant salamanders would have lower levels of diversity than Cope's giant salamanders due to the greater dispersal capability of Coastal giant salamanders, which theoretically should reduce genetic drift and corresponding loss of alleles. Also surprising is the variability in F_{IS}, especially among sites that are very close together. Basin pairs 3110/3111 and 5595N/5595S are adjacent to each other, but have quite different inbreeding coefficients from each other, although the site pairs are not differentiated from each other. In addition, these results suggest that this variability must be considered in the post-treatment analysis.

B. Effective population sizes

The effective population sizes of Coastal giant salamanders associated with the respective treatment sites are much smaller than those found for either tailed frogs or Cope's giant salamanders, averaging only 450 based on the coalescent analyses, or roughly one third the mean value for Cope's giant salamanders. Current effective sizes are generally lower, with most sites less than 150. As with Cope's giant salamander, site 6000 has the highest population size. The overall low N_e is consistent with the high number of siblings detected for Coastal giant salamanders, which already demonstrates that census size is misleading for this species. The small population sizes may be largely due to late Pleistocene glaciations, as Steele and Storfer (2006) concluded that the area around the Columbia River valley was a refugium for this species, and populations expanded from this small refugium when glaciers receded. We would expect that population size would be small in a refugial area, and that perhaps local population sizes have not grown considerably since this historic bottleneck. However, presumably the glacial history should have affected Cope's giant salamander and tailed frogs similarly, but low population sizes are not seen for these species. Currently, these lower estimates of effective population size suggest that if Type N treatments do have a strong impact on stream amphibian populations, we might expect to see the greatest genetic change in Coastal giant salamanders.

C. Declines in effective population size

Widespread evidence of population declines based on M-ratios as well as two sites that had shifted allelic distributions was observed (Table 8). However, no significant heterozygosity excess was found at any sites, and only site 3437 had even a trend of heterozygosity excess. Interestingly, basin 3437 was the only site that had a significant heterozygosity excess for Cope's giant salamanders as well, suggesting that some recent event led to declines in giant salamanders in general at that site. Overall, however, strong evidence exists that Coastal giant salamanders in southern Washington have undergone a decline in the past, and that populations have not recovered, as indicated by low allelic diversity, low effective population sizes, heterozygote deficiencies and low M-ratios. These results underscore the possibility that this species may be particularly vulnerable to negative impacts due to chronically low population sizes in this region.

Table 8. Population bottleneck results for the Coastal giant salamander. H_e is the actual expected heterozygosity, H_{eq} is the expected heterozygosity under the assumed two-phase mutation model, and p-value is for the significance test of heterzygosity excess. Italics represent significant values.

Basin	**H_e**	**H_{eq}**	**p-value**	**Shifted?**	**M-ratio**	**M-crit**
3074	0.81	0.81	0.5000	shifted	0.474	0.436
3098	0.68	0.72	0.6563	normal	0.688	0.588
3110	0.61	0.69	0.9766	normal	*0.423*	0.637
3111	0.69	0.84	1.0000	normal	*0.660*	0.677
3437	0.61	0.59	0.1563	shifted	*0.621*	0.708
3576	0.70	0.70	0.5781	normal	*0.474*	0.551
3914	0.67	0.79	0.9219	normal	0.753	0.667
5785	0.67	0.72	0.9609	normal	*0.569*	0.642
5595N	0.57	0.69	0.7813	normal	*0.575*	0.667
5595S	0.63	0.68	0.9219	normal	*0.560*	0.679
5378	0.64	0.72	0.9375	normal	*0.590*	0.642
6000	0.74	0.85	0.9766	normal	*0.610*	0.679

3.4. Objective 3: Comparison of genetic diversity measures among groups

3.4.1. Coastal tailed frogs

A. Comparison among physiographic regions

Little evidence was found for differences among the three physiographic regions in measures of genetic diversity (Table 9). No significant differences were found in allelic richness, inbreeding coefficient or effective population size. The only significant difference is due to observed heterozygosity, although the magnitude of difference among the three regions was not large. Post-hoc pairwise analyses showed that the significant result was due to greater heterozygosity in the Olympics compared to the South Cascades ($p = 0.037$), and that the Willapa Hills was not significantly different from either the Olympics or South Cascades. We have evidence from null alleles and previous work (Nielson et al. 2006) that the three physiographic regions are represented by distinct evolutionary populations; thus it is interesting that whereas allelic frequencies differ between the regions, genetic diversity does not. In all likelihood, this stems from the high effective population sizes observed, which limit changes in genetic diversity caused by drift. Therefore, the different physiographic regions can be considered as replicates with respect to measures of genetic diversity for Coastal tailed frogs.

Table 9. Comparison of genetic diversity measures among regions for Coastal tailed frogs. Abbreviations are as in Table 3. Values under each measure represent the average for that region, with parentheses indicating the standard deviation. P-value represents the result of the permutation comparison test (for A_r, H_o, and F_{IS}) or ANOVA (for N_e). Italics represent a significant value. Note that due to deviations from normality, tests for differences among ONESAMP N_e values were log-transformed for ANOVA analysis; averages of raw values are presented in the table.

Region	A_r	H_o	F_{IS}	MIGRATE N_e	ONESAMP N_e
Olympics	10.35 (0.74)	0.885 (0.03)	0.016 (0.05)	3933 (708)	94 (104)
Willapa Hills	11.745 (0.71)	0.865 (0.02)	0.037 (0.02)	6650 (1948)	9622 (11490)
South Cascades	11.724 (0.36)	0.834 (0.01)	0.021 (0)	5200 (1273)	627143 (886913)
p-value	0.058	*0.027*	0.555	0.089	0.638

B. Comparison among blocks

Not surprisingly, the results for the block comparison were very similar to the regional comparison, as two of the blocks were the same as the regions (Olympics, South Cascades) (Table 10). Again, the only measure that was significantly different was observed heterozygosity. The significant result was based not only on a lower value in the South Cascades than the Olympics (*p*-value = 0.037), but also on a lower heterozygosity in the South Cascades than the Willapa 3 pair (*p*-value = 0.035). No other pairwise comparisons were significantly different for observed heterozygosity. Thus, we can primarily consider the blocks to be replicates, and more importantly, the only two complete blocks in which tailed frogs were found in all four treatments (Willapa 1 and Willapa 2) are not significantly different for any parameter.

Table 10. Comparison of genetic diversity measures among blocks for Coastal tailed frogs. Abbreviations are as in Table 3. Values under each measure represent the average for that region, with parentheses indicating the standard deviation. P-value represents the result of the permutation comparison test (for A_r, H_o, and F_{IS}) or ANOVA (for N_e). Italics represent a significant value. Note that due to deviations from normality, tests for differences among ONESAMP N_e values were log-transformed for ANOVA analysis; averages of raw values are presented in the table.

Block	A_r	H_o	F_{IS}	MIGRATE N_e	ONESAMP N_e
Olympic	10.35 (0.74)	0.885 (0.03)	0.016 (0.05)	3933 (708)	94 (104)
Willapa 1	11.624 (0.86)	0.848 (0.01)	0.053 (0.03)	5375 (1835)	9556 (13335)
Willapa 2	11.66 (0.77)	0.873 (0.02)	0.026 (0.01)	7212 (1993)	10506 (13150)
Willapa 3	12.157 (0.30)	0.89 (0)	0.022 (0.01)	8075 (530)	7986 (11293)
South Cascades	11.724 (0.36)	0.834 (0.01)	0.021 (0)	5200 (1273)	627143 (886913)

Block	A_r	H_o	F_{IS}	MIGRATE N_e	ONESAMP N_e
p-value	0.134	*0.024*	0.537	0.072	0.761

C. Comparison among anticipated treatments

Among anticipated treatment types, no differences were found in any genetic diversity parameter (Table 11), including observed heterozygosity. This important result indicates that the sites chosen for different treatments are statistically equivalent with respect to levels of genetic diversity. Thus, we can be reasonably confident that any significant post-treatment effects in genetic diversity are due to that treatment and not due to confounding site-specific factor(s). Variance was low for measures of allelic richness and heterozygosity, but higher for F_{IS} and N_e. Thus, allelic richness and heterozygosity might be the most likely variables to detect statistical treatment effects. However, simulations suggest that a large reduction in effective population size is necessary to detect changes in allelic richness and heterozygosity. A reduction to an effective population size of 34 or lower is necessary to significantly reduce the number of alleles in one generation, and the reduction would have to be to seven individuals for a statistically significant change in heterozygosity in one generation. Thus, allelic richness is more likely to respond to a population change than heterozygosity, but there would still have to be a severe bottleneck given the large effective population sizes in tailed frogs. Thus, more subtle genetic measures, such as increasing proportion of full siblings, might be a better immediate measure of treatment effects. If a change in genetic diversity is detected, it would be a rather unequivocal signature of a strong change in the population.

Table 11. Comparison of genetic diversity measures among anticipated treatments for Coastal tailed frogs. Abbreviations are as in Table 3. Values under each measure represent the average for that region, with parentheses indicating the standard deviation. P-value represents the result of the permutation comparison test (for A_r, H_o, and F_{IS}) or ANOVA (for N_e). Italics represent a significant value. Note that due to deviations from normality, tests for differences among ONESAMP N_e values were log-transformed for ANOVA analysis; averages of raw values are presented in the table.

Anticipated Treatment	A_r	H_o	F_{IS}	MIGRATE N_e	ONESAMP N_e
reference	11.867 (0.42)	0.865 (0.02)	0.039 (0.02)	6350 (1244)	257543 (557334)
100%	11.515 (0.63)	0.863 (0.02)	0.017 (0.01)	5925 (1911)	13209 (12550)
FFR	11.428 (1.39)	0.867 (0.04)	0.039 (0.05)	6583 (2954)	3370 (2743)
0%	10.745 (1.04)	0.872 (0.04)	0.014 (0.02)	4500 (2458)	43 (15)
p-value	0.384	0.981	0.466	0.598	0.750

3.4.2. Cope's giant salamanders

A. Comparison among regions

Significant differences existed in all genetic diversity measures among regions for Cope's giant salamanders (Table 12), with the exception of recent effective population size. These differences were all due to lower levels of all measures for basins on the Olympic Peninsula. Significant differences existed for all variables in pairwise comparisons between the Olympics and Willapa Hills (allelic richness *p*-value = 0.001, heterozygosity *p*-value = 0.001, F_{IS} *p*-value = 0.02, historic N_e *p*-value = 0.0004), but not for any comparisons between the Olympics and South Cascades or between the Willapa Hills and South Cascades. Interestingly, the Olympics had a lower level of inbreeding, despite having uniformly lower allelic richness, lower heterozygosity and lower effective population sizes. Generally, low levels of these three parameters would be associated with higher inbreeding coefficients. This discrepancy may be explained by evolved behavioral avoidance of inbreeding due to prolonged small population sizes, as mentioned previously. Nonetheless, it appears that the Olympic Peninsula cannot be considered a replicate for genetic diversity measures in the Type N Study, and highlights the importance of the paired sampling approach of the study to disentangle regional effects.

Table 12. Comparison of genetic diversity measures among regions for Cope's giant salamanders. Abbreviations are as in Table 3. Values under each measure represent the average for that region, with parentheses indicating the standard deviation. P-value represents the result of the permutation comparison test (for A_r, H_o, and F_{IS}) or ANOVA (for N_e). Italics represent a significant value. Note that due to deviations from normality, tests for differences among ONESAMP N_e values were log-transformed for ANOVA analysis; averages of raw values are presented in the table.

Region	A_r	H_o	F_{IS}	MIGRATE N_e	ONESAMP N_e
Olympics	3.722 (0.46)	0.625 (0.08)	0.018 (0.06)	300 (122)	62 (65)
Willapa Hills	5.909 (0.60)	0.748 (0.05)	0.077 (0.04)	1915 (984)	219 (261)
South Cascades	5.204 (0.72)	0.732 (0.05)	0.079 (0.02)	1025 (671)	781 (1345)
p-value	*0.0018*	*0.0109*	*0.0429*	*0.012535*	0.315

B. Comparison among blocks

Results of among block comparisons demonstrated that allelic richness and historic effective population size were significantly different among blocks (Table 13). Pairwise comparison of the Olympic block and Willapa 1 block was significant (p = 0.002), as well as between Olympic block and Willapa 2 (p = 0.001). There were no significant pairwise comparisons after Bonferroni correction (adjusted *p*-value = 0.005)

for N_e, although Olympic v. Willapa 1 (*p*-value = 0.02) and Olympic v. Willapa 2 (*p*-value = 0.02) were significantly different if multiple comparisons were not accounted for. No differences existed between the South Cascades block and any of the Willapa Hills blocks.

Table 13. Comparison of genetic diversity measures among blocks for Cope's giant salamanders. Abbreviations are as in Table 3. Values under each measure represent the average for that region, with parentheses indicating the standard deviation. P-value represents the result of the permutation comparison test (for A_r, H_o, and F_{IS}) or ANOVA (for N_e). Italics represent a significant value. Note that due to deviations from normality, tests for differences among ONESAMP N_e values were log-transformed for ANOVA analysis; averages of raw values are presented in the table.

Block	A_r	H_o	F_{IS}	MIGRATE N_e	ONESAMP N_e
Olympic	3.722 (0.46)	0.625 (0.08)	0.018 (0.06)	300 (122)	62 (65)
Willapa 1	5.801 (0.73)	0.741 (0.04)	0.053 (0.04)	2338 (1215)	79 (75)
Willapa 2	5.954 (0.70)	0.754 (0.08)	0.087 (0.04)	1475 (712)	136 (811)
Willapa 3	6.034 (0.28)	0.75 (0.01)	0.109 (0.01)	1950 (1131)	376 (518)
South Cascades	5.204 (0.72)	0.732 (0.05)	0.079 (0.02)	1025 (671)	781 (1345)
p-value	*0.0142*	0.1602	0.0699	*0.03516*	0.491

C. Comparison among anticipated treatments

As with tailed frogs, no significant differences were found in genetic diversity among anticipated treatment types (Table 14). Therefore, no pre-existing conditions appear to exist that might bias overall comparisons among pre- and post-treatment. Once again, allelic richness and heterozygosity have the lowest variance within treatments, although the variance is slightly higher than seen in tailed frogs. This could mean that treatment effects will be easier to detect in tailed frogs. In fact, simulations indicate that effective size would need to decrease to 12 for a detectable change in allelic richness and only two individuals to detect a change in heterozygosity. However, on average, effective sizes are smaller in giant salamanders, and thus a strong treatment effect may be more likely to be detected in giant salamanders as the proportion of population reduction would be smaller than in tailed frogs.

Table 14. Comparison of genetic diversity measures among treatments for Cope's giant salamanders. Abbreviations are as in Table 3. Values under each measure represent the average for that region, with parentheses indicating the standard deviation. P-value represents the result of the permutation comparison test (for A_r, H_o, and F_{IS}) or ANOVA (for N_e). Italics represent a significant value. Note that due to deviations from normality, tests for differences among ONESAMP N_e values were log-transformed for ANOVA analysis; averages of raw values are presented in the table.

Anticipated Treatment	A_r	H_o	F_{IS}	MIGRATE N_e	ONESAMP N_e
reference	5.33 (1.15)	0.701 (0.07)	0.049 (0.04)	1800 (1295)	218 (298)
100%	5.129 (1.29)	0.695 (0.12)	0.099 (0.02)	1230 (1171)	657 (1205)
FFR	5.516 (1.13)	0.779 (0.08)	0.055 (0.04)	1300 (970)	204 (268)
0%	5.108 (0.99)	0.711 (0.05)	0.063 (0.06)	1025 (733)	94 (77)
p-value	0.9433	0.3162	0.3575	0.425461	0.944

3.4.3. Coastal giant salamanders

A. Comparison between regions

As no Coastal giant salamanders were detected in the Olympics in this study, our regional comparison was restricted to the Willapa Hills and South Cascades. The only significant difference between the two was in historic N_e, which, though low in both regions, was higher in the Willapa Hills (Table 15).

Table 15. Comparison of genetic diversity measures between regions for the Coastal giant salamander. Abbreviations are as in Table 3. Values under each measure represent the average for that region, with parentheses indicating the standard deviation. P-value represents the result of the permutation comparison test (for A_r, H_o, and F_{IS}) or ANOVA (for N_e). Italics represent a significant value. Note that due to deviations from normality, tests for differences among ONESAMP N_e values were log-transformed for ANOVA analysis; averages of raw values are presented in the table.

Region	A_r	H_o	F_{IS}	MIGRATE N_e	ONESAMP N_e
Willapa Hills	3.57 (0.53)	0.604 (0.09)	0.097 (0.22)	456 (192)	128 (174)
South Cascades	3.444 (0.73)	0.602 (0.12)	0.056 (0.06)	438 (232)	624 (1092)
p-value	0.760	0.983	0.596	*0.022*	0.311

B. Comparison among blocks

No significant differences were found for any genetic diversity parameter for Coastal giant salamanders, despite some large differences in F_{IS} (Table 16). Thus, this

statistical technique may have low power to detect differences in the inbreeding coefficient; low power does not seem to be a problem for the other measures of genetic diversity across the three species. Lower power might be expected for this species because of the lower number of loci used. Overall, though, it appears that the blocks can be considered replicates for genetic diversity for Coastal giant salamanders.

Table 16. Comparison of genetic diversity measures among blocks for the Coastal giant salamander. Abbreviations are as in Table 3. Values under each measure represent the average for that region, with parentheses indicating the standard deviation. P-value represents the result of the permutation comparison test (for A_r, H_o, and F_{IS}) or ANOVA (for N_e). Italics represent a significant value. Note that due to deviations from normality, tests for differences among ONESAMP N_e values were log-transformed for ANOVA analysis; averages of raw values are presented in the table.

Block	A_r	H_o	F_{IS}	MIGRATE N_e	ONESAMP N_e
Willapa 1	3.722 (0.3)	0.645 (0.03)	0.04 (0.05)	467 (104)	145 (176)
Willapa 2	3.283 (0.83)	0.637 (0.14)	-0.017 (0.31)	400 (304)	10 (7)
Willapa 3	3.773 (0.03)	0.54 (0.07)	0.198 (0.11)	525 (177)	280 (247)
South Cascades	3.444 (0.73)	0.602 (0.12)	0.056 (0.06)	438 (232)	624 (1092)
p-value	0.781	0.686	0.088	0.135	0.111

C. Comparison among anticipated treatments

We also found little significant difference by anticipated treatment, which is consistent with the finding for the other two species (Table 17). The only exception is recent effective population size, which is due to much larger population sizes in the 100% buffer treatment sites. Therefore, the process for choosing basins, and treatments assigned to those basins, was generally successful in creating an initial baseline without significant genetic differences among the experimental treatments for all taxa involved. However, inferences related to population size in Coastal giant salamanders should consider the baseline higher levels in the 100% treatment. Levels of variance are similar to Cope's giant salamander, except for lower variation in allelic richness. Due to the lower genetic diversity found in Coastal giant salamanders as compared to the other two species, a reduction to an effective size of six would be necessary to detect differences in allelic richness, and an effective size of a single individual would be required to detect changes in heterozygosity. Thus, an extreme bottleneck in giant salamanders would likely need to occur. This further supports the argument that genetic measures such as proportion of siblings detected and potentially direct estimates of effective population size may be more useful in detecting changes.

Table 17. Comparison of genetic diversity measures among anticipated treatments for the Coastal giant salamander. Abbreviations are as in Table 3. Values under each measure represent the average for that region, with parentheses indicating the standard deviation. P-value represents the result of the permutation comparison test (for A_r, H_o, and F_{IS}) or ANOVA (for N_e). Italics represent a significant value. Note that due to deviations from normality, tests for differences among ONESAMP N_e values were log-transformed for ANOVA analysis; averages of raw values are presented in the table.

Anticipated Treatment	A_r	H_o	F_{IS}	MIGRATE N_e	ONESAMP N_e
reference	3.519 (0.57)	0.589 (0.15)	0.036 (0.13)	438 (180)	40 (45)
100%	4.062 (0.33)	0.624 (0.09)	0.112 (0.1)	633 (126)	1021 (1075)
FFR	3.045 (0.62)	0.625 (0.05)	-0.021 (0.24)	283 (208)	54 (46)
0%	3.469 (0.3)	0.51 (0.05)	0.138 (0.03)	450 (141)	69 (72)
p-value	0.191	0.543	0.330	0.173	*0.033*

3.5. Objective 4: Genetic cluster size

3.5.1. Cluster site selection

Altogether, we sampled individuals from 38 streams to use as genetic cluster sites in the Olympics and South Cascades (Table 18). We did not detect both tailed frogs and giant salamanders at all sites, and overall we were more successful capturing tailed frogs than giant salamanders. We sampled tailed frogs from 32 sites (84%) and Cope's giant salamanders from 12 sites (32%). We also collected Coastal giant salamanders at 3 sites in small numbers. While we had relatively uniform success in sampling tailed frogs from both the Olympics and South Cascades, we had much greater sampling success in the Olympics for giant salamanders. This was primarily due to sampling effort; we had difficulty collecting giant salamanders in both regions, but spent much more time in the Olympics. This was because Steele et al. (2009) previously sampled 11 streams in a small region in the South Cascades that was located near basin 6000. They found high subdivision in Cope's giant salamander, as most individual streams clustered separately. In contrast, Coastal giant salamanders formed a single cluster over all 11 sites. Therefore, collecting giant salamanders from additional sites in the South Cascades was a lower priority than the Olympics because we already had a good idea of the genetic cluster size in the South Cascades from Steele et al. (2009).

Table 18. Sites used in genetic cluster analysis. Number is the site number in figures of clustering analysis. The species column represents which species were collected at each site (ASTR=tailed frog, DICO=Cope's giant salamander, DITE=Coastal giant salamander). UTM coordinates are in NAD83, Zone 10.

#	Site	Region	Species	UTME	UTMN
5	H1	Olympic	ASTR/DICO	443429	5254134
6	H11	Olympic	DICO	434932	5247868
7	H12	Olympic	DICO	443962	5255347
8	H3	Olympic	ASTR/DICO	438186	5246262
9	JB1	Olympic	DICO	418302	5297451
10	JB2	Olympic	DICO	469843	5247483
11	S1	Olympic	ASTR/DICO	461328	5249413
12	SAM	Olympic	DICO	430771	5273240
13	W1	Olympic	ASTR/DICO	449711	5246092
27	KC	South Cascades	DICO/DITE	558530	5063399
28	PC	South Cascades	ASTR/DICO/DITE	568972	5083368
29	Y1	South Cascades	ASTR/DICO/DITE	552106	5063310
31	BIG	Olympic	ASTR	450086	5250444
32	C1	Olympic	ASTR	462657	5244349
33	H2	Olympic	ASTR	434725	5243275
34	H5	Olympic	ASTR	441202	5247319
35	H6	Olympic	ASTR	442440	5250902
36	H7	Olympic	ASTR	445133	5248623
37	PETE	Olympic	ASTR	444970	5255907
38	RB	Olympic	ASTR	437876	5242411
39	SP	Olympic	ASTR	457945	5244876
40	T1	Olympic	ASTR	454565	5254620
41	W2	Olympic	ASTR	460196	5256203
42	WFH	Olympic	ASTR	448541	5258877
43	WY	Olympic	ASTR	460734	5260262
44	BR	South Cascades	ASTR	592624	5070774
45	CR	South Cascades	ASTR	575040	5077317
46	GF	South Cascades	ASTR	566425	5075823
47	GRS	South Cascades	ASTR	556202	5063905
48	HB	South Cascades	ASTR	591216	5080242
49	JC	South Cascades	ASTR	564180	5083242
50	L1	South Cascades	ASTR	584835	5110676
51	L3	South Cascades	ASTR	588866	5116446
52	M	South Cascades	ASTR	591480	5077681

#	Site	Region	Species	UTME	UTMN
53	PL	South Cascades	ASTR	566096	5082021
54	TL1	South Cascades	ASTR	605038	5101361
55	WC	South Cascades	ASTR	563891	5088589
56	WD	South Cascades	ASTR	583136	5091665

3.5.2. Coastal tailed frogs

Consistent with the patterns of null alleles, we found Coastal tailed frog sites to be strongly structured by region (Fig. 8). Values of ΔK clearly indicate that 3 clusters is the primary subdivision among sampled tailed frogs. The three clusters corresponded exactly to the three regions of the Olympics, Willapa Hills, and South Cascades (Fig. 8). There does appear to be some limited migration between the Willapa Hills and South Cascades, as evidenced by individuals assigned to one region being found in the other region. Further subdivision was only detected in the Willapa Hills (Fig. 9). Both the Olympics and South Cascades had all individuals assigned evenly to two clusters, strongly suggesting that on the basis of sampled alleles, no genetic structure exists in either region. The Willapa Hills has the greatest ΔK and log likelihood at $K = 3$, although visualizing the assignments revealed only two clear clusters (Fig. 9). Additionally, it appeared that basin 2468 (15) may have individuals belonging to the cluster represented by basin 2260 (14), as well as to the larger cluster containing the remaining Willapa sites. Therefore, we included 2468 (15) in a run with 2260 (14), and also included 2468 (15) in a separate run with the other Willapa sites. The results demonstrated that 2260 (14) and 2468 (15) clustered separately (Fig. 10), but with a number of migrants from 2468 (15) into 2260 (14) (but not the other direction; see relative locations in Fig. 3). There was no subdivision detected between 2468 (15) and all remaining Willapa sites. The AMOVA results supported the presence of only 4 clusters (Olympics, 2260, remaining Willapa Hills, and South Cascades) as only 0.43% of the variation was between sites within groups, whereas 4.8% of variation was between groups (the vast majority of variation was within sites, which is a common result in AMOVA analyses).

Tailed frogs have very high levels of gene flow even over broad geographic distances, indicating a very large genetic cluster size for a small amphibian at a regional scale, extending for 20-30 km (Spear and Storfer 2008). Indeed, it appears that genetic clusters are best defined by large tracts of open lowlands that form the divisions between the Olympics, Willapa Hills and South Cascades. The only apparent exception to this is basin 2260 (14), which is subdivided from the other Willapa Hills sites, and while receiving migrants from 2468 (15), does not seem to produce migrants to 2468 (15). These results are evident in the bar plot (Fig. 10) because 2260 (14) has several predominantly green bars, which indicate genetic assignment to basin 2468 (15). However, 2468 (15) has a very small proportion of individuals belonging to the red cluster suggesting unidirectional migration from 2468 (15) to 2260 (14). Therefore, basin

2260 (14) may represent a sink for tailed frogs (i.e., a site that relies on outside immigration to maintain a population; Pulliam 1988). Additional support for this comes from the fact that site 2260 (14) has the lowest N_e of all basins. The high migration of tailed frog across sites may complicate conclusions of the Type N Study because movement from individuals at other sites into treatment basins may obscure treatment effects. It is difficult to assess whether this connectivity is due to consistent high migration between sites every generation, or whether the high effective population size prevents rapid differentiation even if there is not constant migration between sites, or a combination of both. However, if statistically significant post-treatment differences in genetic diversity or genetic cluster size are detected for tailed frogs, it likely represents a very strong treatment effect that disrupted migration into the experimental basin or influenced the surrounding landscape. Thus, we would consider tailed frogs to serve as a conservative test of experimental buffer effects.

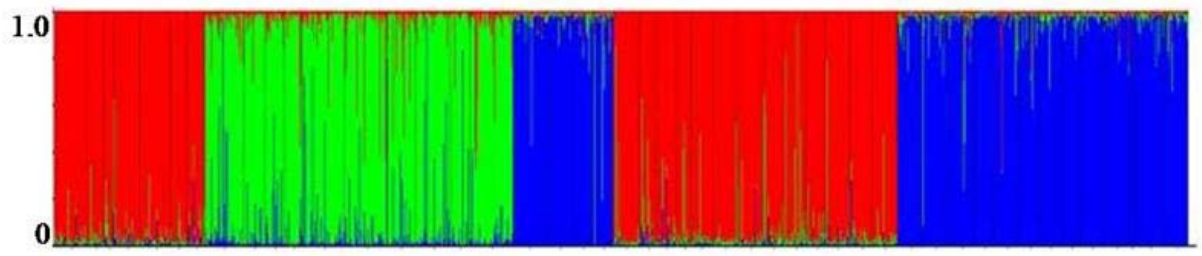

Figure 8. STRUCTURE bar plots for all tailed frog sites (suggesting *K*=3). Each color represents a population cluster. Red represents Olympic sites, green are Willapa Hills sites and blue are South Cascades sites. The y-axis represents the proportion of membership of each individual to each cluster. Each individual is represented by a different vertical line. That is, if an individual (represented by a vertical line) is primarily green, then its genotype indicates that it is most likely from the Willapa Hills. Similarly, if a line representing an individual's genotype is half green and half red, then it is equivocal whether that individual is from the Olympics or Willapa Hills.

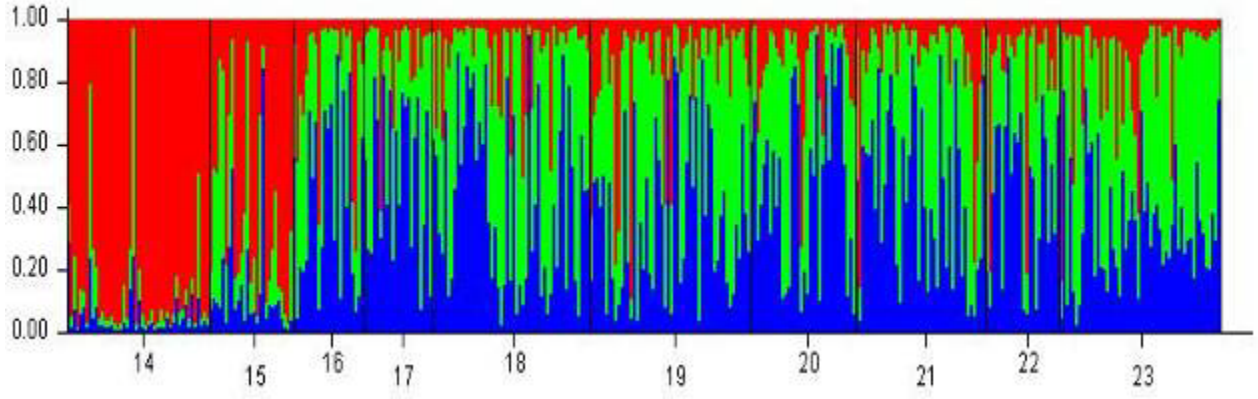

Figure 9. STRUCTURE bar plots for Willapa Hills tailed frog sites. Site numbers are as in Tables 1 and 18. This plot suggests two genetic clusters, sites 14 and 15 seem distinct from 16-23.

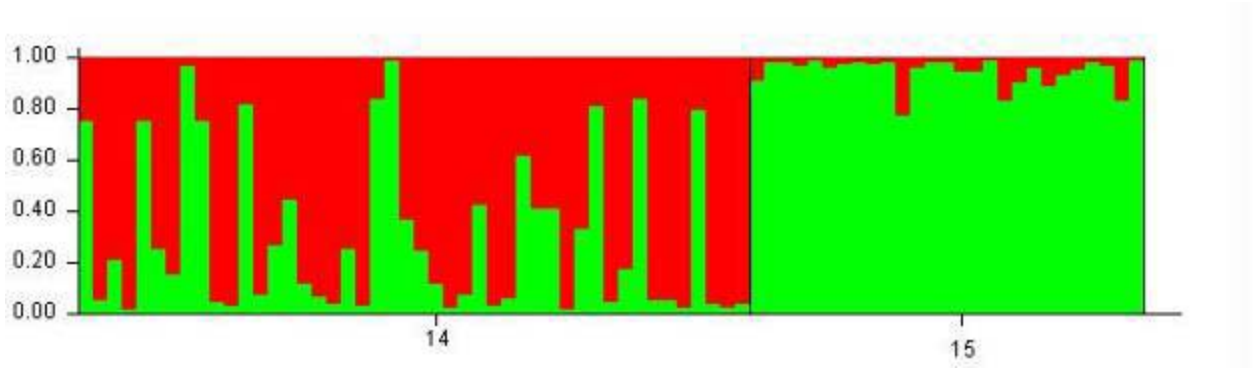

Figure 10. STRUCTURE bar plots for tailed frogs at basins 2260 (14) and 2468 (15). This plot suggests site 14 is distinct from 15, but there is a reasonable amount of gene flow from 15 into 14, but not vice versa (as indicated by the small amount of red for each individual in site 15).

3.5.3. Cope's giant salamanders

The first main population subdivision (which probably describes historic differentiation) in Cope's giant salamander sites was a split into two clusters, roughly corresponding to the Olympics and Willapa Hills/S. Cascades (Fig. 11). However, the two northern Willapa Hills basins, 2260 (14) and 2468 (15), cluster with the Olympic sites, and not with other Willapa Hills sites. This result differs from that for tailed frogs in two major ways. First, in tailed frogs, 2260 (14) and 2468 (15) cluster with the

Willapa Hills. Second, tailed frogs first partition into three major regions instead of the initial two clusters for Cope's giant salamander.

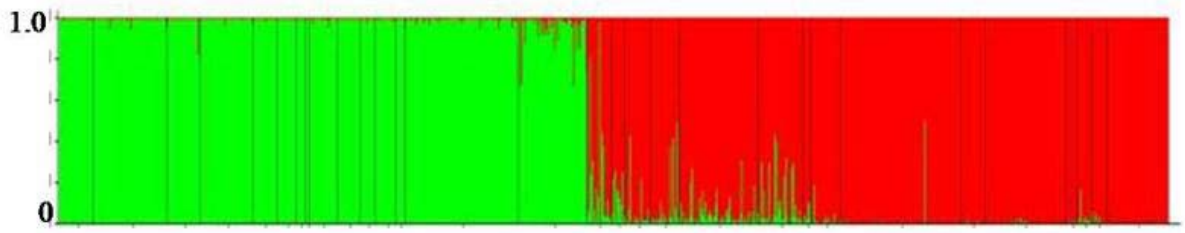

Figure 11. STRUCTURE bar plots for all Cope's giant salamander sites. Each color represents a population cluster. Green represents the Olympic sites as well as 2260 (14) and 2468 (15), whereas red is the remaining Willapa Hills sites and all South Cascade sites.

The AMOVA analysis based on the two groups suggested that there was further subdivision, as there was a greater percentage of variation among sites within groups (10%) than among groups (9%). Further subdividing the Olympic/2260/2468 cluster revealed an intuitive split, with the Olympic sites in one cluster and the 2260/2468 basins in the other (Fig. 12). While this division is expected, what is somewhat surprising is that there appears to be some migration from 2260/2468 into the Olympic sites, but less in the other direction. The other main cluster (Willapa Hills and South Cascades) breaks out into three different clusters (Fig. 13). One of these includes all the Willapa Hills (except 2260/2468) and the other two divide the South Cascades sites. One cluster includes basins 5378 (30) and 6000 (24), and the other cluster is comprised of 5595N (25), 5595S (26) and two of the cluster sites. One cluster site (28) appears to be highly admixed with all three clusters (Fig. 13). This leads to a total of five clusters; the AMOVA analysis did indicate more variation explained among groups (10%), but still that 6% of variation was due to differences between sites within groups, suggesting that further subdivision may be appropriate.

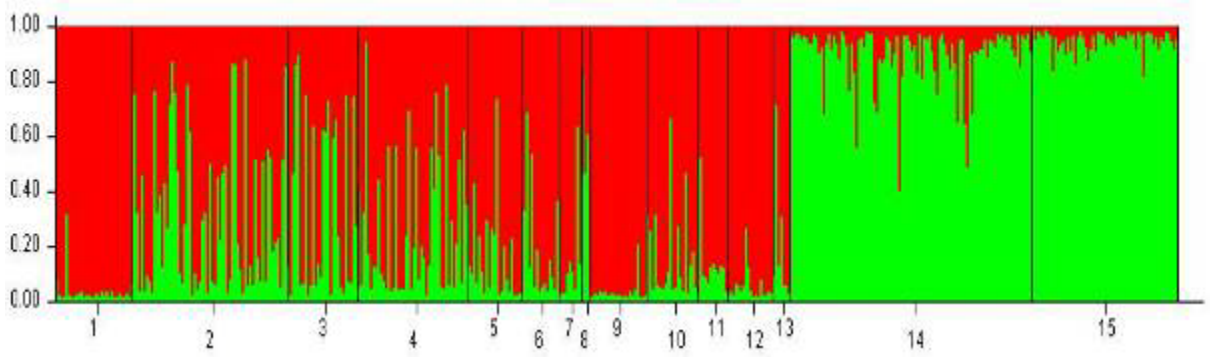

Figure 12. STRUCTURE bar plots for all Olympic Cope's giant salamander sites as well as basins 2260/2468. Site numbers are as in Tables 1 and 18.

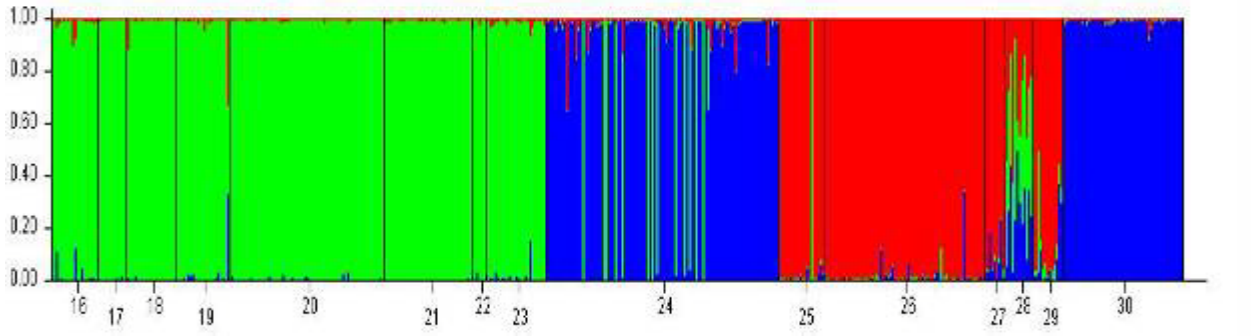

Figure 13. STRUCTURE bar plots for all Willapa Hills and South Cascades Cope's giant salamander sites. Site numbers are as in Tables 1 and 18.

A. Subdivision within the Olympic region

After 2260 (14) and 2468 (15) are split off from the Olympic sites, the first main subdivision within the Olympics is into two clusters: one cluster containing basin 363 (1) and cluster sites JB1 (9), JB2 (10), S1 (11) and SAM (12) and the other containing basins 1099 (2), 1197 (3), 1236 (4) and the remaining cluster sites (Fig. 14). The grouping of this split is interesting because 363 (1), JB1 (9) and SAM (12) form one geographical group in the northwest portion of the Olympic sampling area, whereas JB2 (10) and S1 (11) are a geographically disjunct group on the east side of the study area (Fig. 2). The fact that this disjunct group is genetically distinct from most of the central Olympic sites suggests that occasional dispersal might occur through the southern part of Olympic National Park to connect these two areas. However, the next level of subdivision divides 363/JB1/SAM from JB2/S1 (Fig. 15), so these two regions must only occasionally exchange migrants. Finally, the next round of hierarchical subdivision reveals that these two groups can be further split, so that 363 (1) is its own cluster and JB1 (9) and SAM

(12) are connected (Fig. 19), and JB2 (10) and S1 (11) are each split into unique clusters (Fig. 17). Therefore, the green cluster in Fig. 14 can eventually be almost completely reduced to each stream as a separate population, with the exception of JB1 (9) and SAM (12), which form one population despite a distance of 27 km between them.

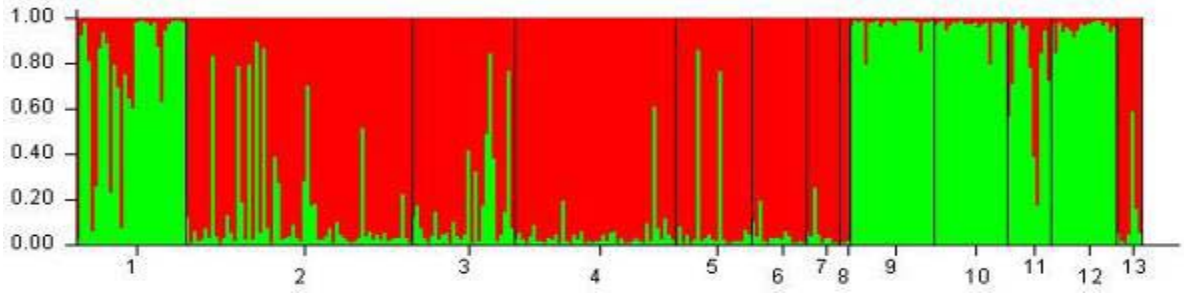

Figure 14. STRUCTURE bar plots for all Olympic Cope's giant salamander sites. Site numbers are as in Tables 1 and 18.

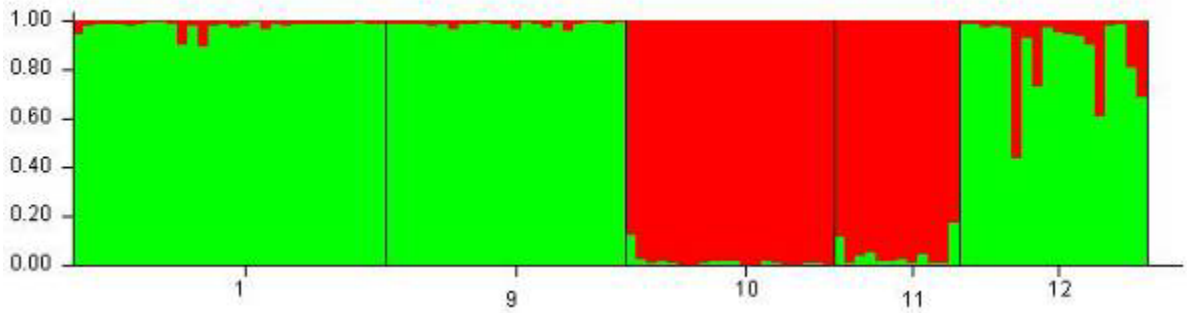

Figure 15. STRUCTURE bar plots for all Olympic sites in the green cluster (Fig. 17) for Cope's giant salamander. Site numbers are as in Tables 1 and 18.

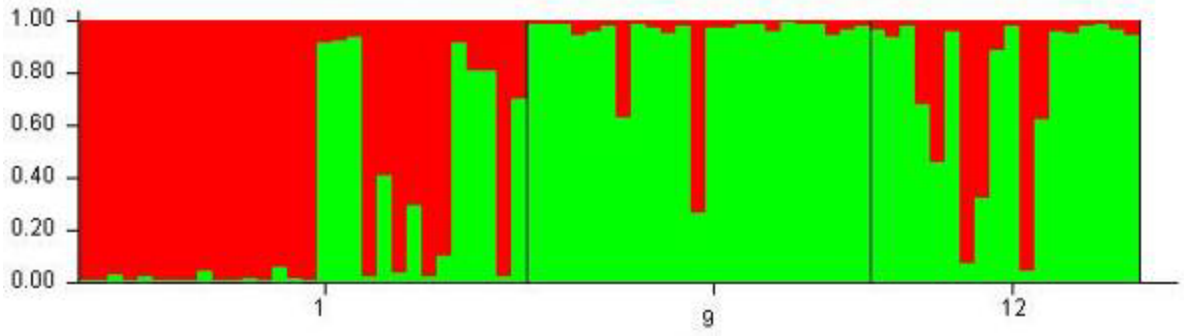

Figure 16. STRUCTURE bar plots for sites 363 (1), JB1 (9) and SAM (12) for Cope's giant salamander.

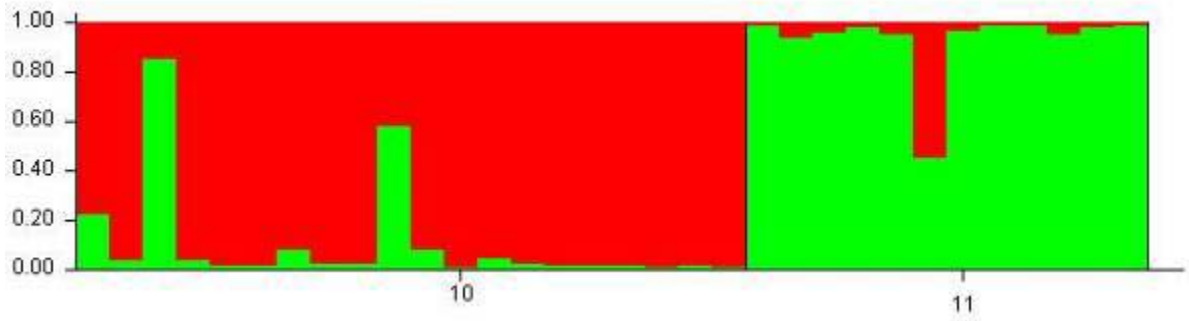

Figure 17. STRUCTURE bar plots for sites JB2 (10) and S1 (11) for Cope's giant salamander.

The other cluster of Olympic sites (the red cluster in Fig. 14) is similarly reduced into more structured populations. The first genetic break occurs into two clusters and results in one site splitting off (basin 1236 (4)), and basin 1197 (3) appears to be admixed between the two clusters (Fig. 18). Further analysis of the green cluster in Fig.18 revealed one last division in which 1099 (2), 1197 (3) and W1 (13) form one group, and the remaining Olympic cluster sites form the second group (Fig. 19). No further subdivision was found within this group of sites, and Fig. 19 indicates only weak genetic structure as mixing exists between the two groups. The maximum genetic cluster size observed in this portion is between 10-15 km. However, it is interesting that basin 1236 (4) is genetically separate from all other sites, despite the fact that it is very geographically close to 1099 (2) and 1197 (3). To summarize genetic patterns across the Olympic region, we detect a high degree of population structuring, as predicted by Steele et al. (2009), but we also observe occasional evidence of long-distance dispersal (Fig. 20). Two of the Type N Study basins (363 (1) and 1236 (4)) are isolated from all other sampled sites, whereas 1099 (2) and 1197 (3) are genetically connected, along with one other cluster site 13 km away.

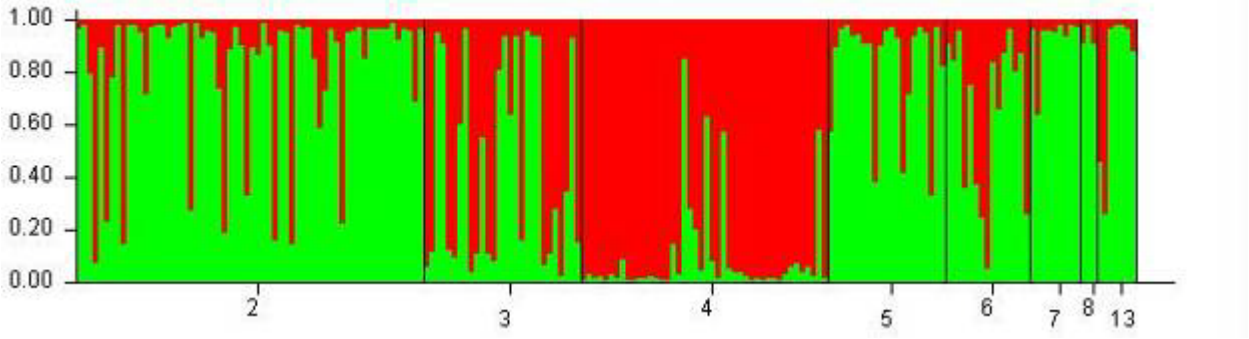

Figure 18. STRUCTURE bar plots further dividing the red cluster from Fig. 14 in the Olympic region for Cope's giant salamander. Site numbers are as in Tables 1 and 18.

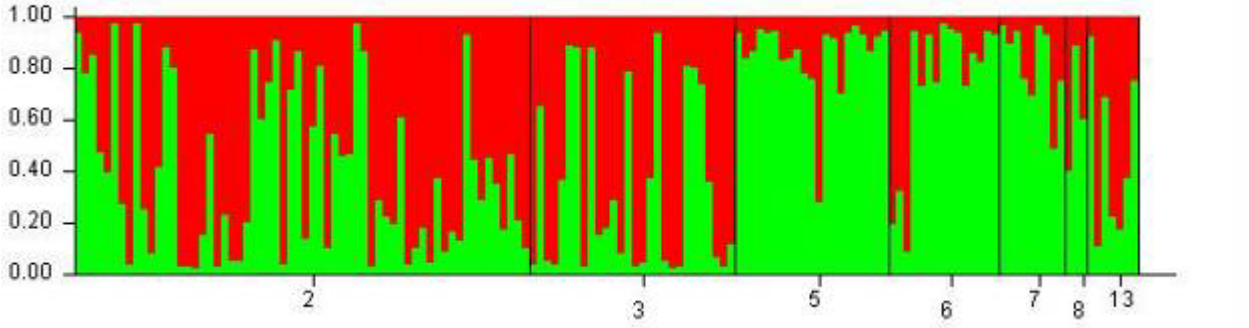

Figure 19. STRUCTURE bar plots further dividing the green cluster from Fig. 18 in the Olympic region for Cope's giant salamander. Site numbers are as in Tables 1 and 18.

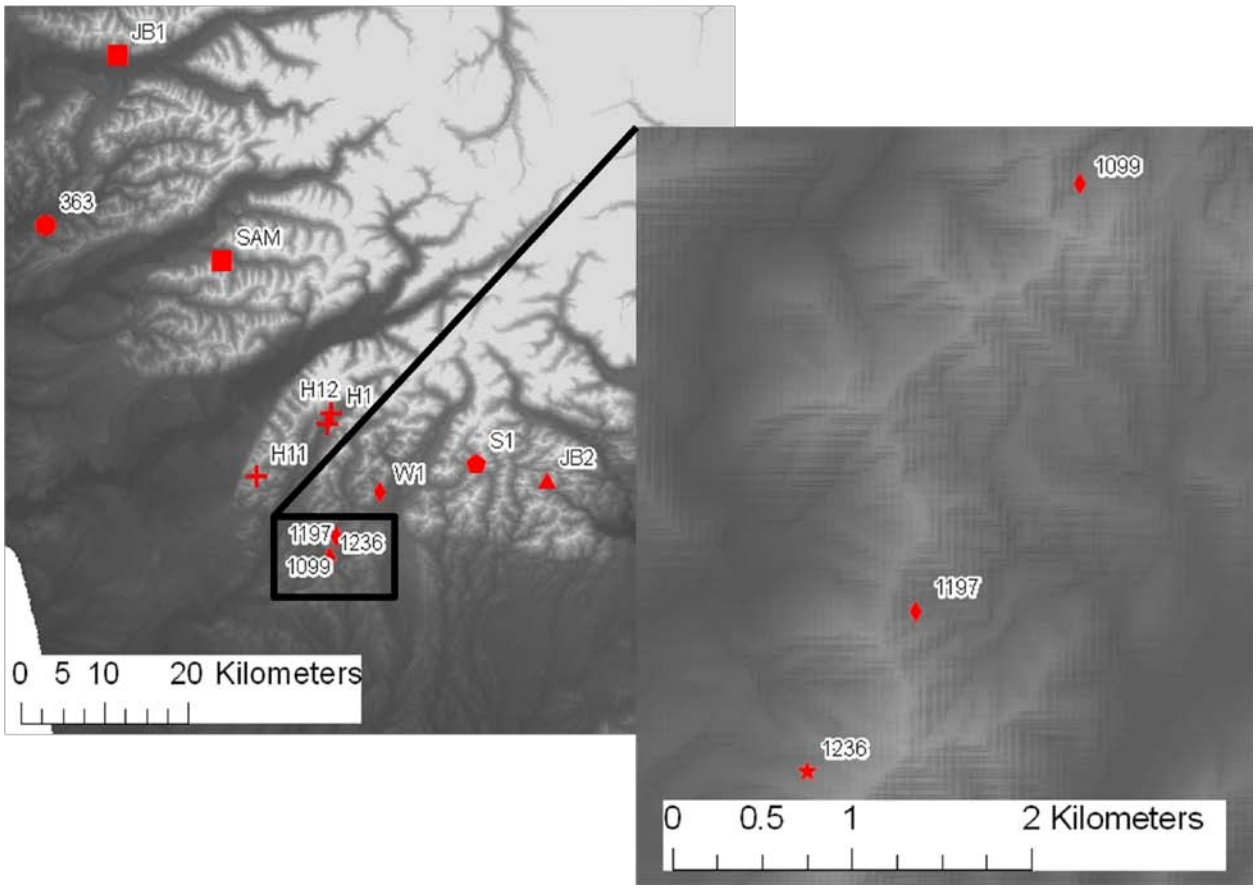

Figure 20. Map of *D. copei* genetic clusters in the Olympic region. Each shape represents a unique cluster.

B. Subdivision within the Willapa Hills region

We have already identified that basins 2260 (14) and 2468 (15) are separate from the other Willapa Hills sites (and are more closely related to the Olympics) (Fig. 12), and that the remaining Willapa Hills basins can be separated as a whole from the South Cascades (Fig. 13). We detected genetic differentiation between 2260 (14) and 2468 (15) when they were analyzed separately (Fig. 21) and similar to the pattern in tailed frogs, there appears to be more migration into 2260 (14) from 2468 (15) than vice versa. The remaining Willapa Hill sites break into three clusters that align well with geography (Fig. 3; Fig. 22). Basins 3074 (16), 3098 (17), 3110 (18) and 3111 (19) all cluster together as green, 3437 (20) and 3576 (21) form a group as red, and the third cluster is 3914 (22) and 5785 (23) in blue. The pair of 3914 (22) and 5785 (23) is most distinct, while there is a fair degree of genetic mixing between the other two groups (Fig. 22). The only further divisions identified within these subgroups are the differentiation of many individuals in basin 3110 (18) from the remaining group (Fig. 23) and the division of 3437 (20) from 3576 (21) (Fig. 24). However gene flow occurs from 3576 (21) into 3437 (20), but very

little evidence of flow exists in the opposite direction (Fig. 24). Therefore 3437 (20) may be located in a sink area for Cope's giant salamanders, and this was the only basin to show evidence of a significant heterozygosity excess in the bottleneck tests. Overall the genetic cluster size of the Willapa Hills is quite restricted, with a maximum detected cluster extent of only 2-6 kilometers (Fig. 25).

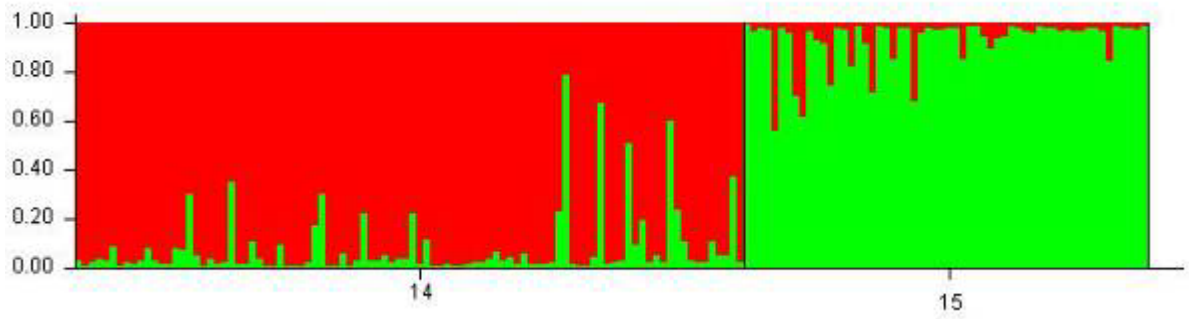

Figure 21. STRUCTURE bar plots demonstrating the division of site 2260 (14) and 2468 (15) for Cope's giant salamander.

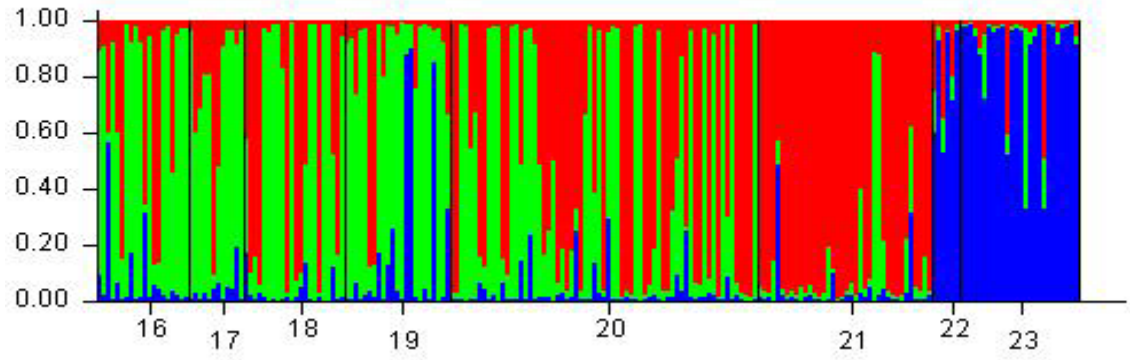

Figure 22. STRUCTURE bar plots for Willapa Hill sites for Cope's giant salamander. Site numbers are as in Tables 1 and 18.

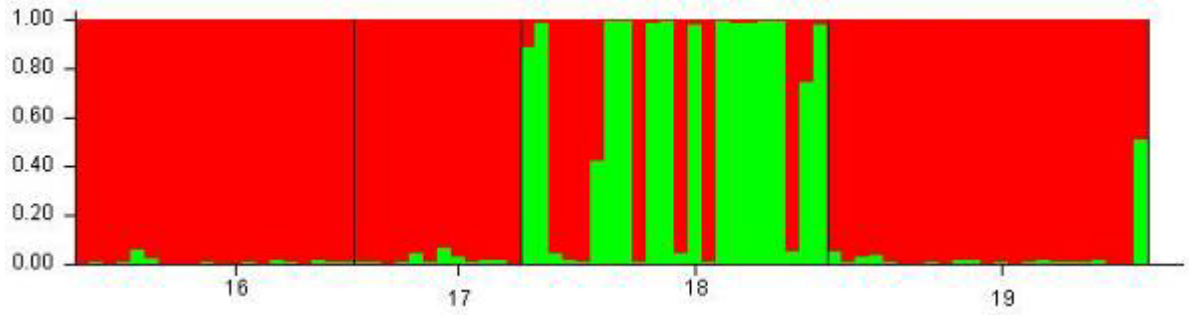

Figure 23. STRUCTURE bar plots for 3074 (16), 3098 (17), 3110 (18) and 3111 (19) for Cope's giant salamander.

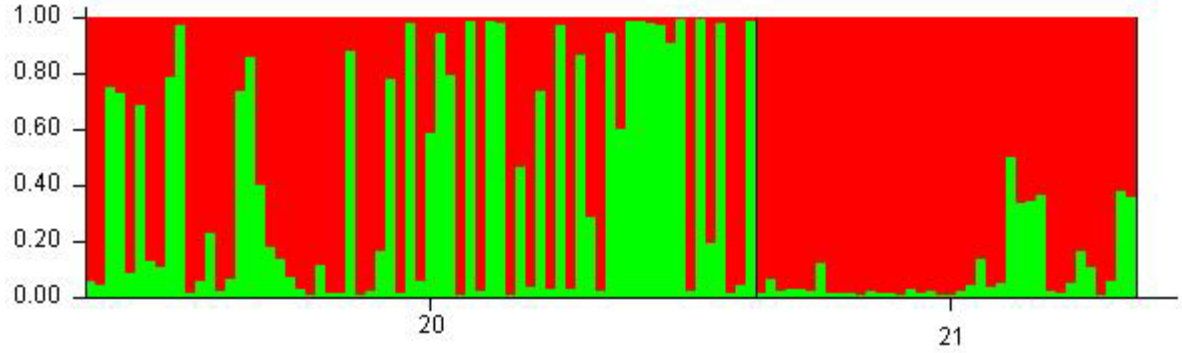

Figure 24. STRUCTURE bar plots for 3437 (20) and 3576 (21) for Cope's giant salamander.

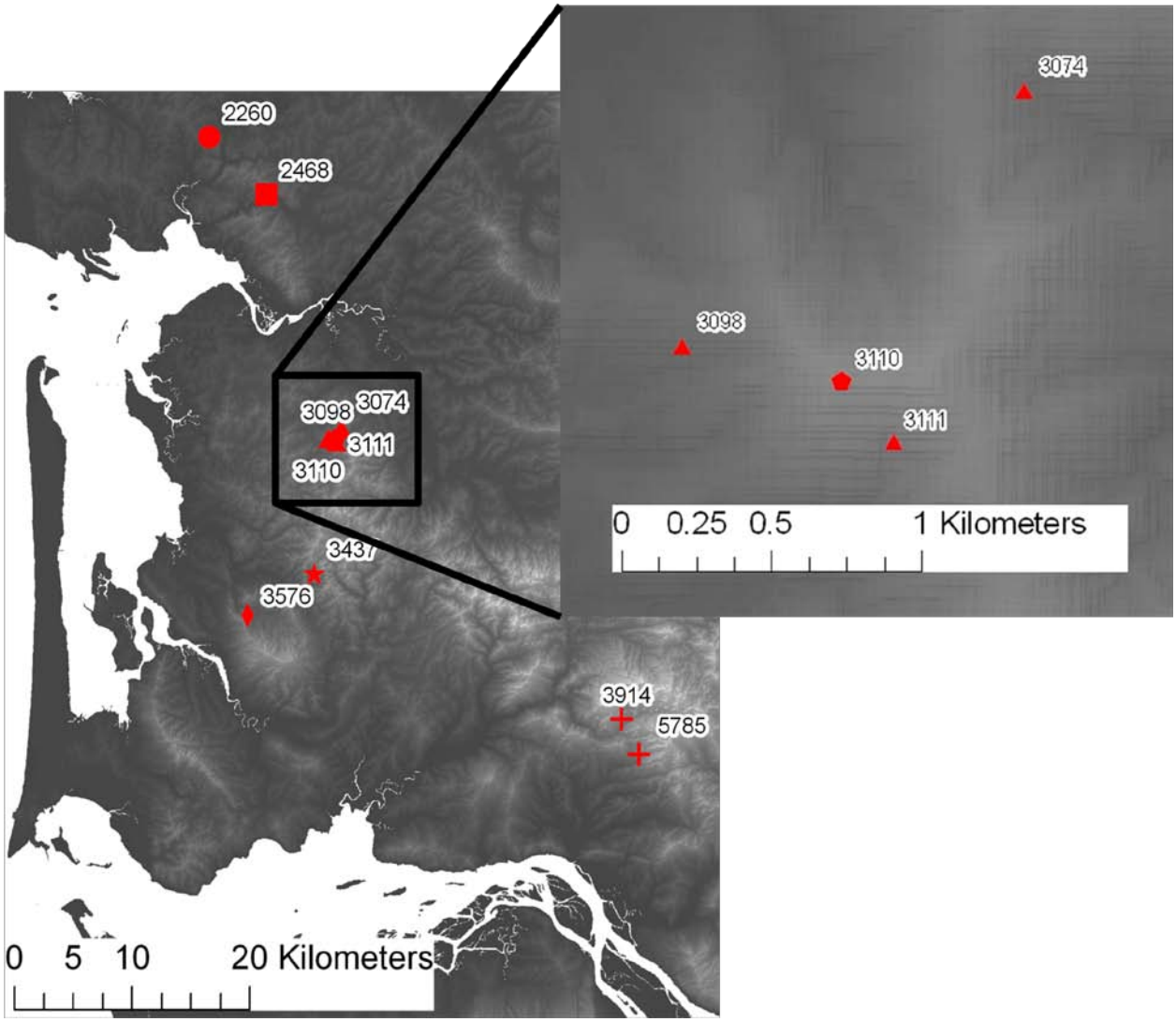

Figure 25. Map of *D. copei* genetic clusters in the Willapa Hills region. Each shape represents a unique cluster.

C. Subdivision within the South Cascades region

We have already identified two genetic clusters in the South Cascades in Fig. 13. The two basins comprising the first cluster, 5378 (30) and 6000 (24), are clearly differentiated from each other (Fig. 26). The second South Cascades cluster in Fig. 13 included basins 5595N (25), 5595S (26) and the three cluster sites. When this cluster is analyzed alone, two of the cluster sites (PC (28) and Y1 (29)) split off, while there is connectivity among 5595N (25), 5595S (26) and the cluster site KC (27) (Fig 27), which

is 5 km north of the 5595 basins. No further subdivision was found among 5595N (25), 5595S (26) and KC (27), but PC (28) and Y1 (29) are genetically differentiated (Fig. 28). Thus, our findings with these sampled sites agree well with the results of Steele et al. (2009) that show very low gene flow among Cope's giant salamander in the South Cascades (Fig. 29).

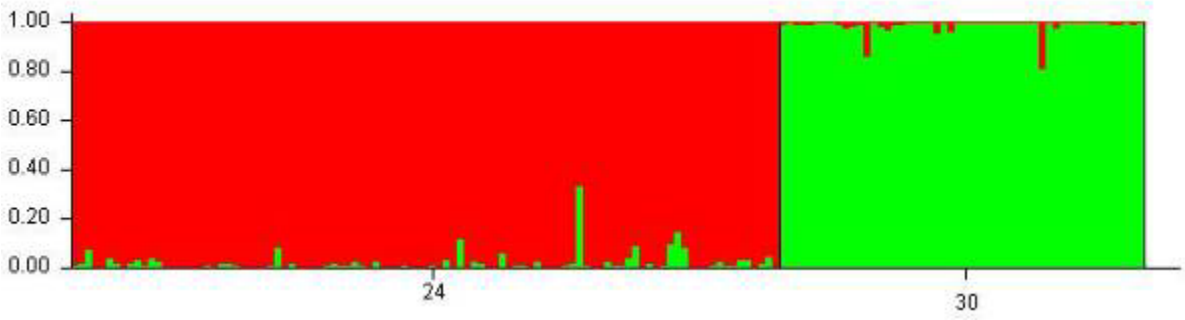

Figure 26. STRUCTURE bar plots for 6000 (24) and 5378 (30) for Cope's giant salamanders in the South Cascades.

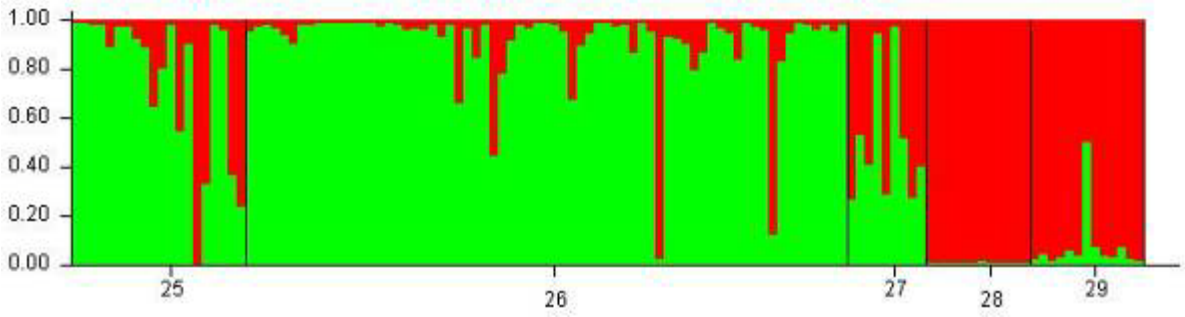

Figure 27. STRUCTURE bar plots for 5595N (25) and 5595S (26), as well as three cluster sites for Cope's giant salamanders in the South Cascades.

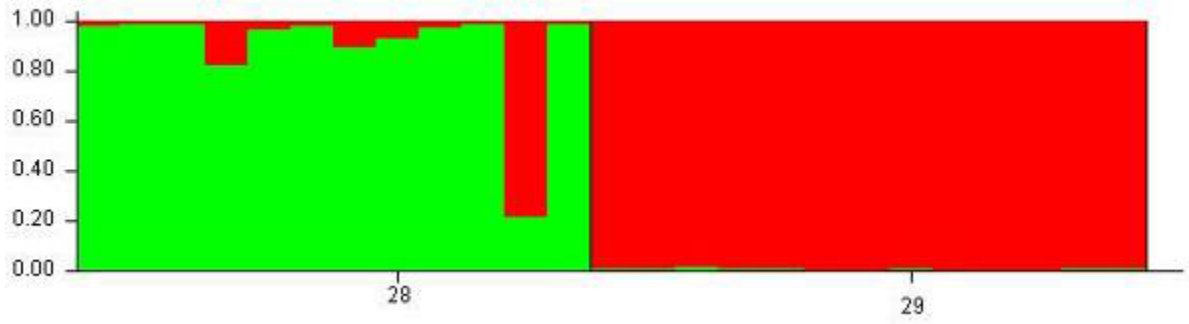

Figure 28. STRUCTURE bar plots for two cluster sites, PC (28) and Y1 (29), for Cope's giant salamanders in the South Cascades.

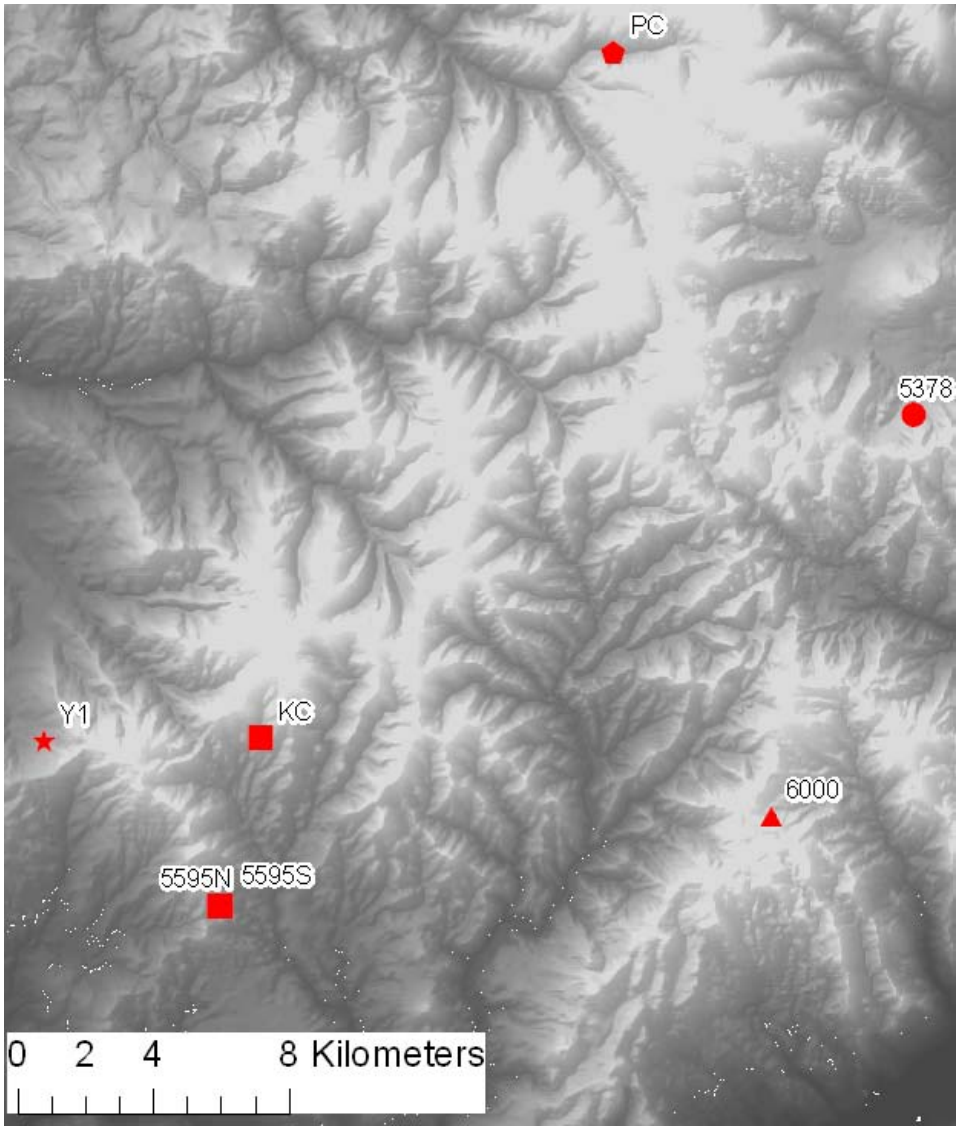

Figure 29. Map of *D. copei* genetic clusters in the South Cascades region. Each shape represents a unique cluster. 5595 N and S are both represented by the square cluster.

The AMOVA supports the full hierarchical clustering across all three regions, as described above, which leads to 18 different genetic groups for Cope's giant salamander. This highly partitioned genetic grouping has only 1.9% of variation due to among sites within groups, versus 13% of variation among groups. Across all regions, a gradient of increased genetic connectivity in Cope's giant salamanders appears to exist as one moves from the South Cascades to the Olympics. The genetic cluster size in the South Cascades is 5 km at most, increases to around 10-15 km in the Willapa Hills, and finally reaches a maximum close to 30 km in the Olympics. This broadly correlates with the precipitation gradient that increases to the Olympics, which may allow periodic overland dispersal, but

explaining this pattern remains a topic for further study. However, even in the Olympics, some proximate sites are differentiated, so a constant genetic cluster size cannot be assumed across an entire region. One consequence of the increased differentiation seen in Cope's giant salamanders is that migration is less likely to influence Type N Study results. Therefore, we predict that if treatments do have an effect, it will likely be most easily detected for Cope's giant salamanders.

3.5.4. Coastal giant salamanders

In contrast to tailed frogs and Cope's giant salamanders, the Coastal giant salamander does not have complete separation between the Willapa Hills and South Cascades (Fig. 30). In fact, basin 3437 (20) grouped with South Cascades sites and South Cascade genetic cluster sites KC (27) and PC (28) grouped with Willapa Hills. Notably, all three of these sites had a low sample size, which may have biased the analysis. Analysis of the predominantly Willapa Hills cluster produced two additional groups, of which one of the clusters contained 3111 (19) and 3576 (21), counter to geographical proximity (Fig. 31). We removed 3111 (19) and 3576 (21) and found two more clusters with a high degree of admixture that grouped 3074 (16), 3098 (17), 3110 (18) in one cluster and 3914 (22), 5785 (23) and the two South Cascades cluster sites in the other cluster (Fig. 32). No further subdivision was detected in the Willapa Hills group. A highly uneven genetic cluster size exists in the Willapa Hills (Fig. 33). Adjacent basins 3110 (18) and 3111 (19) were divergent, but 3111 (19) and 3576 (21) were genetically continuous despite being 10 km apart, and even more surprising, 3914 (22) and 5785 (23) are more than 100 km away from the South Cascades cluster sites. Across the South Cascades, no evidence was found for genetic differentiation by site (Fig. 34) and basin 3437 (20) continued to be included with South Cascades sites. This is also consistent with the findings of Steele et al. (2009), as they found no genetic structure for Coastal giant salamanders. Based on the four clusters indicated by the clustering analysis, the AMOVA that the same percentage of variation (3%) was explained by both among groups and among sites within groups. In concert, with the nonintuitive geographical clusters, this is consistent with the hypothesis that recent colonization by the Coastal giant salamander from a common refugium has not allowed enough time for genetic structure to become evident.

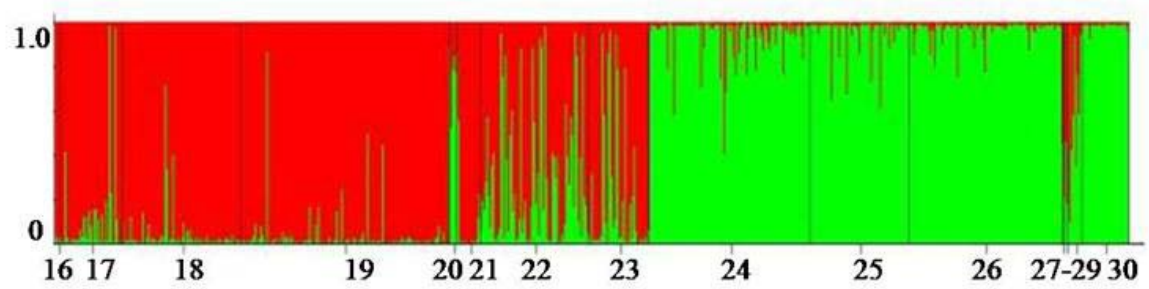

Figure 30. STRUCTURE bar plots for all Coastal giant salamander sites. Site numbers are as in Tables 1 and 18.

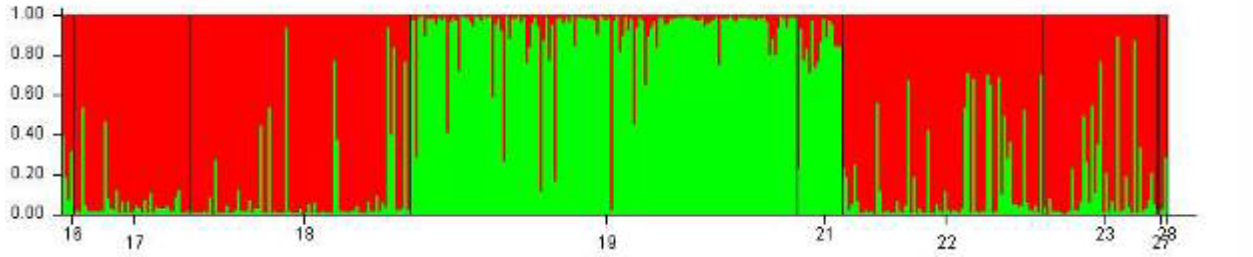

Figure 31. STRUCTURE bar plots for Coastal giant salamander sites in the predominantly Willapa Hills cluster. Site numbers are as in Tables 1 and 18.

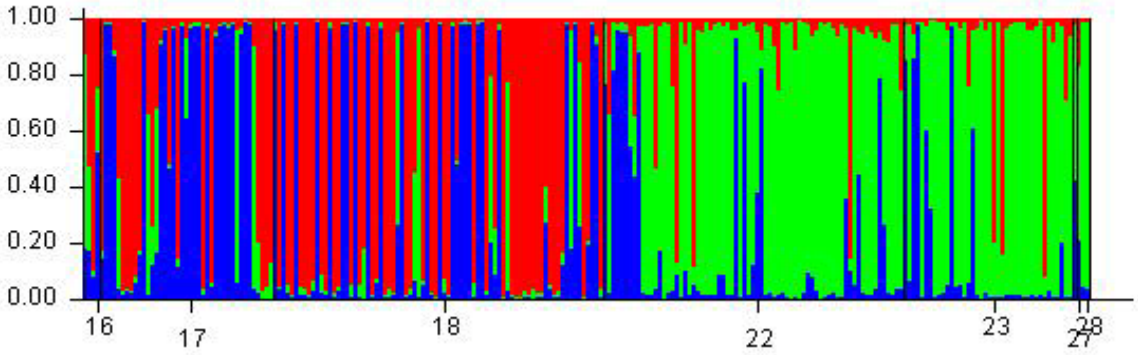

Figure 32. STRUCTURE bar plots for the Coastal giant salamander red cluster depicted in Fig. 32. Site numbers are as in Tables 1 and 18.

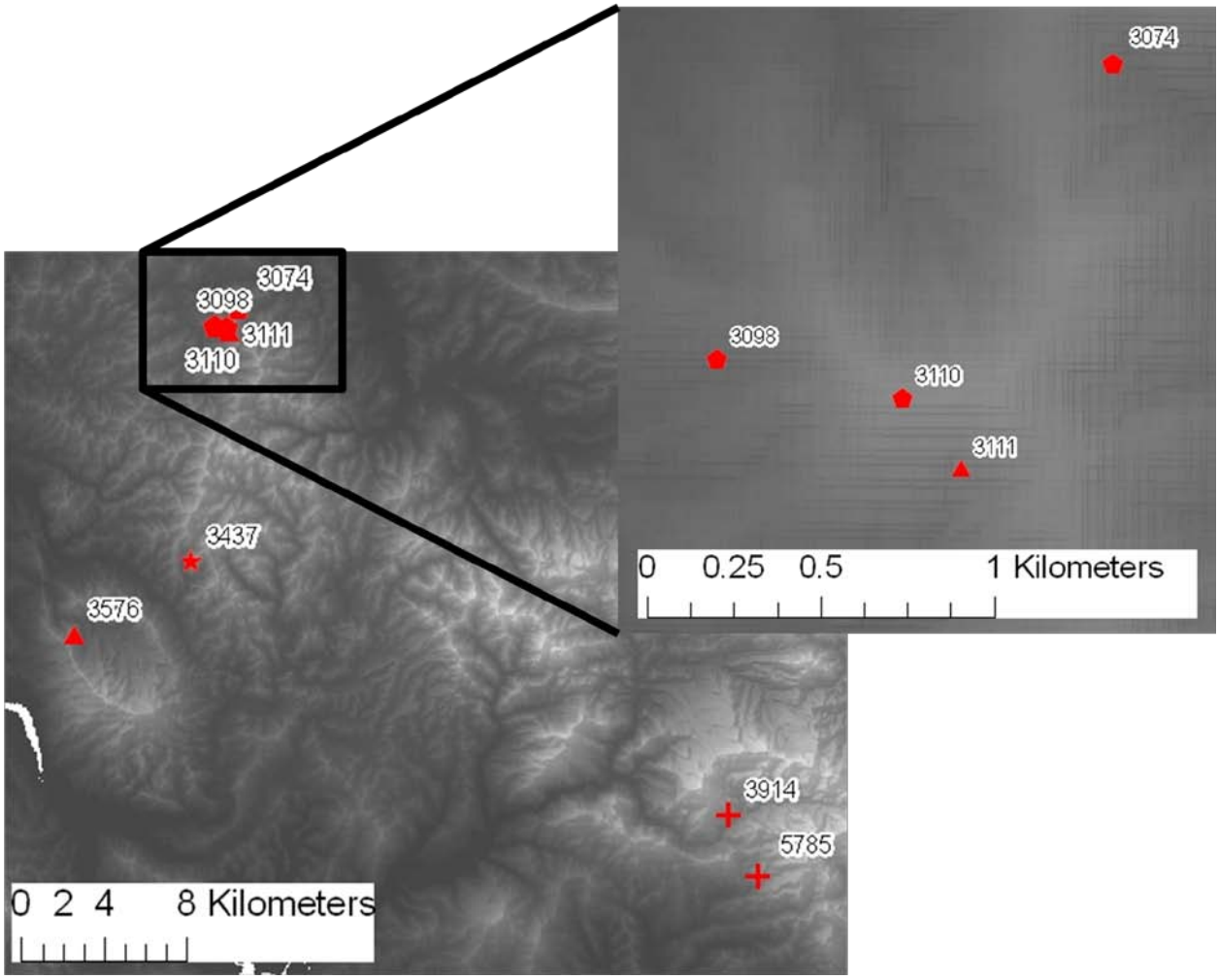

Figure 33. Map of *D. tenebrosus* genetic clusters in the Willapa Hills region. Each shape represents a unique cluster.

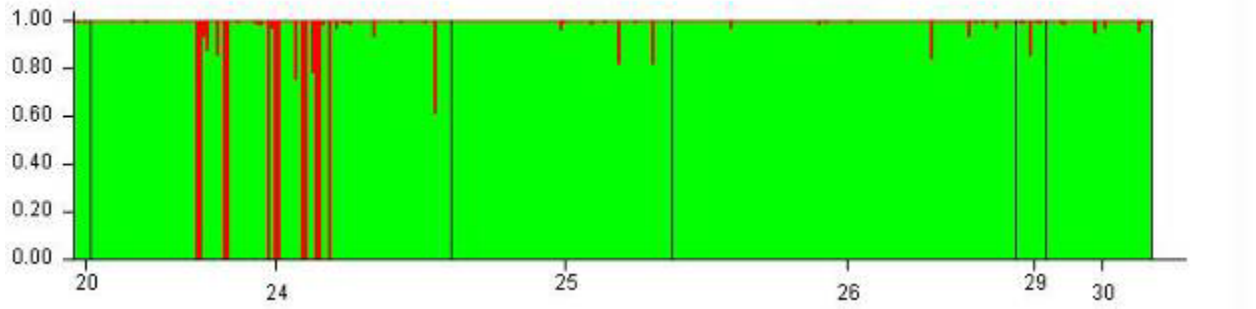

Figure 34. STRUCTURE bar plots for the predominantly South Cascades cluster. Site numbers are as in Tables 1 and 18.

4. Final Conclusions and Synthesis

4.1. Coastal tailed frogs synthesis

Collectively, Coastal tailed frogs are a genetically diverse group of populations, among which we found limited evidence for recent declines. Tailed frogs have among the highest genetic diversity seen in frogs, relatively large effective population sizes and very high rates of gene flow. These characteristics suggest that tailed frogs have been stable for a long time, are resistant to the negative effects of drift and should be buffered from large declines. However, large patches of open habitat are barriers to population connectivity of tailed frogs, as evidenced at a broad scale by the observed regional differentiation. Another study supports this result, as populations in Olympic National Park were separated by alpine meadows (Spear and Storfer 2008). Second, evidence of recent declines exists in the Olympics and two sites in the Willapa Hills and pairwise genetic distance values were negatively correlated with timber harvest in the Olympics (Spear and Storfer 2008), suggesting possible genetic effects of timber management. Whether forest management will have serious long-term effects on tailed frog persistence and genetic diversity is unclear. Results of the Type N Study will be extremely useful in this regard. If detectable changes resulting from a particular buffer treatment occur, then effects are likely to be quite strong due to such high baseline effective population sizes and connectivity in this species. Finally, our genetic analyses suggest that despite a large larval sample, abundance measures are probably representative of population size for this species. We detected few siblings, so most individuals belonged to unique families.

4.2. Cope's giant salamanders

Cope's giant salamander populations are characterized by levels of heterozygosity intermediate between tailed frogs and Coastal giant salamanders (but high in comparison with other salamander studies), large effective population sizes, and little evidence of recent declines, but very restricted genetic connectivity. Significant differences existed in genetic diversity by region, and this is likely due to effects of genetic drift resulting from isolation. However, we observed a consistent pattern of lower diversity and population size on the Olympic Peninsula, which is counterintuitive as there is a larger genetic cluster size in the Olympics, as well as a lack of Coastal giant salamanders as potential competitors. In total, we predict that Cope's giant salamanders are the most likely of the three study species to show a response to harvest treatments. Although our simulations indicated that a larger reduction in absolute numbers of individuals would be required as compared to tailed frogs, it is a lower proportion of average current effective population size than for tailed frogs. Most individuals will be restricted to their home basin and thus are less likely to evade any negative effects. Furthermore, "genetic rescue" from individuals emigrating from other streams is less likely due to low overall genetic cluster sizes and neoteny in most populations. Lastly, genetic data are especially important for estimating Cope's giant salamander population response in this study

because there were a number of full siblings detected. As a result, abundance counts will likely not accurately estimate adult population sizes.

4.3. Coastal giant salamanders

Genetic results for Coastal giant salamanders were perhaps the most surprising. Coastal giant salamanders have been characterized as highly abundant and the most resilient to disturbance of stream-associated amphibians (Welsh 2005). However, we detected low levels of genetic diversity and small effective population sizes, as well as widespread evidence for declines. High gene flow was found, as expected, but some of the geographical patterns of genetic clustering confounded effective interpretation based on available information. Simple abundance surveys for Coastal giant salamanders are also likely to be unreliable because of the great number of siblings detected, at least for our sample basins. Low diversity and population sizes may simply represent the residual effect of contraction resulting from Pleistocene glaciations and recent re-expansion from refugia across Washington landscapes. If this is true, it suggests that populations have not greatly increased in size since expanding across their range, as bottleneck effects are still observable. Therefore, in one aspect, this species could be considered highly vulnerable to habitat disturbance due to low effective population sizes. Nonetheless, the high dispersal capacity of this species might also keep the species from becoming extirpated locally in any streams. Clearly further studies are needed to disentangle these possibilities.

4.4. Final conclusions

An important aspect of all the analyses for the Type N Study is that the three stream amphibian species are not interchangeable: all have different genetic characteristics. Therefore, to truly understand how forest management affects the entire stream amphibian community, each species needs to be examined individually. This is clearly one of the strengths of the Type N study, as it allows for the inclusion of multiple species. Second, the baseline analyses presented here are absolutely necessary to obtain meaningful conclusions from post-treatment data. Genetic diversity was unequal across blocks (especially for Cope's giant salamanders), providing strong support for the importance of blocking; thus, baseline data for each block must be accounted for when analyzing post-treatment effects. Fortunately, however, no consistent differences were detected among treatment types for any species, so treatment assignments should not lead to any significant bias in the results. However, there would need to be strong treatment effects for genetic parameters to significantly vary in only one generation. Despite this, we would like to reiterate the importance of including genetic data after harvest for a thorough evaluation of post-treatment effects for each species. Tailed frog census surveys may be an accurate assessment of the breeding population, but without knowledge of the genetic connectivity of the focal basin with other sites, sampled individuals may simply represent immigrants from nearby sites not exposed to the treatment, although it is unclear the average number of immigrants per generation into a

site. On the other hand, abundance counts do not seem wholly accurate for both species of giant salamanders due to high numbers of sampled full siblings and a high degree of difficulty in identifying specimens to species in the field. Thus, genetic data are needed to determine effective population sizes, as well as for species identification, respectively.

5. Acknowledgments

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7. Glossary

Alleles – the different forms of a gene.

Allelic richness – The number of alleles in the sampled population, adjusted for unequal sample sizes among populations.

Effective population size – The population size that represents the number of breeding individuals in a population, and therefore is a more meaningful population size for predicting population response to change and long-term viability

Heterozygosity – For diploid organisms (two sets of chromosomes), heterozygous individuals have two different alleles at a single locus.

Heterozygosity excess test – A test for recent large population declines, in which an excess of heterozygotes (relative to equilibrium expectations) is expected because allelic diversity is lost faster than heterozygosity. However, this excess signature only exists for 5-10 generations after a decline, and historic declines would actually be expected to have a heterozygote deficiency.

Inbreeding coefficient (F_{IS}) – A metric based on the difference between observed and expected heterozygosity that is generally indicative of non-random mating within a population. Positive values of F_{IS} represent the results of mating between individuals more closely related than random, and negative values represent the results of mating between individuals more distantly related than random.

Linkage disequilibrium – A statistical association between two loci such that alleles at one locus are correlated with presence of particular alleles at another locus. If linkage disequilibrium exists, loci cannot be considered independent. Linkage disequlibrium could be due to several factors ranging from proximity on the same chromosome to population substructure.

Locus (plural: loci) – the location on a chromosome where a gene is located.

M-ratio – A test to detect population declines that is based on the ratio of number of alleles to allele size range. Allelic size range is the difference in size of the longest microsatellite allele from the size of the smallest allele. As a population bottleneck or decline occurs, alleles of all sizes are randomly lost, and thus the number of alleles is reduced faster relative to the total size range, and a lowered M-ratio results. A critical value based on effective population size can be calculated to determine if a M-ratio value signifies a decline.

Microsatellite DNA marker – A locus that is characterized by alleles with multiple repeats of a short sequence of nucleotides. These loci do not code for proteins (i.e. are selectively neutral) and mutate rapidly. Therefore, these loci have high genetic variability and are excellent for investigating fine-scale genetic differences.

Appendix 1**:**

Multiplex PCR conditions for *Ascaphus* (locus names are as in Spear et al. 2008)
All runs have 10 µl total volumes/sample
All multiplexes have: 5ul Qiagen Master Mix/sample
0.5 µl Q solution/sample
1 µl template DNA/sample
All primer volumes are equal for both forward and reverse primers, and are at 5 µM stock concentrations.

Multiplex 1- [96 deg 15 min, (94 deg 30 s, 55 deg 90 s, 72 deg 60 s)29X, 60 deg 30 min]
A2 – 0.3 µl
A4 – 0.5 µl
A12 – 0.05 µl
A31 – 0.05 µl
1.7 µl water

Multiplex 2 – [96 deg 15 min, (94 deg 30 s, 55 deg 90 s, 72 deg 60 s)28X, 60 deg 30 min]
A15 – 0.3 µl
A24 – 0.15 µl
A26 – 0.25 µl
A29 – 0.1 µl
1.9 µl water

Multiplex 3 – [96 deg 15 min, (94 deg 30 s, 60 deg 90 s, 72 deg 60 s)26X, 60 deg 30 min]
A1 – 0.075 µl
A3 – 0.45 µl
A13 – 0.1 µl
A14 – 0.15 µl
A17 – 0.15 µl
1.65 µl water

Dilute PCR product 1:15 (1 µl template PCR product, 0.25 µl LIZ500 size standard, 13.75 µl water)

Appendix 2:

Multiplex PCR conditions for *Dicamptodon* (locus names are as in Steele et al. 2008)
All runs have 10 µl total volumes/sample
All multiplexes have: 5ul Qiagen Master Mix/sample
0.5 µl Q solution/sample
1 µl template DNA/sample
1 µl primer mix (both forward and reverse mixed at a concentration of 2 µM each)
2.5 µl water

Multiplex 1- [95 deg 15 min, (94 deg 30 s, 53 deg 90 s, 72 deg 60 s)30X, 60 deg 30 min]
D18 – 6FAM
D13 – VIC
D04 – PET
D25 - NED

Multiplex 2 – [95 deg 15 min, (94 deg 30 s, 60 deg 90 s, 72 deg 60 s)30X, 60 deg 30 min]
D24 – 6FAM
D17 – PET
D07- NED
D20 - VIC

Multiplex 3 – [95 deg 15 min, (94 deg 30 s, 60 deg 90 s, 72 deg 60 s)30X, 60 deg 30 min]
D23 – VIC
D05 – NED
D14 – 6FAM
D06 – PET
D15 – 6FAM

Dilute PCR product 1:20 (1 µl template PCR product, 0.25 µl LIZ500 size standard, 18.75 µl water)

Appendix 3. F_{ST} pairwise values for Coastal tailed frogs across all Type N basins. Bold, italicized value indicates sites that are significantly genetically differentiated.

	1099	1197	1236	2260	2468	3074	3098	3110	3111	3437	3576	3914	5785	5378
1197	***0.01***													
1236	0.00	0.01												
2260	***0.05***	***0.06***	***0.05***											
2468	***0.05***	***0.06***	***0.04***	***0.03***										
3074	***0.04***	***0.05***	***0.04***	***0.03***	***0.02***									
3098	***0.03***	***0.04***	***0.03***	***0.03***	***0.02***	0.00								
3110	***0.03***	***0.04***	***0.03***	***0.02***	***0.02***	0.01	0.00							
3111	***0.04***	***0.04***	***0.03***	***0.03***	***0.02***	0.01	0.00	0.00						
3437	***0.03***	***0.04***	***0.03***	***0.03***	***0.02***	***0.01***	0.01	0.01	0.01					
3576	***0.04***	***0.05***	***0.04***	***0.04***	***0.02***	***0.01***	***0.01***	0.01	***0.01***	0.01				
3914	***0.04***	***0.04***	***0.03***	***0.02***	***0.01***	0.01	0.00	0.00	0.00	0.00	***0.00***			
5785	***0.03***	***0.04***	***0.03***	***0.03***	***0.02***	***0.01***	0.01	0.00	0.00	0.01	***0.01***	0.00		
5378	***0.08***	***0.09***	***0.08***	***0.08***	***0.07***	***0.04***	***0.06***	***0.04***	***0.06***	***0.05***	***0.05***	***0.05***	***0.05***	
6000	***0.07***	***0.09***	***0.07***	***0.07***	***0.06***	***0.05***	***0.06***	***0.04***	***0.05***	***0.05***	***0.05***	***0.05***	***0.04***	0.00

Appendix 4. F_{ST} pairwise values for Cope's giant salamander across all Type N basins. Bold, italicized value indicates sites that are significantly genetically differentiated.

	363	1099	1197	1236	2260	2468	3074	3098	3110	3111	3437	3576	3914	5378	5785	6000	5595N
1099	***0.0946***																
1197	***0.1271***	***0.0329***															
1236	***0.1048***	***0.0441***	***0.0522***														
2260	***0.1403***	***0.0569***	***0.055***	***0.0897***													
2468	***0.1651***	***0.074***	***0.0712***	***0.0916***	***0.0406***												
3074	***0.1781***	***0.1272***	***0.1129***	***0.142***	***0.065***	***0.0626***											
3098	***0.2048***	***0.1429***	***0.1289***	***0.1497***	***0.0757***	***0.0846***	0.0064										
3110	***0.2099***	***0.1562***	***0.1446***	***0.1541***	***0.0893***	***0.0927***	***0.0397***	0.0172									
3111	***0.196***	***0.1451***	***0.1283***	***0.1435***	***0.0894***	***0.0818***	***0.029***	0.0162	***0.0338***								
3437	***0.1679***	***0.1331***	***0.1155***	***0.1351***	***0.0802***	***0.0821***	***0.016***	***0.0196***	***0.0333***	***0.0225***							
3576	***0.2068***	***0.1526***	***0.1274***	***0.1421***	***0.0971***	***0.092***	***0.0314***	***0.0232***	***0.0467***	***0.0429***	***0.0214***						
3914	***0.2309***	***0.1602***	***0.1629***	***0.1662***	***0.0924***	***0.0911***	***0.0456***	***0.042***	***0.0592***	***0.0601***	***0.0481***	***0.0531***					
5378	***0.3397***	***0.2827***	***0.2816***	***0.2919***	***0.2432***	***0.2278***	***0.2041***	***0.2128***	***0.213***	***0.1929***	***0.1845***	***0.2055***	***0.2212***				
5785	***0.1891***	***0.1477***	***0.1382***	***0.1514***	***0.09***	***0.0945***	***0.0352***	***0.0385***	***0.0448***	***0.0488***	***0.0366***	***0.0512***	***0.0174***	***0.1894***			
6000	***0.2552***	***0.2197***	***0.2085***	***0.2206***	***0.1742***	***0.16***	***0.119***	***0.1441***	***0.1479***	***0.1311***	***0.1159***	***0.1365***	***0.1466***	***0.1152***	***0.1195***		
5595N	***0.27***	***0.2287***	***0.2087***	***0.2338***	***0.165***	***0.1521***	***0.1015***	***0.1158***	***0.1336***	***0.0977***	***0.0915***	***0.1134***	***0.1384***	***0.1688***	***0.1095***	***0.099***	
5595S	***0.2488***	***0.2087***	***0.197***	***0.215***	***0.1586***	***0.1486***	***0.1003***	***0.1208***	***0.1318***	***0.0976***	***0.0923***	***0.1076***	***0.1257***	***0.1586***	***0.1038***	***0.1051***	0.0101

Appendix 5. F_{ST} pairwise values for Coastal giant salamanders across all Type N basins. Bold, italicized value indicates sites that are significantly genetically differentiated.

	3074	3098	3110	3111	3437	3576	3914	5378	5785	6000	5595N
3098	-0.0029										
3110	0.0137	0.0168									
3111	0.005	0.0321	0.0654								
3437	0.1891	0.1228	0.1957	***0.1116***							
3576	0.0117	0.019	0.0708	0.0281	0.1226						
3914	0.0145	0.0174	***0.0389***	***0.0647***	0.1286	0.0354					
5378	0.1769	***0.1612***	***0.1973***	***0.1211***	***0.1581***	0.1732	***0.1415***				
5785	0.0279	0.0083	***0.0324***	***0.0454***	0.135	***0.0307***	0.0126	***0.1133***			
6000	0.0232	0.0337	***0.072***	***0.0315***	0.0912	0.0509	***0.0538***	0.0862	***0.0412***		
5595N	***0.0918***	***0.0939***	***0.1358***	***0.0843***	0.1315	***0.1143***	***0.096***	***0.0445***	***0.0743***	***0.0404***	
5595S	***0.0403***	***0.0407***	***0.0719***	***0.0466***	***0.1196***	***0.0637***	***0.0648***	***0.095***	***0.0402***	***0.0214***	0.0199