AN ABSTRACT OF THE DISSERTATION OF

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Susan M. Haig

Phylogeographic studies of six Pacific Northwest forest-associated salamanders provide insight into historical and contemporary processes on population genetic structure. Among Larch Mountain Salamanders (*Plethodon larselli*), cytochrome b mitochondrial (mtDNA) sequences (381 bp) and random amplified polymorphic DNA (RAPDs; 34 loci) supported separate Management Units for northern and southern populations (12 populations, N = 184 individuals) as delineated by the Columbia River. Southern populations exhibited significantly reduced expected heterozygosity at RAPD loci, which may be a consequence of a founder event or bottleneck. Similarly, significant population structure was found in Oregon Slender Salamanders (*Batrachoseps wrighti*). Cytochrome b sequences (744 bp) revealed two historical lineages among 22 populations (N = 339 individuals). RAPD markers further differentiated mid-range populations. Therefore, overlapping Management Units are warranted for *northern-most*, *mid-range,* and *southern-most* populations. Phylogenetic relationships, taxonomic identity, and population differentiation was examined among four morphologically

conserved Torrent Salamanders species (Family Rhyacotritonidae). Analysis of three mitochondrial genes (cytochrome b, 16S, and 12S ribosomal RNA) indicated each species represented a well-supported monophyletic group. Results agreed with allozyme data (Good et. al. 1987, Good and Wake 1992) suggesting three groups of Torrent Salamanders (*Rhyacotriton variegatus*, *R. cascadae*, and the ancestor of *R. olympicus* and *R. kezeri*) diverged during the Miocene. A more recent divergence appears to have occurred between *R. olympicus* and *R. kezeri* during the late Pliocene/early Pleistocene. Populations within *R. variegatus* appear to be as diverged as *R. olympicus* and *R. kezeri,* supporting conservation unit designation within *R. variegatus*. MtDNA 16S ribosomal RNA sequences and allozymes (5 loci) identified Cascade and Southern Torrent Salamanders recently discovered in the Central Oregon Cascades. Results indicate a range extension for both species and suggest the Middle Fork of the Willamette River may provide a geographic barrier to dispersal. Phylogenetic analyses of Southern Torrent Salamanders (72 localities) based upon cytochrome b sequences revealed three divergent clades (*north coast*, *Oregon,* and *California*) that coincide with possible geographic barriers to dispersal. Merging mtDNA results with previous allozyme studies provides support for an Evolutionary Significant Unit for the *California* clade and separate Management Units for the *north coast* and *Oregon* clades.

Phylogeography, Evolution, and Conservation in Forest-associated Pacific Northwest Salamanders.

by

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

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Contribution of Authors

The work was performed in the laboratory of Dr. Susan Haig who provided financial support, assisted with the project proposal, and writing of each manuscript. Charles Crisafulli assisted in the research conducted in Chapter 2 by supplying samples and contributing to the design of the project.

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PHYLOGEOGRAPHY, EVOLUTION, AND CONSERVATION IN FOREST-ASSOCIATED PACIFIC NORTHWEST SALAMANDERS.

CHAPTER 1

INTRODUCTION

General Introduction

Understanding the phylogeographic distribution of species provides insight into how historical versus contemporary events influence differentiation and genetic structure of populations (Avise 1987, Avise 1994). In the U.S. Pacific Northwest, geographic barriers arise by a complex history of glaciation, flooding, and volcanism that fragment forest communities throughout the region. Not only are these forests fragmented by historical geologic and ecological processes, but they are increasingly fragmented by forest management practices (i.e., timber harvest) and rural development (Spies et al. 1994). Thus, phylogeographic studies can allow us to determine the appropriate scale at which to focus management efforts to avoid the loss of genetic diversity.

The classic paradigm for most species conservation efforts is to maintain gene flow among populations to avoid loss of genetic diversity through random genetic drift (Lande and Barrowclough 1987). However, many species with limited dispersal capabilities are vulnerable to vicariant events that isolate populations for long periods and lead to genetic divergence. This is most pronounced in amphibians where a

general pattern is one of low gene flow and extreme differentiation among populations (Highton et al. 1989, Good and Wake 1992, Tilley and Mahoney 1996). Therefore, threats to their persistence are not necessarily a consequence of low gene flow but instead are threatened by loss of unique genetic lineages when single populations go extinct.

To gain an understanding of how historical versus contemporary processes may influence the evolutionary history of species, this dissertation is focused on phylogeographic variation, population genetic structure, and the evolutionary relationships of several late-successional coniferous forest-associated salamanders endemic to the Pacific Northwest (Larch Mountain Salamander, *Plethodon larselli*; Oregon Slender Salamander, *Batrachoseps wrighti*; Olympic Torrent Salamander, *Rhyacotriton olympicus*; Columbia Torrent Salamander, *R. kezeri*; Cascade Torrent Salamander, *R. cascadae*; and the Southern Torrent Salamander, *R. variegatus*). Each is considered a species of concern and is managed with respect to the Northwest Forest Plan (U.S. Forest Service and U.S. Bureau of Land Management 1994). Therefore, the results of these studies are presented in the context of conservation units in order to provide guidance in prioritizing conservation efforts.

The conservation unit concept, namely identification of Evolutionary Significant Units (ESUs) and Management Units (MUs), provides a framework for determining the scale at which to focus management efforts and preserve historical lineages. There has been intense debate over how conservation units should be defined (Ryder 1986; Waples 1991; Dizon et al. 1992; Moritz 1994a,b; Vogler and Desalle 1994; Bowen 1998; Crandall et al. 2000). However, the most widely used

conservation unit designations are those described by Moritz (1994a,b; see also Moritz et al. 1995), which operationally defines ESUs to reflect long-term reproductive isolation by requiring reciprocal monophyly of mitochondrial alleles *and* divergence of nuclear alleles. Further, MUs, subunits that comprise ESUs, are designed for short-term or demographic focus and are defined by divergence of either mitochondrial alleles *or* nuclear alleles. Application of these definitions to three species examined in this dissertation will provide perspective to those implementing the Northwest Forest Plan and USFW biologists considering listing options under the Endangered Species Act.

Dissertation Organization

The research in this dissertation consists of five manuscripts written as chapters. Chapters 2 and 3 stress the importance of considering historical influences on population differentiation and genetic structure, and proposes conservation unit designations to aid in management efforts for two terrestrial salamanders (Larch Mountain Salamander and Oregon Slender Salamander). Chapter 2 presents a study of Larch Mountain Salamanders, completely terrestrial plethodontids, which until recently were considered a declining relict species, with very specific habitat needs, restricted to the Columbia River Gorge. However, recent discovery of several populations found further north of the Gorge has greatly extended their range into the southern and central Cascade Range of Washington (Aubry et al. 1987; Darda and Garvey-Darda 1995; C. Crisafulli, unpublished). Thus, we describe geographic

variation and population structure in the Larch Mountain Salamanders (12 populations) using mitochondrial DNA (mtDNA) cytochrome b sequence data (381 bp) and randomly amplified polymorphic DNA sequence data (RAPDs; 34 loci).

Chapter 3 is focused on the Oregon Slender Salamander, another completely terrestrial plethodontid salamander, which is associated with mesic forests of the western slopes of the Cascades. They are patchily distributed and forest management practices may lead to local extirpation that could affect the overall viability of the species (Marshall et al. 1992, Vesely et al. submitted). Thus, we used mtDNA cytochrome b sequence data and RAPD markers (46 loci) to analyze 22 populations across their range to assess the relative impact of historical processes on population structure and differentiation.

Chapters 4, 5 and 6 examine how vicariant events and phylogeographic barriers have contributed to the distribution, population divergence, and speciation among the Torrent Salamanders. Torrent Salamander species are remarkably morphologically conserved, have similar life histories and occupy ecologically similar habitats (Good and Wake 1992). In fact, they were considered a monotypic genus until allozyme studies revealed large genetic divergences both within and among the currently recognized taxa (Good et al. 1989, Good and Wake 1992). Torrent Salamander have an aquatic larval stage and more terrestrial adult stage; however, both life stages are found in cold, clear, small streams and headwaters associated with

late-successional forests. Currently, they are suggested to be impacted by timber harvest and related disturbance activities (Bury and Corn 1988a, Welsh and Lind 1988, Corn and Bury 1989, Bury et al. 1991, Diller and Wallace 1997).

Chapter 4 stresses how historical vicariant events have contributed to species divergences among Torrent Salamanders (Family Rhyacotritonidae). Three different mitochondrial gene regions (cytochrome b, 12S and 16S ribosomal RNA) were used to infer phylogenetic relationships among species and estimate their divergence times. These relationships give perspective to the amount of divergence among Torrent Salamander species and provide further support for designation of conservation units described for Southern Torrent Salamanders in Chapter 6.

Chapter 5 analyzes a potential contact zone among Southern Torrent and Cascade Torrent Salamanders using maternally inherited mtDNA 16S ribosomal RNA sequences (499 bp), and allozymes (6 loci). These markers define the taxonomic identity of recently discovered Torrent salamander populations found in the central Cascade mountain range of Oregon and extends the previously described ranges of both species. This is particularly important considering issues surrounding recent Endangered Species Act listing concerns of the Southern Torrent Salamander.

Chapter 6 describes the phylogeography and evolutionary history of populations within the Southern Torrent Salamander. Recently, the Southern Torrent Salamander was denied listing under the U.S. Endangered Species Act (Federal Register 60:33785) due to a lack of information regarding population fragmentation and gene flow. Therefore, this study of fine-scale population differentiation among 72

localities of Southern Torrent Salamanders using the cytochrome b gene sequences (779 bp) results in identifying conservation units that can be considered for management, listing or recovery efforts.

CHAPTER 2

GEOGRAPHIC VARIATION, GENETIC STRUCTURE AND CONSERVATION UNIT DESIGNATION IN THE LARCH MOUNTAIN SALAMANDER (*Plethodon larselli*).

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Accepted *Biological Conservation*

Abstract

Larch Mountain salamanders (*Plethodon larselli*) are associated with late-successional forests in North America's Pacific Northwest and face threats related to habitat destruction and fragmentation. To prioritize conservation strategies, we used mitochondrial DNA sequences and random amplified polymorphic DNA (RAPDs) to examine differences in 12 populations (184 individuals) of Larch Mountain salamander. Phylogenetic inferences, using cytochrome *b* sequences (381bp), based upon three methods indicated significant differences between northern and southern populations separated by the Columbia River, and a greater difference between southeast and southwest populations located on the south-bank of the Columbia River. This result was confirmed by RAPD analyses (34 loci) using phylogenetic analyses, non-metric multidimensional scaling and analysis of molecular variance. Southern populations exhibited significantly ($p = 0.003$) reduced expected heterozygosity (average $H_e = 0.17$) compared to northern populations (average $H_e = 0.22$). Further, gene flow is inferred to be lower among populations on the south-bank compared to northern populations. Finally, based upon Moritz's definitions for conservation units, we suggest separate Management Unit designations for northern, south-west and south-east populations.

Introduction

Phylogeographic studies aid in identifying historic barriers to dispersal and gene flow, which contribute to understanding the relative effects of natural and anthropogenic impacts to habitat fragmentation (Avise 1994). Amphibians often have specific ecological requirements and low dispersal rates making them susceptible to fragmentation by historic and current processes. Genetic studies of amphibians often reveal significant amounts of cryptic genetic diversity attributable to the influence of vicariant events in shaping population structure (Good and Wake, 1992; Highton, 1995; Jockusch, 1996; Tilley and Mahoney, 1996). Therefore, to enhance amphibian conservation efforts, it is important to understand the role of fragmentation in population differentiation and implement management plans that preserve within-species genetic diversity.

The conservation-unit concept provides a framework for prioritizing management of intra-specific genetic diversity (Ryder, 1986). However, conservation units have rarely, if ever, been described for amphibians (Wagner and Haig, in review). In this paper, we describe application of the conservation-unit concept, designation of evolutionary significant units (ESUs) and management units (MUs), for Larch Mountain salamanders (*Plethodon larselli*) using mitochondrial DNA (mtDNA) and random amplified polymorphic DNA (RAPD) data sets.

Although there has been debate regarding diagnosis of conservation units (Ryder, 1986; Waples, 1991; Dizon et al., 1992; Dowling et al., 1992; Moritz, 1994a,b; Moritz, et al., 1995; Vogler and Desalle, 1994; Pennock and Dimmick, 1997;

Bowen, 1998; Dimmick, et al. 1999), the most widely used concept is Moritz's ESU (1994a,b; Moritz et al., 1995). Designed to reflect long-term reproductive isolation, an ESU is defined by a 2-fold test: populations must show reciprocal monophyly of mitochondrial DNA alleles *and* show significant divergence at nuclear alleles. Failing to meet both criteria for the ESU designation, populations can be defined as Management Units (MUs) based on the significant divergence of mtDNA alleles *or* nuclear alleles. The MU designation reflects demographic isolation or short-term focus. Conservation units can be used to define "distinct population segments" for listing or recovery under the U.S. Endangered Species Act or IUCN (Waples, 1991; IUCN, 1997).

Larch Mountain salamanders are a completely terrestrial mature forest-associated species in the Pacific Northwestern United States. Major threats to this species include habitat loss and population fragmentation due to logging, recreational activities, and housing development (Herrington and Larsen, 1985). The states of Washington and Oregon have designated Larch Mountain salamanders as "sensitive" and "sensitive-vulnerable", respectively. They are considered "survey and manage" species with respect to the federal Northwest Forest Management Plan (U.S. Forest Service and U.S. Bureau of Land Management, 1994). Further, they are listed in the IUCN Redbook (1997) as Data Deficient, citing insufficient population or distribution data to make an assessment of extinction threat. Finally, The Nature Conservancy lists them as globally and sub-nationally "imperiled".

Until recently, these salamanders were considered the rarest amphibian in the Pacific Northwest due to their restricted range along a narrow corridor of the Columbia River Gorge (Kirk, 1983; Howard et al., 1983; Herrington and Larsen, 1985). Previously, they were described as occurring only in isolated patches restricted to specific forested, steep, talus slopes within the Columbia River Gorge (Burns, 1954; Burns, 1962; Burns, 1964; Herrington and Larsen, 1985). However, many new populations have recently been discovered in the southern and central Cascade Mountain Range of Washington (Aubry et al., 1987; Darda and Garvey-Darda, 1995; C. Crisafulli, unpublished data). In order to assess the historic impact of fragmentation on the Larch Mountain salamander, we examined population differentiation and genetic structure of 12 populations throughout their range.

To examine geographic variation and designate conservation units, we used mtDNA cytochrome *b* sequences and RAPD markers. Cytochrome *b* sequences have been used in a wide variety of taxa for designating conservation units (e.g., Baker et al., 1995; Lento et al., 1997; Mundy et al., 1997; Castilla et al., 1998; Walker et al., 1998) and in several salamander species to infer intra-specific phylogeny (e.g., Hedges et al., 1992; Moritz et al., 1992; Jackman et al., 1997; Tan and Wake, 1995). The RAPD technique, a method to sample large numbers of segregating nuclear loci from the genome, has been increasingly used in vertebrate conservation studies (e.g., Haig et al., 1994; Fleischer et al., 1995; Haig et al., 1996; Kimberling et al., 1996; Nusser et al., 1996; Haig et al., 1997, Haig, 1998; Haig et al., in review) and in herpetological studies (e.g., Gibbs et al., 1994; Prior et al., 1997). The technique has advantages of being a simple, expedient and cost-effective procedure.

Materials and Methods

Tissue sampling and DNA isolation

Larch Mountain salamanders were hand-captured and the distal 1cm of tail was removed using sterile surgical scissors for each individual from 12 populations throughout their range. Individuals were (Figure 2.1, Table 2.1). Each sample was placed in a cryogenic vial and stored in liquid nitrogen or on dry ice until transferred to an ultra-cold freezer (-80°C).

DNA was isolated by digesting 2 μg of tissue in 400 μl of extraction buffer (100mM Tris-HCl pH 7.5, 100mM EDTA, 250mM NaCl, 600μg/ml of Proteinase K) in a 1.5 ml microtube. Samples were vortexed for 1 min. and then incubated overnight at 55°C. Samples were extracted twice using equal volumes of phenol saturated with Tris-HCl buffer (pH 7.5) and then once with chloroform/isoamyl alcohol (25:1). DNA was concentrated using a micron-50 filter (Millipore). The concentration of each sample was determined by fluorimetry (Hoefer TKO 100) and the quality of extraction was checked on an agarose gel.

Figure 2.1 Sampling locations of Larch Mountain salamanders: (1) Bridal Veil Falls, OR, (2) Multnomah Falls, OR, (3) Herman Creek, OR, (4) Wyeth, OR, (5) Starvation Falls, OR, (6) Cape Horn, WA, (7) Lower Copper, WA, (8) Zig Zag, WA, (9) Ole's Cave, WA, (10) Straight Creek, WA, (11) Quartz Creek, WA, (12) Packwood Palisades, WA.

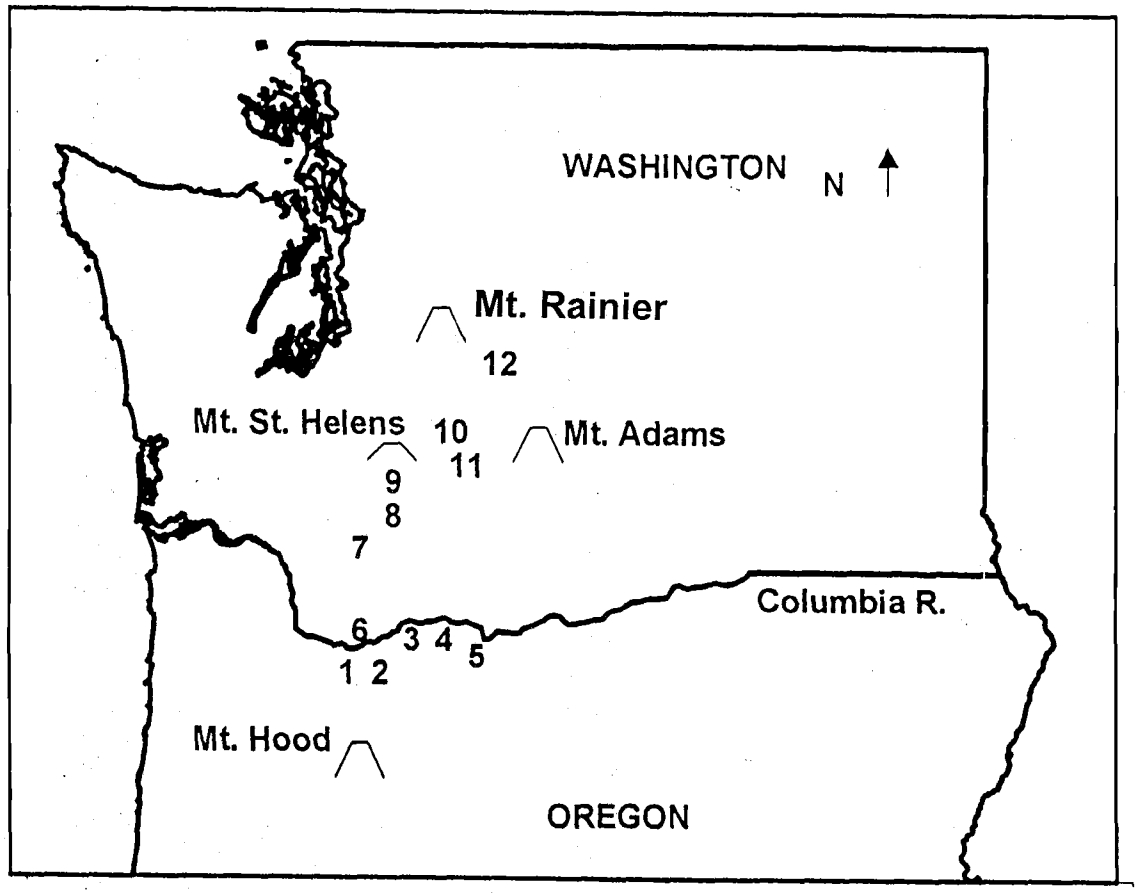
WASHINGTON
N
Mt. Rainier
12
Mt. St. Helens
10
11
Mt. Adams
9
8
7
Columbia R.
6
3
4
5
1
2
Mt. Hood
OREGON

Figure 2.1

Table 2.1 Locations and abbreviations for Larch Mountain Salamander populations sampled. See Figure 1 for map locations.

Population	Code	Legal Location
1. Bridal Veil Falls(OR)	BDVF	T1N,R5E,S24,SW1/4
2. Multnomah Falls(OR)	MHFL	T1N,R6E,S10,SW1/4
3. Herman Creek(OR)	HMCK	T2N,R8E,S4,SW1/4
4. Wyeth Campground(OR)	WYTH	T2N,R8E,S1,SW1/4
5. Starvation Falls(OR)	STVF	T2N,R9E,S3,NW1/4
6. Cape Horn(WA)	CAPE	T1N,R5E,S16,NE1/4
7. Lower Copper Creek(WA)	LCCK	T4N,R5E,S32,SE1/4
8. Zig Zag Creek(WA)	ZIGZ	T4N,R6E,S8,NW1/4
9. Ole's Cave(WA)	OLEC	T7N,R5E,S17,SW1/4
10. Straight Creek(WA)	STCK	T9N,R8E,S32,NE1/4
11. Quartz Creek(WA)	QZCK	T8N,R8E,S8,SW1/4
12. Packwood Palisades(WA)	PKPL	T14N,R10E,S22,SE1/4

Amplification and DNA Sequencing

The polymerase chain reaction (PCR) was used to amplify a ~850bp fragment of the cytochrome *b* gene, using the following primers designed for vertebrates: MVZ15 5'-GAACTAATGGCCCACAC(A/T)(A/T)TACGNAA-3' and MVZ16 5'-AAATA-GGAAATATCATTCTGGTTTAAT-3' (Kocher et. al., 1989). Fragments were amplified using a MJ Research thermal cycler (PTC 100) with the following steps: initial denaturation for 10 min. at 93°C, followed by 40 cycles of 1 min. denaturation at 93°C, annealing for 1 min. at 52°C and extending at 72°C for 2 min. A final extension at 72°C for 10 min. completed the reaction. Each reaction was conducted using 100 ng of sample in a 50-µl volume. The reaction cocktail used 0.5 units of Taq Gold (Perkin Elmer) with the supplied reaction buffer, 2mM MgCl, and 1mM of each primer. Amplifications were extracted from a 1% agarose gel using an ultra-free-mc 0.45 filter (Millipore) from which the supernatant was transferred to a micron-50 filter (millipore) to prepare templates for sequencing. Sequencing primers included MVZ-15, MVZ-16 and cytb2 (5'-AAACTGCAGCCCCTCAGAATGATAT-TTGTCCTCA3'; Moritz et al., 1992). Automated sequencing was performed at Oregon State University Central Services Laboratory with an Applied Biosystems (373A) sequencer. Sequences from fragments were aligned by eye using the Genetic Data Environment (Smith et al., 1992) and compared to a Genbank archived cytchrome *b* sequence of *Plethodon elongatus* (L75821; Moritz et al., 1992).

RAPD procedure and scoring

RAPD profiles were generated, as described in Aagaard et al. (1995), using the polymerase chain reaction. PCR reactions were setup using the following concentrations (25 µl volume): 10X buffer (50mM KCl; 10mM Tris-HCl at pH 9.0; 0.1% Triton X-100); 1.8mM $MgCl_2$; 100µM for each of dATP, dCTP, dGTP, dTTP; 0.2µM primer; 2 ng template DNA; and 1 unit of Taq Polymerase (Promega). Reactions were conducted using a MJ Research thermal cycler (PTC-100) programmed with the following parameters: first denaturation for 3 min. at 93°C, then 45 cycles of; denaturation for 1 min. at 93°C, annealing for 1 min. at 45°C, and elongation for 2 min. at 72°C. A final 10 min. elongation at 72°C completed the reaction, which was then held at a constant 4°C until removed from the cycler. Then 15 µl of each reaction was loaded in a 2.0% agarose gel (GibcoBRL; Ultrapure) and electrophoresed for 4 hours (100 V) in TBE (90mM Tris base, 90mM Boric acid, 2mM EDTA, pH 8.0). Amplification products were sized using a 1 Kb DNA ladder (GibcoBRL). The gels were then stained with ethidium bromide (1µg/ml) for 30 min. and destained for 2 hours in deionized H_2O.

Preliminary screening of 235 primers (10-mers from the Oligonucleotide Synthesis Laboratory, University of British Columbia), for variable bands used two individuals from each of four populations (Straight Creek, WA.; Cape Horn, WA; Herman Creek, OR; Multnomah Falls, OR; Figure 1), were assessed. RAPD profiles

with distinct, well separated, and reproducible bands were chosen for the final analyses. Reproducibility was assessed in replicate side-by-side RAPD reactions and in multiple RAPD runs. Negative controls were run with all amplifications to check for contamination.

Analyses of mitochondrial DNA Sequences

Three distinct methods of phylogenetic reconstruction were used to estimate relationships among cytochrome *b* sequences, including distance (minimum evolution), maximum likelihood, and parsimony phylogenetic trees using the program PAUP* 4.0b1 (Swofford, 1998). The strengths, weaknesses, and assumptions of each method have been discussed previously (Hasegawa and Fujiwara, 1993; Huelsenbeck and Hillis, 1993; Kuhner and Felsenstein, 1994; Tateno, et al. 1994; Gaut and Lewis, 1995; Pagel, 1999); however, similar tree topologies derived from different methods are expected reflect true phylogenetic relationships (Kim, 1993). Kimura 2-parameter (Kimura, 1980) distances using a heuristic search were used to generate minimum evolution trees. For maximum parsimony, heuristic searches and the tree bisection-reconnection algorithm were used to find trees of shortest length (Swofford, 1998). Maximum-likelihood reconstructions were performed using the general-time-reversible model (Yang, 1994) with maximum likelihood estimated nucleotide frequencies and substitution rate-matrix parameters. Site-specific rates were estimated to account for rate heterogeneity among codon positions with starting trees obtained by stepwise random addition. In addition, we performed bootstrap re-sampling

(Felsenstein, 1985) of 100 iterations for all methods to assess reliability of the data to derive the same tree. Alternative topologies of phylogenetic trees were compared for significant differences using the Kishino-Hasegawa test in PAUP* 4.0b1 (Swofford, 1998). *Plethodon elongatus* (Genbank accession L75821; Moritz et al., 1992) was used as an outgroup for each tree.

Analyses of random amplified polymorphic DNA markers

RAPDs were analyzed directly as phenotypes due to dominance of RAPDs markers. Homozygous dominant (presence/presence) and heterozygous (presence/absence) individuals are indistinguishable because of the dominant band, so both were scored as a (1) phenotype, while null-allele homozygous recessive individuals (absence/absence) were scored as a (0) phenotype. All scored loci were assumed to be in Hardy-Weinberg equilibrium and non-allelic.

Dominance can cause bias in estimation of null allele frequency and subsequent population genetic parameters (Lynch and Milligan, 1994; Zhivotovsky, 1999). Therefore, allele frequencies were calculated using the Lynch and Milligan (1994) Taylor-expansion-correction incorporated in the program TFPGA (Miller, 1998b). Calculations of expected heterozygosity (H_e; Nei, 1978) using Lynch and Milligan's (1994) Taylor expansion from the program TFPGA (Miller, 1998b), and uncorrected estimates from POPGENE (Yeh et al., 1997) were compared. Further, POPGENE was used to estimate percent of polymorphic loci (P_e, 95% and 99%

criteria) as well as mean (A) and effective number of alleles per locus (A_e). Non-parametric Mann-Whitney rank sum tests (Wilcoxon, 1945; Mann and Whitney, 1947) were then used to compare genetic diversity parameters.

Each scored individual locus was evaluated for its contribution to population differentiation. Exact tests of population differentiation per locus were performed with TFPGA using 2000 permutations (Raymond and Rousset, 1995). The extent of genetic differentiation within and among populations was estimated using the following statistics: Wright's F_{st} (1931), Weir and Cockerham's θ_w (1984), and Lynch and Milligan's (1994) F_{st} were calculated using RAPDFST (Black, 1998). For comparison Nei's G_{st}, (Nei, 1973) was calculated by POPGENE. Further, F-statistics θ_P were calculated by jackknifing over all loci using TFPGA.

Analysis of molecular variance (AMOVA) was used to further describe subdivision of genetic variation among populations within groups (northern and southern), and between groups. AMOVA-PREP (Miller, 1998a) was used to prepare input files for WINAMOVA (Excoffier et al., 1992; Excoffier, 1993), which calculated within and among population variance components, and the F-statistic analog (ϕ_{st}).

A quantitative non-parametric assessment was performed with multi-response permutation procedures (MRPP) to compare groups (northern and southern) using PC-ORD (version 4.28 beta; McCune and Mefford, 1999). Within-group heterogeneity was compared to that expected by chance, using Jaccard's distances (1908), and evaluated as chance corrected within-group agreement values (*A*-values) and their associated significance (Mielke, 1984).

A more qualitative assessment of relationships among populations was evaluated with non-metric multidimensional scaling. PC-ORD was used to scale all loci using Jaccard's distances (Jaccard, 1908; Kruskal, 1964a,b; Mather, 1976). First, the relationship between overall stress (opposite of goodness-of-fit) and increasing number of dimensions was plotted to evaluate the appropriate number of dimensions needed for the final solution. The plot indicated that three dimensions were sufficient. Next, a plot of stress versus iteration number was used to evaluate stability of the solution for the given data. Stress reached a minimum after 20 iterations; therefore, the 100 iterations for the final solution should have been sufficient. Kendall correlation coefficients were used to examine the relationship among the final three ordination axes and all variable loci.

The phylogenetic relationships among populations, using RAPD phenotypes, were compared by constructing neighbor-joining distance trees (Saitou and Nei, 1987). First, Manhattan distances (Prevosti distance in Wright 1978) were calculated among populations using RAPDDIST (Black, 1998). Then, bootstrap matrices (100 replications) were calculated with RAPDDIST and analyzed using NEIGHBOR and CONSENSE options in PHYLIP v 3.5C (Felsenstein, 1993).

To examine the hypothesis of isolation-by-distance (Wright, 1954), genetic distance (Manhattan distance) was evaluated with respect to geographic distance using a Mantel (1967) test in NTSYS-PC (Rohlf, 1994). Resulting *r*-values, normalized Mantel Z statistics, are interpreted as correlation coefficients and examined for significance by permutation procedures (100 permutations; Smouse et al., 1986). Mantel tests were performed for all populations, and separately for northern and southern groups.

Results

MtDNA sequence analyses

Cytochome *b* sequence analyses showed considerable differentiation among populations (Table 2.2). Nucleotide sequences based on 381 base pairs of the cytochrome *b* gene (5'-region), were characterized by 28 variable sites with pair-wise sequence differences ranging between 0.0 to 5.3 % (Table 2.3). All substitutions were synonymous with 2 first, 7 second and 19 third position codon substitutions. Eleven distinct haplotypes were found among twelve populations, with two southern

populations (Herman Creek and Starvation Falls) showing identical haplotypes. Because sequencing of at least three individuals from each population yielded identical haplotypes, within-population haplotype diversity appears to be insignificant compared to among-population haplotype diversity. Analyses showed significant differences among haplotypes from populations found north of the Columbia River compared to populations found south of the Columbia River. Further, differences were found between south-west and south-east populations found on the south-bank Kimura 2-parameter distances among all populations varied from 2.4 to 12.1%, while among southern populations distances varied 0.0% to 5.5%, and among northern populations from 0.26% to 2.1% (Table 2.3).

Phylogenetic trees based upon aligned cytochrome *b* gene sequences showed similar topologies for all three methods of inference (Figure 2.2). Four Kimura 2-parameter distance (minimum evolution) based trees were found to have a tree length of 0.53. A consensus (50% majority) minimum-evolution bootstrap tree indicated strong support for monophyletic grouping of southeastern populations (Wyeth, Starvation Falls, Herman Creek), while southwestern populations (Multnomah Falls, Bridal Veil Falls) were paraphyletic with respect to a northern clade (Lower Copper Creek, Zig Zag, Packwood Palisades, Cape Horn, Ole's Cave, Straight Creek; Figure 2.2A). Similarly, four most-parsimonious trees were found, based upon 17 parsimony

informative sites, each comprised of 100 steps. A consensus parsimony bootstrap tree showed the same topology as the distance tree (Consistency Index 0.90, Retention Index 0.95, Figure 2.2B). Finally, a maximum-likelihood (Figure 2.2C) consensus tree showed a similar topology with a –ln likelihood score of 908.85.

Table 2.2 Mitochondrial DNA sequence variation from 381 base pairs of the cytochrome *b* gene in Larch Mountain salamanders. Dots indicate sequence identity. Only the 28 variable sites are shown, identified by three digits (above) corresponding to its sequence location. *Plethodon elongatus* is an outgroup species.

Location	000000000111111112222333333 045667788235788991368003355 958232768440769018172460 1225
1. Bridal Veil Falls(OR)	TGACTACGCCCATTTCGGCGATCCGTGA
2. Multnomah Falls(OR)	..T...TT.....A...
3. Herman Creek(OR)	..TT..TTAT...C.....CT.A...
4. Wyeth(OR)	..TT..TTATG..C.....GCT.A...
5. Starvation Falls(OR)	..TT..TTAT...C.....CT.A...
6. Cape Horn(WA)	.TT.C.TT...G.....TA.....C..
7. Lower Copper Ck(WA)	.TT.C.TT...G.....ATA...T..C.
8. Zig Zag(WA)	.TT.C.TT...G.....TA.....
9. Ole's Cave(WA)	.TT.C.TT...G.....TA...GCG.C
10. Straight Creek(WA)	CTT.C.TT...GC.CGC.TA.....A..
11. Quartz Creek(WA)	.TT.C.TT...G.....ATA.....
12. Packwood Palisades(WA)	.TT.CGTT...G...T..TAG.....
13. *P. elongatus*	NAC.A.TTT...CCC....T..TTA...

Table 2.3 Kimura 2-parameter distances (below diagonal, distances multiplied by 100) and percentage of sequence difference (above diagonal) based upon cytochrome *b* sequence (381 bp) data for Larch Mountain salamanders.

Location	1 BDVF	2 MHFL	3 HMCK	4 WYTH	5 STVF	6 CAPE	7 LCCK	8 ZIGZ	9 OLEC	10 STCK	11 QZCK	12 PKPL	13 PLEL
1. Bridal Veil Falls(OR)	-	1.1	3.2	3.7	3.2	1.8	2.4	1.6	2.1	3.1	1.8	2.4	20.7
2. Multnomah Falls(OR)	1.0	-	2.1	2.6	2.1	1.3	1.8	1.1	1.6	2.6	1.3	1.8	19.9
3. Herman Creek(OR)	3.2	2.1	-	0.52	0.0	3.4	3.9	3.1	3.7	4.7	3.4	3.9	19.6
4. Wyeth(OR)	3.8	2.7	0.5	-	0.53	3.9	4.5	3.7	4.2	5.3	3.9	3.9	20.1
5. Starvation Falls(OR)	3.2	2.1	0.0	0.5	-	3.4	3.9	3.6	3.7	4.7	3.4	3.9	19.6
6. Cape Horn(WA)	1.9	1.3	3.5	4.1	3.5	-	1.1	2.6	0.26	1.6	0.52	1.1	20.4
7. Lower Copper Ck(WA)	2.4	1.9	4.1	4.6	4.1	1.1	-	0.79	1.3	2.4	1.1	1.6	20.4
8. Zig Zag(WA)	1.6	1.1	3.2	3.8	3.2	0.26	0.79	-	0.52	1.6	0.26	0.79	20.6
9. Ole's Cave(WA)	2.1	1.6	3.8	4.4	3.8	0.26	1.3	0.53	-	1.8	0.79	1.3	20.7
10. Straight Creek(WA)	3.2	2.7	4.9	5.5	4.9	1.6	2.4	1.6	1.9	-	1.8	2.1	20.7
11. Quartz Creek(WA)	1.9	1.3	3.5	4.1	3.5	0.53	1.1	0.26	0.79	1.9	-	1.1	20.2
12. Packwood Palisades(WA)	2.4	1.9	4.1	4.1	4.1	1.1	1.6	0.79	1.3	2.1	1.1	-	21.0
13. *P. elongatus*	24.7	23.6	23.2	23.9	23.2	24.4	24.4	24.0	25.0	24.3	24.0	25.2	-

Figure 2.2 Phylogenetic relationships among Larch Mountain salamanders based upon cytochrome *b* sequences (381bp). A. Consensus distance (minimum evolution) tree based upon Kimura 2-parameter distances (distances above branches, bootstrap values below). B. Consensus maximum parsimony tree (number of steps above branches, bootstraps values below). C. Consensus maximum likelihood tree (distances above branches, bootstrap values below).

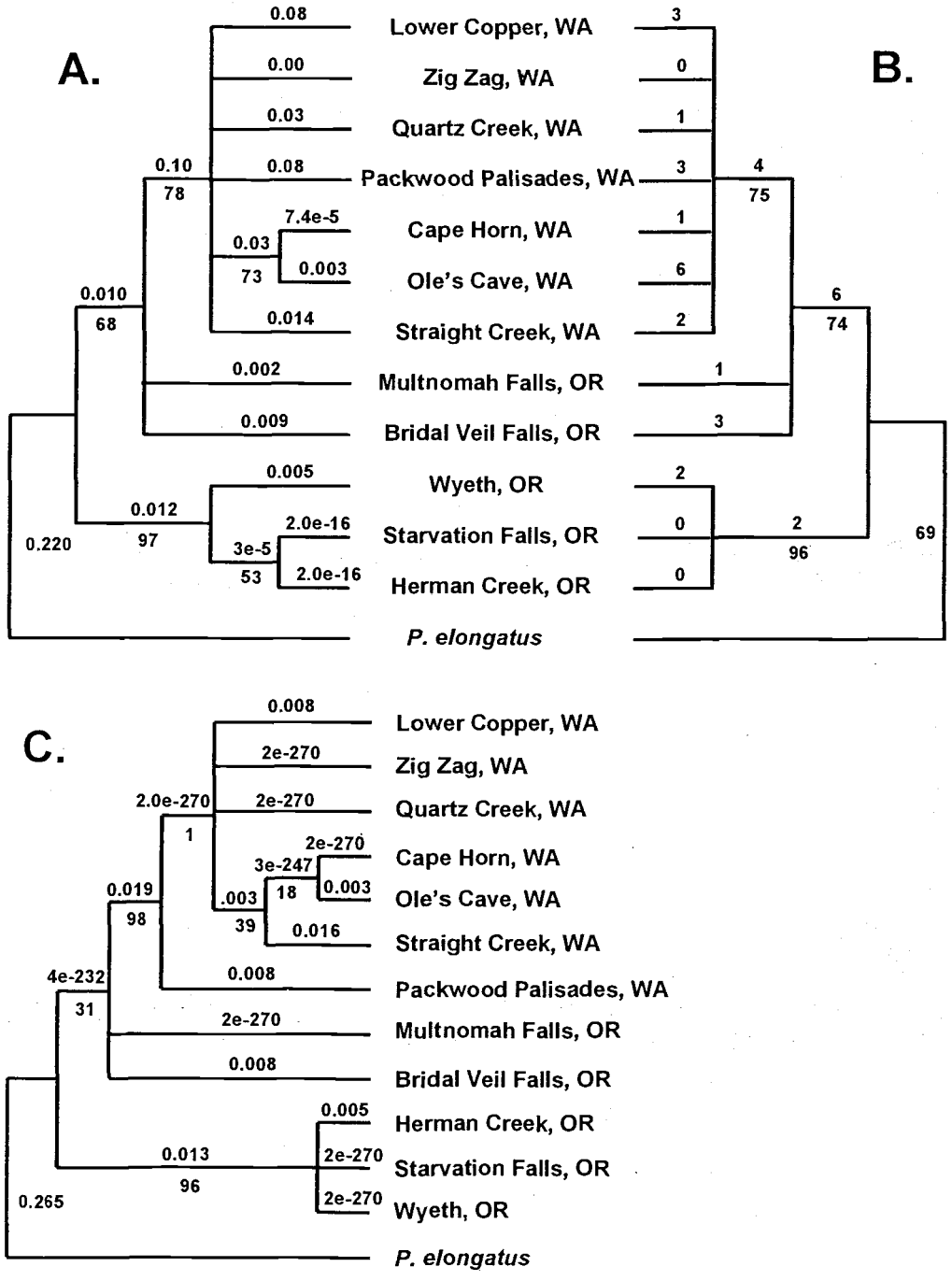
A.
B.
C.
Lower Copper, WA
Zig Zag, WA
Quartz Creek, WA
Packwood Palisades, WA
Cape Horn, WA
Ole's Cave, WA
Straight Creek, WA
Multnomah Falls, OR
Bridal Veil Falls, OR
Wyeth, OR
Starvation Falls, OR
Herman Creek, OR
P. elongatus
0.08
0.00
0.03
0.10
0.08
78
7.4e-5
0.03
0.003
73
0.010
68
0.014
0.002
0.009
0.005
0.012
2.0e-16
0.220
97
3e-5
53
2.0e-16
3
0
1
4
75
1
6
2
6
74
2
0
2
96
69
0
0.008
2e-270
2.0e-270
2e-270
1
2e-270
3e-247
0.003
0.019
98
.003
18
39
0.016
4e-232
31
0.008
2e-270
0.008
0.005
0.013
2e-270
96
0.265
2e-270

Figure 2.2

The relationship among southwestern haplotypes (Bridal Veil and Multnomah Falls) is difficult to resolve. Bootstrap resampling values for the cluster supporting the grouping of southwestern haplotypes with the northern group is significant at 68% and 75% for distance and parsimony trees, respectively (Figure 2.2A and 2.2B). However, support for this cluster in maximum likelihood analyses is less than 50% (Figure 2.2C). Comparison with a tree derived by constraining southwestern haplotypes to form a monophyletic cluster with southeastern populations (Wyeth, Herman Creek, and Starvation Falls) showed no significant differences, using the Kishino-Hasegawa test, for either maximum likelihood (differences in –ln likelihood = 0.0000 ± 0.0004 S.D, T = 0.0000, p = 1.0000) or maximum parsimony (difference in tree length = 2 ± 1.41 S.D steps, T = 1.41, p = 0.16) tree. The difficulty in resolving this relationship may be due to the short branch uniting southwestern populations to either northern or southeastern populations.

RAPD analyses

Of 235 primers screened, 14 primers produced 34 variable bands for final analyses. Frequency of these bands varied considerably within and among 12 populations sampled (Table 2.4). A number of population-specific bands were identified. Two bands (loci 3 and 8) were specific (fixed) for populations occurring south of the Columbia River and one band (locus 12) was variable in two southern populations (Bridal Veil Falls, Multnomah Falls) while specific for remaining southern populations. Northern populations were specific for locus 9. Southeastern

populations (Starvation Falls, Wyeth, and Herman Creek) were specific for locus 34. Exact tests of population differentiation indicated 33 loci were significant ($p < 0.05$) except for locus 5 ($p = 0.14$; Raymond and Rousset, 1995). Further, pair-wise exact tests between populations revealed significant differences between a majority of populations, suggesting considerable fine-scale structure among populations (Table 2.5).

Multi-dimensional scaling along three dimensions for 34 loci indicated that most variation was contained within the first axis (46.5%, $p < 0.05$, $R^2 = 0.40$), while the second (26.1%, $p < 0.05$, $R^2 = 0.06$) and third (19.9%, $p < 0.05$, $R^2 = 0.02$) axes accounted for the remainder. The cumulative R^2 among the first three ordination axes was 0.48. Plots of first and second axes show distinct clustering of southern populations, while most of the variation appears to be found within northern group with 43.2 % of the variation among populations (Figure 2.3). Kendall correlations of loci with each axis revealed three loci significantly correlated with the first axes (Locus 3, 8, and 9 each have R^2 values of 0.62, $p < 0.05$). Each of these loci shows fixed differences between northern and southern groups.

Table 2.4. Estimates of dominant (+) RAPD marker frequencies using Lynch & Milligan's (1994) Taylor Expansion method for 12 populations of Larch Mountain salamander. UBC# is the University of British Columbia RAPD primer set number, followed by fragment size of the locus scored. See Figure 2.1 for locations.

Locus	UBC code	1 BDVF	2 MHFL	3 HMCK	4 WYTH	5 STVF	6 CAPE	7 LCCK	8 ZIGZ	9 OLEC	10 STCK	11 QZCK	12 PKPL
Locus-1	UBC#100-795bp	0.000	0.000	0.000	0.000	0.000	0.370	0.116	0.289	0.000	0.000	0.000	0.000
Locus-2	UBC#108-790bp	0.000	0.000	0.000	0.071	0.000	0.370	0.116	0.289	0.000	0.000	0.000	0.000
Locus-3	UBC#135-350bp	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Locus-4	UBC#189-670bp	0.071	0.044	0.175	0.000	0.071	0.120	0.028	0.070	0.000	0.161	0.110	0.386
Locus-5	UBC#189-885bp	0.441	0.404	0.083	0.000	0.329	0.260	0.288	0.289	0.175	0.288	0.386	0.345
Locus-6	UBC#192-425bp	1.000	0.625	0.000	0.000	0.000	1.000	0.328	0.470	0.398	1.000	0.530	0.588
Locus-7	UBC#192-450bp	0.000	0.000	1.000	1.000	1.000	0.260	0.288	0.289	0.278	0.051	0.170	0.170
Locus-8	UBC#203-1590bp	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Locus-9	UBC#210-320bp	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Locus-10	UBC#210-770bp	0.577	0.164	0.000	0.234	0.234	0.504	0.328	0.228	0.175	0.104	0.236	0.140
Locus-11	UBC#220-500bp	0.000	0.245	1.000	0.234	0.329	0.164	0.251	0.228	0.083	0.191	0.270	0.110
Locus-12	UBC#220-630bp	0.234	0.022	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Locus-13	UBC#220-650bp	0.329	0.763	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Locus-14	UBC#225-550bp	1.000	1.000	1.000	1.000	1.000	0.687	0.215	0.355	0.000	0.746	0.236	1.000
Locus-15	UBC#225-580bp	0.149	0.090	0.000	0.000	0.000	0.164	0.517	0.145	0.175	0.288	0.270	1.000
Locus-16	UBC#254-795bp	0.577	0.763	0.175	0.234	0.071	0.313	0.215	0.391	0.544	0.222	0.170	0.000
Locus-17	UBC#264-550bp	0.234	0.625	1.000	0.577	1.000	1.000	0.415	0.470	0.544	0.444	0.270	0.386

Table 2.4 Continued.

Locus	UBC code	1 BDVF	2 MHFL	3 HMCK	4 WYTH	5 STVF	6 CAPE	7 LCCK	8 ZIGZ	9 OLEC	10 STCK	11 QZCK	12 PKPL
Locus-18	UBC#264-600bp	1.000	0.763	1.000	0.577	1.000	0.370	0.328	0.321	0.544	0.599	0.386	0.236
Locus-19	UBC#264-700bp	0.577	0.164	0.398	0.329	0.329	0.313	0.148	0.562	0.398	0.324	0.236	0.202
Locus-20	UBC#264-850bp	0.441	0.687	1.000	0.441	1.000	0.120	0.056	0.046	0.398	0.324	0.202	0.053
Locus-21	UBC#264-950bp	0.329	0.481	0.175	0.000	0.000	0.433	0.463	0.172	0.398	0.599	0.656	0.307
Locus-22	UBC#264-1000bp	1.000	0.687	1.000	1.000	1.000	0.433	0.086	0.289	0.398	0.541	0.477	0.170
Locus-23	UBC#264-1100bp	0.234	0.687	0.175	1.000	1.000	0.211	0.517	0.321	0.398	0.161	0.588	0.270
Locus-24	UBC#264-1800bp	0.577	0.572	1.000	0.441	1.000	0.211	0.148	0.289	0.398	0.541	0.386	0.236
Locus-25	UBC#278-850bp	1.000	0.763	1.000	1.000	1.000	0.586	0.251	0.470	0.544	0.746	0.430	0.477
Locus-26	UBC#278-950bp	1.000	0.763	1.000	0.577	1.000	0.370	0.116	0.258	1.000	0.746	0.477	0.202
Locus-27	UBC#278-1100bp	0.441	0.763	0.398	0.234	1.000	0.504	0.181	0.120	1.000	0.401	0.202	0.270
Locus-28	UBC#278-1200bp	0.577	1.000	1.000	1.000	0.577	0.039	0.215	0.120	0.175	0.191	0.110	0.110
Locus-29	UBC#320-410bp	0.441	0.217	0.544	0.149	0.234	0.504	0.116	0.258	0.175	0.324	0.140	0.081
Locus-30	UBC#320-570bp	1.000	0.625	0.544	0.329	0.577	0.164	0.370	0.070	0.544	0.077	0.110	0.140
Locus-31	UBC#320-850bp	0.071	0.044	0.544	0.071	0.000	0.164	0.215	0.258	0.083	0.104	0.202	0.053
Locus-32	UBC#320-870bp	1.000	1.000	1.000	0.577	0.441	0.164	0.181	0.145	0.544	0.288	0.477	0.236
Locus-33	UBC#320-900bp	1.000	1.000	1.000	1.000	0.329	0.079	0.116	0.258	0.398	0.362	0.170	0.170
Locus-34	UBC#372-820bp	0.000	0.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 2.5 Exact tests of population differentiation for Larch Mountain salamander based upon 34 RAPD loci ($p < 0.05$ are considered significant).

Location	1 BDVF	2 MHFL	3 HMCK	4 WYTH	5 STVF	6 CAPE	7 LCCK	8 ZIGZ	9 OLEC	10 STCK	11 QZCK	12 PKPL
1. Bridal Veil Falls(OR)	-											
2. Multnomah Falls(OR)	0.542	-										
3. Herman Creek(OR)	0.009	<0.001	-									
4. Wyeth(OR)	<0.001	<0.001	1.000	-								
5. Starvation Falls(OR)	<0.001	<0.001	0.989	0.996	-							
6. Cape Horn(WA)	<0.001	<0.001	<0.001	<0.001	<0.001	-						
7. Lower Copper Ck(WA)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	-					
8. Zig Zag(WA)	<0.001	<0.001	<0.001	<0.001	<0.001	0.409	0.013	-				
9. Ole's Cave(WA)	<0.001	<0.001	<0.001	<0.001	<0.001	0.073	0.111	0.075	-			
10. Straight Creek(WA)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.185	-		
11. Quartz Creek(WA)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.014	<0.001	0.832	0.028	-	
12. Packwood Palisades(WA)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.008	-

The analysis of molecular variance (nested AMOVA) results agreed with those for multi-dimensional scaling. The partition of variance was 52.9% within populations ($\phi_{ST} = 0.47$, $p < 0.01$), 34.0% between groups (northern versus southern populations, $\phi_{CT} = 0.34$, $p < 0.01$), and 13.1% among populations within groups ($\phi_{CS} = 0.20$, $p < 0.01$, Table 2.6). Analyses for the northern group alone indicated that 13.2% of variation is contained among populations, contrasted to the southern populations, which indicated 43.2% of the variation was among populations. These results suggest that gene flow is reduced among southern populations compared to northern populations and is consistent with the mtDNA results, indicated a greater difference among southern haplotypes.

The AMOVA results were consistent with those obtained from multi-response permutation procedures using Jaccard's distances, which showed a significant amount of heterogeneity between northern and southern populations ($A = 0.10$, $p < 0.00$). Less heterogeneity was observed when all populations were considered independently ($A = 0.19$, $p < 0.00$, a lower agreement value indicates greater heterogeneity). Furthermore, examination of population differentiation using hierarchical analyses for different estimators (F_{ST}, G_{ST}, θ_W, Table 2.7) produced similar results. θ_p calculated with TFPGA was identical to Weir and Cockeram's (1984) θ_W derived from RAPDFST. The greatest population differentiation occurred among southern populations, followed by differentiation among all populations, with the least amount of differentiation observed among northern populations.

Population relationships inferred with the neighbor-joining method revealed two distinct clades (Figure 2.4). Northern and southern populations each

represent monophyletic groupings, supported by high bootstrap values. Clustering of populations within the southern group was consistent with geography; however, the pattern is less clear among northern populations.

Figure 2.3. Non-metric multidimensional scaling of individual Larch Mountain salamanders (N = 184) using 34 variable RAPD loci. The plot shows the two most significant axes derived using Jaccard's distance. Solid symbols represent individuals found south of the Columbia River, while open symbols are from individuals sampled north of the Columbia River (■ Bridal Veil Falls, ● Multnomah Falls, □Herman Creek, □Wyeth , ◆ Starvation Falls, O Cape Horn, □ Lower Copper Creek,△ Zig Zag, ▽ Ole's Cave,◇ Straight Creek, X Quartz Creek, + Packwood Palisades).

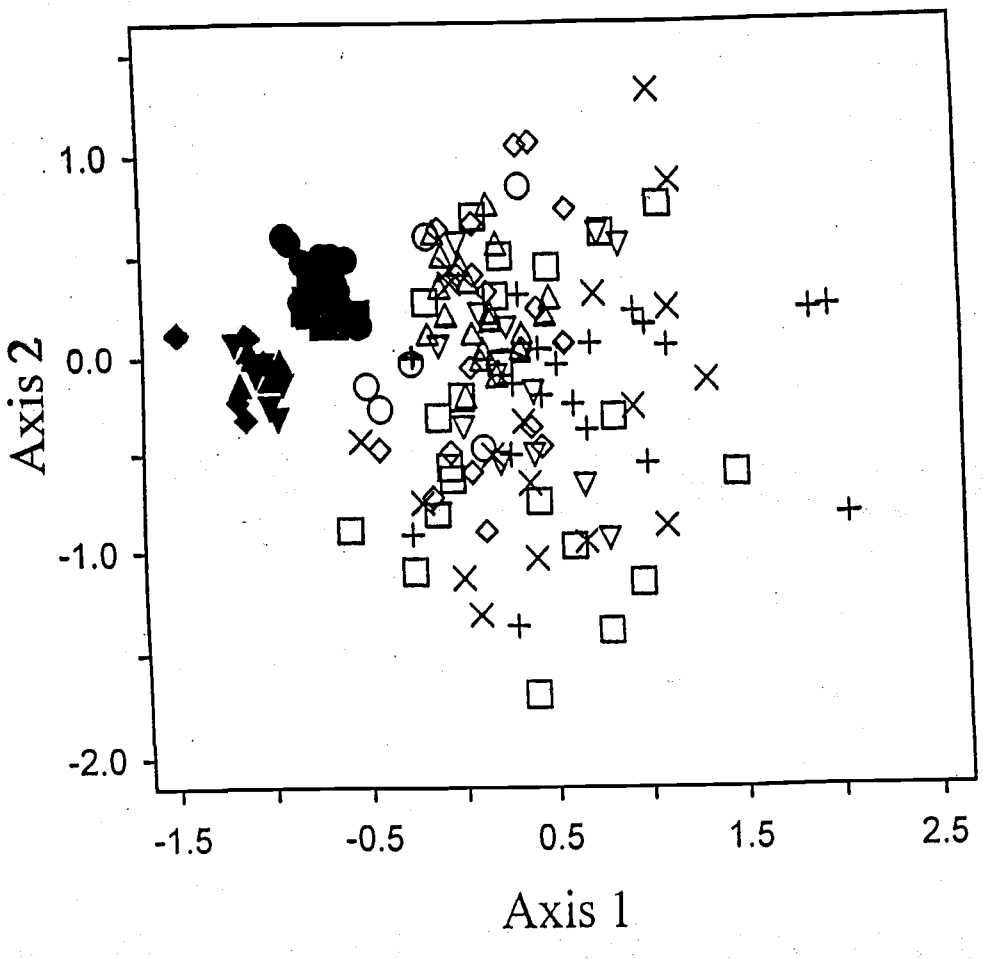

Figure 2.3

Table 2.6 Analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) to estimate genetic variation within and among populations, and groups of Larch Mountain salamanders using variable RAPD markers. Tests of significance (p-value) for variance component statistics (Φ) were calculated using 100 permutations.

Variance component	df	%var	Φ	p
Nested Analysis				
Among groups	1	34.03%	Φ_{CT} = 0.34	p < 0.01
Among populations within groups	10	13.05%	Φ_{SC} = 0.20	
Within populations	154	52.92%	Φ_{ST} = 0.47	
Northern Group				
Among populations	6	13.22%	Φ_{ST} = 0.13	p < 0.01
Within populations	115	86.78%		
Southern Group				
Among populations	4	43.18%	Φ_{ST} = 0.43	p < 0.01
Within populations	49	56.82%		
Pooled				
Among populations	11	35.37%	Φ_{ST} = 0.35	p < 0.01
Within populations	154	64.63%		

Table 2.7 Population subdivision (F_{ST}, θ_W, and G_{ST}) and gene flow (Nm) estimates among larch mountain salamanders based on 34 variable RAPD markers.

	Wright (1931)		Lynch & Milligan (1994)		Weir & Cockerham (1984)		Nei (1973)	
Groupings	$F_{ST} \pm$ SE	Nm^1	$F_{ST} \pm$ SE	Nm^1	$\theta_W \pm$ SE	Nm^1	$G_{ST} \pm$ SE	Nm^2
Northern only	0.21 ± 0.08	0.9	0.15 ± 0.02	1.5	0.12 ± 0.02	1.9	0.16 ± 0.02	2.7
Southern only	0.40 ± 0.09	0.4	0.51 ± 0.09	0.2	0.33 ± 0.07	0.3	0.41 ± 0.11	0.7
Southern vs. Northern	0.19 ± 0.07	1.1	0.36 ± 0.08	0.4	0.37 ± 0.07	0.4	0.26 ± 0.04	1.4
All Populations	0.39 ± 0.08	0.4	0.45 ± 0.07	0.3	0.39 ± 0.05	0.4	0.26 ± 0.05	1.4

[1]Nm = (1- F_{ST})/4 F_{ST}
[2]Nm = 0.5 (1 – G_{ST})/ G_{ST}

Mantel tests supported the inferred phylogenetic relationships. There was little correlation between genetic (Manhattan) and geographic distance when analyzed for all populations (Mantel $R^2 = 0.12$, $p = 0.20$) and northern populations (Mantel $R^2 = 0.18$, $p = 0.15$). In contrast, significant correlation of genetic and geographic distance among southern populations (Mantel $R^2 = 0.88$, $p = 0.01$) was observed. The distances derived from RAPD loci varied considerably from 0.11 to 0.57 among all populations, 0.11 to 0.24 for northern populations, and 0.16 to 0.33 for southern populations (Table 2.8). Furthermore, these distances are significantly correlated with distances derived from mtDNA haplotypes (Mantel $R^2 = 0.67$, $p = 0.01$).

Genetic diversity estimates within populations show considerable variation (Table 2.9). Observed number of alleles A, effective number of alleles A_E, number of polymorphic loci P, and expected heterozygosity H_e are significantly reduced for southern populations compared to northern populations (H_e; Mann-Whitney $Z = -2.8$, $p = 0.003$). This might be a consequence of southern populations showing fixation (presence or absence) of 1.94 times the average number of alleles per population than northern populations.

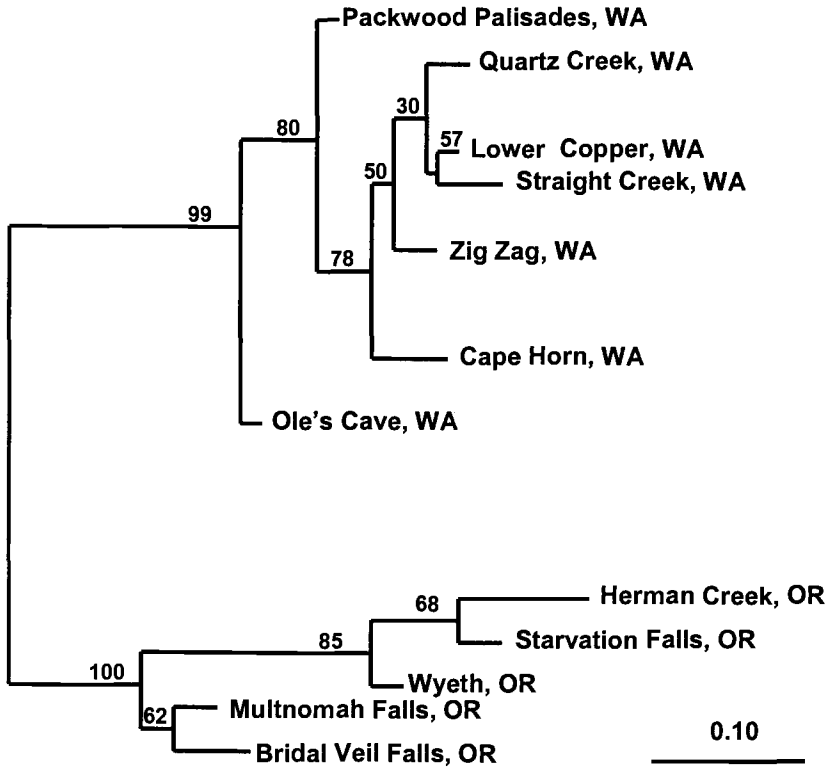

Figure 2.4. Neighbor-joining phylogenetic tree based on Manhattan distances using 34 variable RAPD loci for Larch Mountain salamander. Bootstrap values are indicated above the line.

Table 2.8 Manhattan distances (below diagonal) and geographic distances (Km, above diagonal) based upon 34 RAPD loci for Larch Mountain salamanders.

Location	1 BDVF	2 MHFL	3 HMCK	4 WYTH	5 STVF	6 CAPE	7 LCCK	8 ZIGZ	9 OLEC	10 STCK	11 QZCK	12 PKPL
1. Bridal Veil Falls(OR)	-	4.2	21.7	31.0	31.8	1.8	20.1	24.0	43.2	58.2	57.6	100.8
2. Multnomah Falls(OR)	0.175	-	18.0	21.0	27.6	4.8	19.8	22.8	42.0	55.8	54.9	97.2
3. Herman Creek(OR)	0.305	0.321	-	3.9	45.0	21.7	25.8	21.0	40.8	45.0	43.8	85.2
4. Wyeth(OR)	0.308	0.269	0.189	-	9.4	24.6	29.4	22.8	47.4	44.4	43.0	84.6
5. Starvation Falls(OR)	0.328	0.303	0.164	0.157	-	42.6	36.0	28.8	39.5	44.4	42.6	82.2
6. Cape Horn(WA)	0.398	0.404	0.518	0.460	0.479	-	18.0	22.8	42.6	56.4	56.4	99.6
7. Lower Copper Ck(WA)	0.463	0.417	0.568	0.437	0.501	0.179	-	9.0	24.0	42.6	43.2	84.0
8. Zig Zag(WA)	0.422	0.414	0.534	0.427	0.488	0.132	0.115	-	21.0	35.5	34.2	76.8
9. Ole's Cave(WA)	0.338	0.300	0.465	0.388	0.389	0.219	0.198	0.185	-	26.1	26.4	64.8
10. Straight Creek(WA)	0.312	0.312	0.458	0.391	0.424	0.144	0.203	0.175	0.166	-	3.6	42.6
11. Quartz Creek(WA)	0.392	0.356	0.528	0.402	0.455	0.172	0.114	0.126	0.163	0.133	-	43.2
12. Packwood Palisades, WA.	0.427	0.406	0.555	0.451	0.497	0.180	0.138	0.150	0.237	0.168	0.141	-

Table 2.9 Genetic diversity parameters (± SE) within populations of Larch Mountain salamanders based on 34 variable RAPD markers. n^1 is number of individuals analyzed. Expected heterozygosity within populations or groups is estimated using Lynch & Milligan's (1994) Taylor expansion corrected allele frequencies.

Location (n^1)	Observed alleles	Effective alleles	Polymorphic alleles	%Polymorphic alleles	Expected Heterozygosity
Southern Group					
1. Bridal Veil Falls(OR)(7)	1.50 ± 0.26	1.37 ± 0.18	17	50.00	0.20 ± 0.05
2. Multnomah Falls(OR)(23)	1.68 ± 0.22	1.38 ± 0.12	23	67.65	0.23 ± 0.04
3. Herman Creek(OR)(15)	1.29 ± 0.21	1.19 ± 0.12	10	29.41	0.11 ± 0.03
4. Wyeth(OR)(10)	1.44 ± 0.25	1.30 ± 0.15	15	44.12	0.17 ± 0.04
5. Starvation Falls(OR)(12)	1.32 ± 0.22	1.22 ± 0.13	11	32.35	0.12 ± 0.04
Southern Mean	1.45 ± 0.23	1.29 ± 0.14	15	44.71	0.17 ± 0.04
Northern Group					
6. Cape Horn(WA)(13)	1.82 ± 0.15	1.48 ± 0.13	28	82.35	0.28 ± 0.03
7. Lower Copper Ck(WA)(18)	1.82 ± 0.15	1.45 ± 0.11	28	82.35	0.27 ± 0.03
8. Zig Zag(WA)(22)	1.82 ± 0.15	1.50 ± 0.12	28	82.35	0.30 ± 0.03
9. Ole's Cave(WA)(6)	1.65 ± 0.23	1.48 ± 0.18	22	64.71	0.27 ± 0.05
10. Straight Creek(WA)(20)	1.74 ± 0.20	1.46 ± 0.13	25	73.53	0.27 ± 0.04
11. Quartz Creek(WA)(19)	1.76 ± 0.19	1.49 ± 0.13	26	76.47	0.29 ± 0.03
12. Packwood Palisades(WA)(19)	1.68 ± 0.23	1.36 ± 0.11	23	67.65	0.22 ± 0.03
Northern Mean	1.76 ± 0.19	1.46 ± 0.15	26	75.63	0.27 ± 0.03
Pooled	1.60 ± 0.21	1.37 ± 0.14	21	62.75	0.22 ± 0.04

Discussion

Narrow physiological tolerances, short dispersal ranges, and extreme site fidelity of amphibians in general and salamanders in particular, may limit dispersal among populations or into vacant suitable habitat (Blaustein et al., 1994). These life-history traits also suggest they may be highly susceptible to habitat fragmentation. Our results show considerable genetic differentiation among Larch Mountain salamander populations at local and regional scales, with both mtDNA and RAPD markers, which may be reflective of low dispersal abilities. Dispersal and movement of terrestrial salamanders has not been well studied. Among five studies of western plethodontid salamander home range and movement, the distance individuals moved ranged from 1.7m (*Batrachoseps attenuatus*; Hendrickson, 1954) to 23m (*Ensatina eschscholtzii*; Stebbins, 1954), with a mean distance of 2.5m (*Plethodon vehiculum*; Hendrickson, 1954; Stebbins, 1954; Barbour, 1969; Barthalmus, 1972; Ovaska, 1988). Given the probable low dispersal rate of terrestrial salamanders, many natural and anthropogenic factors may contribute to population differentiation and fragmentation.

Phylogeographic Structure

MtDNA and RAPD markers have different inheritance patterns (uni-parental vs. bi-parental) and mutation rates which can influence their rate of fixation or loss in a population. Our mtDNA results show northern haplotypes nested within the southern haplotypes suggesting a more recent radiation of northern populations compared to southern populations. The southern region may represent their relict

distribution with an expansion northward during the early pleistocene with the retreat of glaciers. The pattern of population relationships indicated by the RAPD data suggests two major groups (northern and southern) are defined by the Columbia River.

The impact of phylogeographic barriers on population differentiation and fragmentation can be significant (Avise, 1994). The long-term consequences of isolation can lead to differentiation through random drift and differential selection (Mayr 1954). Our data suggests the Columbia River may acts as a barrier for gene flow.

The efficacy of rivers as effective barriers to the dispersal of terrestrial plethodontid salamanders has been questioned (Highton, 1972). However, the Columbia River appears, based upon distributional data, to be a barrier for a number of terrestrial salamanders including the Oregon Slender Salamander (*Batrachoseps wrighti*) and Clouded Salamander (*Aneides ferreus*), for whom the river appears to be the northern boundary of their range (Corkran and Thoms, 1996). Morphological differences have been suggested between northern and southern populations based on variation in number of vomerine teeth and melanophore pigmentation observed among (Brodie, 1970) Larch Mountain salamanders. Howard et al. (1983) suggested that populations of Larch Mountain salamanders from each side of the Columbia Gorge were relatively recently diverged (between 4,000-43,000 years ago) based upon low allozyme divergence of two pairs (four populations) of populations located directly

across the river from one another. They found this result surprising because the river has existed in its present location since the Miocene epoch, and may have presented a barrier to dispersal for millions of years. Our results suggest a longer divergence time for populations separated by the river.

Estimated divergence rates for vertebrate mtDNA cytochrome *b* sequences vary typically from 1-3% per million years (Hasegawa et al., 1985; Irwin et al., 1991). Although molecular clock estimates tend to be inexact without calibration (Moritz et al., 1987), they can be important for relative comparisons. Based on this rate the Bridal Veil Falls and Cape Horn haplotypes, from populations located directly across the Columbia River from one another, are estimated to have a divergence time ranging from 0.63- 1.9 million years ago. The divergence between Cape Horn and Starvation Falls, 1.2-3.5 million years ago, is greater. Cape Horn clearly phylogenetically clusters with the rest of the northern populations. Evidence for the northern grouping of the Cape Horn population is provided by presence of northern-specific RAPD loci and a northern-specific mtDNA haplotype for Cape Horn. However, because of our limited sampling on the northern slope within the Columbia River Gorge, the phylogenetic relationship and population structure of northeastern (within Gorge) populations is uncertain.

Population Structure

Herrington and Larsen (1985) described Larch Mountain salamander populations found within the Columbia River Gorge as small, isolated and restricted to specific habitats. They suggest that populations are 'relict' and on the decline; however, discovery of Larch Mountain salamanders further north and in other habitats (Aubry et al., 1987; D. Darda and Darda-Garvey, 1995; Crisafulli, 1999a,b) suggests a re-visiting of these conclusions. Our mtDNA and RAPD results suggest considerable fine-scale population structure. The observed population structure for Larch Mountain salamanders is consistent with the hypothesis that these salamanders are patchily distributed across the landscape, perhaps as a consequence of combined influences of habitat specificity, isolation factors (geographic barriers) resulting from natural disturbances (volcanism, catastrophic wildfire or flooding) and limited dispersal rates.

Based upon RAPD markers, gene flow within and between northern and southern populations appears to be on the lower end of the range considered necessary to offset possible effects of random genetic drift for small populations (Table 2.7). Although estimates of population subdivision (F_{ST}) and subsequently inferred gene flow estimates (Nm) have been criticized for not reflecting current vs. historic processes of random drift, mutation, natural selection or gene flow model (e.g., island model; Wright, 1969; Slatkin, 1994; Templeton et al, 1995; Hedrick, 1999). The relative estimates are nonetheless informative. Traditionally, the migration of one individual per generation was considered adequate to offset the negative effects of

random drift (Wright 1931). However, Mills and Allendorf (1996) suggested one migrant per generation was a minimal value and that up to ten migrants per generation may be needed in some populations to counteract the loss of alleles due to drift.

Low migration combined with reductions in effective population size can cause loss of rare alleles and fixation of common alleles due to inbreeding (Nei, 1975). Southern populations of Larch Mountain salamander exhibit reduced heterozygosity, a lower number of polymorphic alleles and are fixed for a greater number of alleles compared to northern populations. This pattern is possibly the result of a historic reduction in effective population size or a population bottleneck. A catastrophic event (e.g., flooding, fire, volcanic eruption, etc.) or loss of suitable habitat may have decreased the effective population size and led to increased inbreeding.

In an allozyme study of four populations within the Columbia River Gorge, Howard et al. (1983) suggested a similar pattern of significant population differentiation (G_{ST}= 0.25), reduced gene flow among populations and reduced within population heterozygosity (0.0017-0.019). Larch Mountain salamander heterozygosity values were substantially lower than those observed from 13 other salamander species, which averaged 0.079 (Nevo, 1978). RAPD studies for terrestrial salamanders have been limited, but average expected heterozygosity ($H_E = 0.22 \pm 0.04$) for Larch Mountain salamanders is lower but not significantly different than the expected heterozygosity ($H_E = 0.28 \pm 0.03$) for populations of Oregon Slender Salamander (*Batrachoseps wrighti*; Wagner and Haig; in review).

All populations of Larch Mountain salamanders appear to be highly genetically structured. Southern populations in addition to exhibiting reduced heterozygosity are highly structured within the Columbia River Gorge showing a correlation of geographic distance with genetic distance. This structure is evidenced by both a high variance in RAPD markers and a significant amount of mtDNA haplotype divergence among populations. Among the southern group, populations show reduced gene flow compared to northern populations. In contrast, northern populations do not show a correlation of geographic distance with genetic distance. This may have resulted from northern populations expanding rapidly, compared to southern populations, as evidenced by the lack of geographic structure among the RAPD data and lower amount of sequence divergence among northern haplotypes. These results suggest a differential influence of factors contributing to population structure and dispersal among these populations; for example, by the influence of habitat availability or geographic barriers affecting dispersal within the northern and southern groups.

Conservation-unit designations

Based on the operational definition of conservation units proposed by Moritz (1994a,b; 1995), we suggest northern and southern populations as demarcated by the Columbia River warrant separate conservation-unit designations. Reciprocal

monophyly for northern and southern groups is not supported by the mtDNA results. However, the RAPD loci show significant differentiation among northern and southern groups and include several loci specific for northern and southern populations.

Based on use of an alternative operational ESU definition (e.g., Vogler and Desalle, 1994), an argument could be made for designation of these groups as separate ESUs. However, we prefer to take a conservative approach to designation of ESUs (Haig et al., in review; Wagner and Haig, in prep) by the strict use of Moritz's criteria for reciprocal monophyly of mtDNA. Therefore, we suggest designation of separate Management Units for the northern and southern geographic areas we sampled.

Conservation implications

Designation of conservation units for Larch Mountain salamanders can significantly influence their conservation status. Northern and southern populations may face differential threats to their persistence, and designation of separate Management Units provides flexibility in prioritizing specific populations for conservation efforts. For example, both groups show significant differentiation and limited gene flow among populations but may have habitat destruction (i.e. timber harvest) and fragmentation threats that could serve to further isolate these populations.

In addition to the effects of fragmentation, a number of factors related to the reproductive biology of the Larch Mountain salamander could influence species viability. For example, their reproductive rate may be low because females reach

sexual maturity only after 4 years of age and appear to have a biennial ovarian cycle with an average clutch size of 7.33 (Herrington and Larsen, 1987). Variance in hatching success, juvenile survival, and adult survival is unknown; however, it is expected to be low. Furthermore, is it thought the amount of suitable habitat is limited, particularly within the Columbia River Gorge (Herrington and Larsen, 1985). These factors combined with increasing fragmentation or habitat destruction could affect persistence of these populations.

While it appears all populations of Larch Mountain salamanders are significantly fragmented, specific additional concern for southern populations may be warranted. The extent of population subdivision along with their lower heterozygosity could particularly influence their viability. A separate conservation unit designation for southern populations could fulfill the "distinct population segment" criteria for federal listing under the Endangered Species Act (Waples 1991). However, populations from both sides of the river continue to have threats from housing development and recreational activities as suggested by Herrington and Larsen (1985). In addition, northern populations outside of the Columbia River Gorge face threats related to timber harvest practices. Because populations of Larch Mountain salamander will most likely continue to be fragmented with loss of western forest habitat and rural development, designation of conservation units and prioritizing of conservation efforts may benefit species viability, and serve to protect the genetic component of biodiversity.

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CHAPTER 3

PHYLOGEOGRAPHY, GENETIC STRUCTURE AND CONSERVATION IN THE FOREST-ASSOCIATED OREGON SLENDER SALAMANDER *(Batrachoseps wrighti).*

R. Steven Wagner and Susan M. Haig

Abstract

We studied phylogeography and genetic structure of the Oregon slender salamander (*Batrachoseps wrighti*) in order to assess the impact of historic versus current fragmentation processes. Endemic to Oregon in the northwestern U.S., the Oregon slender salamander is a completely terrestrial plethodontid found mainly associated with coarse woody debris in mature forests. Subsequently, alteration of their habitat by forest management practices may impact their persistence. Therefore, as a first step to infer possible affects of these practices on population structure and differentiation, we used mitochondrial DNA sequences (cytochrome *b*) and RAPD markers to analyze 22 populations across their range. Phylogenetic inferences, based on sequence data (774 bp), using three distinct methods indicated two historical lineages, northern and southern, are contained within Oregon slender salamander. Relationships among haplotypes suggest the northern region may have more recently been colonized compared to the southern region. Neighbor-joining phylogenetic analyses based upon RAPD markers (46 loci) confirm divergence of northern and southern populations and are supported by non-metric multidimensional scaling. In addition, these analyses further suggest differentiation of mid-range populations. Analyses of pairwise-F_{ST} estimates versus geographic distances suggest genetic drift may contribute more to population structure compared to gene flow. Finally, using Moritz's criteria (1994a,b) for conservation units we propose designation of three overlapping Management Units corresponding to *northern-most*, *mid-range* and *southern-most* populations.

Introduction

The dynamics of population divergence and reticulation can be revealed through phylogeographic studies (Avise *et al.* 1987; Avise 1994). These studies focus on relationships among populations and provide information concerning historic patterns of diversity, and often identify geographic features as the prime source of genetic structuring (Lamb *et al.* 1989; Avise 1992; Phillips 1994; Routman *et al.* 1994; Phillips *et al.* 2000). In the Pacific Northwest region of the United States, geographic barriers are provided by a complex history of glaciation, flooding, and volcanism that fragment Douglas fir (*Pseudotsuga menziesii*) dominant forest communities on the western slopes of the Cascade Range.

Pacific Northwest forests are further fragmented by forest management practices (i.e., timber harvesting) and rural development. Mature forest-associate species with limited dispersal capabilities may be impacted by this increased fragmentation. As a first step towards understanding fragmentation in the context of historic processes, we investigated geographic variation and population genetic structure in the mature forest-associated Oregon slender salamander (*Batrachoseps wrighti*).

Endemic to the western slopes of the Oregon Cascades, the Oregon slender salamander is a species of concern with respect to the Northwest Forest Management Plan (U.S. Forest Service & U.S. Bureau of Land Management 1994). Further, they are classified as "sensitive" in Oregon (Oregon Department of Fish &

Wildlife 1997). Characterized by a completely terrestrial life history, they are mostly associated with moist woody debris, older decay classes of logs, and occasionally found in talus slopes (Nussbaum *et. al.* 1983; Bury & Corn 1988; Gilbert & Allwine 1991; Vesely *et al.,* submitted). The Oregon slender salamander can be locally abundant in mature forests; however, the species is rare in second growth or clearcuts (Bury & Corn 1988; Gilbert & Allwine 1991; Vesely *et al.,* submitted). Consequently, forest management practices may potentially lead to local extirpation and could affect overall viability of the species (Marshall *et al.* 1992; Vesely *et al.,* submitted).

Many aspects of Oregon slender salamander life history may influence their susceptibility to habitat fragmentation and resulting persistence. These traits include low reproductive rate (clutch size averages 6.3 eggs, clutch frequency and survivorship is unknown; Tanner 1953) and low rates of dispersal. There have been few studies of dispersal, movement, and home range size in terrestrial salamanders (*Genus Batrachoseps*, summarized in Stebbins & Cohen 1995). For example, the home range of a congener, the California slender salamander (*B. attenuates*), was observed to have a diameter 1.7 m (Hendrickson 1954). Thus, home range size and dispersal is thought to be limited in Oregon slender salamander.

In order to provide guidance for the management Oregon slender salamander, we evaluated population differentiation and structure in the context of conservation units. A number of conflicting definitions for conservation units have

been proposed for prioritizing conservation efforts (Ryder 1986; Waples 1991; Dizon *et al.* 1992; Moritz 1994 a,b; Vogler & Desalle 1994; Bowen 1998, Crandall *et al.* 2000). However, the most frequently applied concept is the operational definition of Moritz (1994a,b; Moritz *et al.* 1995) which defines an Evolutionary Significant Unit (ESU) as requiring reciprocal monophyly of mitochondrial DNA (mtDNA) alleles *and* significant divergence at nuclear alleles. Subunits of ESUs, Management Units (MU) are diagnosed based upon significant divergence of mtDNA alleles *or* nuclear alleles. ESUs are defined to reflect long-term reproductive isolation and MUs are for short-term or demographic isolation.

To examine the phylogeographic divergence, population structure and diagnose conservation units, we used two molecular markers: mtDNA cytochrome *b* sequence data and random amplified polymorphic DNA (RAPD) data. Cytochrome *b* sequences have proven to be a useful metric to infer intra-specific phylogeny in many salamander species (e.g., Moritz *et al.* 1992; Tan & Wake 1995; Jockusch 1996; Jackman *et al.* 1997; Alexandrino *et al.* 2000) and in a number of other taxa to define conservation units (Walker *et al.* 1998, Doukakis *et al.* 1999, Wood & Raley 2000). The RAPD technique is a simple and cost effective procedure to sample large numbers of segregating nuclear. Increasingly, RAPDs are used in vertebrate conservation studies to investigate population structure (e.g., Kimberling *et al.* 1996; Prior *et al.* 1997; Cooper 2000; Haig *et al.*, submitted; Wagner *et al.*, submitted; Chapter 2).

Materials and methods

Tissue sampling and DNA isolation

Oregon slender salamanders (n = 339) were sampled from 22 localities throughout their known range (Figure 3.1, Table 3.1). Salamanders were hand-captured and sample tissue was taken by non-lethal tail clipping (approximately 1 cm), using a different sterile surgical scissors for each individual. After sampling, animals were released promptly at the exact site they were captured. Sample tissue was placed immediately in a cryogenic tube containing buffer solution (100 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 10 mM NaCl, 0.5% SDS) until transferred to an ultracold freezer (-80 °C).

Figure 3.1 Sampling locations of Oregon slender salamanders. See Table 3.1 for location identification. Locator map shows the putative range of the Oregon slender salamanders.

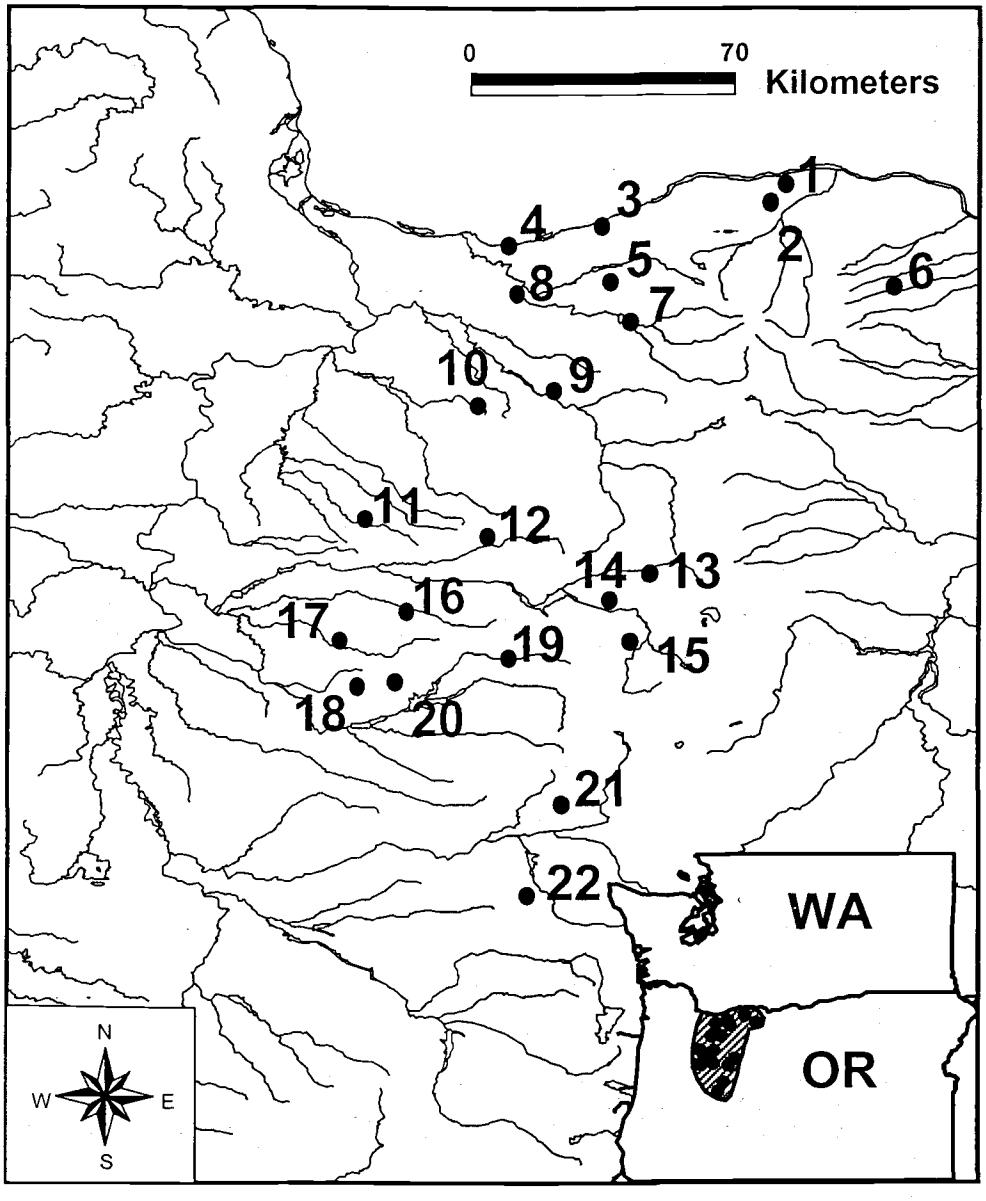
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Figure 3.1

Table 3.1 Locations and abbreviations for Oregon slender salamander populations sampled. *M* and *R* are the number of individuals analyzed for mitochondrial haplotype and RAPD loci, respectively. See Figure 3.1 for map locations.

Population	*M/R*	Code	Location Long,Lat	County
1. Post Canyon	3/16	PCRD	-121.616,45.6713	Hood River, OR
2. Viento State Park	3/0	VWBW	-121.657,45.6278	Hood River, OR
3. Ainsworth State Park	3/0	AWBW	-122.380,45.5480	Multnomah, OR
4. Train Tunnel	3/7	TRTN	-122.280,45.5380	Multnomah, OR
5. Bull Run	3/17	BULL	-122.034,45.4543	Multnomah, OR
6. East Mt. Hood	3/15	EMTH	-121.368,45.4252	Multnomah, OR
7. Wildwood	3/16	WILD	-121.993,45.3536	Wasco, OR
8. N. Eagle Creek	3/13	NECK	-122.264,45.4223	Clackamas, OR
9. Estacada	3/19	ESTC	-122.276,45.6380	Clackamas, OR
10. Jackson Five	3/26	JACK	-122.347,45.1617	Clackamas, OR
11. Silver Creek Falls	3/9	SLCF	-122.630,44.8899	Marion, OR
12. Detroit Lake	3/13	DELK	-122.275,44.5676	Marion, OR
13. Little Santiam	3/19	LISR	-122.329,44.8499	Marion, OR
14. Breitenbush	3/0	BTNB	-121.941,44.7604	Marion, OR
15. Bugaboo	3/10	BUGB	-121.981,44.6040	Linn, OR
16. Thomas Creek	3/26	TMCK	-122.532,44.6834	Linn, OR
17. Church Creek	3/15	CHCK	-122.696,44.6100	Linn, OR
18. Keel Over	3/28	KEOV	-122.651,44.5137	Linn, OR
19. Quartzville	3/20	QVCK	-122.275,44.5676	Linn, OR
20. Withycomb	3/21	WITH	-122.555,44.5187	Linn, OR
21. H.J. Andrews	3/21	HJAN	-122.155,44.2322	Lane, OR
22. Hidden Lake	3/28	HDLK	-122.235,44.0144	Lane, OR

DNA was isolated using a modified phenol/chloroform extraction procedure (Sambrook *et al.* 1989) and collected over a microcon-50 filter (Millipore). First, 2 µg of tissue was digested in 400 µl of extraction buffer (100 mM Tris-HCl pH 7.5, 100 mM EDTA, 250 mM NaCl, Proteinase K 600 µg/ml) overnight at 55 °C. Each sample was extracted twice using equal volumes of phenol equilibrated with Tris-HCl buffer (pH 7.5), followed by two chloroform/isoamyl alcohol (25:1) extractions. Finally, the aqueous layer was placed in a microcon-50 filter (Millipore), washed twice with 400 µl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), centrifuged again for 5 min. (14,000 x g), then inverted and centrifuged for 30 s to elute the final DNA solution. Extraction quality was checked using agarose gel electrophoresis. The concentration for each sample was estimated by fluorimetry (Hoefer TKO 100).

MtDNA amplification and analyses

The polymerase chain reaction (PCR) was used to amplify a 774 bp fragment of the cytochrome *b* gene, using the following primers designed for vertebrates: MVZ15 (5'-GAACTAATGGCCCACAC(A/T)(A/T)TACGNAA-3') and MVZ16 (5'-AAATAGGAAATATCATTCTGGTTTAAT-3', Kocher *et al.* 1989). Each reaction was carried out using the following concentrations (50 µl): 0.5 units of Taq Gold (Perkin Elmer) with the supplied reaction buffer (5 µl); 100 µM for each of dATP, dCTP, dGTP, dTTP; 2 mM MgCl and 1 mM of each primer.

Fragments were amplified using a MJ Research programmable thermocycler (PTC 100) with the following parameters: an initial denaturation at 93 °C (10 min.), followed by 40 cycles of denaturation at 93 °C (1 min.), annealing at 52 °C (1 min.) and extending at 72 °C (2 min.). Following a final extension at 72 °C (10 min.), reactions were held at 4 °C until removed from the cycler. Fragments were extracted from a 1 % agarose gel using an ultra-free-mc 0.45 filter (Millipore) from which the supernatant was transferred to microcon-50 filter (Millipore). Automated sequencing was performed at Oregon State University Central Services Laboratory with an Applied Biosystems (373A) sequencer. Sequencing primers included MVZ-15, MVZ-16 and cytb2 (5'-AAACTGCAGCCCCTCA-GAATGATATTTGTCCTCA-3', Moritz *et al.* 1992). Sequences from fragments were aligned by eye using the Genetic Data Environment (Smith *et al.* 1992) and compared to a GenBank archived cytochrome *b* sequence of Oregon slender salamander (U89625; Jackman *et al.* 1997).

Three distinct methods of phylogenetic inference were used to examine relationships among cytochrome *b* haplotypes that included: distance (minimum evolution), maximum parsimony, and maximum likelihood methods (PAUP* 4.0b1; Swofford 1998). A comparison of the merits of each method has been discussed previously (Hasegawa & Fujiwara 1993; Huelsenbeck & Hillis 1993; Kuhner & Felsenstein 1994; Tateno *et al.* 1994; Gaut & Lewis 1995); however, concordance of tree topologies inferred from different methods is expected to be more reflective of true phylogenetic relationships (Kim 1993). Distance (minimum

evolution) trees were calculated using the Kimura 2-parameter model (Kimura 1980) and an empirically derived transition:transversion ratio. Maximum parsimony was used to search for trees of shortest length, trees were evaluated using a heuristic search and the tree bisection-reconnection algorithm. Maximum likelihood reconstructions accounted for rate heterogeneity among codon positions using a 0.5 gamma distribution, an empirically derived transition:transversion ratio, and the Hasegawa-Kishino-Yano substitution model (Hasegawa *et al.* 1985). For each method, a consensus bootstrap tree (100 replicates) was used to assess reliability of support for each node (Felsenstein 1985). A cytochrome b sequence from the Inyo Mountain salamander (*B. campi;* GenBank accession U89626; Jackman *et al.* 1997), one of the closest extant phylogenetic relatives of the Oregon slender salamander (Yanev 1978; Marlow *et al.* 1979; Jockusch 1996), was used as an outgroup in each tree.

RAPD procedure and analyses

RAPD profiles were generated using a polymerase chain reaction protocol as described in Aagaard *et al.* (1995). PCR reactions were setup using the following concentrations (25 µl volume): 10X buffer (50 mM KCl; 10 mM Tris-HCl pH 9.0; 0.1 % Triton X-100); 1.8 mM $MgCl_2$; 100 µM for each of dATP, dCTP, dGTP, dTTP; 0.2 µM primer; 2 ng template DNA; and 1 unit of Taq Polymerase (Promega). Reactions were run using a MJ Research thermal cycler (PTC-100) with the following parameters: 1 cycle at 93 °C (3 min.) followed by 45

cycles of denaturation at 93 °C (1 min.), annealing at 45 °C (1 min.), and elongation at 72 °C (2 min.). A final elongation at 72 °C (10 min.) completed the reaction, which was held at a constant 4 °C until removed from the cycler. Fifteen µl of each reaction was loaded in a 2.0 % agarose gel (GibcoBRL; Ultrapure) and electrophoresed for 4 hours (100 V) in TBE (90 mM Tris base, 90 mM Boric acid, 2 mM EDTA, pH 8.0). Amplification products were sized using a 1 Kb DNA ladder (GibcoBRL). Gels were stained with ethidium bromide (1 µg/ml) for 30 min. and destained for 2 hours in de-ionized H_2O.

RAPD profiles were assessed for variable bands by preliminary screening of 235 primers (from the Oligonucleotide Synthesis Laboratory, University of British Columbia) utilizing two individuals from four populations (Post Canyon Road, Wildwood, Quartzville, Hidden Lake). Only distinct, well separated, and reproducible bands were chosen for final analyses. Reproducibility was assessed in multiple RAPD runs and in side-by-side RAPD reactions. Negative controls were run with each reaction to check for contamination products.

RAPDs, a dominant marker, were analyzed directly as phenotypes. Homozygous dominant (presence/presence) and heterozygous (presence/absence) individuals are indistinguishable because of the presence of a dominant band, so they were both scored as a (1) phenotype, while null allele individuals (absence/absence) were scored as a (0) phenotype. Each locus was assumed to be non-allelic and in Hardy-Weinberg equilibrium.

Dominance can cause bias in the estimation of null allele frequency and subsequent population genetic parameters (Lynch & Milligan 1994; Zhivotovsky 1999). Therefore, the Lynch & Milligan (1994) Taylor expansion correction was used to estimate allele frequencies and expected heterozygosity (H_e; Nei 1978) with the program TFPGA (Miller 1998b). Estimates of genetic diversity parameters were obtained from POPGENE (Yeh *et al.* 1997) which included: mean (A) and effective (A_e) number of alleles per locus, number of polymorphic loci (P), and percentage of polymorphic loci (P_e, 95% criteria). Genetic diversity parameters were compared using non-parametric Mann-Whitney Rank Sum tests (Wilcoxon 1945; Mann & Whitney 1947).

Exact tests (Raymond & Rousset 1995) were performed to analyze each scored locus for population differentiation and also for pairwise population comparisons of differentiation using TFPGA (Miller 1998b). Significant ($p < 0.01$) loci were identified using 2000 permutations. Estimates of population subdivision were obtained by the program RAPDFST (Black 1996) using the following statistics: Wright's F_{ST} (1931), Weir & Cockerham's θ_W (1984), and Lynch & Milligan's F_{ST} (1994). Analysis of molecular variance (AMOVA) was used to describe subdivision of genetic variation within and among populations, and between groups. Input files were generated using AMOVA-PREP (Miller 1998a) for the analysis program WINAMOVA (Excoffier *et al.* 1992; Excoffier 1993), which was used to calculate variance components and Φ_{ST}, the F-statistics analog.

Traditional population subdivision (F_{ST} estimates) and inferred gene flow estimates applied to natural populations often violate the assumptions of equilibrium in gene flow and genetic drift upon which the "island model" of gene flow is based. Furthermore, it is difficult to assess the influences of gene flow and genetic drift on population structure because they are confounded in the product *Nm* when utilizing the equation $F_{ST} \cong 1/(4Nm + 1)$ (Wright 1931). However, we used the approach of Hutchison & Templeton (1999) to assess the relative influences of gene flow and genetic drift on population structure, by correlation analyses of pairwise-F_{ST} values and geographic distances.

Pairwise-F_{ST} values were calculated using RAPDFST (Black 1996) for northern populations, southern populations, and among all populations and plotted against pairwise geographic distances. To assess if scatter increased with geographic distance, residuals obtained from simple linear regression of pairwise-F_{ST} values versus geographic distances were plotted against geographic distances. Mantel (1967) tests using NTSYS-PC (Rohlf 1994) were used to estimate correlation coefficients between the pairwise F_{ST} matrices, residual F_{ST} matrices, and pairwise geographic distances. The resulting *r*-values, normalized Mantel Z statistics, were interpreted as correlation coefficients and examined for significance by permutation procedures (100 permutations; Smouse *et al.* 1986).

Quantitative non-parametric assessments by multi-response permutation procedures (MRPP) were used to compare heterogeneity among populations using PC-ORD (version 4.28 beta; McCune & Mefford 1999). Within population heterogeneity was compared to that expected by chance, using Jaccard's distances (Jaccard 1908), and evaluated as chance corrected within-group agreement values (A-values; Mielke 1984). Jaccard's distance is useful for two-state data (+/-), calculated by $F = M_{xy} /(M_t-M_{xyo})$ where M_{xy} is the number of shared fragments between individuals, M_{xyo} is the number not shared, and M_t is the total number of bands scored. Genetic distance was calculated as 1-F.

Non-metric multidimensional scaling was used as a qualitative comparison of relationships among populations with Jaccard's distances using PC-ORD (Kruskal 1964a,b; Mather 1976). To assess the number of dimensions most appropriate to explain the variation, overall stress (opposite of goodness-of-fit) was plotted versus an increasing number of dimensions. Further, to determine the minimum number of iterations needed to reach a stable solution for the data, stress was plotted versus iteration number. Finally, each variable locus was evaluated for significant correlation with respect to final ordination axes by Kendall correlation.

Neighbor-joining trees, using RAPD phenotypes, were constructed to evaluate phylogenetic relationships (Saitou & Nei 1987). RAPDDIST (Black 1996) was used to calculate Manhattan distances (Prevosti distance in Wright 1978) and bootstrap matrices (100 replications) among populations. A final consensus tree was constructed by analyzing the matrices with the NEIGHBOR and CONSENSE options in PHYLIP v 3.5C (Felsenstein 1993).

Results

MtDNA sequence analyses

Cytochrome *b* sequence analyses indicated significant differentiation among 22 populations (N = 69, Table 3.2). Sequences (774 base pairs) were characterized by 44 variable sites, with pairwise sequence differences (uncorrected) ranging from 0.0 to 4.01 % (Table 3.3). Seventeen distinct haplotypes were found, with identical haplotypes occurring among five northern-most populations (Train Tunnel, Ainsworth State Park, Viento State Park, Bull Run, Wildwood) and two southern populations (Keel Over, Quartzville). The only-within population variation found among haplotypes sequenced for each population occurred in the Thomas Creek

samples, characterized by a single synonymous substitution in the third codon position of sequence position 220. Therefore, haplotype diversity within populations appears to be trivial compared to among population haplotype diversity.

Table 3.2 Mitochondrial DNA sequence variation in 774 base pairs of the cytochrome b gene in Oregon slender salamanders. Only the 44 variable sites are shown, identified by three digits (above) corresponding to its sequence location (see Table 3.1 for site locations of 1-22).

	000000011111112222222333344445555556666777777
	011456914457790112568012733690355581138015666
Location	567024382512512470320705446935801706919438 25
Northern	
1. Post Canyon	TGAATCTTCCCCGATAATCCGGGCTGCTGGCAAGTAGCGTTCGG
2. Viento	.....G.....C.....C.
3. Ainsworth	.....G.....C.....C.
4. Train Tunnel	.....G.....C.....C.
5. Bull Run	.....G.....C.....C.
6. East Mt. Hood	.....G.....C.....
7. Wildwood	.....G.....C.....C.
8. N. Eagle Creek	.....T.G..G.....T.....C.....C.
9. Estacada	.....T.GC.....A..T.....C.....C.
10. Jackson Five	.....G.....A..T.....C.....C.
Southern	
11. Silver Creek	CAGTCTCC.TTT.GCCG.T..A.T.A.CAATGG.CGCT.CC.C.
12. Detroit Lake	CAGTCTC..TTT.GCCG.TT.A.TCA.CAATGG.CGCT.CC.C.
13. Little Santiam	CAGTCTC..TT..GCC..TT.A.TCA.CAATGG.CGCT.CC.C.
14. Breitenbush	CAGTCTC..TTT.GCCG.TT.A.T.A.CAATGG.CGCT.CC.C.
15. Bugaboo	CAGTCTC..TTT.GCCG.T..A.T.A.CAATGG.CGCT.CC.C.
16a.Thomas Creek	CAGTC.C..T.TCGCCGCT....T.A.CAATGGACGCTACCAC.
16b.Thomas Creek	CAGTC.C..T.TCGCCG.T....T.A.CAATGGACGCTACCAC.
17. Church Creek	CAGTC.C..TTT.GCCG.T....T...CA.TGGACGCTACCACT
18. Keel Over	CAGTCTCC.TTT.GCCG.T..A.T...CAATGGACGCTACC.C.
19. Quartzville	CAGTCTC..TTT.GCCG.T..A.T...CAATGGACGCTACC.C.
20. Withycomb	CAGTCTC..TTT.GCCG.T..A.T...CAATGGACGCTACC.C.
21. H.J. Andrews	CA.TC.C.TT.T.GCCGCT...AT..TC..TGG.C.CT.CC.C.
22. Hidden Lake	CA.TC.C..T.T.GCCGCT...AT..TC..TGG.C.CT.CC.C.
23. *P. campi*	N...CT.....G....T.AA.T.....T.G.NNNNNNNNN

Table 3.3 Kimura 2-parameter distances (below diagonal, distances multiplied by 100) and percentage of uncorrected sequence differences (above diagonal) based upon cytochrome *b* sequence (774 base pairs) for Oregon slender salamanders (see Table 3.1 for site identification).

		1 PCRD	2 VWBW	3 AWBW	4 TRTN	5 BULL	6 EMTH	7 WILD	8 NECK	9 ESTC	10 JACK	11 SLCF	12 DELK	13 LISR	14 BTNB	15 BUGB	16a TMCA	16b TMCB	17 CHCK	18 KEOV	19 QVCK	20 WYTH	21 HJAN	22 HDLK	23 BACA
1.	PostCRd	-	0.39	0.39	0.39	0.39	.004	0.39	0.78	0.90	0.65	4.13	4.26	4.01	4.13	4.01	4.26	4.13	4.39	4.26	4.13	4.13	3.62	3.49	9.01
2.	Viento	0.39	-	0.00	0.00	0.00	0.00	0.00	0.39	0.52	0.26	3.75	3.88	3.62	3.75	3.62	3.88	3.75	4.01	3.88	3.75	3.75	3.23	3.10	8.83
3.	Ainsworth	0.39	0.00	-	0.00	0.00	0.00	0.00	0.39	0.52	0.26	3.75	3.88	3.62	3.75	3.62	3.88	3.75	4.01	3.88	3.75	3.75	3.23	3.10	8.83
4.	TrainTl	0.39	0.00	0.00	-	0.00	0.00	0.00	0.39	0.52	0.26	3.75	3.88	3.62	3.75	3.62	3.88	3.75	4.01	3.88	3.75	3.75	3.23	3.10	8.83
5.	BullRun	0.39	0.00	0.00	0.00	-	.001	0.00	0.39	0.52	0.26	3.75	3.88	3.62	3.75	3.62	3.88	3.75	4.01	3.88	3.75	3.75	3.23	3.10	8.83
6.	EastHood	0.15	0.24	0.24	0.24	0.24	-	0.13	0.52	0.65	0.39	3.88	4.01	3.75	3.88	3.75	4.01	3.88	4.13	4.01	3.88	3.88	3.36	3.23	8.83
7.	Wildwood	0.39	0.00	0.00	0.00	0.00	0.24	-	0.39	0.52	0.26	3.75	3.88	3.62	3.75	3.62	3.88	3.75	4.01	3.88	3.75	3.75	3.23	3.10	8.83
8.	NEagleCk	0.52	0.12	0.12	0.12	0.12	0.37	0.12	-	0.39	0.39	3.36	3.49	3.75	3.36	3.23	3.49	3.36	3.62	3.49	3.36	3.36	2.84	2.71	9.01
9.	Estacada	0.64	0.24	0.24	0.24	0.24	0.49	0.24	0.12	-	0.26	3.49	3.62	3.62	3.49	3.36	3.62	3.49	3.75	3.62	3.49	3.49	2.97	2.84	8.83
10.	Jackson5	0.64	0.24	0.24	0.24	0.24	0.49	0.24	0.12	0.00	-	3.75	3.88	3.62	3.75	3.62	3.88	3.75	4.01	3.88	3.75	3.75	3.23	3.10	8.47
11.	SilverCk	3.34	2.91	2.91	2.91	2.91	3.18	2.91	2.77	2.91	2.91	-	0.39	0.65	0.26	0.13	1.16	1.03	1.55	0.39	0.52	0.52	1.81	1.68	10.4
12.	DetroitLk	3.37	2.94	2.94	2.94	2.94	3.21	2.94	2.80	2.94	2.94	0.27	-	0.26	0.13	0.26	1.29	1.16	1.68	0.78	0.65	0.65	1.94	1.81	10.6
13.	LSantiam	3.37	2.94	2.94	2.94	2.94	3.21	2.94	2.80	2.94	2.94	0.27	0.00	-	0.39	0.52	1.55	1.42	1.94	1.03	0.90	0.90	2.20	2.07	10.3
14.	BreitenBh	3.34	2.91	2.91	2.91	2.91	3.18	2.91	2.77	2.91	2.91	0.24	0.02	0.02	-	0.13	1.16	1.03	1.55	0.65	0.52	0.52	1.81	1.68	10.4
15.	Bugaboo	3.22	2.77	2.77	2.77	2.77	3.04	2.77	2.63	2.77	2.77	0.12	0.15	0.15	0.12	-	1.03	0.90	1.42	0.52	0.39	0.39	1.68	1.55	10.3
16a.	ThomasCkA	3.13	2.71	2.71	2.71	2.71	2.97	2.71	2.57	2.71	2.71	0.82	0.84	0.84	0.82	0.69	-	0.13	1.16	1.03	0.90	0.90	1.68	1.55	11.0
16b.	ThomasCkB	2.99	2.57	2.57	2.57	2.57	2.83	2.57	2.43	2.57	2.57	0.69	0.72	0.72	0.69	0.57	0.12	-	1.03	0.90	0.78	0.78	1.81	1.68	10.8
17.	ChurchCk	3.55	3.12	3.12	3.12	3.12	3.39	3.12	2.98	3.12	3.12	1.25	1.28	1.28	1.25	1.12	1.17	1.04	-	1.16	1.03	1.03	2.07	1.94	10.4
18.	KeelOver	3.37	2.94	2.94	2.94	2.94	3.21	2.94	2.80	2.94	2.94	0.07	0.34	0.34	0.32	0.20	0.79	0.67	1.17	-	0.13	0.13	1.94	1.81	10.4
19.	Quartz	3.23	2.80	2.80	2.80	2.80	3.07	2.80	2.66	2.80	2.80	0.20	0.22	0.22	0.20	0.07	0.67	0.54	1.05	0.12	-	0.00	1.81	1.68	10.3
20.	Withycom	3.23	2.80	2.80	2.80	2.80	3.07	2.80	2.66	2.80	2.80	0.20	0.22	0.22	0.20	0.07	0.67	0.54	1.05	0.12	0.00	-	1.81	1.68	10.3
21.	HJAndrews	3.00	2.57	2.57	2.57	2.57	2.84	2.57	2.44	2.57	2.57	1.58	1.61	1.61	1.58	1.45	1.13	1.26	1.78	1.61	1.47	1.47	-	0.13	10.4
22.	HiddenLk	2.86	2.44	2.44	2.44	2.44	2.70	2.44	2.30	2.44	2.44	1.45	1.47	1.47	1.45	1.31	1.00	1.10	1.65	1.47	1.34	1.34	0.12	-	10.3
23.	*B.campi*	9.28	9.23	9.23	9.23	9.23	9.23	9.23	8.96	8.70	8.70	10.8	10.9	10.9	10.8	10.6	11.4	11.1	10.8	10.8	10.6	10.6	11.3	11.0	-

There were 7 first position, 2 second position and 35 third position synonymous substitutions. In addition, there were three non-synonymous first codon position substitutions occurring at sites 17, 434 and 551. Substitutions at sites 17 and 434 distinguish northern haplotypes (Post Creek Road, Train Tunnel, Ainsworth State Park, Viento State Park, Bull Run, East Mt. Hood, Wildwood, N. Eagle Creek, Estacada, Jackson Five) from southern haplotypes (Silver Creek Falls, Detroit Lake Area, Little Santiam River, Brietenbush, Bugaboo, Thomas Creek, Church Creek, Keel Over, Quartzville, Withycomb, H.J. Andrews, Hidden Lake).

Phylogenetic analyses based upon aligned cytochrome *b* sequences showed similar topologies for all three methods of inference. For maximum parsimony analyses, twenty-four most parsimonious trees were found, based upon 36 parsimony informative sites, each comprised of 357 steps. A parsimony consensus bootstrap (100 replicates), using the 50% majority rule consensus option, yielded a single tree of 365 steps (Consistency Index 0.91, Retention Index 0.96; Figure 3.2A). A bootstrap minimum evolution tree, using Kimura 2-parameter distances, had a tree score of 0.27 (Figure 3.2B). Finally, a maximum likelihood consensus bootstrap tree, allowing for rate heterogeneity among codon position, yielded a –ln likelihood score of 1,554 (Figure 3.2C).

All three methods showed nearly identical topologies indicating support for two major clades among Oregon slender salamanders. The first clade was comprised of haplotypes from northern populations (Post Creek Road, East Mt. Hood, Train Tunnel, Ainsworth State Park, Viento State Park, Bull Run,

Wildwood, N. Eagle Creek, Estacada, Jackson Five) and the second was a cluster of southern populations (Silver Creek Falls, Keel Over, Detroit Lake Area, Little Santiam River, Brietenbush, Bugaboo, Quartzville, Withycomb, H.J. Andrews, Hidden Lake). Within the northern clade, the northern-most geographic populations showed a tight cluster (Post Creek Road, East Mt. Hood, Train Tunnel, Ainsworth State Park, Viento State Park, Bull Run, Wildwood). For the southern group, Thomas Creek and Church Creek formed a sister clade to a group that includes: Silver Creek Falls, Keel Over, Detroit Lake Area, Little Santiam River, Brietenbush, Bugaboo, Quartzville, and Withycomb. The southern-most populations (H.J. Andrews, Hidden Lake) outgroup the rest of the southern populations.

RAPD analyses

RAPD profiles were generated from 14 primers, of which 46 variable bands were scored (Table 3.4). Allele frequency varied considerably within and among the 19 populations (N = 339) sampled. No population specific bands were identified; however, exact tests by locus showed significant population differentiation for each locus ($X^2 = 892.70$, $p < 0.0001$). Estimates of genetic diversity parameters within populations showed considerable variation (Table 3.5).

Figure 3.2 Phylogenetic relationships among Oregon slender salamander populations based upon cytochrome *b* sequences (774bp): (A) Consensus distance (minimum evolution) tree based upon Kimura 2-parameter distances (distances above the branches, bootstrap values below), (B) Consensus maximum parsimony tree (number of steps above the branches, bootstraps values below), (C) Maximum likelihood tree (distances above branches, bootstrap values below).

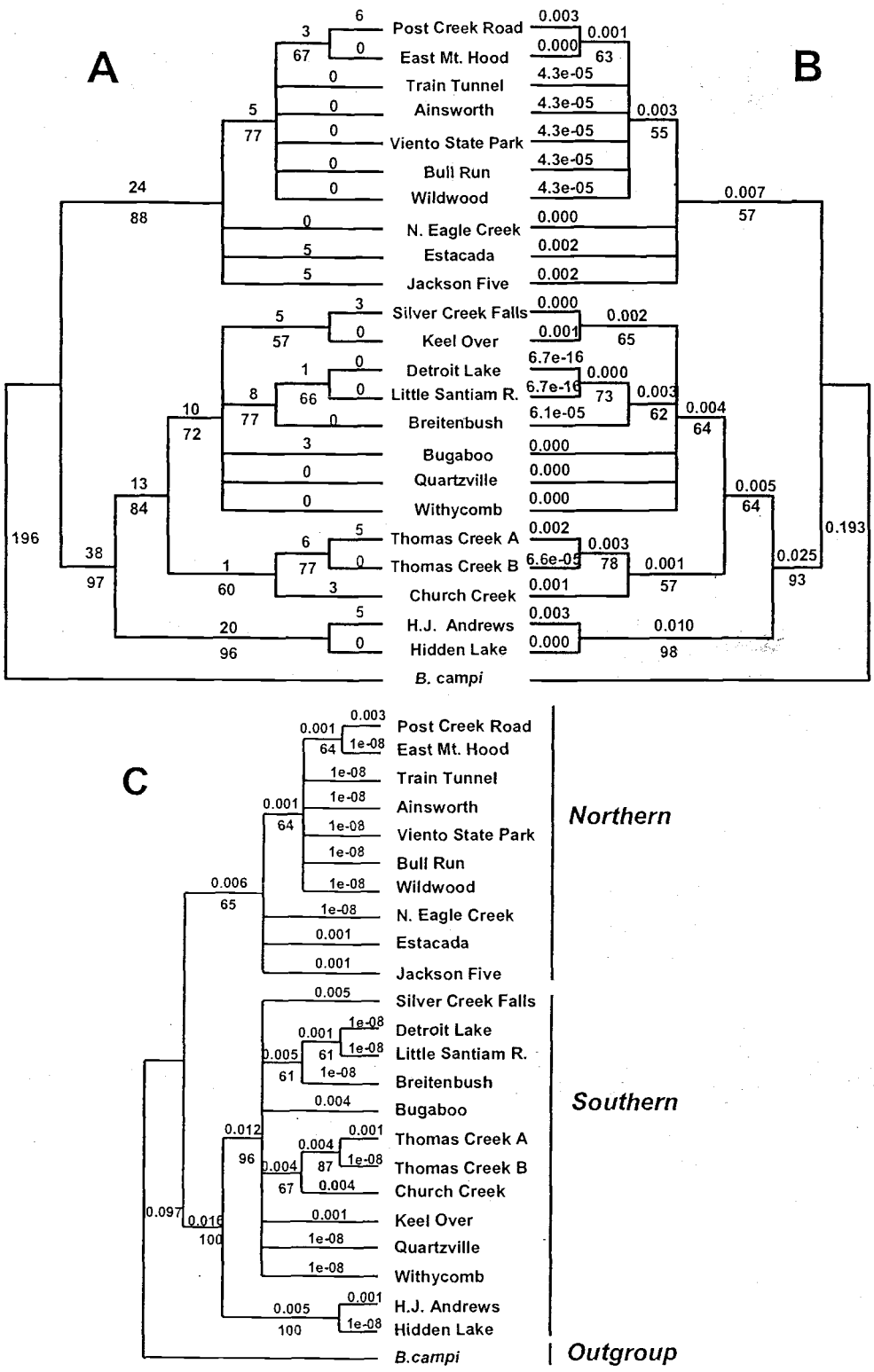

A
B
C
Post Creek Road
East Mt. Hood
Train Tunnel
Ainsworth
Viento State Park
Bull Run
Wildwood
N. Eagle Creek
Estacada
Jackson Five
Silver Creek Falls
Keel Over
Detroit Lake
Little Santiam R.
Breitenbush
Bugaboo
Quartzville
Withycomb
Thomas Creek A
Thomas Creek B
Church Creek
H.J. Andrews
Hidden Lake
B. campi
Northern
Southern
Outgroup

Figure 3.2

Table 3.4 Dominant (+) RAPD band frequencies for 34 variable loci from 19 Oregon Slender Salamander populations estimatedby using Lynch & Milligan's (1994) Taylor expansion. UBC is the University of British Columbia RAPD primer set number, followed by fragment size of the locus scored. See Table 3.1 for location identification.

Locus	UBC Code	1 PCRD	4 TRTN	5 BULL	6 EMTH	7 WILD	8 NECK	9 ESTC	10 JACK	11 SLCF	12 DELK	13 LISR	15 BUGB	16 TMCK	17 CHCK	18 KEOV	19 QVCK	20 WITH	21 HJAN	22 HDLK
Locus1	UBC#102-700bp	0.43	0.15	0.00	0.03	0.38	0.50	0.03	0.10	0.00	0.00	0.00	0.00	0.04	0.00	0.34	0.38	0.15	0.21	0.37
Locus2	UBC#102-850bp	1.00	0.58	1.00	1.00	0.03	0.59	0.75	0.10	1.00	0.45	0.00	0.58	0.00	0.18	0.37	0.46	0.76	0.00	0.00
Locus3	UBC#102-1100bp	0.55	0.58	1.00	0.54	0.72	0.59	0.75	0.78	0.63	0.45	1.00	0.58	1.00	0.41	0.66	0.55	0.62	0.46	0.37
Locus4	UBC#112-480bp	1.00	1.00	1.00	0.71	1.00	0.50	1.00	0.78	1.00	0.15	0.43	1.00	1.00	1.00	1.00	1.00	0.76	0.75	0.72
Locus5	UBC#112-510bp	0.63	0.58	0.73	0.54	1.00	0.37	1.00	0.60	1.00	0.52	0.24	1.00	0.78	0.31	0.61	0.55	0.62	0.24	0.22
Locus6	UBC#112-520bp	0.72	0.58	1.00	0.61	1.00	0.37	0.44	0.78	1.00	0.39	0.17	1.00	0.65	0.14	0.57	0.46	0.68	0.18	0.22
Locus7	UBC#112-540bp	0.55	1.00	1.00	1.00	0.72	0.43	0.49	0.21	0.63	0.45	0.03	0.44	0.78	0.47	1.00	0.50	0.76	0.34	0.40
Locus8	UBC#119-800bp	1.00	1.00	1.00	0.71	1.00	1.00	0.13	1.00	1.00	0.00	0.03	1.00	0.71	1.00	0.29	0.61	0.43	1.00	0.79
Locus9	UBC#121-550bp	0.63	1.00	0.64	0.31	1.00	0.59	1.00	1.00	1.00	0.11	0.03	1.00	0.38	0.36	0.61	1.00	0.62	0.75	1.00
Locus10	UBC#121-700bp	0.49	0.33	0.57	0.47	0.25	0.50	0.60	0.65	0.00	0.29	0.20	0.33	0.41	0.36	0.46	0.67	0.62	0.21	0.53
Locus11	UBC#121-900bp	0.72	0.58	1.00	0.31	0.63	0.50	0.54	0.55	0.32	0.24	0.66	0.07	0.41	0.07	0.27	0.38	0.43	0.24	0.46
Locus12	UBC#121-1000bp	0.03	0.33	0.00	0.10	0.17	0.08	0.13	0.08	0.11	0.19	0.20	0.15	0.08	0.10	0.27	0.10	0.20	0.15	0.04
Locus13	UBC#126-420bp	0.29	0.15	0.64	0.41	0.29	0.43	0.16	0.35	0.25	0.11	0.20	0.58	0.65	0.36	0.46	0.13	0.36	0.02	0.02
Locus14	UBC#131-1100bp	1.00	1.00	1.00	1.00	1.00	0.69	1.00	1.00	0.63	0.52	0.48	1.00	0.47	0.18	0.13	1.00	0.36	1.00	0.24
Locus15	UBC#131-1600bp	0.33	0.44	0.64	0.31	0.55	0.50	0.44	0.38	0.18	0.39	0.24	0.44	0.44	0.18	0.37	0.15	0.36	0.34	0.15
Locus16	UBC#131-1700bp	0.00	0.00	0.00	0.00	0.49	0.12	0.08	0.02	0.00	0.29	0.05	0.44	0.47	0.31	1.00	0.55	0.76	0.46	0.79
Locus17	UBC#133-780bp	0.72	0.58	1.00	0.54	0.63	0.04	0.29	0.55	1.00	0.39	0.48	0.58	0.35	0.54	0.32	0.18	0.07	0.27	0.00
Locus18	UBC#145-650bp	0.72	0.44	0.50	0.36	1.00	0.37	0.00	0.32	0.25	0.00	0.31	0.23	0.26	0.10	0.29	0.00	0.05	0.07	0.02
Locus19	UBC#145-700bp	0.72	0.23	0.57	0.54	0.63	0.26	0.54	0.17	0.00	0.60	0.66	0.33	0.47	0.47	0.66	0.50	0.47	0.50	0.5
Locus20	UBC#145-820bp	0.00	0.00	0.00	0.00	0.72	0.00	0.05	0.00	0.00	0.00	0.03	0.00	0.55	0.61	0.72	0.50	0.62	0.55	1.00
Locus21	UBC#145-980bp	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.04	0.63	0.45	0.00	1.00	0.00	0.00	0.40	0.13	0.20	0.07	0.00
Locus22	UBC#175-310bp	0.00	0.00	0.00	0.00	0.00	0.00	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.46	0.38	0.56	0.05	0.13
Locus23	UBC#175-600bp	0.72	1.00	1.00	0.36	0.49	0.50	0.36	0.12	0.41	0.39	0.27	1.00	0.24	0.36	0.32	0.55	0.20	0.21	0.57

Table 3.4 Continued.

Locus	UBC Code	1 PCRD	4 TRTN	5 BULL	6 EMTH	7 WILD	8 NECK	9 ESTC	10 JACK	11 SLCF	12 DELK	13 LISR	15 BUGB	16 TMCK	17 CHCK	18 KEOV	19 QVCK	20 WITH	21 HJAN	22 HDLK
Locus24	UBC#175-720bp	0.72	0.00	0.40	0.31	0.43	0.50	0.05	0.47	0.41	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00
Locus25	UBC#175-835bp	1.00	0.44	0.57	0.71	1.00	0.37	0.66	0.78	0.00	0.45	0.48	0.58	0.12	0.54	0.79	0.67	0.76	0.42	0.53
Locus26	UBC#175-900bp	0.63	1.00	0.64	0.71	0.49	0.43	0.32	0.65	0.63	0.52	0.43	1.00	0.32	0.47	0.53	0.13	0.51	0.38	0.04
Locus27	UBC#177-750bp	0.55	0.00	0.00	0.00	0.00	0.04	0.60	0.04	0.00	0.00	0.00	0.00	0.02	0.00	0.05	0.00	0.00	0.00	0.00
Locus28	UBC#177-800bp	0.72	1.00	1.00	0.71	1.00	0.69	1.00	1.00	1.00	0.60	0.39	0.00	0.71	0.07	0.53	0.55	0.76	0.38	0.29
Locus29	UBC#177-820bp	0.29	1.00	1.00	0.41	0.29	0.31	0.75	0.78	0.25	0.52	0.39	1.00	0.78	0.26	0.46	1.00	0.68	0.38	0.37
Locus30	UBC#177-1000bp	0.49	1.00	0.45	0.36	1.00	0.31	0.44	0.55	0.32	0.52	0.53	0.58	0.65	0.54	0.32	0.18	0.36	0.07	0.17
Locus31	UBC#177-1070bp	0.72	1.00	0.64	1.00	0.55	0.59	0.66	0.44	1.00	1.00	0.43	1.00	0.32	1.00	0.57	0.61	0.76	0.31	0.49
Locus32	UBC#187-610bp	0.55	0.44	1.00	0.61	0.38	0.50	0.32	0.41	0.41	0.29	0.48	0.44	0.04	0.47	0.00	0.31	0.43	0.07	0.07
Locus33	UBC#187-750bp	0.10	0.00	0.64	0.22	0.72	0.37	0.49	0.65	0.06	0.29	0.11	0.00	0.29	0.26	0.15	0.00	0.26	0.38	0.00
Locus34	UBC#187-830bp	0.43	0.44	0.57	0.41	0.72	0.26	0.44	0.51	0.25	0.34	0.14	1.00	0.51	0.36	0.43	0.27	0.56	0.24	0.27
Locus35	UBC#190-200bp	0.72	0.58	0.73	0.71	0.72	0.31	0.54	0.71	0.25	0.52	0.17	0.44	0.47	0.41	0.46	0.55	0.56	0.50	0.32
Locus36	UBC#190-700bp	0.06	0.00	0.19	0.07	0.21	0.16	0.10	0.17	0.06	0.19	0.00	0.00	0.14	0.54	0.61	1.00	0.62	0.24	0.34
Locus37	UBC#190-800bp	0.03	0.07	0.19	0.00	0.00	0.00	0.75	0.35	0.50	1.00	0.66	0.00	0.44	0.07	0.05	0.05	0.05	0.02	0.07
Locus38	UBC#193-510bp	1.00	1.00	0.73	1.00	1.00	0.69	1.00	1.00	0.32	0.00	0.11	0.23	0.24	0.10	0.29	0.05	0.29	0.15	0.13
Locus39	UBC#193-600bp	0.49	1.00	0.73	0.14	1.00	0.50	0.00	0.55	0.32	1.00	0.14	1.00	1.00	0.54	1.00	0.61	0.68	0.27	0.72
Locus40	UBC#193-700bp	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.74	1.00	1.00	1.00	0.66	1.00	0.76	0.46	0.20
Locus41	UBC#193-850bp	1.00	1.00	1.00	1.00	1.00	0.59	0.00	1.00	1.00	1.00	0.59	1.00	1.00	0.54	0.29	0.15	0.68	0.34	0.29
Locus42	UBC#193-900bp	1.00	1.00	0.73	0.71	0.72	1.00	0.40	0.51	0.63	0.70	0.48	1.00	0.47	0.61	0.46	0.00	0.47	0.27	0.53
Locus43	UBC#193-1700bp	0.55	0.33	0.50	0.71	1.00	0.43	0.29	0.41	0.25	0.34	0.20	0.44	0.26	0.36	0.37	0.10	0.47	0.24	0.00
Locus44	UBC#199-800bp	0.63	1.00	0.19	0.14	0.55	0.04	0.25	0.60	0.25	0.11	0.24	0.58	0.21	0.07	0.15	0.21	0.20	0.13	0.07
Locus45	UBC#199-1050bp	0.03	0.15	0.09	0.10	0.10	0.04	0.66	0.04	0.06	0.24	1.00	0.00	0.12	0.36	0.72	0.67	0.62	0.55	0.40
Locus46	UBC#229-600bp	0.03	0.15	0.03	0.00	0.17	0.04	0.44	0.47	0.63	0.60	0.66	0.58	0.60	0.18	0.04	0.07	0.05	0.00	0.05

Table 3.5 Genetic diversity parameters (± SE) within populations of Oregon slender salamanders based on variable 46 RAPD markers. n^1 is number of individuals analyzed. Expected heterozygosity within populations is estimated using Lynch & Milligan's (1994) Taylor expansion correction for dominant markers. See Table 3.1 for site locations.

Population (n^1)	Observed alleles	Effective alleles	Polymorphic alleles	%polymorphic alleles	Expected heterozygosity
Northern					
1. Post Canyon (16)	1.71 ± 0.46	1.48 ± 0.40	33	71.74	0.27 ± 0.03
2. Train Tunnel (7)	1.50 ± 0.50	1.34 ± 0.42	21	45.65	0.19 ± 0.05
3. Bull Run (17)	1.52 ± 0.50	1.40 ± 0.40	24	52.17	0.21 ± 0.04
4. East Mt. Hood (15)	1.70 ± 0.47	1.50 ± 0.40	32	69.57	0.27 ± 0.05
5. Wildwood (16)	1.60 ± 0.50	1.41 ± 0.40	27	58.70	0.24 ± 0.04
6. N. Eagle Creek (13)	1.84 ± 0.40	1.62 ± 0.40	39	84.78	0.34 ± 0.03
7. Estacada (19)	1.78 ± 0.42	1.52 ± 0.40	36	78.26	0.29 ± 0.03
8. Jackson Five (26)	1.80 ± 0.40	1.52 ± 0.39	37	80.43	0.29 ± 0.03
Mean (129)	1.68 ± 0.46	1.47 ± 0.40	31	67.66	0.26 ± 0.04
Southern					
9. Silver Creek Falls (9)	1.60 ± 0.50	1.40 ± 0.40	27	58.70	0.22 ± 0.04
10. Detroit Lake (13)	1.73 ± 0.44	1.60 ± 0.40	34	73.91	0.31 ± 0.03
11. Little Santiam (19)	1.80 ± 0.40	1.50 ± 0.38	37	80.43	0.28 ± 0.03
12. Bugaboo (10)	1.43 ± 0.50	1.40 ± 0.44	20	43.48	0.19 ± 0.05
13. Thomas Creek (26)	1.80 ± 0.40	1.54 ± 0.40	37	80.43	0.31 ± 0.03
14. Church Creek (15)	1.82 ± 0.40	1.60 ± 0.40	38	82.61	0.31 ± 0.03
15. Keel Over (28)	1.87 ± 0.34	1.64 ± 0.35	40	86.96	0.36 ± 0.03
16. Quartzville (20)	1.80 ± 0.43	1.51 ± 0.40	35	76.09	0.29 ± 0.03
17. Withycomb (21)	1.78 ± 0.42	1.54 ± 0.37	36	78.26	0.38 ± 0.03
18. H.J. Andrews (21)	1.89 ± 0.31	1.53 ± 0.35	41	89.13	0.31 ± 0.03
19. Hidden Lake (28)	1.80 ± 0.40	1.47 ± 0.38	37	80.43	0.27 ± 0.03
Mean (210)	1.76 ± 0.41	1.52 ± 0.39	35	75.49	0.29 ± 0.03
Pooled	1.72 ± 0.43	1.49 ± 0.39	33	71.57	0.27 ± 0.03

However, there were no significant differences among northern and southern groups, with groups defined according to their northern and southern mtDNA haplotypes, for observed number of alleles *A*, effective number of alleles A_e, number of polymorphic loci *P*, and number of polymorphic loci P_e (95% criteria), (*A*: $Z = -1.44$, $p < 0.07$; A_e: $Z = -1.34$ $p < 0.08$; P: $Z = -1.43$, $p < 0.07$; P_e: $Z = -1.44$, $p < 0.07$). Expected heterozygosity varied among populations from 0.19 ± 0.05 SE to 0.38 ± 0.03 SE. There is suggestive but inclusive evidence for a difference ($Z = -1.61$, $p < 0.053$) in average expected heterozygosity between northern (average $H_e = 0.26 \pm 0.04$ SE) and southern groups (average $H_e = 0.29 \pm$ 0.03 SE).

For the multi-dimensional scaling analyses, plots of overall stress versus increasing number of dimensions indicated three dimensions were sufficient to explain most of the variation. Scaling for 46 RAPD loci indicated most of the variation was contained within the first axis (46.1 %, $p < 0.05$, $R^2 = 0.18$), while the second (30.8 %, $p < 0.05$, $R^2 = 0.06$) and third axes (23.4 %, $p < 0.05$, $R^2 = 0.08$) accounted for the rest. The cumulative R^2 was 0.33. Plots were evaluated using three different groups: all individuals coded for populations separately, regional grouping, and mtDNA haplotype grouping. Plots of all individuals coded separately for population of origin was too difficult to interpret for 19 populations.

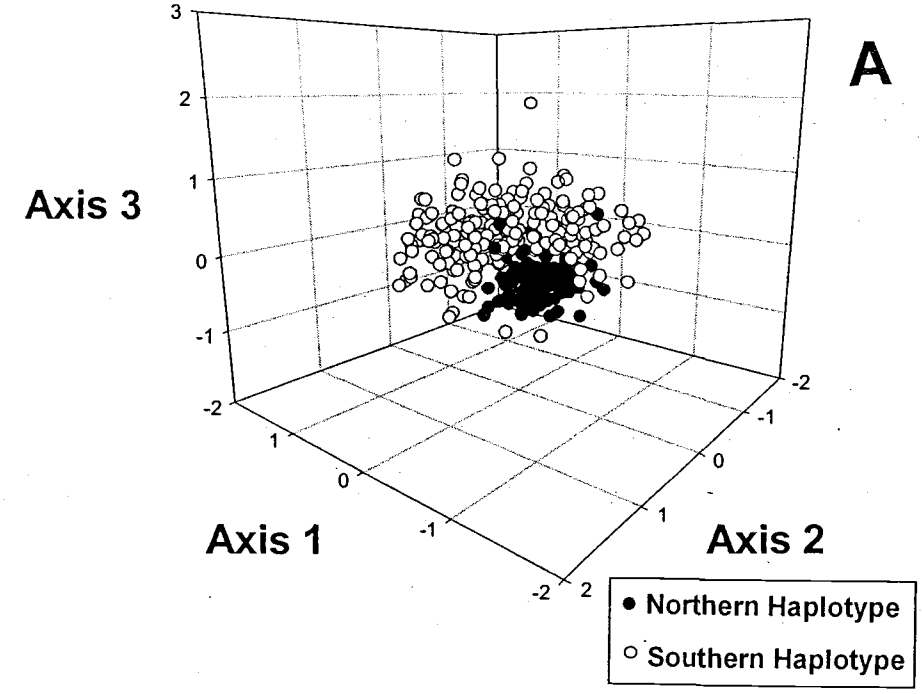
Therefore, plots were evaluated based upon regional population distributions. The first group was comprised of *northern-most* populations (Post Creek Road, Train Tunnel, East Mt. Hood, Bull Run, Wildwood, N. Eagle Creek, Jackson Five), a second group encompassed *mid-range* populations (Estacada, Detroit Lake Area, Little Santiam River Area, Silver Creek Falls, Bugaboo), and the third group contained *southern-most* populations (Thomas Creek, Church Creek, Keel Over, Withycomb, Quartzville, H.J. Andrews, Hidden Lake). Distinct clustering of each regional group was evident in a three dimensional plot of all axis (Figure 3.3A). Similarly, distinct clustering of northern individuals occurred in a plot with individuals coded for northern and southern mitochondrial DNA haplotypes (Figure 3.3B). Kendall correlation of individual loci with each axis revealed no significant correlation.

Neighbor-joining analyses based on the RAPD data revealed a topology consistent with clustering of the three regional groups (Figure 3.4). The northern-most clade, which included Post Creek Road, East Mt. Hood, Train Tunnel, Wildwood, Bull Run, Estacada and N. Eagle Creek, was supported by 66% bootstrap resampling. A mid-range clade formed a non-supported (bootstrap resampling support less than 50%) sister clade with the northern-most clade, which included the following populations: Little Santiam River, Detroit Lake, Bugaboo, Jackson Five, Thomas Creek, and Silver Creek Falls. Finally, a basal clade

composed of southern populations (Keel Over, Withycomb, Church Creek, Quartzville, H.J. Andrews, and Hidden Lake) was not well supported, except for a sub-clade within this group (Quartzville, H.J. Andrews and Hidden Lake), which was supported by 72% re-sampling.

Although the overall phylogenetic relationships of the three major groups is not well-supported, exact tests revealed significant differences ($p < 0.01$) between a majority of population pairwise comparisons. The only combinations that did not show significant differences ($p > 0.05$) occurred between Train Tunnel and the following populations: Post Canyon Road (chi-squared = 108.74, $p = 0.11$), East Mt. Hood (chi-squared = 89.80, $p = 0.55$), and Bugaboo (chi-squared = 65.27, $p = 0.98$). In sum, differences among populations and low support for some regional clusters by phylogenetic analyses suggested a complex pattern potentially resulting from the confounding influences of localized gene flow and random drift.

Figure 3.3 Non-metric multidimensional scaling of individual Oregon slender salamanders ($N = 339$) using 46 variable RAPD loci: (A) Individuals are coded for the major mtDNA lineage, northern or southern cytochrome b haplotype, (B) Individuals are coded for regional geographic grouping.


A
Axis 3
Axis 1
Axis 2
Northern Haplotype
Southern Haplotype

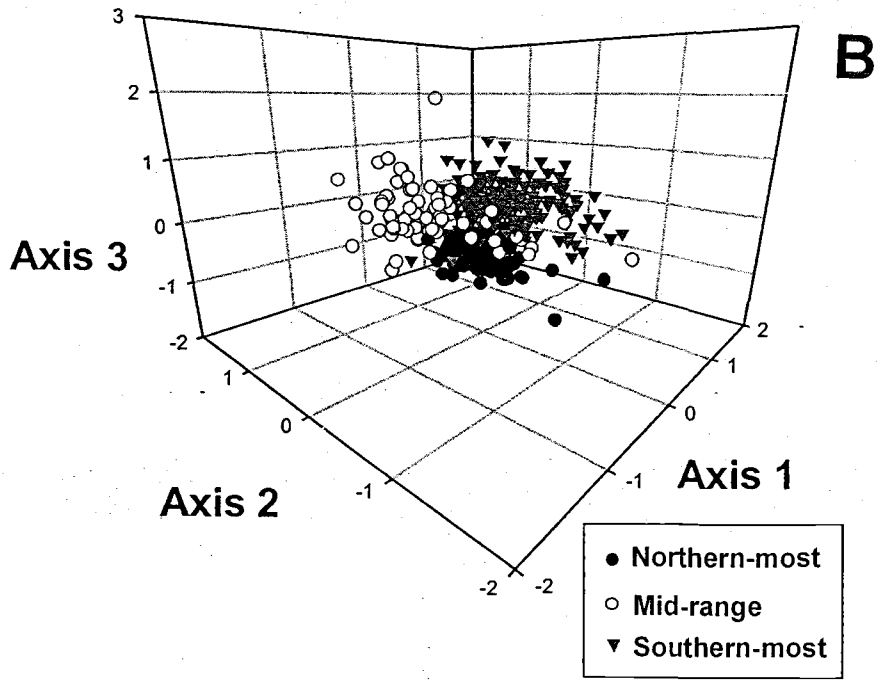

B
Axis 3
Axis 2
Axis 1
Northern-most
Mid-range
Southern-most

Figure 3.3

Figure 3.4 Neighbor-joining phenogram derived from Manhattan distances using 46 variable RAPD loci in Oregon slender salamanders. The tree was rooted at the midpoint between taxa pairs with the greatest patristic distance. Bootstrap resampling values, based on 100 replicates, with support greater than 50% shown above branches.

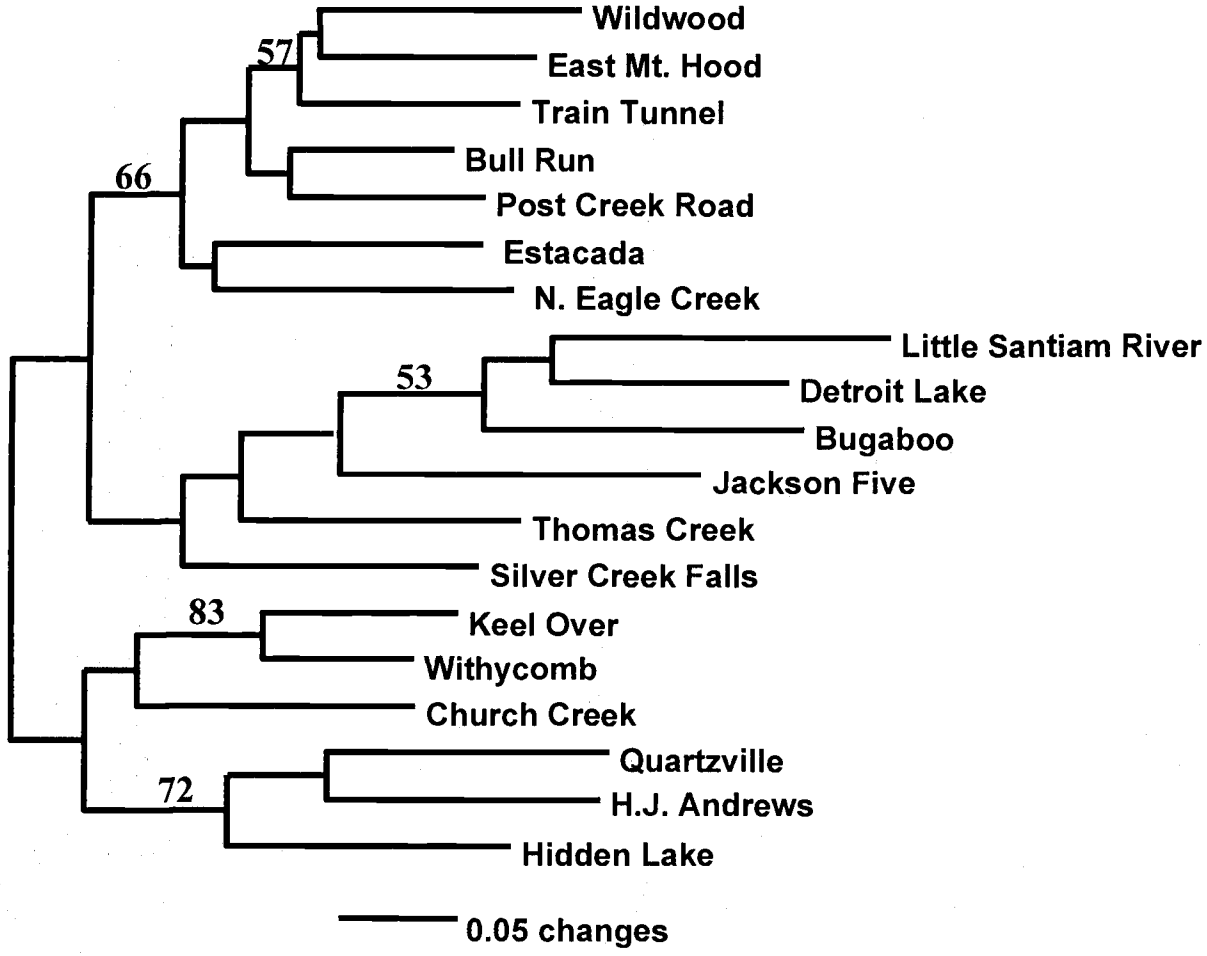

Wildwood
East Mt. Hood
Train Tunnel
Bull Run
Post Creek Road
Estacada
N. Eagle Creek
Little Santiam River
Detroit Lake
Bugaboo
Jackson Five
Thomas Creek
Silver Creek Falls
Keel Over
Withycomb
Church Creek
Quartzville
H.J. Andrews
Hidden Lake
0.05 changes
57
66
53
83
72

Figure 3.4

Among all populations, there was a significant association between pairwise F_{ST}-values and geographic distance (Table 3.6, Figure 3.5A, r = 0.38, p = 0.01). Additionally, there was a significant association between degree of scatter and geographic distance (r = 0.92, p = 0.01). The F_{ST} estimate (Lynch & Milligan 1994) across all populations was 0.35 ± 0.03 SE. Therefore, the null hypothesis of equilibrium between gene flow and random genetic drift was not rejected. In contrast, the null hypothesis of equilibrium is rejected for northern and southern regions. For the northern populations (grouped based on geography and northern mtDNA haplotypes), both the scatterplot and correlation analysis show no association between pairwise F_{ST}-values and geographic distances (Figure 3.5B, r = -0.23, p = 0.20). Similarly, there was no association between degree of scatter with geographic distance (r = -0.080, p = 0.43). The F_{ST} estimate (based on the Lynch & Milligan (1994) correction) for the northern region was 0.29 ± 0.06 SE. In the southern region (grouped based upon geography and southern mtDNA haplotypes), pairwise F_{ST}-values did not correlate with geographic distance (Figure 3.5C, r = 0.29, p = 0.37) nor was there an association between degree of scatter and geographic distances (r = -0.00002, p = 0.37). The F_{ST} estimate for the southern region was 0.33 ± 0.05 SE. At the regional scale, random drift and low gene flow may predominate in contributing to population structure. Moreover, similarly large population subdivision values were found using different estimators (Table 3.7).

Table 3.6 Pairwise geographic distances (Km, below diagonal) and *Fst* estimates (multiplied by 100, above diagonal) based upon 46 RAPD loci for Oregon slender salamanders (see Table 3.1 for location identification).

		1 PCRD	2 TRTN	3 BULL	4 EMTH	5 WILD	6 NECK	7 ESTC	8 JACK	9 SLCF	10 DELK	11 LISR	12 BUGB	13 TMCK	14 CHCK	15 KEOV	16 QVCK	17 WITH	18 HJAN	19 HDLK
1.	PostCRd	-	20.4	19.1	15.8	21.2	19.7	29.2	20.5	26.4	33.9	37.9	28.5	33.5	31.2	34.7	34.2	30.8	35.5	39.5
2.	TrainTl	53.9	-	20.8	21.0	23.4	25.2	29.5	20.9	26.6	30.5	38.3	18.7	28.5	31.4	34.5	32.3	30.0	36.2	39.5
3.	BullRun	40.6	21.4	-	18.9	23.8	24.5	30.1	20.5	26.4	34.6	40.1	27.2	27.5	34.3	36.4	36.3	28.2	38.7	43.3
4.	EstHood	33.5	72.4	52.2	-	26.2	19.0	26.3	20.6	23.2	25.0	31.1	28.8	26.6	24.4	29.0	31.4	24.9	29.2	36.4
5.	Wildwd	46.0	30.4	11.6	49.6	-	28.1	33.0	20.4	31.2	35.9	41.5	29.2	27.3	32.2	32.8	35.6	30.6	33.5	37.7
6.	NeagleC	57.7	12.9	18.3	70.1	22.6	-	28.4	21.9	25.8	26.0	28.7	30.5	27.1	22.7	29.0	27.9	29.4	24.1	26.7
7.	Estacad	67.9	39.4	30.5	67.0	21.9	26.7	-	23.0	29.6	27.3	28.4	35.2	28.0	31.9	29.9	23.5	26.9	29.1	35.4
8.	Jackson	80.5	42.1	40.7	82.2	35.0	29.7	153	-	24.3	29.6	30.6	27.0	22.9	29.5	34.4	31.8	29.0	29.7	36.6
9.	SilverC	118	77.1	78.3	116	71.9	65.8	50.2	37.5	-	26.7	31.6	24.3	27.2	29.1	36.3	35.4	31.8	34.0	38.8
10.	Detroit	112	94.3	83.1	95.8	85.7	81.5	55.0	56.1	51.0	-	28.6	22.7	22.4	27.5	30.2	29.7	28.0	32.6	21.7
11.	LSantm	107	76.5	71.0	99.0	62.0	63.8	40.6	34.7	24.2	28.0	-	39.8	25.9	24.8	31.5	34.1	36.9	24.4	29.7
12.	Bugaboo	122	106	94.6	103	83.2	93.6	67.0	68.4	60.4	12.3	38.8	-	27.3	28.4	35.1	33.6	32.8	36.4	40.4
13.	ThomasC	131	97.0	94.2	123	85.7	84.8	64.0	55.1	24.2	39.2	24.5	44.6	-	23.3	23.2	28.3	24.6	24.8	29.1
14.	ChurchC	145	108	107	138	99.5	96.4	77.6	67.2	31.5	53.2	39.4	56.7	15.4	-	22.7	24.3	24.6	19.3	20.6
15.	KeelOr	152	117	115	143	107	105	85.0	75.9	41.8	53.1	45.2	54.2	21.1	11.3	-	21.3	15.7	23.8	23.2
16.	Quartz	133	108	100	119	90.1	95.0	70.2	66.2	45.5	24.2	31.7	23.7	24.1	33.8	30.5	-	22.1	20.7	19.6
17.	Withyco	148	115	112	137	103	103	81.3	73.3	41.7	45.9	40.9	46.6	18.4	15.1	7.7	22.9	-	30.2	30.1
18.	HJAndre	165	145	136	146	125	132	107	104	82.2	53.5	70.0	43.6	58.4	60.1	50.4	38.5	45.0	-	16.0
19.	HiddenL	190	169	161	171	150	156	131	128	102	78.4	93.1	68.6	78.0	75.7	64.6	61.5	61.6	25.0	-

Figure 3.5 Scatterplots based on 46 variable RAPD loci of pairwise-F_{ST} estimates versus geographic distance in Oregon slender salamanders: (A) All populations, (B) Northern clade populations, and (C) Southern clade populations.

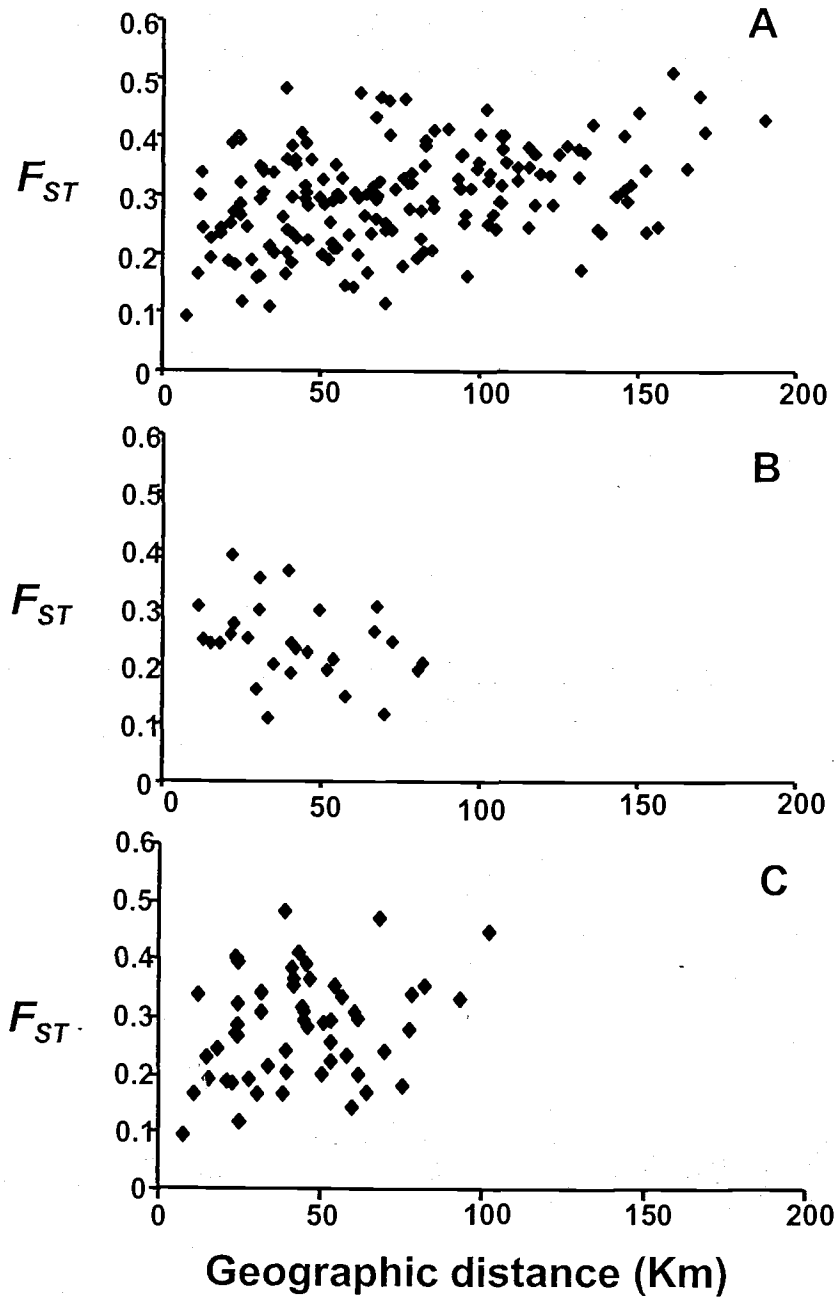

A
B
C
F_{ST}
F_{ST}
F_{ST}
0.6
0.5
0.4
0.3
0.2
0.1
0
0
50
100
150
200
Geographic distance (Km)

Figure 3.5

The analysis of molecular variance (nested AMOVA) agreed with the multi-dimensional scaling, pairwise exact test, and pairwise-F_{ST} analyses. Most of the variance was contained within populations (58.6%), followed by among populations within groups (27.0%), and finally among groups (14.4%; Table 3.8). Analyses for the northern group alone indicated 29.6% of the variation was contained among populations and 70.4% within populations. The distribution of variance was quite similar for the southern populations with 29.3% of the variation among populations and 70.7% within populations. Multi-response permutation procedures gave similar results indicating a significant amount of heterogeneity within groups than expected by chance ($A = 0.04$, $p < 0.000$).

Table 3.7 Population differentiation (F_{ST}, θ_W, G_{ST}) and gene flow (Nm) estimates among Oregon slender salamanders based on 46 variable RAPD markers.

	Wright (1931)		Lynch & Milligan (1994)		Weir & Cockerham (1984)		Nei (1973)	
Groupings	F_{ST} ± SE	Nm	F_{ST} ± SE	Nm	θ_W ± SE	Nm	G_{ST}	Nm
Northern	0.26 ± 0.06	0.7	0.29 ± 0.06	0.6	0.26 ± 0.07	0.7	0.30	1.2
Southern	0.26 ± 0.05	0.7	0.33 ± 0.16	0.5	0.27 ± 0.05	0.7	0.25	1.5
All populations	0.32 ± 0.03	0.5	0.35 ± 0.03	0.5	0.31 ± 0.04	0.6	0.27	1.3

Table 3.8 Analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) to estimate genetic variation within and among populations, and groups of Oregon slender salamanders using 46 variable RAPD markers. Tests of significance (p-value) for variance component statistics (Φ) were calculated using 100 permutations.

	df	%var	Φ	p
Nested Analysis				
Among groups	1	14.4%	Φ_{CT} = 0.14	p < 0.01
Among populations within groups	17	27.0%	Φ_{SC} = 0.32	p < 0.01
Within populations	337	58.6%	Φ_{ST} = 0.41	p < 0.01
Northern Group				
Among populations	10	29.6%	Φ_{ST} = 0.30	p < 0.01
Within populations	198	70.4%		
Southern Group				
Among populations	7	29.3%	Φ_{ST} = 0.43	p < 0.01
Within populations	122	70.7%		

Discussion

Phylogeography

Overall phylogeographic structure suggests a complex history of divergence within the Oregon slender salamander. The most significant differences in phylogeographic structure occur among northern and southern populations. However, the level of population divergence is lower than that shown in members of the *attenuate* clade of Slender salamanders (Genus *Batrachoseps*), in which recent molecular studies revealed a remarkable number of cryptic species (Yanev 1980; Jockusch 1996; Jackman *et al.* 1997).

Slender salamanders are comprised of two deep branching lineages (diverging about 30 million years ago), the *attenuate* and *robust* clades (Wake 1996). The Oregon slender and Inyo Mountain salamander (*B. campi*; found in eastern California) belong to the *robust* clade (Brame & Murray 1968; Jockusch 1996; Wake 1996). Among the *attenuate* clade of Slender salamanders, found mainly in California, Jockusch (1996) showed cytochrome b sequence divergence between populations within recognized species was considerable. The smallest divergence (0.2 %) occurred between populations of the San Gabriel Mountain slender salamander (*B. gabrieli*; see also Wake 1996) and the largest (13.9 %)

between populations of the Relictual slender salamander (*B. relictus*) which is potentially a species complex. Given the large amount of divergence and extreme population subdivision seen in other Slender salamanders, significant amounts of cryptic diversity were expected within the Oregon slender salamander.

Initially, Jockusch (1996) reported a relatively low amount of cytochrome b divergence (0 - 1.6 %) between three southern Oregon slender salamander populations, which was low compared to extreme values seen among the attenuate clade. However, our results based upon more extensive sampling, which included populations from the northern extent of their range (up to 190 km further north), revealed two distinct well-supported mtDNA clades, with between population divergence ranging from 2.07 - 4.26 % (uncorrected; Table 3.3). While this level of differentiation may not be sufficient warrant taxonomic changes (other evidence should also be considered than just mtDNA divergence alone), it suggests this divergence occurred some time ago.

We used the cytochrome b sequence diverge rate of 1.7 % per million years, estimated for the *attenuate* clade (Jockusch 1996), which is close to the commonly used vertebrate rate of 2 % per million years (Brown & Simpson 1982). Although estimates of divergence time among mtDNA can be inexact without calibration, they can be useful for relative comparisons (Moritz *et al.* 1987, Hasegawa *et al.* 1985; Irwin *et al.* 1991). The combined evidence from the mtDNA and RAPD data suggest a scenario of a basal split between the northern and southern Oregon slender clades between 2.1 – 3.0 million years ago (based upon relative rate

estimates; see Li & Graur 1991). The northern part of their current range may have been recently colonized following divergence of the two major lineages. Considering the low divergence (0 – 0.78 %) of haplotypes among northern populations, this may have occurred within the past 0.5 million years. Further, the shared haplotypes among the Columbia River Gorge populations (Viento, Ainsworth, Train Tunnel) and Bull Run suggests there has been insufficient time for lineage sorting of mtDNA haplotypes. However, a selective sweep or high rate of gene flow could account for lack of divergence, but these arguments are countered by the high degree of population subdivision shown by the RAPD analyses among these populations.

Oregon slender salamanders found in the area of the East Mt. Hood population, occupying a dry Ponderosa Pine (*Pinus ponderosa*) dominant forest habitat, are the only plethodontids to occur east of the Cascade Crest outside of the Columbia River Gorge in Oregon. Of interest is whether the Mt. Hood populations were founded from western populations migrating across the Cascade Crest or from the Columbia River Gorge region to the north. Our results suggested the later with the East Mt. Hood population most closely related to the Gorge Post Creek Road population. It shows mtDNA haplotype most closely related to the Post Creek Road haplotype and showed the lowest genetic distance (Manhattan distance =

0.15), based on the RAPD analyses, with Post Creek Road. Further, Kirk & Forbes (1991) hypothesized that more populations of Oregon slender salamander may occur in the region between those found east of the Cascade crest and the Columbia River Gorge. In sum, the low mtDNA divergence and decreased population subdivision supports their view.

In contrast to the north, the southern clade shows greater divergence between populations (0.26 – 2.20 %). The southern region has had a dynamic geological (e.g., volcanism, flooding) and ecological history and any number of factors may have contributed to vicariance in the southern region and contributed to mtDNA lineage sorting. Currently, however, there is possible secondary contact between the two major clades (northern and southern) in the geographic area between the Jackson Five and Silver Creek populations. The Jackson Five population does not cluster with the rest of the members of the northern mtDNA clade in the RAPD neighbor-joining phylogenetic tree, but instead clusters with *mid-range* populations, all of which have a southern haplotype. This could be the result of male-mediated gene flow resulting from contact in the region or the inability of the RAPD markers to infer these phylogenetic relationships due to homoplasy. Moreover, there may be a phylogeographic barrier in this region; however, more extensive geographic sampling will be needed in this region to resolve these questions.

Population Structure

In addition to the regional differences in phylogeographic structure, our results suggest a considerable amount of fine-scale local population genetic structure within the Oregon slender salamander. Population structure is influenced by both gene flow and random drift; however, in studies of natural populations it is often difficult determine their relative contributions, and gene flow estimates from F_{ST} estimates are often inappropriate. Our pairwise-F_{ST} analyses across all populations suggests equilibrium between random genetic drift and gene flow, with gene flow predominating at local scales and genetic drift predominating at larger geographic scales. Although given the different histories of the two major lineages as shown by the mtDNA analyses, the regional pairwise-F_{ST} analyses may be more reflective of the actual population structure.

For regional analyses, by considering the northern and southern groups separately, the hypothesis of equilibrium between gene flow and random genetic drift was rejected. Generally, this result would suggest that inferences of gene flow would be inappropriate; but if either gene flow or genetic drift dominate population structure such an estimate may be more accurate (Hutchinson & Templeton 1999). For example, if gene flow (Nm) estimates are large (greater than four migrants per generation) or when F_{ST} estimates are very large ($Nm < 1$ migrant per generation) then the conversion is acceptable.

The inferred gene flow estimates, for both the northern and southern populations, based on regional F_{ST} are low (Nm = 0.6 and 0.5 respectively). Classically, the migration of one individual per generation was considered adequate to offset the negative effects of drift; however, it has recently been suggest that up to 10 individuals may be needed to offset drift (Wright 1931, Mills & Allendorf 1996). Therefore, the overall pattern suggested by the pairwise-F_{ST} analyses indicates that genetic drift may contribute more to population structure than gene flow for both the northern and southern groups.

Oregon slender salamanders occur sympatrically with Larch Mountain salamanders (*Plethodon larselli*) on the south bank of the Columbia River. Along the south bank, Larch Mountain salamanders showed considerable differentiation in cytochrome b haplotypes (0 - 8.9 %) among populations (Chapter 2). In addition, they showed extreme population subdivision (F_{ST} = 0.51, Nm = 0.2) among populations and reduced expected heterozygosity (H_e = 0.17) within populations using RAPD markers (34 loci), suggesting southern population structure may have resulted from a founder event by dispersal of salamanders from the north. In contrast, as described previously Oregon slender salamanders appear to have more recently expanded northward from the south into the Gorge. The difference between the Oregon slender and Larch Mountain salamander illustrates how historical events (founder effect vs. expansion) can contribute to population structure.

Conservation Unit Designation

It is imperative for conservation units to be defined rigorously based upon an operational definition or they run the risk of becoming an arbitrary taxonomic unit. Based upon the Management Unit definition requiring significant divergence of mitochondrial alleles or nuclear alleles as suggested by Moritz (1994a,b; Moritz et al. 1995), there is strong evidence for at least the northern and southern groups to be considered separate Managements Units. In fact, an argument could be made to consider these groups separate Evolutionary Significant Units; however, given our limited sampling in the region between Jackson Five and Silver Creek and the absence of any apparent phylogeographic barriers, such designation may not be prudent at this time. Therefore, we suggest three overlapping Management Units be recognized corresponding to the *northern-most*, *mid-range*, and *southern-most* groups. If future studies confirm reciprocal monophyly of the northern and southern clades based upon the mtDNA analyses, a revision to ESU status may be warranted.

Conservation Implications

Our results indicate Oregon slender salamanders are comprised of two historic lineages and three regional sub-groupings. Populations within these groups are highly structured across the landscape as a consequence of limited gene flow among populations, which may be reflective of their limited dispersal, low reproductive and specific habitat characteristics. Although Oregon slender

salamanders show historic differentiation and population subdivision, increasing habitat alteration and rural development may further fragment their habitat and decrease their population viability. Therefore, designation of three Management Units will provide an important framework for prioritizing conservation efforts for the species under the Northwest Forest Plan (U.S. Forest Service & U.S. Bureau of Land Management 1994). The spatial arrangement of federal late-successional forest reserves, designed to enhance the persistence of Northern spotted owls (*Strix occidentalis*), may not be adequate to preserve the genetic diversity contained within the Oregon slender salamander. However, by focusing management efforts with respect to management unit designations, it may be possible to mitigate for differential threats to their persistence across their range.

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CHAPTER 4

PHYLOGENETIC RELATIONSHIPS AMONG THE TORRENT SALAMANDERS (Genus: *Rhyacotriton*).

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Abstract

We used three mitochondrial genes to infer phylogenetic relationships among species within the morphologically conserved Torrent salamanders (Family Rhyacotritonidae). Cytochrome b (778 bp), 12S ribosomal RNA (360 bp), and 16S ribosomal RNA (560 bp) sequences were obtained from four Torrent salamander species (*Rhyacotriton olympicus*, *R. kezeri*, *R. variegatus*, and *R. cascadae*) sampled from 26 localities (n = 78 individuals). Each recognized species represented a well-supported monophyletic group based on analyses with each gene. The greatest pairwise sequence divergences occurred among taxa using the cytochrome b gene which indicated differences ranging from 3.5 % between *R. olympicus* and *R. kezeri* to 11.8 % between *R. kezeri* and *R. variegatus*. Ribosomal gene substitutions were lower, with pairwise differences among taxa about half that of cytochrome b. Three methods of inference (maximum parsimony, minimum evolution, and maximum likelihood) were used to construct phylogenetic trees. Trees constructed using cytochrome b sequences (separately) and combined analyses using all three gene regions were most fully resolved; 16S sequences yielded the least resolved trees. Overall, there were only minor differences in support and topology for trees constructed using different evolutionary models or weighting schemes. Maximum parsimony and maximum likelihood methods produced trees with a higher number of supported branches, each with higher support values (bootstrap values) per branch, compared to minimum evolution

methods. Results are consistent with those based on allozymes suggesting that three main groups of Torrent salamanders (*R. variegatus*, *R. cascadae,* and the ancestor of *R. olympicus* and *R. kezeri*) became isolated during the late Miocene. *R. olympicus* and *R. kezeri* apparently diverged about 4.5 MYA. Divergence among major clades (*north coast*, *Oregon* clade and *California* clade) within *R. variegatus* also occurred during this period between (1.8 – 4.7 MYA). These results further support the need for conservation units to be recognized within *R. variegatus* as management, listing and recovery efforts are currently being prioritized.

Introduction

Accurate phylogenetic reconstruction is very gene dependent. It is well documented that different genes from the same taxa can yield different phylogenies (Hedges 1994, Russo et al. 1996). Namely, phylogenies not reflective of true species relationships can result from a number of factors including homoplasy of quickly evolving genes, a weak phylogenetic signal from slowly evolving genes, or substitution rate heterogeneity among lineages. Moreover, even within the mitochondrial (mtDNA) genome, which consists of a single non-recombining linkage group, the rate and pattern of substitution among genes and even within genes can vary considerably (Brown et al. 1982, Miyata et al. 1982, Moritz et al. 1987, Edwards et al. 1991). For example, the mtDNA protein coding genes have

high rates of substitution and can quickly become saturated (Roe et al. 1985, Desjardins and Morias 1990, Moritz et al. 1992), while ribosomal genes often have one-half to one-third slower substitution rates and may not resolve closely related groups (Moritz et al. 1987). Subsequently, gene substitution rates and potential divergence time among taxa must be considered carefully in order to make robust inferences about taxonomic relationships.

Salamanders are highly morphologically conserved yet show deep genetic divergences among and within families or conspecifics (Wake 1991, Tilley and Mahoney 1996, Camp et al. 2000). Subsequently, a number of mitochondrial genes have been particularly useful for inferring both intra-specific and inter-specific phylogeny in salamanders (Chapter 2; Chapter 3; Chapter 6; Hay et al. 1995; Alexandrino et al. 2000; Garcia-Paris and Wake 2000; Wagner and Haig, in review; Wagner et al., in review). Arguably, the most extensively used locus for population and species relationship studies in salamanders is the protein coding cytochrome b gene that has an estimated sequence divergence rate between 0.7 - 1.0 % per million years (Spolsky et al. 1995, Tan and Wake 1995, Jockrusch 1996, Caccone et al. 1997, Alexandrino et al. 2000). However, this locus can quickly become saturated and bias phylogenetic inferences when there is substantial divergence among taxa (Graybeal 1993, 1994). The divergence rate of mtDNA ribosomal genes (12S and 16S) is slower, between 0.3-0.6 % per million years; therefore they have been used to infer relationships among species and families within salamanders (Hedges and Maxson 1993, Hay et al. 1995, Caccone et al. 1997).

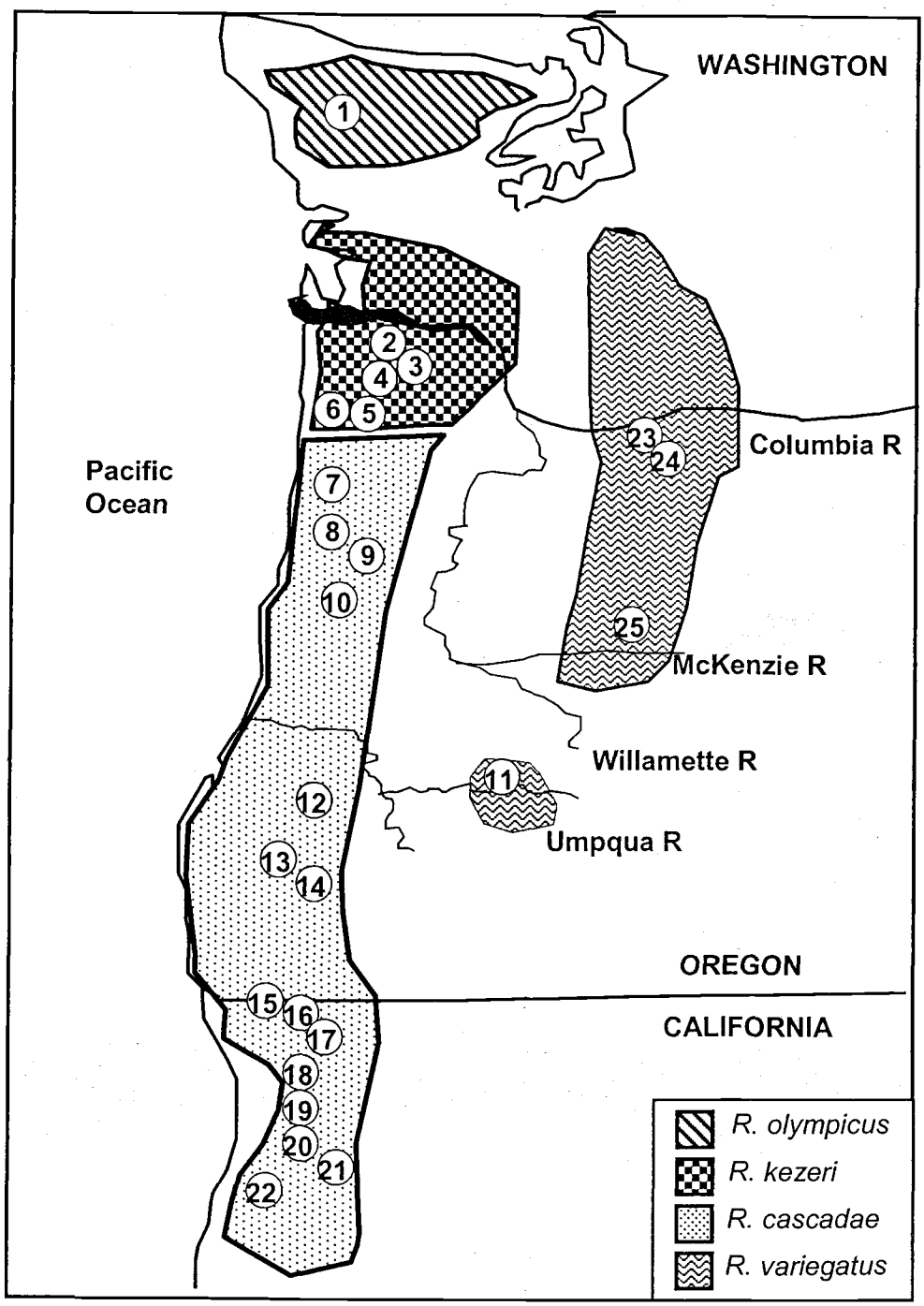
Highly variable substitution rates among genes can make it difficult for a single gene to fully resolve phylogenetic relationships among groups that have variable levels of divergence. Therefore, we compared three mtDNA genes (cytochrome b, 12S ribosomal RNA, and 16S ribosomal RNA) in separate and combined analyses to resolve relationships among Torrent salamander species (Family Rhyacotritonidae: *Rhyacotriton olympicus*, *R. kezeri*, *R. variegatus*, *R. cascadae*). Previous allozyme analyses indicated Torrent salamanders had extremely variable levels of divergence among lineages (Good et al. 1987, Good and Wake 1992).

Torrent salamanders represent a deeply divergent monophyletic family comprised of four recognized species endemic to the U.S. Pacific Northwest (Figure 4.1; Good et al. 1987, Good and Wake 1992). Two vicariant events are hypothesized to have resulted in the present pattern of speciation among the Torrent Salamanders (Good and Wake 1992). First, volcanic activity during the Miocene is suggested to have isolated present day *R. cascadae*, *R. variegatus*, and the ancestor of *R. olympicus* and *R. kezeri*. Next, *R. olympicus* and *R. kezeri* are thought to have been isolated by a large river created by glacial expansion during the late Pliocene/early Pleistocene.

Despite the variable timing of divergence among Torrent salamander species, they are remarkably morphologically conserved, have similar life histories, and occupy ecologically similar habitats (Good and Wake 1992). Primarily found in cold, clear, fast-flowing small streams and headwater areas associated with late-

successional forests, they appear to be sensitive to timber harvest and related disturbances (Bury and Corn 1988, Corn and Bury 1989, Bury et al. 1991, Welsh and Lind 1992, Diller and Wallace 1996). Currently, they are provided protection under the Northwest Forest Plan (U.S. Forest Service and U.S. Bureau of Land Management 1994); however, *R. variegatus* was recently denied protection via listing under the U.S. Endangered Species Act due to lack of information about the genetic status of populations (Federal Register 60: 33785). A recent extensive cytochrome b study of intra-specific phylogeny within *R. variegatus* revealed three historic lineages (*north coast*, *Oregon*, and *California* clades) which are suggested to have separate conservation unit status when considered for management or listing options (Chapter 6; Wagner and Haig, in review). Examination of phylogenetic relationships among Torrent salamander species will give perspective to the amount of divergence observed within Torrent salamander species and aid in designing management strategies and assigning conservation unit designations.

Figure 4.1 Sampling locations and putative ranges of Torrent salamanders. See Table 4.1 for site identification.


WASHINGTON
Pacific Ocean
Columbia R
McKenzie R
Willamette R
Umpqua R
OREGON
CALIFORNIA
1
2
3
4
5
6
7
8
9
10
11
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17
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20
21
22
23
24
25
R. olympicus
R. kezeri
R. cascadae
R. variegatus

Figure 4.1

Materials and methods

Mitochondrial DNA amplification and sequencing

We sampled three individual Torrent Salamanders from each locality (Table 4.1, Figure 4.1). Animals were non-lethally sampled by clipping approximately 1 cm of tissue from the distal end of the tail. All samples were placed in a cryogenic tube with 1 ml of buffer (100 mM Tris HCl pH 8.0, 100 mM EDTA pH 8.0, 10 mM NaCl, 0.5 % SDS) and stored at ambient temperatures until transferred to a -80°C ultra-cold freezer upon arrival in the laboratory.

DNA was extracted and purified by a modified phenol/chloroform extraction procedure (Maniatis et al. 1982). First, tissue (2 μg) was digested in buffer (400 mM Tris-HCl pH 7.5, 100 mM EDTA, 250 mM NaCl, Proteinase K 600 μg/ml) overnight at 55°C. Then two phenol extractions were performed followed by two chloroform/isoamyl alcohol (25:1) extractions. DNA was concentrated and cleaned by centrifugation dialysis using a microcon-50 filter (Millipore). Samples were washed twice in the filter with 400 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Finally, extraction quality was checked by agarose gel electrophoresis, and concentration estimated by fluorimetry using a Hoefer TKO 100.

The polymerase chain reaction was used to amplify three different mitochondrial DNA gene regions. Primers used for cytochrome b fragment (~850 bp) included MVZ15 (5'-GAACTAATGGCCCACAC(A/T)(A/T)TACGNAA-3') and MVZ16 (5'-AAATAGGAAATATCATTCTGGTTTAAT-3'), 12SA-5' (5'-

AAACTGGGA-TTAGATACCCCACTAT-3') and 12SB-3' (5'-GAGGGTGA-CGGGCGGTGTGT-3') for the 12S fragment (~360bp), and 16SA-5' (5'-ACAAGTGATTACCTTTGCAT-AATACCG-3') and 16SB-3' (5'-TTTAGTAA-ATTAAGCTTTGACGCTATTT-AGTAAG-3') for the 16S region (~380bp; Kocher et al. 1989, Palumbi et al. 1991, Moritz et al. 1992). Each PCR reaction used 100 ng of DNA in a 50 µl reaction volume with the following cocktail concentrations: 0.5 units of Taq Polymerase Gold (Perkin Elmer), 5 µl of the supplied 10X reaction buffer; 100 µM of each nucleotide (dATP, dCTP, dGTP, dTTP); 2 mM MgCl and 1 mM of each primer. A MJ Research programmable thermocycler (PTC 100) was used for all amplifications with the following steps: an initial 10 min. denaturation at 93°C, followed by 40 cycles of denaturation for 1 min. (93°C), annealing for 1 min. (52°C), and extending for 2 min. (72°C). A final extension for 10 min. (72°C) followed the cycles and the reaction was held at 4°C until removed from the cycler. Amplifications were prepared for sequencing by extracting fragments from 1% agarose gels using an ultra-free-mc 0.45 filter (Millipore). The template was concentrated by washing the supernatant using a microcon-50 filter (Millipore). Sequences generated by Big-Dye Terminator cycle sequencing (Perkin Elmer) based on the Sanger method, were read using an

Applied Biosystems (373A) sequencer at the Oregon State University Central Services Laboratory. All fragments were bi-directionally sequenced by terminal priming with the amplification primers. Alignments of sequences were made by eye, using the Genetic Data Environment (Smith et al. 1992). Sequence gaps were aligned based upon inferred secondary structures for mitochondrial 16S genes (Guttell et al. 1994).

Table 4.1. Sampling localities (latitude and longitude) for Torrent salamanders (Genus *Rhyacotriton*) and corresponding Genbank accession numbers for mitochondrial sequence data (cytochrome b, 12S ribosomal RNA, and 16S ribosomal RNA).

Species Population	Locality Lat	Long	County, State
R. olympicus			
1. Olympic	-124.276	48.044	Clallam, WA
R. kezeri			
2. Astoria	-123.433	46.163	Clatsop, OR
3. Ranch Ck	-123.519	45.793	Clatsop, OR
4. Falls Ck	-123.390	45.614	Tillamook, OR
5. Tillamook	-123.453	45.643	Tillamook, OR
6. Little Nestucca	-123.892	45.137	Tillamook, OR
R. variegatus			
7. Ball Mountain	-123.940	44.920	Tillamook, OR
8. Siletz	-123.941	44.656	Lincoln, OR
9. Mary's Peak	-123.551	44.495	Benton, OR
10. Little Lobster Ck	-123.704	44.310	Benton, OR
11. N. Scaredman	-122.794	43.397	Douglas, OR
12. Cow Creek	-123.632	42.904	Douglas, OR
13. N. Galice	-123.694	42.539	Douglas, OR
14. Galice	-123.631	42.543	Douglas, OR
15. Lower Division Rd	-124.025	41.870	Del Norte, CA
16. M. Fork Smith R	-124.012	41.770	Del Norte, CA

Table 4.1 continued

	17. S. Fork Smith R	-123.887 41.550	Del Norte, CA
	18. Omagar	-123.974 41.455	Humboldt, CA
	19. Dry Ck	-124.019 40.843	Humboldt, CA
	20. Graham Ck	-123.847 40.714	Humboldt, CA
	21. University Hills	-123.472 40.650	Trinity, CA
	22. Chadbourne	-123.761 39.628	Mendocino, CA
R. cascadae			
	23. Wahkeena	-122.114 45.569	Multnomah, OR
	24. Larch Mountain	-122.078 45.522	Multnomah, OR
	25. HJ. Andrews	-121.054 45.456	Lane, OR
Outgroup			
	26. *Plethodon larselli*	-122.123 45.643	Hood River, OR

Phylogenetic methods

Three methods of phylogenetic inference were used to evaluate relationships among haplotypes for mitochondrial gene regions (cytochrome b, 12S, and 16S) in separate and combined analyses. Phylogenetic trees were constructed using maximum parsimony (Hennig 1966, Swofford et al. 1998), minimum evolution distance (Swofford 1998), and maximum likelihood (Felsenstein 1981, Huelsenbeck and Crandall 1997) methods. The merits and evolutionary assumptions of each method have been debated previously; however, trees yielding similar topologies based on different methods are suggested to more likely represent the true phylogenetic relationships (Hasegawa and Fujiwara 1993, Huelsenbeck and Hillis 1993, Kuhner and Felsenstein 1994, Tateno et al. 1994).

All phylogenetic trees were generated using the program PAUP* 4.0b1 (Swofford 1998). Maximum parsimony was used to search for trees of shortest length by heuristic searches made using random stepwise addition with 10 replications, tree-bisection-reconnection branch-swapping, and branches collapsed to zero-length using the MULPARS option. To evaluate if homoplasy at individual codon positions in cytochrome b influenced tree topology and support, trees were compared using equally weighted character positions and differential weighting at each codon position: weightings were 3:6:1 for first, second, and third positions with a transition: transversion ratio of 3:1. For 12S and 16S sequence alignments, effects of insertions/deletions (indels) on tree topology were evaluated by

comparing trees generated by treating gaps either as missing data or as a 5th character. Finally, the consistency (CI, Kluge and Farris 1989), retention (RI, Farris 1989) and homoplasy (HI, Farris 1989) indices were calculated to evaluate tree support.

Distance trees were calculated using the minimum evolution algorithm (Swofford 1998). Heuristic searches were performed based on Kimura 2-parameter distances with empirically derived substitution rates and a 0.5 gamma distribution, tree-bisection-reconnection branch swapping and zero-length branches were collapsed for tree score calculations.

Fifty-six models of DNA substitution for the data were compared using the program MODELTEST v3.0 (Posada and Crandall 1998) to estimate parameters for the final maximum likelihood tree. The program compares each model by two methods: either by nesting models and evaluating likelihood scores or by Akaike information criterion (Akaike 1974). We compared trees generated from model parameters suggested by both methods.

Consensus bootstrap trees were constructed for each phylogenetic method using the 50 % majority consensus option in order to analyze support for each branch (100 replications, Felsenstein 1985). Branches supported by bootstrap values greater than 70% were found to have a 95% probability of recovering the correct topology (Hillis and Bull 1993); therefore, we considered branch values greater than 70% to be "well-supported" and trees with values greater than 50% to be "supported".

Degree of sequence saturation was evaluated for the cytochrome b gene region based on plots of total distance versus percent sequence divergence for each codon position, and for transitions and transversions at each codon position. Partition homogeneity tests were used to test for significant differences among genes regions. Alternative topologies of phylogenetic trees were compared for significant differences using the Kishino-Hasegawa test in PAUP* 4.0b1 (Swofford 1998). Outgroups species were comprised of sequences from a representative individual of the Larch Mountain salamander (*Plethodon larselli)*. Torrent salamander secondary structures for the most variable region of the 16S gene were inferred by overlaying sequences on published vertebrate mtDNA secondary structures (Gutell 1994).

Results

Sequence variation

There were significant differences in sequence variation among genes and among Torrent salamander species. The greatest differences among Torrent salamanders occurred in the cytochrome b gene (Table 4.2; 778 bp, 180 variable sites), followed by 12S (Table 4.3; 360 bp, 44 variable sites including 13 indels) and then 16S (Table 4.4; 560 bp, 68 variable sites including 26 indels) genes. Cytochrome b sequences included 41 synonymous and 139 non-synonymous

substitutions among Torrent salamander sequences (excluding the outgroup). The substitution ratio among codon positions was 2.8:1:6 for first, second and third positions. Sequence divergence (uncorrected) among Torrent salamander species was substantial. The greatest pairwise distances occurred among cytochrome b haplotypes and ranged from 3.5 % between *R. olympicus* and *R. kezeri* to 11.8% between *R. kezeri* and *R. variegatus* (Table 4.5), about half the amount of divergence seen among ribosomal haplotypes.

Table 4.2 Mitochondrial DNA sequence variation (180 variable sites) in 778 base pairs of the cytochrome *b* gene for Torrent salamanders (see Table 4.1 and Figure 4.1 for locations).

		Sequence Position
		3479112334455566778999911111111111111111111111122222222222222222222233333333333333444444444
		0546959045045810137012223344444455677788899001112233355577788999122345567778990223333
	Population	1135280123474654570360814568281470250395817885748473235738236023 45
1.	Olympic	TCTCCTCTTTGCTTTTCCCAACCAACCTTCACACATACTGTTCTTGCTGGAATTTAAGGTTCGTTACCCGTCAACTATAGCGCAAGAATC
2.	Astoria	C..AA.....G...C.TG.....T.....G.....
3.	Ranch Ck	C..AA.....G...C.TG.....T.....G.....
4.	Falls Ck	C..AA.....G...C.TG.....T.....G.....
5.	Tillamook	C..AA.....G...C.TG.....T.....G.....
6.	L. Nestucca	C..AA.....G...C.TG.....T.....G.....
7.	Ball Mt	CT.AA.TC.A.T..CATTATGTTGC.T.CT.TT....TCAG.TC...CC.G.AC..C.A..G...C.TTACT.....CGA.....T..T
8.	Siletz	CT.AA.TC.A.T..CATTATGTTGC.T.CT.TT....TCAG.TC...CC.G.AC..C.A..G...C.TTACT.....CGA.A....T..T
9.	Mary Pk	CT.AA....A.T....TTA..TTGC.T.CT.TT....TCAG.TC...CC...AC..C.A..AA..C.TT.CT.....CGA.....T
10.	L. Lobster	CT.AA.TC.A.T..ACTTA..TTGC.T.CT.TT.G..TCAG.TC...CC.G.AC..C.A..AA..CTTT.CT.....CGA.....T
11.	N.Scaredman	CT.AA.TC.A.T..A.TTA..TTGT.T.CT.TT....TCAG.TC...CC.G.AC..CAA..AA..CTTT.CT.....CGA.....T
12.	Cow Ck	CT.AA..C.A.T....TTA..TTGCTT..T.TT....TCAG.TC...CC.G.AC..C.A..AA..C.TT.CT..T..CGA.....T
13.	N.Galice	CT.AA..C.A.T....TTA..TTGC.T..T.TT....TCAG.TC...CC.G.AC..C.A..AA..C.TT.CT..T..CGA.....T
14.	Galice	CT.AA..C.A.T....TTA..TTGC.T..T.TT....TCAG.TC...CC.G.AC..C.A..AA..C.TT.CT..T..CGA.....T
15.	Lower Div.	CT.AA..C.A.T....TTA..TTGC.T.CT.TT....TCAG.TC...CC.GGAC..C.A..AA..C.TT.CT...C.CGA.....T
16.	M.ForkSmith	CT.AA..C.A.T....TTA..TTGC.T.CT.TT....TCAA.TC...CC.G.AC..C.A..AA..C.TT.CT....GCGA.....CT
17.	S.ForkSmith	CT.AA.TC.A.T....TTA..T.GC...CT.TT....TCAG.TC...CC.G.A..GC.A..A..CC.TTACT....GCGA....G....T
18.	Omagar	CT.AA.TC.A.T....TTA.GT.GC...CT.TT....TCAG.TC...CC.G.A.CGC.A..AA.CC.TTACTG....CGA.....T
19.	Dry Ck	CT.AA.TC.A.T....TTA.GT.GC...CT.TT....TCAG.TC...CC.G.A.CGC.A..AA.CC.TTACTG....CGA.....T
20.	Graham	CT.AA.TC.A.T....TTA.GT.GC...CT.TT....TCAG.TC...CC.G.A..GC.A..AA.CC.TTACTG....CGA.....T
21.	Chadbourne	CT.AA.TC.A.T....TTA..T.GC...CT.TT....TCAG.TC...CC.G.A..GC.A..GA.CC.TTACT.....CGA.....T
22.	Univ. Hill	CT.AA.TC.A.T....TTA.GT.GC...CT.TT....TCAG.TC...CC.G.A.CGC.A..AA.CC.TTACTG....CGA.....T
23.	Wahkeena	CTCAAC.CC.A..C..TT.T..T.CTA..TTT.T.C...AG.T.C.TC....AC.GC.ACC..C.C.TT.CT.C....GAT.TG...G..
24.	Larch Mt	ATCAACTCC.A.CC..TT.T..T.CTA..TTT.T.CG..TG.T.C.TC....AC.GC.ACC..C.C.TT.CT.C....GAT.TG...G..
25.	HJ Andrews	A.CAACTCC.A.CC..TT.T..T.CTA..TTT.T.C...TG.T.CATCAA..AC.GC.AC...C.C.TT.CT.C....GAT.TG.A.G..
26.	*P.larselli*	...AA.TC.AT.....TATC....TTA....T...A.A.TGATC...A.....C.CCAT..A...GTAT.CT..AATCCAA.TCT.T...

Table 4.2 Continued

	Sequence Position
	4444444444444444444555555555555555555555556666666666666666666666777777777777777777777777
	3344456666667778999001112444455666677889990011122334556667778889000111122233444555666667777
	8924800235681477289460392456919045706281271624514062043690231480268245703402579269134566678
1. Olympic	ATAAGTAGTAGTGGGGGATCGCTTCGCCATGTCCAGCAGAATAAACTCTCCATCCTATTGAATAAATTACACGTGTATCCTTTACTGGCT
2. Astoria	G.....C.....A...C.....C..C.....C.T..GC...C...G..A.....A..C.C.....AGA...
3. Ranch Ck	G.....C.....A...C.....C..C.....C.T..GC...C...G..A.....A..C.C.....AGA...
4. Falls Ck	G.....C.....A...C.....C..C.....C.T..GC...C...G..A.....A..C.C.....AGA...
5. Tillamook	G.....C.....A...C.....C..C.....C.T..GC...C...G..A.....A..C.C.....AGA...
6. L. Nestucca	G.....C.....A...C.....C..C.....C.T..GC...C...G..A.....A..C.C.....AGA...
7. Ball Mt	..TT.CC...A...T..CC..T.C.ATTTC.....AT....C.T..A.CTT...AAG.....CG.TA....TAC.C.C..CC....AAT.
8. Siletz	..TTACC...A...T..CC.AT.C.ATTTC.....AT..T.C.T..A.CTT...AAG.....CG.TA....TACACCC..CC....AAT.
9. Mary Pk	..TT.CC.....CC..TCCTAT..C...T.AT....C.T..C.CTT...AGG.....GGTA....TAC.C.CA.CA....TATC
10. L. Lobster	..TT.CC...A.A.A..CC..TCC.AT..C..TT.AT.A..C.T.T..CTT...AGG.....GGTA....TAC.C.C..CA....AATC
11. N. Scaredman	..TT.CC...A.A...ACC..TCC.AT..C..T..AT.A..C.T..C.CTT...AGG.....GGTA....TAC.C.C..CAG...AATA
12. Cow Ck	.CTT.CC...A.....CC..TCC.AT..C.....AT.A.GC.T..C.CTTG..AAG.C....G.TA....TAC.C.C..CA.G.CTATG
13. N. Galice	..TT.CC...A.....CC..TCC.AT..C.....AT.A..C.T..C.CTTG..AAG.C....G.TA....TACAA.C..CA...CAATA
14. Galice	..TT.CC...A....A.CC..TCC.AT..C.....AT.A..C.T..C.CTTG..AAG.C....G.TA....TACAA.C..CA...CAATA
15. Lower Div.	..TT.CC..GA.....CC..TCC.AT..C.....AT.A..C.T....CTT...AAG....G...TAC...TAC.C.C..CA...CAATA
16. M.ForkSmith	..TT.CC..GA..A...CC..TCC.AT..C.....AT.A..C.T..C.CTT...AAG....G...TAC...TAC.C.C..CA...CT.TG
17. S.ForkSmith	..TT.CCA....A....CCA.TCC.AT..C.....AT.A..C.T..C.CTT...AAG..A...G.TA....TAC.C.C..CC....T.TG
18. Omagar	..TT.CC.....A....CCA.TCC.ATT.C.....AT.A..C.T..C..TT...AAG.....G.TA....TAC.C.C..CC....A.T.
19. Dry Ck	..TT.CC.....A....CCA.TCC.AT..C.....AT.A..C.T..C.CTT...AAG.....G.TA....TAC.C.C..CC....T.T.
20. Graham	..TT.CC.....AA...CCA.TCC.AT..C.....AT.A..C.T..C.CTT...AAG..A...G.TA....TAC.C.C..CC....AAG.
21. Chadbourne	..TT.CCA....AA...CCA.TCC.AT..C.....AT.A..C.T..C.CTT...AAG..A...G.TA....TAC.C.C..CC....AAG.
22. Univ. Hill	..TT.CC.....A....CCA.TCC.AT..C.....AT.A..C.T..C.CTT...AAG.....G.TA....TAC.C.C.GCC....A.T.
23. Wahkeena	..T..CC....C.....C.....T.CA...GAT.A....T....C.T.CTGA...AG....CCC.....C...C..C.....ACT.
24. Larch Mt	..T..CC.A..C.....C.....T.CA...GATGA....T....C.T.CTGA...AG....CCCCAC..C...C..C.....AC..
25. HJ Andrews	..T..CCC...C.....C.....T.CA...GAT.A....T....C.T.CTGA.A.AG....CCC.....C...C..C.....A..G
26. *P.larselli*	T.....C...ACA...ACC..T..T.TTGCA...CATGCC....C.AT.TTTCTTA...T.T...CA....TCA????????????????

Table 4.3 Mitochondrial DNA sequence variation (44 variable sites including indels (-)) in 360 base pairs of the 12S rRNA gene for Torrent salamanders (see Table 4.1 and Figure 4.1 for locations).

Population	Sequence position 3122556666791111111222222222222333333333333 6570713490803456682222255567889000123455555 1495343156784573218301247230346 7
1. Olympic	CCC-G-TCAC-CACTCTACAATGAGAATA-AG---CGA-ATTTA
2. Astoria	A..-.-.....C.....G-G.---...-.....
3. Ranch Ck	A..-.-....C.....C.....-..---...-.....
4. Falls Ck	A..-.-....-.....C.....G-G.---...-.....
5. Tillamook	A..-.-....C.....C.....-..---...-.....
6. L. Nestucca	A..-.-....C.....C.....-..---...-.....
7. Ball Mt	...-.CCT.--.CTC.C...GA.G..GA.-.A---TAG-.ACGG
8. Siletz	...-.CCT.--.CTC.C...GA.G..GA.-.A---TAG-.ACGG
9. Mary Pk	...-.CCT.--.CTC.....GA.G..GA.-.A---.AG-.ACGG
10. L. Lobster	...-.CCT.--.CTC.....GA.G..GA.-.A---.AG-.ACGG
11. N. Scaredman	...-.CCT.--.CTC.....GA.G..GA.-.A---.AG-.ACGG
12. Cow Ck	...-.CCT.--.CTC.....GA.G..GA.-.A---.AG-.ACGG
13. N. Galice	...-.CCT.--.CTC.....GA.G..GA.-.A---.AG-.ACGG
14. Galice	...-.CCT.--.CTC.....GA.G..GA.-.A---.AG-.ACGG
15. Lower Div	...-.CCT.--.CTC.....GA.G..GA.-.A---.AG-.ACGG
16. M.ForkSmith	...-.CCT.--.CTC.....GA.G..GA.-.A---.AG-.ACGG
17. S.ForkSmith	...-.CCT.--.CTC..T..GA.G...A.-.A---TAG-.ACGG
18. Omagar	...-.CCT.--.CTC..T..GA.G...A.-.A---TAG-.ACGG
19. Dry Ck	...-.CCT.--.CTC..T..GA.G...A.-.A---TAG-.ACGG
20. Graham	...-.CCT.--.CTC..T..GA.G...A.-.A---TAG-.ACGG
21. Chadbourne	...-.CCT.--.CTC..T..GA.G...A.-.A---TAG-.ACGG
22. Univ. Hill	...-.CCT.--.CTC..T..GA.G..GA.-.A---.AG-.ACGG
23. Wahkeena	ATA-ACC----TC.CAC.TC.A.G...A.-.---TAG-GA..G
24. Larch Mt	ATA-ACC----TC.CAC.TC.A.G...A.-.---TAG-GA..G
25. HJ Andrew	ATA-ACC----TC.CA..TCGAAG...A.-.---TAG-GA..G
26. *P.larselli*	A..C.CC.C--TC.CT..A.GAAG--TAG-.A---AAG-GA.G.

Table 4.4 Mitochondrial DNA sequence variation (68 variable sites including indels (-)) in 560 base pairs of the 16S rRNA gene for Torrent salamanders (see Table 4.1 and Figure 4.1 for locations).

Sequence Position

```
                  7344788888111111111222222222222222222222222222222222222222222222222222233333333333333333334444444444455
                  2897013451111557890011122222244444445555555666666666677777777778888990111134477788888990112233566O3
                          0158349050938901268901235673456789012345678901234567890145126248950523556789576O1691592530

1.  Olympic       CTTATCTTTAAAGCTCACAAT------TTTATTTAAAATA--------------ATAAAAATAGTGA-GGAATT--GCACATTCCC-ATCCCTTACAAT
2.  Astoria       ............T..CT...---------.....C...-----------------------.......-A.....--AA.A.--TTT-.AAATA..GT.C
3.  Ranch Ck      ............T..CT...---------.....C...-----------------------.......-AA.....--AA.A.--TTT-.AAATA..GT.C
4.  Falls Ck      ............T..CT...---------.....C...-----------------------.......-A.....--AA.A.--TTT-.AAATA..GT.C
5.  Tillamook     ............T..CT...---------.....C...-----------------------.......-A.....--AA.A.--TTT-.AAATA..GT.C
6.  L Nestucc     ............T..CT...---------.....C...-----------------------.......-A.....--AA.A.--TTT-.AAATA..GT.C
7.  Ball Mt       A....T.....A...TT...-------.A.....---TA.------------------...TA.T-..-A...C.--AA.A---TTTT.AAATA..GT..
8.  Siletz        A....T.....A...TT...-------.A.....---TA.------------------...TA.T-..-A...C.--AA.A---TTTT.AAATA..GT..
9.  Mary Pk       ............A...TT...-------.G.....---TA.------------------..GTA.T-..-A...C.--AA.A---TTTT.AAATA..GT..
10. L Lobster     A...........A...CT...-------.A.....---TA.------------------..GTA.T-..-A...C.--AA.A---TTTT.AAATA..GT..
11. N Scared      ............A...TT...-------.G.....---TA.------------------..GTA.T-..-AA.....--AA.A---TTTT.AAATA..GT..
12. Cow Ck        ............A...CT...-------.A.....---TA.------------------..GTA.T-..-A...C.--AA.A---TTTT.AAATA..GT..
13. N Galice      ............A...CT...-------.A.....---TA.------------------..GTA.T-..-A...C.--AA.A---TTTT.AAATA..GT..
14. Galice        ............A...CT...-------.A.....---TA.------------------..GTA.T-..-A...C.--AA.A---TTTT.AAATA..GT..
15. Lower Div     ............A...TT...-------.A.....---TA.------------------..GTA.T-..-A...C.--AA.A---TTTT.AAATA..GT..
16. MFSmith       ............A...TT...-------.A.....---TA.------------------..GTA.T-..-A...C.--AA.A---TTTT.AAATA..GT..
17. SF Smith      ............A...TT...-------.A.....---TA.------------------..GTA.T-..-A.G.CC--AA.A---TTTT.AAATA..GT..
18. Omagar        ............A...TT...-------.A.....---TA.------------------..GTA.T-..-A.G.CC--AA.A---TTTT.AAATA..GT..
19. Dry Ck        ............A...TT...-------.A.....---TA.------------------..GTA.T-..-A.G.CC--AA.A---TTTT.AAATA..GT..
20. Graham        ............A...TT...-------.A.....---TA.------------------..GTA.T-..-A.G.CC--AA.A---TTTT.AAATA..GT..
21. Chadbour      ............A...TT...-------.A.....---TA.------------------..GTA.T-..-A.G.CC--AA.A---TTTT.AAATA..GT..
22. UnivHill      ............A...TT...-------.A.....---TA.------------------..GTA.T-..-A.G.CC--AA.A---TTTT.AAATA..GT..
23. Wahkeena      ............A...TT.GC------CA.....CC-TA.------------------..GTA.T...-AA..C.A-AATA.A-TTTT.AAATACTGT.C
24. Larch Mt      ............A...TT.GC------CA.....CC-TA.------------------..GTA.T...-AA..C.A-AATA.A-TTTT.AAATACTGT.C
25. HJ Andrew     ............A...TT.GC-------.A.....CC-TA.------------------..GTA.T...-AA..C.A-AATA---TTTT.AAATACTGTTC
26. P.larselli    ..C...C.....A..A.A...AAGCTACGC----CC.TAGAGCAGACAGACTACGC.-----------TAC.TC.TA.A.G.--AATA-AAATA..GT.C
```

Table 4.5 Range of percent sequence divergences (uncorrected) between haplotypes for Torrent Salamanders. Upper matrix is based on mtDNA cytochrome b (778 bp) sequence differences. Lower matrix is based on mtDNA 12S rRNA/16S rRNA sequence (360 bp and 560 bp, respectively) differences. Values are derived from minimum and maximum pairwise haplotype differences observed.

	1	2	3	4
1. *R. olympicus*	___	3.5	10.9-11.7	9.9-10.8
2. *R. kezeri*	0.9-1.4/4.0-5.5	____	10.7-11.8	10.3-11.1
3. *R. variegatus*	4.8-5.1/5.5-5.7	5.7-6.6/1.9-2.7	____	9.5-11.1
4. *R. cascadae*	6.3-6.6/7.3-7.5	5.5-6.9/3.5-3.7	4.6-5.1/1.6-2.3	___

Intra-specific variation was slight for all genes except among cytochrome b haplotypes in *R. variegatus* and *R. cascadae,* which had pairwise differences ranging from 0.1 – 4.4% and 1.4 – 2.1%, respectively. 12S sequences indicated the least divergence within species: there were two unique haplotypes (0.6 % different) among *R. kezeri*, 15 unique haplotypes (0.0 – 0.9 % different) among *R. variegatus*, and two unique haplotypes among *R. cascadae* (0.9 %). Finally, 16S sequences revealed four unique haplotypes (0.2 – 2.0%) among *R. kezeri*, seven unique haplotypes (0.6 - 1.0%) among *R. variegatus*, and two unique haplotypes (0.2 – 0.4%) among *R. cascadae*. All individuals sampled per locality contained the same haplotypes for each gene. Intra-specific variation was slight, therefore, we focused our analyses on inter-specific differences.

Phylogenetic analyses

We compared phylogenetic analyses based on single gene analyses and in combined analyses. Each gene region contained a phylogenetic signal based on a random sample of 10,000 trees for each dataset, where the distribution of tree scores was right-hand skewed (cytochrome b g_1 = - 0.731, 12S g_1 = - 0.836, 16S g_1 = - 1.308). Partition homogeneity tests indicated there were significant differences

($p < 0.03$) among different gene regions in resolving relationships within Torrent salamanders, resulting in trees with slightly different topologies and support, particularly among terminal branches. However, the monophyly of each species and the sister relationships of *R. olympicus* and *R. kezeri* was indicated in each case.

Cytochrome b analyses

Trees constructed using different phylogenetic methods indicated different basal branching among species. Maximum parsimony analyses revealed four most-parsimonious trees (Figure 4.2, 147 parsimony informative characters). Topologies among most-parsimonious trees were identical except for instability of terminal branches between Graham Creek and Chadbourne haplotypes *R. variegatus*. Maximum parsimony trees resulting from differential weighting of codon positions appeared to have no effect on tree topology or support (670 steps, CI 0.814, RI 779, HI 0.178).

The only difference in topology among maximum likelihood trees (hierarchical and AIC) occurred within *R. variegatus* where the *north coast* clade clustered with either the *Oregon* clade or *California* clade. Hierarchical likelihood ratio tests indicated the Hasegawa-Kishino-Yano model was the least rich with the smallest –ln likelihood value of 3,041 [gamma = 0.3645, transition:transversion ratio of 2.6476, and fixed base frequencies (A = 0.3172, C = 0.1970, G = 0.1262, T = 0.3596)]. The best model selected using the AIC criteria was the transversion

rate matrix model with a –ln likelihood value of 3,048 [R (a) = 4.4009, R (b) = 14.4119, R (c) = 2.5272, R (d) = 1.4868, R (e) = 14.4119, R (f) = 1.000; gamma = 0.9080, invariable sites = 0.3133, and fixed base frequencies (A = 0.3060, C = 0.1927, G = 0.1336, T = 0.3677)].

Maximum parsimony and maximum likelihood analyses indicated similar basal branching relationships where *R. cascadae* formed a sister group to the *olympicus- kezeri* clade (60% and 75% bootstrap support, respectively). In contrast, the minimum evolution analyses (minimum evolution score = 0.804) showed *R. variegatus* was a sister group (bootstrap value less than 50%) to the *olympicus-kezeri* clade.

12S ribosomal RNA analyses

Trees based on 12S sequences, using different phylogenetic methods, were similar in basal branching topology and resulted in minor differences in terminal branch support. 12S sequences revealed 18 most-parsimonious trees (37 parsimony informative characters) with gaps treated as a 5th base (Figure 4.3). Differences among trees revealed alternative branching among haplotypes within the *Oregon clade* of *R. variegatus*. The maximum parsimony consensus tree agreed with the cytochrome b minimum evolution tree resulting in *R. variegatus* as a supported (60% bootstrap value) sister group to the *olympicus-kezeri* clade. This same topology is supported by analyses conducted with gaps treated as missing data based on 33 parsimony informative characters (90 steps, CI 0.878, RI 0.934, HI

0.122). Moreover, maximum parsimony basal branching agreed with minimum evolution (minimum evolution score = 0.480) and maximum likelihood consensus trees, except for minor differences in resolution among terminal branches.

For maximum likelihood analyses, hierarchical likelihood ratio tests indicated the Hasegawa-Kishino-Yano model was the least rich with the smallest – ln value of 904.1 [gamma = 0.2683, fixed transition:transversion ratio of 1.942, and fixed base frequencies (A = 0.3773, C = 0.2196, G = 0.1707, T = 0.2324)]. The best model selected using the AIC criteria (AIC score =1813) was an unrealistic transversion rate matrix model that assumed a C to G rate of zero; therefore, it was not compared.

Figure 4.2 Maximum parsimony consensus (50% majority) of four most-parsimonious trees (420 steps, CI 0.802, RI 0.893, HI 0.189) based on mitochondrial cytochrome b sequences (778 bp) for Torrent salamanders. Bootstrap values (greater than 50%) supporting the same branching order are shown for maximum parsimony (above branches), minimum evolution (below branches), and maximum likelihood (below branches in parentheses) methods.

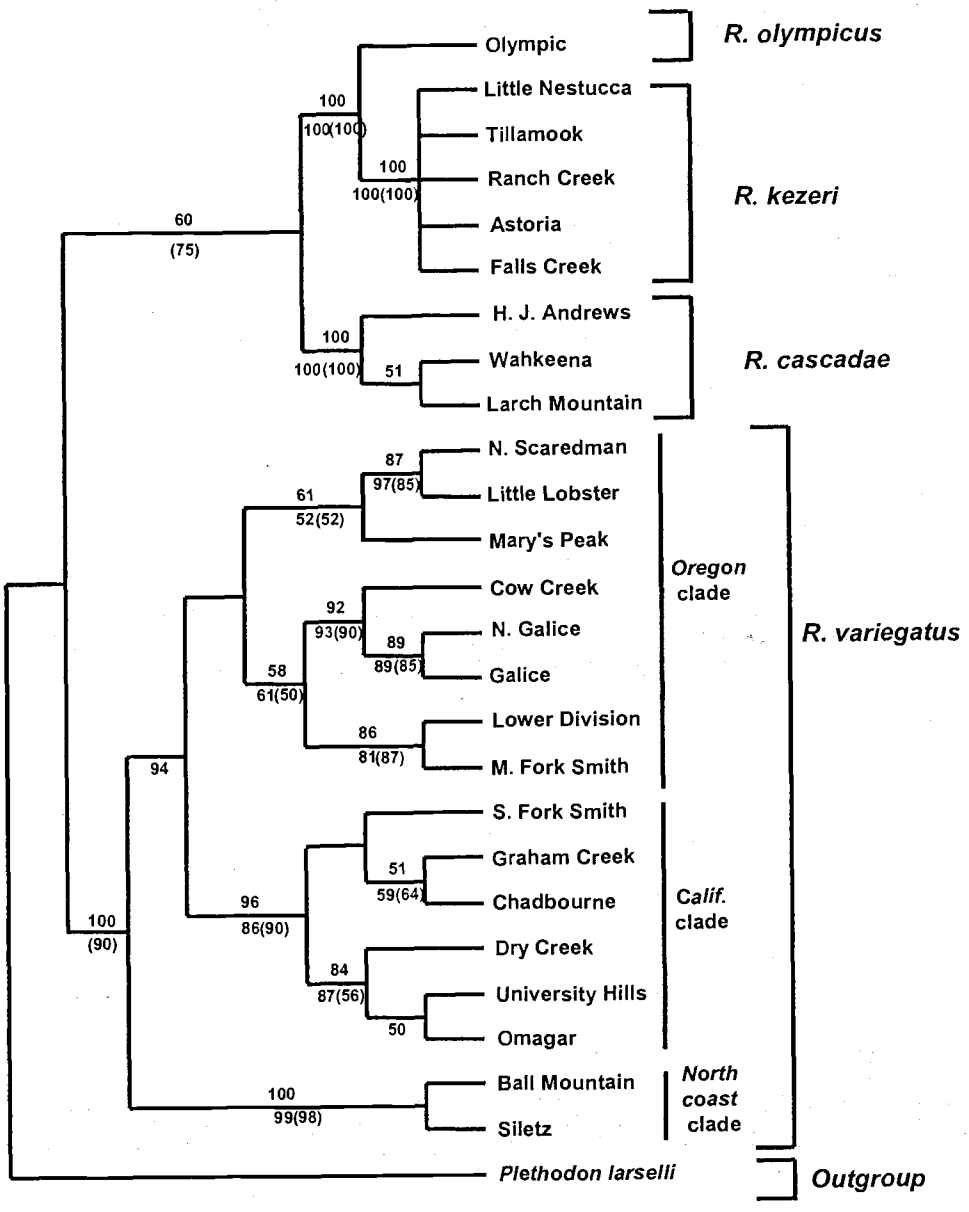
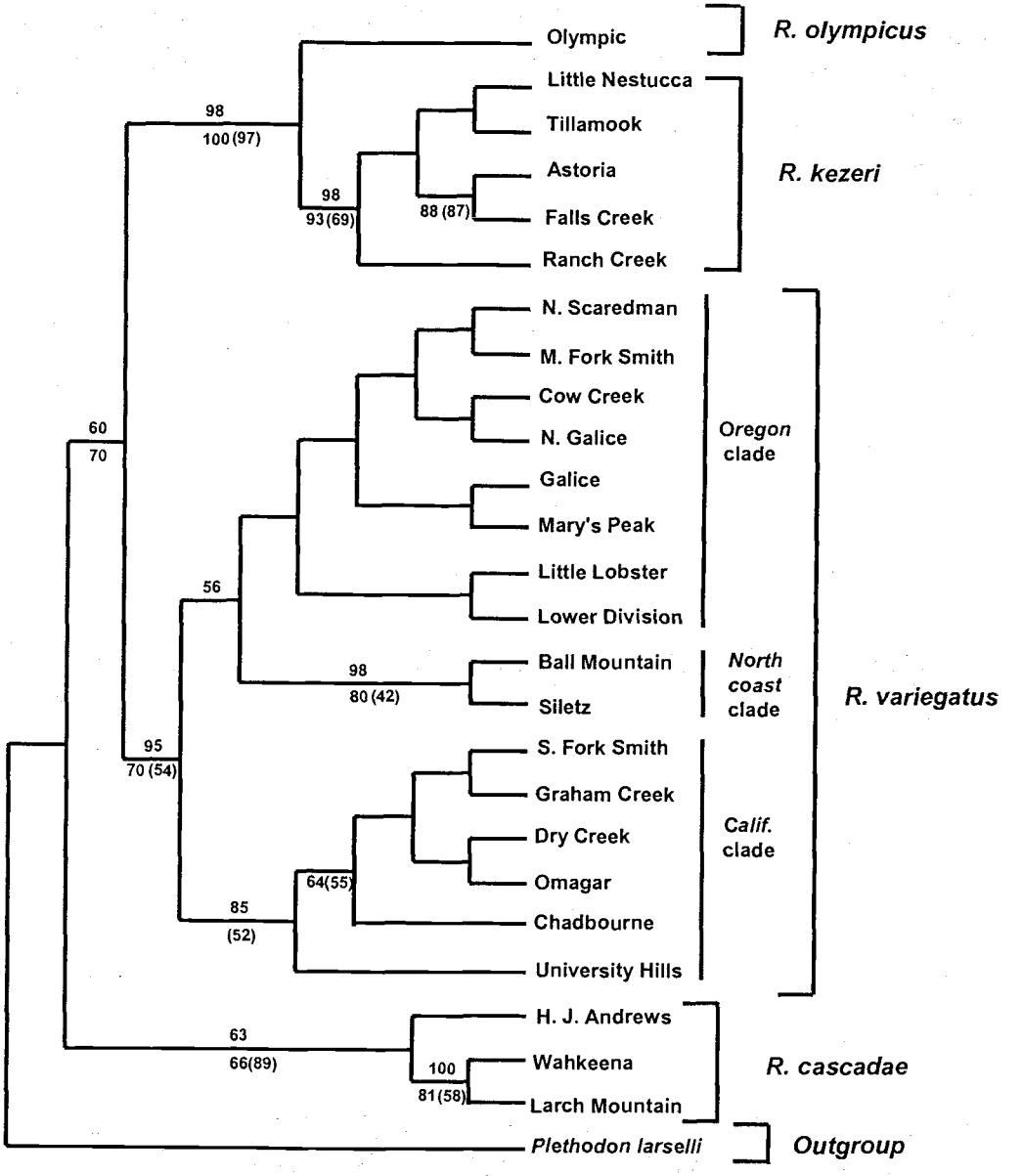

Olympic
Little Nestucca
Tillamook
Ranch Creek
Astoria
Falls Creek
H. J. Andrews
Wahkeena
Larch Mountain
N. Scaredman
Little Lobster
Mary's Peak
Cow Creek
N. Galice
Galice
Lower Division
M. Fork Smith
S. Fork Smith
Graham Creek
Chadbourne
Dry Creek
University Hills
Omagar
Ball Mountain
Siletz
Plethodon larselli
R. olympicus
R. kezeri
R. cascadae
R. variegatus
Oregon clade
Calif. clade
North coast clade
Outgroup
100
100(100)
100
100(100)
60
(75)
100
100(100)
51
87
97(85)
61
52(52)
92
93(90)
89
89(85)
58
61(50)
86
81(87)
94
51
59(64)
96
86(90)
100
(90)
84
87(56)
50
100
99(98)

Figure 4.2

Figure 4.3 Maximum parsimony consensus (50% majority) of 18 most parsimonious trees (99 steps, CI 0.879, RI 0.934, HI 0.121) based on mitochondrial 12S ribosomal RNA sequences (360 bp including indels) for Torrent salamanders. Bootstrap values (greater than 50%) supporting the same branching order are shown for maximum parsimony (above branches), minimum evolution (below branches), and maximum likelihood (below branches in parentheses) methods.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Astoria
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Ranch Creek
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M. Fork Smith
Cow Creek
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Mary's Peak
Little Lobster
Lower Division
Ball Mountain
Siletz
S. Fork Smith
Graham Creek
Dry Creek
Omagar
Chadbourne
University Hills
H. J. Andrews
Wahkeena
Larch Mountain
Plethodon larselli
98
100 (97)
98
93 (69)
88 (87)
60
70
56
98
80 (42)
95
70 (54)
64(55)
85
(52)
63
66(89)
100
81 (58)
R. olympicus
R. kezeri
Oregon clade
North coast clade
Calif. clade
R. variegatus
R. cascadae
Outgroup

Figure 4.3

16S ribosomal RNA analyses

Compared to the cytochrome b and 12S genes, the 16S gene resulted in less resolved trees with fewer supported branches regardless of phylogenetic method. 16S sequences showed a large number of indels, mainly between position 197 and 422 of the sequence. Therefore, we constructed secondary structures to aid in making sequence alignments for phylogenetic analyses (Figure 4.5). Most of the indels occurred in the large loop region between postitions 242 and 262 of the alignment, with *R. olympicus* having a three base pair insertion in the large loop at positions 242-243. Stems were more conserved than loop regions. For example, only *R. cascadae* had a compensatory fixed G:C pair (position 206 and 411) compared to an A:U pair for all other species in a stem region. Within *R. variegatus*, the *north coast* clade populations had a deletion at position 204 and the *California* clade had fixed transitions at positions 310 and 316 compared to the *north coast* and *Oregon* clades.

Maximum parsimony analyses of 16S sequences yielded 12 most-parsimonious trees with gaps treated as a 5th character (36 parsimony-informative characters). Among most-parsimonious trees, alternative branching of terminal branches occured within the *R. kezeri* clade and *R. variegatus* clade. In contrast to the cytochrome b and 12S maximum parsimony trees, the 12S maximum parsimony consensus bootstrap tree indicated *R. cascadae* was a sister group to *R. variegatus* (Figure 4.5, 79 % bootstrap support). Haplotype relationships within *R. variegatus* were poorly resolved in comparison to the cytochrome b and 12S trees.

Trees constructed with gaps missing based on 25 parsimony-informative characters equally lacked support (122 steps, CI 0.918, RI 0.896, HI 0.082). Further, masking (exclusion) of most the variable and difficult to align region (position 242-262) yielded a tree that was similar in topology and support for major groups, except for greater support of the monophyly of *R. variegatus* (90% bootstrap, 32 parsimony informative characters, 153 steps, CI 0.904, RI 0.883, HI 0.096).

Many branches were not supported or resolved, particularly terminal branches, in minimum evolution (minimum evolution score = 0.310) and maximum likelihood consensus bootstrap trees. The same evolutionary model, general time reversible, was chosen for maximum likelihood analyses with the hierarchical likelihood ratio test and the AIC method (AIC score = 2576) with a –ln likelihood value of 1,279 [R (a) = 3.7164, R (b) = 8.3010, R (c) = 7.3326, R (d) = 1.3677 R (e) = 20.2000, R (f) = 1.000; g = 0.5246, invariable sites = 0, fixed base frequencies (A = 0.3773, C = 0.2196, G = 0.1707, T = 0.2324)].

Figure 4.4 Inferred mitochondrial 16S ribosomal RNA secondary structures showing the variable region from sequence position 363 – 462 among Torrent salamanders (Genus *Rhyacotriton*): A) *R. olympicus*, B) *R. kezeri*, C) *R. variegatus*, and D) *R. cascadae*. Base pairs that are variable among haplotypes are circled.

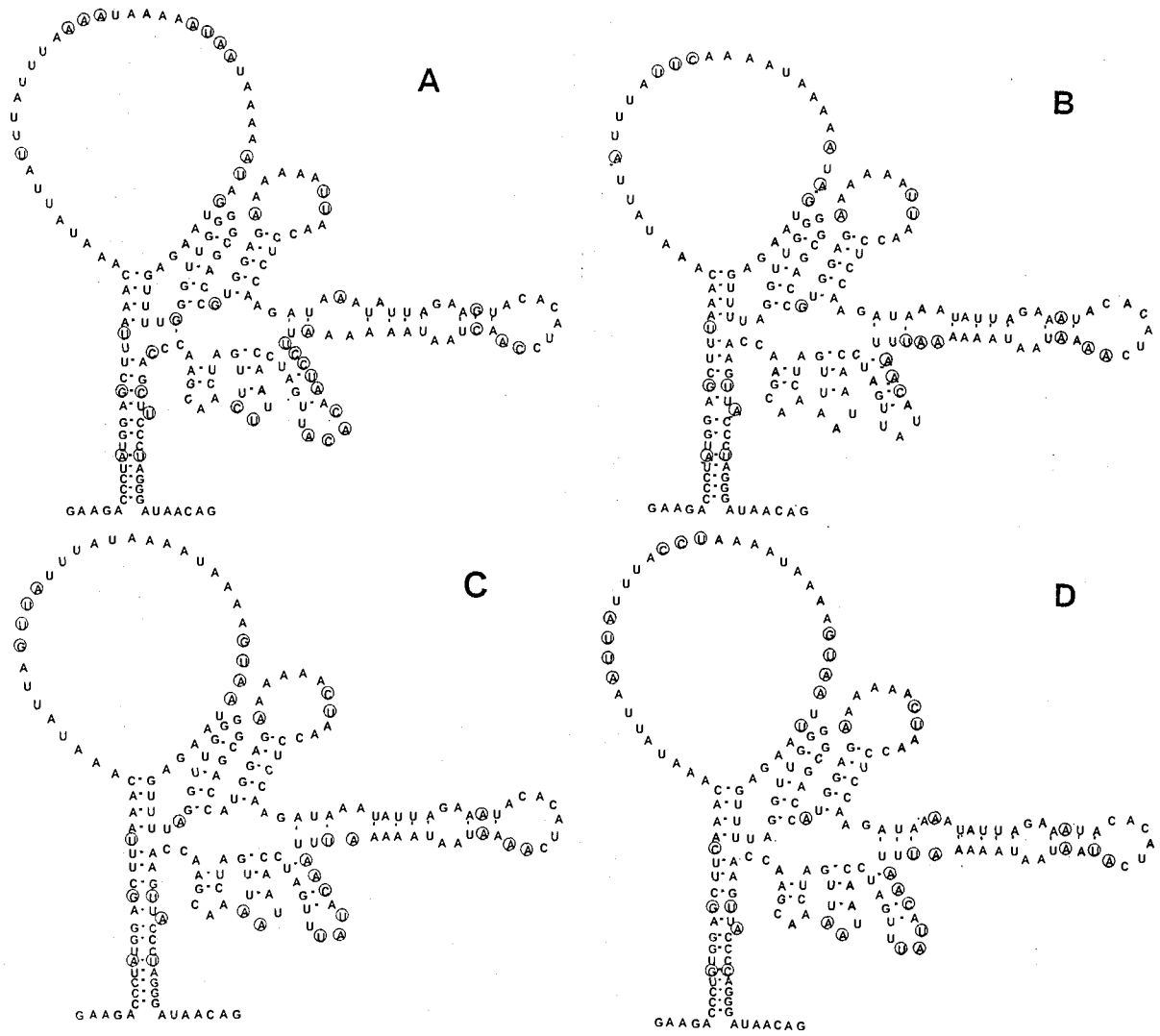
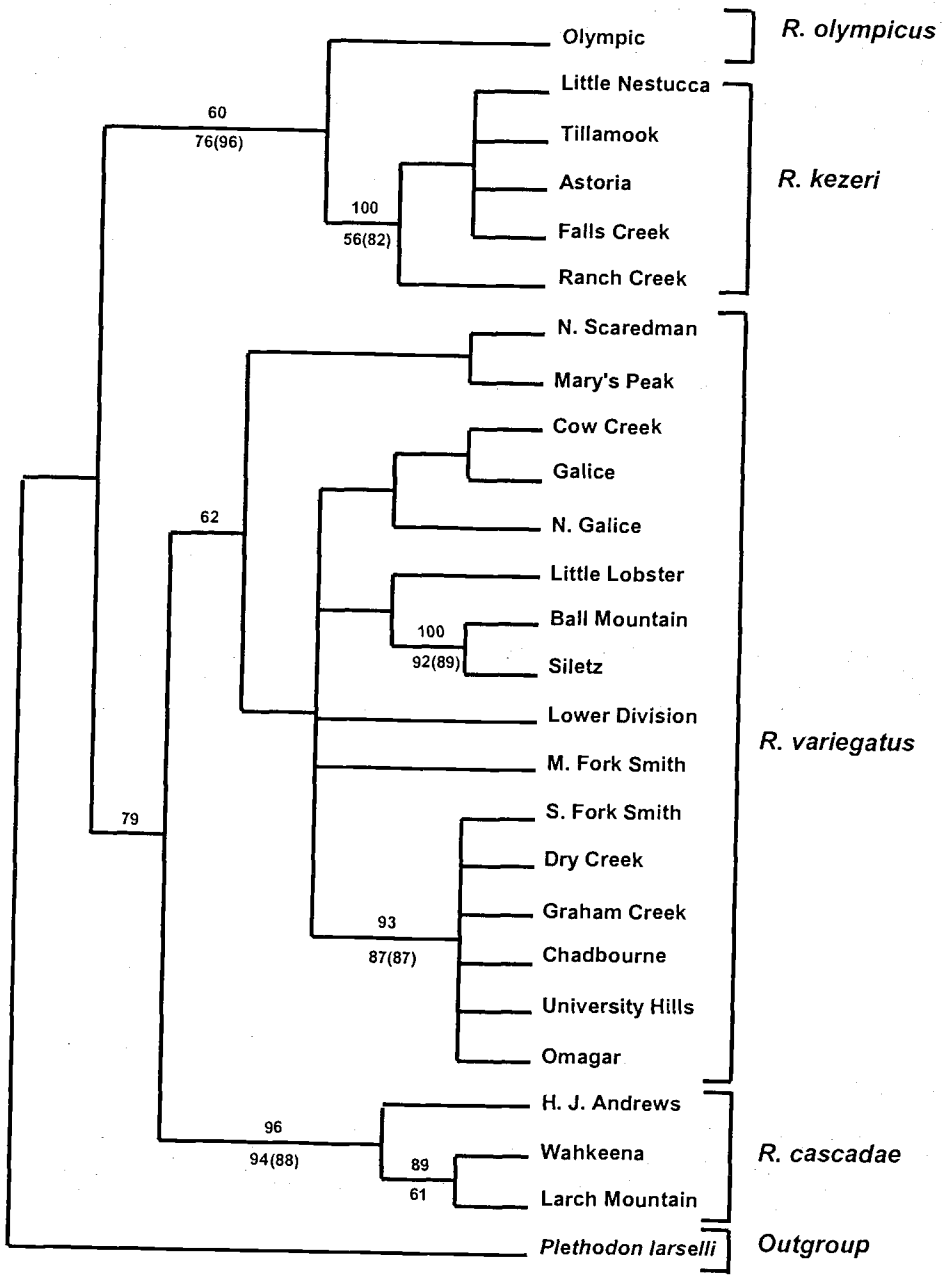

A
B
C
D
GAAGA AUAACAG
GAAGA AUAACAG
GAAGA AUAACAG
GAAGA AUAACAG

Figure 4.4

Figure 4.5 Maximum parsimony consensus of 18 most parsimonious trees (181 steps, CI 0.917, RI 0.910, HI 0.083) for 16S ribosomal RNA sequences (560 bp including indels) for Torrent salamanders. Bootstrap values (greater than 50%) supporting the same branching order are shown for maximum parsimony (above branches), minimum evolution (below branches), and maximum likelihood (below branches in parentheses) methods.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Little Lobster
Ball Mountain
Siletz
Lower Division
M. Fork Smith
S. Fork Smith
Dry Creek
Graham Creek
Chadbourne
University Hills
Omagar
H. J. Andrews
Wahkeena
Larch Mountain
Plethodon larselli
60
76(96)
100
56(82)
62
79
100
92(89)
93
87(87)
96
94(88)
89
61
R. olympicus
R. kezeri
R. variegatus
R. cascadae
Outgroup

Figure 4.5

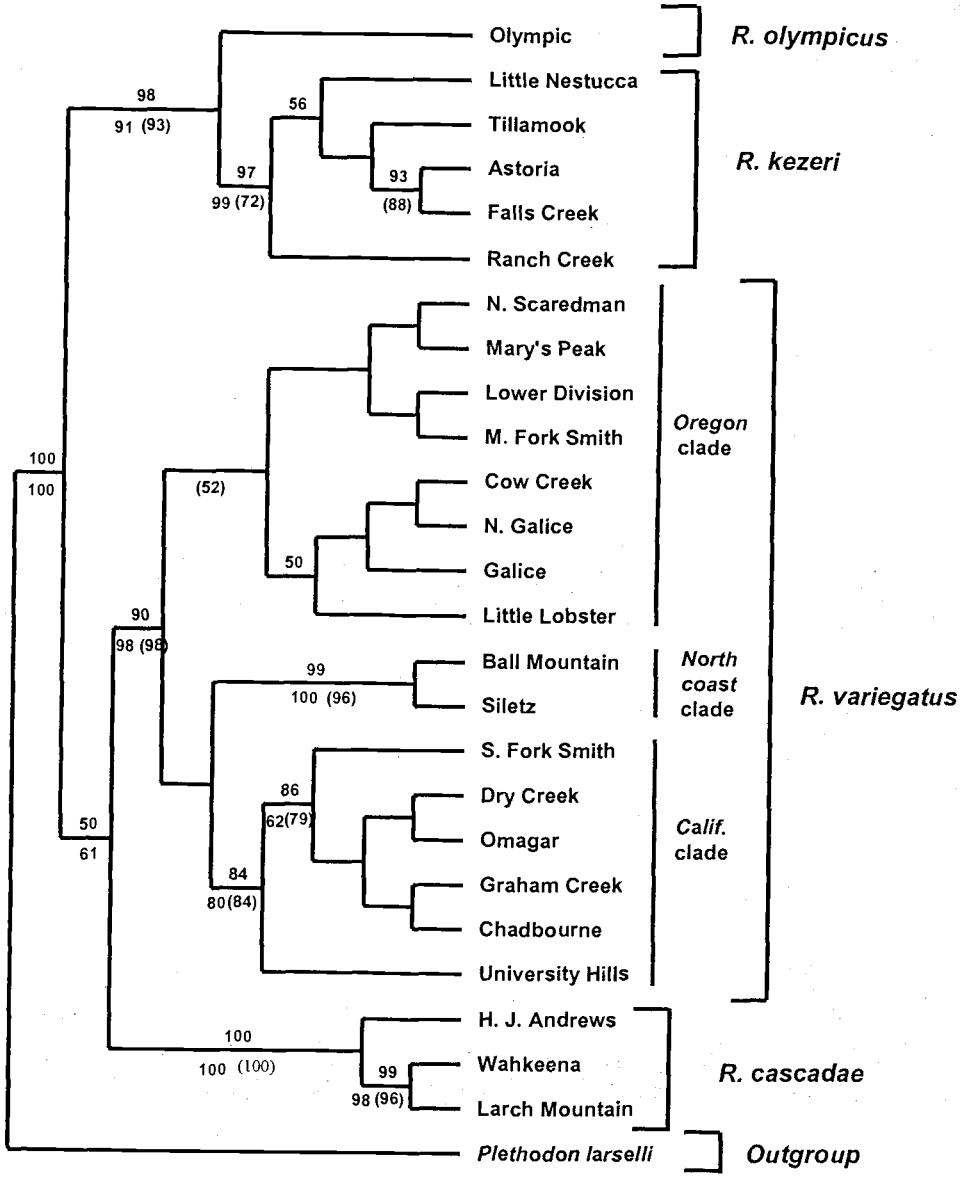
Combined 12S and 16S ribosomal RNA analyses

12S and 16S were combined for analyses (906 bp including indels) because they have similar rates of substitution since both are non-translated genes coding for ribosomal subunits. Phylogenetic analyses yielded trees with basal branching similar to 16S trees, with *R. variegatus* and *R. cascadae* as supported sister groups. Ten most-parsimonious trees (76 parsimony-informative characters) were found with gaps treated as a 5th character (Figure 4.6). No difference in topology or support occurred with gaps treated as missing data (58 parsimony informative characters, 214 steps, CI 0.893, RI 0.912, HI 0.107).

Similar topologies were obtained for minimum evolution (minimum evolution score 0.475) and maximum likelihood consensus bootstrap trees; however, differences did occur among terminal branches within *R. variegatus*. For maximum likelihood analyses, the hierarchical method supported (60 % bootstrap values) the *California* clade and *Oregon* clade as sister groups while the AIC method supported grouping of the *north coast* and *California* clade. The hierarchical likelihood ratio tests selected a transition rate matrix model with a –ln likelihood of 2,917 [R (a) = 1.0000, R (b) = 2.6766, R (c) = 1.0000, R (d) = 1.0000, R (e) = 5.5149, R (f) = 1.000; gamma = 0.3868, invariable sites = 0, and fixed base frequencies (A = 0.3489, C = 0.2188, G = 0.1912, T = 0.2411)]. The AIC method

(AIC score = 51819) selected a general time reversible rate matrix model a –ln likelihood value of 2,900 [R (a) = 3.5221, R(b) = 6.2350, R (c) = 3.9564, R (d) = 0.2503 R (e) = 13.6295, R (f) = 1.0000; gamma = 0.4552, invariable = 0, fixed base frequencies (A = 0.3489, C = 0.2188, G = 0.1912, T = 0.2411)].

Figure 4.6 Maximum parsimony consensus of ten most parsimonious trees (282 steps, CI 0.897, RI 0.917, HI 0.107) for combined 12S and 16S ribosomal RNA sequences (920 bp including indels) for Torrent salamanders. Boostrap values (greater than 50%) supporting the same branching order are shown for maximum parsimony (above branches), minimum evolution (below branches), and maximum likelihood (below branches in parentheses) methods.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Ball Mountain
Siletz
S. Fork Smith
Dry Creek
Omagar
Graham Creek
Chadbourne
University Hills
H. J. Andrews
Wahkeena
Larch Mountain
Plethodon larselli
R. olympicus
R. kezeri
Oregon clade
North coast clade
Calif. clade
R. variegatus
R. cascadae
Outgroup
98
91 (93)
56
97
99 (72)
93
(88)
100
100
(52)
50
90
98 (98)
99
100 (96)
86
62(79)
84
80(84)
50
61
100
100 (100)
99
98 (96)

Figure 4.6

Combined analyses for all genes

Differences in basal branching occurred between methods, with slight differences in terminal branches, for combined analyses using all three regions. The maximum parsimony search yielded five equally parsimonious trees with gaps treated as a 5th character (Figure 4.7, 225 parsimony informative characters) with alternative branching of terminal groups within *R. kezeri* and *R. variegatus*. The same topology and support was recovered for a maximum parsimony consensus tree with gaps treated as missing data yielded (637 steps, CI 0.829, RI 0.895, HI 0.171, 205 parsimony informative characters).

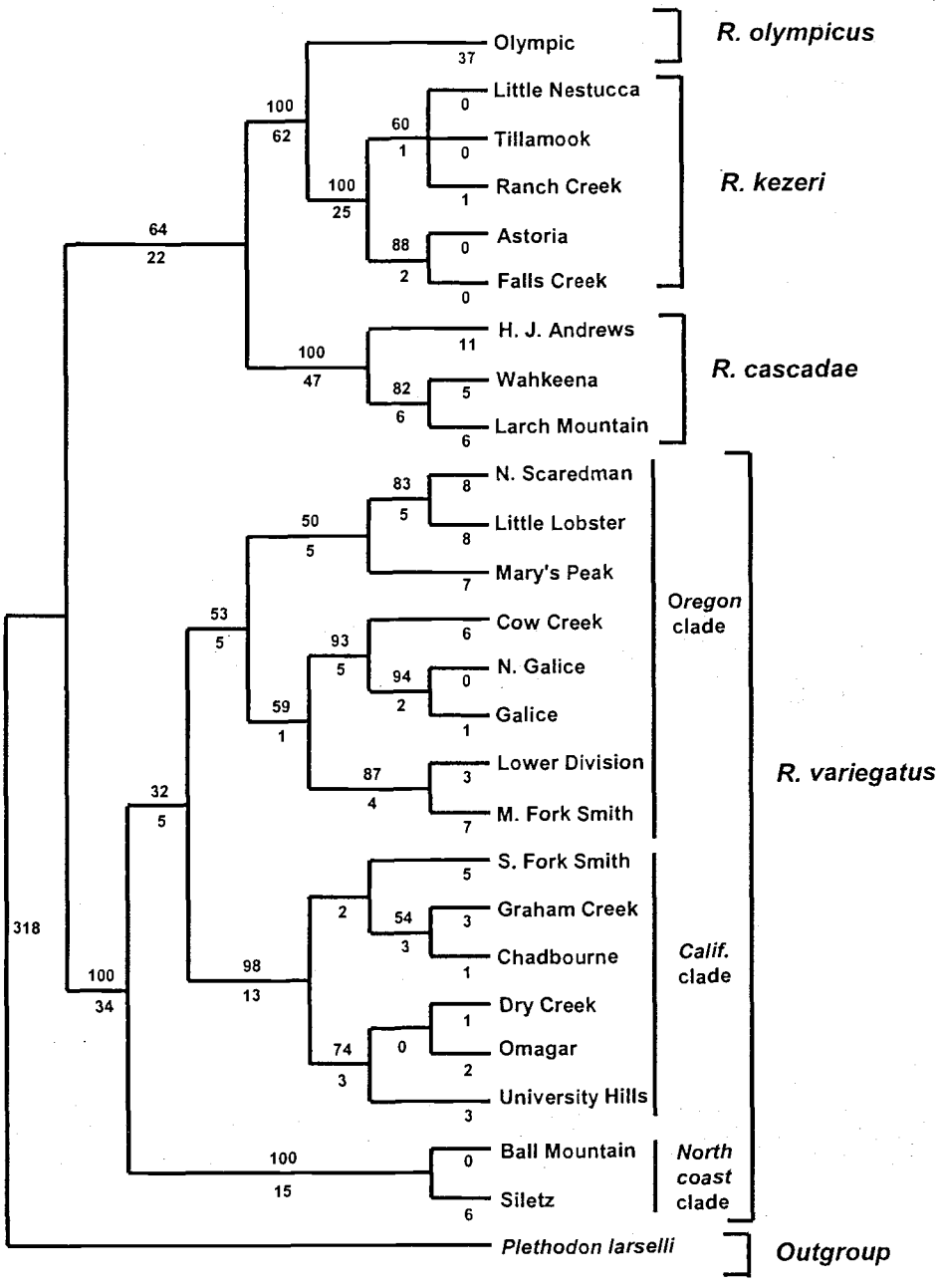
For maximum likelihood analyses, the hierarchical and AIC models yielded trees with similar support and branching order. The hierarchical likelihood ratio tests selected a Hasegawa-Kishino-Yano model as the least rich the smallest –ln likelihood value of 5,368 [Figure 4.8; gamma = 0.727, invariable sites = 0.418, a fixed transition:transversion ratio of 2.478, and fixed base frequencies (A = 0.3416, C = 0.2032, G = 0.1472, T = 0.308)]. The selected AIC model (AIC score = 10746) was a general time reversible rate matrix model with a –ln likelihood value of 5363 [R (a) = 4.7708, R (b) = 13.4093, R (c) = 3.8288, R (d) = 1.1495 R (e) = 18.1429, R (f) = 1.000; gamma = 0.7339, invariable sites = 0, fixed base frequencies (A = 0.3327, C = 0.1985, G = 0.1616, T = 0.3072)].

Maximum parsimony and maximum likelihood methods showed similar basal branching order, indicating support for a sister relationship between the *R. cascadae* and *olympicus- kezeri* clades. There were only minor differences in terminal branch topology showing the *Oregon clade* as a well-supported outgroup to the rest of *R. variegatus* in the maximum parsimony tree, and as a non-supported sister group to the *California clade* in the maximum likelihood analyses. In contrast, the minimum evolution consensus tree (minimum evolution score = 0.409) showed *R. variegatus* as a sister group to the *R. olympicus-R. kezeri* clade, and the *north coast* clade as a non-supported sister group to the *Oregon* clade.

Trees were compared for significant differences in topology using the Kishino-Hasegawa test. First, analyses were conducted using all genes for a total evidence approach. A significant difference (difference in tree length = 31 ± 8.03 SD steps, T = 3.86, p = 0.0001) was indicated between maximum parsimony trees with alternative branching topology: trees were compared with either *R. cascadae* as a sister group to the *olympicus- kezeri* clade (as seen in Figure 4.2) or to *R. variegatus* (as seen in Figure 4). The topology as represented in Figure 4.2 was the best with 710 steps (223 parsimony-informative characters, CI 0.832, RI 0.894, HI 0.168), suggesting *R. cascadae* is more likely to be a sister group to the *olympicus-kezeri* clade. However, comparisons of the same topologies based on the combined 12S and 16S genes indicated no significant difference (difference in tree length = 2 ± 3.16 SD steps, T = 0.63, p = 0.52) in branching topology between the two alternatives, with shortest length tree (287 steps, 76 parsimony-informative

characters, CI 0.882, RI 0.902, HI 0.118) suggesting *R. variegatus* and *R. cascadae* are sister groups. The cytochrome b region contains more informative characters and may be better able to resolve this relationship; however, our inability to resolve this branching order may be due to the divergence of these lineages at similar times.

Figure 4.7 Maximum parsimony consensus of 5 most parsimonious trees (706 steps, CI 0.837, RI 0.897, HI 0.163) for Torrent salamanders based on three gene regions (cytochrome b, 12S and 16S rRNA, 1702 bp). Bootstrap values (greater than 50%) are shown above branches and the minimum number of steps supporting each are shown below.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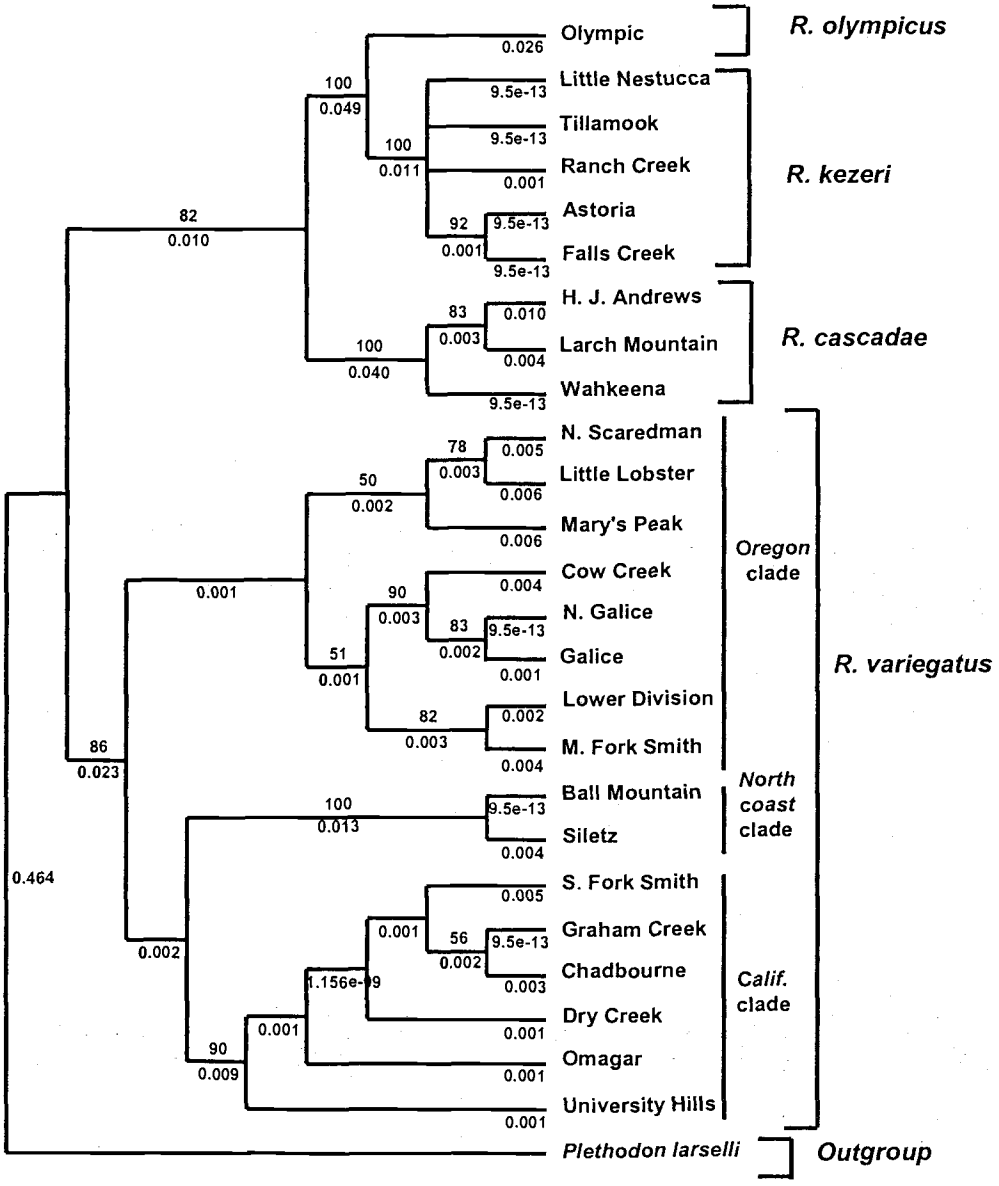
Cow Creek
N. Galice
Galice
Lower Division
M. Fork Smith
S. Fork Smith
Graham Creek
Chadbourne
Dry Creek
Omagar
University Hills
Ball Mountain
Siletz
Plethodon larselli
R. olympicus
R. kezeri
R. cascadae
Oregon clade
Calif. clade
North coast clade
R. variegatus
Outgroup

Figure 4.7

Figure 4.8 Maximum likelihood consensus bootstrap tree (-ln likelihood = 5368) for Torrent salamanders based on three gene regions (cytochrome b, 12S and 16S rRNA, 1702 bp). Bootstrap values (greater than 50 %) are shown above branches and the maximum likelihood distances of each branch are shown below.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Mary's Peak
Cow Creek
N. Galice
Galice
Lower Division
M. Fork Smith
Ball Mountain
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S. Fork Smith
Graham Creek
Chadbourne
Dry Creek
Omagar
University Hills
Plethodon larselli
R. olympicus
R. kezeri
R. cascadae
R. variegatus
Oregon clade
North coast clade
Calif. clade
Outgroup

Figure 4.8

Discussion

Comparisons among genes

Homoplasy, rate substitution heterogeneity among lineages, and "long-branch" attraction can lead to inconsistencies in tree recovery (Hendy and Penny 1989, Graybeal 1993). These problems can be compounded in cases where relationships are asymmetric due to deep branching and shallow branching taxa (Graybeal 1994). Thus, congruence of phylogenetic trees using different genes should provide the strongest evidence for the relationships (Vidal et al. 2000). Therefore, we used three mitochondrial genes with different rates of substitution in order to minimize problems in inferring relationships among the Torrent Salamanders.

Our results indicated significant differences in basal topology and support for single gene analyses and in combined analyses. Overall, cytochrome b and combined analyses (using all three gene regions) performed best resulting in a greater number of well-supported branches (greater than 70%) with higher bootstrap support values per branch compared to other analyses. The combined 12S-16S analyses performed next best followed by 12S analyses. 16S analyses showed support for few branches.

More fully resolved trees, with a greater number of well-supported terminal branches, were obtained with cytochrome b and combined analyses using all three genes. However, many common basal branches were supported with all methods despite low support for terminal branches with ribosomal genes. For example, the

monophyly of each species and the sister relationship *R. olympicus* and *R. kezeri* was well-supported in all single and combined analyses. There was a general lack of support, regardless of gene region, for a sister group relationship of *R. cascadae* with either *R. variegatus* or the *R. olympicus-R. kezeri* clade. This instability was also indicated among trees derived using different evolutionary models for the same gene region (separate gene analyses) or regions (combined analyses). Most likely the relationship of *R. cascadae* is either polyphyletic with respect to *R. variegatus* and the *olympicus-kezeri* clade or the relationship is the result of a poorly resolved short branch with its sister group.

The majority of informative sites (134 parsimonious sites) were found in the cytochrome b gene; therefore it contributed more to obtaining fully resolved trees in separate and combined analyses of all gene regions. In comparison, the number of informative characters was lower for 16S (39 characters) and 12S (54 characters) ribosomal sequences. One possible explanation is that cytochrome sequences are longer and sequence length has been shown to have a great effect on phylogeny reconstruction (Russo et al. 1996). However, combined 12S-16S sequences (930 bp) did not perform as well as the shorter length cytochrome b analyses (778 bp) for Torrent salamanders. Thus, the greatest effect appears to be due to the number of informative characters.

There was little difference in branch support among single gene or combined trees derived using maximum parsimony and maximum likelihood phylogenetic methods. For example, trees derived from cytochrome b sequences

were equally supported with both methods; the converse is true for 16S sequences that showed equally poor trees regardless of method. Further, trees derived using different weighting schemes or evolutionary models yielded only slight differences in support and topology. It has been shown that the probability of obtaining the correct topology for a complicated model is equal to that of a simple model unless the extent of divergence is large (Gaut and Lewis 1995).

Overall, the cytochrome b gene alone provides as fully resolved and equally supported trees as the combined analyses for Torrent salamanders. Saturation of cytochrome b does not appear to bias phylogenetic inferences because different evolutionary models perform equally well. On the other hand, the ribosomal genes support basal branches but do not adequately resolve terminal branches, suggesting their substitution rate is too low to make robust inferences concerning Torrent salamander phylogeny.

Evolutionary relationships among Torrent salamanders

Our results are consistent with the originally hypotheses proposed suggesting that two major vicariant events contributed to the present Torrent salamander relationships (Good et al. 1987, Good and Wake 1992). Three major groups of Torrent salamanders appear to have diverged first which included *R. variegatus*, *R. cascadae*, and the ancestor to *R. olympicus* and *R. kezeri*). Subsequently, isolation of *R. olympicus* and *R. kezeri* appears to have occurred more recently along with three major clades observed among *R. variegatus*.

Estimates of divergence time based upon mtDNA sequence differences can be inexact without calibration using some independent event (i.e., geologic); however, they can provide useful relative comparisons for dating divergence times among taxa (Moritz et al. 1987, Irwin et al. 1991). Isolation of the three main groups, assuming a constant molecular clock, appears to have occurred between 10.9 – 13.6 million years ago (MYA) based on the cytochrome b, 9.6 – 13.2 MYA for the 12S, and 11.0 – 15.0 MYA for the 16S. These estimates agree with those based on allozymes that suggest a divergence between 6.0 – 11.0 MYA (Good and Wake 1992). Dating divergence between *R. olympicus* and *R. kezeri* based on cytochrome b results (4.5 MYA) is consistent with allozyme results; however, divergence estimates based on ribosomal genes (1.8-2.8 MYA for the 12S, and 8.0 – 11.0 for 16S) have a much greater spread. The low substitution rate of ribosomal genes may not provide enough resolution over this time scale. Further, the higher 16S divergence times may be due substitution rate heterogeneity among *R. olympicus*.

The overall pattern of divergence, however, supports the previously proposed hypothesis concerning the evolutionary history of *Rhyacotriton* (Good et al. 1989, Good and Wake 1992). In sum, they suggest the ancestor to presently recognized groups occurred in the area of the Cascade Mountain Range in Oregon and moved into the Coastal Range as it uplifted (15 – 22 MYA). During the Miocene, basalt flows and flooding from ancient rivers as a result of volcanic activity is thought to have isolated the three deep branching groups: with *R.*

cascadae becoming isolated in the Cascades by the Willamette Valley, and *R. kezeri* and *R. variegatus* becoming isolated in the area where they are currently in contact near the Little Nestucca River, Oregon. The split between *R. olympicus* and *R. kezeri* is more recent, attributed to the massive river that formed the Chehalis River Valley, southwestern Washington, during the last glacial period. It appears that populations within *R. variegatus* may have also become isolated during this period.

Substantial divergence appears to have occurred among lineages within *R. variegatus*. Based on a larger cytochrome b study (78 populations), *R. variegatus* is comprised of three major clades (*north coast* clade, *Oregon* clade and *California* clade), each of which appear to be influenced by historic geographic barriers to dispersal (Chapter 6; Wagner and Haig, in review). These clades were also supported as monophyletic groups for each separate gene and in combined analyses (except for 16S single gene analyses). However, clade relationships are uncertain. The *north coast* clade alternatively groups with either the *Oregon clade* or the *California clade*. Most likely these clades diverged at about the same time, between 1.8 – 4.7 MYA, which is within the range of divergence between *R. olympicus* and *R. kezeri*.

Given recent conservation concerns for *R. variegatus*, this study puts into perspective divergence among Torrent Salamander species relative to that among populations. It further confirms substantial divergence among the three major clades of *R. variegatus* and supports the need for these clades to be recognized as separate conservation units with respect to management, listing, and recovery efforts.

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CHAPTER 5

PHYLOGEOGRAPHY OF TORRENT SALAMANDERS (*Rhyacotriton cascadae* and *R. variegatus*) IN THE CASCADES.

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Abstract

A potential contact zone among Southern Torrent salamanders (*Rhyacotriton variegatus*) and Cascade torrent salamanders (*R. cascadae*) was investigated for taxonomic identity, hybridization and sympatry. Torrent salamanders (Family Rhyacotritonidae) are extremely morphologically conserved, subsequently, taxonomic identification based upon morphology is problematic for populations discovered intermediate between their previously described ranges. We used mitochondrial (mtDNA) 16S ribosomal RNA sequences (499 bp) and allozymes (6 loci) to taxonomically identify and investigate the distribution of recently discovered Torrent salamander populations found in the central Cascade mountain range of Oregon (USA). Phylogenetic inferences based upon mtDNA sequences with maximum parsimony and maximum likelihood methods indicated two distinct clades, with each clade corresponding to the allopatric distribution of Cascade and Southern torrent salamander haplotypes. Similarly, allozyme analyses revealed allopatric distribution of allele variants diagnostic for each species. The results suggest the middle fork of the Willamette River may be a phylogeographic barrier in the central Cascades, limiting either the southern or northern distribution of the Cascade torrent or Southern torrent salamanders, respectively. Finally, this study extends the previously described ranges of both the Cascade torrent and Southern torrent salamander.

Introduction

The accurate identification of taxa is essential for determining conservation status, assessing population viability, and designing management plans for threatened species. This has particular relevance for amphibians, which are notoriously morphologically conserved, yet genetic studies often reveal significant amounts genetic differentiation both within and among species (Camp et al. 2000, Highton et al. 1989, Jockusch 1996, Tilley and Mahoney 1996). Molecular markers can greatly aid in identifying individuals of uncertain specific taxonomy, in investigating hybridization at contact zones among congeneric species, and assessing the limits of species distribution (Avise 1994, Lamb et al. 2000).

In order to investigate taxonomic identity of newly discovered populations of Torrent salamanders (Family Rhyacotritonidae) in the central Oregon Cascades, we used mitochondrial DNA and allozyme markers. The previously described range of the Cascade torrent salamander (*Rhyacotriton cascadae*) was considered to extend just south of the McKenzie River (Lane Co.) in the Cascade Mountain Range (Figure 5.1). Moreover, the Southern Torrent salamander (*R. variegatus*) was thought restricted to the Coastal mountain range except for an isolated population found in the central Cascades near Steamboat Springs (Bury B, personal communication, Good and Wake 1992). Recently, populations of Torrent

salamanders were found in the gap between the aforementioned ranges (*contact zone* populations, Table 5.1). However, the taxonomic status of these populations was uncertain due to morphological conservation (Weddell L and Wagner RS, personal observation).

Figure 5.1 A. Putative ranges of Torrent salamander species. B. Sampling locations of control and *contact zone* populations in the central Oregon Cascades. See Table 5.1 for location identification of 1-12.

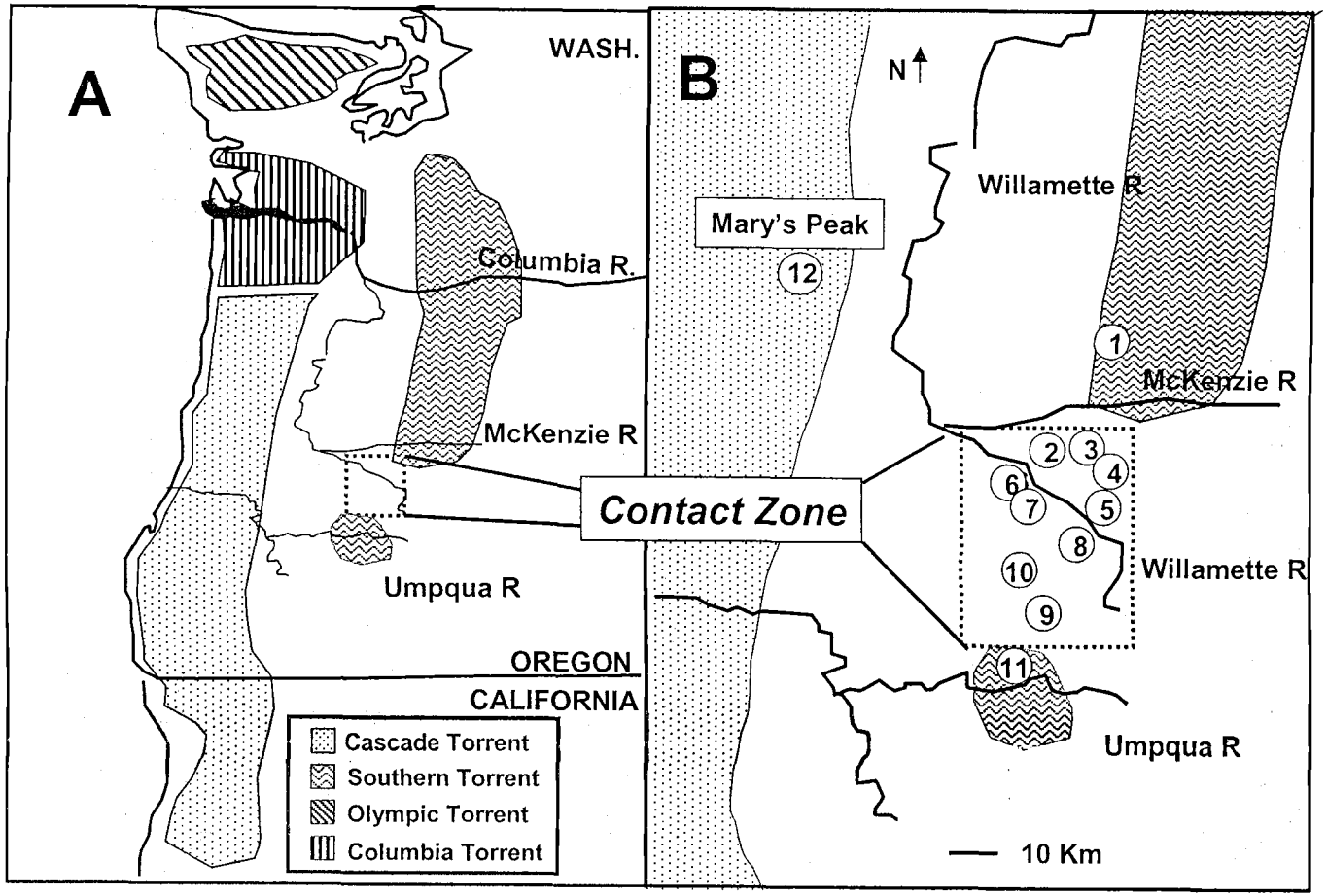
A
WASH.
Columbia R.
McKenzie R
Umpqua R
OREGON
CALIFORNIA
Cascade Torrent
Southern Torrent
Olympic Torrent
Columbia Torrent
B
N
Mary's Peak
12
Willamette R
1
McKenzie R
Contact Zone
2
3
4
6
7
5
8
10
9
Willamette R
11
Umpqua R
10 Km

Figure 5.1

Table 5.1 Sites sampled for mitochondrial DNA and allozyme analyses in Torrent salamanders for *contact zone* and control populations. M is the number of individuals analyzed for mitochondrial DNA haplotype. A is the number of individuals analyzed for allozyme variants. *Contact zone* populations are taxonomically unidentified Torrent salamanders found in the Central Cascades.

Population	M/A	Legal locality	County
Control (Cascade Torrent)			
1. H.J. Andrews	10/5	T18S,R5E,S39	Lane,OR
Contact zone			
2. Alder Creek	10/0	T19S,R1E,S13 NW	Lane,OR
3. Gold Point	10/5	T18S,R3E,S33	Lane,OR
4. Jones Creek Trail	10/5	T18S,R2E,S14 NW	Lane,OR
5. Barrow Pit	5/5	T20S,R3E,S11 NE	Lane,OR
6. Goodman Ck #1	10/5	T20S,R1E,S15	Lane,OR
7. Goodman Ck #2	10/5	T20S,R1E,S16	Lane,OR
8. Patterson Mtn	10/5	T21S,R2E,S6	Lane,OR
9. Rainbow Mine	3/3	T23S,R1E,S14 SE	Lane,OR
10. Middle Bryce Ck	10/5	T22S,R1E,S22 SE	Lane,OR
11. N. Scaredman	3/0	T35S,R8W,S10	Douglas,OR
Control (Southern)			
12. Mary's Peak	10/5	T12S,R7W,S28 NW	Benton,OR
Outgroup			
13. Columbia Torrent Tillamook,OR	1/0	T4N,R7W,S26, NE	
14. Olympic Torrent	1/0	T29,R12W,S5	Clallam,WA

In fact, Torrent salamanders provide one of the most extreme examples of morphological conservation and genetic divergence in any vertebrate (Good and Wake 1992). They were described as a monotypic species, the Olympic Salamander (*R. olympicus*), until allozyme studies identified four deeply divergent species within the family (Good and Wake 1992; see also Good et al. 1987; Figure 5.1). These results suggested Cascade and Southern Torrent salamanders diverged from a common ancestor between 6 – 11 million years ago. However, despite the long divergence time, phenotypic characters to make taxonomic assignments for these species can be unreliable. Furthermore, hybridization between these species could confound identification.

To identify taxa in the central Cascades (*contact zone*), we used mitochondrial DNA 16S ribosomal RNA (16S rRNA) sequences because they have been shown to resolve differences between species in a larger study of Torrent salamander phylogeny (Chapter 6; Wagner et al., in review). However, hybridization resulting from male-mediated migration could be wrongly characterized as allopatry due to maternal inheritance of mtDNA; therefore, we also used bi-parentally inherited allozyme markers. We surveyed *contact zone* populations for allozyme loci previously shown to be diagnostic for either Cascade torrent salamanders or Southern Torrent salamanders (Good et al. 1987).

Finally, Cascade torrent and Southern Torrent salamanders occupy similar habitat in small streams and headwaters associated with mature-forests sensitive to timber harvest and other disturbance activities (Bury and Corn 1988, Bury et al.

1991, Corn and Bury 1989, Welsh 1990). Currently, they are protected under the Northwest Forest Plan (U.S. Forest Service & U.S. Bureau of Land Management 1994). However, the Southern torrent salamander was recently petitioned for listing under the U.S. Endangered Species Act (Federal Register 60-33785). Therefore, understanding the identity and distribution of these species is of vital importance for management, listing, and recovery objectives.

Materials and Methods

Mitochondrial DNA sequencing and analyses

Eight populations of Torrent salamanders (Table 5.1) were sampled from the region intermediate between the known ranges of Cascade torrent and Southern torrent salamanders in the central Cascades (*contact zone*, Figure 1). Also included were two control populations: a Southern Torrent salamander site (Mary's Peak) and a recognized Cascade torrent salamander site (H.J. Andrews). Individuals were hand-captured and sampled by tail clipping using a single sterile surgical scissor for each salamander. Approximately 1 cm of tissue from the distal end of the tail was placed in a cryogenic tube containing buffer solution (100mM Tris HCl pH 8.0, 100mM EDTA pH 8.0, 10mM NaCl, 0.5% SDS) and stored at ambient temperatures until transferred to an -80°C ultra-cold freezer upon arrival in the laboratory.

DNA was isolated by a modified phenol/chloroform extraction procedure (Sambrook et al. 1989). Tissue (2 μg) was digested in extraction buffer (400mM Tris-HCl pH 7.5, 100mM EDTA, 250mM NaCl, Proteinase K 600μg/ml) overnight at 55°C. Extractions were first performed with two equal volumes of phenol (equalibrated with Tris-HCl pH 7.5) and then extracted twice with chloroform/isoamyl alcohol (25:1). A microcon-50 filter (Millipore) was used for concentrating DNA in the aqueous layer by washing the sample twice in the filter with 400 μl of TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0). DNA extraction quality was checked by agarose gel electrophoresis, and the concentration estimated by fluorimetry using a Hoefer TKO 100.

The polymerase chain reaction (PCR) was used to amplify a ~550 base pair fragment of the mtDNA 16S rRNA gene locus using the following primers designed for salamanders: 16SA-5' (5'-ACAAGTGATTACCTTTG-CATAATACCG-3') and 16SB-3' (5'-TTTAGTAAATTAAGCTT-TGACGCTATTTAGTAAG-3'. PCR reactions were carried using a 50 μl reaction volume and 100 ng of DNA with the following cocktail concentrations: 0.5 units of Taq Polymerase Gold (Perkin Elmer) with 5 μl of the supplied reaction buffer; 100 μM of each nucleotide (dATP, dCTP, dGTP, dTTP); 2mM MgCl; and 1 mM of each primer. A MJ Research programmable thermocycler (PTC 100) was used for all amplifications with the following steps: an initial 10 min. denaturation at 93°C, followed by 40 cycles of denaturation for 1 min. (93°C), annealing for 1 min. (52°C) and extending for 2 min. (72°C). A final extension for 10 min. (72°C)

followed the cycles and then the reaction was held at 4°C until removed from the cycler. Amplifications were prepared for sequencing by extracting fragments from 1% agarose gels using an ultra-free-mc 0.45 filter (Millipore). The template was concentrated by washing the supernatent using a microcon-50 filter (Millipore). Sequences were generated using Big-Dye Terminator cycle sequencing (Perkin Elmer) based on the Sanger method and read with an Applied Biosystems (373A) sequencer at the Oregon State University Central Services Laboratory. Sequencing primers included 16SA-5' and 16SB-3'. Alignments of sequences were made by eye using the Genetic Data Environment (Smith et al. 1992). Sequence gaps were aligned based upon inferred secondary structures for 16S rRNA genes (Chapter 4).

Maximum parsimony (Camin and Sokal 1965, Hennig 1966, Swofford et al. 1998) and maximum likelihood phylogenetic analyses were used to infer relationships among the mtDNA 16S rRNA haplotypes (Felsenstein 1981, Huelsenbeck and Crandall 1997). Comparisons of each method have been discussed previously (Hasegawa and Fujiwara 1993, Huelsenbeck and Hillis 1993, Kuhner and Felsenstein 1994, Tateno et al. 1994), but it has been suggested that trees yielding similar topologies based on different methods are more likely to reflect true phylogenetic relationships (Kim 1993).

All phylogenetic trees were generated using the program PAUP* 4.0b1 (Swofford 1998). Maximum parsimony heuristic searches were made to search for trees of the shortest length using the tree bisection-reconnection algorithm with all characters weighted equally and gaps treated as a 5^{th} character. Maximum

likelihood reconstructions accounted for rate heterogeneity in transversion/transition ratio using a gamma distribution of 0.5, empirically derived nucleotide frequencies, and the Hasagawa-Kishino-Yano substitution model (Hasagawa et al. 1985). A consensus bootstrap tree (100 or 1000 replicates) was used to assess the reliability of support for each node (Felsenstein 1985). Outgroups species were comprised of sequences from individuals representative of the Columbia torrent salamander (*R. kezeri*) and the Olympic torrent salamander (*R. olympicus*).

Allozyme Analyses

Eight populations (Table 5.1) were examined for 6 presumptive allozyme loci fixed for diagnostic alleles in each species (Cascade torrent and Southern torrent salamanders) as indicated by Good et al. (1987). Allozymes were surveyed from liver tissue of adult animals sacrificed using 10 % chlorotone in accordance with established protocols for amphibians (McDiarmid 1993). We used horizontal starch gel electrophoresis to examine loci using two different buffer systems: 1) RW (Ridgway et al., 1970) and 2) Tris-Citrate II (Selander et al. 1971). The RW buffer system was used to examine AAT-1, EST, SOD, and the Tris II was used for MDH, IDDH, and ME.

Results

Mitochondrial DNA analyses

The *contact zone* populations contained either Cascade or Southern Torrent salamander haplotypes, which were allopatrically distributed. There were five unique haplotypes with 47 variable sites (including gaps) among the *contact zone* populations (Table 5.2). Individuals within each population yielded identical haplotypes. Four northern populations haplotypes (Alder Creek, Gold Point, Jones Creek Trail, and Barrow Pit) showed less than a 0.2 % sequence difference with the control Cascade torrent salamander (H.J. Andrews). Three of these sites had identical haplotypes: Gold Point, Jones Creek Trail, and Barrow Pit. In contrast, three southern populations (Goodman Creek #1, Goodman Creek #2, and Patterson Mtn) had identical haplotypes with the control Southern Torrent salamander (Mary's Peak) and three other southern populations (Rainbow Mine, Middle Bryce Ck, and N. Scaredman) had identical haplotypes less than 0.2% different from the control. The difference between the Cascade and Southern torrent salamander haplotypes was significantly greater ranging between 2.28 – 2.69 %.

The existence of Cascade and Southern Torrent haplotypes and their allopatric distribution in the *contact zone* is further supported by phylogenetic analyses. Both phylogenetic methods yielded trees showing two major clades, with each clade corresponding to either the control Cascade torrent (H.J. Andrews) or Southern torrent salamander (Mary's Peak). For maximum parsimony analyses, a

Table 5.2 Mitochondrial DNA 16S rRNA (499bp) sequence variation in Torrent salamanders from control and *contact zone* populations.

	Population	Haplotype	Sequence Location 1111112222222222222222222223333333334444444444 1117790122223333333334445678123334478001167899 9 2566776957890123456784799335002342460470483178 9
1.	H.J. Andrews	Cascade torrent	ACTTTGCATCC-----TAAAAGATATACAT---T-ACTGT-AC-TAG
2.	Alder Ck	Cascade torrent	.....----.....---.-.....T..G...
3.	Gold Point	Cascade torrent	.....-----.....---.-.....-T.-...
4.	Jones Ck Trail	Cascade torrent	.....-----.....---.-.....-T.-...
5.	Barrow Pit	Cascade torrent	.....-----.....---.-.....-T.-...
6.	Goodman Ck #1	Southern torrent	..-..ATG.-------.....-..G..A---.T.TA..T.TGATC
7.	Goodman Ck #2	Southern torrent	..-..ATG.-------.....-..G..A---.T.TA..T.TGATC
8.	Patterson Mtn	Southern torrent	..-..ATG.-------.....-..G..A---.T.TA..T.TGATC
9.	Rainbow Mine	Southern torrent	...C.ATG.-------.....-..G..A---.T.TA..T.TGATC
10.	Middle Bryce Ck	Southern torrent	...C.ATG.-------.....-..G..A---.T.TA..T.TGATC
11.	N. Scaredman	Southern torrent	...C.ATG.-------.....-..G..A---.T.TA..T.TGATC
12.	Mary's Peak	Southern torrent	..-..ATG.-------.....-..G..A---.T.TA..T.TGATC
13.	Columbia torrent	Columbia torrent	GT.C.ATTCAA----------AG-..GT.AA--.-.TA..T..GG.T
14.	Olympic torrent	Olympic torrent	G..ACATT.AAATAAAA.T..AG-G-GTGAA--CTTTACAT.TGG.T

heuristic search resulted in three equally parsimonious trees each composed of 48 steps based on 21 parsimony informative characters. A maximum parsimony bootstrap tree (1000 replications) yielded a tree with a length of 48 (consistency index 0.96, retention index 0.97, Figure 5.2). The divergence of the two major clades was well supported with 93% support. The heuristic maximum likelihood search generated a single tree with a negative ln likelihood score of 903, while a bootstrap search (100 replications) yielded a tree with a negative ln-likelihood of 861 (Figure 5.3). Divergence of the two clades is supported by a 99% bootstrap value.

Allozyme analyses

Similar to mtDNA results, *contact zone* populations indicated allele patterns diagnostic for either Cascade torrent or Southern torrent salamanders. Northern populations (Gold Point, Jones Ck Trail, Barrow Pit) resulted in a fixed diagnostic allele pattern identical to the control Cascade torrent salamander population (H.J. Andrews) for the following variants AAT-1 (*c*), EST-2 (*b*), SOD *(a)*, MDH-1 (*b*) and ME (*d*) (Table 5.3). The IDDH locus showed variation in the Gold Point and Jones Ck Trail for the (*e*) and (*f*) allele variants; however, they did not have the (*g*) variant found in Southern torrent salamanders (Table 5.3). In contrast to the northern populations, southern populations (Goodman Ck #1, Goodman Ck #2, Patterson Mtn, Rainbow Mine, Middle Bryce Ck) showed a fixed allele pattern

identical to the control Southern Torrent salamander population (Mary's Peak) which included AAT-1 (*e*), EST-2 (*c*), SOD (*b*), MDH-1 (*a*) and ME (*f*). In sum, the results suggested northern populations are taxonomically Cascade torrent salamanders and southern populations Southern Torrent salamanders.

Figure 5.2 Maximum parsimony tree based on mtDNA 16S rRNA sequences (499bp) of Torrent salamanders from control and *contact zone* populations (number of steps above branches, bootstrap values below).

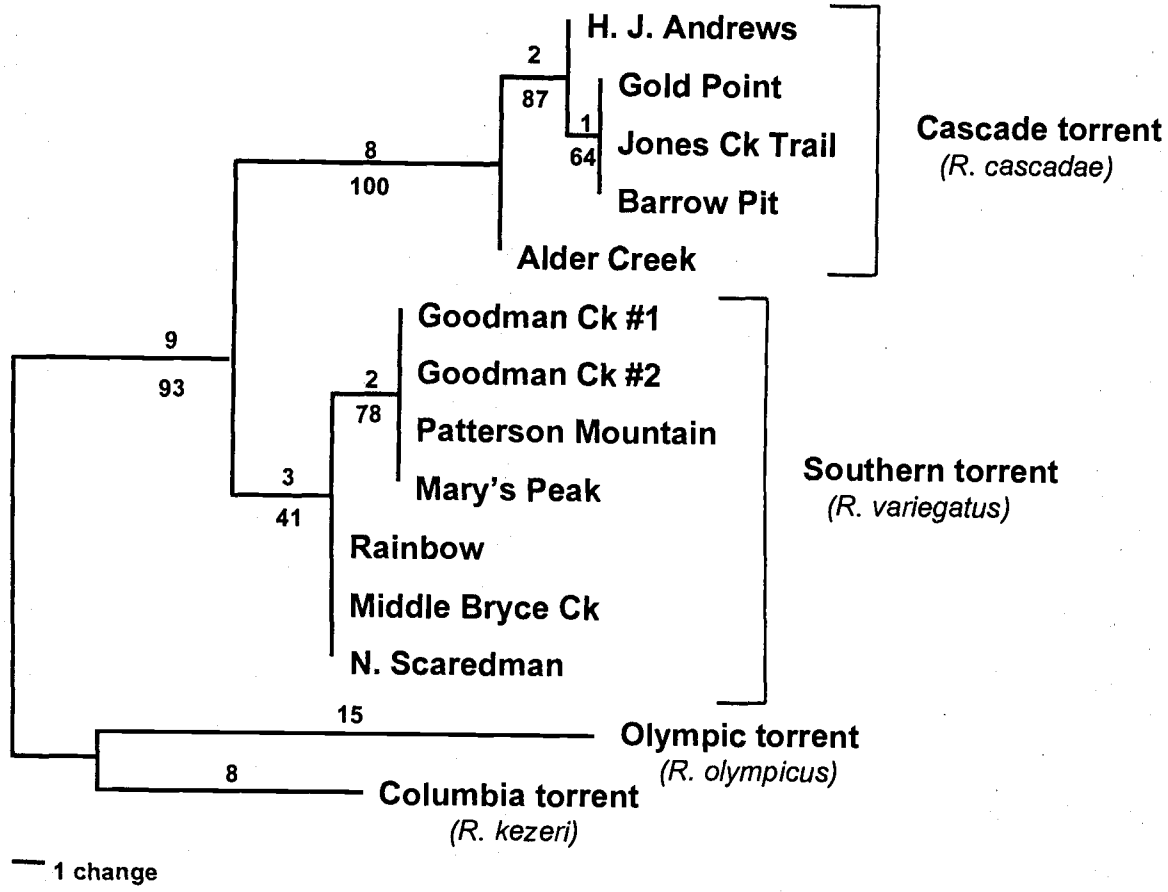
H. J. Andrews
Gold Point
Jones Ck Trail
Barrow Pit
Alder Creek
Cascade torrent
(R. cascadae)
Goodman Ck #1
Goodman Ck #2
Patterson Mountain
Mary's Peak
Rainbow
Middle Bryce Ck
N. Scaredman
Southern torrent
(R. variegatus)
Olympic torrent
(R. olympicus)
Columbia torrent
(R. kezeri)
2
87
1
64
8
100
9
93
2
78
3
41
15
8
1 change

Figure 5.2

Figure 5.3 Maximum likelihood tree based on mtDNA 16 rRNA sequences (499bp) of Torrent salamanders from control and *contact zo*ne populations (number of steps above branches, bootstrap values below).

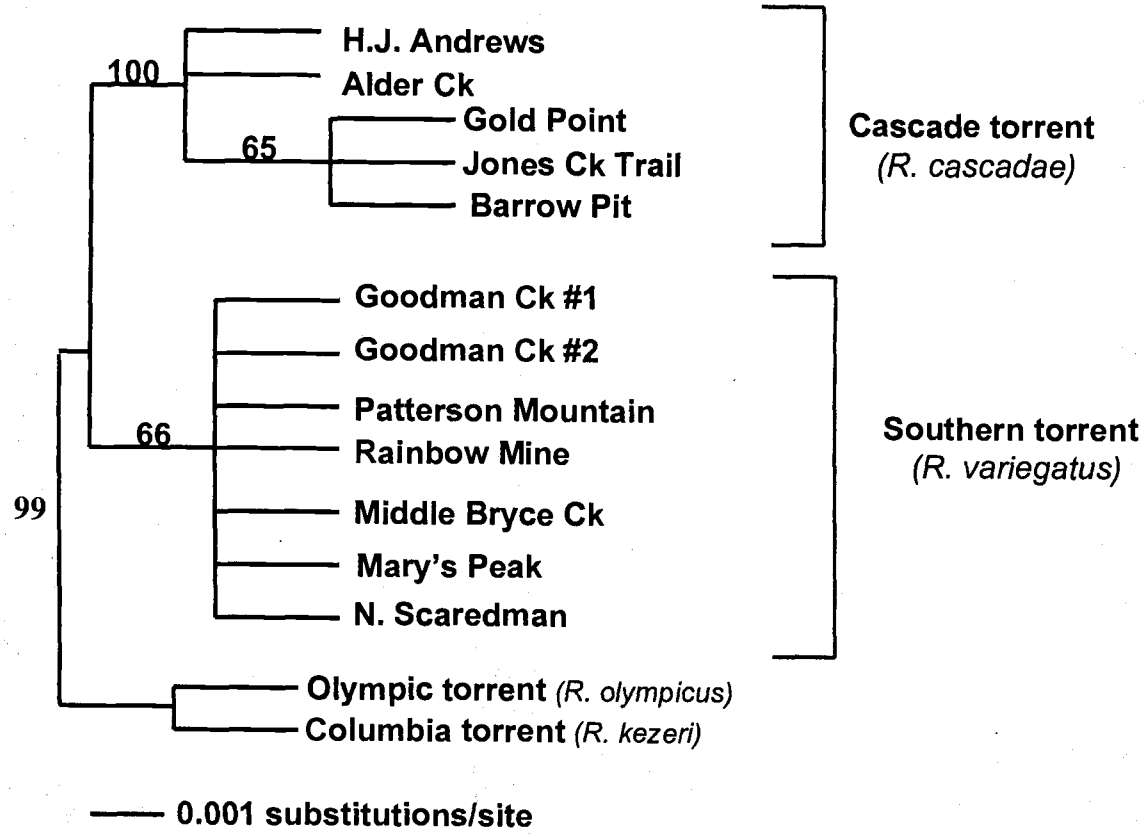
H.J. Andrews
Alder Ck
Gold Point
Jones Ck Trail
Barrow Pit
100
65
Cascade torrent
(R. cascadae)
Goodman Ck #1
Goodman Ck #2
Patterson Mountain
Rainbow Mine
Middle Bryce Ck
Mary's Peak
N. Scaredman
66
99
Southern torrent
(R. variegatus)
Olympic torrent (R. olympicus)
Columbia torrent (R. kezeri)
0.001 substitutions/site

Figure 5.3

Table 5.3 Allele frequencies of six allozyme loci from Torrent Salamanders in control and *contact zone* populations. Allele variants labeled using the system of Good et al. (1989).

	AAT-1		EST-2		SOD		MDH-1		IDDH			ME	
Population	c	e	b	c	a	b	a	b	e	f	g	d	f
Cascade torrent													
1. H.J. Andrews	1.0	_	1.0	_	1.0	_	_	1.0	_	1.0	_	1.0	_
2. Alder Creek	N/A		N/A		N/A		N/A			N/A		N/A	
3. Gold Point	1.0	_	1.0	_	1.0	_	_	1.0	0.2	0.8	_	1.0	_
4. Jones Creek Trail	1.0	_	1.0	_	1.0	_	_	1.0	0.1	0.9	_	1.0	_
5. Barrow Pit	1.0	_	1.0	_	1.0	_	_	1.0	_	1.0	_	1.0	_
Southern torrent													
6. Goodman Ck #1	_	1.0	_	1.0	_	1.0	1.0	_	_	_	1.0	_	1.0
7. Goodman Ck #2	_	1.0	_	1.0	_	1.0	1.0	_	_	_	1.0	_	1.0
8. Patterson Mountain	_	1.0	_	1.0	_	1.0	1.0	_	_	_	1.0	_	1.0
9. Rainbow Mine	_	1.0	_	1.0	_	1.0	1.0	_	_	_	1.0	_	1.0
10. Middle Bryce Ck	_	1.0	_	1.0	_	1.0	1.0	_	_	_	1.0	_	1.0
11. Mary's Peak	_	1.0	_	1.0	_	1.0	1.0	_	_	_	1.0	_	1.0

Discussion

Good and Wake (1992) speculated on the existence of a possible contact zone among the Cascade and Southern Torrent salamander in the central Cascades because of the existence of apparently suitable habitat. Our results confirm their inference and extend the range of both species in the central Cascades, which appear to be allopatrically distributed. Neither molecular marker (mtDNA or allozyme) supported sympatry or hybridization between these species. Further, the middle fork of the Willamette River may be a phylogeographic barrier limiting the distribution of both species.

The range of the Cascade torrent salamander is extended 25 km south to just north of the middle fork of the Willamette River, while the range of the Southern Torrent salamander is extended 40 km north in the central Cascades to just south of the south-bank of the middle fork of the Willamette River. The occurrence of this distribution begs the question of whether the Willamette River provides a geographic barrier to dispersal or some other factor is responsible.

In a similar contact zone study between Southern Torrent and Columbia Torrent salamanders (*R. kezeri*) occurring in the Coastal mountain range of Oregon, allozymes did not indicate hybridization or sympatry occurred, yet these species are separated by less than 100 meters by the Little Nestucca River (Good and Wake

1992). Since both species appear to cross larger rivers and should not be limited by the Little Nestucca River, Good and Wake (1992) discussed alternative reasons for the distribution which included: a selection gradient for the loci sampled, populations are in recent contact, or there is active exclusion of one species by the other.

For our study in the central Cascades, we can rule out their first hypothesis because a selection gradient is not expect to act on a nearly neutral marker such as the mitochondrial 16S RNA gene. Therefore, if hybridization was occurring, reciprocal monophyly should not be expected for this marker. For the second alternative, phylogenetic analyses of the more quickly evolving the mtDNA cytochrome *b* locus indicate salamanders found in the central Cascades appear to have diverged from populations found in the Coast range to the northwest (Chapter 6). This is contrary to expectations of the recent contact hypothesis, in which Cascade populations of Southern torrent salamanders should be more closely related to southern Umpqua River populations were there appears to be a continuous bridge of habitat that allows for dispersal from the south. Active exclusion by pre-occupancy could occur, but reciprocal transplant experiments need to be conducted to test this hypothesis. Finally, we cannot rule out the

possibility the Willamette River may provide an important historic geographic barrier to dispersal of both species. Moreover, it may be a barrier for other taxa as well. For example, the southern distribution of the terrestrial Oregon Slender salamander (*Batrachoseps wrighti*) is poorly known and River may play a role in limiting its dispersal.

Although among population variation appears to be great, there appears to be some correlation with color pattern variation and taxonomic identity for *contact zone* populations (Weddell L and Wagner RS, unpublished). Individuals identified by molecular methods as Cascade torrent salamanders appear to have distinct dorsal spotting with a lighter dorsal background coloration. In contrast, individuals identified as Southern torrent salamanders have larger, less distinct dorsal spotting and a darker background coloration with a more distinct demarcation between the dorsum and venter.

In sum, this study identifies and provides a significant range extension for both the Cascade torrent salamander and the Southern torrent salamander. However, our study reports on the only known localities occupied by Southern torrent salamanders that have been found in the region between the Willamette River and the Steamboat Springs area in the Cascade Range. These populations

appear to be patchily distributed and may face threats related to timber harvest practices. Therefore, further surveys need to be carried out to determine the distribution and abundance of the Southern Torrent salamanders in this region. This is particularly important considering the recent concern for the Southern Torrent salamander.

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CHAPTER 6

PHYLOGEOGRAPHY AND CONSERVATION IN THE SOUTHERN TORRENT SALAMANDER (*Rhyacotriton variegatus*).

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Abstract

The Southern Torrent Salamander (*Rhyacotriton variegatus*) has recently been overturned for listing under the U.S. Endangered Species Act due to lack of information regarding population fragmentation and gene flow. Mainly found in small order streams and headwaters associated with late-successional coniferous forests of the U.S Pacific Northwest, potential threats to their persistence include disturbance activities related to timber harvest. Therefore, we conducted a study of fine-scale population differentiation in an effort to understand the potential impact of natural versus anthropogenic contributions to population fragmentation in the Southern Torrent Salamander. Sequence variation in the mitochondrial cytochrome *b* gene locus (779 bp) was examined among 72 localities sampled across their range. There were significant differences in sequence variation at local and regional scales, yielding 49 distinct haplotypes. Three methods of phylogenetic inference revealed three major deeply diverging clades that included a *north coast clade*, *Oregon clade* and *California clade*. The Yaquina River, Oregon, may provide a phylogeographic barrier between the *north coast clade* and *Oregon clade*; while the Smith River, in northern California, corresponds to the haplotype break between the *Oregon clade,* and *California clade*. Merging these results with those of previous genetic studies using allozymes (Good et al. 1989, Good and Wake 1992) suggest gene flow among populations is low and may be exacerbated by factors related to habitat and population fragmentation, we suggest Evolutionary

Significant Unit (ESUs) designation for the *California clade* and separate Management Unit designations for the *north coast clade* and the *Oregon clade.* Recognition of conservation units can aid in management, listing, and recovery of Southern Torrent Salamanders at the appropriate scale, by focusing management efforts on the most threatened portions of the species range and avoiding actions that might unnecessarily impact the whole species range.

Introduction

The Southern Torrent Salamander (*Rhyacotriton variegatus*) was recently denied listing under the U.S. Endangered Species Act (Federal Register 60:33785). U.S. Fish and Wildlife Service concluded there was a "lack of information (that) the species is threatened by low gene flow and low genetic diversity across its range". This statement reflects the classic paradigm that species conservation efforts need to maintain gene flow among populations to avoid loss of genetic diversity (Lande and Barrowclough 1987). While this is an appropriate goal for most species conservation efforts, many amphibians have low rates of dispersal and are subject to historical vicariant events that can isolate populations for long periods; therefore, a general pattern for amphibians is one of low gene flow and extreme genetic differentiation among populations (Highton et al. 1989, Good and Wake 1992, Tilley and Mahoney 1996). Subsequently, species may be threatened

by loss of unique lineages rather than limited gene flow. However, at the local scale reduced gene flow may decrease viability in populations impacted by habitat fragmentation processes. Therefore, conservation efforts need to consider issues of scale in developing management strategies.

The conservation unit concept, namely Evolutionary Significant Units (ESUs) and Management Units (MUs), can provide a framework for determining the scale at which management efforts should be targeted. ESUs can be used to define "distinct population segments" for listing under the U.S. Endangered Species Act (Waples 1991). There has been intense debate over how conservation units should be defined (Ryder 1986; Waples 1991; Dizon et al. 1992; Moritz 1994a,b; Vogler and Desalle 1994; Bowen 1998; Crandall et al. 2000). Recently, it has been suggested that ESU criteria should include ecologically and adaptively significant traits (Crandall et al. 2000). However, the ecological or adaptive significance of a given trait is difficult to determine, let alone predict if it will be "adaptive" in the future (Gould and Lewontin 1979). While these traits should be considered when evaluating management and listing decisions, conservation units should be based on an operational genetically determined definition, otherwise they run the risk being an arbitrary unit, similar to the classic subspecies taxonomy (Wilson and Brown 1953, O'Brien and Mayr 1991).

The most widely used conservation unit designations are those described by Moritz (1994a,b; see also Moritz et al. 1995), which define ESUs to reflect long-term reproductive isolation by requiring reciprocal monophyly of mitochondrial alleles *and* divergence of nuclear alleles. Further, MUs, subunits that comprise ESUs, are designed for short-term or demographic focus and are defined by the divergence of either mitochondrial alleles *or* nuclear alleles. These definitions provide a framework for determining the scale at which to focus management efforts and preserve historical lineages.

The Southern Torrent Salamander is widely but patchily distributed throughout the Pacific Coast mountain range of the U.S. Pacific Northwest, extending from Tillamook County, Oregon, south to Mendicino County, California. While they are limited primarily to the Pacific Coast range, they do extend eastward into the Central Cascade Range of Oregon (Figure 6.1; Leonard et al. 1994; Chapter 5). Mostly found in small streams and headwaters associated with late-successional forests, they are impacted by timber harvest and related disturbance activities (Bury and Corn 1988a, Welsh and Lind 1988, Corn and Bury 1989, Bury et al. 1991, Diller and Wallace 1997). Juvenile larvae are restricted to cold, clear, fast-flowing streams and adults are rarely found more than a few meters from these stream-banks. Both age classes appear sensitive to loss of body water and heat shock, and require low ambient temperatures (Brattstrom 1963, Nussbaum and Tait 1977, Nussbaum et al. 1983). Subsequently, removal of the forest canopy may lead to increased mean stream temperatures and stream

sedimentation leading to extirpation of local populations (Bury and Corn 1988b, Corn and Bury 1989, Welsh 1990, Welsh and Lind 1992, Welsh et al. 1992, Welsh and Ollivier 1992). As a consequence, re-colonization following extirpation is thought to be low, due to these ecological factors and their apparent limited dispersal abilities (Nussbaum and Tait 1977, Nijhuis and Kaplan 1998).

In this paper, we demonstrate how genetic differentiation in the Southern Torrent Salamander can be framed in the context of conservation units and used to better evaluate potential ESA listing options. We used mitochondrial (mtDNA) cytochrome b gene sequences to investigate population differentiation across their range and compare results to allozyme studies (Good et al. 1987, Good and Wake 1992). MtDNA cytochrome *b* has been used widely as a metric in both intra-specific and inter-specific phylogenetic studies of salamanders (Spolsky et al. 1992, Hedges et al. 1992, Moritz et al. 1992, Tan and Wake 1995, Jockusch 1996, Jackman et al. 1997, Garcia-Paris and Wake 2000).

Figure 6.1 Sampling locations of Southern Torrent Salamanders. See Table 6.1 for site identification. Inset (A) shows an expanded view of localities where samples were collected in the Smith River Area along the Oregon-California border.

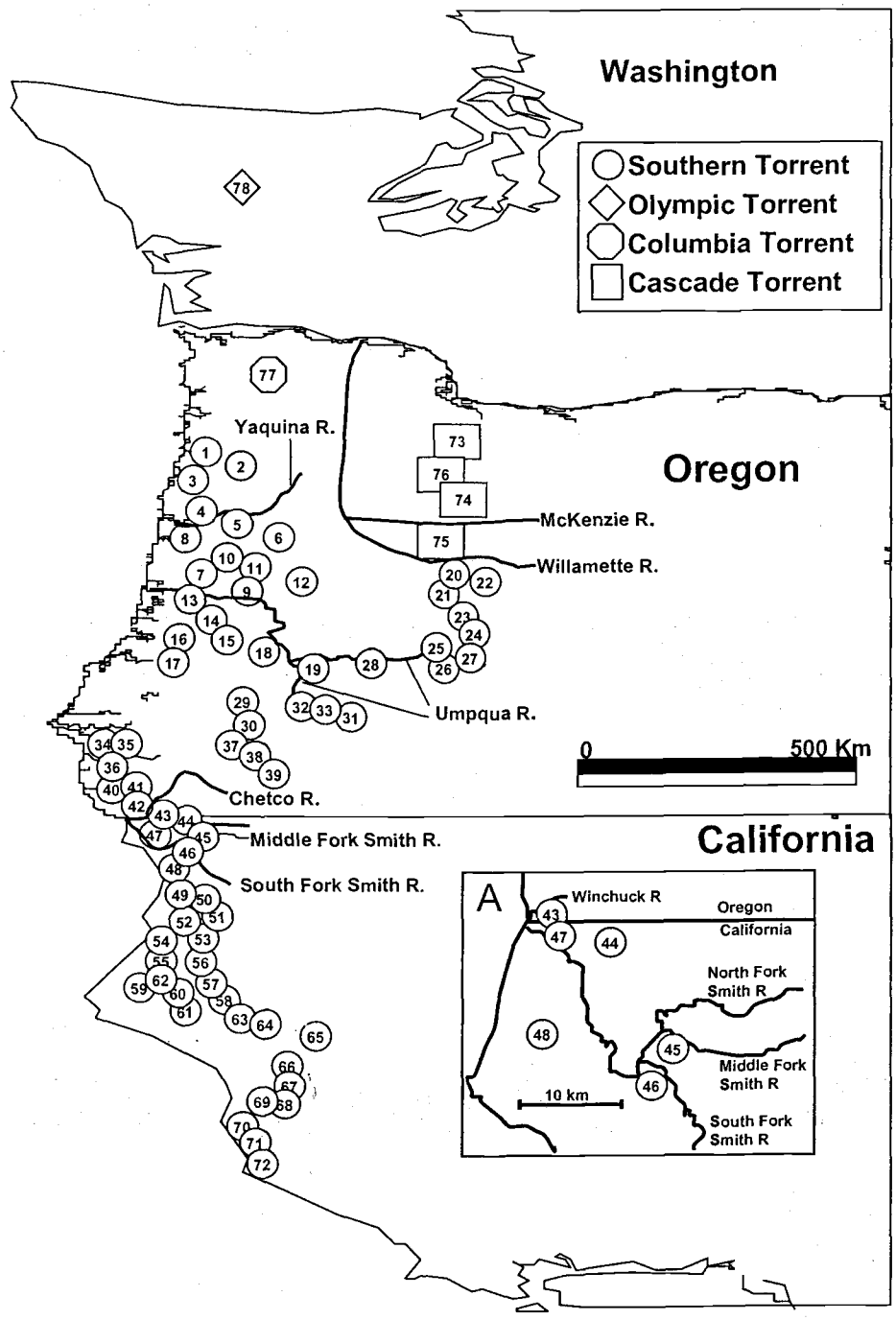
Washington
Southern Torrent
Olympic Torrent
Columbia Torrent
Cascade Torrent
Yaquina R.
Oregon
McKenzie R.
Willamette R.
Umpqua R.
0
500 Km
Chetco R.
Middle Fork Smith R.
South Fork Smith R.
California
A
Winchuck R
Oregon
California
North Fork Smith R
Middle Fork Smith R
South Fork Smith R
10 km

Figure 6.1

Methods

Southern Torrent Salamanders were sampled from 72 localities (n = 2-5/location) throughout their known range (Figure 6.1, Table 6.1). Sample tissue was taken by non-lethal tail clipping (approximately 1 cm) from hand-captured adults. Individual sterilized surgical scissors were used to sample each salamander. Sample tissue was stored immediately in a cryogenic tube containing buffer solution (100mM Tris HCl pH 8.0, 100mM EDTA pH 8.0, 10mM NaCl, 0.5% SDS) until transferred to an ultracold freezer (-80°C).

A modified phenol/chloroform extraction procedure was used to isolate and purify DNA (Sambrook et al. 1989). First, 2 μg of tissue was digested in 400 μl of extraction buffer (100mM Tris-HCl pH 7.5, 100mM EDTA, 250mM NaCl, Proteinase K 600ug/ml) overnight at 55°C. A second aliquot of Proteinase K was added if the tissue was not fully digested. Samples were extracted twice with equal volumes of phenol (equilibrated with Tris-HCl buffer pH 7.5) and then two chloroform/isoamyl alcohol (25:1) extractions. Finally, the aqueous layer was placed in a microcon-50 filter (Millipore) and washed twice with 400 μl of TE buffer (10mM Tris HCl, 0.1 mM EDTA, pH 8.0) to purify and concentrate DNA. The concentration for each sample was estimated using a Hoefer TKO 100 fluorimeter.

Table 6.1 Locations of Southern Torrent Salamander populations sampled. Numbers refer to locations in Figure 6.1.

	Population	Locality (Long/Lat)		County, State		Population	Locality (Lat/long)		County, State
1.	E. Little Nestucca	-123.892	45.123	Tillamook,OR	40.	Pistol R.	-124.313	42.284	Curry,OR
2.	W. Little Nestucca	-123.819	45.107	Tillamook,OR	41.	Little Redwood	-124.143	42.145	Curry,OR
3.	Ball Mountain	-123.940	44.920	Tillamook,OR	42.	Chetco R.	-124.173	42.130	Curry,OR
4.	Siletz	-123.941	44.656	Lincoln,OR	43.	Winchuck R.	-124.101	42.024	Curry,OR
5.	Salmon Ck	-123.728	44.587	Lincoln,OR	44.	L. Division Rd.	-124.025	41.870	Del Norte,CA
6.	Mary's Peak	-123.551	44.495	Benton,OR	45.	M. Fork Smith R.	-124.012	41.770	Del Norte,CA
7.	Alsea Area Trib.	-123.546	44.306	Benton,OR	46.	S. Fork Smith R.	-123.887	41.550	Del Norte,CA
8.	Risley Ck	-124.064	44.411	Lincoln,OR	47.	Dominie Ck.	-124.130	41.963	Del Norte,CA
9.	Bear Ck Trib.	-123.790	44.349	Benton,OR	48.	Miller Rellium	-124.054	41.748	Del Norte,CA
10.	Mossy Falls	-123.749	44.350	Benton,OR	49.	Hunter Ck	-124.029	41.575	Humboldt,CA
11.	Little Lobster Ck	-123.704	44.310	Benton,OR	50.	Turwer Ck #1	-123.950	41.590	Humboldt,CA
12.	Heidi Ck	-123.461	44.252	Lane,OR	51.	Turwer Ck #2	-123.970	41.590	Humboldt,CA
13.	Madera's Grave	-123.928	44.218	Lane,OR	52.	Omagar	-123.974	41.455	Humboldt,CA
14.	Mapleton	-123.856	43.920	Lane,OR	53.	Morek Ck	-123.826	41.269	Humboldt,CA
15.	Kentucky Falls	-123.820	43.890	Lane,OR	54.	McDonald Ck	-124.091	41.221	Humboldt,CA
16.	Elliot SF #1	-124.026	43.589	Douglas,OR	55.	Mitsui Ck	-124.052	40.978	Humboldt,CA
17.	Elliot SF #2	-124.034	43.492	Douglas,OR	56.	Wire Grass	-123.902	41.020	Humboldt,CA
18.	Bear Ck	-123.618	43.320	Douglas,OR	57.	Cannon Ck #1	-123.847	40.714	Humboldt,CA
19.	No Name	-123.440	43.480	Douglas,OR	58.	Cannon Ck #2	-123.888	40.711	Humboldt,CA
20.	Goodman #1	-122.676	43.831	Lane,OR	59.	Jacoby Ck	-124.034	40.817	Humboldt,CA
21.	Goodman #2	-122.696	43.831	Lane,OR	60.	M. Trib.	-124.019	40.843	Humboldt,CA
22.	Patterson Mountain	-122.616	43.776	Lane,OR	61.	Dry Ck	-124.019	40.843	Humboldt,CA
23.	M. Bryce Ck	-122.681	43.642	Lane,OR	62.	Black Dog	-124.018	40.858	Humboldt,CA
24.	Rainbow Mine	-122.656	43.573	Lane,OR	63.	Goodman Praire	-123.888	40.711	Humboldt,CA
25.	N. Scaredman	-122.794	43.397	Douglas,OR	64.	Graham Ck	-123.847	40.714	Humboldt,CA
26.	W. Scaredman	-122.754	43.368	Douglas,OR	65.	University Hills	-123.472	40.650	Trinity,CA
27.	E. Scaredman	-122.794	43.368	Douglas,OR	66.	Ten Mile	-123.598	39.753	Mendocino,CA
28.	Scott Mountain	-123.063	43.348	Douglas,OR	67.	Fox Ck	-123.594	39.741	Mendocino,CA
29.	Cow Creek	-123.632	42.904	Douglas,OR	68.	Elder Ck	-123.617	39.736	Mendocino,CA
30.	Ollala Ck	-123.546	44.306	Douglas,OR	69.	Skunk Ck	-123.615	39.738	Mendocino,CA
31.	Canyon Ck	-123.257	42.876	Douglas,OR	70.	Chadbourne	-123.761	39.628	Mendocino,CA
32.	Shoestring #1	-123.396	42.905	Douglas,OR	71.	Dark Gulch	-123.773	39.236	Mendocino,CA
33.	O'Shea Ck	-123.316	42.877	Douglas,OR	72.	M. Alder Ck	-123.639	39.005	Mendocino,CA
34.	Elk #1	-124.327	42.702	Curry,OR	73.	*R.cascadae* (T)	-122.059	45.122	Clackamas,OR
35.	Elk #2	-124.365	42.710	Curry,OR	74.	*R.cascadae* (Y)	-122.434	44.594	Linn,OR
36.	Qoutsana	-124.236	42.485	Curry,OR	75.	*R.cascadae* (A)	-122.640	43.914	Lane,OR
37.	N. Galice	-123.694	42.539	Douglas,OR	76.	R.cascadae (D)	-122.162	45.136	Clakamas,OR
38.	Galice	-123.631	42.543	Douglas,OR	77.	R.kezeri (R)	-123.519	45.794	Tillamook,OR
39.	Limpy Ck	-123.439	42.423	Douglas,OR	78.	*R.olympicus*	-124.276	48.044	Clallam,WA

A ~850 base pair (bp) fragment of the cytochrome *b* gene was amplified using the following primers designed for vertebrates: MVZ15 5'-GAACTAATGGCC-CACAC(AA/TT)TACGNAA-3' and MVZ16 5'-AAATAGGAAATATCATTCT-GGTTTA-AT-3' (Kocher et al. 1989). Each polymerase chain reaction was carried out with 100 ng of sample DNA in a 50 µl volume using the following cocktail concentrations: 0.5 units of Taq Gold (Perkin Elmer) with the supplied reaction buffer (5 µl); 100 µM for each of dATP, dCTP, dGTP, dTTP; 2mM MgCl and 1mM of each primer. A MJ Research thermocycler (PTC 100) was used for the amplifications programmed with the following parameters: an initial denaturation of 10 min. at 93°C, followed by 40 cycles of denaturation for 1 min. at 93°C, annealing for 1 min. at 52°C and extending at 72°C for 2 min. A final extension at 72°C for 10 min. completed the reaction that was then held at 4°C until removed from the cycler. Reaction products were run on 1% agarose gels and amplified cytochrome *b* fragments were extracted from gel slices using an ultra-free-mc 0.45 filter (Millipore). The supernatant was then transferred to micron-50 filter (Millipore) and washed twice with 400 µl distilled deionized water. Sequencing was performed at the Oregon State University Central Services Laboratory with an Applied Biosystems (373A) sequencer. Sequencing primers included MVZ-15, MVZ-16 and cytb2 (5'-AAACTGCAGCCCCTCAG-AATGATATTTGTCCTCA3', Moritz et al. 1992). Sequences from fragments were aligned by hand using the Genetic Data Environment (Smith et al. 1992) and those with indels were re-sequenced.

The following genetic diversity parameters were calculated using Arlequin (Schneider et al. 1997): number of unique haplotypes, transitions and transversions, polymorphic sites, and nucleotide diversity indices. The degree of sequence saturation was evaluated based on plots of total maximum likelihood distance versus percent sequence divergence for each codon position, and for transitions and transversions at each codon position.

Phylogenetic relationships among haplotypes were evaluated using three different inferential methods: distance (minimum evolution, Swofford 1998), maximum parsimony (Hennig 1966), and maximum likelihood (Felsenstein 1981). All phylogenetic relationships were calculated using PAUP* 4.0b1 (Swofford 1998). There have been several discussions comparing the merits of each method (Hasegawa and Fujiwara 1993, Huelsenbeck and Hillis 1993, Kuhner and Felsenstein 1994, Tateno et al. 1994); however, similar topologies derived using different methods are more likely to reflect the true phylogenetic relationships (Kim 1993).

Distance (minimum evolution) trees were calculated using the Kimura 2-parameter model (Kimura 1980) and 0.5 gamma distribution to account for rate heterogeneity among sites. Maximum parsimony was used to search for trees of shortest length. Trees were evaluated using a heuristic search, an empirically derived transversion:transition ratio, and the tree bisection-reconnection algorithm. Maximum parsimony trees were calculated using two different weighting schemes to evaluate if homoplasy at third positions influenced tree topology: (a) all codon

positions weighted equally and (b) first and second codon positions weighted five and three times the third codon positions. For maximum likelihood analyses, a skeletal data set (40 haplotypes) was constructed to reduce computational time by removing identical haplotypes and haplotypes with percent sequence divergences of less than 0.3. Maximum likelihood reconstructions accounted for rate heterogeneity among codon positions using a 0.5 gamma distribution, an empirically derived transition:transversion ratio, and the Hasegawa-Kishino-Yano substitution model (Hasegawa et al. 1985). For maximum likelihood, phylogenetic trees were calculated utilizing the unique haplotypes in order to minimize computational time.

For each method, a consensus bootstrap tree (100 replicates) was used to assess reliability of support for each node (Felsenstein 1985). Outgroup species were comprised of individuals representative of other taxa within the family Rhyacotritonidae which included: Cascade Torrent Salamander (*R. cascadae*), Columbia Torrent Salamander (*R. kezeri*), and Olympic Salamander (*R. olympicus*).

Genetic distances were plotted against geographic distances in order to investigate if population differentiation fit an isolation-by-distance model. Mantel (1967) tests using NTSYS-PC (Rohlf 1994) were used to estimate correlation coefficients between genetic distance and geographic distance matrices. Correlation coefficients were derived from r-values, normalized Z statistics, and examined for significance by permutation procedures (100 permutations; Smouse et al. 1986). Genetic distances were calculated using the Kimura 2-parameter model.

Results

There was significant variation in haplotype diversity among Southern Torrent Salamander populations at the regional and local scale. Nucleotide sequences (779 bp) were characterized by 123 polymorphic sites, a mean number of 22 pairwise differences among haplotypes, and a calculated nucleotide diversity of 0.028 ± 0.014 S.D (Table 6.2). Pairwise sequence differences (uncorrected) ranged considerably from 0.0 to 5.4%. Forty-nine distinct haplotypes were found among 72 populations (Table 6.2). Cytochrome *b* sequence differences among individuals within populations appears to be small, most with less than 0.03 % difference based on least two individuals per population. Therefore, only one representative sequence was chosen per locality for analyses in order to minimize computer time.

Most substitutions were synonymous with 35 first position, 27 second position, and 55 third position synonymous substitutions. There were nine non-synonymous amino acid substitutions based on a vertebrate mitochondrion codon table (Smith et al. 1991), with six 1st position and three 2nd codon position substitution. One non-synonymous substitution unambiguously differentiates populations based on geography; site 64 differentiates northern California populations from all others (Figure 6.2).

Table 6.2 Mitochondrial DNA sequence variation (125 variable sites) in 779 base pairs of the cytochrome *b* gene for Southern Torrent Salamanders (see Table 6.1 and Figure 6.1 for locations). H is the haplotype code.

Codon Position		313331331113133312323331123323133123113331332333211333133313232333323133112232313133312223231213311123132122223121231232323 1
		Sequence Position
		000000000011111111222222222222222233333333333344444444444444445555555555555666666 6666666666666666677777777777777777777777777777
		233466899901223478111233355577788922445566779902333456667778990012446668990111222 2446677778888888900122233344455566666666677777
Population	H	446804401791132458689814501835908756895736023826249802561477244632690458127145045 2369234701345680262129027569259011456783467 8
1. ELNestucca	A	TGCCCATTGTGCCTCAGGGATCTGGGTTGGGTTCATAGGCTAGACGCATTTGCGAAGGTGGCCGTCTTTCCGAAATCAGC AATAGTGTATATATTCGATTCTAGCAACCCACTTACTACCCAATT
2. WLNestucca	A	....
3. Ball Mt.	B	...T.....A.....A. .....
4. Siletz	C	...T.....A.....A.....A.....A.....T.....A. .....A..C.....
5. Salmon Ck	D	C.TTTT.AA.....A....A....A.A...G.....A.....G..G.....C.CA.TT.....CA. .....T.....A..A.....T.....C
6. Mary's Pk	E	C.TTTT.AA.....A....A....A.A...G.....A.....G..G.....CTCA..T.....CA. ...G.....T.G.....A..A.....T.....C
7. AlseaArea	F	C..TTT.AA.....A....A.A...G...A.....A.....A.....C.CA.TTA.....TA. .....T.GC.....A.....C
8. RisleyCk	G	C.TTTT.AA.....A....A....A.A...G.....A.....G..G.....C.CA.TT.....CA. ...G....G.....T.....A..A.....T.....C
9. BearCkTrib	H	C..TTT.AA.....T.....A....A.AC..G....T.....A.....A.G.....C.CA.T.A.....TA. ...G....G.....T.G....G.....A.....T...G..C
10.MossyFalls	I	..TTAT.AA..T.....A....A.A..TG.....A.....A.G.....C.CA.T.A.....TA. ...G....G.....T.G....G.....A.....T...G..C
11.LittLobster	J	...TAC.AA.....G.....A....A.A..TG.....A.....A.A.....C.CA.TTA....TTA. ...G.....T.G.....A.....C
12.Heidi Ck	J	...TAC.AA.....G.....A....A.A..TG.....A.....A.A.....C.CA.TTA....TTA. ...G.....T.G.....A.....C
13.MaderasG	K	..TTAT.AA..T.....A....A.A..TG.....A.....A.G.....C.CA.T.A.....TA. ...G....G.....T.G....G.....A.....T...G..C
14.Mapleton	L	...TAT.AA..T.....A....A.A..TG.....A.....A.A.....C.CA.T.A.....TA. ...G...G.....T.GC.....T.A.....
15.KYFalls	M	C..TAT.AA..T.....A...AA.A..TG.....A.....A.A.....C.CA.T.A.....TA. ...G...G.....T.GC.....T.A.....
16.ElliotSF1	N	C..TTT.AA.....T.....A....A.A...GG.....A.....A.G.....C.CA...A.....CA. .CG.....A...T.....A.....T.....A
17.ElliotSF2	N	C..TTT.AA.....T.....A....A.A...GG.....A.....A.G.....C.CA...A.....CA. .CG.....A...T.....A.....T.....A
18.BearCk	O	C..TAT.AA.....A.AC.A.A...G.....A...A...T.G...G.....C.CA...A.....CA. .G..A....A..G..TA..C.....A....C.....A
19.NoName	P	C..TAT.AA.....A.A..A.A...G.....A.....G.....C.CA...A.....CA. .....C.....T.....A.....T.....A
20.Goodman1	Q	...TAT.AA..T.....A.A..A.A..TG.....A.....A.G.A...CACA.T.A.....CA. ...G.....T.G.....A.....T.....A
21.Goodman2	Q	...TAT.AA..T.....A.A..A.A..TG.....A.....A.G.A...CACA.T.A.....CA. ...G.....T.G.....A.....T.....A
22.PattersonMt	Q	...TAT.AA..T.....A.A..A.A..TG.....A.....A.G.A...CACA.T.A.....CA. ...G.....T.G.....A.....T.....A
23.M.BryceCk	R	...TAT.AA..T.....A.....ACA..AAA..TG.....T.....A.....A.G.A...CACA.T.....CA. ...G.....T.G.....G.....A.....A
24.RainbowMine	S	...TAT.AA..T.....A.....ACA..AAA..TG.....T...G.A.....A.G.A...CACA.T.A.....CA. ...G.....T.G.....G...G.A.....A
25.N.Scaredman	T	...TAT.AA..T.....A.A..A.A..TG.....A.....A.G.A...C.CA.T.A.....CA. ...G.....T.G.....A.G.....A
26.W.Scaredman	T	...TAT.AA..T.....A.A..A.A..TG.....A.....A.G.A...C.CA.T.A.....CA. ...G.....T.G.....A.G.....A
27.E.Scaredman	T	...TAT.AA..T.....A.A..A.A..TG.....A.....A.G.A...C.CA.T.A.....CA. ...G.....T.G.....A.G.....A
28.Scott Mtn	T	...TAT.AA..T.....A.A..A.A..TG.....A.....A.G.A...C.CA.T.A.....CA. ...G.....T.G.....A.G.....A
29.Cow Creek	U	C..TTT.AA...T.T.....A....A.A...G....T.....A.C.....G.....C.CA...A.G...CA. .G...C.....T.....A..G.CT.....G
30.Ollala Ck	U	C..TTT.AA...T.T.....A....A.A...G....T.....A.C.....G.....C.CA...A.G...CA. .G...C.....T.....A..G.CT.....G
31.Canyon Ck	V	C..TTT.AA.....T.....A....A.A...G....T.....A.....G.....C.CA...A.....CA. .G...C.....T.....AC...C.....
32a.Shoestrg1	W	C..TTT.AA.....T.....A....A.A...G..A.T.....A.....G.....C.CA...A.....CA. .G...C.....T.....AC...C.....A
32b.Shoestrg2	X	CA.TTT.AA.....T.....A....A.A...G....T.....A.....G.....C.CA...A.....CA. .G...C.....T.....A..G.C.....A
33.O'Shea Ck	Y	C..TTT.AA.....T.....A....A.A...G....T.....A.....G.....C.CA...A.....CA. .G...C.....AT.....A....C.....A
34.Elk #1	Z	C..T.T.AA.....A.AC.A.A...G.....A...A...T.G...G.....C.CA...A.....CA. ....A....A..G..TA..C.....A....C.....A
35.Elk #2	AA	C..T.T.AA.....A.AC.A.A...G.....A...A...T.G...G.....C.CA...A.....CA. ....A..G....G..TA..C.....TTCA....C.....A
36.Qousatana	AB	C..TTT.AA.....C..A....A.....G.....A.....GG..G.....C.CA...A.....CA. .....G.G..TA.....A....C.....A

Table 6.2 continued

Codon Position		313331331113133312323333112332313312311333133233321133313331323233332313311223231313331222323121331112313212222312123123232312
		Sequence Position
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		233466899901223478111233355577788922445566779902333456667778990012446668990111222446677778888888900122233344455566666666677777
Population	H	446804401791132458689814501835908756895736023826249802561477244632690458127145045236923470134568026212902756925901145678346781
37.N.Galice	AC	C..TTT.AA.....T........A....A.A...G....T........A.........G.....C.CA...A.....CA. ...........G..TA..C..........A....C.......A
38.Galice	AD	C..TTT.AA.....T........A....A.A...G....T........A.........GA....C.CA...A.....CA. ...........G..TA..C..........A....C.......A
39.LimpyCk	AE	C..TTT.AA.T...T........A....A.A...G....T........A.........G.....C.CA...A.....CA. ...........G..TA..C..........A....C.......A
40.Pistol R.	AF	C..TTT.AA.............A....A.A...G......G......A.....G...G..G..C.CA...A..T..TA. ...........GG.TA..C.........G.A....C.......A
41.LRedwood	AF	C..TTT.AA.............A....A.A...G......G......A.....G...G..G..C.CA...A..T..TA. ...........G..TA..C..........A....C.......A
42.Chetco R.	AG	C..TTTCAA.............A....A.A...G............A.....G...G.....C.CA...A..T..TA. ...........G..TA..C..........A....C.......A
43.WinchuckR	AH	C..TTT.AA.............A....A.A...G............A.....G...G.....C.CAC..A.....TA. ......A........T.....................T....G.G
44.L.Division	AI	C..TTT.AA.........G...A....A.A...G.....C.......A.....G...G.....C.CA...A.....TA. ...........G..TA.............A....C.....G.G
45.MForkSmithR	AJ	C..TTT.AA.......A......A....A.A...G.........G....AC....G..AG.....C.CA...A.....CA. ......A........T.....................T....G.G
46.SForkSmithR	AK	...TTT.AAC...C.......T......A...C..........G...GA....A.GA.G...A.C.CA...A.....CA. G.....................T.....................T....G..
47.DominieCk.	AL	C..TTT.AA.............A....A.A...G............A.....G...G.....C.CA...A.....TA. G.....................T.....................T....G..
48.MillerRell	AK	...TTT.AAC...C.......T......A...C..........G...GA....A.GA.G...A.C.CA...A.....CA. G.....................T.....................T....G..
49.Hunter Ck	AM	C..TTT.AAC...C.......T......A.A.C.G............A.....G.A.G...A.C.CA...A.....CA. :.....................T.....................G..
50.TurwerCk#1	AM	C..TTT.AAC...C.......T......A.A.C.G............A.....G.A.G...A.C.CA...A.....CA. .....................T.....................G.G
51.TurwerCk#2	AM	C..TTT.AAC...C.......T......A.A.C.G............A.....G.A.G...A.C.CA...A.....CA. .....................T.....................T....G..
52.Omagar	AN	...TTT.A.C...C.......TC.....A.A.C...G..........A......GA.G...A.C..A...A.....CAT .....................T.....................T....G..
53.Morek Ck	AO	...TTT.AAC...C..............A.A.C..........G....A.....G.A.G...A.C.CA...A...C.CAT .....................T.....................T....G..
54.Mad Dog	AS	...TTT.A.C...C.......TC.....A.A.C...G..........A......GA.G...A.C.CA...A.....CA. .....................T.....................T....G..
55.MitsuiCk	AP	...TTT.AAC...C..............A.A.C..............A.....G.A.G...A.C.CA...A.....CA. .....................T..........T..........T....G..
56.WireGrass	AR	...TTT.A.C...C.......TC.....A.A.C...G..........A......GA.G...A.C.CA...A.....CA. .....................T..........T..........T....G..
57.CannonCk1	AS	...TTT.A.C...C.......TC.....A.A.C...G..........A......GA.G...A.C.CA...A.....CA. .....................T.....................T....G..
58.CannonCk2	AS	...TTT.A.C...C.......TC.....A.A.C...G..........A......GA.G...A.C.CA...A.....CA. .....................T.....................T....G..
59.JacobyCk	AS	...TTT.A.C...C.......TC.....A.A.C...G..........A......GA.G...A.C.CA...A.....CA. .....................T.....................T....G..
60.M. Trib.	AT	...TTT.A.C...C.......TC.....A.A.C...G..........A......GA.G...A.C.CA...A.....CA. .....................T....T.....................G.G
61.Dry Ck	AR	...TTT.A.C...C.......TC.....A.A.C...G..........A......GA.G...A.C.CA...A.....CA. .....................T.....................G..
62.Black Dog	AU	...TTT.A.C...C.......TC.....A.A.C...G..........A......GA.G...A.C.CA...A.....CA. .....A...............T.....................G.
63.Goodman,CA	AV	...TTT.A.C...C.......TC.....A.A.C...G..........A......GA.G...A.C.CA...A.....CA. .....................T.....................G..
64.Graham Ck	AY	...TTT.A.C...C.......T......A.A.C...G..........A......GAAG...A.C.CA...A.....CA.G.....A.....T.....G....G..........TA...G.
65.UnivHills	AV	...TTT.A.C...C.......TC.....A.A.C...G..........A......GA.G...A.C.CA...A.....CA.G.....A.....T.....G....G..........TA...G.
66.Ten Mile	AW	...TTT.AAC...C.......T........A.C..............A....A.GAAG...A.C.CA...A.....CA.G.....A.....T.....G....G..........TA...G.
67.Fox Ck	AW	...TTT.AAC...C.......T........A.C..............A....A.GAAG...A.C.CA...A.....CA.G.....A.....T.....G....G..........TA...G.
68.Elder Ck	AW	...TTT.AAC...C.......T........A.C..............A....A.GAAG...A.C.CA...A.....CA. .....A.....T.....................G.
69.Skunk Ck	AW	...TTT.AAC...C.......T........A.C..............A....A.GAAG...A.C.CA...A.....CA. .....................T.....................A.G.G
70.Chadbourne	AX	...TTT.AAC...C.......T........A.C..............A....A.GAAG...A.C.CA...A.....CA. .....A.....T.....................T.....G.
71.Dark Gulch	AY	...TTT.A.C...C.......TC.....A.A.C...G..........A......GA.G...A.C.CA...A.....CA. ....A.A...G....TA.CC....T......T..........C..
72.M.Alder Ck	AZ	...TTT.AAC...C.......T........A.C..............A....A.GAAG...A.C.CA...A.....CA. ....A.A...G....TA.CCT...T......T..........C.C
73.*R.cascadaeT*	BA	...TTT..A.A.TAT...A.........C.....G.........T.T.A.....G..G.........A...A.....TA..G...C.........T.......AA......A....C.......A
74.*R.cascadaeY*	BB	...TTT..A.A.TAT...A.........C.....G.........T.T.A.....G..GC.......A...A.....TA..G...C.........T.......AA......A....C.......A
75.*R.cascadaeA*	BC	...TTT..A.A.TAT...A.........C.....G..A......T.T.A.....G..G.........A...A.....TA..G.............T.G.............A....C.......A

Table 6.2. continued

Codon Position	313331331113133312323333112332313312311333133233321133313331323233332313311223231313331222323121331112313212222312123123232312
	Sequence Position
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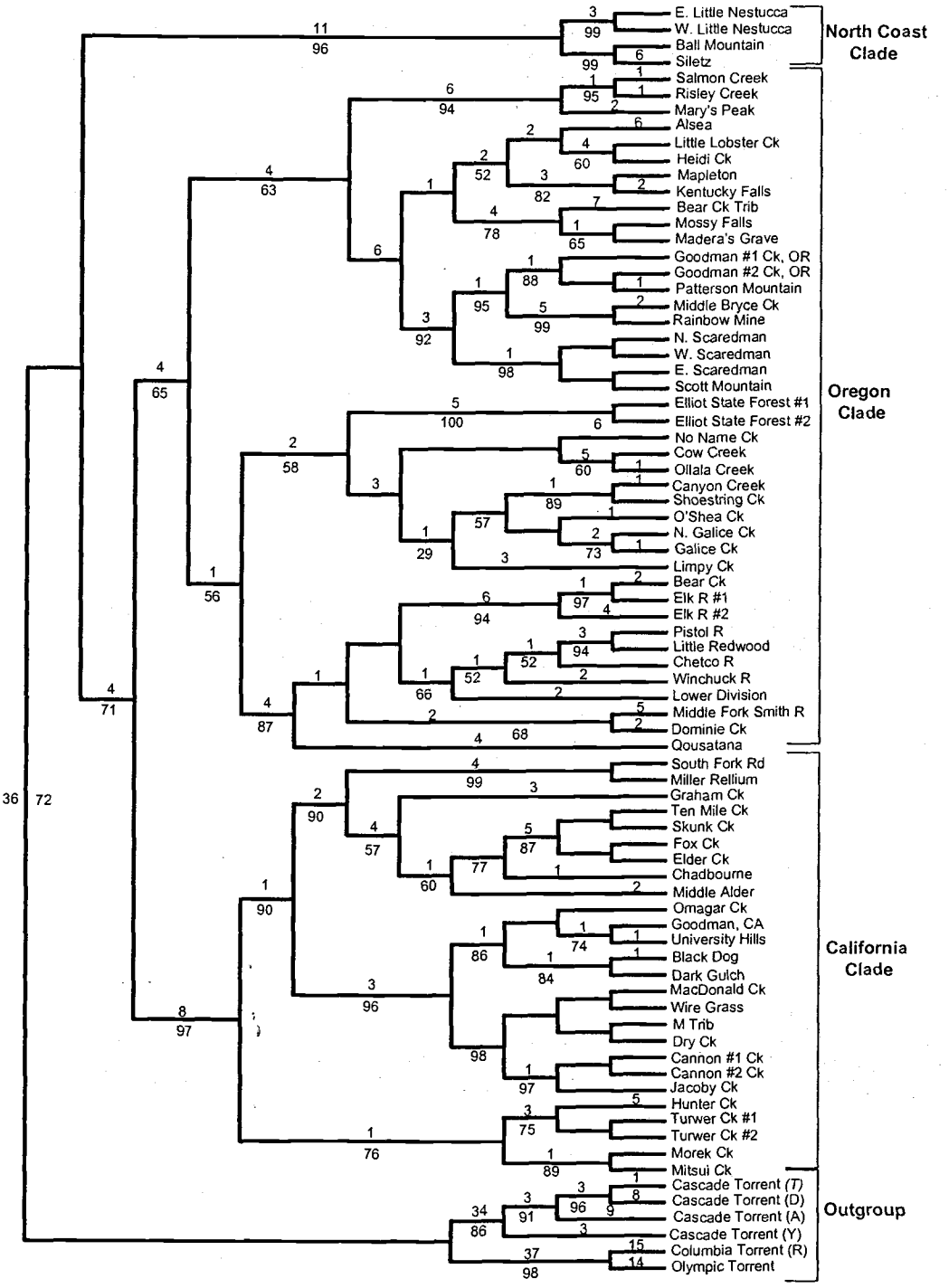
	233466899901223478111233355577788922445566779902333456667778990012446668990111222446677778888888900122233344455566666666777777
Population H	446804401791132458689814501835908756895736023826249802561477244632690458127145045236923470134568026212902756925901145678346 78
76.*R.cascadae*D BD	...TTT..A.A.TAT...A.........C.....G.........T.T.A......G..G........A...A.....TA. ....A.A...G....TA.CC....T......T..........CC.
77.R.kezeri(R) BE	C.TTTT.A.CA...T.T.A..T.A....C.....G.............A...T..G..G...A...CAC........TA. ...T..A.........TA.A.............T...AG.....GC.
78.*R.olympicus* BF	C.TTTT.AACAA.CT.T.A..T.A....C.....G.............A...T..G..G.......CA.........TAT...TA..........TA.A.....T......T.....?....GC.

Overall, there were 2.4 times as many transitions as transversions. A plot of uncorrected (p) DNA divergences for transitions and transversions at each codon position versus maximum likelihood DNA distances was used to evaluate the degree of saturation. Rates of substitution appear to increase linearly with maximum likelihood distances suggesting saturation effects and homoplasy should not influence phylogenetic inferences.

Southern Torrent Salamanders appear to be composed of three major clades based on the results of the phylogenetic analyses. Maximum parsimony and distance based trees showed similar topologies (Figure 6.2, Figure 6.3, Figure 6.4). For maximum parsimony trees, there was no difference between topologies of weighted and unweighted trees. Eight most-parsimonious trees each with a tree length of 253 (Consistency Index 0.68, Retention Index 0.87), showed differences only in the alternative branching of the Elliot State Forest and Southern Umpqua clades. Minimum evolution (Kimura-2 parameter) based methods yielded 36 trees each with a minimum evolution score of 0.507. For each method, a group comprised of northern coastal populations (*north coast clade*) was basal to two sister clades; identified as an *Oregon clade* and a *California clade*. Branching order of the sister clades is supported (bootstrap values > 71) by the maximum parsimony but not the minimum evolution method. Within the *Oregon clade*, two groups are supported (values > 61): the first group includes mid-Oregon populations, north Umpqua and central Cascade populations, while the second group includes southern coastal and southern Umpqua populations (Figure 3A).

The *California clade* is composed of three groups: one group of mostly northern populations (north-California) is basal to two sister groups distinguished by differentiation of mid-coastal (mid-California) and south-California

Figure 6.2 Maximum parsimony consensus tree (50% majority rule) derived from eight most-parsimonious trees (253 steps, consistency index = 0.67, retention index = 0.89) based on mitochondrial cytochrome b sequences (779bp) from Southern Torrent Salamanders. Each codon position was equally weighted and there were 89 parsimonious sites. Number of steps are above and bootstrap values greater than 50 are below each branch.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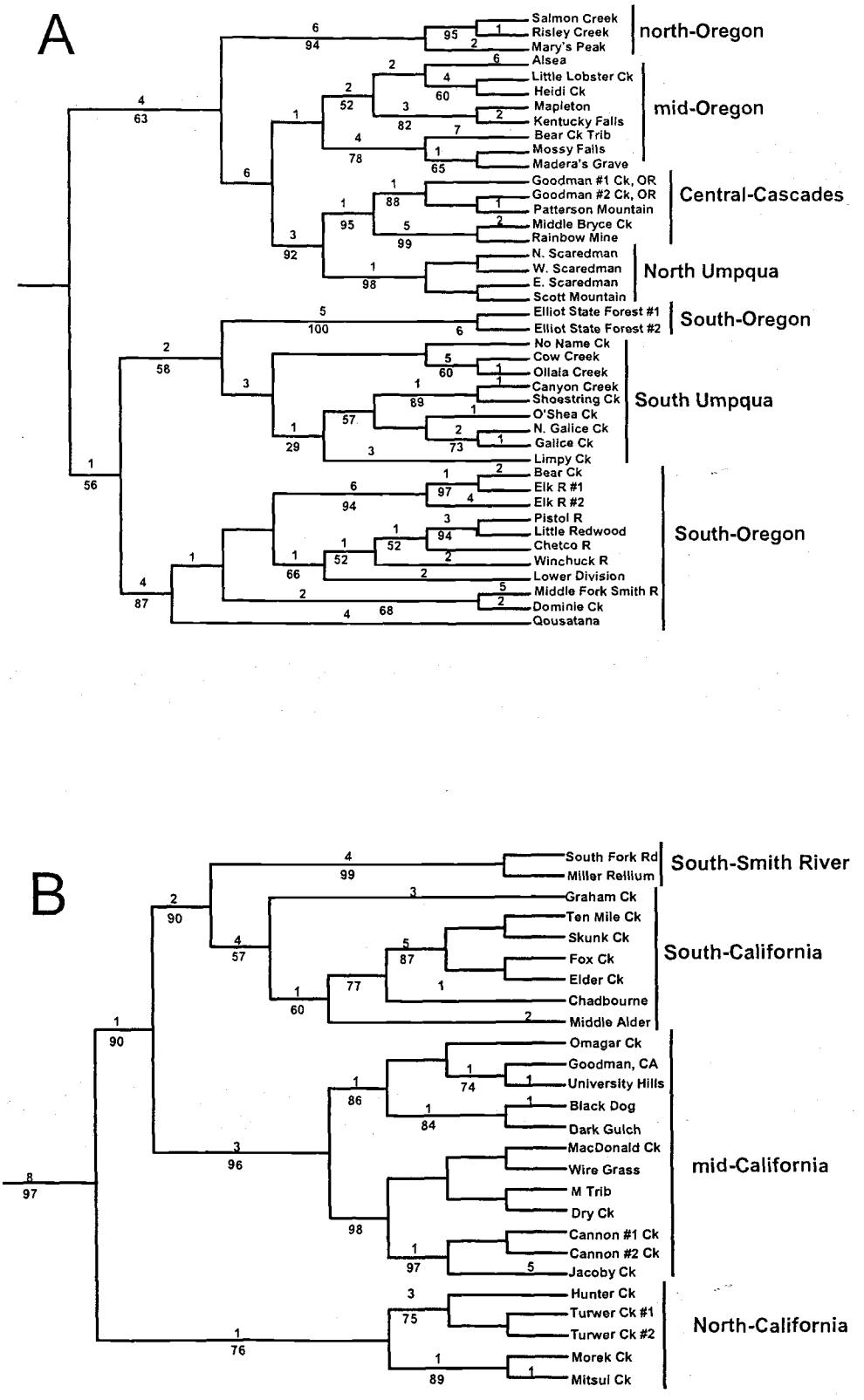
W. Little Nestucca
Ball Mountain
Siletz
North Coast Clade
Salmon Creek
Risley Creek
Mary's Peak
Alsea
Little Lobster Ck
Heidi Ck
Mapleton
Kentucky Falls
Bear Ck Trib
Mossy Falls
Madera's Grave
Goodman #1 Ck, OR
Goodman #2 Ck, OR
Patterson Mountain
Middle Bryce Ck
Rainbow Mine
N. Scaredman
W. Scaredman
E. Scaredman
Scott Mountain
Elliot State Forest #1
Elliot State Forest #2
Oregon Clade
No Name Ck
Cow Creek
Ollala Creek
Canyon Creek
Shoestring Ck
O'Shea Ck
N. Galice Ck
Galice Ck
Limpy Ck
Bear Ck
Elk R #1
Elk R #2
Pistol R
Little Redwood
Chetco R
Winchuck R
Lower Division
Middle Fork Smith R
Dominie Ck
Qousatana
South Fork Rd
Miller Rellium
Graham Ck
Ten Mile Ck
Skunk Ck
Fox Ck
Elder Ck
Chadbourne
Middle Alder
Omagar Ck
Goodman, CA
University Hills
Black Dog
Dark Gulch
MacDonald Ck
Wire Grass
M Trib
Dry Ck
Cannon #1 Ck
Cannon #2 Ck
Jacoby Ck
Hunter Ck
Turwer Ck #1
Turwer Ck #2
Morek Ck
Mitsui Ck
California Clade
Cascade Torrent (T)
Cascade Torrent (D)
Cascade Torrent (A)
Cascade Torrent (Y)
Columbia Torrent (R)
Olympic Torrent
Outgroup
36
72

Figure 6.2

Figure 6.3 Subclade identifications within the (A) *Oregon Clade* and (B) *California Clade* based upon maximum parsimony tree in Figure 2.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Salmon Creek
Risley Creek
Mary's Peak
north-Oregon
Alsea
Little Lobster Ck
Heidi Ck
Mapleton
Kentucky Falls
Bear Ck Trib
Mossy Falls
Madera's Grave
mid-Oregon
Goodman #1 Ck, OR
Goodman #2 Ck, OR
Patterson Mountain
Middle Bryce Ck
Rainbow Mine
Central-Cascades
N. Scaredman
W. Scaredman
E. Scaredman
Scott Mountain
North Umpqua
Elliot State Forest #1
Elliot State Forest #2
South-Oregon
No Name Ck
Cow Creek
Ollala Creek
Canyon Creek
Shoestring Ck
O'Shea Ck
N. Galice Ck
Galice Ck
Limpy Ck
South Umpqua
Bear Ck
Elk R #1
Elk R #2
Pistol R
Little Redwood
Chetco R
Winchuck R
Lower Division
Middle Fork Smith R
Dominie Ck
Qousatana
South-Oregon
B
South Fork Rd
Miller Rellium
South-Smith River
Graham Ck
Ten Mile Ck
Skunk Ck
Fox Ck
Elder Ck
Chadbourne
Middle Alder
South-California
Omagar Ck
Goodman, CA
University Hills
Black Dog
Dark Gulch
MacDonald Ck
Wire Grass
M Trib
Dry Ck
Cannon #1 Ck
Cannon #2 Ck
Jacoby Ck
mid-California
Hunter Ck
Turwer Ck #1
Turwer Ck #2
Morek Ck
Mitsui Ck
North-California

Figure 6.3

Figure 6.4 Minimum evolution (Kimura 2-parameter) consensus tree derived from 36 most-parsimonious trees (ME score = 0.46, Rohlf's consistency index 0.96) based on mitochondrial cytochrome b sequences (779bp) from Southern Torrent Salamanders. Branch distances are above and bootstrap values greater than 50 are below each branch.

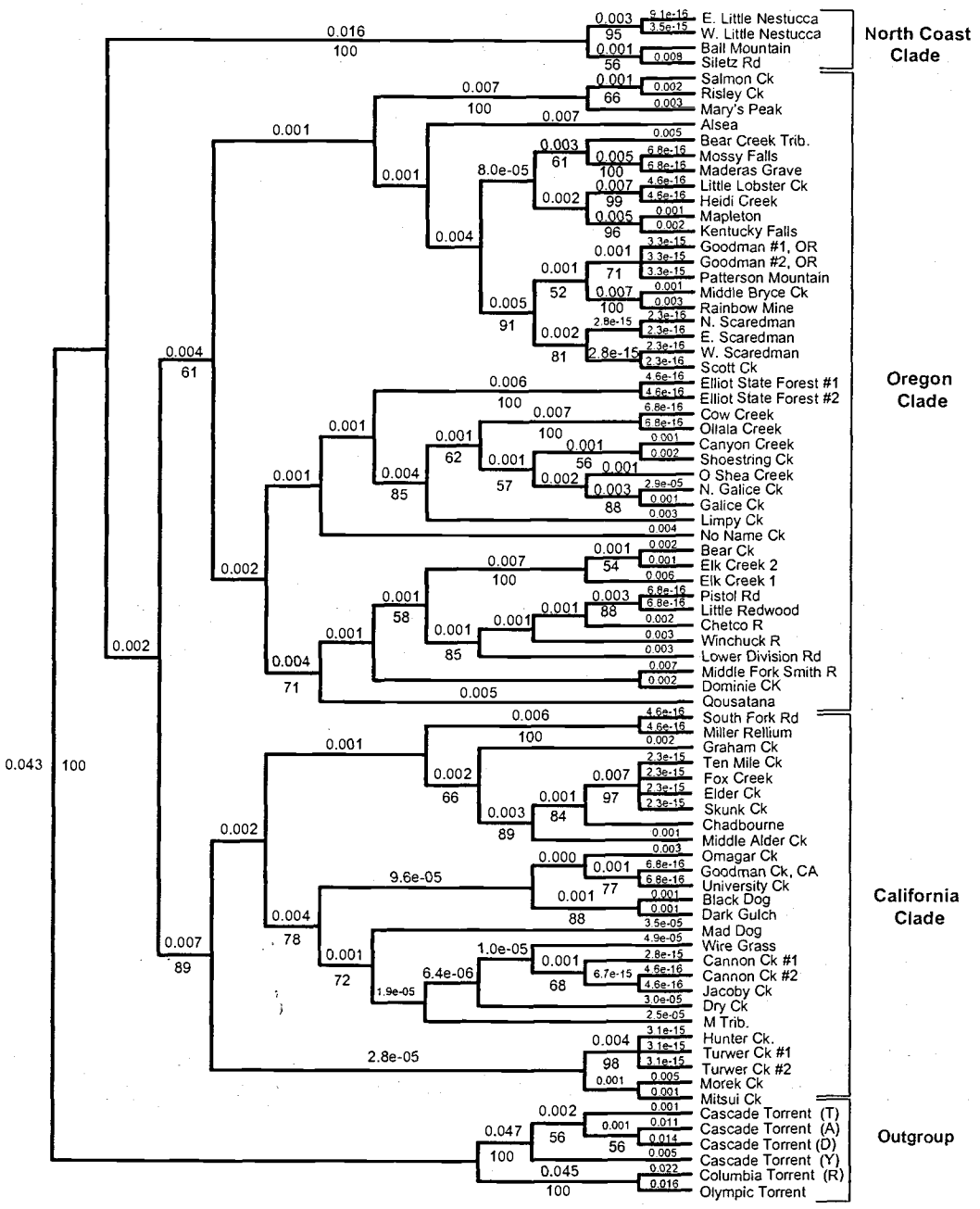
E. Little Nestucca
W. Little Nestucca
Ball Mountain
Siletz Rd
Salmon Ck
Risley Ck
Mary's Peak
Alsea
Bear Creek Trib.
Mossy Falls
Maderas Grave
Little Lobster Ck
Heidi Creek
Mapleton
Kentucky Falls
Goodman #1, OR
Goodman #2, OR
Patterson Mountain
Middle Bryce Ck
Rainbow Mine
N. Scaredman
E. Scaredman
W. Scaredman
Scott Ck
Elliot State Forest #1
Elliot State Forest #2
Cow Creek
Ollala Creek
Canyon Creek
Shoestring Ck
O Shea Creek
N. Galice Ck
Galice Ck
Limpy Ck
No Name Ck
Bear Ck
Elk Creek 2
Elk Creek 1
Pistol Rd
Little Redwood
Chetco R
Winchuck R
Lower Division Rd
Middle Fork Smith R
Dominie CK
Qousatana
South Fork Rd
Miller Rellium
Graham Ck
Ten Mile Ck
Fox Creek
Elder Ck
Skunk Ck
Chadbourne
Middle Alder Ck
Omagar Ck
Goodman Ck, CA
University Ck
Black Dog
Dark Gulch
Mad Dog
Wire Grass
Cannon Ck #1
Cannon Ck #2
Jacoby Ck
Dry Ck
M Trib.
Hunter Ck.
Turwer Ck #1
Turwer Ck #2
Morek Ck
Mitsui Ck
Cascade Torrent (T)
Cascade Torrent (A)
Cascade Torrent (D)
Cascade Torrent (Y)
Columbia Torrent (R)
Olympic Torrent
North Coast Clade
Oregon Clade
California Clade
Outgroup

Figure 6.4

populations (Figure 6.3B). However, the South Fork Smith River/Miller Rellium (South-Smith River) haplotype clusters with the south-California populations contrary to hypothesized expectations based on isolation-by-distance.

In contrast, the maximum likelihood tree shows the *north coast clade* is a sister group to the *Oregon clade* instead of forming a basal group (Figure 6.5) as was found in the maximum parsimony and distance based trees. This difference may be a consequence of the short internal branch among the three clades or rapid radiation among these three lineages.

Although there are clear regional groupings for haplotypes, there is support for differentiation based on an isolation-by-distance model, with a significant correlation between genetic and geographic distance among Southern Torrent Salamander haplotypes (Mantel $R^2 = 0.67$, $p = 0.01$). In contrast, there was little support for an isolation-by-distance model among the two of the major clades identified, the *Oregon clade* (Mantel $R^2 = 0.47$, $p = 0.11$) and *California clade* (Mantel $R^2 = 0.23$, $p = 0.16$).

Figure 6.5 Maximum likelihood phylogenetic tree based on Southern Torrent Salamander cytochrome *b* sequences (779bp, -ln likelihood score = 3002). Branch distances above and bootstrap values greater than 50 are below each branch.

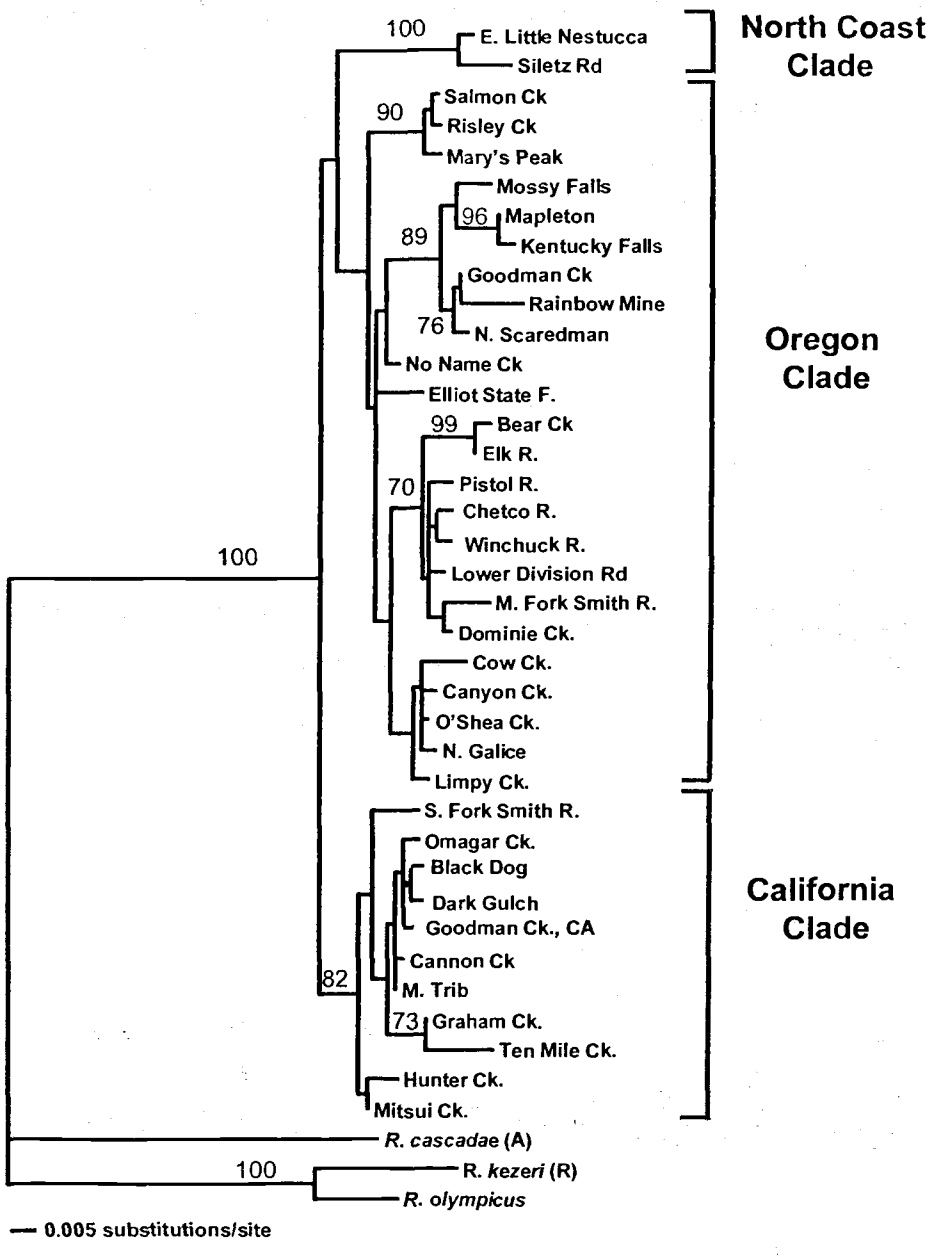
100
E. Little Nestucca
Siletz Rd
North Coast Clade
90
Salmon Ck
Risley Ck
Mary's Peak
Mossy Falls
96
Mapleton
89
Kentucky Falls
Goodman Ck
Rainbow Mine
76
N. Scaredman
No Name Ck
Elliot State F.
99
Bear Ck
Elk R.
70
Pistol R.
Chetco R.
Winchuck R.
Lower Division Rd
M. Fork Smith R.
Dominie Ck.
Oregon Clade
100
Cow Ck.
Canyon Ck.
O'Shea Ck.
N. Galice
Limpy Ck.
S. Fork Smith R.
Omagar Ck.
Black Dog
Dark Gulch
Goodman Ck., CA
Cannon Ck
82
M. Trib
73
Graham Ck.
Ten Mile Ck.
Hunter Ck.
Mitsui Ck.
California Clade
R. cascadae (A)
100
R. kezeri (R)
R. olympicus
0.005 substitutions/site

Figure 6.5

Discussion

The patchy distribution of Southern Torrent Salamanders combined with results of high cytochrome b differentiation among populations suggests a number of historic events have contributed to population structure and provides insight into how current habitat fragmentation may influence population structure. The divergence of Southern Torrent Salamander populations is most likely influenced by their limited dispersal capabilities. For example, movement patterns are limited to stream and streamside habitats, with slight linear movement per individual (0.08 m/month or 0.003 m/day, Welsh and Lind 1992). Studies of the Cascade Torrent Salamander (*R. cascadae*), a sister species, also suggests movements are limited with a mean distance moved per day of 0.36 meters and an average linear movement per individual of 2.4 meters over a three month period (Nijhuis and Kaplan 1998). However, these studies are limited and there is a lack of information concerning juvenile dispersal distances or site-fidelity. But given their apparent limited dispersal capabilities it is not surprising Southern Torrent salamanders appear to have been fragmented by a number of vicariant events and may be influenced by a number of geographic barriers to dispersal.

Population Differentiation

Our data suggest regional and local population differentiation in the Southern Torrent Salamander. This is consistent with the high degree of population subdivision and reduced gene flow among populations reported for allozymes (Good et al. 1989). In fact, Good and Wake (1992) hypothesized gene flow between extreme northern and southern populations would be non-existent and it would take an allele longer than the lifetime of the species to travel that distance. Further, they suggested gene flow among local populations is what holds the species together as a unit, with isolation-by-distance as the overall model of genetic structure. While our results support isolation-by-distance, the main factor contributing to population differentiation may have been a series of vicariant events resulting in the divergence of three major lineages (*north coast clade*, *Oregon clade* and *California clade*).

The three clades appear to have diverged at about the same time. Although estimates of divergence time based upon mtDNA sequence differences can be inexact, they can provide useful relative comparisons (Moritz et al. 1987, Irwin et al. 1991). We compared divergences based upon molecular clock estimates of 2% per million years for vertebrate mtDNA (Brown & Simpson 1982) and divergences calibrated (Li and Graur 1991) to allozyme divergences among the Southern Torrent and Cascade Torrent Salamanders (Good et al. 1989). The *north coast clade* appears to have diverged from the other two clades between 1.5 - 2.25 million years ago (based on a 2% divergence rate) and between 1.8 - 4.7 million

years ago for a calibrated divergence rate. The *Oregon clade* and *California clade* diverged approximately 0.7 - 2.4 million years ago (based on 2% divergence) and between 0.9 - 4.8 million years ago (calibrated). The divergences among haplotypes from the three clades is as great as the difference seen between haplotypes of the Olympic Torrent Salamander and Columbia Torrent Salamander (3.7%, Table 6.3). The relationship and divergence time among the three clades is also supported by mitochondrial 16S ribosomal RNA and 12S ribosomal RNA sequences (Chapter 4).

In addition to differences among clades, variation within each clade appears to be significant. The maximum divergence among haplotypes within the *north coast clade*, which occupies the smallest geographic region, is lowest with 1.2 %, while the greatest divergence (3.7 %) occurs in the *Oregon clade,* which has the largest geographic range. Finally, the maximum divergence among the *California clade* is 2.3 %. Consequently, it appears that even populations within each clade have been significantly isolated.

Gene flow appears to be limited across the range of the Southern Torrent Salamander based on the overall pattern of mtDNA divergence and population structure based upon allozyme analyses (Good et al. 1989, Good and Wake 1992). Random drift and founder events (populations founded by a small number of individuals) may be responsible for the pattern of lineage sorting and resulting high degree of population differentiation, which is evidenced within each clade. Currently, distribution of three historical lineages may be maintained by geographic barriers to dispersal.

Table 6.3 Range of percent sequence divergences (uncorrected) for cytochrome b sequences (779bp) between major clades of Southern Torrent Salamanders (*North*, *Oregon* and *California clades*) and other Torrent Salamanders. Values are derived from minimum and maximum pairwise haplotype differences.

	1	2	3	4	5	6
1. *North Clade*	-					
2. *Oregon Clade*	3.0-5.4	-				
3. *California Clade*	3.0-4.5	1.4-4.1	-			
4. Cascade Torrent	9.9-11.1	9.4-12.5	9.5-11.3	-		
5. Columbia Torrent	11.7-12.5	11.1-12.9	11.3-12.0	10.9-11.8	-	
6. Olympic Torrent	11.3-12.1	10.3-12.5	10.8-11.6	10.3-11.1	3.7	-

Phylogeography

Vicariance, geography, and factors related to climate change influence the genetic structure of populations across a species range by historically restricting gene flow or allowing for range expansion and colonization of new areas (Templeton et al. 1995, Bernatchez and Wilson 1998, Phillips et al. 2000). These factors combined with the limited vagility of some species may contribute to population fragmentation (Larson et al. 1984). Patterns of divergence also may be the result of, or maintained by, phylogeographic barriers. Divergences among three major clades of Southern Torrent Salamanders appear to correspond to potential phylogeographic barriers. The range of each clade appears to correspond to a major river.

The Yaquina River appears to be a geographic barrier between the *north coast clade* and the *Oregon clade*. However, support for this divergence is based upon a maternally inherited marker, thus male mediated gene flow could occur among these clades. Therefore, further studies need to be conducted in this region to investigate if the Yaquina River represents a true geographic barrier. The northern limited of the *north coast clade* appears to be in the vicinity of the Little Nestucca River where it is parapatric with the Columbia Torrent Salamander. Allozymes originally determined no hybridization occurred along this contact zone

(Good and Wake 1992). Our results are consistent with theirs and supports of reproductive isolation of each species along the Little Nestucca River, with all phenotypically Southern Torrent Salamanders exhibiting a Southern Torrent Salamander mtDNA haplotype and all phenotypic Columbia Torrent Salamanders sampled having the appropriate mtDNA haplotype.

The Middle Fork of the Willamette River appears to be a phylogeographic barrier for populations within the *Oregon clade* found in the Central Cascades. Distribution of Southern Torrent Salamanders has recently been extended in the central Cascades north to the Middle Fork of the Willamette River, which may provide a historic barrier limiting contact with the Cascade Torrent Salamander (Wagner and Haig in review). The north Umpqua and central Cascade populations appear to have diverged most recently from mid-Oregon populations suggesting a colonization of this region from the northwest instead of from more proximate closer populations to the southwest.

The *Oregon clade* and *California clade* division corresponds to two groups identified by allozymes (Good et al. 1987). Good and Wake (1992) further examined populations within these groups to see if differences required separate taxonomic treatment. They found a zone of integradation in two allozyme loci (AAT-2, ME) between the Pistol River (OR), Chetco River (OR), and Winchuck River (OR). AAT-2 were ME fixed among the further north and south populations. However, Good and Wake (1992) concluded separate species designation was not warranted because of the intergrade of allozyme loci, even though genetic distances

among the groups was high (D_N = 0.46). Separate taxonomic treatment has been suggested for amphibian populations differentiated by more than a D_N of 0.15 (Nei's genetic distance from allozymes; Highton et al. 1989, Highton 1990). However, Good and Wake (1992) suggested a more conservative approach based on the Biological Species Concept and recognized both of these groups as Southern Torrent Salamander.

Our results indicate the haplotype break for the Oregon and California clades occurs between the Middle Fork of the Smith River, CA, and the South Fork of the Smith River, CA. Populations north of the Middle Fork have the *Oregon clade* haplotype, while populations found south of the South Fork have the *California clade* haplotype (Figure 6.1A). This region of divergence appears to correspond to an area of phylogeographic importance for a variety of taxa. Jackman (1998) recently described a species level divergence within the genus *Aneides*. He found a zone of hybridization occurring directly south of the South Fork between Clouded Salamanders (*A. ferreus*) and the newly identified Wandering Salamander (*A. vagrans*). Similarly, taxonomically differentiated species have been recognized among Red Tree Voles (*Phenycomys sp.*), with a chromosomal inversion occurring between the Oregon and California populations in the northern California coastal region (Johnson and George 1991). Additionally, Dunn's Salamander (*Plethodon dunni*) is found only directly north of the Smith River drainage, with its distribution not extending into California.

In sum, these patterns suggest that historic geologic or biogeographic events in this region may have contributed to the phylogenetic divergences among a wide range of taxa. Furthermore, this region may currently be an area of secondary contact following a historic vicariant event, as evidence by hybridization observed among Clouded and Wandering Salamanders, Oregon and California Red Tree Voles, and Del Norte (*P. elongatus*) and Sisykou Mountain Salamanders (*P. stormi*). The importance of how shared historical biogeographic factors in the Smith River region have shaped both intra-specific phylogeny and species distribution needs to be further explored. In addition, comparative phylogeographic studies in this area may aid in regional conservation planning efforts to preserve genetic diversity across multiple forest-associated taxa with limited dispersal characteristics.

Conservation unit designation

Utilizing operational definitions of conservation units suggested by Moritz (1994), we suggest the *California clade* be recognized as an Evolutionary Significant Unit (ESU). The ESU designation is supported by the reciprocal monophyly of mtDNA cytochrome *b* haplotypes from the California populations and the significant amount of divergence observed among allozyme loci (Good et al. 1989, Good and Wake 1992).

The evidence for assigning ESU designations for the other groups, specifically the *north coast* and *Oregon clade*, is less clear. The criteria for reciprocal monophyly is met among the populations we sampled; however, it is possible there is introgression among the clades along the Yaquina River through male-mediated gene flow. Therefore, until evidence is available for significant differentiation of the *north coast* from the *Oregon clade* populations using nuclear loci is available, the clade should be recognized as separate Management Unit from the *Oregon clade*. However, the *north clade* represents a deep divergence and it is expected that nuclear data will raise this to ESU status.

Conservation implications

Designation of conservation units within Southern Torrent Salamanders could significantly influence their conservation status in light of differential threats to their persistence across their range. Results of our mtDNA study suggest Southern Torrent Salamanders have been historically fragmented into three major lineages; therefore, populations are highly differentiated across their range. Our results as well as allozyme studies (Good et al. 1989, Good and Wake 1992) suggested limited gene flow across their range and even among local populations.

Subsequently, Southern Torrent Salamanders may face threats at both the local scale were habitat fragmentation can lead to further isolation and subdivision, and at the regional scale where local extirpation can lead to the loss of historical lineages.

Currently, the Southern Torrent Salamander is protected by the matrix of federal lands reserved for the preservation of the Northern Spotted Owl (*Strix occidentalis*) under the Northwest Forest Plan (U.S. Forest Service and U.S. Bureau of Land Management 1994). This conservation strategy may not be adequate to provide for the maintenance of genetic diversity found in the Southern Torrent Salamanders across its range. Therefore, management efforts should be focused at re-examining their status with respect to conservation unit designations.

Recognition of conservation units can greatly improve management efforts under the Northwest Forest Plan and for listing and recovery under the U.S. Endangered Species Act. For example, the strategic management or listing of ESUs as distinct population segments could be effective in avoiding the "train wreck" scenarios of listing a widespread species throughout it's entire range, but instead focus efforts on the most critically threatened populations or regions. This is particularly relevant for species such as the Southern Torrent Salamanders that have deeply divergent genetic lineages and an extensive geographic range.

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CHAPTER 7

CONCLUSIONS

Summary

The results of this dissertation clearly stress the importance of vicariant events and phylogeographic barriers in influencing the population differentiation and genetic structure of forest-associated Pacific Northwest Salamanders. Gene flow across the range of each of the species studied appears to be historically limited resulting in significant divergence of a number of lineages within populations. Therefore, species management efforts with respect to the Northwest Forest Plan and U.S. Endangered Species listing actions should prioritize conserving this genetic diversity.

Key Results

Larch Mountain Salamander

- Mitochondrial cytochrome b analyses and RAPD analyses support significant differences between northern and southern populations of Larch Mountain Salamanders as delineated by the Columbia River.

- Reduced expected heterozygosity of southern populations, compared to northern populations of Larch Mountain Salamanders, suggests that southern population structure may be the result of a founder event from the north.

- Separate Management Unit designations are suggested for northern, south-west, and south-east groups of Larch Mountain Salamanders based upon the significant differentiation of RAPD markers.

Oregon Slender Salamander

- Cytochrome b analyses revealed two historical lineages (northern and southern) among Oregon Slender Salamanders suggesting the northern region may have more recently been colonized compared to the southern region.

- RAPD markers revealed divergence of three clades within Oregon Slender Salamander corresponding to *northern-most*, *mid-range* and *southern-most* populations.

- Genetic drift is suggested to have contributed more to population structure compared to gene flow in Oregon Slender Salamander based upon analyses of pairwise-F_{ST} estimates for RAPD markers versus geographic distances.

- Three overlapping Management Units are suggested to be recognized within Oregon Slender Salamander corresponding to the *northern-most*, *mid-range* and *southern-most* groups based on the significant divergence of RAPD markers. However, if reciprocal monophyly is supported between the region of the northern-most and mid-range groups in future studies an Evolutionary Significant Unit designation should be considered for the northern and southern groups as defined by the mitochondrial results.

Phylogenetic relationships among the Torrent Salamanders

- Each species represented a well-supported monophyletic based on analyses of each mitochondrial gene region (cytochrome b, 12S rRNA and 16S rRNA).

• The mitochondrial DNA analyses agreed with those based on allozymes (Good et. al. 1987, Good and Wake 1992) suggesting three main groups of Torrent Salamanders (*R. variegates*, *R. cascadae*, and the ancestor of *R. olympicus* and *R. kezeri*) diverged during the Miocene. A more recent divergence appears to occurred between *R. olympicus* and *R. kezeri* during the late Pliocene/ early Pleistocene.

• Some populations within *R. variegatus* appear to be as diverged as *R. olympicus* and *R. kezeri* lending support to recognition of conservation units within *R. variegatus* for management efforts.

Torrent Salamanders

• Based upon mtDNA markers (16S ribosomal RNA sequences) and allozymes (5 loci) there appears to be no hybridization or sympatry of Southern Torrent or Cascade Torrent Salamanders in the Central Oregon Cascades. These results indicate a significant range extension for both species and suggest the Middle Fork of the Willamette River may provide a geographic barrier to dispersal of these species. Southern Torrent Salamanders appear to occur south of the river and Cascade Torrent Salamanders north of the river.

- Results from the mitochondrial cytochrome b analyses indicate there are historical differences among Southern Torrent Salamanders populations at the regional and local scale.

- On a regional scale, there appears to be three major clades of Southern Torrent Salamanders. The groups appear to have diverged between 1.5-4.7 million years ago. The first group, the *north coast* clade, is found between the Little Nestucca River, OR, and the Yaquina River, OR. The second group, the *Oregon* clade, appears to occur between the Yaquina River, OR, and the middle fork of the Smith River, CA. The final group, the *California* Clade, ranges from just south of the middle fork of the Smith River, CA to the southern extent of their distribution in California.

- The distribution of each of these groups appears to correspond to a geographic barrier (e.g. the Yaquina River, OR, or Smith River, CA) that may limit dispersal among these groups. The Smith River drainage may also be an important historical biogeographic region for a number of species, for example, Clouded (*A. ferreus*) and Wandering Salamanders (*Aneides vagrans*) are demarcated by the river. Also, the river may have played a role in the divergence of Red Tree Vole (*Phyenycomys sp.*) populations that have chromosome differences (chromosomal inversion) that occurs in the Smith River area.

• On a local scale patterns of differentiation suggest that gene flow among Southern Torrent Salamander populations is limited, and is perhaps non-existent among the three major clades. Therefore, local extirpation of populations could significantly affect population structure and long-term viability of each of these clades.

• An Evolutionary Significant Unit designation may be warranted for the *California* clade based on significant divergence of mitochondrial and nuclear alleles (Good et al. 1987). Management Unit designations for the *north coast* clade and *Oregon* clade are evidenced by significant divergence of mitochondrial DNA alleles. Differentiation of the *north coast* clade and *Oregon* clade at nuclear alleles has not been investigated, thus evaluation of ESU designations cannot be completed at this time.

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