Geographic variation, genetic structure, and conservation unit designation in the Larch Mountain salamander (*Plethodon larselli*)

R. Steven Wagner, Mark P. Miller, Charles M. Crisafulli, and Susan M. Haig

Abstract: The Larch Mountain salamander (*Plethodon larselli* Burns, 1954) is an endemic species in the Pacific northwestern United States facing threats related to habitat destruction. To facilitate development of conservation strategies, we used DNA sequences and RAPDs (random amplified polymorphic DNA) to examine differences among populations of this species. Phylogenetic analyses of cytochrome *b* revealed a clade of haplotypes from populations north of the Columbia River derived from a clade containing haplotypes from the river's southwestern region. Haplotypes from southeastern populations formed a separate clade. Nucleotide diversity was reduced in northern populations relative to southern populations. These results were corroborated by analyses of RAPD loci, which revealed similar patterns of clustering and diversity. Network analyses suggested that northern populations were colonized following a range expansion mediated by individuals from populations located southwest of the river. Changes in the Columbia River's location during the Pliocene and Pleistocene likely released distributional constraints on this species, permitting their northern range expansion. Based on the barrier presented by the Columbia River's present location and differences in haplotype diversity and population structure observed between northern and southern populations, we suggest that designation of separate management units encompassing each region may assist with mitigating different threats to this species.

Résumé : La salamandre de Larch Mountain (*Plethodon larselli* Burns, 1954) est une espèce endémique de la région du nord-ouest pacifique des États-Unis qui est menacée par la destruction de son habitat. Les séquences d'ADN et les RAPD (amplifications aléatoires d'ADN polymorphe) nous ont servi à étudier les différences entre les populations de l'espèce, afin de faciliter la mise au point de stratégies de conservation. Les analyses phylogéniquea du cytochrome *b* indiquent l'existence d'un clade d'haplotypes provenant des populations au nord du Columbia dérivés d'un clade contenant des haplotypes provenant de la région du sud-ouest du fleuve. Les haplotypes de la région sud-est forment un clade séparé. La diversité des nucléotides est plus faible dans les populations du nord que dans celles du sud. Les analyses des locus RAPD confirment les résultats et présentent des patrons similaires de regroupement et de diversité. Des analyses de réseau laissent croire que les populations du nord ont fait leur colonisation après une extension d'aire réalisée par des individus provenant des populations établies au sud-ouest du fleuve. Les déplacements du lit du Columbia durant le pliocène et le pléistocène ont réduit certaines contraintes biogéographiques pour l'espèce et ont permis l'expansion d'aire vers le nord. Compte tenu de la barrière que forme le Columbia dans son emplacement actuel et des différences de diversité des haplotypes et de structure de population entre les populations du nord et du sud, nous proposons l'établissement d'unités de gestion distinctes pour chacune des régions pour atténuer les diverses menaces auxquelles fait face cette espèce.

[Traduit par la Rédaction]

Introduction

Phylogeographic studies aid in identifying historical barriers to dispersal and gene flow. Such analyses can substantially contribute to our understanding of the effects of natural and anthropogenic impacts on habitat and population fragmentation (Avise 1994). Amphibians often have specific ecological requirements and low dispersal rates that make them susceptible to fragmentation by historical and current processes (Blaustein et al. 1994). Indeed, genetic studies of amphibians frequently reveal substantial cryptic genetic diversity attributable to the influence of vicariant events

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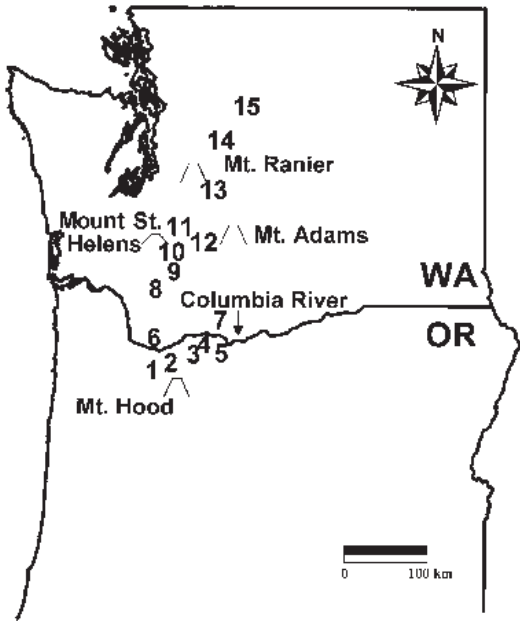
(Good and Wake 1992; Highton 1995; Jockusch 1996; Tilley and Mahoney 1996; Miller et al. 2005). Therefore, to aid amphibian conservation efforts, it is important to understand the role of fragmentation in population differentiation and implement management plans that preserve within-species genetic diversity.

The Larch Mountain salamander (*Plethodon larselli* Burns, 1954) is a completely terrestrial species associated with a variety of habitat types in the Pacific northwestern United States (Crisafulli 1999). This species is considered to be one of the rarest amphibian species in the Pacific Northwest because of its restricted range along a narrow corridor of the Columbia River Gorge and patchy distribution in the northern Oregon Cascade Range and south and central Washington Cascade Range (Howard et al. 1983; Kirk 1983; Herrington and Larsen 1985; Crisafulli 1999). As of 2004, there are only 137 extant sites. They occur in isolated patches of forested, steep talus within the Columbia River Gorge (Burns 1954, 1962, 1964; Herrington and Larsen 1985) and at rock outcrops, cave entrances, and a wide variety of forest and shrub habitats in the Cascade Mountains (Aubry et al. 1987; Darda and Garvey-Darda 1995; Crisafulli 1999). These regions have a substantial disturbance history primarily because of volcanism, glaciation, and flooding during the late Pliocene Epoch (McGroder 1991) and the Quaternary Period (Harris 1988) that could influence contemporary patterns of genetic diversity and population genetic structure in Larch Mountain salamanders.

Major threats to this species include habitat loss and population fragmentation caused by logging, recreational activities, and housing development (Herrington and Larsen 1985). Larch Mountain salamanders are considered a species of concern by federal and state agencies. They are listed by Oregon State as a Sensitive species, Vulnerable status, and by Washington State as a Sensitive species. In Oregon and Washington the species receives special management consideration from the US Forest Service as a Sensitive species and the US Bureau of Land Management as an Assessment species (US Forest Service and US Bureau of Land Management 1994). The Nature Conservancy has classified this species as both globally imperiled and subnationally imperiled. Furthermore, the species is listed in the *IUCN Red List* (International Union for the Conservation of Nature 2004) as Near Threatened. Thus, given contemporary management concerns over the Larch Mountain salamander, we examined population differentiation and genetic structure (both nuclear and mitochondrial) of 15 Larch Mountain salamander populations throughout their range. Furthermore, we evaluate possible historical biogeographical factors that may also influence Larch Mountain salamander genetic structure.

We used mtDNA cytochrome *b* sequences and RAPD (random amplified polymorphic DNA) markers to assess patterns of genetic diversity across the salamander's range. 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 intraspecific phylogenies (e.g., Hedges et al. 1992; Moritz et al. 1992; Tan and Wake 1995; Jackman et al. 1997). The RAPD technique, a procedure that permits examination of large numbers of segregating, putative nuclear loci from the genome, has been frequently 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) and in herpetological studies (e.g., Gibbs et al. 1994; Prior et al. 1997).

Fig. 1. Map showing the locations of Larch Mountain salamander (*Plethodon larselli*) populations examined in this study.



Materials and methods

Tissue sampling and DNA isolation

Larch Mountain salamanders were hand-captured at 15 sites throughout their range (Fig. 1). The distal 1 cm of tail was removed from each individual and stored in liquid nitrogen or on dry ice until it was transferred to an ultra-cold freezer (–80 °C). Salamanders from 7 of the sites were returned to their exact collection location after tissue sampling. Individuals captured at the remaining 8 sites were killed with chlorotone solution, fixed in formalin, and stored in buffered ethanol for use as voucher specimens and for studies of salamander morphology and diet. All sampling was performed in accordance with approved Institutional Animal Care and Use Committee protocols under collection permits issued by the US Forest Service. DNA was isolated from each sample using standard phenol–chloroform extraction protocols (Sambrook et al. 1989). The quantity and quality of each DNA sample were determined by fluorimetry and agarose gel electrophoresis, respectively.

Mitochondrial DNA sequencing and analyses

The polymerase chain reaction (PCR) was used to amplify an ~850 bp fragment of the cytochrome *b* gene, using the following primers designed for vertebrates: MVZ-15 (5′-GAACTAATGGCCCACACWWTACGNAA-3′) and MVZ-16 (5′-AAATAGGAAATATCATTCTGGTTTAAT-3′) (Moritz et al. 1992). Three individuals from each of 14 sampling locations and two individuals from an additional location were randomly selected for sequence analysis (Table 1). Frag-

Table 1. Locations and abbreviations for Larch Mountain salamander (*Plethodon larselli*) populations sampled for genetic analyses.

Site number	Site name	*M*/*R*	Haplotype	UTM Northing	UTM Easting
1	Bridal Veil Falls, Oreg.	3/7	A	5044609	564084
2	Multnomah Falls, Oreg.	3/23	B	5047459	569864
3	Herman Creek, Oreg.	3/6	C	5060052	593158
4	Wyeth Campground, Oreg.	3/7	D	5060141	595314
5	Starvation Falls, Oreg.	3/7	C	5060132	602222
6	Cape Horn, Wash.	3/13	E	5047071	563095
7	Dog Creek, Wash.	3/0	F	5062753	603369
8	Lower Copper Creek, Wash.	3/18	G	5070324	560795
9	Zig Zag Creek, Wash.	3/22	H	5077306	569269
10	Ole's Cave, Wash.	3/6	J	5103836	560258
11	Straight Creek, Wash.	3/20	I	5119422	590086
12	Quartz Creek, Wash.	3/19	K	5117283	592217
13	Packwood Palisades, Wash.	3/19	L	5171614	610412
14	Lake Kachess, Wash.	3/0	M	5236995	638500
15	Box Canyon, Wash.	2/0	M	5251500	628100

Note: *M* and *R* are the number of individuals used for mtDNA sequence and RAPD analyses, respectively. Site number corresponds to map location in Fig. 1.

ments were amplified using the following thermalcycling parameters: initial denaturation for 10 min at 93 °C, followed by 40 cycles of 1 min at 93 °C, 1 min at 52 °C, and 2 min at 72 °C. A final extension at 72 °C for 10 min completed the reaction. Each 50-µL reaction was conducted using 100 ng of template, 0.5 U (1 U ≈ 16.67 nkat) of AmpliTaq Gold® DNA polymerase (Perkin-Elmer) with the supplied reaction buffer, 2 mmol/L $MgCl_2$, 1 µmol/L of each primer, and 100 µmol/L of each dNTP. PCR amplicons were extracted from a 1% agarose gel using 0.45-µm Ultrafree-MC centrifugal filters (Millipore); supernatants were transferred to 50-µm filters (Millipore) to prepare templates for sequencing. A ~400 bp section of the PCR product was sequenced using the primers MVZ-15 and cytb2 (5′-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3′; Kocher et al. 1989) and run on an Applied Biosystems 373A DNA sequencer. Sequences from fragments were aligned by eye using the Genetic Data Environment (Smith et al. 1992) and compared with a GenBank archived cytochrome *b* sequence of *Plethodon elongatus* Van Denburgh, 1916 (accession No. L75821; Moritz et al. 1992).

We used Molecular Evolutionary Genetic Analysis (MEGA) version 2.1 (Kumar et al. 2001) to perform preliminary sequence data explorations and to obtain simple descriptive statistics (average pairwise genetic distances and standard errors) from the haplotypic data. In addition, we used three methods to infer phylogenetic relationships among haplotypes observed in this study. First, we reconstructed a neighbor-joining phylogeny (Saitou and Nei 1987) of haplotypes using PAUP* 4.0b10 (Swofford 1998). Pairwise Jukes–Cantor genetic distances calculated for the haplotypes detected in our analyses were all small (0.002–0.054 substitutions/site) and clearly suggested that relatively short-term evolutionary processes were being observed within *P. larselli*. This indicated that use of more complicated nucleotide substitution models (with their associated greater variances) to account for multiple hits at nucleotide sites was not necessary (Nei and Kumar 2000, p. 112). As a result, we relied on Jukes–Cantor genetic distances for the neighbor-joining analysis. Second, we used PAUP* to perform maximum parsimony based analyses of our data. Trees were evaluated using a heuristic search and the tree bisection–reconnection algorithm with starting trees obtained from 10 replicates of a random stepwise addition procedure. Bootstrap support for both neighbor-joining and maximum parsimony topologies was obtained from 1000 bootstrap replicates (Felsenstein 1985). A cytochrome *b* sequence from *P. elongatus* (GenBank accession No. L75821; Moritz et al. 1992) was used to root each phylogenetic tree. Finally, we used the computer program TCS (Clement et al. 2000) to produce a haplotype network from the set of sequences observed in *P. larselli*. This procedure, based on the statistical parsimony approach of Templeton et al. (1992), allowed us to infer the genealogy of the set of haplotypes observed among populations.

RAPD procedure and data analyses

RAPD profiles were generated as described in Aagaard et al. (1995). PCR was performed using the following reagent concentrations (25 µL volume): 1× PCR buffer (Promega), 1.8 mmol/L MgCl, 100 µmol/L of each dNTP, 0.2 µmol/L primer, 2 ng of template DNA, and 1 U of *Taq* DNA Polymerase (Promega). Thermalcycling was performed using the following parameters: denaturation for 3 min at 93 °C, followed by 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. Fifteen microlitres of each reaction were loaded in a 2.0% agarose gel and electrophoresed for 4 h (100 V) in TBE (90 mmol/L Tris Base, 90 mmol/L boric acid, 2 mmol/L EDTA, pH 8.0). Amplification products were sized by comparison with a 1-kb DNA ladder (Gibco BRL). The gels were then stained with ethidium bromide (1 µg/mL) for 30 min and destained for 2 h in deionized water.

Two salamanders from each of four different populations (Straight Creek, Cape Horn, Herman Creek, and Multnomah

Falls; Fig. 1, Table 1) were used to perform preliminary screening of 235 RAPD primers (Oligonucleotide Synthesis Laboratory, University of British Columbia, Vancouver; www.biotech.ubc.ca). This preliminary screening allowed us to identify RAPD primers that yielded distinct, well-separated, and reproducible bands, which were subsequently chosen for final analyses. Reproducibility was assessed in side-by-side RAPD reactions and over multiple RAPD runs. Negative controls were run with all amplifications to check for contamination. Ultimately, 14 RAPD primers that produced a total of 34 distinct, reproducible bands were used for analysis of the complete data set. Information on the specific RAPD primers used for analysis can be obtained from Wagner (2001). Sample sizes from each location for the final data set are provided in Table 1.

We used two congruent approaches for our analyses of the RAPD data. First, owing to the dominant nature of RAPD markers (Lynch and Milligan 1994; Zhivotovsky 1999), we obtained estimates of allele frequencies from the dominant marker data at each locus using the allele frequency estimator of Lynch and Milligan (1994). For purposes of these estimates, each RAPD marker was assumed to be a Mendelian locus whose underlying genotypes corresponded to Hardy–Weinberg expectations. Using these allele frequency estimates, we quantified the extent of genetic differentiation among sampling locations using the F_{ST} analog θ (Weir and Cockerham 1984) with the computer program TFPGA (Miller 1998*a*). Estimates of θ were obtained for all populations simultaneously (θ_T) as well as separately for groups of populations found north (θ_N) and south (θ_S) of the Columbia River. Ninety-five percent confidence limits for each θ value were obtained through the use of a bootstrap procedure over loci consisting of 1000 resampling replications. In addition, we used TFPGA to calculate pairwise genetic distances between populations using the coancestry distance measure of Reynolds et al. (1983; a logarithmic transformation of pairwise θ estimates), which were subsequently used to perform a UPGMA (unweighted pair group method with arithmetic means) analysis of the interpopulational genetic distance matrix. Node support for the resulting UPGMA dendrogram was obtained through the use of a bootstrap procedure over loci (sensu Felsenstein 1985) based on 1000 replicates.

Second, we performed a congruent set of analyses based solely on variation in RAPD marker phenotypes. Rather than relying on potentially biased allele frequencies estimated from dominant marker data (Lynch and Milligan 1994; Zhivotovsky 1999), in these analyses we used only information on the presence or absence of RAPD markers within and among populations. For these analyses, we used the computer program AMOVA-PREP (Miller 1998*b*) to help prepare input files that were subsequently analyzed using Arlequin 2.001 (Schneider et al. 2000). In Arlequin, we performed an analysis of molecular variance (AMOVA; Excoffier et al. 1992) of marker phenotype variation to obtain an additional measure of overall and pairwise interpopulational genetic differentiation (i.e., F_{ST} values). F_{ST} estimates were calculated based on interindividual genetic distances determined by the number of mismatched RAPD marker phenotypes between individuals. As with the calculation of θ estimates (see above), F_{ST} estimates were obtained for all populations simultaneously (F_{ST}^T) and for sets of populations located north (F_{ST}^N) and south (F_{ST}^S) of the Columbia River. Furthermore, we obtained F_{ST} estimates for each pairwise combination of populations under analysis. The significance of F_{ST} values (overall and pairwise) was determined via random allocation of individuals and RAPD marker genotypes to populations using 3000 randomization replicates. *P* values from pairwise contrasts were evaluated at the $\alpha = 0.004$ level to account for nonindependence of genetic distances from the 12 populations examined in the RAPD analyses. As with coancestry distances obtained from TFPGA, pairwise F_{ST} values obtained for populations were subsequently used for UPGMA analyses performed by NTSYS-PC (Rohlf 1994).

Results

mtDNA sequence analyses

Our analyses of cytochrome *b* sequence variation revealed 13 unique haplotypes among the 15 populations examined (Table 2). These sequences were characterized by the presence of 31 variable nucleotide sites (14 parsimony informative) and pairwise percent sequence divergences ranging from 0.2% to 5.2%. In general, haplotypes were location specific with the exception of two sequences (Table 1): haplotype C was found at both Herman Creek and Starvation Falls (Oregon, sites 3 and 5), while Lake Kachess and Box Canyon (Washington, sites 14 and 15) both contained haplotype M. No mtDNA sequence variation was detected within populations. Thus, despite limited sample sizes for sequencing, our analyses suggest that significant differences in haplotype frequencies generally exist among populations. To illustrate, haplotype A (from population 1; Table 1) occurs at a frequency of 0.068 in our data set (three copies of the haplotype from a total of 44 sampled individuals). Under a simple null hypothesis of genetic panmixia, the probability of observing all three copies of this haplotype at the same location is ~0.068^3, or ~0.003. Clearly, by extension, the probability of observing 11 location-specific haplotypes (Table 1) becomes exceedingly small. Overall, this pattern is consistent with other genetic analyses of salamanders from the northwestern United States (e.g., Jockusch and Wake 2002; Mahoney 2004; Miller et al. 2005) and suggests little contemporary gene flow among populations.

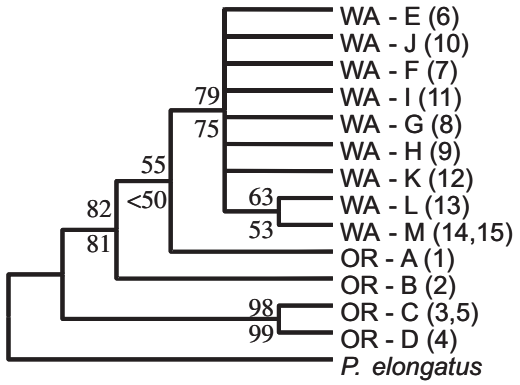
In maximum parsimony analyses, we found three most parsimonious trees (tree length = 103, consistency index = 0.94) that were mainly characterized by rearrangements of haplotypes joined by short branches. Neighbor-joining analysis revealed a single tree with a sum of branch lengths of 0.308. Bootstrap analyses based on both inferential methods produced extremely similar topologies, especially with respect to the relationships of haplotypes found in populations north and south of the Columbia River (Fig. 2). Specifically, southern haplotypes C (from Oregon populations 3 and 5) and D (from Oregon population 4) formed a monophyletic group that was supported by over 98% of bootstrap replicates for both phylogenetic reconstruction techniques. In contrast, all haplotypes detected in populations north of the Columbia River (Washington haplotypes E–M) formed a separate monophyletic group that was supported by over 75% of bootstrap replicates. Interestingly, two southern haplotypes (haplotypes A and B) from populations 1 and 2

Table 2. Nucleotide variation observed among 13 cytochrome *b* haplotypes (381 bp) detected in Larch Mountain salamanders.

	Nucleotide position																															
	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	3
	0	4	5	6	6	7	7	8	8	2	3	5	7	8	9	0	1	1	1	3	6	8	8	0	0	3	3	3	5	2	2	5
Haplotype	9	5	8	2	3	2	7	6	8	3	4	4	0	7	6	9	0	1	8	1	7	1	2	4	6	0	1	2	2	2	5	5
A	T	G	A	C	T	A	C	G	C	T	C	C	G	T	T	T	C	G	A	G	T	G	G	A	T	C	C	G	T	G	G	A
B	.	.	T	.	.	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
C	.	.	T	T	.	.	T	T	A	.	.	T	.	C	.	.	.	.	.	C	.	.	.	C	T	.	.	A	.	.	.	.
D	.	.	T	T	.	.	T	T	A	.	.	T	G	A	.	C	.	.	.	C	.	G	C	T	.	.	.	A	.	.	.	.
E	.	T	T	.	C	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	.	.	.	.	.	C	.	.	.
F	.	T	T	.	C	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
G	.	T	T	.	C	.	T	T	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.	.	.	T	.	.	.	C	.	.
H	.	T	T	.	C	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
I	C	T	T	.	C	.	T	T	.	.	.	.	C	.	C	G	C	.	.	.	A	.	.	.	.	.	.	A	.	.	.	.
J	.	T	T	.	C	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	C
K	.	T	T	.	C	.	T	T	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	.	.	.	.	.	.	.	.	.
L	.	T	T	.	C	G	T	T	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	.
M	.	T	T	.	C	.	T	T	.	C	T	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	.	.	.

Note: All variants are listed relative to haplotype A (GenBank accession No. T174972).

Fig. 2. Majority rules bootstrap consensus tree (1000 replicates) illustrating relationships of 13 Larch Mountain salamander cytochrome *b* haplotypes detected in this study. Haplotype codes (A–M), states (OR, Oregon; WA, Washington), and population numbers (in parentheses; Fig. 1, Table 1) are presented at branch termini. Numbers along branches reflect the percentage of 1000 bootstrap replicates that supported a given node in maximum parsimony (above branch) and neighbor-joining (below branch) analyses, respectively. Neighbor-joining analyses revealed an additional well-supported node (72%) that included haplotypes E, J, and F.



were part of a larger, well-supported monophyletic group (supported by over 80% of bootstrap replicates) that also included all haplotypes detected in northern populations. Patterns observed in phylogenetic analyses were consistent with inferred genealogies obtained from the haplotype network (Fig. 3), which also suggested that haplotypes C and D were the most divergent alleles detected.

Despite observing more alleles among populations north of the Columbia River, we note that nucleotide diversity was lower among haplotypes from these populations compared with populations south of the river. Among the 9 northern haplotypes, the average pairwise nucleotide difference among haplotypes (d = 4.33, SE = 1.10) was approximately two times lower than that seen among the 4 haplotypes found in southern populations (d = 8.33, SE = 2.09). Diversity observed among southern populations was in fact comparable to the overall average diversity of all 13 haplotypes detected in our analyses (d = 7.77, SE = 1.44).

RAPD analyses

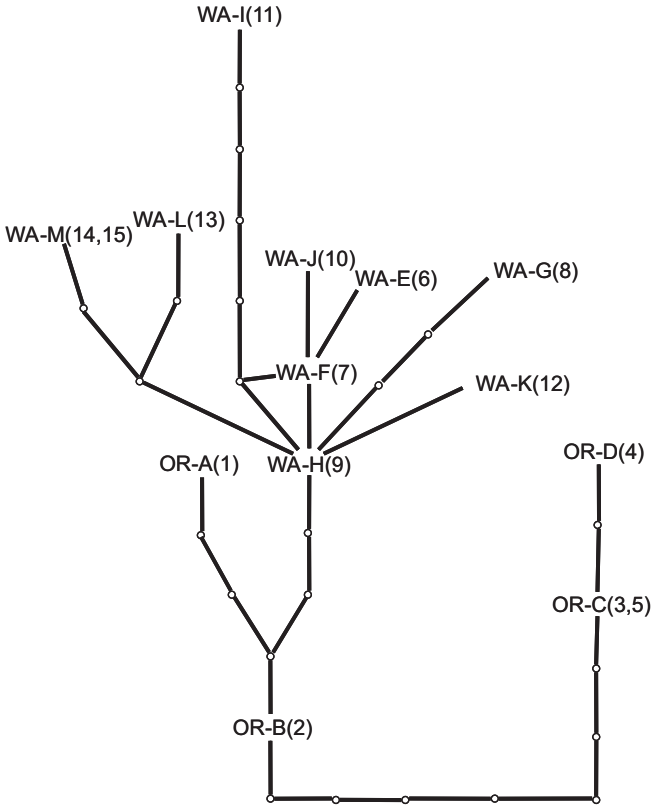
Our analyses of 34 nuclear RAPD loci indicated that there is substantial genetic variation among locations. Indeed, when allele frequencies were estimated from the dominant marker data, we obtained an overall θ estimate (θ_T) of 0.38 (upper confidence limit (UCL) = 0.47, lower confidence limit (LCL) = 0.29). This value was comparable to the overall F_{ST} estimate obtained when raw marker phenotype data were used for analyses (F_{ST}^T = 0.36, $P < 0.001$). Despite these high overall indicators of population differentiation, we note that the relative contributions of northern and southern populations were not similar. Specifically, analyses of seven populations north of the Columbia River yielded relatively low θ and F_{ST} values (θ_N = 0.13, UCL = 0.18, LCL = 0.09; F_{ST}^N = 0.13, $P < 0.001$) that were, based on the comparison of confidence limits obtained from θ estimates, significantly

Table 3. Pairwise F_{ST} values reflecting the extent of genetic differentiation among 12 Larch Mountain salamander populations used for RAPD analyses in this study.

Population	1	2	3	4	5	6	8	9	10	11	12
1	—										
2	0.18	—									
3	0.51	0.56	—								
4	0.44	0.51	0.21	—							
5	0.47	0.52	0.26	**0.08**	—						
6	0.42	0.54	0.55	0.51	0.53	—					
8	0.46	0.53	0.54	0.48	0.51	0.15	—				
9	0.41	0.51	0.49	0.44	0.48	**0.05**	0.08	—			
10	0.38	0.47	0.54	0.48	0.51	0.19	0.16	0.16	—		
11	0.37	0.46	0.52	0.5	0.52	0.14	0.23	0.16	0.14	—	
12	0.39	0.47	0.51	0.46	0.48	0.14	0.08	0.1	**0.08**	0.08	—
13	0.49	0.57	0.59	0.54	0.56	0.19	0.1	0.15	0.28	0.15	0.12

Note: All values are significantly different from zero at the $\alpha = 0.004$ level, with the exception of those values in bold. These values are nonetheless significant at the $\alpha = 0.05$ level. F_{ST} values are also highly correlated with coancestry distances estimated from the data ($r = 0.95$).

Fig. 3. Haplotype network based on sequence differences among 13 cytochrome *b* haplotypes observed in Larch Mountain salamander. Letters (A–M) indicate one of 13 haplotypes (Table 2) found among 15 populations examined (Fig. 1, Table 1) in Oregon (OR) and Washington (WA). Numbers in parentheses indicate population(s) where haplotypes were detected. Small circles indicate inferred haplotypes that are extinct or were not detected in analyses. Each segment (letter or circle) of the network reflects a single base-pair difference between haplotypes.



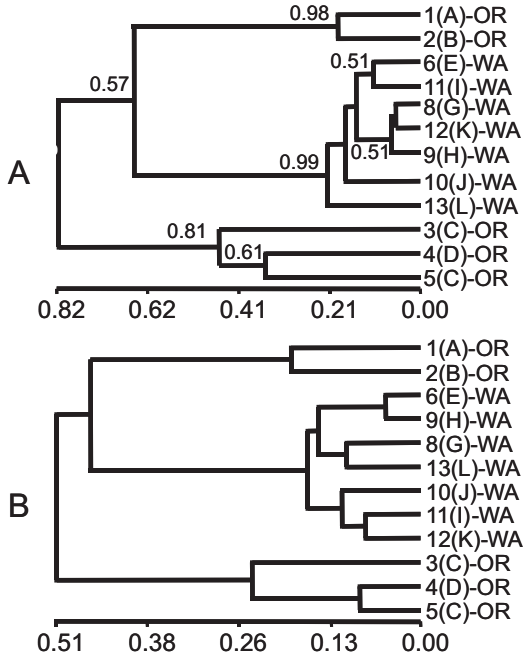
smaller than comparable values obtained for the five southern populations analyzed ($\theta_S = 0.41$, UCL = 0.54, LCL = 0.28; $F_{ST}^S = 0.44$, $P < 0.001$).

In addition to high overall statistical indicators of genetic structure, we also observed considerable variation in pairwise genetic distances (coancestry distances and F_{ST} values) between populations. Coancestry distances ranged from 0.05 to 1.1, while pairwise F_{ST} values ranged from 0.05 to 0.59 (Table 3). All pairwise F_{ST} values were significantly different from zero at the $\alpha = 0.004$ level, with the exception of those for Oregon populations 4 and 5 ($F_{ST} = 0.085$, $P = 0.044$), Washington populations 6 and 9 ($F_{ST} = 0.049$, $P = 0.015$), and Washington populations 10 and 12 ($F_{ST} = 0.083$, $P = 0.015$), which nonetheless were significant at the $\alpha = 0.05$ level (Table 3). Pairwise coancestry distances and F_{ST} estimates, however, were highly correlated ($r = 0.95$), indicating that analyses based on estimated allele frequencies and raw marker phenotypes gave qualitatively similar results. Furthermore, UPGMA analyses based on both distance matrices gave comparable results, especially with respect to detection of major population clusters (Fig. 4). UPGMA dendrograms suggested three main clusters of populations with substantial bootstrap support; these clusters largely coincided with well-supported nodes obtained from the phylogenetic relationships of haplotypes detected among populations (Fig. 2). For example, as in our phylogenetic analyses of mtDNA sequence variation, a well-supported cluster (99% of bootstrap replicates) contained all populations north of the Columbia River (Fig. 4). Likewise, Oregon populations 3, 4, and 5, which contained haplotypes C and D (Fig. 2), clustered together in over 80% of bootstrap replicates (Fig. 4). Overall, the main difference between topologies obtained from mtDNA sequence analyses and RAPD markers was the placement of Oregon populations 1 and 2 (containing haplotypes A and B). In UPGMA analyses, these populations clustered together in over 98% of bootstrap replicates (Fig. 4) and, in contrast to the mtDNA sequences, did not show any particularly substantial alliance with northern populations.

Discussion

Our analyses of nuclear and mitochondrial DNA data produced remarkably similar overall patterns (Figs. 2, 4). Specifically, although pairwise analyses of RAPD data

Fig. 4. Midpoint-rooted UPGMA (unweighted pair group method with arithmetic means) dendrograms of (A) pairwise coancestry genetic distances and (B) pairwise F_{ST} values between 12 Larch Mountain salamander populations based on 34 RAPD (random amplified polymorphic DNA) loci. Numbers at branch termini indicate the populations listed in Table 1 and Fig. 1. Letters in parentheses indicate the mitochondrial haplotype (Tables 1, 2) detected at each site and state (OR, Oregon; WA, Washington) for each population. Numbers on branches of the coancestry distance tree reflect the proportions of 1000 bootstrap replicates that supported the topology. Only bootstrap values greater than 0.5 are shown.



suggested that most populations significantly differed from one another, we note that there was substantially less differentiation among populations located north of the Columbia River than among populations to the south. In RAPD analyses, statistical indicators of genetic differentiation for northern populations were low (θ_N = 0.13, UCL = 0.18, LCL = 0.09; F_{ST}^N = 0.13, $P < 0.001$) compared with those for southern populations (θ_S = 0.41, UCL = 0.54, LCL = 0.28; F_{ST}^S = 0.44, $P < 0.001$). This pattern was also reflected by mtDNA sequence data: average pairwise nucleotide differences among haplotypes were small in the north compared with the south (north, d = 4.33, SE = 1.10; south, d = 8.33, SE = 2.09). Overall, this pattern may reflect one of two nonexclusive possibilities. First, our data may suggest that the northern region of *P. larselli*'s distribution was more recently established via range expansion, perhaps during the late Pleistocene in concert with glacial retreat. Alternatively, despite detection of significant nuclear differences among populations, our data may also suggest that contemporary gene flow among northern populations is greater and perhaps facilitated through more substantial connectivity among suitable habitats. We propose, however, that the former hypothesis is likely more tenable given the assumed low dispersal ability of many terrestrial salamanders (and *P. larselli* by analogy; see below) and the more patchy distribution of habitat (and likely salamander populations) in the north compared with the south (Crisafulli 2005). Furthermore, consistent with the range expansion hypothesis, our phylogenetic analyses of mtDNA sequence data show a well-supported northern haplotype clade nested within the southern haplotypes (Fig. 2), with haplotypes from the northern clade forming the distinctive starlike pattern commonly attributed to demographic expansions that occur in concert with range expansions (Avise 2000; Fig. 3). Based on inferences made from the haplotype network (Fig. 3), our data also indicate that individuals from the southwestern populations (or closely related and geographically proximal ones) primarily facilitated the northern range expansion. Overall, our data are extremely consistent with other phylogeographical studies of North American taxa that have detected genetic signatures of northern range expansions following glacial retreat (e.g., Nason et al. 2002; Masta et al. 2003; Starkey et al. 2003; Zamudio and Savage 2003; Ripplinger and Wagner 2005).

Although analyses suggest that individuals from the southwestern region of our study area primarily contributed to the range expansion, we must further provide a mechanism to explain how the Columbia River itself was traversed in this process. The efficacy of rivers as barriers to dispersal of terrestrial plethodontid salamanders has been questioned (Highton 1972). However, based on distributional data, the Columbia River appears to be a barrier for a number of terrestrial salamanders including the Oregon slender salamander, *Batrachoseps wrighti* (Bishop, 1937) (= *B. wrightorum*), and the clouded salamander, *Aneides ferreus* Cope, 1869, for whom the river demarks the northern boundary of their range (Corkran and Thoms 1996). Likewise, the Columbia River defines the southwestern extent of the range of Van Dyke's salamander (*Plethodon vandykei* Van Denburgh, 1906; Jones and Crisafulli 2005). Furthermore, we note that the location of the Columbia River has changed over the long-term course of evolutionary history. The river became repeatedly blocked during the Pliocene and the Pleistocene, a process that significantly deflected the main river channel both north and south of the current main stem on several different occasions (Fecht et al. 1987; Heller et al. 1987). One extreme reroute of the channel altered the course of the river southward towards Mt. Hood (Fig. 1), which may have caused the river to bisect the region between the southwestern (populations 1 and 2) and southeastern populations (populations 3–5) analyzed in this study. Therefore, this process may have released distributional constraints (limits imposed by the river) on southwestern populations, providing an opportunity for a northern range expansion. This hypothesis is also consistent with the high degree of both nuclear and mitochondrial genetic differentiation of southwestern and southeastern populations examined in our study (Figs. 2, 3, 4), as this southern reroute of the river may have acted as a formidable barrier to gene flow that promoted the strong contemporary pattern of divergence between southeastern and southwestern populations. Furthermore, this dramatic hydrogeological event may have subjected populations along the southern bank of the Columbia River to strong bottlenecks that effectively reduced allelic diversity within populations and exacerbated the overall pattern of genetic differentiation in the region. Thus, we suggest that our data

point to the Columbia River as an important overall determinant of patterns of genetic diversity in *P. larselli*. This conclusion is consistent with those reached in a recent study of genetic variation in *B. wrighti* (Miller et al. 2005), which also suggested that changes in the location of the Columbia River may have permitted an expansion of this species' range up to the river's southern bank.

Conservation implications

Larch Mountain salamanders face various threats across their range depending on land ownership and land management objectives. Anthropogenic habitat destruction and fragmentation may have important effects (Herrington and Larsen 1985). In the Columbia River Gorge, road and house construction and development of rock quarries are primary concerns, whereas in the Cascade Mountains, forestry practices are the chief concern. Logging is a particularly acute threat in areas east and northeast of Mount St. Helens, where deep tephra deposits, accumulated over millennia, seal off the salamander's access to cool, moist subterranean microhabitats (Crisafulli et al. 2004). In this tephra fall zone, forest canopy ameliorates forest floor conditions through shading, reduction of desiccating winds, and maintenance of high relative humidity, which provides the microclimatic conditions required by the salamander. In areas outside of deep tephra deposition, unconsolidated surface rock is prevalent and provides the salamanders with access to subterranean retreats during periods of unfavorable surface conditions (i.e., summer drought period). In such rocky areas, forest canopy does not appear to be a critical attribute of Larch Mountain salamander habitat and logging probably poses fewer problems for the salamander, provided that surface rocks and associated microclimates are not altered.

Given their reliance on sites with cool, moist microhabitats (Crisafulli 1999), their narrow physiological tolerances (as with most *Plethodon* species; see Feder 1983; Spotila 1972), and their putative low dispersal capabilities, Larch Mountain salamanders may be constrained in their ability to respond to contemporary disturbances. Indeed, although dispersal and movement of terrestrial salamanders are poorly understood and infrequently quantified, five studies of similar western plethodontid salamanders revealed typical distances moved by individuals, ranging from 1.7 m (*Batrachoseps attenuatus* (Eschscholtz, 1833); Hendrickson 1954) to 23 m (*Ensatina eschscholtzii* Gray, 1850; Stebbins 1954), with a mean distance of 2.5 m (*Plethodon vehiculum* (Cooper, 1860); Hendrickson 1954; Stebbins 1954; Barbour et al. 1969; Barthalmus and Bellis 1972; Ovaska 1988). In addition to the potential for low dispersal, a number of factors related to the reproductive biology of Larch Mountain salamanders could influence species viability when extensive habitat disturbances occur. For example, their reproductive rate may be low because females reach sexual maturity only after 4 years of age, and they 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 are unknown. Furthermore, it has been suggested that the amount of suitable habitat for Larch Mountain salamanders is limited within the Columbia River Gorge itself (Herrington and Larsen 1985) and the central Washington Cascade Range (Crisafulli 1999). Therefore, assignment of conservation units to allow for different management options across the range of the Larch Mountain salamander may assist with mitigation of threats to this species.

Previous studies of multiple species have demonstrated strong patterns of genetic differentiation across the Columbia River (Soltis et al. 1997; Arbogast et al. 2001; Monsen and Blouin 2003; Spruell et al. 2003). In contrast, Howard et al. (1983) suggested that populations of Larch Mountain salamanders from each side of the Columbia Gorge were relatively recently diverged (between 4000 and 43 000 years ago) based on low allozyme divergence of two pairs of populations (four populations) located directly across the river from one another. However, their analyses (based on less polymorphic allozyme data) also detected unique alleles for each location examined. Thus, our results may indicate that the Columbia River provides a long-term barrier to dispersal for Larch Mountain salamanders that will ultimately reinforce genetic differentiation of northern and southern populations. Furthermore, we note that morphological differences have been described between northern and southern populations, including variation in number of vomerine teeth and melanophore pigmentation (Brodie 1970). Subsequently, given the concordance of genetic and morphological patterns of divergence, in combination with the river as a long-term barrier to dispersal, we suggest that northern and southern populations are currently on separate evolutionary trajectories, despite the paraphyly of southwestern populations revealed through phylogenetic analyses of mtDNA sequence data (Fig. 2). In addition, our data clearly indicate that northern populations are substantially less differentiated than southern populations. This suggests that extirpation of a southern population would, on average, result in the loss of appreciably more genetic diversity in *P. larselli* relative to extirpation of a northern population. This is especially important considering that although most Columbia River Gorge populations are nominally protected within National Scenic Area reserves, increasing recreational use in this area has the potential to affect already fragmented southern populations (Herrington and Larsen 1985). Furthermore, northern populations are also genetically differentiated (albeit less so than southern populations; Table 3) and face threats from timber harvesting practices. Therefore, designation of separate management strategies for northern versus southern populations may be beneficial to allow for flexibility in mitigating different threats to *P. larselli* across its range.

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