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ALLOZYME AND MITOCHONDRIAL DNA VARIATION IN THE TAILED FROG (ANURA: *ASCAPHUS*): THE INFLUENCE OF GEOGRAPHY AND GENE FLOW

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Abstract: In this paper we support the division of the previously monotypic genus *Ascaphus* into two species based on an analysis of 23 allozyme loci from 34 populations. We use maximum likelihood to estimate the *Ascaphus* phylogeny from the allozyme data and find strongly supported monophyletic Rocky Mountain and Pacific clades. In a nonhierarchal, model-based cluster analysis of the data, each of the 1085 individual genotypes is correctly assigned to either *Ascaphus montanus* or *A. truei* with a high probability. We also find a virtually fixed difference between the species at the *Pgm*-2 locus. Within *A. truei*, we find a lack of significant pairwise F_{ST} values among populations from the Coast and central Cascades Mountains, suggesting relatively recent range expansion or contemporary gene flow among these populations. Olympic Mountains populations form a discrete clade in the allozyme topology and are fixed for a unique allele at the *Lap* locus. These populations remain isolated from the remainder of the species' range based on pairwise F_{ST} values. The four southernmost *A. truei* populations each show significant allelic divergence from the remaining populations (based on pairwise F_{ST} values), suggesting climate-induced isolation. In addition. we extend mtDNA sampling within the Rocky Mountains and sequence 530 nucleotides from the mtDNA Cytochrome *b* (cyt b) gene in 12 previously unsampled *A. montanus* populations. This additional sampling defines the geographic extent of a southern mtDNA clade distinguished on average by 0.024 substitutions per site from the northern clade. We use nested clade analysis, a coalescent-based divergence by isolation with migration model (MDIV), maximum-likelihood estimation of the mtDNA topology, and Bayesian model-based genotype assignment, to test predictions from two hypotheses: the western refugia hypothesis, which claims that *A. montanus* persisted in refugia west of the Snake River during Pleistocene glacial maxima, and the dual refugia hypothesis, which asserts that *A. montanus* occupied refugia within the Salmon and Clearwater River Valleys during glacial maxima. Our data do not support predictions of the western refugia hypothesis. Nested-clade analysis, estimated dates of lineage divergence supplied by MDIV, and the mtDNA topology support predictions of the dual refugia hypothesis; however, the allozyme topology fails to support some of these predictions. Allozyme and mtDNA data endorse the preliminary recognition of a minimum of two Evolutionarily Significant Units (ESUs) within *A. truei*: (1) populations from the Olympic Mountains and (2) populations south of the Umpqua River. Two ESUs are also suggested within *A. montanus*: (1) populations south of the South Fork of the Salmon River, and (2) populations to the north and west of the Salmon River (including the Blue, Wallowa and Seven Devils Mountains). The MDIV analysis indicates an exchange rate of 10 migrants per generation between the northern ESU and the southern ESU.

Key words: Amphibians; Biogeography; Conservation; Genetic structure; Pacific Northwest; Pacific tailed frog; Rocky Mountain tailed frog

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TAILED frogs inhabit forested headwater streams with clean cobble substrates and temperatures below 21 C (Adams and Frissell, 2001) in the mountains of the northwestern United States and southwestern Canada. These frogs display a number of morphological adaptations to their unique environments. Both tadpoles and adults are ventrally flattened, tadpoles possess suctorial mouthparts that allow them to maneuver in high velocity currents, and unlike most anurans, fertilization in tailed frogs is internal and male ascaphids have a copulatory organ.

Mitochondrial DNA (mtDNA) sequences reveal considerable variation within *Ascaphus* (Nielson et al., 2001). The largest divergence (7.8 to 9.1% uncorrected sequence divergence) distinguishes Rocky Mountain populations from Coastal and Cascades Mountain populations. Nielson et al. (2001) proposed that this divergence dates to the rise of the Cascades Mountain Range and the ensuing rain shadow that currently bifurcates the tailed frogs' range. Based on mtDNA divergence, Nielson et al. (2001) proposed that Rocky Mountain populations represent a distinct species, *Ascaphus montanus*, a designation that is generally accepted (e.g., Frost, 2004).

Morphological studies of the genus (Pauken and Metter, 1971) originally suggested that *Ascaphus* colonized the northern Rocky Mountains via the highlands of central and eastern Oregon at the close of the Pleistocene. However, the ancient vicariance hypothesis that Nielson et al. (2001) invoked to explain the depth of mtDNA divergence within *Ascaphus* has recently been substantiated by statistical tests within the lineage, as well as for other amphibians with similar distributions (Carstens et al., 2005). The ancient vicariance hypothesis predicts that genetic structure—originating from historic climatic events—will be found within *A. montanus*, constrasting with a lack of significant structure expected if colonization of the Rocky Mountains occurred at the close of the Pleistocene (Brunsfeld et al., 2001). Two potential corollaries to the ancient vicariance hypothesis explain contemporary genetic structure in terms of the alternative locations of ancient glacial refugia.

The first hypothesis (western refugia hypothesis) posits that habitats west of the Snake River served as a refugium for *A. montanus* through the Pleistocene while the body of their current range in the northern Rocky Mountains was glaciated. Under this hypothesis, populations from west of the Snake River served as a source for colonization of the remainder of the current range. Predictions of this hypothesis include: (1) a grouping of haplotypes from the Blue and Wallowa Mountains at the origin of the *A. montanus* phylogeny, (2) higher mean heterozygosity within Blue and Wallowa Mountains populations, and (3) evidence of range expansion from west of the Snake River.

The second hypothesis (dual refugia hypothesis) is suggested by recent phylogeographic studies of *Plethodon vandykei* (Carstens et al., 2004*a*) and *Dicamptodon aterrimus* (Carstens et al., 2004*b*), which indicate that the Clearwater River and South Fork Salmon River valleys, respectively, served as refugia for these species during Pleistocene glaciation. These salamander species have ecological tolerances similar to that of the tailed frog; therefore, we propose that both refugia were used by *A. montanus*. Predictions under this hypothesis include: (1) the presence of two monophyletic clades within *A. montanus*, (2) geographic association of these clades with the purported refugia, (3) evidence of range expansion from two refugia, and (4) an estimated divergence time separating these clades that dates to the Pleistocene.

In this paper, we have four objectives. First, we present an extensive allozyme data set from across the range of *Ascaphus* to evaluate the taxonomic hypothesis proposed by Nielson et al. (2001) using independent nuclear data. Second, we examine genetic structure within *A. truei* using the allozyme data. Third, we test the western refugia and dual refugia hypotheses using mtDNA samples from 12 populations not examined previously, as well as allozyme samples from 14 localities within the northern Rocky Mountains. Finally, we integrate the information from the mtDNA and allozyme data sets to provide a phylogenetic framework for conservation and management.

METHODS

Allozymes

Sample collection and scoring.—We examined allozymes in 1085 individuals from 34 localities; 14 localities were within the range of *A. montanus* and 20 were within the range of *A. truei* (Table 1; Fig. 1; Appendix I). Allozyme sample collection took place between 1973–1976 prior to the rise of concern over conservation of the tailed frog. All sampled individuals were larvae, with the exception of several adults from population 36 that were sacrificed for other aspects of Daugherty's (1979) dissertation research. Many of the sampled larvae would not be expected to survive to maturity, and this sampling is not known to have negatively affected the populations in question. Twenty-three loci were appraised using three buffer systems (Appendix II). Details of collection and assessment of electromorph homology are given in Daugherty (1979).

Phylogenetic analysis.—To estimate the population phylogeny from allozyme data, we used the CONTML program in PHYLIP. This program implements restricted maximum-likelihood estimation under a Brownian motion model of allele frequency change designed to approximate independent genetic drift among loci (Felsenstein, 1981). The *Gus* locus was not resolved in the Dollar Creek population (pop. 42); we therefore estimated topologies both with the Dollar Creek population but without the *Gus* locus and without the Dollar Creek population but with the *Gus* locus. Bootstrap estimates of nodal support for each phylogeny were calculated using 200 replicate data sets. The CONTML program is unable to analyze data sets with identical populations, and such populations are an unavoidable outcome given a reasonable number of replicate data sets (J. Felsenstein, personal communication). Therefore, we used the bootstrap data sets to calculate distance matrices (Cavalli-Sforza's chord measure; Cavalli-Sforza and Edwards, 1967) in the Phylip program GENDIST, and generated 200 neighbor-joining trees using the program NEIGHBOR. Topologies were rooted at the midpoint because a suitable outgroup is not clear. Tailed frogs are considered sister to all other anuran lineages and appear highly divergent based on several ancestral characteristics retained in *Ascaphus* that have been lost in all other anuran lineages (Cannatella and Hillis, 1993; Ford and Cannatella, 1993; but see Hay et al., 1995).

Population level analyses.—We employed analysis of molecular variance (AMOVA; Cockerham, 1969, 1973) to examine structure in the allozyme data using Arlequin (Schneider et al., 2000). We first classified populations as *A. montanus* or *A. truei* based on collection locality and then calculated pairwise F_{ST} values for all populations and tested the significance of divergence among populations; pairwise F_{ST} values were not corrected for multiple comparisons. Arlequin was also used to test for deviations from Hardy–Weinberg equilibrium (Guo and Thompson, 1992) and to calculate mean heterozygosity.

mtDNA

Collection and sequencing.—We examined mtDNA in 60 larvae from 12 populations within the range of *A. montanus* (Appendix I; Fig. 1). There was no overlap between these localities and the sampling of Nielson et al. (2001). We extracted DNA using the same methods described in Nielson et al. (2001; i.e., Puregene Kit with subsequent cleaning on glass beads).

We initially amplified and sequenced the Cytochrome *b* (cyt *b*) gene using primers L14115 and SUV as detailed in Nielson et al. (2001); however, we were unable to produce high quality sequences using the SUV primer in samples from the southeastern portion of the species' range (pops. 12a, 12b and 24–32). We replaced this primer with another *Ascaphus* specific primer (929V 5′-ATGGAAAGC-GAAAAATCGTG-3′) that amplified 530 nucleotides. We precipitated double stranded PCR products, prepared cycle sequencing reactions and sequenced the cyt b gene using the methods described in Nielson et al. (2001). Sequences are deposited in the GenBank database under accession numbers DQ087511–DQ087517.

Phylogenetic analyses.—We aligned and edited sequences using Sequencher (Gene-Codes, Inc.) and conducted all mtDNA phylogenetic analyses using PAUP* (version 4.0; Swofford, 1998). For these analyses, we

TABLE 1.—Allele frequencies and average heterozygosity for 14 loci in 34 populations across the range of Ascaphus. Population numbers correspond with those in Appendix I and Figures 1 and 2. Twenty-three loci were originally examined; nine showed no variation in these.

A. montanus

		Northern Rocky Mts.						Salmon R Mts.	7 Devils		Wallowa Mts.			Blue Mts.	
Population		37	43	36	41	5	33	32	42	34	39	40	35	14	38
Number of individuals		40	44	29	40	40	20	40	40	40	11	5	60	40	22
Locus (allele)															
Aat-1	a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	b	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Aat-2	a	1.000	0.990	1.000	0.990	0.750	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	b	—	0.010	—	0.010	0.250	—	—	—	—	—	—	—	—	—
	c	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	d	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Agp-1	a	1.000	1.000	0.980	0.980	0.900	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	b	—	—	0.020	0.030	0.100	—	—	—	—	—	—	—	—	—
Gus	a	1.000	0.990	1.000	0.900	1.000	1.000	1.000	ND	1.000	—	—	0.020	—	—
	b	—	0.010	—	0.100	—	—	—	ND	—	1.000	1.000	0.980	1.000	1.000
	c	—	—	—	—	—	—	—	ND	—	—	—	—	—	—
Idh-1	a	1.000	1.000	1.000	1.000	0.960	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	b	—	—	—	—	0.040	—	—	—	—	—	—	—	—	—
Idh-2	a	1.000	0.930	0.930	0.910	0.840	1.000	1.000	0.880	1.000	1.000	1.000	1.000	1.000	1.000
	b	—	0.070	0.070	0.090	0.160	—	—	0.130	—	—	—	—	—	—
Lap	a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	b	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ldh-2	a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	b	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Mdh-2	a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	b	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	c	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	d	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Pep-2	a	1.000	0.990	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	b	—	0.010	—	—	—	—	—	—	—	—	—	—	—	—
Pgi-1	a	0.770	0.820	0.960	0.990	1.000	0.140	1.000	1.000	—	1.000	1.000	0.600	0.900	1.000
	b	—	—	0.040	0.010	—	—	—	—	—	—	—	—	0.100	—
	c	0.230	0.180	—	—	—	0.860	—	—	1.000	—	—	—	—	—
	d	—	—	—	—	—	—	—	—	—	—	—	0.400	—	—

A. truei

		Northern Cascades Mts.					Central Cascades Mts.			Coast Mts.		Olympic Mts.				Southern Cascades Mts.				Siskiyou Mts.	
Population		59	46	23	48	45	44	49	47	58	17	52	18	50	51	53	54	55	56	15	57
Number of individuals		40	40	40	11	7	54	15	40	6	46	61	43	5	19	40	40	40	21	40	6
Locus (allele)																					
Aat-1	a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.990	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	b	—	—	—	—	—	—	—	—	—	0.01	—	—	—	—	—	—	—	—	—	—
Aat-2	a	1.000	1.000	1.000	1.000	1.000	0.360	0.400	0.430	0.170	0.120	0.000	0.010	0.000	0.000	0.500	0.980	0.990	0.740	0.990	1.000
	b	—	—	—	—	—	—	0.830	0.600	0.830	0.870	0.260	1.000	0.970	1.000	0.970	0.030	0.000	0.580	0.010	—
	c	—	—	—	—	—	—	—	—	—	0.010	—	0.020	—	0.030	—	—	0.000	—	—	—
	d	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.010	—	—	—
Agp-1	a	1.000	1.000	1.000	0.820	0.930	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.670
	b	—	—	—	0.180	0.070	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.330
Gus	a	0.990	1.000	1.000	—	0.290	—	—	0.090	—	0.040	—	—	—	—	—	—	—	—	—	—
	b	0.010	—	—	1.000	0.710	1.000	1.000	0.910	1.000	0.960	0.990	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	c	—	—	—	—	—	—	—	—	—	—	0.010	—	—	—	—	—	—	—	—	—
Idh-1	a	—	—	—	—	—	—	—	0.010	0.080	0.650	1.000	1.000	1.000	1.000	0.850	0.440	0.840	0.600	1.000	1.000
	b	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.990	0.920	0.350	—	—	—	—	0.150	0.560	0.160	0.400	—	—
Idh-2	a	1.000	1.000	1.000	1.000	1.000	0.880	0.870	1.000	0.750	0.870	0.380	0.190	—	0.630	0.880	0.680	0.230	0.930	—	—
	b	—	—	—	—	—	0.120	0.130	—	0.250	0.130	0.630	0.810	1.000	0.370	0.130	0.330	0.780	0.070	1.000	1.000
Lap	a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	—	—	—	—	1.000	1.000	1.000	1.000	1.000	1.000
	b	—	—	—	—	—	—	—	—	—	—	1.000	1.000	1.000	1.000	—	—	—	—	—	—
Ldh-2	a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.930	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	b	—	—	—	—	—	—	—	—	—	—	—	0.070	—	—	—	—	—	—	—	—
Mdh-2	a	1.000	1.000	1.000	1.000	1.000	0.960	1.000	0.970	1.000	1.000	0.530	0.490	0.700	0.580	0.840	1.000	1.000	0.050	1.000	1.000
	b	—	—	—	—	—	0.040	—	0.030	—	—	0.470	0.510	0.300	0.420	0.160	—	—	0.950	—	—
	c	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	d	—	—	—	—	—	—	—	—	—	—	0.010	—	—	—	—	—	—	—	—	—
Pep-2	a	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	b	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Pgi-1	a	1.000	1.000	1.000	1.000	1.000	0.990	1.000	0.920	1.000	0.990	0.970	0.850	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	b	—	—	—	—	—	0.010	—	—	—	0.010	0.030	0.150	—	—	—	—	—	—	—	—
	c	—	—	—	—	—	—	—	0.080	—	—	—	—	—	—	—	—	—	—	—	—
	d	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table 1.—Continued.

		A. montanus														*A. truei*																			
		Northern Rocky Mts.						Salmon R Mts.	7 Devils		Wallowa Mts.			Blue Mts.		Northern Cascades Mts.				Central Cascades Mts.			Coast Mts.		Olympic Mts.				Southern Cascades Mts.					Siskiyou Mts.	
Pgm-2	a	1.000	1.000	0.980	0.850	1.000	1.000	0.960	1.000	1.000	1.000	1.000	1.000	1.000	1.000	—	—	—	—	—	0.010	—	—	—	0.010	0.010	—	—	—	—	—	—	0.020	—	—
	b	—	—	0.020	0.090	—	—	0.040	—	—	—	—	—	—	—	1.000	1.000	1.000	1.000	1.000	0.990	0.970	1.000	1.000	0.990	0.990	1.000	1.000	1.000	1.000	1.000	0.070	0.980	1.000	1.000
	c	—	—	—	0.060	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	d	—	—	—	—	—	—	—	—	—	—	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.900	0.900	—	—	—
	e	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.020	—	—	—
	f	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.030	—	—	—	—	—	—	—	—	—	0.000	—	—	—
Pmi-2	a	—	0.920	0.750	0.700	0.840	1.000	0.240	0.040	1.000	0.360	—	0.950	—	—	—	0.010	—	0.050	0.070	0.010	—	0.010	—	0.030	0.180	—	0.200	0.210	0.030	—	—	0.020	0.090	—
	b	0.010	—	0.150	0.210	0.160	—	0.760	0.700	—	—	—	0.020	—	—	1.000	0.990	1.000	0.950	0.930	0.990	1.000	0.980	1.000	0.880	0.640	0.920	0.700	0.760	0.980	1.000	1.000	0.980	0.830	1.000
	c	0.830	0.080	0.040	0.090	—	—	—	—	—	—	—	—	1.000	1.000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	d	—	—	0.020	—	—	—	—	0.260	—	—	—	—	—	—	—	—	—	—	—	—	—	0.010	—	0.030	0.060	—	—	—	—	—	—	—	0.090	—
	e	0.010	—	0.040	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	f	—	—	—	—	—	—	—	—	—	0.640	1.000	0.030	—	—	—	—	—	—	—	—	—	—	—	0.030	0.030	—	0.100	—	—	—	—	—	—	—
	g	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.020	0.090	0.080	—	0.030	—	—	—	—	—	—
Sod	a	0.670	0.710	0.770	0.740	0.530	0.750	0.690	0.530	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	b	0.330	0.290	0.230	0.260	0.460	0.250	—	—	1.000	—	—	1.000	1.000	1.000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.080	—	—	—
	c	—	—	—	—	—	—	0.310	0.470	—	1.000	1.000	—	—	—	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.930	0.020	1.000	1.000
	d	—	—	—	—	0.010	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.980	—	—
mean heterozygosity		0.015	0.010	0.013	0.012	0.016	0.014	0.015	0.020	0.000	0.016	0.000	0.010	0.000	0.000	0.001	0.001	0.000	0.011	0.010	0.008	0.014	0.008	0.017	0.010	0.015	0.014	0.027	0.019	0.017	0.016	0.010	0.010	0.005	0.029

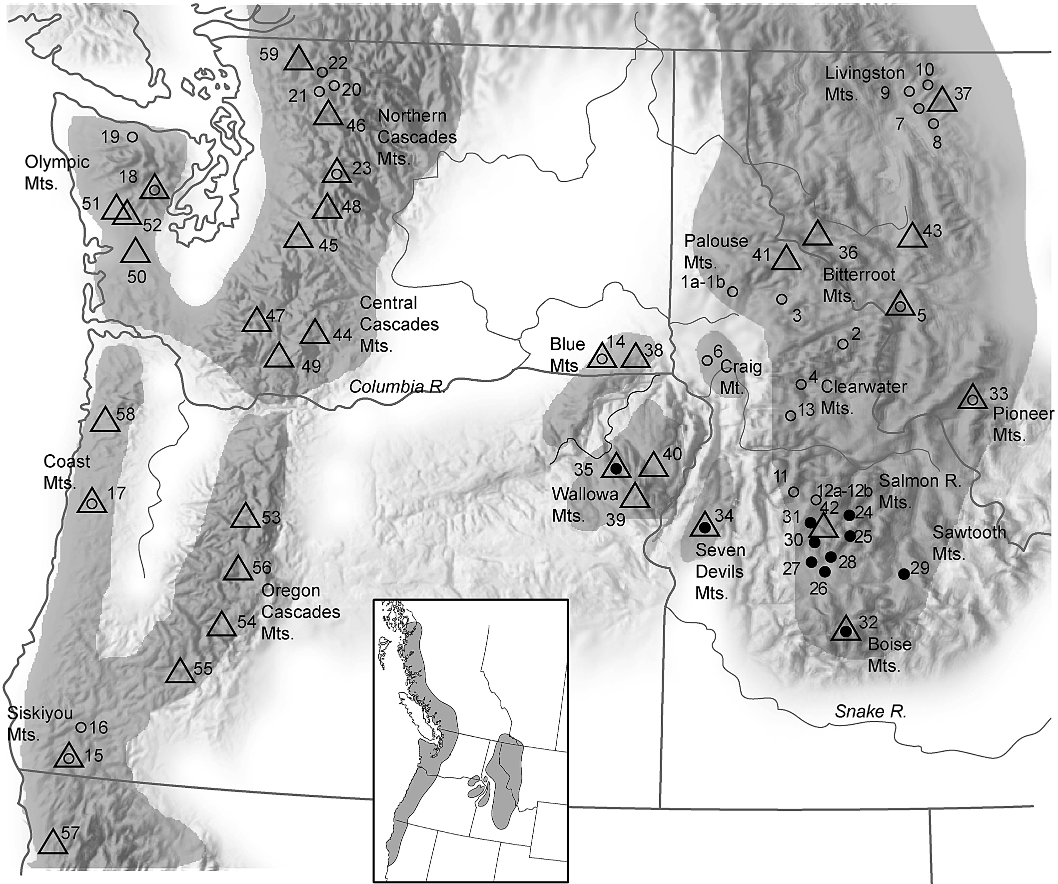

FIG. 1.—Collection localities for *Ascaphus montanus* and *Ascaphus truei* (range shown within inset map; modified from Metter and Pauken, 1969 and Green and Campbell, 1984). Numbers correspond with the collection localities given in Appendix I. Sites with a dot indicate mtDNA data. Solid dots indicate localities with newly collected mtDNA, open dots indicate localities with mtDNA sequences reported in Nielson et al. (2001). Open triangles indicate localities where allozyme data were collected.

combined the new haplotypes discovered in our sampling with the corresponding mtDNA sequences from the 16 *A. montanus* haplotypes characterized by Nielson et al. (2001) and we included nine representative *A. truei* sequences. Parsimony analyses provided initial topologies for subsequent evaluation of ML models and estimation of model parameters (Sullivan and Swofford, 1997; Swofford et al., 1996). We arbitrarily chose one of ten optimal trees saved from parsimony searches (equal weights, heuristic search, 10 random stepwise addition replicates, TBR branch swapping), and held this topology constant in model selection using DT-ModSel (Minin et al., 2003) to select from among 56 substitution models. Each model represents a special case of the GTR+I+Γ model of nucleotide evolution, and these are the same models evaluated by Modeltest (Posada and Crandall, 1998). Abdo et al. (2005) have demonstrated that this approach selects simpler models than likelihood ratio tests or the AIC, that nevertheless estimate phylogenies accurately. This approach led to the selection of HKY+Γ for the 530 bp data set, and ML searches were performed with the model fully defined (heuristic search with 10 replicate random addition sequences to generate stepwise addition trees & TBR branch swapping). ML bootstrap analysis (100 replicates with a maximum of one tree in each replicate; Felsen-

stein, 1985) was used to estimate nodal support for the phylogeny. In addition, we used a Bayesian approach to estimate posterior probabilities for each node. We used uniform priors on all parameters and ran 4 chains of 4×10^6 generations each (MRBayes; Huelsenbeck and Ronquist, 2001). We discarded the first 10,000 generations as burn-in after determining that the chains had reached stable plateaus.

Analysis of Congruence

We used a Bayesian approach to examine congruence among the allozyme and mtDNA data. We analyzed the mtDNA data matrix using MRBayes (Huelsenbeck and Ronquist, 2001) under the previously selected HKY+Γ model of sequence evolution. A run of 6.7×10^6 generations was started with a random tree and we discarded the first 10,000 generations as burn-in. We then sampled every 1000 generations to generate the posterior probability distribution. We filtered the resulting sample of 5,700 trees to find the portion consistent with the constraint that haplotypes from the coast (*A. truei*) and those from the northern Rocky Mountains (*A. montanus*) exhibit reciprocal monophyly (indicated in both allozyme topologies).

We also used the program STRUCTURE (Pritchard, Stephens and Donnelly, 2000) to implement a non-hierarchal test of the hypothesis that *A. montanus* and *A. truei* represent distinct species. STRUCTURE applies a Bayesian, model-based clustering method to probabilistically assign individual genotypes to one of K populations. Populations are assumed to be in Hardy-Weinberg equilibrium and linkage equilibrium. A Markov chain Monte Carlo (MCMC) approach is used to estimate model parameters.

We ran STRUCTURE using the "No Admixture Model" of individual ancestry. This model assumes that each sampled individual is drawn discretely from one population, and calculates the posterior probability that a given individual is from each of K populations. We chose this model based on the lack of long-distance dispersal documented during mark-recapture studies of the tailed frog (Adams and Frissell, 2001; Daugherty and Sheldon, 1982*a*). For similar reasons we used a model that assumes independent allele frequencies among populations. All runs were 10^6 generations in length, preceded by a burn-in period of 10,000 generations (during which parameters were verified to have reached stationarity). To test the hypothesis that *A. montanus* and *A. truei* represent discrete species we examined the assignment of individual genotypes with K set equal to two.

Phylogeographic Hypothesis Tests Within A. montanus

We tested three predictions of the western refugia hypothesis. We used a two-tailed student's *t*-test to test the prediction that populations from the Blue and Wallowa Mountains show a higher mean allozyme heterozygosity. A Bayesian analysis was used to test the prediction that haplotypes from the Blue and Wallowa Mountains group at the origin of the *A. montanus* phylogeny. We followed the methods described above for the analysis of congruence (i.e., 6.7×10^{-6} generations, 10,000 generations discarded as burn-in). We filtered the resulting sample of 5700 trees to find the portion consistent with the constraint that haplotypes from west of the Snake River are basal to the remaining Rocky Mountains haplotypes. The proportion of trees in the sample consistent with the hypothesis represents the probability that the hypothesis is correct, given the data, model and priors.

We used nested clade analysis to examine the mtDNA data for signs of a range expansion from west of the Snake River (Templeton, 1998; Templeton et al., 1995). We constructed our haplotype network and grouped clades using guidelines presented in Templeton et al. (1987) and Templeton and Sing (1993). We used ParsProb (Posada et al., 2000) to determine the number of substitutional steps connecting haplotypes parsimoniously. GeoDis (Posada et al., 2000) was used to calculate the statistics used in nested clade analysis (D_C, D_N, and I-T) and to test the observed statistics against a null distribution. One thousand permutations of the haplotype network provided a null distribution, representing a random association of haplotypes with geography, against which to test the empirical values of D_C, D_N, and I-T for

significance. We use the updated inference key (Templeton, 2004) developed in response to recent critiques of the method (e.g. Knowles and Maddison, 2002) to interpret the nested clade analysis in terms of phylogeographic processes.

We used three approaches to test predictions of the dual refugia hypothesis. To test for the association of haplotypes with the purported refugia (and subsequent range expansion), we used the nested clade analysis described above. To examine the allozyme data set for structure consistent with two historic refugia, we ran STRUCTURE using the *A. montanus* data set for values of K from one to five. The number of discrete populations (K) can be approximated by running the model for a range of values, calculating the probability for each K (from Pr (X|K), and selecting the smallest K from those of similar probability (because the probability of K often plateaus). Three runs were completed for each value of K. The number of generations appears to be adequate for approximating K because only slight differences in the value of ln Pr (X|K) were seen within a given K. Model settings, the number of generations and the burn-in period are the same as those described above for the analysis of congruence.

We used MDIV to test whether the date of lineage divergence falls within the Pleistocene. This program uses a coalescent-based, divergence by isolation with migration model (Nielsen and Wakeley, 2001). Haplotype samples were partitioned into two geographically defined populations. We posited that samples collected north of the arid Salmon River canyon were derived from a Pleistocene refuge in the Clearwater River valley. Samples collected south of this break were assumed to have arisen from a refuge in the South Fork of the Salmon River. Samples collected from the Seven Devils, Blue, and Wallowa Mountains and Craig Mountain were omitted because their predicted origin is unclear with respect the purported refugia (i.e., they represent geographic isolates of the main range).

The model applied by MDIV uses an MCMC methodology to approximate the posterior distribution of three parameters; the divergence time between populations in generations (T), the migration rate in number of migrants per generation (M), and theta (θ; a function of the effective population size and the nucleotide substitution rate). We used uniform priors and experimented with truncation following the recommendations of Nielsen and Wakeley (2001). We completed two runs of 2×10^7 generations to assess stationarity. We assumed a nucleotide substitution rate of 10^{-7} (based on the value used for amphibian taxa in Carstens et al., 2005) and a generation time of six years (Daugherty and Sheldon, 1982*a*) to calculate the divergence time between the geographically delimited populations. We summed the frequencies of the posterior probabilities in the distribution for each parameter to define the boundaries that contain 95% of the estimates. The 95% credibility intervals for T served to test the hypothesis that the populations diverged due to Pleistocene isolation. Estimates of N_e and M are discussed in the context of conservation.

Results

Allozymes

Description of loci.—We scored 23 allozyme loci in 34 populations; of these, 14 were polymorphic (Table 1). Heterozygote deficiencies were detected at five loci in 11 populations (*Pgi-1*, pops. 14 and 37; *Gus*, pop. 41; *Pmi-2*, pops. 42, 35, 47, 15, and 17; *Idh-2*, pops. 43 and 44; *Aat-2*, pop. 18).

Several geographically clustered groups of populations exhibit differentiation from the remaining populations at one or more loci. For example, the Cascades Mountains populations are fixed for the "b" allele at the *Idh-1* locus, whereas other populations carry this allele at lower frequencies (Table 1). In addition, populations from the Olympic Mountains are fixed for the "b" allele at the *Lap* locus; this allele does not occur in any of the samples from other populations (Table 1). We also found a fixed difference (with negligible leakage) between *A. montanus* and *A. truei* populations at the *Pgm-2* locus (Table 1). The *A. montanus* sample (n = 1120 alleles) contains 12 "*A. truei* alleles" (frequency = 1.07%), whereas the *A. truei* sample (n = 1600 alleles) contains 4 "*A. montanus* alleles" (frequency = 0.25%).

Phylogeny estimates.—Phylogenetic analysis of the allozyme data yields two topologies; Figure 2a corresponds with the analysis that excluded the *Gus* locus, Figure 2b corresponds with the analysis that included the *Gus* locus, but excluded population 42 from the Salmon River Mts. Both topologies contain two, well-supported clades corresponding to Pacific and Rocky Mountain populations (83% and 71% bootstrap values, respectively; Fig. 2). The relationships among populations within these major clades differ somewhat between topology 2a and 2b. Considering only *A. truei*, in topology 2a, population 56 is sister to all populations found to the north; however in topology 2b population 56 is sister only to the group formed by populations from the northern Cascades Mountains, and the group formed by the central Cascades Mountains populations and population 58 from the Coast Mountains. Two groupings are well supported in both topologies: the group formed by populations 15 and 57 from the Siskiyou Mountains, and the group formed by populations 18, 50, 51 and 52 from the Olympic Mountains (Fig. 2).

Likewise, the relationships among *A. montanus* populations differ slightly depending on whether locus *Gus* is included and population 42 is excluded or vice versa. In the analysis excluding population 42 (but retaining the *Gus* locus; Fig. 2b), the two populations from the Wallowa Mountains (pops. 39 and 40) form a clade sister to the rest of *A. montanus*. Populations from the Blue Mountains (pops. 14 and 38) form a clade sister to the remaining Rocky Mountains populations. Thus, *A. montanus* populations from west of the Snake River are paraphyletic to populations east of the river. However, inclusion of population 42 and the elimination of the *Gus* locus alters this conclusion (Fig. 2a). Well supported clades in both allozyme topologies include: populations 39 and 40 from the Wallowa Mountains, populations 14 and 38 from the Blue Mountains, and populations 33 and 34 from the Pioneer and Seven Devils Mountains (Fig. 2).

Population level parameters.—We removed three loci (*Gus*, *Aat-2* and *Ldh-2*) from all AMOVA runs because Arlequin cannot perform an AMOVA with loci resolved in fewer than 95% of the individuals genotyped. Fifty-five percent of the allozyme variation partitions between *A. montanus* and *A. truei* populations, (F_{CT} = 0.55; P < 0.001), 28% occurs among populations within groups (F_{SC} = 0.63; P < 0.001), and 17% occurs within populations (F_{ST} = 0.83; P < 0.001).

Out of 572 comparisons, 20 pairwise F_{ST} values are not significant (P > 0.05). Weakly differentiated *A. truei* populations include the three northernmost populations in the Cascades Mountains (pops. 23, 48 and 59), three populations from the western Olympic Mountains (pops. 50, 51 and 52), and populations from the Coast Mountains in Oregon and the central Cascades Mountains (pops. 17, 44, 45, 48, 49, 53, 56, and 58; Fig. 3). Weakly differentiated *A. montanus* populations include two from the Blue Mountains (pops. 14 and 38), two from the Seven-Devils Mountains (pops. 39 and 40), and two from the Bitterroot Mountains (pops. 41 and 36; Fig. 3).

mtDNA Within A. montanus

Our sampling identifies seven additional *A. montanus* haplotypes distinct from the 16 previously characterized (Nielson et al., 2001). With this additional sampling, we define a southern clade within *A. montanus* distinguished by up to 0.031 substitutions per site from northern populations (Fig. 4).

All parsimony trees measure 90 steps (70 variable sites; 52 parsimony-informative; consistency index [CI] = 0.833; retention index [RI] = 0.959; rescaled consistency index [RC] = 0.799). The HKY+Γ model, which allows unequal base frequencies, and in which all transitions are assigned a different rate of substitution from all transversions, is the model chosen by decision theory (Minin et al., 2003). Parameter estimates for the ML tree under the HKY+Γ model are as follows: transition to transversion rate ratio = 4.2; shape parameter for the Γ−distribution = 1.6; base frequencies $\pi_A = 0.272$; $\pi_C = 0.243$; $\pi_G = 0.162$; $\pi_T = 0.323$.

All optimal parsimony topologies, and the ML topology contain two major haplotype clades. The northern clade is comprised of haplotypes from populations north of the South Fork of the Salmon River and populations in the Blue, Wallowa, and Seven Devils

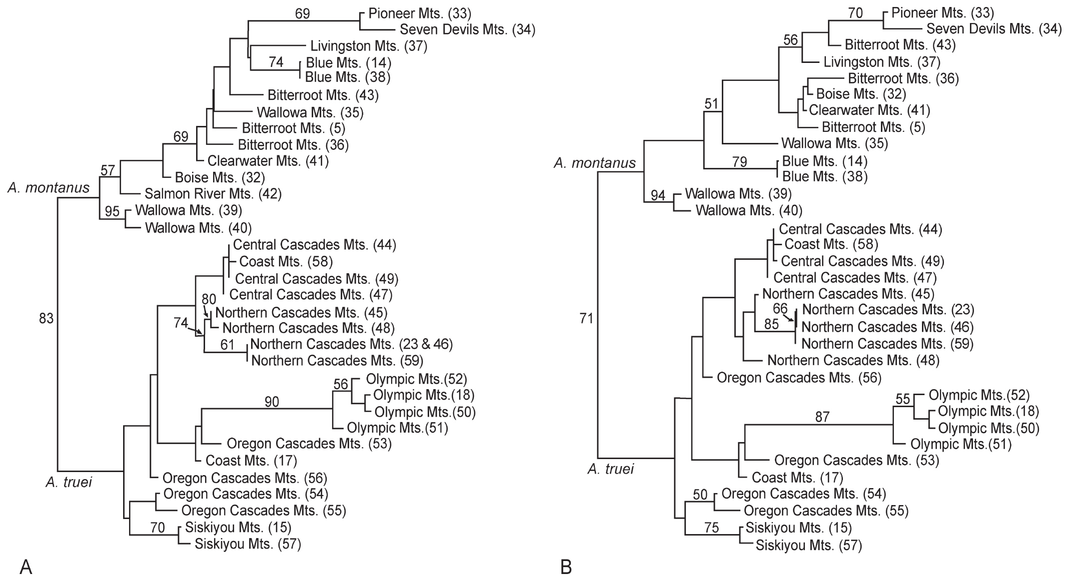

Fig. 2.—(A) Maximum-likelihood tree estimated from the allozyme frequency data using CONTML with locus *Gus* omitted and rooted at the midpoint. (B) Maximum-likelihood tree estimated from the allozyme frequency data using CONTML with the Dollar Cr. population (42) omitted. Population numbers (given in parentheses) and geographic designations correspond with Appendix I and Fig. 1. Neighbor-joining bootstrap values (200 replicates) based on Cavalli-Sforza chord distances (Cavalli-Sforza and Edwards, 1967) are shown above branches for those clades with greater than 50 percent support.

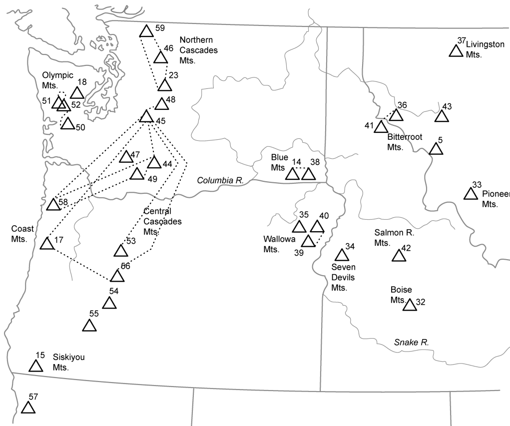

Fig. 3.—Map showing populations with nonsignificant allozyme F_{ST} values. Dashed lines connect populations without significant F_{ST} values. Population numbers correspond with Appendix A and Fig. 1. A table of F_{ST} values is given in Appendix III.

Mountains. The southern clade is composed of populations south of the South Fork Salmon River in the Boise and Salmon River Mountains. Alternative parsimony topologies differ from each other and the ML topology only in the resolution of internal branches within each clade (in particular haplotype FF is alternatively placed sister to the rest of the northern clade; Fig. 4). Bootstrap values and Bayesian posterior probabilities strongly support monophyly of haplotypes from southern Idaho (98% and 1.00, respectively). The remaining *A. montanus* haplotypes group with low support because of the alternate positioning of haplotype FF (12% bootstrap, 0.41 posterior probability; Fig. 4). Maximum-likelihood corrected genetic distances (HKY+Γ) between these groups average 0.024 substitutions per site (range 0.018–0.031). The mean corrected genetic distance within the southern haplotype group is 0.004 (range 0.002–0.006); mean distance within the northern clade is 0.006 (range 0.002–0.012).

Analysis of Congruence

Each of the 5700 trees in the Bayesian posterior distribution exhibits reciprocal monophyly between Rocky Mountains and Pacific populations. Thus, the two character systems (mtDNA and allozymes) are strongly

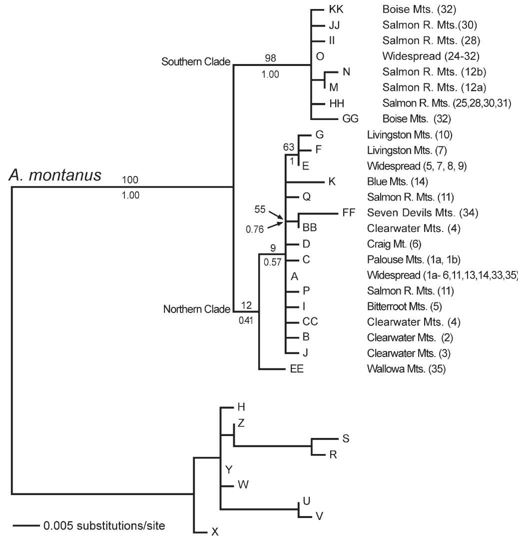

FIG. 4.—One of five *A. montanus* maximum-likelihood trees estimated from the cyt *b* data set under the objectively determined HKY+Γ model of sequence evolution and rooted using nine representative *A. truei* sequences. This tree has a lnL of −1273.85 and differs from the other maximum-likelihood and parsimony trees only in alternative resolutions of internal branches. Numbers above branches represent maximum-likelihood bootstrap values (100 replicates). Those below branches represent Bayesian estimates of nodal support (4 chains of 10^7 generations each). The populations where each haplotype was found are indicated in parentheses. Population numbers and geographic designations correspond with Fig. 1 and Appendix I.

concordant ($P = 1.0$) with respect to reciprocal monophyly across the disjunction. Comparison of the mtDNA phylogeny to the allozyme topologies is not straight-forward because the tips of the allozyme topology represent populations and those of the mtDNA phylogeny represent unique haplotypes. Nevertheless, haplotypes from the same locale or population as those represented in the allozyme-based phylogeny do not group in the same manner (Figs. 2 and 4).

When the number of populations (K) is set equal to two, each of the 1085 allozyme genotypes is assigned with high probability to either the *A. montanus* or the *A. truei* group. Out of 471 individuals sampled from the Rocky Mountains, 455 group with *A. montanus* with a probability of one; the remaining 16 genotypes are assigned to *A. montanus* with probabilities ranging from 0.999 to 0.998. Thirteen individuals assigned with probabilities < 1.0 originate from populations 32 and 42; one originates from each of populations 36, 39, and 40. Out of 614 individuals sampled from the Coast and Cascades Mountains, 607 are assigned to *A. truei* with a probability of one. The remaining seven (all from population 55) are assigned with probabilities ranging from 0.869 to 0.999.

Phylogeographic Hypothesis Tests Within A. montanus

We examined three predictions under the western refugia hypothesis. The first, that mtDNA haplotypes from the Blue and Wallowa Mountains will group at the origin of the *A. montanus* phylogeny, is not supported by our analysis. Only twelve of 5700 trees generated for the Bayesian analysis are consistent with colonization of the northern Rocky Mountains from populations west of the Snake River ($P = 0.002$).

The second prediction, that mean allozyme heterozygosity will be higher within populations from the Blue and Wallowa Mountains, is not supported by our data either. Mean heterozygosity is actually lower within populations from the Blue and Wallowa Mountains when compared with populations from the remainder of the species' range ($P = 0.05$).

The third prediction, that mtDNA halotypes will show evidence of a range expansion from west of the Snake River is not validated by the nested clade analysis. The nested clade network includes haplotypes connected by up to eight substitutional steps (Fig. 5). Haplotypes K, A and EE are found in populations from the Blue and Wallowa Mountains. These haplotypes group within the northern clade (clade 2-2). With respect to the northern group, haplotypes K and EE appear geographically restricted ($P < 0.001$). Haplotype A, which is interior, and thus ancestral, in the northern group's haplotype network, appears widespread geographically within the northern portion of *A. montanus'* range ($P < 0.001$; Fig. 5). The group of haplotypes closely related to haplotype A (clade 1-1), shows a pattern consistent with restricted gene flow with isolation by distance. Based on the updated nested-clade inference key (Templeton, 2004), the restricted ranges of haplotypes K and EE suggest one or more past fragmentation events.

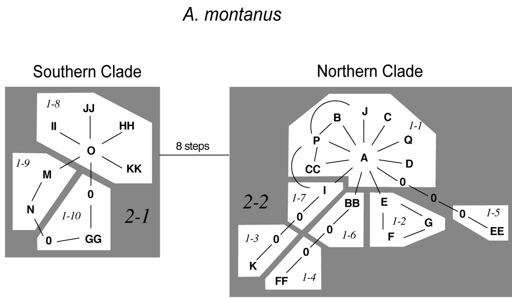

FIG. 5.—The minimum-spanning network of haplotypes grouped into nesting clades following procedures given in Templeton et al. (1987) and Templeton and Sing (1993). Each dash represents a substitution. Zeros indicate ancestral haplotypes that were not sampled. Connections up to 9 steps have a 95% probability of a parsimonious connection. White cells represent one-step clades and shading encloses two-step clades. Clade numbers are given within each enclosure with the level of the nesting clade followed by the number within that level (i.e. 2-1, nesting level two, grouping number one). Haplotype letters correspond with those given in Figs. 4 and 6.

We examined four predictions under the dual refugia hypothesis. The first, that *A. montanus* will contain two monophyletic clades, is consistent with the mtDNA topology (Fig. 4). The allozyme topology, however, does not show two clades within *A. montanus* (Fig. 2). When the number of populations (K) is set to two, individuals are not clearly assigned to northern or southern clades. Individuals from the four northernmost populations (5, 36, 37, 41, and 43) are assigned in a mixed manner to both groups without high probabilities. Individuals from three populations in the Blue and Wallowa Mountains (14, 38, and 35) are assigned exclusively to group one, while individuals from populations 39 and 40 are assigned exclusively to group two. The remaining individuals from populations in the central and southern portion of the species range are assigned in a clear manner to either group one or two; however, this assignment shows no geographic pattern. Values of K from one to five do not produce an optimal ln Pr(X|K). This indicates the presence of more than five populations in the sampled genotypes. This result is not unexpected given the number of significant pairwise F_{ST} values for *A. montanus* (Appendix III).

The second and third predictions, that the mtDNA haplotypes will show a geographic association consistent with their purported refugia and that the clades will show evidence of range expansion, were examined using nested clade analysis. Haplotypes are divided into two major nesting clades that correspond with the northern and southern clades identified in the ML topology (Fig. 6). Random association of haplotypes with geography could be rejected for the northern group (clade 2-2; $P < 0.001$). The dispersion of haplotypes did not appear strongly geographically structured in the southern group (clade 2-1; $P = 0.073$). With respect to the northern group, haplotypes D, C, K and FF and the E, F, G group appear geographically restricted ($P = 0.008$, $P = 0.006$, $P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively). The group formed by haplotypes one-step removed from haplotype A (clade 1-1) shows a restricted geographic range clustered near the center of the northern group ($P < 0.001$; Fig. 5).

Using the updated inference key (Templeton, 2004), four clades contained sufficient sampling and genetic structure to infer processes. The group of haplotypes closely related to haplotype A (clade 1-1), and those closely related to haplotype O (clade 1-8) show patterns consistent with restricted gene flow with isolation by distance. Within the northern clade (clade 2-2) the restricted ranges of several geographically peripheral haplotypes (FF, E, F, G, K, EE) indicate one or more past fragmentation events. When the northern and southern clades (clade 2-2 and 2-1, respectively) are considered together the geographic pattern is consistent with past fragmentation followed by range expansion. Thus, the nested clade analysis supports the second and third predictions of the dual refugia hypothesis.

The final prediction, that the divergence between northern and southern populations will date to the Pleistocene, was examined using MDIV. Our estimate of the timing of lineage divergence, 999,902 years ago (95% credibility interval 5.4 mya to 92,892 years ago), supports a Pleistocene split. This is consistent with the dual refugia hypothesis.

DISCUSSION

The allozyme data we present provides further support for the recognition of *A. montanus* as originally proposed by Nielson

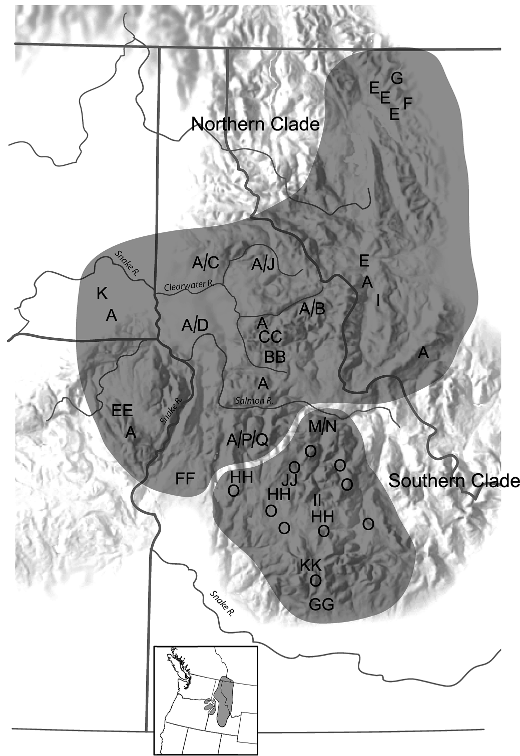

FIG. 6.—The dispersion of *A. montanus* haplotypes on geography. Haplotypes are indicated at their collection locality and correspond with the designations given in Fig. 4. Collection localities correspond with Fig. 1 and Appendix I. The northern and southern clades are enclosed by shading, and the range of the Rocky Mountain tailed frog is indicated in the inset map.

et al. (2001) based on monophyly within the mtDNA lineage. According to the unified general lineage concept of species (de Queiroz, 1998), the strongest support for a given species hypothesis will rise from multiple species criteria. Monophyly within the mtDNA phylogeny represents one line of evidence under the general lineage concept of species. The monophyly criterion is also met by the allozyme phylogeny. Fixation of the *Pgm-2* locus within *A. montanus* meets the diagnosability criterion (again using terminology introduced by de Queiroz, 1998). The correct assignment of each of the 1085 allozyme genotypes to either *A. montanus* or *A. truei* with high probability also supports this criterion. Furthermore, the majority (55%) of allozyme variation within tailed frogs partitions between *A. montanus* and *A. truei* as indicated by the AMOVA. Congruence between the allozyme and mtDNA phylogenies (with respect to the interspecies divergence) suggests concordance between the multiple gene geneaolgies approximated by the allozyme phylogeny and the mtDNA gene lineage. These independent phylogenies provide evidence that both mtDNA and nuclear genes coalesce more recently within *A. montanus* than between *A. montanus* and *A. truei*, and thus satisfy the concordant coalescence criterion.

Additional differences in the nuclear genome were found by Ritland et al. (2000) in comparisons of *A. truei* and *A. montanus* in British Columbia using randomly amplified polymorphic DNA (RAPD); the deepest divergence in RAPDs also occurred between Rocky Mountain and Pacific samples. Although limitations imposed by a lack of understanding of the mechanisms behind RAPD evolution prevent direct comparisons with our mtDNA and allozyme data, these differences between Pacific and Rocky Mountain populations in British Columbia are consistent with recognition of *A. montanus* as a distinct species.

Allozyme Variation Within A. truei

Both allozyme topologies show three well-supported geographic groupings within *A. truei*: the clade formed by two populations from the Siskiyou Mountains of Oregon (populations 15 and 57), the clade formed by populations 23, 46, and 59 from the northern Cascades Mountains, and the clade formed by populations from the Olympic Mountains (populations 18, 50, 51, and 52; Fig. 2). F_{ST} values indicate gene flow among populations within the northern Cascades Mountains clade and the Olympic Mountains clade, and indicate a lack of genetic exchange among each of these three clades and the remaining populations from the central Cascades Mountains and Coast Mountains of Oregon (Fig. 3).

Populations from the Coast Mountains and central Cascades Mountains of Oregon and Washington (populations 17, 44, 45, 47, 48, 49, 53, 56, and 58) do not group distinctly by geography in the allozyme topology. F_{ST} values indicate a lack of divergence in allozyme frequencies among these popula-

tions. Nielson et al. (2001) did not sample within this central part of the species' range and could not determine whether mtDNA patterns were consistent with early Pleistocene refugia or limited dispersal and isolation by distance. The presence of non-significant F_{ST} values and lack of geographic grouping within the core of the Pacific tailed frog's range suggests that relatively recent dispersal may have occurred and/or that contemporary gene flow has not been limited.

Unlike many populations to the north, pairwise F_{ST} values among populations from the Siskiyou Mountains and Southern Cascades Mountains in Oregon show significant differentiation of allele frequencies (Fig. 3; Appendix III). Significant F_{ST} values may reflect reduced gene flow due to climate-induced limitations on dispersal. Tailed frogs exhibit greater mobility over land during precipitation events and their habitats become increasingly fragmented in dry climates (Daugherty and Sheldon, 1982*b*). Similarities in precipitation and climate within the southern portion of the Pacific tailed frog's range and the range of the Rocky Mountain tailed frog may explain why F_{ST} values within these southernmost populations exhibit a pattern similar to that seen within *A. montanus*, where significant differentiation is found among nearly all population pairs. An alternative (although not exclusive) explanation for this pattern may be that a range expansion occurred earlier within the southern portion of the Pacific tailed frog's range as compared to the northern part, such that allozyme frequencies have had additional time to diverge among southern populations. This explanation is consistent with the direction of glacial retreat. Under this hypothesis, contemporary isolation could be similar throughout all parts of the species' range.

Phylogeographic Hypothesis Tests Within A. montanus

Nielson et al. (2001) postulated that *A. montanus* populations persisted within the northern Rocky Mountains through the Pleistocene. Carstens et al. (2005) estimated the mean time of divergence between the two *Ascaphus* lineages at 3.1×10^6 years ago, further supporting this hypothesis. Pliocene drought and subsequent Pleistocene glacial cycles provide a context for describing contemporary genetic patterns within *A. montanus*. During the Pliocene the northern Rocky Mountains began to experience summer drought and lower mean annual temperatures (Wolfe, 1969, 1978). Pleistocene glacial cycles followed these climate changes. The Cordilleran ice-sheet periodically covered the Rocky Mountain region north of Coeur d'Alene, Idaho, and montane glaciers extended south throughout the Rocky Mountain tailed frog's range during glacial maxima from 2 million to 12,000 years ago (Alt and Hyndman, 1995).

The western refugia hypothesis asserts that habitats west of the Snake River served as a refuge for *A. montanus* through the Pleistocene while the body of their current range in the northern Rocky Mountains was glaciated. This hypothesis is not supported by our data. The placement of populations from the Blue and Wallowa Mountains at the origin of *A. montanus* within allozyme topology 2b appears consistent with the western refugia hypothesis; however, this placement is not well supported, and is not stable to inclusion/omisision of the *Gus* locus. Furthermore, our Bayesian analysis indicated that haplotypes from the Blue and Wallowa Mountains do not consistently group basally in the mtDNA phylogeny. Mean allozyme heterozygosity is lower within populations west of the Snake River compared to the remainder of the species' range, rather than higher as would be expected for populations with a longer history in the area (given that population size and interpopulation genetic exchange appear to be similar). Nested clade analysis indicates that unique haplotypes from the Blue and Wallowa Mountains arose as a result of historic fragmentation and isolation; signals of range expansion from west of the Snake River are not evident in the data.

The Clearwater River drainage has been suggested as a potential refuge for mesic forest species during the Pleistocene because of the large numbers of endemic and coastal disjunct plant species found here (Daubenmire, 1975). Explicit hypothesis testing based on mtDNA sequence data within the Coeur d'Alene salamander (*Plethodon idahoensis*) supports expansion of this species from the

Clearwater drainage at the close of the Pleistocene (Carstens et al., 2004*a*). Similar analysis of sequences from the Idaho giant salamander (*Dicamptodon atterimus*) indicates expansion from the South Fork of the Salmon River (Carstens et al., 2004*b*). Both the Coeur d'Alene salamander and the Idaho giant salamander have ecological tolerances similar to those of the tailed frog. The dual refugia hypothesis posits that isolated populations of the Rocky Mountain tailed frog persisted within the Clearwater and Salmon River Valleys during glacial maxima. Much of the existing data support this hypothesis.

The dual refugia hypothesis predicts that allozyme and mtDNA data will be partitioned into two groups, that these groups will show a geographic affinity for the purported refugia, that signals of a range expansion from the refugia will be evident in the data, and that the timing of divergence between the clades will correspond with a Pleistocene division of the lineage. The *A. montnaus* mtDNA phylogeny is composed of two monophyletic clades, distinguished by 1.8–3.1 percent sequence divergence, with a strong north/south geographic association. Nested clade analysis indicates that these groups arose due to historic fragmentation and subsequent range expansion. Post-glacial expansion is reflected in the dispersion of haplotypes within each clade. Both clades are represented by one common haplotype: A in the northern clade and O in the southern clade. These haplotypes are the most frequently occurring in each clade and are found in almost every locality sampled. Haplotypes A and O are ancestral to multiple derived haplotypes in the phylogeny, and the derived haplotypes are found to a large extent along the periphery of the sampled range (Figs. 5, 6). Because little contemporary gene flow occurs among populations within each clade (based on the significant differentiation indicated by allozyme F_{ST} analyses; Fig. 3; Appendix II) the distribution of each common haplotype suggests a range expansion consistent with colonization during favorable post-glacial periods.

The timing of lineage divergence between the northern and southern clades is consistent with glacially induced isolation. Our point estimate of the timing of lineage divergence (999,902 years ago) falls in the mid-Pleistocene. The upper bound of the credibility interval (92,829 years ago) remains within the Pleistocene. The lower bound (5.4 million - years ago) extends into the late Pliocene. The dual refugia hypothesis emphasizes the role that glacial cycles and cooling trends played to foster geographic isolation. Based on our credibility interval we cannot exclude the role that increasing aridity through the Pliocene may have also played in isolating these groups.

In contrast, the allozyme phylogeny does not show two distinct clades. Only populations 32 and 42 are from localities within the range of the southern mtDNA clade. These populations do not group together in the allozyme topologies, nor do they carry unique alleles distinguishing them from the remaining *A. montanus* populations (Fig. 2; Table 1). Furthermore, a nonhierarchical analysis of the allozyme data showed that individual genotypes could not be clearly assigned to two groups with a north/south geographic affinity. Our data show no indication that a southern allozyme clade would emerge given more extensive sampling south of the Salmon River Mountains; populations 32 and 42 carry the most common allele at 13 of the 14 loci. Secondary contact and/or male mediated gene flow could explain the absence of distinct allozyme clades. However F_{ST}values among populations 32 and 42 and all remaining populations indicate a lack of current gene flow (Fig. 3; Fig. 6).

Conservation

Conservation within A. truei.—The Pacific tailed frog is listed as a species of special concern by state and provincial governments in California, Oregon, Washington and British Columbia. Although local distribution depends on a number of variables, *Ascaphus* populations are sensitive to increased siltation and elevated water temperatures that may accompany land management activities such as timber harvest and road building (Corn and Bury, 1989; Walls et al., 1992; Welsh, 1990). This has generated concern over the loss and fragmentation of old growth habitat in the Pacific Northwest and the effect this may have on populations of tailed frogs (Blaustein et al.,

1994; Bury, 1983; Corn and Bury, 1989; Walls et al., 1992; Welsh, 1990).

The concept of evolutionarily significant units (ESUs) is designed to aid long-term conservation planning at an intraspecific level by delineating population groups that represent significant elements of genetic diversity in the lineage (Avise, 1989; Ryder, 1986). The ESU concept is valuable and practical for the Pacific tailed frog because much of its habitat is publicly administered by agencies with a mandate to conserve biological diversity (and limited resources to do so). Furthermore, substantial genetic diversity is present within the lineage. Pairwise F_{ST} values show a minimum of seven discrete groups within *A. truei*, and data from Nielson et. al. (2001) show four mtDNA clades distinguished by one to three percent sequence divergence.

Several defining criteria have been proposed to delimit ESUs, these include: unique ecological adaptations (Dizon et al., 1992; Waples, 1991), diagnosable characters (Vogler and DeSalle, 1994), geographic isolation, and a distinct phylogenetic lineage (Vogler and DeSalle, 1994). These criteria may overlap with those used to delimit species under some phylogenetic species concepts (Cracraft, 1991). We choose to describe ESUs using the following criteria introduced by Moritz (1994): *ESUs should be reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci*. Under these criteria nuclear and mitochondrial evidence is required to prevent misclassifying populations linked by male-mediated gene flow and, because of the large number of generations necessary to acquire reciprocal monophyly, only divergence of allele frequencies is required for nuclear loci.

The lack of uniform geographic sampling for mtDNA creates an obstacle to defining ESUs within the Pacific tailed frog; however we present a preliminary working delineation of these groups pending further sampling. This species appears to be represented by a minimum of two ESUs: (1) Olympic Mountains populations and (2) populations from the Umpqua River south through the Siskiyou Mountains and into northern California. The Olympic Mountains ESU exhibits a unique fixed allele at the *Idh-1* locus and is represented by a monophyletic clade in the mtDNA phylogeny (Nielson et al., 2001). The Siskiyou Mountains/southern Cascades Mountains clade is supported by significant F_{ST} values, divergence of frequencies at the *Aat-2* and *Idh-1* loci, and a monophyletic mtDNA clade (Nielson et al., 2001). F_{ST} values indicate significant divergence of allozyme frequencies between these two ESUs and the remaining populations in the Coast and Cascades Mountains; however it is not clear whether the remaining populations also form a single ESU. Haplotypes from the northern Cascades Mountains and Coast Mountains do not form a clade in the mtDNA phylogeny, and mtDNA sequences are not available for populations from the central Cascades (Nielson et al., 2001).

In addition to ESUs, pairwise F_{ST} values may communicate useful conservation information because they provide an indicator of genetic connectivity among populations. Nonsignificant F_{ST} values indicate contemporary or recent historical association among populations. Based on pairwise F_{ST} values the three northernmost populations (23, 46, and 59) and the Olympic Mountains populations (18, 50, 51, and 52) of the Pacific tailed frog are isolated from the remaining populations in the central Cascades Mountains and Coast Mountains. Recent historical or contemporary genetic exchange appears to connect populations from the Coast and central Cascades Mountains. In contrast, each population sampled south of the Umpqua River appears isolated. As described earlier, this pattern may be due to the effects of an increasingly arid climate on dispersal in the southern latitudes.

Conservation within A. montanus.—The Rocky Mountain tailed frog is listed as a state species of concern in Washington and is federally protected as endangered in British Columbia. The species has yet to garner conservation priority in Idaho or Montana. The range of *A. montanus* is characterized by multiple mountain ranges separated by increasingly xeric valleys toward its southern extent. Presumably due to geographic and climatic isolation, pairwise F_{ST} values show significant genetic differentiation for all but the nearest neighboring populations (Fig. 3; Appendix III). This genetic isolation, in

combination with minimal dispersal documented in mark recapture studies (Adams and Frissell, 2001; Daugherty and Sheldon, 1982*b*) emphasizes the need to manage populations on a local watershed or subwatershed scale.

Under Moritz's (1994) criteria the Rocky Mountain tailed frog is represented by two ESUs: (1) the northern clade that includes populations from the Blue, Wallowa and Seven Devils Mountains and the northern Rocky Mountains from the Sesech River north, and (2) the southern clade that encompasses the species range south of the South Fork of the Salmon River. Each of these groups forms a monophyletic clade in the mtDNA phylogeny. F_{ST} values (which reflect allele frequencies at multiple nuclear loci) indicate significant divergence of allozyme frequencies between northern and southern populations. The recognition of two ESUs within *A. montanus* could be questioned because almost all population pairs show significant F_{ST} values and the allozyme phylogeny does not contain distinct northern and southern clades. Nevertheless, we choose to delimit these groups because each contains a unique and significant portion of the Rocky Mountain tailed frog's mtDNA diversity, and our data indicate a lack of genetic exchange between the groups. Furthermore, allozyme sampling included only two populations within the geographic boundaries of the southern ESU, which may have confounded our analysis.

We were able to estimate the effective population size for the Rocky Mountain tailed frog and the number of migrants exchanged between the northern and southern clades using a coalescent-based, divergence by isolation with migration model implemented by MDIV (Nielsen and Wakeley, 2001). Based on this model (and assuming a mutation rate of 1×10^{-7}, used in Carstens et al., 2005), the Rocky Mountain tailed frog is represented by an effective population size of 51,436 females (95 percent credibility interval extending from 22,118 to 92,584). The census number of adults would be much higher than this estimate of effective population size for several reasons. First, a maximum of half of the female population breeds each year, such that the number of females in the population is at least twice the effective population size (Daugherty and Sheldon, 1982*a*). Furthermore, the model assumes low variance in reproductive success, a stable population size, and no assortative mating; violations of these assumptions could certainly result in a census population an order of magnitude greater than the effective population size. We suspect that reproductive success varies widely among years and between streams in response to the magnitude and timing of flow events (Lohman, 2002; Metter, 1968), but strong documentation for this is lacking. Similarly, there have likely been fluctuations in population size in response to changes in climatic conditions and habitat availability. Unfortunately, evaluating our estimate of effective population size in relation to an estimate of the actual number of tailed frogs in the northern Rockies is hampered, as it is for most amphibians, by the general scarcity of population data. A few studies have focused on defining the range of *A. montanus* in a given area (e.g., Dupuis et al., 2000; Marnell, 1997). A number of studies have estimated larval densities for tailed frogs (Dupuis and Stevenson, 1999; Hawkins et al, 1988; Lohman, 2002), but estimates of adult numbers have been rare. Daugherty and Sheldon (1982*a*) marked 543 individuals in an 80 m section of Butler Creek (Montana) and Metter (1964) reported capture rates as high as 0.4–1.1 individuals/m in sections of streams in the Touchet River (Washington) and Palouse River (Idaho) drainages. Although these estimates do suggest that tailed frogs can be locally abundant, an estimate of population size will require a much more comprehensive understanding of densities across the range.

Based on MDIV results 10 migrants are exchanged between the northern and southern clades each year (95% credibility interval 6–35). This estimate appears reasonable based on the high degree of philopatry that has been observed in the Rocky Mountain tailed frog (Adams and Frissell, 2001; Daugherty and Sheldon, 1982*b*) and the relatively fragmented nature of mesic forests along the zone of contact between these clades. This estimate of exchange is consistent with the significant pairwise F_{ST} values found in this species.

Based on minimal recaptures of newly metamorphosed frogs (Daugherty and Sheldon, 1982*b*), some uncertainty has existed regarding long-distance dispersal in juvenile tailed frogs. Both genetic markers confirm that long-range dispersal either does not occur within this juvenile cohort or does not contribute significantly to genetic exchange.

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APPENDIX I

Collection localities. Population numbers correspond to those from Figures 1, 2 and 4, and where applicable, to collection localities given in Nielson et al. (2001). Populations 1a and 1b and 12a and 12b were considered single populations in Nielson et al. (2001). Populations are marked in column D for mtDNA data and column A for allozyme data.

Population #	Data		County	Mountain Range	Watershed	Stream
	D	A				
1a	X		Idaho. Latah Co.	Palouse Mountains	Palouse R.	Mountain Gulch
1b	X		Idaho. Latah Co.	Palouse Mountains	Palouse R.	Mannering Cr.
2	X		Idaho. Idaho Co.	Clearwater Mts.	Lochsa R.	Indian Post Office Cr.
3	X		Idaho. Clearwater Co.	Clearwater Mts.	Little N. Fk. Clearwater	Unnamed Tributary
4	X		Idaho. Idaho Co.	Clearwater Mts.	S. Fk Clearwater	Legett Cr.
5	X	X	Montana. Missoula Co.	Bitterroot Mts.	Clark Fk. R.	East Fork Lolo Cr.
6	X		Idaho. Nez Perce Co.	Craig Mt.	Snake R.	Eagle Cr.
7	X		Montana. Flathead Co.	Livingston Mts.	Middle Fk. Flathead R.	Lower Rubridge Cr.
8	X		Montana. Flathead Co.	Livingston Mts.	Middle Fk. Flathead R.	Autumn Cr.
9	X		Montana. Flathead Co.	Livingston Mts.	Middle Fk. Flathead R.	Upper Fern Cr.
10	X		Montana. Glacier Co.	Livingston Mts.	St. Mary R.	Reynolds Cr.
11	X		Idaho. Valley Co.	Salmon River Mts.	Sesech R.	Maverick Cr.
12a	X		Idaho. Valley Co.	Salmon River Mts.	East Fk. of the S. Fk. Salmon R.	Parks Cr.
12b	X		Idaho. Valley Co.	Salmon River Mts.	East Fk. of the S. Fk. Salmon R.	Reegan Cr.
13	X		Idaho. Idaho Co.	Salmon River Mts.	Salmon R.	Slate Cr.
14	X	X	Washington. Columbia Co.	Blue Mts.	Touchet R.	Headwaters Touchet R.
15	X	X	Oregon. Josephine Co.	Siskiyou Mts.	Illinois R.	Sucker Cr.
16	X		Oregon. Josephine Co.	Siskiyou Mts.	Illinois R.	Lost Canyon Cr.
17	X	X	Oregon. Benton Co.	Oregon Coast Mts.	Alsea R.	Parker Cr.

APPENDIX I
Continued.

Population #	Data		County	Mountain Range	Watershed	Stream
18	X	X	Washington. Mason Co.	Olympic Mts.	Hamma Hamma R.	Jefferson Cr.
19	X		Washington. Clallam Co.	Olympic Mts.	Drains to Pacific Ocean	Ennis Cr.
20	X		Washington. Skagit Co.	Cascade Mts.	Skagit R.	Happy Cr.
21	X		Washington. Chelan Co.	Cascade Mts.	Stehekin R.	Bridge Cr.
22	X		Washington. Skagit Co.	Cascade Mts.	Stehekin R.	McAllister Cr.
23	X	X	Washington. Chelan Co.	Cascade Mts.	Wenatchee R.	Smith Br.
24	X		Idaho. Valley Co.	Salmon River Mts.	E. Fk. of the S. Fk. Salmon R.	Moose Cr.
25	X		Idaho. Valley Co.	Salmon River Mts.	E. Fk. of the S. Fk. Salmon R.	Sheep Cr.
26	X		Idaho. Valley Co.	Salmon River Mts.	Middle Fk. Payette R.	Unnamed headwater tributary
27	X		Idaho. Valley Co.	Salmon River Mts.	N. Fk. Payette R.	Clear Cr.
28	X		Idaho. Valley Co.	Salmon River Mts.	S. Fk. of the Salmon R.	Curtis Cr.
29	X		Idaho. Custer Co.	Sawtooth Range	Yankee Fk. of the Salmon R.	Five Mile Cr.
30	X		Idaho. Valley Co.	Salmon River Mts.	S. Fk. of the Salmon R.	Four Mile Cr.
31	X		Idaho. Valley Co.	Salmon River Mts.	S. Fk. of the Salmon R.	Buckhorn Cr.
32	X	X	Idaho. Elmore Co.	Boise Mts.	Middle Fk. Boise R.	Unnamed tributary near Rocky Bar
33	X	X	Montana. Beaverhead Co.	Pioneer Mts.	Big Hole R.	Steele Cr.
34	X	X	Idaho. Washington Co.	Seven Devils Mts.	Snake R.	Brownlee Cr.
35	X	X	Oregon. Wallowa Co.	Wallowa Mts.	Imnaha R.	Lick Cr.
36		X	Montana. Mineral Co.	Bitterroot Mts.	Clark Fk. R.	Oregon Falls Cr.
37		X	Montana. Glacier Co.	Livingston Mts.	Flathead R.	Sprague Cr.
38		X	Washington. Garfield Co.	Blue Mts.	Snake R.	Tucannon R.
39		X	Oregon. Baker Co.	Wallowa Mts.	Powder R.	O'Brien Cr.
40		X	Oregon. Wallowa Co.	Wallowa Mts.	Grand Ronde R.	Lostine R.
41		X	Idaho. Shoshone Co.	Clearwater Mts.	St. Joe R.	Bird Cr.
42		X	Idaho. Valley Co.	Salmon River Mts.	S. Fk. Salmon	Dollar Cr.
43		X	Montana. Missoula Co.	Bitterroot Mts.	Clark Fk. R.	Butler Cr.
44		X	Washington. Skamania Co.	Cascade Mts.	White Salmon R.	Mosquito Cr.
45		X	Washington. Kittitas. Co.	Cascade Mts.	Keechelus Lk.	Hyak Cr.
46		X	Washington. Skagit Co.	Cascade Mts.	S. Fk. Sauk R.	Weden Cr.
47		X	Washington. Cowlitz Co.	Cascade Mts.	Toutle R.	Coldwater Cr.
48		X	Washington. Kittitas Co.	Cascade Mts.	S. Fk. Snoqualmie R.	Surveyor Cr.
49		X	Washington. Cowlitz Co.	Cascade Mts.	Toutle R.	Elk Cr.
50		X	Washington. Grays Harbor Co.	Olympic Mts.	Humptulips R.	W. Fk. Humptulips R.
51		X	Washington. Grays Harbor Co.	Olympic Mts.	Quinault R.	Fletcher Canyon
52		X	Washington. Grays Harbor Co.	Olympic Mts.	Quinault R.	Merriman Cr.
53		X	Oregon. Linn Co.	Cascade Mts.	Lost Lk.	Hackleman Cr.
54		X	Oregon. Douglas Co.	Cascade Mts.	Umpqua R.	Bear Cr.
55		X	Oregon. Douglas/Jackson Co. line	Cascade Mts.	Rogue R.	Bert Cr.
56		X	Oregon. Lane Co.	Cascade Mts.	S. Fk. McKenzie R.	Starr Cr.
57		X	California. Humboldt Co.	Coast Mts.		Redwood Cr.
58		X	Oregon. Tillamook Co.	Coast Mts.	Nestucca R.	Mt. Hebo
59		X	Washington. Glacier Co.	Cascades Mts.	Nooksack R.	Boyd Cr.

APPENDIX II

Summary of electrophoretic conditions of the allozyme loci included in this study. The following abbreviations are used for the tissues: L = liver, M = muscle, T = tadpole.

Enzyme System	Locus Designation	Enzyme Commission Number	Buffer System[1]	Tissue	Alleles identified
Asparatate aminotransferase	*Aat-1*	2.6.1.1	A	M, T	*Aat-1* (a, b)
	Aat-2		A	M, T	*Aat-2* (a, b, c, d)
a-Glycerophosphate dehydrogenase	*Agp-1*	1.1.1.8	C	M, T	*Agp-1* (a, b)
B-glucuronidase	*Gus*	3.2.2.31	B	L, T	*Gus* (a, b, c)
Isocitrate dehydrogenase	*Idh-1*	1.1.1.42	C	M, T	*Idh-1* (a, b)
	Idh-2		B	L, T	*Idh-2* (a, b)
Leucine aminopeptidase	*Lap*	3.4.11.1	A	M, T	*Lap* (a, b)
Lactate dehydrogenase	*Ldh-2*	1.1.1.27	C	L, T	*Ldh-2* (a, b)
Malate dehydrogenase	*Mdh-2*	1.1.1.37	B	L, T	*Mdh-2* (a, b, c, d)
Peptidase	*Pep-2*	3.4.–.–	A	L, T	*Pep-2* (a, b)
Phosphoglucose isomerase	*Pgi-1*	5.3.1.9	A	L, T	*Pgi-1* (a, b, c, d)
Phosphoglucomutase	*Pgm-2*	5.4.2.2	A	M, T	*Pgm-2* (a, b, c, d, e, f)
Phosphomannose isomerase	*Pmi-2*	5.3.1.8	A	M, T	*Pmi-2* (a, b, c, d, e, f, g)
Superoxide dismutase	*Sod*	1.15.1.1	A	L, T	*Sod* (a, b, c, d)

[1] *Buffer A* (Ridgway et al., 1970) gel: 0.03 M Tris, 0.005 M citric acid, pH 8.5; electrode: 0.06 M lithium hydroxide, 0.3 M boric acid, pH 8.1; gels were made using 99% gel buffer and 1% electrode buffer; *Buffer B* (Clayton and Tretiak, 1972) gel: 0.002 M citric acid, pH 6.0; electrode: 0.04 M citric acid, pH 6.1; both buffers are pH adjusted with N-(3-Aminopropyl)-Morpholine; *Buffer C* (Siciliano and Shaw, 1976) gel: 0.009 M Tris, 0.003 M citric acid, pH 7.0. Gels were made using 14% starch in the appropriate gel buffer.

Appendix III

Pairwise F_{ST} values for all populations sampled for allozymes. Population numbers correspond with those in Fig. 1 and Appendix I. Significant F_{ST} values shown in bold.

	5	14	15	17	18	23	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58
5																																	
14	**0.61**																																
15	**0.78**	**0.94**																															
17	**0.69**	**0.84**	**0.59**																														
18	**0.76**	**0.87**	**0.63**	**0.64**																													
23	**0.84**	**0.98**	**0.93**	**0.47**	**0.82**																												
32	**0.35**	**0.75**	**0.8**	**0.62**	**0.69**	**0.84**																											
33	**0.44**	**0.86**	**0.91**	**0.81**	**0.84**	**0.95**	**0.64**																										
34	**0.67**	**0.95**	**0.97**	**0.89**	**0.9**	**1**	**0.84**	**0.72**																									
35	**0.4**	**0.84**	**0.95**	**0.86**	**0.89**	**0.98**	**0.75**	**0.73**	**0.79**																								
36	**0.06**	**0.68**	**0.83**	**0.71**	**0.78**	**0.88**	**0.28**	**0.47**	**0.79**	**0.61**																							
37	**0.37**	**0.44**	**0.84**	**0.73**	**0.8**	**0.87**	**0.45**	**0.55**	**0.77**	**0.65**	**0.28**																						
38	**0.57**	0.06	**0.95**	**0.83**	**0.86**	**1**	**0.73**	**0.88**	**1**	**0.9**	**0.66**	**0.43**																					
39	**0.54**	**0.9**	**0.9**	**0.7**	**0.78**	**0.98**	**0.57**	**0.81**	**0.98**	**0.91**	**0.59**	**0.62**	**0.95**																				
40	**0.57**	**0.92**	**0.91**	**0.71**	**0.78**	**1**	**0.6**	**0.83**	**1**	**0.94**	**0.62**	**0.63**	**1**	0.07																			
41	**0.06**	**0.62**	**0.78**	**0.66**	**0.76**	**0.83**	**0.25**	**0.44**	**0.72**	**0.53**	0	**0.29**	**0.59**	**0.54**	**0.56**																		
42	**0.37**	**0.7**	**0.74**	**0.58**	**0.72**	**0.8**	**0.07**	**0.63**	**0.81**	**0.72**	**0.32**	**0.44**	**0.67**	**0.47**	**0.49**	**0.3**																	
43	**0.06**	**0.71**	**0.85**	**0.75**	**0.81**	**0.89**	**0.43**	**0.35**	**0.71**	**0.52**	**0.07**	**0.36**	**0.7**	**0.65**	**0.68**	**0.08**	**0.46**																
44	**0.81**	**0.94**	**0.85**	**0.4**	**0.79**	**0.07**	**0.8**	**0.91**	**0.96**	**0.94**	**0.85**	**0.85**	**0.94**	**0.89**	**0.9**	**0.81**	**0.77**	**0.86**															
45	**0.67**	**0.94**	**0.79**	−0.15	**0.65**	**0.17**	**0.65**	**0.87**	**0.99**	**0.95**	**0.72**	**0.74**	**0.98**	**0.86**	**0.92**	**0.65**	**0.58**	**0.77**	0.03														
46	**0.83**	**0.97**	**0.92**	**0.46**	**0.82**	0	**0.84**	**0.95**	**1**	**0.98**	**0.88**	**0.87**	**1**	**0.97**	**0.99**	**0.83**	**0.8**	**0.89**	**0.06**	**0.09**													

APPENDIX III
Continued.

	5	14	15	17	18	23	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58
47	0.81	0.95	0.88	0.41	0.79	0.07	0.8	0.92	0.97	0.95	0.85	0.85	0.96	0.91	0.93	0.8	0.77	0.86	0.06	0.77	0.06												
48	0.74	0.95	0.86	0.35	0.74	0.28	0.75	0.89	0.98	0.96	0.79	0.8	0.97	0.89	0.91	0.74	0.7	0.82	0.1	−0.02	0.23	0.11											
49	0.76	0.95	0.85	0.34	0.74	0.17	0.76	0.9	0.98	0.96	0.8	0.81	0.97	0.9	0.93	0.75	0.71	0.83	−0.01	0.04	0.14	0.08	0.1										
50	0.74	0.93	0.71	0.68	0.14	0.94	0.72	0.86	0.97	0.95	0.77	0.8	0.93	0.81	0.79	0.74	0.72	0.81	0.86	0.7	0.93	0.88	0.78	0.81									
51	0.72	0.88	0.69	0.58	0.11	0.85	0.63	0.82	0.92	0.9	0.73	0.77	0.87	0.75	0.74	0.71	0.68	0.78	0.79	0.58	0.84	0.79	0.7	0.72	0.17								
52	0.7	0.81	0.56	0.56	0.06	0.74	0.61	0.78	0.84	0.82	0.71	0.74	0.79	0.69	0.69	0.69	0.66	0.74	0.72	0.55	0.74	0.71	0.66	0.66	−0.12	0.01							
53	0.71	0.87	0.63	0.04	0.63	0.64	0.64	0.84	0.92	0.89	0.73	0.75	0.87	0.75	0.76	0.68	0.6	0.77	0.55	0.06	0.63	0.56	0.5	0.5	0.7	0.58	0.54						
54	0.7	0.86	0.71	0.49	0.71	0.71	0.65	0.82	0.9	0.87	0.72	0.75	0.84	0.72	0.73	0.69	0.6	0.76	0.64	0.49	0.7	0.65	0.58	0.57	0.72	0.68	0.65	0.54					
55	0.69	0.88	0.54	0.53	0.65	0.82	0.66	0.85	0.92	0.89	0.73	0.76	0.87	0.77	0.78	0.68	0.58	0.77	0.74	0.63	0.82	0.77	0.72	0.7	0.69	0.67	0.6	0.57	0.23				
56	0.69	0.9	0.71	−0.01	0.66	0.59	0.65	0.85	0.95	0.92	0.73	0.75	0.91	0.79	0.8	0.67	0.59	0.78	0.42	−0.15	0.58	0.44	0.38	0.38	0.72	0.6	0.56	0.06	0.51	0.59			
57	0.72	0.95	0.26	0.54	0.55	0.97	0.74	0.89	0.99	0.96	0.77	0.79	0.98	0.89	0.92	0.72	0.67	0.81	0.84	0.76	0.96	0.88	0.82	0.84	0.52	0.57	0.5	0.58	0.65	0.5	0.65		
58	0.72	0.94	0.81	0.25	0.68	0.48	0.72	0.88	0.99	0.96	0.76	0.78	0.97	0.87	0.9	0.71	0.66	0.81	0.02	−0.06	0.41	0.18	0.14	0	0.68	0.63	0.6	0.41	0.51	0.63	0.26	0.73	
59	0.84	0.98	0.93	0.47	0.82	0	0.84	0.95	1	0.98	0.88	0.87	1	0.98	1	0.83	0.8	0.89	0.07	0.17	0	0.07	0.28	0.17	0.94	0.85	0.74	0.64	0.71	0.82	0.59	0.97	0.48