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PHYLOGEOGRAPHY OF THE TAILED FROG (*ASCAPHUS TRUEI*): IMPLICATIONS FOR THE BIOGEOGRAPHY OF THE PACIFIC NORTHWEST

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Abstract.—Tailed frogs are distributed in high-gradient streams within the disjunct mesic forests of the Pacific Northwest and represent the basal lineage of the anurans. We sequenced 1530 nucleotides of the mitochondrial cytochrome *b* and NADH dehydrogenase subunit two genes from 23 populations and used parsimony, maximum-likelihood, and nested-clade analyses to estimate relationships among populations and infer evolutionary processes. We found two divergent haplotype clades corresponding with inland Rocky Mountain populations and coastal populations and separated by up to 0.133 substitutions per site. Within the coastal assemblage, haplotypes formed clades by mountain range with 0.010–0.024 substitutions per site divergence among populations. Inland haplotypes exhibited minimal genetic structure, with the exception of 0.021 substitutions per site distance between populations from the East Fork of the South Fork of the Salmon River and all other inland haplotypes. The magnitude of divergence between inland and coastal populations, as well as the paleobotanical record, suggest isolation of these lineages occurred during the late Miocene to early Pliocene, probably in response to the rise of the Cascade Mountains. Genetic structure within coastal and inland populations is consistent with isolation in refugia during the late Pliocene and early Pleistocene. Closely related inland haplotypes reflect range expansion following glaciation. The depth of divergence between inland and coastal populations supports the persistence of mesic forests within the inland Pacific Northwest throughout the Pleistocene and is congruent with patterns found in several other mesic forest species. Based on mitochondrial divergence and previous allozyme and morphological data, we recommend recognition of inland populations as a distinct species, *Ascaphus montanus.*

Key words.—*Ascaphus truei,* biogeography, maximum likelihood, mesic forests, nested clade, Pacific Northwest.

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Regional phylogeography examines the effects of geographic history on genetic variation and thus is essential to understanding evolutionary processes (reviewed in Avise 1994). Comparing the phylogenies of codistributed taxa provides insight into how factors such as population structure and life history influence the genetic signature left by geographic events. Beyond descriptive biogeography, understanding the factors that affect the evolution of taxa in a region allows for the development of testable predictions about patterns of genetic diversity. In turn, regional phylogeography provides insight into the response of biotic communities to historic events (e.g., Sullivan et al. 2000).

The Pacific Northwest region of North America (40–52° N, 113–126° W) provides abundant opportunities to examine phylogenetic patterns in the context of historic geology and climate. The region has experienced dramatic geological events including the uplift of several mountain ranges, multiple glaciations, and scouring postglacial floods (Alt and Hyndman 1995). Furthermore, biotic communities in this region range from temperate rain forest to xeric sagebrush steppe (Daubenmire 1975). In this paper, we begin to address regional phylogeography in the Pacific Northwest by examining patterns of molecular variation in the tailed frog, *Ascaphus truei.*

This species represents an excellent starting point for the examination of regional phylogeography in the Pacific Northwest for several reasons. First, *Ascaphus* inhabits forested headwater streams in the coastal ranges and Cascade Mountains from British Columbia south to northern California. Disjunct populations occur in the northern Rocky, Blue, Wallowa, and Seven Devils Mountains (Metter and Pauken 1969; Nussbaum et al. 1983; Green and Campbell 1984; Fig. 1). Although local distribution depends on a number of variables, *Ascaphus* populations are sensitive to the increased siltation and water temperatures that may accompany timber harvest; thus, they are found most often in old growth reaches (Bury 1983; Corn and Bury 1989; Welsh 1990; Walls et al. 1992). 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 (Bury 1983; Corn and Bury 1989; Welsh 1990; Walls et al. 1992; Blaustein et al. 1994).

Second, several ecological characteristics of *Ascaphus* may influence genetic variation. Savage (1960, 1973) suggested that the progenitors of tailed frogs inhabited the North American continent by the Jurassic, thus genetic structure within this species may reflect ancient events. In addition, strong geographic structure might be expected because these frogs are restricted to stream habitats and have limited dispersal abilities (Daugherty 1979). Conversely, a long generation time (6–8 years) and low metabolic rate may result in a slow evolutionary rate and therefore reduced genetic divergence (Kocher et al. 1989; Martin and Palumbi 1993; Rand 1994).

Third, the tailed frog is among 156 species, including bryophytes, fungi, vascular plants, worms, insects, and vertebrates, endemic to the Pacific Northwest that share this disjunct distribution in association with mesic coniferous forests (compiled from McCune 1984; Johnson 1987; Lorrain 1988). Numerous authors (e.g., FAUNMAP Working Group 1996; Avise et al. 1998; Bernatchez and Wilson 1998) have focused on Pleistocene glaciations and their effect on the fauna of North America, however, earlier events undoubtedly influenced intraspecific patterns (Riddle 1996a,b; Zink 1996; Waits et al. 1998). Savage (1960, 1973) included tailed frogs

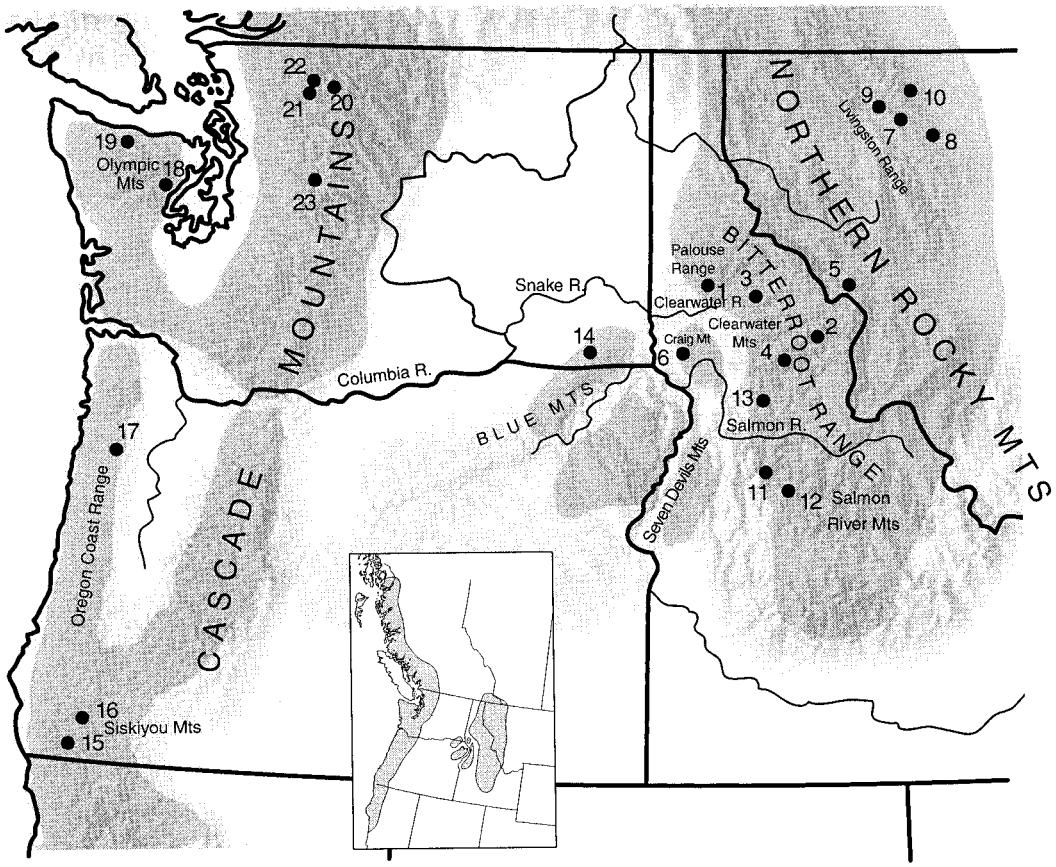

FIG. 1. Collection localities for *Ascaphus truei* (range shown within inset map; modified from Metter and Pauken 1969). Sites are as follows: 1, Mountain Gulch; 2, Indian Post Office Creek; 3, unnamed tributary, Little North Fork Clearwater River; 4, Legett Creek; 5, East Fork Lolo Creek; 6, Eagle Creek; 7, Lower Rubridge Creek; 8, Autumn Creek; 9, Upper Fern Creek; 10, Reynolds Creek; 11, Maverick Creek; 12, Parks and Reegan Creeks; 13, Slate Creek; 14, Touchet River; 15, Sucker Creek; 16, Lost Canyon Creek; 17, Parker Creek; 18, Lena Creek; 19, Ennis Creek; 20, Happy Creek; 21, Bridge Creek; 22, McAllister Creek; 23, Smith Brook.

as members of the ''old northern element'' and postulated that this element evolved in close association with the mesic flora of the Miocene, responding in concert as the range of this flora contracted in response to geologic events. Others have agreed that elements of the mesic forest community may have responded similarly to changes in climate (Johnson 1987; Lorraine 1988).

In addition to phylogeographic considerations, *Ascaphus* represents the monotypic basal lineage of anurans (Green et al. 1980; Cannatella and Hillis 1993; Ford and Cannatella 1993; but see Hay et al. 1995). It is characterized by the retention of many primitive morphological features (e.g., ascending process of the palatoquadrate, braincase articulating to the palatoquadrate through a basal process, root of facial nerve exiting braincase via anterior acoustic foramen into auditory capsule, and short arms on the sternum) that have been lost in all other extant anuran lineages (Ford and Cannatella 1993). Along with these ancestral characters, tailed frogs possess uniquely derived adaptations for life in cold, high-gradient streams. *Ascaphus* is the only North American frog that practices internal fertilization. Breeding occurs in late summer and fall, but egg deposition is delayed until the cessation of high flows the following summer, thus minimizing egg loss (Metter 1964). Moreover, larval mouth parts form a suction disc that anchors tadpoles firmly to the substrate and prevents them from being flushed downstream during their 1–3-year larval period (Metter 1964).

Based on multivariate analysis of morphological characters, Metter and Pauken (1969) and Pauken and Metter (1971) distinguished between western and disjunct inland populations. In addition, they hypothesized that these disjunct segments were most recently connected at the close of the Pleistocene by gene flow through the highlands of central Oregon. Daugherty (1979) examined allele frequencies at 16 allozyme loci in seven *Ascaphus* populations and found populations to cluster according to the geographic relationships of their respective mountain ranges. Differences in allele frequencies at two loci led Daugherty (1979) to hypothesize that coastal and inland populations represented independent and long-isolated gene pools.

More powerful methods now exist to estimate intraspecific relationships using DNA sequence data (Avise 1994). Examination of the relationships among tailed frog populations in the context of their disjunct mesic forest community may provide insight into the biogeographic and evolutionary processes acting in the Pacific Northwest. We used sequences from the mitochondrial cytochrome *b* (cyt *b*) and NADH dehydrogenase subunit 2 (ND2) genes to examine genetic

TABLE 1. Collection localities for *Ascaphus truei* larvae by mountain range and river system. Population numbers correspond with those from Figure 1. Number of cytochrome *b* sequences is followed by the number of NADH dehydrogenase subunit 2 sequences in parentheses. Haplotype letters correspond with those in Figure 2. The number in the parentheses following the haplotype letter indicates the number of that haplotype represented in the sample.

Pop	Range	Drainage	Stream	Number sequenced	Haplotypes
1	Palouse	Palouse R.	Mountain Gulch	7(2)	A(4), C(3)
2	Clearwater	Lochsa R.	Indian Post Office Cr.	2(1)	A(1), B(1)
3	Clearwater	Little N. Fork Clearwater R.	Unnamed creek	2(2)	A(1), J(1)
4	Clearwater	S. Fork Clearwater R.	Legett Cr.	5(5)	AA(1), BB(1), CC(1), DD(2)
5	Bitterroot	Clark Fork R.	East Fork Lolo Cr.	5(0)	A(3), I(1), E(1)
6	Craig Mts.	Snake R.	Eagle Cr.	5(2)	A(2), D(3)
7	Livingston	Middle Fork Flathead R.	Lower Rubridge Cr.	2(2)	E(1), F(1)
8	Livingston	Middle Fork Flathead R.	Autumn Cr.	2(2)	E(2)
9	Livingston	Middle Fork Flathead R.	Upper Fern Cr.	2(2)	E(2)
10	Livingston	St. Mary R.	Reynolds Cr.	1(1)	G(1)
11	Salmon R. Mts.	Sesech R.	Maverick Cr.	6(2)	A(4), P(1), Q(1)
12	Salmon R. Mts.	E. Fork of the S. Fork	Parks and Reegan Cr.	5(3)	O(3), M(1), N(1)
13	Salmon R. Mts.	Salmon R.	Slate Cr.	5(5)	A(5)
14	Blue Mts.	Touchet R.	Headwaters Touchet R.	7(2)	K(5), L(2)
15	Siskiyou Mts.	Illinois R.	Sucker Cr.	1(1)	R(1)
16	Siskiyou Mts.	Illinois R.	Lost Canyon Cr.	3(3)	R(1), S(1), T(1)
17	Oregon Coast	Alsea R.	Parker Cr.	5(3)	U(3), V(2)
18	Olympic Mts.	Hamma Hamma R.	Lena Cr.	1(1)	W(1)
19	Olympic Mts.	Ennis Cr.	Ennis Cr.	4(4)	W(2), X(1), Y(1)
20	Northern Cascades	Skagit R.	Happy Cr.	2(2)	Z(2)
21	Northern Cascades	Stehekin R.	Bridge Cr.	2(1)	Z(2)
22	Northern Cascades	Stehekin R.	McAllister Cr.	1(0)	Z(1)
23	Northern Cascades	Wenatchee R.	Smith Brook	5(2)	Z(4), H(1)

structure within *Ascaphus*, estimate the mitochondrial DNA (mtDNA) phylogeny, and infer the geological and ecological processes that may have structured genetic variation. We interpreted the relationships inferred among populations within the framework of conservation, taxonomy, and biogeography.

METHODS

Data Collection

We collected 80 larval tailed frogs from 23 sites across the range of *Ascaphus* (Table 1, Fig. 1) and extracted DNA from tail clips (10–20 mg) using the Puregene kit (Gentra Systems, Inc., Minneapolis, MN). We subsequently cleaned samples on glass beads to remove residual polymerase-chain-reaction (PCR) inhibitors (BIO 101, Inc., Vista, CA).

The mitochondrial cyt *b* and ND2 genes have been used to interpret phylogenetic relationships both within species and between closely related species for a variety of taxa (e.g., Moritz et al. 1992; Bernardi and Powers 1995; Schmidt and Gold 1995; Sullivan et al. 1997; Demboski et al. 1998). We sequenced approximately 730 nucleotides from the cyt *b* gene and 800 nucleotides from the ND2 gene. Cyt *b* was initially amplified and sequenced using primers L14115 and H14963 from Sullivan et al. (1997). We designed an *Ascaphus*-specific primer (SUV 5′-AGGGGGAGTAACTAGGGGGTTGGCTGGC-3′) to replace H14963 in subsequent amplifications. We used primers L4437 and H5934 from Macey et al. (1997) to amplify and sequence ND2, and subsequently designed an *Ascaphus*-specific primer (ND2C 5′-TTGTAGGTGAGTCGGAGGTA-3′) to replace the heavy-strand primer. Double-stranded PCR products were precipitated using polyethylene glycol as a carrier, and 15–45 ng of PCR product were used as template for 10-μl cycle sequencing reactions with the BigDye Kit (Applied Biosystems, Inc., Palo Alto, CA). We used CentriSep columns (Princeton Separations, Inc., Adelphia, NJ) to clean sequencing reactions and ran them on an ABI 377 automated sequencer using 4% Long Ranger (FMC BioProducts, Rockland, ME) gels. Ninety percent of the data for both ND2 and cyt *b* were sequenced either in both directions or were within 200 bp of the sequencing primers. Cyt *b* and ND2 sequences are deposited in the GenBank database under accession numbers AF277324–AF277352 and AF277353–AF277370, respectively.

Data Analysis

We conducted two complimentary sets of analyses. To examine deep phylogenetic structure, we estimated the phylogeny using both parsimony and maximum-likelihood (ML) criteria. In addition, we used nested-clade analyses to examine shallow genetic structure and infer population processes that may have influenced genetic structure. We aligned and edited sequences using Sequencher (GeneCodes, Inc., Ann Arbor, MI), and estimated phylogenies using PAUP* (vers. 4.0ba; Swofford 1998). We performed identical analyses with cyt *b*, ND2, and the combined datasets. We used GeoDis 2.0 (Posada et al. 2000) to conduct nested-clade analysis on the combined dataset. In addition, we used Arlequin 2.0 (Schneider et al. 2000) to conduct an analysis of molecular variance (AMOVA; Excoffier et al. 1992) and test for the significance of genetic differentiation among populations.

Phylogenetic analyses

We conducted phylogenetic analyses under ML and parsimony criteria. The explicit model-based approach of ML is consistent under a wide range of conditions (e.g., Huelsenbeck 1995), however, the computational demands make

simultaneous optimization of all model parameters on each tree impractical during a search. Therefore, we used a successive-approximations approach in which parsimony analyses provided initial topologies for subsequent evaluation of ML models and estimation of model parameters (Swofford et al. 1996; Sullivan and Swofford 1997). We arbitrarily chose tree 5 from 10 optimal trees saved during parsimony searches (equal weights, heuristic search, 100 random stepwise addition replicates, TBR branch swapping), and held this topology constant to assess the relative fit of 16 alternative models of sequence evolution using likelihood ratio tests. Our models are identical to those used in Sullivan et al. (1997; GTR + I + Γ, GTR + I, GTR + Γ, GTR, HKY + I + Γ, HKY + I, HKY + Γ, HKY85, K2P + I + Γ, K2P + I, K2P + Γ, K2P, JC + I + Γ, JC + I, JC + Γ, JC). Explicit assumptions of each model are given in Sullivan and Swofford (1997). A justification for using the χ^2 approximation of the null distribution is given in Yang et al. (1995; but see Whelan and Goldman 1999).

We then performed ML searches (heuristic search with 10 random stepwise addition trees, TBR branch swapping) under the fully defined, best-fit model. We conducted analyses of cyt *b* sequences both with and without *Xenopus laevis* (Roe et al. 1985; GenBank ascession number NC001573.1) and *Ensatina eschscholtzii* (Moritz et al. 1992; GenBank accession number L75804) as outgroups (see below). We reoptimized model parameters on the ML trees, and used the likelihood-ratio test (Felsenstein 1988) to compare the scores of unconstrained trees with topologies constrained to fit the molecular clock. We estimated nodal support under both parsimony and ML criteria using bootstrap analysis (Felsenstein 1985). For parsimony bootstrap analysis, we used 500 replicates with equal weights (stepwise-addition, TBR branch swapping). Because of computational demands, we limited the ML bootstrap analyses to 100 replicates, with a maximum of one tree in each replicate (i.e., MAXTREES = 1) and used nearest-neighbor interchange branch swapping. This method has been shown to produce estimates of bootstrap support indistinguishable from those analyses in which multiple trees are held at each step (DeBry and Olmstead 2000).

Nested-clade analyses

We used nested-clade analysis (Templeton et al. 1995; Templeton 1998) to test the significance of haplotype association with geographic area and to infer population processes. We restricted our analysis to the combined dataset; the numbers of individuals with each haplotype were taken from the cyt *b* dataset. We implemented the formula given in Templeton et al. (1992) using ParsProb (Posada et al. 2000) to determine the maximum number of substitutional steps with 95% or greater probability of a parsimonious connection between two haplotypes. An unrooted minimum-spanning network was constructed from the matrix of absolute character differences and partitioned into nested clades (Templeton et al. 1987; see Fig. 3). Ambiguities in the network were resolved using guidelines provided in Templeton and Sing (1993). Although a minimum-spanning network was created for all unique haplotypes, we confined our analysis to inland populations because limited sampling among coastal populations restricted the inferences we could make. We used GeoDis (Posada et al. 2000) to perform the following analyses. We determined the geographic center of each clade by calculating a weighted average of latitude and longitude for all individuals within that clade. We then calculated the average geographic distance of the haplotypes from the center of each clade (D_c), and determined the average distances of the haplotypes from the geographic center of the next most inclusive clade in the nesting hierarchy (D_n). In addition, we determined the contrast in D_c and D_n between tip clades and clades representing interior nodes in the network (abbreviated I − T) to determine the dispersion of recent haplotypes relative to older ones (Castelloe and Templeton 1994). We then tested for the significant association of haplotype clades with locality by using a categorical permutational contingency analysis, as well as the more complex analysis described in Templeton et al. (1995) that incorporates geographic distance. One thousand permutations of the haplotype network were conducted to provide 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. Significance was determined at the 5% level using the null distribution of random association of haplotypes with geography. We used the key provided in Templeton (1998) to interpret significant patterns.

Ensatina and *Xenopus* were the only amphibians with cyt *b* and ND2 sequences available on GenBank. However, a Blast search of the GenBank database indicated that *Ascaphus* sequences were no more similar to these sequences than to mammal sequences. Thus, available outgroups are too divergent from tailed frogs to provide a reliable root.

RESULTS

Cyt b Phylogeny

We identified 28 *Ascaphus* haplotypes with up to 7.8% uncorrected sequence divergence. The shortest parsimony trees measured 103 steps (consistency index [CI] = 0.864; retention index [RI] = 0.971; rescaled consistency index [RC] = 0.839) with 84 sites observed to vary and 70 parsimony-informative sites. Six percent of first-codon positions, 3% of second-codon positions, and 26% of third-codon positions were observed to vary. As expected, the GTR + I + Γ model had the best likelihood score (ln L = −1606.252). This model allows a unique instantaneous relative rate for each of the six reversible substitution types and for variable (yet stationary) base frequencies and uses a mixed distribution model to account for rate heterogeneity among sites. However, the simpler HKY + Γ model, in which all transitions have the same instantaneous rate of substitution and all transversions have the same instantaneous rate of substitution, was the simplest model that we could not reject (lnL = −1610.062; $\chi^2_5 = 7.62$, $P > 0.1$). We could reject all simpler models ($P < 0.001$). The most likely unrooted topology was not significantly different when constrained to fit the molecular clock ($\chi^2_{29} = 27.54$, $P > 0.5$), thus the ML tree was rooted at the midpoint. Parameter estimates for the HKY + Γ model and midpoint-rooted ML topology are given in Table 2.

TABLE 2. Parameter estimates under selected models. Parameters were optimized under midpoint-rooted topologies. T_i/T_v represents the rate ratio between transitions and transversions. The substitution rate between nucleotides is represented by r_{XY}. Substitution rates are relative with r_{GT} set to one. π_i represents the estimated base frequency under the model. The proportion of invariable sites is given by p_{inv}, and α represents the shape parameter of the gamma distribution. Cyt *b*, cytochrome *b*; ND2, NADH dehydrogenase subunit 2.

Dataset:	Cyt *b*		ND2	Combined
Model:	HKY + Γ		GTR + I	GTR + 1
T_i/T_v	4.069	r_{AC}	1395.062	1.680
		r_{AG}	79,165.790	34.716
		r_{AT}	5151.079	2.561
		r_{CG}	10,356.249	4.724
		r_{CT}	35,565.452	17.083
p_{inv}	—		0.782	0.778
α	0.206		—	—
π_A	0.271		0.322	0.294
π_C	0.246		0.250	0.248
π_G	0.146		0.121	0.133
π_T	0.338		0.307	0.325

ND2 Phylogeny

Among ND2 haplotypes, 91 nucleotides were observed to vary (77 parsimony informative) of 830 base pairs sequenced. Seven percent of first-codon positions, 1% of second-codon positions, and 25% of third-codon positions were observed to vary. We excluded haplotype I from the analysis because DNA degradation prevented us from sequencing the individual in which it was found. Individuals with cyt *b* haplotypes A, D, C, E, G, F, O, N, M, P, J, CC, and AA were identical at ND2. The individual with haplotype BB varied at ND2, although ND2 was identical to individuals with haplotype A at cyt *b*. Thus, the ND2 dataset contained only 18 unique haplotypes with uncorrected sequence divergences ranging to 9.1%. Again, we arbitrarily chose tree 5 from the shortest parsimony trees (113 steps, CI = 0.858, RI = 0.963, RC = 0.827) for model selection. As in the earlier analysis, the GTR + I + Γ model had the best likelihood score (ln L = −1734.327), however the GTR + I and GTR + Γ models were not significantly different (ln L = −1734.676, χ^2_1 = 0.698, $P > 0.1$; ln L = −1735.107, χ^2_1 = 1.560, $P > 0.1$, respectively). We could not make comparisons between the GTR + I and GTR + Γ models using the likelihood-ratio test because they are not special cases of the same model, however GTR + I was chosen for subsequent ML searches because its likelihood score was higher. We could reject all simpler models ($P < 0.005$). Again, we could not reject the molecular clock hypothesis for the ND2 data (χ^2_{16} = 19.012, $P > 0.1$). Parameter estimates for ND2 under the GTR + I model with the molecular clock enforced are given in Table 2.

Combined Data Phylogeny

We combined all unique cyt *b* and ND2 sequences from corresponding individuals (with the exception of haplotype I) for a total of 29 unique haplotypes. Optimal parsimony trees measured 214 steps (CI = 0.865, RI = 0.972, RC = 0.841). Again, the GTR + I + Γ model had the highest likelihood score (ln L = −3308.923) and both the GTR + I and GTR + Γ models were the simplest models that we could not reject (ln L = −3309.180; χ^2_1 = 0.515, $P > 0.1$; ln L = −3309.625; χ^2_1 = 1.405, $P > 0.1$, respectively). We chose GTR + I because it had a higher likelihood score; this model fit the data better than any of the simpler models ($P < 0.005$) and was used for subsequent ML searches. For the combined data, we could not reject the molecular clock hypothesis (χ^2_{27} = 30.376, $P > 0.1$), and therefore rooted the ML tree at the midpoint. Parameter estimates for this tree under the GTR + I model are presented in Table 2.

Major haplotype clades were identical for cyt *b*, ND2, and the combined datasets (Fig. 2). In general, likelihood bootstrap values were slightly lower than the corresponding parsimony values, however, both parsimony and ML bootstrap values supported reciprocal monophyly of inland haplotypes (populations 1–14) and coastal haplotypes (populations 15–23). ML-corrected genetic distances (GTR + I) between these populations ranged up to 0.133 substitutions per site (Table 3). Coastal haplotypes formed clades by mountain ranges with strong bootstrap support. Within inland populations, sequences from the East Fork of the South Fork of the Salmon River (population 12) grouped separately from all other haplotypes with strong support (Fig. 2).

Nested-Clade Analyses

With 1530 nucleotides, haplotypes connected by up to 17 substitutional steps had a 95% probability of a parsimonious connection. The minimum-spanning network contained five clades connected by greater than 17 substitutional steps, three within coastal populations and two within inland populations (Fig. 3). To analyze the haplotype network using nested-clade methods, clades must contain both genetic and geographic variation. Of the two inland clades encompassing 95% probable connections, only nesting clade 4-1 exhibited sufficient geographic variation to conduct statistical tests of patterns of association (Figs. 3, 4). Within clade 4-1, clades 2-3, 2-4, 2-5, 1-2, and 2-2 contained no geographic variation and were excluded from individual analysis; the haplotypes they contain were incorporated at higher levels of nesting (e.g., haplotypes L, K, and BB were incorporated into clade 3–1). The nested-contingency analysis, which did not incorporate geographic distance, indicated clustering of genetic variation with geographic location for clades 1-1 and 4-1 ($P = 0.014$; $P < 0.001$). When geographic distance was included in the analysis, haplotypes C, A, and D within clade 1-1 showed restricted geographic ranges (Fig. 5; $P = 0.016$, $P = 0.004$, and $P = 0.014$, respectively). Haplotype A also showed a limited distance from the geographic center of the clade (Fig. 5; $P = 0.017$) and haplotype E was distant from the clade center (Fig. 5; $P = 0.003$). Clade 1-3 had no significant values of D_c or D_n. Within clade 3-2 tip clades were more restricted in their distribution than interior clades (Fig. 5; $P = 0.029$). Clade 3-1 exhibited a restricted range and was distant from the center of clade 4-1 (Fig. 5; $P = 0.001$ and $P = 0.037$, respectively).

The AMOVA indicated that both coastal (populations 15–23) and inland (populations 1–14) groups exhibited genetic structure; 88.7% of genetic variation within the coastal group and 82.7% of genetic variation within the inland group was partitioned among populations ($P < 0.001$ and $P < 0.001$,

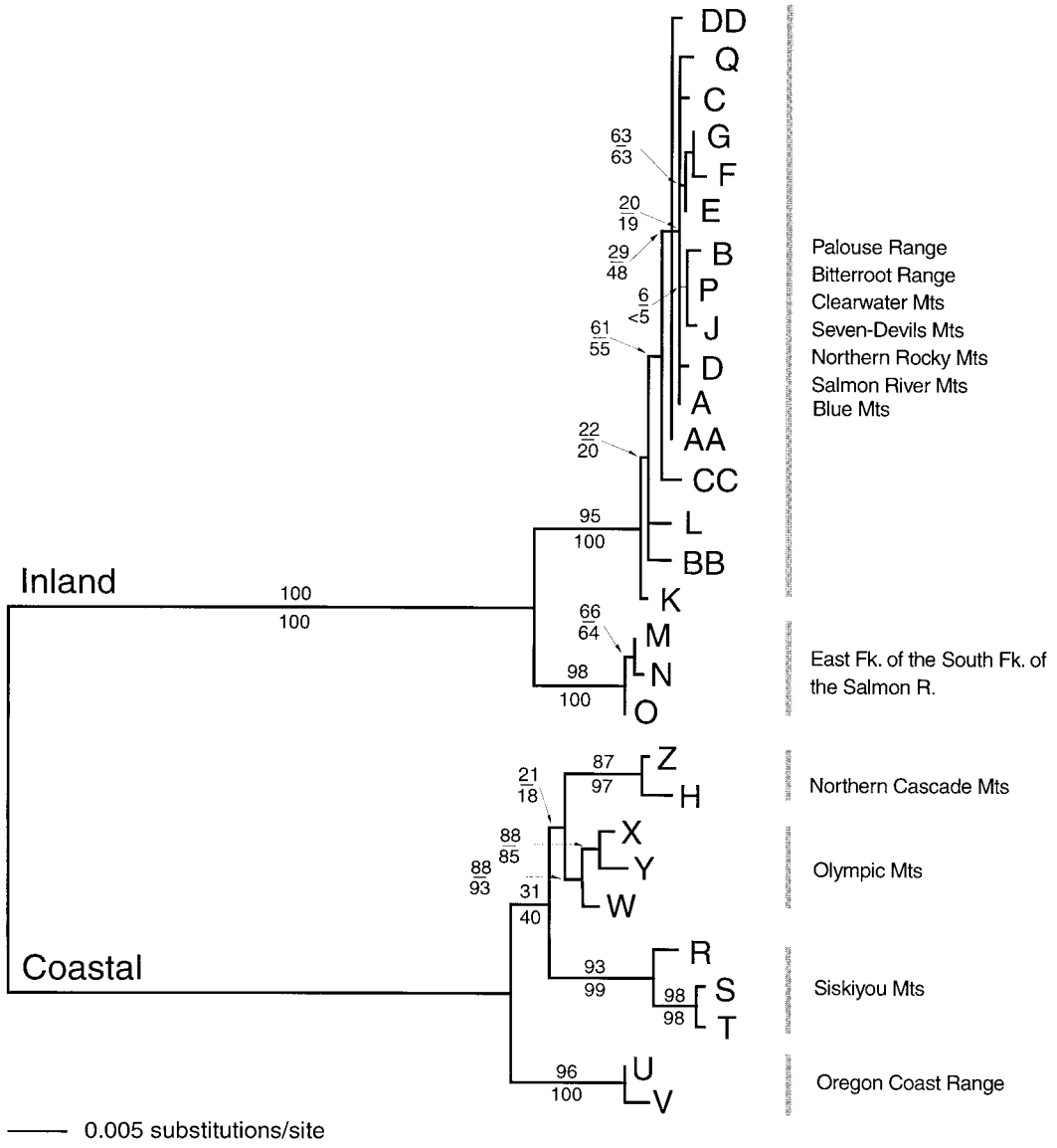

FIG. 2. One of four maximum-likelihood trees estimated from the combined dataset under the objectively determined GTR + I model of sequence evolution and rooted at the midpoint. This tree has a lnL of −3309.180 and differs from the other maximum-likelihood and parsimony trees only by alternative resolutions of internal branches within the inland Rocky Mountain group and weakly supported relationships among the major haplotype clusters of the coastal group. Numbers above branches represent maximum-likelihood bootstrap values (100 replicates); numbers below branches represent parsimony bootstrap values (500 replicates). Haplotype letters correspond with those in Table 1, Figure 3, and Figure 4.

TABLE 3. Genetic distances (substitutions per site) among monophyletic lineages within *Ascaphus* using 1530 bp from the combined dataset (730 bp cyt *b*, 800 bp ND2). Distances were estimated with maximum likelihood under the GTR + I model of nucleotide substitution. Mean distances are given above the diagonal with range below. Mean distances within each population are given in bold on the diagonal.

	N. Cascades Pop. 20–23	Olympics Pop. 18, 19	Coast Range Pop. 17	Siskiyous Pop. 15, 16	E. Fork S. Fork Salmon R. Pop. 12	Rockies Pop. 1–11, 13, 14
N. Cascades	**0.003**	0.013	0.023	0.019	0.101	0.110
Olympics	0.010–0.016	**0.004**	0.020	0.018	0.102	0.114
Coast Range	0.021–0.024	0.017–0.022	**0.002**	0.026	0.102	0.113
Siskiyous	0.017–0.020	0.014–0.020	0.022–0.029	**0.005**	0.104	0.121
E. Fork S. Fork Salmon R.	0.099–0.103	0.098–0.106	0.100–0.104	0.100–0.111	**0.001**	0.121
Rockies	0.011–0.121	0.107–0.126	0.109–0.122	0.111–0.133	0.018–0.023	**0.003**

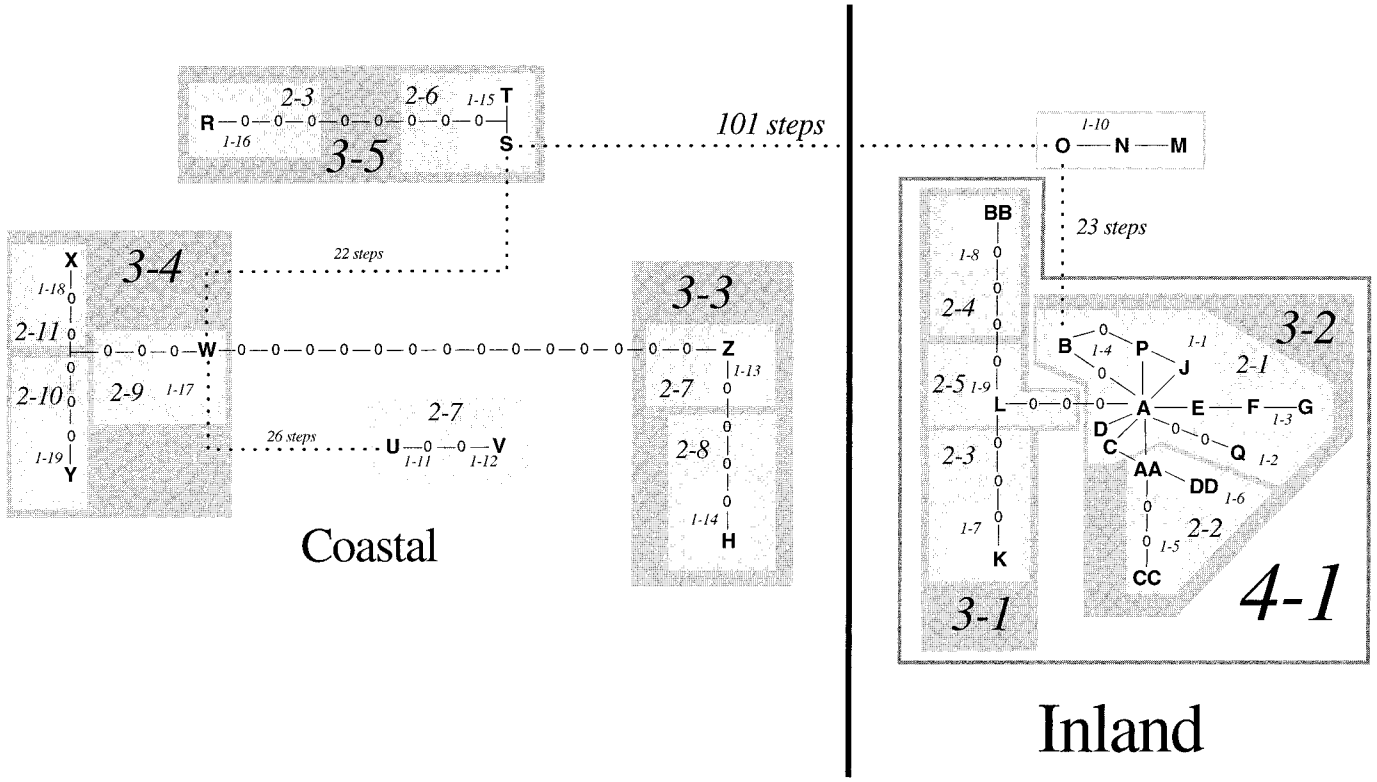

FIG. 3. The minimum-spanning network of haplotypes grouped into nesting clades following procedures given in Templeton et al. (1987) and Templeton and Sing (1993). Each dash represents a substitution. Zeros indicate ancestral haplotypes that were not sampled. Dotted lines indicate connections of more than 17 steps. Connections up to 17 steps have a 95% probability of a parsimonious connection. White cells represent one-step clades, light shading encloses two-step clades, and dark shading encompasses three-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 Table 1 and Figure 1.

respectively). Significant differentiation occurred even within clade 4-1, which encompasses 14 populations yet has only 0.003 substitutions per site mean divergence ($\Phi_{ST} = 0.571$, $P < 0.001$).

DISCUSSION

Phylogeography

Late Miocene and early Pliocene events provide the best supported explanation of the deepest bifurcation in *Ascaphus.* Under this scenario, the major divergence in the lineage emerged during the late Miocene in response to the rise of the Cascade Mountain range. The Miocene (28–10 million years ago) and earlier Eocene of the Pacific Northwest were characterized by a mesic equable climate that supported broadleaf deciduous and evergreen forests with an increasing conifer component (Axelrod 1968; Wolfe 1969, 1978); such forests were likely similar to those that modern tailed frogs inhabit. The effect of the rain shadow created by the high Cascade range became increasingly apparent in the paleoflora through the Pliocene (Wolfe 1969); some plant lineages attained a distribution restricted to the east or west of the early Cascades (approximately 1000 m in elevation) during the late Miocene (Wolfe 1969). If the climatic influences of the early Cascades did not split contiguous populations of *Ascaphus* into eastern and western groups, they were in all likelihood divided by the rain shadow of the high Cascade Mountains, which currently partitions their range (Fig. 1).

Metter and Pauken (1969) and Pauken and Metter (1971) suggested that inland and coastal populations of *Ascaphus* were connected by gene flow through the mountains of central Oregon and did not diverge until the close of the Pleistocene (10,000 years ago). There is substantial evidence, however to refute a Pleistocene interpretation of the deepest divergence in *Ascaphus.* Assuming clocklike evolution (which cannot be rejected for our data), the magnitude of divergence observed between coastal and inland haplotypes would require a minimum rate of 0.0616 substitutions per site per million years to have occurred during the Pleistocene. This is a much faster rate of evolution than the 0.0278 substitutions per site per million years typically estimated for mammals (e.g., Arbogast and Slowinski 1998). We strongly doubt that *Ascaphus* sequences are evolving at such a rapid rate. A generation time of six or more years and a cold-water habitat have probably slowed the evolutionary rate of *Ascaphus* even below that of other ectotherms, which are hypothesized to evolve four to five times slower than mammals (Kocher et al. 1989; Thomas and Bechenbach 1989; Adachi et al. 1993; Martin and Palumbi 1993; Rand 1994). Thus, a Pleistocene or post-Pleistocene divergence between inland and coastal populations is unlikely.

This view is also supported by the paloebotanical record.

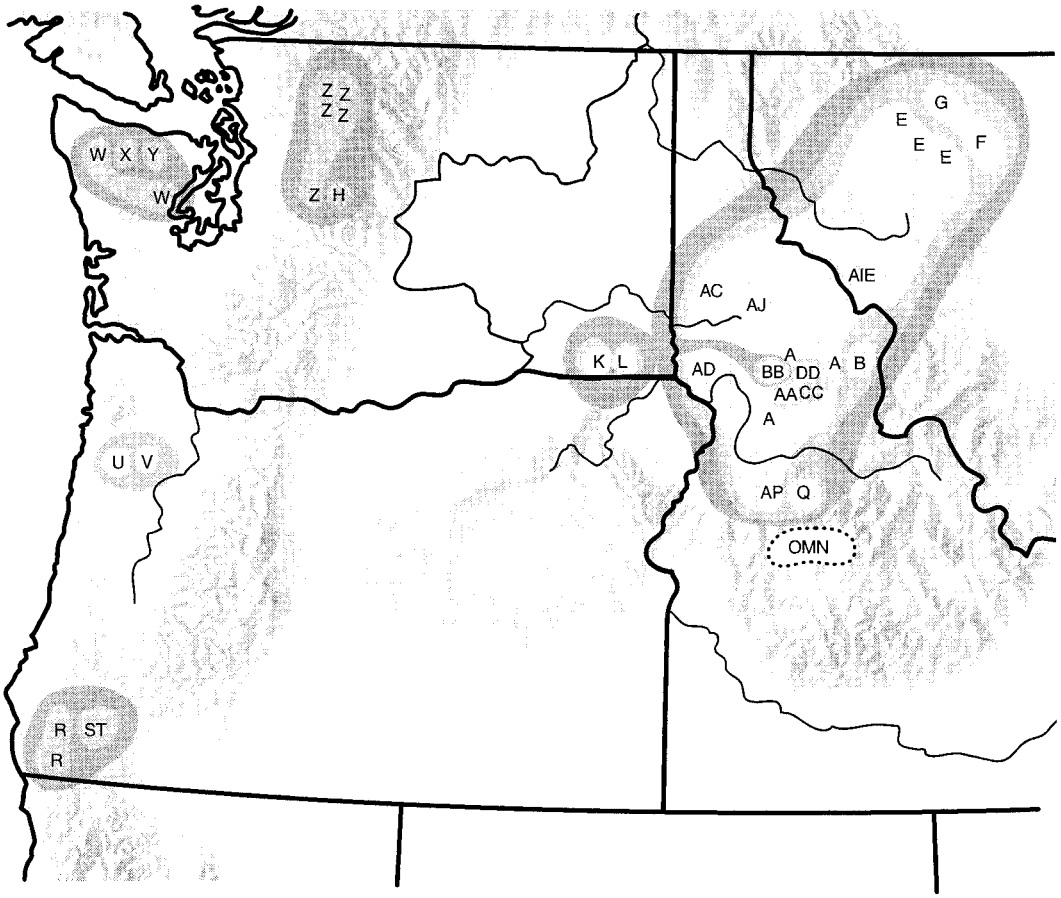

FIG. 4. An overlay of nested clades on geography. White cells enclose one-step clades, light shading encloses two-step clades, and dark shading encompasses three-step clades. Haplotypes present in each clade are indicated at the collection locality and correspond with the letters given in Table 1, Figure 2, and Figure 3. Collection localities correspond with those in Figure 1. Shading does not represent distribution information, and *Ascaphus* is absent from the lowlands between collection localities.

The most recent continuity of mesic forest between inland and coastal sites appears to have occurred during the late Miocene to early Pliocene (Wolfe 1969). Some xeric-adapted forest plant species, however, may have moved through the highlands of central Oregon during the Pleistocene (Lorrain 1988), as Metter and Pauken (1969) and Pauken and Metter (1971) suggested for tailed frogs. Most plant species hypothesized to have taken this route, however, are presently distributed in more xeric areas than is *Ascaphus* (Lorrain 1988). An alternative recent dispersal route for tailed frogs is across the mountains of southern British Columbia as glaciers retreated. Some dominant forest species may have dispersed via this route, however, these species usually exhibit remnant populations across southern British Columbia unlike the tailed frog's distribution (Lorrain 1988; Soltis et al. 1997). Furthermore, pollen records indicate that dispersal of forest species into this area occurred very recently (3000 years; Mack et al. 1978). The depth of divergence between inland and coastal populations of *Ascaphus* is inconsistent with such recent gene flow.

Among coastal populations, sequences from the Olympic Mountains, North Cascades, Coast Range, and Siskiyou Mountains all form discrete groups with strong bootstrap support, although the relationships among them are poorly resolved. If the late Miocene timing of east-west vicariance is adopted, divergences within the coastal clade fall within the late Pliocene to early Pleistocene. During the Pliocene, the Pacific Northwest began to experience summer drought, a decline in mean annual temperature, zonation of vegetation by latitude and altitude, and a less equable climate (Wolfe 1969, 1978). These changes could have fragmented a previously panmictic western population. Glacial refugia during the early Pleistocene may have also played a role in fostering divergence among coastal haplotypes. Soltis et al. (1997) documented genetic variation in Pacific Northwest plant taxa consistent with early Pleistocene refugia along the coast. Phylogeographic patterns within the coastal group could also be the result of limited dispersal and contemporary isolation by distance, however, because our sampling was not extensive along these mountain ranges and intermediate haplotypes may fill the spaces between sampled populations (see Avise 1994; Templeton 1998).

Similar climatic changes during the Pliocene may have effectively isolated inland populations as well. Although haplotypes from the region spanning the northern Rockies (populations 1–11, 13, 14) show minimal variation, haplotypes

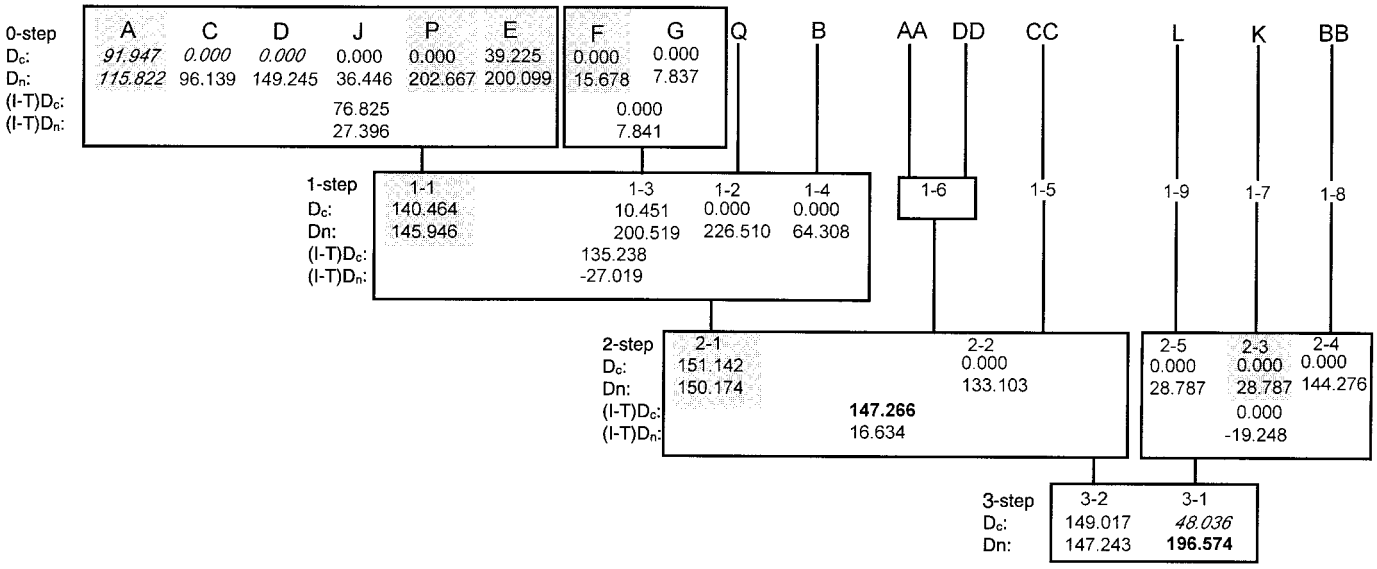

FIG. 5. Results of the nested-clade analysis. Haplotype letters are given at the top and are boxed together to reflect one-step clades given in Figure 3. These one-step clades are combined into higher level nesting groups through boxes lower down the figure. The clade distance (D_c) and nested-clade distance (D_n) are given immediately below each clade designation. If the clade contains both tip and interior groups, the contrast between them (I − T) is given for both D_c and D_n in the next two lines. Significance was determined at the 5% level using the null hypothesis of random association of haplotypes with geography. Significantly small values are italicized and significantly large values are in bold; interior clades are shaded.

from the East Fork of the South Fork of the Salmon River (population 12) are highly divergent. Samples collected 17 km away on the same river system (population 11) fall within the major inland group, and no obvious geographic barriers exist between populations 11 and 12. This phylogenetic pattern may be explained by historic divergence of the two haplotypes within separate refugia associated with Pliocene drying and secondary contact. Early Pleistocene cooling may have also contributed to isolation as vegetation zones shifted south and lower in elevation (Butler 1972). Northern populations may have remained along the Clearwater River drainage, as has been suggested for other mesic forest species (Daubenmire 1975), whereas southern populations could have been isolated in the valleys along the southern front of the Salmon River Mountains.

Along with earlier events, glaciation within the past million years influenced the genetic structure in *Ascaphus.* The most distinct feature of northern Rocky Mountain populations (populations 1–11, 13, and 14; clade 4-1) is their relative lack of structure (Fig. 2). The Φ_{ST} value for this group of 13 populations is lower than that for the nine coastal populations (Φ_{ST} = 57.110 and Φ_{ST} = 88.710, respectively). This is particularly interesting because inland populations currently occur in isolated moist mountain habitats separated by dry lowlands, whereas coastal populations occur within more contiguous mesic forest habitat.

Because inland sequences exhibited very low divergence levels and therefore weak phylogenetic signal, nested-clade analysis was useful for detecting and interpreting significant relationships. Clades 1-1, 2-1, and 3-2 occur in formerly glaciated regions of *Ascaphus*'s range (Fig. 4). Haplotype A is widespread and found in most populations within these clades; this suggests it was a founding haplotype that spread during the range expansion that must have occurred as *Ascaphus* colonized these areas subsequent to glacial retreat (Fig. 4). Each of these clades also contain geographically restricted haplotypes closely related to A, many of which represent tips (recent haplotypes) in the minimum spanning network (Fig. 3). This pattern implies restricted current gene flow, which is consistent with the xerification of valley floors as the Holocene progressed. Hewitt (1996) described similar patterns in other species that have undergone range expansions.

Patterns within clade 3-1, which contains haplotypes L and K from the Blue Mountains as well as haplotype BB from the South Fork of Clearwater River (Fig. 4), may also reflect glacial effects on inland populations. The relatively high number of substitutional steps separating haplotype BB from L and K (Fig. 3), in combination with the large geographic distance between them and a lack of intermediate haplotypes, suggests historic fragmentation of these populations. The Blue Mountain populations may have become isolated earlier than other inland populations, with the xerification of the deep Snake River Canyon prior to the drying of more shallow inland valleys. When significant patterns in the northern Rocky Mountain populations (clade 4-1) are considered as a whole, we cannot differentiate between range expansion and restricted gene flow using Templeton's (1998) inference key. This is not surprising, however, because both processes appear to be generating patterns within the clade. This result is consistent with an expanding range following glaciation and subsequent isolation by the currently dry intervening lowlands.

Conservation and Taxonomy

Ascaphus truei is listed as a species of special concern in California, a vulnerable species in Oregon, a monitored spe-

cies in Washington, and a blue-listed species in British Columbia. Moritz (1994) suggested that reciprocal monophyly of mtDNA haplotypes and significant divergence of allele frequencies at nuclear loci are criteria for establishing evolutionarily significant units (ESUs), which have been proposed to establish long-term conservation strategies (Ryder 1986; Moritz 1994). Based on our data and Daugherty's (1979) allozyme data, inland and coastal populations represent unique conservation units. In addition, populations from the Olympic Mountains, North Cascades, Coast Range, and Siskiyou Mountains exhibit reciprocal monophyly at the mitochondrial genes examined. Samples from the East Fork of the South Fork of the Salmon River are also monophyletic. Each of these lineages may represent an ESU. As the geographic extent of haplotypes are further defined and examined with independent markers, each of these groups may warrant individual conservation consideration.

Mittleman and Myers (1949) partitioned *A. truei* into Rocky Mountain, northern California, and coastal subspecies based on eye diameter, head width : body length ratios, and the number of vomerine teeth. Metter (1967) did not detect geographic patterns in morphological characters, however, and discounted Mittleman and Myers's (1949) classifications. Subsequently, Daugherty's (1979) allozyme study demonstrated the divergence between inland and coastal populations.

We identify a minimum of two distinct, major lineages in *Ascaphus.* The degree of divergence between inland and coastal populations is similar to that found between subspecies or sister-species of other taxa (Moritz et al. 1992; Bernardi and Powers 1995; Schmidt and Gold 1995). Given the potentially slow rate of molecular evolution in ectothermic vertebrates, this divergence may represent a much longer isolation than found between other similarly divergent species. The concordant east-west division in *Ascaphus* is seen in morphological (Metter 1969), nuclear (Daugherty 1979), and mitochondrial markers. Inland and coastal groups fill the requirements of a phylogenetic approach to species delimitation such as the genealogical concordance species concept (Baum and Shaw 1994), although monophyly has yet to be fully examined for nuclear loci. Thus, we recommend inland populations of *Ascaphus* be recognized as a distinct species, *Ascaphus montanus* (using the epithet of Mittleman and Myers 1949), to reflect an independent evolutionary history and the diversity within this lineage. Under this taxonomic hypothesis, we predict that future studies will discover additional fixed differences between coastal and inland populations.

Regional Biogeography

Ascaphus is a member of the unique disjunct mesic forests of the Pacific Northwest characterized by high winter precipitation and cool temperatures (Lorrain 1988). At least 156 invertebrates, bryophytes, lichens, and vascular plants share a similar distribution and many retain distinct inland forms (McCune 1984; Johnson 1987; Lorrain 1988). Although several species with more general habitat requirements and continuous distributions across the West show unique coastal forms (e.g., Green et al. 1996; Demboski et al. 1999), *Ascaphus, Dicamptodon* (giant salamanders), and the *Plethodon vandykei*/*P. idahoensis* complex (Van Dyke's and Coeur d'Alene salamanders) are the only vertebrate members of this disjunct mesic biota that have been examined for genetic variation.

Dicamptodon species are limited to the same forested stream habitats as *Ascaphus.* Daugherty et al. (1983) examined allozyme variation at 22 loci in *Dicamptodon* from 23 populations across their range (five sites in common with our collection localities). Using Nei's genetic identity, they constructed a phenogram using the weighted pair-group arithmetic average method (WPGMA). Although their methods are not directly comparable with those we used for *Ascaphus,* the two topologies share common features (Fig. 6). Less structure is found among inland populations in both groups. In general, coastal populations cluster by mountain range, and the deepest divergence is between inland and coastal populations. Good (1989) examined coastal populations and supported recognition of four *Dicamptodon* species roughly corresponding with the Olympic Mountains; Cascades and Coast Range of Oregon; coastal mountains of California; and northern Rocky Mountains.

The *Plethodon vandykei* species group has a similar distribution, although it is found in moist unconsolidated talus along streams (Wilson and Larsen 1998). Howard et al. (1993) examined nine populations at 24 allozyme loci and recommended that inland populations be recognized as a distinct species (*Plethodon idahoensis*). They constructed dendrograms (using FREQPARS and UPGMA) that are consistent with the patterns found in *Dicamptodon* and *Ascaphus* (Fig. 6). Thus, it appears that *Ascaphus, Dicamptodon,* and the *P. vandykei* complex responded similarly to ancient climatic and geological fluctuations. Along the coast, populations of *Ascaphus, Dicamptodon,* and *Plethodon* appear monophyletic by mountain range, but the relationships among these populations are unclear and may not be concordant (Daugherty 1983; Good 1989; Howard et al. 1993; Fig. 6). These three amphibian species appear to have responded similarly to initial fragmentation of their distribution caused by the rise of the Cascade Mountains, but it is unclear whether they responded similarly to Pliocene drying and Pleistocene glacial flux. We predict major divergences will be found in the phylogenies of other species endemic to these disjunct mesic forests corresponding with inland and coastal types.

Daubenmire (1952) initially posited that disjunct mesic Rocky Mountain communities represent relicts of a continuous historic distribution. His arguments were disputed by pollen cores from glaciated regions in Idaho, Montana, and southern British Columbia, which showed a lack of mesic forest species up to about 3000 years ago (Mack et al. 1978; Baker 1983; Mehringer 1985). The molecular evidence we present for tailed frogs, however, provides strong support for the persistence of mesic forests in the northern Rocky Mountains during glaciation. The divergence between inland and coastal populations is not consistent with dispersal since the Pleistocene. Furthermore, divergences between East Fork of the South Fork of the Salmon River populations and northern Rocky Mountain populations suggests the presence of refugia during this period. Daubenmire (1975) proposed the Clear-

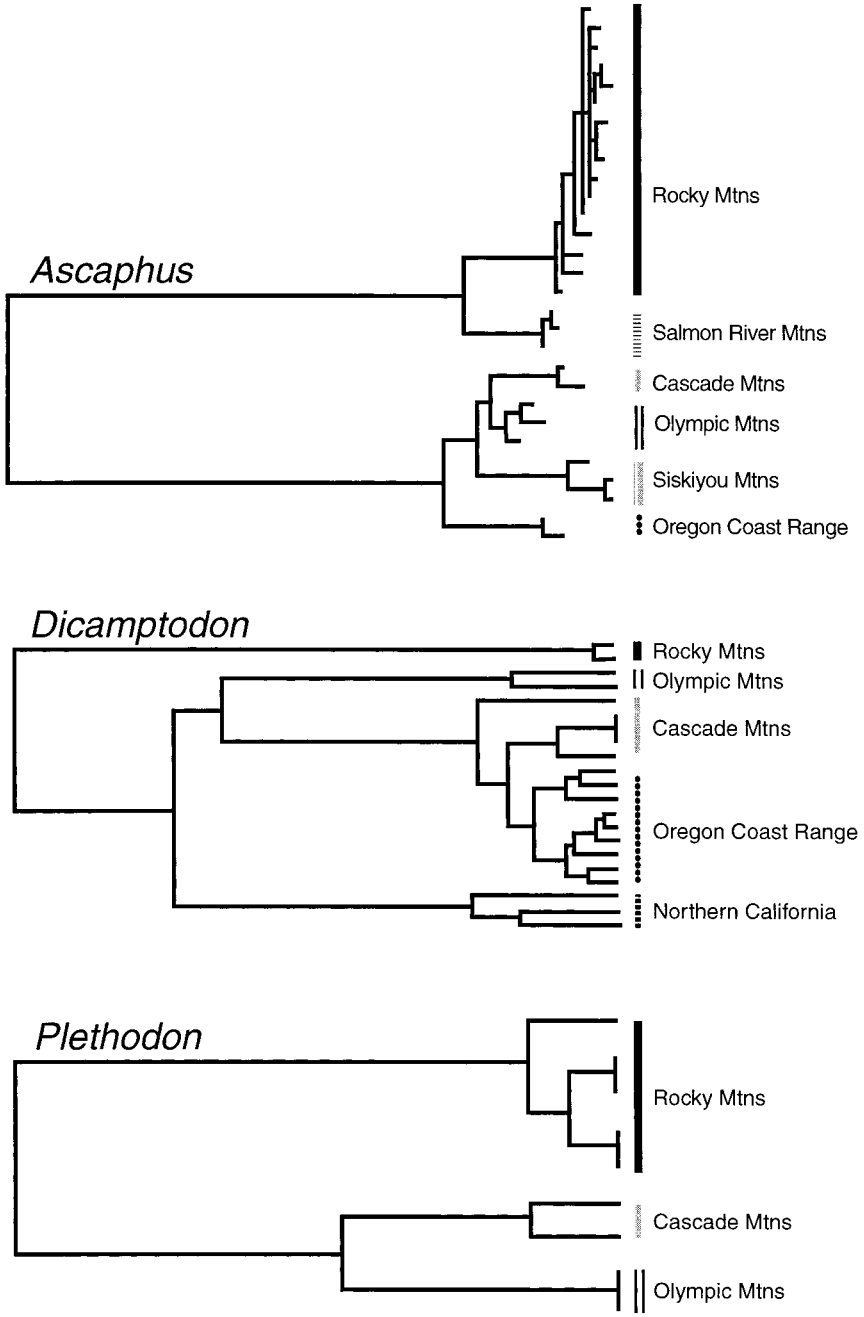

FIG. 6. Dendrograms depicting relationships among populations of *Ascaphus* (ML), *Dicamptidon aterrimus*/*D. copei*/*D. ensatus*/*D. tenebrosus* (UPGMA; Good 1989), and *Plethodon vandykei*/*P. idahoensis* (UPGMA; Howard et al. 1993). Figures are not drawn to the same scale.

water River basin as a likely refuge based on the large number of rare endemic and coastal disjunct species located here. The lack of genetic structure among Rocky Mountain populations of tailed frogs suggests that they expanded northward following glaciation, presumably along with other components of the mesic forest community.

Conclusions

Three major patterns emerge in the *Ascaphus* phylogeny. First, large genetic divergences in mtDNA separate inland and coastal populations. These divergences correspond with earlier allozyme and morphological examinations and are comparable to the patterns found in other endemic amphibians from this region. The magnitude of this divergence and the paleobotanical record suggest inland populations have been evolving independently since the late Miocene to early Pliocene and represent a cryptic species. Second, coastal haplotypes formed clades by mountain range and potentially diverged within mesic refugia during the Pliocene to early Pleistocene. Each of these major lineages represents a unique

evolutionary unit. Third, inland populations exhibit minimal variation, with the exception of haplotypes from the East Fork of the South Fork of the Salmon River. Isolation within Pliocene or early Pleistocene refugia may explain the divergence of these populations. The shallow divergences among the remaining inland haplotypes suggest range expansion occurred following glacial retreat with contemporary isolation. Populations from the East Fork of the South Fork of the Salmon River may also represent evolutionarily significant units for conservation. *Ascaphus* is one of a large number of taxa sharing a disjunct distribution in mesic forests of the Pacific Northwest. The inland forms of many of these taxa are recognized as distinct species or subspecies and appear to have responded similarly to the rise of the Cascade Range and changes in climate during the Pliocene and early Pleistocene. Further examination of species endemic to the Pacific Northwest will allow for refinement of these biogeographic hypotheses.

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