Coalescent-based hypothesis testing supports multiple Pleistocene refugia in the Pacific Northwest for the Pacific giant salamander (Dicamptodon tenebrosus)

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Abstract

Phylogeographic patterns of many taxa are explained by Pleistocene glaciation. The temperate rainforests within the Pacific Northwest of North America provide an excellent example of this phenomenon, and competing phylogenetic hypotheses exist regarding the number of Pleistocene refugia influencing genetic variation of endemic organisms. One such endemic is the Pacific giant salamander, Dicamptodon tenebrosus. In this study, we estimate this species’ phylogeny and use a coalescent modeling approach to test five hypotheses concerning the number, location and divergence times of purported Pleistocene refugia. Single refugium hypotheses include: a northern refugium in the Columbia River Valley and a southern refugium in the Klamath-Siskiyou Mountains. Dual refugia hypotheses include these same refugia but separated at varying times: last glacial maximum (20 000 years ago), mid-Pleistocene (800 000 years ago) and early Pleistocene (1.7 million years ago). Phylogenetic analyses and inferences from nested clade analysis reveal distinct northern and southern lineages expanding from the Columbia River Valley and the Klamath-Siskiyou Mountains, respectively. Results of coalescent simulations reject both single refugium hypotheses and the hypothesis of dual refugia with a separation date in the late Pleistocene but not hypotheses predicting dual refugia with separation in early or mid-Pleistocene. Estimates of time since divergence between northern and southern lineages also indicate separation since early to mid-Pleistocene. Tests for expanding populations using mismatch distributions and ‘g’ distributions reveal demographic growth in the northern and southern lineages. The combination of these results provides strong evidence that this species was restricted into, and subsequently expanded from, at least two Pleistocene refugia in the Pacific Northwest.

Keywords: coalescence, Columbia River, Dicamptodon, dispersal, Klamath-Siskiyou, phylogeography

Introduction

One of the main objectives of phylogeography is to infer the processes that have lead to the genetic patterns observed in populations across the landscape (Avise 2000). The cycles of glacial advance and retreat during the Pleistocene had an undeniable effect on genetic structuring within species (Hewitt 1996; Ibrahim et al. 1996; Avise et al. 1998) and among species groups (Brunsfeld et al. 2001; Carstens et al. 2005a). The role of Pleistocene refugia during glacial advances was especially important in generating and maintaining genetic diversity. The separation of ancestral populations into isolated refugia allowed for the formation of distinct evolutionary lineages within species (Hewitt 2000). Identifying the number and location of Pleistocene refugia is important in determining the patterns of postglacial expansion from Pleistocene refugia (Hewitt 1999), identifying distinct lineages or populations for conservation or management purposes (Wagner et al. 2005) and providing insights into the evolutionary history of ecosystems (Carstens et al. 2005a).

The world’s largest expanse of temperate rainforest occurs within the Pacific Northwest of North America and provides a prime example of an ecosystem shaped by Pleistocene glacial processes. Within this ecosystem are a multitude of endemic organisms for which several competing
phylogeographic hypotheses exist regarding the number of Pleistocene refugia in structuring genetic variation of these species (Brunsfeld et al. 2001). One such endemic of this coniferous rainforest ecosystem is the Pacific giant salamander, *Dicamptodon tenebrosus*. Its widespread range (Fig. 1) from southwestern British Columbia to northwestern California makes it an ideal organism for testing hypotheses on the number and location of Pleistocene refugia in the Pacific Northwest, as well as investigating postglacial expansion routes from these refugia. Previous studies have confirmed the monophyly of *D. tenebrosus* and revealed some geographic structure (Daugherty et al. 1983; Good 1989; Steele et al. 2005), but relationships among these lineages are not well resolved. In this study we use a coalescent modelling approach to test statistically competing phylogeographic hypotheses concerning the number, location and divergence time among Pleistocene refugia in the Pacific Northwest for this species.

Specific hypotheses proposed by Brunsfeld et al. (2001) include the possibility of single or dual refugia. Location of the purported refugia is uncertain because postglacial expansion from refugia resulted in a contiguous distribution across the landscape, thereby removing any clues as to the location of the refugia. However, genetic patterns revealed in previous studies suggest at least two refugia. A southern refugium is thought to exist in the Klamath-Siskiyou Mountains based on a study of six plant species (Soltis et al. 1997). Evidence also suggests another refugium located farther north. Proposed locations of a northern refugium have included the Olympic Peninsula, Vancouver Island and Haida Gwaii (Queen Charlotte Islands; Byun et al. 1997; Soltis et al. 1997; Demboski et al. 1999). All of these localities are unlikely northern refugia for *D. tenebrosus* because these areas are well outside its known distribution. However, another purported northern refugium is the Columbia River Valley. Genetic studies conducted in a variety of fish species have identified the lower Columbia River and its tributaries as a probable refugium (Brown et al. 1992; Bickham et al. 1995; Taylor et al. 1999; McCusker et al. 2000; Haas & McPhail 2001). Considering that *D. tenebrosus* is a stream-breeding salamander and that terrestrial adults are closely associated with streams, we propose the Columbia River Valley to also be a plausible refugium for this species.

In this study, we test five hypotheses concerning the number, location and divergence times of Pleistocene refugia for *D. tenebrosus*. Our hypotheses (Fig. 2) include: (i) a single northern refugium in the Columbia River Valley; (ii) a single southern refugium in the Klamath-Siskiyou Mountains; (iii) two refugia, one in the Columbia River Valley and the other in the Klamath-Siskiyou Mountains, separated at last glacial maximum (20 000 years ago); (iv) these same two refugia but separated since the mid-Pleistocene (800 000 years ago); and (v) the two refugia separated since the early Pleistocene (1.7 million years ago). By constructing evolutionary models based on these hypotheses and then coalescing simulated data under these models, we can determine the probability that the observed data are generated by these evolutionary scenarios.

**Materials and methods**

**Sample collection and DNA amplification**

We obtained tissue samples of 82 individuals from 31 localities throughout the range of *Dicamptodon tenebrosus* (Fig. 1 and Table 1), including localities in purported refugia of the Columbia River Valley and the Klamath-Siskiyou Mountains. Samples were obtained primarily from the Museum of Vertebrate Zoology at Berkeley but were supplemented with field-collected tissues. Sequences of the three remaining members of the genus (*Dicamptodon*...
aterrimus, Dicamptodon ensatus and Dicamptodon copei) were used as outgroups (Steele et al. 2005).

DNA was extracted using standard phenol–chloroform extractions (Sambrook et al. 1989). Thirty-nine sequences for a ~1100-bp section of the cytochrome b gene (cyt b) were obtained from an earlier study (Steele et al. 2005) and are deposited in GenBank. Amplification of the same cyt b region from an additional 43 samples was performed using the two primer sets in Carstens et al. (2005b): tRNA-Threonine (5′-TTCAGCTTACAAGGCTGATGTTTT-3′) with a reverse internal (5′-TAATTAGTGGATTTGCTGGTGTAA-3′) and tRNA-Glutamine (5′-TTGTATTCAACTATAAAAAC-3′) with a forward internal (5′-TCCACCATCTTTTTCTATTAAGA-3′). We also amplified a ~750-bp portion of the mitochondrial control region (CR) for all 82 samples using a modified 007 primer (5′-GGACCCCAAGGGCGAAATTCTTC-3′) and the 651 primer (5′-GTAAGATTAGGCCAAATCT-3′) (Shaffer & McKnight 1996). Amplicons were purified using centrifugal filters (Millipore) and sequencing reactions were performed using BigDye Kit version 3.1 (Applied Biosystems) with 20–40 ng of polymerase chain reaction product in 10-µL reaction volumes. Sequencing reactions for cyt b and CR were performed in both 5′ and 3′ directions, purified with a 70% isopropyl wash and run on either an ABI 377 or ABI 3730 automated sequencer. Sequences were aligned and edited with Sequencher 4.1 (Gene Codes). Sequences are deposited in GenBank (Appendix).

**Phylogeny reconstruction**

We analysed the data using maximum parsimony (MP), maximum likelihood (ML) and Bayesian analyses. Redundant haplotypes were removed and we used dt-modesl (Minin et al. 2003) to select a model of evolution. MP and ML analyses were performed with PAUP* 4.0.b10 (Swofford 2002) using a heuristic search with TBR and 10 random-addition replicates. For the MP analysis, we weighted all sites equally and treated gaps as missing data. The HKY

<table>
<thead>
<tr>
<th>Locality</th>
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<th>Haplotypes</th>
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<td>1</td>
<td>G</td>
<td>Mallardy Crk, Snohomish Co., WA</td>
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<td>4</td>
<td>A</td>
<td>11 Mile Crk, Chelan Co., WA</td>
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<tr>
<td>4</td>
<td>2</td>
<td>A</td>
<td>Mine Crk, King Co., WA</td>
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<tr>
<td>5</td>
<td>2</td>
<td>A</td>
<td>Mosquito Crk, Kittitas Co., WA</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>A</td>
<td>Tributary of West Fork of Little Nisqually River, Lewis Co., WA</td>
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<td>7</td>
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<td>A</td>
<td>West Fork of Elochoman River, Wahkiakum Co., WA</td>
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<td>E, F</td>
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<td>5</td>
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<td>V</td>
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<tr>
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<td>af</td>
<td>Drive-Thru-Tree at Leggett, Mendocino Co., CA, MVZ 187978</td>
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<td>1</td>
<td>ai</td>
<td>Hwy 1 between Fort Bragg and Rockport, Mendocino Co., CA, MVZ 192579</td>
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<tr>
<td>31</td>
<td>2</td>
<td>ae, ad</td>
<td>1.4 mi S of Little Riv, Mendocino Co., CA, MVZ 192639–40</td>
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</table>

Table 1: Locality information, number of captures and haplotypes sampled for localities of Dicamptodon tenebrosus. Locality numbers correspond to those in Fig. 1. Unique haplotype sequences were deposited in GenBank for cytochrome b (DQ387923–DQ387957) and control region (DQ388392–DQ388426)
Two data sets during analysis. Two independent runs were performed simultaneously on the data with each run using one cold and three heated chains. Examination of the posterior probability distributions suggested the Markov chain reached stationarity within 100,000 generations but we discarded the first 25% of the samples (1,125,000 generations) as ‘burn in’ to ensure stationarity. The average standard deviation of split frequencies between the two independent runs at completion was 0.0038 and suggested convergence of the two runs on a stationary distribution.

**Testing Pleistocene hypotheses**

Our approach to hypothesis testing is similar to that of Carstens et al. (2005b) in their testing of competing Pleistocene hypotheses for the Idaho giant salamander (*D. aterrimus*). We used mesquite 1.05 (Maddison & Maddison 2004) to conduct coalescent simulations of the combined data set within each of the five hypotheses of Pleistocene refugia (Fig. 2). We simulated 1000 coalescent genealogies within the predicted population history of each hypothesis and then simulated DNA, using model parameters determined from ingroup sequences only to better reflect intraspecific evolution, on each of the simulated gene trees.

Performing the simulations required an estimate of effective population size (*N*). To estimate *N*, we used a coalescent approach implemented in MIGRATE 2.0.6 (Beerli 2004) to calculate the parameter θ where θ = 2*N*μ. A mutation rate (μ) for the cyt *b* region was estimated as μ = 1.6 × 10−8 based on degree of divergence of this gene region between the Idaho giant salamander (*D. aterrimus*) and the Pacific giant salamander (*D. tenebrosus*) and calibrated to the orogeny of the Cascade Mountains that lead to the separation of the species (Steele et al. 2005). We therefore used cyt *b* data and its associated mutation rate to estimate an *N* for the entire species and for populations in the purported refugia of the Columbia River Valley and the Klamath-Siskiyou Mountains. Simulations under a single refugium hypothesis used an *N* equivalent to the proportion of the *N* estimated for all populations sampled. The three hypotheses with two Pleistocene refugia (see Fig. 2) differ in time since divergence between refugia. Simulations were conducted using three divergence dates that correspond to a split early in the Pleistocene (1.7 million years ago), a mid-Pleistocene split (800,000 years ago) and a recent Pleistocene split (20,000 years ago) corresponding to the last glacial maxima (Watt & Thorson 1983). A generation length of 4 years (Nussbaum et al. 1983) was used to convert divergence times in years to coalescent times in generations.

Slatkin & Maddison’s (1989) S statistic, which measures the discord between a gene tree and subdivision of populations, was used to assess significance of each hypothesis. This statistic treats the defined populations as categorical
variables and is a measure of the minimum number of migration events (i.e. sorting events) between populations as implied by the gene tree. Coalescent simulations of the gene tree within the population tree provided by each hypothesis produced a distribution of expected values of the S statistic under the proposed degree of population subdivision and divergence time. Values of the S statistic calculated from the observed gene tree were compared to this distribution in order to determine the significance of discord between simulated gene trees and the population divisions presented in each hypothesis.

**Nested clade and population level analyses**

To test for significant association of haplotypes with geography we performed a nested clade analysis (NCA) (Templeton et al. 1987, 1995). NCA analyses are a common tool in phylogenetic studies and are often useful in inferring historical phylogeographic processes. NCA has been criticized for lacking statistical assessment among alternative phylogeographic inferences (Knowles & Maddison 2002) but Templeton (2004) maintains that a slightly revised inference key reduces error and provides an accurate assessment of phylogeographic processes, especially when specific a priori scenarios are unknown. We employ the NCA as an opportunity to reinforce results obtained from coalescent simulations. Congruent results among coalescent simulations and NCA increases confidence in the accuracy of inferences made about past phylogenetic processes.

A minimum spanning network was constructed using TCS 1.18 (Clement et al. 2000) and haplotypes were nested using rules of Templeton et al. (1987) and Templeton & Sing (1993). Geographic localities for each population were calculated using latitude and longitude. GEODIS 2.2 (Posada et al. 2000) was used to test for significant association of haplotypes and geography. We followed the inference key in Templeton (2004) for clades with significant geographic associations.

Genetic diversity of haplotypes was explored using AMOVA performed in ARLEQUIN 2.0 (Excoffier et al. 1992; Schneider et al. 2000). We partitioned samples into population groupings based on results of the previous analyses and considered two alternative groupings: one corresponding to the major lineages identified by the phylogeny and the other corresponding to clades identified by NCA.

After major clades of the phylogeny were identified, divergence times between the clades were estimated using MDIV (Nielsen & Wakeley 2001). Estimating divergence time requires a mutation rate and we again used $\mu = 1.6 \times 10^{-8}$ from cyt $b$ data to estimate time since divergence using sequences from this gene. Initial analysis indicated migration among major clades was nearly zero; thus, we reanalysed the data with the migration prior set to $M = 0$ and the max $T = 1$. We conducted $2 \times 10^6$ generations of the Markov chain and repeated the analysis several times to ensure stationary.

Time since divergence was estimated as $t_{\text{div}} = T(\theta)/2\mu$ and a 95% confidence interval was calculated from the distribution of posterior probabilities of $\theta$.

**Analysis of demographic history**

We used FLUCTUATE (Kuhner et al. 1998) to estimate exponential growth rate ($g$) of a population to test for demographic growth in clades indicated by the NCA as having undergone range expansion. We used 10 short chains of 1000 generations and 10 long chains of 20 000 generations with an initial ‘$g$’ value of 0. Each run started with Watterson’s estimate of $\theta$ (Watterson 1975), empirical nucleotide frequencies, and with a transition/transversion ratio (3.0292) and proportion of variable sites (0.8855) determined from DT-ROADS using only ingroup sequences. The program was run several times to ensure consistent estimation of ‘$g$’. Results of the ‘$g$’ distribution can be biased upward (Kuhner et al. 1998); thus, to determine significant deviation from a constant population size ($g = 0$) we used a conservative 99% confidence interval ($\pm 3SD$ around the mean) to infer population growth.

Evidence for population expansion was also tested under the expansion model of Rogers & Harpending (1992) by examining pairwise mismatch distributions. Populations that have had constant size are thought to be multimodal in the pairwise mismatch distribution, while populations that have undergone recent demographic expansion are unimodal. Mismatch distributions were calculated in ARLEQUIN (Excoffier et al. 1992; Schneider et al. 2000) for samples contained in each of the main lineages or clades identified in the phylogeny and the NCA. Harpending (1994) raggedness index was used to evaluate deviation from the null expectation of no population expansion.

**Results**

**Summary of samples**

We sequenced 1847 nucleotides of mitochondrial DNA; 1093 bases of partial cyt $b$ sequence and 754 bases of partial CR sequence. We found 35 distinct haplotypes from 82 individuals; nine haplotypes were found in multiple individuals and 26 haplotypes were represented by single individuals. The most frequently sampled haplotype, designated as ‘A’ in the phylogeny (Fig. 3), was found in 27 of the 82 individuals (33.3%) and was present in 11 of the 31 localities. All localities that contained this widespread haplotype are located either within the Columbia River Valley or north of the valley into Washington state.

**Phylogenetic analyses**

There is clear separation of populations into two main lineages corresponding to northern and southern localities.
This topological pattern is consistent across MP, ML, and Bayesian estimations of the phylogeny and is well supported by MP and ML bootstrap support as well as Bayesian posterior probabilities (Fig. 3). There were 98 parsimony-informative sites in the complete data set and the MP analysis found 38 equally parsimonious MP trees with a tree length of 353 steps. Topology of MP trees was similar to that of the single best ML tree (–ln 4620.7173). The Bayesian topology (Fig. 3) was the most resolved and, except for a minor difference of relationships at the tips within the southern clade, has an identical topology to that of MP and ML analyses.

Within the northern clade, there are two well-supported sister clades. One clade includes an isolated population at Oak Springs, Oregon, and the other includes localities in the Columbia River Valley and throughout Washington state (Fig. 3). The southern clade, which contains the remainder of localities in Oregon and California, is also split into two weakly supported sister clades. One lineage corresponds to coastal localities extending from the Klamath-Siskiyou Mountains along the Oregon Coast Range to the mouth of the Columbia River and includes localities in the Cascade Mountains of Oregon. The other lineage corresponds to localities extending southward from the Klamath-Siskiyou Mountains into California. The overall phylogenetic pattern of two well-supported clades corresponding to northern and southern localities is suggestive of two Pleistocene refugia for this species.

**Pleistocene hypotheses**

Coalescent simulations conducted in mesquite were run using the estimates of \( N_e \) calculated from the population parameter \( \theta \) for the entire population and for populations occurring in each of the purported refugia. Using migrate we calculated: \( \theta_{\text{Total}} = 0.01926, N_e_{\text{Total}} = 601,875; \theta_{\text{Columbia}} = 0.00101, N_e_{\text{Columbia}} = 31,563; \) and \( \theta_{\text{Klam-Sisk}} = 0.00887, N_e_{\text{Klam-Sisk}} = 277,188. \) Slatkin and Madison’s S was calculated in mesquite as S = 1 for the observed data. The model of evolution for ingroup sequences used in the coalescent simulations was: HKY + I + G, I = 0.8855, G = 0.7502, transition/transversion = 3.0292, A = 0.3201 C = 0.1739 G = 0.1564, T = 0.3492.

Results of coalescent simulations indicate that we could reject the hypothesis of a single refugium located in the Columbia River Valley (\( P < 0.0001 \)) or the Klamath-Siskiyou Mountains (\( P < 0.0001 \)). We could also reject the hypothesis of dual refugia with a separation date in the late Pleistocene (\( P < 0.0001 \)) but not hypotheses predicting dual refugia with a separation in the early Pleistocene (\( P > 0.99 \)) or mid-Pleistocene (\( P > 0.99 \)).

**Nested clade analysis**

The haplotype network consisted of two main networks that could only be joined with a nonparsimonious connection of 25 steps (Fig. 4). These two groups corresponded to the northern and southern lineages identified in the phylogeny. Some ambiguous connections caused by loops are present in the network but were resolved using nesting procedures from Templeton et al. (1992) and Templeton & Sing (1993) and ultimately do not affect nesting design or conclusions inferred from the analysis. The overall pattern of the NCA is consistent with phylogenetic results and coalescent hypotheses tests, suggesting two Pleistocene refugia for this species.

Five nested haplotype networks had significant association with geography and inferences for these groups are given in Table 2. Significant associations with geography within the northern lineage include the nested clade 1-2 which was identified as isolation by distance (Fig. 5).
Testing Pleistocene Refugia Hypotheses

The entire northern network (clade 4-1) had significant geographic association defined as allopatric fragmentation of the isolated population at Oak Springs. In the southern clade there were three clades with significant association with geography. Nested clade 4-5, which encompasses Fig. 5 Results of nested clade analysis overlaid on a map of sampled populations. The haplotype network comprised a northern clade (populations 1–12, 15–16) and southern clade (13–14, 17–31) which could only be connected with a nonparsimonious connection of 25 steps. Clade identities are indicated in parentheses.

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Table 2 Results of nested clade analysis on haplotypes of the Pacific giant salamander (*Dicamptodon tenebrosus*). Haplotype networks without significant geographic associations are not listed

<table>
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<tr>
<th>Clade</th>
<th>( \chi^2 ) statistic</th>
<th>Probability</th>
<th>Inference chain</th>
<th>Inferred pattern*</th>
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*RGF, restricted gene flow; IBD, isolation by distance.
Discussion

The role of Pleistocene glaciation in structuring contemporary genetic variation has been an active area of research and phylogenetic patterns are often interpreted in the context of postglacial expansion from glacial refugia (Hewitt 1996; Ibrahim et al. 1996; Avise et al. 1998). Dicamptodon tenebrosus has been extensively studied using morphological (Nussbaum 1976) and electrophoretic methods (Daugherty et al. 1983; Good 1989) but our results are novel in the detection of two distinct lineages corresponding to northern and southern populations. Perhaps the most intriguing implication is the identification of the Columbia River Valley as a Pleistocene refugium for the northern populations. The Columbia River Valley is often implicated as a refugium for fishes (Brown et al. 1992; Taylor et al. 1999), but this has not been the case for terrestrial taxa.

Postglacial expansion

Results of this study show two well-supported lineages corresponding to northern and southern populations. Estimates of divergence between these lineages indicate separation since the early to mid-Pleistocene. The localities of northern populations encompass the purported Pleistocene refugium of the Columbia River Valley, while apparent northward expansion of southern populations supports another purported refugium in the Klamath-Siskiyou Mountains. Coalescent simulations also support the hypothesis of two Pleistocene refugia for this species. The combination of these results provides strong evidence that this species was restricted into at least two Pleistocene refugia in the Pacific Northwest.

Isolation by distance of the northern populations suggests a slow and gradual northward expansion from the Columbia River Valley to the southern banks of the Fraser River in British Columbia which forms the northernmost boundary of the species and apparently limits further expansion. The isolated population at Oak Springs which is included within the northern lineage, was probably connected by suitable habitat to populations in the Columbia River Valley during the initial north–south split and became isolated only relatively recently. Populations in the southern refugium of the Klamath-Siskiyou Mountains expanded northward along either side of the Willamette Valley of Oregon. One route was along the coastal mountain ranges of Oregon to the mouth of the Columbia River while the other was an inland route along the Oregon Cascades. The Columbia River and its gorge appear to be effectively preventing migration and mixing between the two lineages since no southern haplotypes were found north of the Columbia River and no northern haplotypes (excluding Oak Springs) were found south of the Columbia River Valley. The southern limit of the species is defined by a narrow zone of secondary contact.

Table 3 Genetic distances within and between northern and southern lineages. Corrected genetic distances are with the HKY + 1 + G model of sequence evolution

<table>
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<tr>
<th>Populations</th>
<th>Uncorrected</th>
<th>Corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within northern</td>
<td>0.00499</td>
<td>0.00592</td>
</tr>
<tr>
<td>Within southern</td>
<td>0.00957</td>
<td>0.01188</td>
</tr>
<tr>
<td>Between clades</td>
<td>0.01953</td>
<td>0.03163</td>
</tr>
</tbody>
</table>

localities in the Klamath-Siskiyou Mountains, is defined as isolation by distance. Clade 5-1, which corresponds to coastal localities from the Klamath-Siskiyou Mountains to the mouth of the Columbia, is defined as range expansion. Clade 5-2 includes the nested clade 4-5 and additional populations in the Cascade Mountains of Oregon and is also defined as range expansion.

Diversity, divergence, and demographic growth

AMOVA revealed 68.4% of the variation is explained by the north–south split and that 78.3% of the variation is explained by partitioning populations into the four main groups identified by the NCA. The average nucleotide diversity estimated for the southern lineage (π = 0.009) is four-and-a-half times greater than the northern lineage (π = 0.002). Southern populations also had a higher number of haplotypes (25) and polymorphic sites (S = 80) than northern population (haplotypes = 10, S = 27). Genetic distances within and between the two lineages is shown in Table 3.

The cyt b sequence data for the northern and southern lineages identified in the phylogeny and NCA were analysed in MDIV to estimate time since divergence of these populations. Results estimated θ = 10.33 and T = 0.002. Using a mutation rate of μ = 1.6 × 10⁻⁸, an estimate of time since divergence between the northern and southern clades was placed during early to mid-Pleistocene at 645,625 years ago with a 95% confidence interval of 971,875 to 319,373 years ago.

We analysed all sequence data from each of the significant clades identified by the NCA for population growth in FLUCTUATE. Demographic expansion of the entire northern clade (4-1) was not significant when the isolated population at Oak Springs (locality 16) was included (g = 224.82 ± 144.1), but was significant when this allopatric population was removed (clade 3-1; g = 10,000 ± 3045.67). There was also evidence of significant demographic expansion in each southern clade showing range expansion in the NCA: clade 5-1 (g = 871.30 ± 126.86; clade 5-2 (g = 755.54 ± 81.48). Results of mismatch distributions were also consistent with a pattern of demographic growth. The model of population expansion could not be rejected for southern clades 5-1 (P = 0.53), 5-2 (P = 0.72), nor for the northern clade (P = 0.79).
in northern California with the California giant salamander (*Dicamptodon ensatus*) (Good 1989). Populations in the Klamath-Siskiyou refugium may have gradually expanded southward into northern California to form this contact zone with northward expanding *D. ensatus*.

### Pleistocene refugia

Traditionally, the Columbia River Valley has not been considered a Pleistocene refugium for terrestrial organisms. It has only recently been identified as a Pleistocene refugium for the Larch Mountain salamander (*Plethodon larselli*), which has expanded northward along the Cascade Mountains of Washington state (Wagner et al. 2005). Additionally, the northern populations of the Oregon slender salamander (*Batrachoseps wrighti*) that diverge in mitochondrial DNA are closely associated with the Columbia River (Miller et al. 2005). However, these are examples of species with restricted distributions adjacent to the Columbia River Valley. When north–south splits in genetic data attributable to Pleistocene glaciation are discovered in a widely distributed species, the typical refugia proposed for northern populations include the Olympic Peninsula, the Queen Charlotte Islands or southeast Alaska (Soltis et al. 1997; Conroy & Cook 2000; Janzen et al. 2002). However, it is unlikely that Pacific giant salamanders resided in any one of these refugia, because their current distribution is neither in nor near these locations. The Columbia River and its tributaries are more often regarded as a Pleistocene refugium for fish species (Brown et al. 1992; Bickham et al. 1995; Taylor et al. 1999; McCusker et al. 2000; Haas & McPhail 2001). While some fish may prey upon larval salamanders, presumably some of these tributaries in the Columbia River Valley were fishless and would have provided suitable habitat for stream-breeding salamander larvae.

In contrast to the Columbia River Valley, the Klamath-Siskiyou Mountains have been proposed or implicated as a Pleistocene refugium for a variety of organisms (Soltis et al. 1997; Wake 1997; Brunsfeld et al. 2001; Wilke & Duncan 2004; Kuchta & Tan 2005). The area remained unglaciated throughout the Pleistocene and is known for its complex geology and a range of climates which have contributed to the region’s biological diversity and endemism (Whittaker 1960; Noss et al. 1999). The restricted distribution of the Del Norte salamander (*Plethodon elongatus*), Siskiyou salamander (*Plethodon elongatus*) and the recently discovered Scott Bar salamander (*Plethodon asupak*) (Mead et al. 2005) attest to the diversity and endemism of the region.

The detection of two well-defined lineages corresponding to northern and southern populations has also been documented in other codistributed taxa (Soltis et al. 1997; Kuchta & Tan 2005). In these cases, the highest genetic diversity was within the southern populations and lowest genetic diversity in the northern populations. This pattern could result from northward expansion of populations; however, the two highly divergent clades within *D. tenebrosus* and other organisms (Soltis et al. 1997; Kuchta & Tan 2005) suggest separation and isolation into two Pleistocene refugia. The high genetic diversity of southern populations of *D. tenebrosus* encompasses the Klamath-Siskiyou Mountains indicates that the southern refugium was larger than the northern refugium or had a larger ancestral population.

### Regional phylogeography

The patterns observed from this research add to our understanding of the role of Pleistocene refugia in regional phylogeography of the Pacific Northwest. The results provide additional evidence of a Pleistocene refugium in the Klamath-Siskiyou Mountains and further support the importance of a Columbia River Valley refugium for terrestrial taxa. Genetic structure of several taxa with distributions similar to that of the Pacific giant salamander have been examined but do not always show a distinct north–south split corresponding to separation and isolation in two Pleistocene refugia. Phylogenetic patterns in a mollusk (Wilke & Duncan 2004) and the *Ensatina* salamander (Wake 1997) suggest expansion from one or more southern refugia in the Klamath-Siskiyou Mountains, while patterns in a salamander (Wagner et al. 2005) and several fish species (Taylor et al. 1999; McCusker et al. 2000) provide examples of expansion from a northern refugium in the Columbia River Valley. Other taxa such as garter snakes (Janzen et al. 2002), newts (Kuchta & Tan 2005), and a variety of plant species (Soltis et al. 1997) have a more defined north–south split fitting the hypothesis of northern and southern refugia in the Pacific Northwest proposed by Brunsfeld et al. (2001).

Various studies have examined the explicit hypotheses proposed by Brunsfeld et al. (2001), which invoke either ancient vicariance or recent dispersal, for explaining disjunct distributions of mesic forest taxa located in the coastal Pacific Northwest and the inland northern Rocky Mountains (Nielson et al. 2001; Carstens et al. 2004, 2005b; Steele et al. 2005). Results of these studies indicate that codistributed amphibians have a similar pattern of deep divergence between coastal and inland populations consistent with the ancient vicariance hypothesis (Carstens et al. 2005a). Within the northern Rocky Mountains, amphibians also have concordant phylogenetic patterns indicating similar response to Pleistocene glaciation (Carstens et al. 2004, 2005b). The phylogenetic concordance across amphibians in this region suggests that these organisms responded similarly to geological events. Opportunities exist to investigate whether the patterns of concordance in amphibian phylogenies is also apparent in coastal populations of the Pacific Northwest. Within this region are assemblages of codistributed species including the northwestern salamander (*Ambystoma gracile*), the western red-backed salamander...
(Plethodon vehiculum), and the tailed frog (Ascaphus truei).
Examining genetic structure across a variety of organisms and testing for concordant phylogenies will undoubtedly provide insights into the evolution of communities in general and within the Pacific Northwest.

Conclusions

Pleistocene glaciation has often influenced the genetic structure of species and studies of taxa within the Pacific Northwest regularly reveal distinct lineages often attributed to isolation within northern and southern Pleistocene refugia. The location of a southern refugium in the Klamath-Siskiyou Mountains is generally accepted but a variety of locations for northern refugia exist. Results of this study indicate the Columbia River Valley as a refugium from which northern populations of the Pacific giant salamander (Dicamptodon tenebrosus) expanded. This refugium has been generally established in phylogeographic studies of fishes in the Pacific Northwest but is not well recognized as a potential refugium for terrestrial taxa. It still remains to be seen whether codistributed taxa also have genetic patterns suggestive of dispersal from a Columbia River refugium.

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This research was conducted as part of Craig A. Steele’s PhD dissertation at Washington State University. His main research interests include phylogeography and determining the evolutionary or ecological processes that lead to genetic structuring of populations. Andrew Storfer studies limits to species’ ranges and uses population genetics as a tool to understand the factors that shape distributions of species. He is also interested in host–pathogen co-evolution and conservation of amphibians.

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