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Phylogeographic and Phylogenetic Exploration of *Plethodon* (Plethodontidae, Caudata) Salamanders in the Pacific Northwest

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ABSTRACT

An abstract of the thesis of Tara Anne Pelletier for the Master of Science in Biology presented June, 9 2009.

Title: Phylogeographic and Phylogenetic exploration of *Plethodon* (Plethodontidae, Caudata) Salamanders in the Pacific Northwest

Genetic studies of amphibians often reveal substantial population structure due to either historical demographics from changing climate and geographic features over varying timeframes. Eight species of terrestrial salamanders (Family: Plethodontidae, Genus: *Plethodon*) reside in forests of the Pacific Northwest (PNW). *Plethodon vehiculum* is the most widespread and abundant terrestrial salamander in the PNW yet evolutionary studies are lacking. Using mitochondrial DNA (mtDNA) sequence data (D-loop and cytb) questions regarding the phylogeography of *P. vehiculum* and phylogenetics of western *Plethodons* are explored. Two major clades were defined in *P. vehiculum*, a southern clade in the Klamath-Siskiyou region and a northern clade ranging from northern Oregon to British Columbia using parsimony and maximum likelihood trees and a haplotype network. High divergence levels between the north and south clades are observed warranting further investigation into the southern clade's unique evolutionary trajectory. The northern populations were not highly differentiated with high levels of haplotype sharing, not common in other terrestrial salamander species. A large recent range expansion or high habitat connectivity for

these salamanders is suggested. The Columbia River did not act as barrier to dispersal in this species, however, Vancouver Island and the population of Washington's Olympic Peninsula revealed unique haplotypes only to those areas, due to the presence of geographic barriers to dispersal and/or multiple glacial refugia. The D-loop and cytb provided evidence for recent range expansion in the northern clade.

This was the first study to incorporate all western *Plethodon* salamanders in a phylogenetic study. Parsimony and maximum likelihood methods offered strong support for recognized relationships among western *Plethodons*, however relationships between the major groups remain unhighly supported. Lack of genetic diversity in the mtDNA cytb gene in *P. vehiculum* is highly inconsistent with other *Plethodon* salamanders and highlights the importance of understanding mtDNA evolution in ectotherms. Divergence measures were used to estimate divergence times among species, dating all speciation before the Pleistocene glaciations. The southern OR clade of *P. vehiculum* was dated to have been separated from the northern clade at the start of the Pleistocene. The deep phylogeographic break here justifies the possibility of reclassification of the southern clade.

PHYLOGEOGRAPHIC AND PHYLOGENETIC EXPLORATION OF *PLETHODON* (PLETHODONTIDAE, CAUDATA) SALAMANDERS IN THE PACIFIC NORTHWEST

by

TARA ANNE PELLETIER

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

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DEDICATION

This thesis is dedicated to my parents who have always encouraged me to follow my heart and have supported my dreams.

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Table of Contents

List of Tables

List of Figures

Chapter 1: General Introduction

The woodland salamanders, Genus *Plethodon,* (Family: Plethodontidae; Order: Caudata) are fully terrestrial, lungless salamanders. Direct development frees them from the need to breed in water, but all require moist, often relatively undisturbed, forests (Dumas 1956). Plethodontidae includes almost 400 species, which make up almost all species of salamanders (Myers et al. 2008). The subfamily Plethodontinae consists of 25 genera of salamanders found in North and South America, as well as Europe. There are approximately 45 recognized *Plethodon* species in North American forests. Eastern and western counterparts exist and are thought to have begun divergence over 40 MYR ago and both form a monophyletic group (Highton 1995). As a group, *Plethodon* salamanders display extremely high levels of local differentiation (Gibbs 1998). Eight recognized species of these salamanders – *P. vehiculum, P. dunni, P. elongatus, P. stormi, P. asupak, P. larselli, P. vandykei,* and *P. idahoensis* – reside in the Pacific Northwest (PNW), including: British Columbia, Idaho, Washington, Oregon, and northern California (Fig. 1).

The Western Red-backed Salamander (*Plethodon vehiculum*) is the most abundant and widespread terrestrial salamander in the PNW, however, ecological/evolutionary studies are lacking and there are no known data describing the genetic distribution of *P. vehiculum.* In addition, *P. vehiculum* has the highest population density of Plethodontids in the Portland, Oregon region, exceeded only by *Ensatina eschscholtzii,* although their relative abundance tends to fluctuate (Davic and Welsh 2004, Roberts 2005). A long evolutionary past, high population density, wide

distribution, and low powers of dispersal of salamanders make *P. vehiculum* an excellent candidate for the study of the effects of habitat fragmentation and population history on current population genetic structure. Using mitochondrial DNA (mtDNA) sequence data, questions regarding the population structure of *P. vehiculum* can be explored. Many studies of population genetic structure in salamanders have established that substantial subpopulation structure exists (Templeton et al. 1990, Phillips 1994, Donovan et al. 2000, Schaffer et al. 2004, Miller et al. 2005, Miller et al. 2006, Kuchta et al. 2009). Further, previous descriptions of the relationships between species of Plethodontid salamanders have been carried out using morphological, or allozyme data, but these relationships are rather ambiguous (Brodie 1970, Highton and Larson 1979, Wake 1993). Mahoney (2001) further studied these relationships using two mtDNA genes, however conclusions were not strongly supported. With the integration of all known western *Plethodon* species and exploring an mtDNA gene commonly used in phylogenetic studies, these species relationships can be better acknowledged. By using the techniques of population genetics and phylogenetics we can begin to understand the level at which the processes of evolution (i.e. mutation, migration, drift, selection, and vicariance) are contributing to the current abundance and distribution of these species.

Terrestrial salamanders generally occur in rocky outcrop-talus slopes and can be regularly found from sea level to 760 m (2,500 ft), although they have been found up to 1250 m (Dumas 1956). Typically, *Plethodon* salamanders exhibit high site

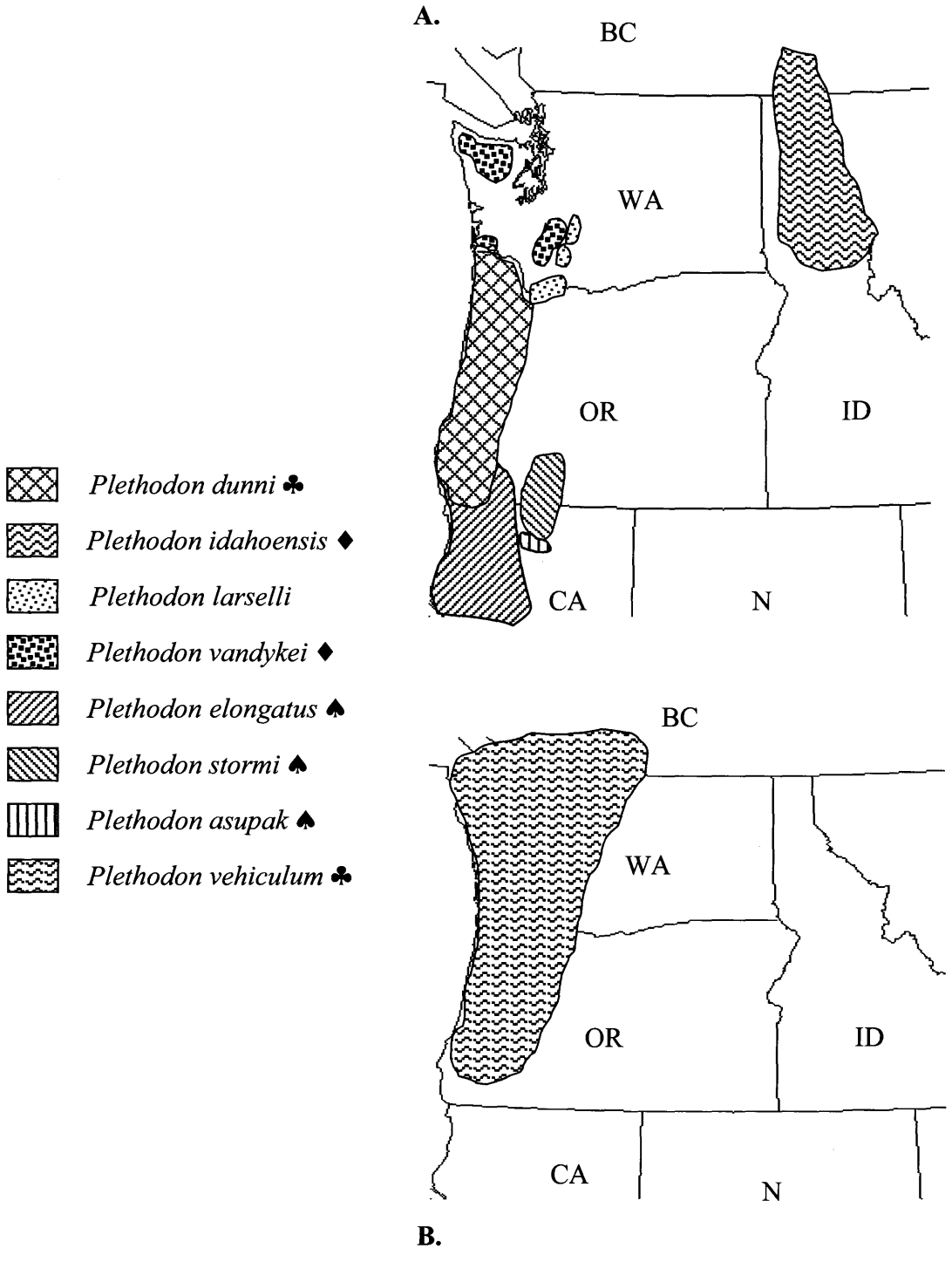


Figure 1. *Plethodon* distribution map in the Pacific Northwest. **A**. All known *Plethodon* species ranges. **B**. The range of *P. vehiculum*. Matching symbols indicate sister relationships.

fidelity and defense of small territories. Studies in eastern North America show a 90% return rate to home range in Red-Backed Salamanders (*P. cinereus*) after displacement of 30 meters (100 ft) and a 25% return rate when displaced to 90 meters (300 ft), even after two weeks (Kleeberger and Werner 1982). Similar results have been obtained for other Plethodontids indicating they have limited capability for long-range movement (Smith and Green 2005), however, during optimum conditions salamanders are able to move into adjacent microstands of similar habitat.

In the forests of the PNW the home range of *P. vehiculum* is quite small (<3 m^2; Ovaska 1998). In particular, it has been shown that *P. vehiculum* displays extreme site specificity and spends most of the time under the same available cover objects (Ovaska 1998), which include rocks, logs, planks, bark, and duff. *Plethodon vehiculum* prefer a moist substratum but are said to avoid standing puddles or running water and are active in areas with a mean relative humidity of roughly 90% (range: 63% to 100%) and a mean temperature of 10.4° C (range: 5° C to 19° C).

Given the climatic constraints on activity, *P. vehiculum* has a fall season (September – November) and a spring season (March – June) of surface activity (rains permitting). Subfreezing temperatures force salamanders underground during the winter months, although it has been known to find them throughout the winter season (Ovaski and Gregory 1989). The summer draught period (July-October) of the PNW also forces them underground due to lack of surface moisture. As of now, the percentage of the population active on the surface at any one time for Plethodontids is unknown. Males may spend more time active on the surface searching for mating

opportunities as males reproduce annually while females often reproduce every second or third year (Ovaski and Gregory 1989). Mating occurs in the fall, winter, and spring, while eggs are laid in the spring and have often been found brooded by females in some Plethodontid species (Stebbins 1985). Juveniles hatch in the fall and are forced to disperse until they establish a territory of their own and therefore tend to be more active on the forest floor during drier conditions in order to find suitable microhabitat.

Salamanders will seldom disperse across habitats that expose them to dryness and heat (Gibbs 1998). Therefore, habitat connections can influence the genetic structure of salamander populations in human-dominated landscapes or areas with major geological/geographical barriers. For example, rivers, roads, or similar large features, will hinder successful migration between populations but the presence of forest corridors may facilitate dispersal between subpopulations, causing them to remain more genetically similar and constraining their divergence. On the other hand, higher rates of dispersal could serve to unite locally disjunct populations. In spite of this, movements of salamanders are poorly recognized and infrequently quantified (Wagner et al. 2005*). Plethodon vehiculum* has been observed to move nine meters, but other *Plethodon* species have been found to move over 100s of meters (Smith and Green 2005). These studies are done using mark-recapture techniques, which can be time-consuming and difficult to assess. At any one time, it is impossible to determine whether the rate of recapture is influenced by migration, death, or limited surface activity. By using molecular techniques we may be better able to investigate their dispersal capabilities.

Due to high site fidelity and long historical presence of Plethodontid salamanders, past population demographics influenced by glacial refugia or changes in climate and geologic arrangement can have profound effects on the genetic structuring of current populations. Certainly, *Plethodon* salamanders, although morphologically, ecologically, and behaviorally constrained, show high levels of genetic differentiation among populations (Larson et al 1984, Wake 1993). The PNW has an extensive history of climatic and geologic changes which have affected the distribution and abundance of many North American plant and animal taxa during the late Pliocene (Pielou 1991) and Quaternary Period (Hewitt 2004), as well as the Miocene Epoch (Cerling 1997) (Fig. 2). Terrestrial salamanders will have been affected by these changes, as the dissemination of suitable habitat and dispersal corridors have continually expanded and retracted during these geologic cycles. Change in topography over time would influence the ability for dispersal among both nearby and distant populations, balanced by multifarious selection pressures, such as, temperature, moisture, canopy cover, competition, and local predators, all of which could further increase the rate of divergence among populations and influence their current distribution.

Pacific Northwest Species Description

Plethodon vehiculum has a long body (37-64 mm SVL), a tail being as long as the snout-vent length, 16 costal grooves and short legs. The dorsal stripe is straight edged and usually red, although sometimes appearing yellowish/gold or orange. The

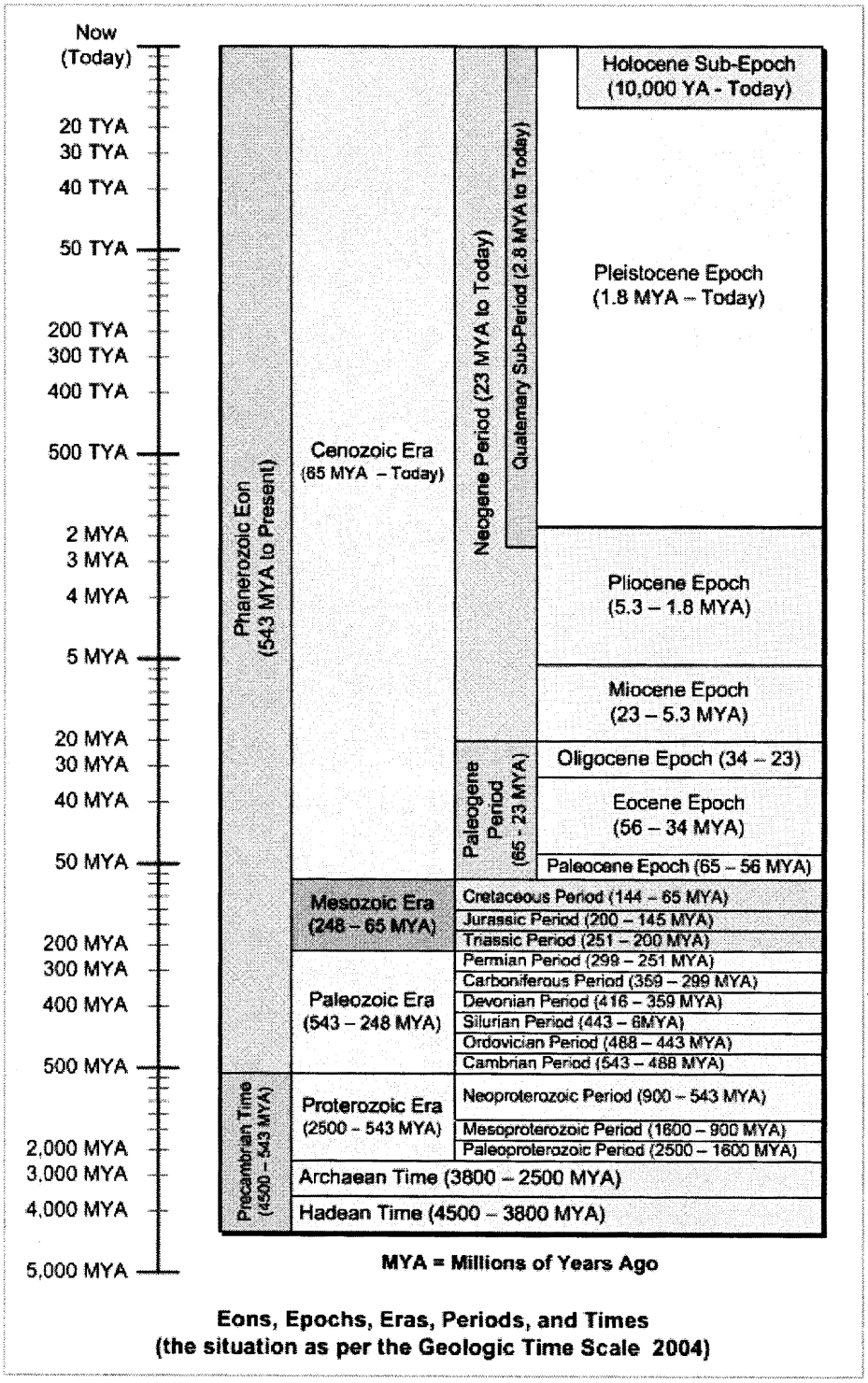


Figure 2. Map of geologic time.

stripe is solid to the tip of the tail and occurs on the upper surfaces of the legs. The sides and belly show salt and pepper markings. The International Union for the Conservation of Nature (IUCN; 2009) lists this species as 'least concern', meaning populations are presumed to be stable.Other species from the Genus *Plethodon* inhabiting the PNW (see Fig. 1) display enough differences in their morphology allowing for nearly unmistakable

identification (Brodie 1970, Stebbins 1985, Corkran and Thoms 2006, personal observation), although this can sometimes be difficult (Highton 1995). Most of these species interestingly have much smaller, disjoint ranges nested within or adjacent to the distribution of *P. vehiculum*. *Plethodon dunni* is the largest (50-75 mm SVL) of these species, and has the second largest distribution. It also has IUCN 'least concern' designation, and is classified as the sister species to *P. vehiculum* (Highton and Larson 1979, Mahoney 2001). Their preferred habitat is similar to that of *P. vehiculum* and they can often be found living in the same geographic area. They are identified by 15 costal grooves and also have a tail similar in length to the body. Their ragged stripe is usually dark yellow to olive colored and does not extend to the tip of the tail, while patches of stripe color appear on the sides of the body and the tops of the legs. The underbelly has white flecks.

With a longer body proportion (60-77mm SVL) than the other species, *P. elongatus* has 18 costal grooves and a tail that is about as long as the body but tends to be wider than other *Plethodons*. The even dorsal stripe is orange but often vague or dull, and the legs and sides of the body may have speckles of stripe color. It's sister

species, *P. stormi* (Mahoney 2001, 2004), also has a long body (60-70mm SVL) but with 17 costal grooves. Adults are pink/tan to light brown with white flecks, and the dorsal stripe is composed of light pink/gold dots. The most recently described species, *P. asupak* (58-67 SVL), is morphologically distinct from *P. elongatus* and *P. stormi* (Mead et al. 2005). It has 17 costal grooves, a brown or bronze dorsal stripe extending to the tip of the tail, a gray chin, and white/yellow flecks all over the body. *Plethodon asupak* has not yet been listed by the IUCN, while *P. elongatus* is listed as 'near threatened' and *P. stormi* as 'endangered'.

Plethodon vandykei (44-60mm SVL) has 14 costal grooves, a semi-solid stripe color of yellow, orange, or pink, and a demarcating pale yellow throat. Its sister species, *P. idahoensis*, is very similar morphologically but tends to have a more narrow dorsal stripe and is usually darker in color. Both appear shorter and stockier than other *Plethodon* salamanders in the PNW. Both are IUCN listed as species of 'least concern'. *Plethodon larselli* (37-53mm SVL) can be easily distinguished by its salmon colored belly; its often speckled dorsal stripe is reddish or tan with a dark line down the middle and extends to the end of the tail. The tail is often shorter than the length of the body and they possess 18-19 costal grooves. *Plethodon lareselli* is currently listed as 'near threatened'.

Population structure

The genetic structure of populations is a product of the balance between mutation, migration, drift, and natural selection and determines the level of genetic

diversity that exists within a population. The most effective means of describing the roles of these processes is through complementary studies revealing current population dynamics and historical patterns of gene flow (Phillips 1994). By themselves, field-based demographic studies often have limited capacity to shed light on historical events or even contemporary levels of gene flow that contribute to existing patterns of genetic variation. For example, Breden (1987) marked 25,000 individual toads and after five years, only 37 individuals were recaptured. Genetic analysis is a useful addition because data can be added that reflects long-term trends that can be used to provide explanations for current genetic structuring among populations.

The long-term survival of species is contingent upon the existence of adequate levels of genetic variation enabling populations to evolve in response to environmental changes (Frankham 1996), and as a consequence of both environmental and demographic stochasticity, small populations inevitably lose genetic variation (Hedrick and Miller 1992). Natural barriers to dispersal can isolate populations and lead to high degrees of genetic distinctiveness among native populations with risk of loss of variation as a result of reduced population numbers. Anthropogenic development can exacerbate such processes and increase extinction probabilities of local populations, and in some cases, entire species (Templeton et al. 1990).

Genetic techniques can be used to assess levels of variation and differentiation within and among populations. Thus, conservation genetics embraces ecology, molecular biology, population genetics, and evolutionary systematics and can be used as an important tool to identify and manage populations of concern (Moritz 1994,

Paetkau 1999). The goal of conservation genetics is to retain enough genetic variation in a population to allow successful future adaptation to environmental changes, as well as to allow for the possibility of expansion and/or the reestablishment of natural populations (Hedrick and Miller 1992). Even when there is adequate natural habitat and species have strong protection, random extrinsic or intrinsic factors could cause populations to become endangered or even extinct.

MtDNA sequence variation is commonly used to examine the geographic structure of populations and to explore patterns of variation within species (Moritz et al. 1992, Avise 1995, Donovan et al. 2000, Gibbs 2001). Studies such as these, use mtDNA sequence data to measure genetic diversity and phylogeography of organisms with the potential for population isolation due to vicariance and/or low vagility. Habitat fragmentation results from the deconstruction of once continuous habitat into smaller isolated habitats and depending upon the dispersal capacities of species, populations in fragments may experience a loss of gene flow (Larson et al. 1984). Fragmentation may occur through human induced changes or naturally as a result of geological processes or climatic events that influence a population over varying time frames. It can result in a reduction of effective population size, the reduction of fitness due to increased possibility of inbreeding depression (Hedrick and Miller 1992), the possible accumulation of deleterious mutations (Lande 1994), and genetic drift is more common in small populations resulting in the random fixation and/or loss of alleles over time (Hedrick and Miller 1992). Eventually, loss of variation will reduce population fitness and limit the ability to respond to changing selective pressures

(Hedrick and Miller 1992, Young et al 1996). The consequence is a continuing spiral of local extinctions eventually leading to the endangerment of a species, which Caughley (1994) describes as the *small population paradigm*. Current increasing rates of extinctions demand that more attention be focused on conservation genetics (Hedrick and Miller 1992, Hedrick 2001).

MtDNA has a mutation rate five to ten times that of nuclear DNA, however, this rate may not be constant across taxa (see Avise and Ellis 1986, Moritz et al. 1987). However, because of its relatively high rate of mutation, genetic variation is common among conspecifics and it is a useful tool in describing gene flow, founder events, and other processes at the population level, especially when females are more sedentary than males (Moritz et al. 1987, Hedrick and Miller 1992). As population success is contingent upon female reproduction, the recovery or reestablishment of a disturbed area is unlikely where females have limited dispersal capabilities (Avise 1995). As a result, it is important to understand the geographic distribution and migration of females. Maternal mode of inheritance makes mtDNA excellent for studies of matrilineal gene flow; a wealth of intraspecific phylogeographic studies have demonstrated significant genetic structuring at the mtDNA level that can be easily explained geographically (see Avise and Waker 1999). The evolutionary and demographic history of a species origin, past bottlenecks, and range expansion may also be inferred through mtDNA analysis (Avise and Ellis 1986).

The D-loop control region is known to undergo a higher mutation rate than other regions of the mitochondrial genome as it is responsible for replication and

transcription, it does not code for protein (Moritz et al. 1987). This region is therefore expected to show a higher resolution of genetic variation within species and shed more light on contemporary patterns of genetic distribution than the very deep phylogenetic relationships that are established using a more highly conserved portion of the genome. Intraspecific plylogeographic studies have commonly used mtDNA sequence data to describe patterns and processes of population structure in salamanders residing in the PNW (Carstens et al. 2004, 2005, Mahoney 2004, Wagner et al. 2005, Miller et al. 2005, 2006, Kutcha et al. 2009).

Phylogenetics

The discovery and delimitation of unique phylogenetic lineages has been used to define monophyletic groups that have undergone irreplaceable evolutionary trajectories (Shaffer et al. 2004). Phylogenetic techniques additionally give us the ability to build hypotheses regarding the relationships among taxa and the timing of speciation events (Barraclough and Nee 2001). This is important for conservation purposes by documenting loss of, and current levels of, diversity (Moritz 1995), and by promoting our understanding of local adaptation and speciation (Barraclough and Nee 2001).

The additional information we gain from the use of genetic approaches, can give important insights on how to protect existing biodiversity at both the population and species levels (Avise et al. 1987, Barraclough and Nee 2001). Pacific Northwest *Plethodon* salamanders are a fascinating group in view of the fact that, unlike the

eastern group, many of the western species occur in sympatry with one another and often times demonstrate extensive overlap in their ranges (see Fig. 1). The most recent phylogenetic study of *Plethodon* salamanders in the PNW was carried out by Mahoney (2001) using the ND4 and tRNA regions of the mtDNA. The relationship between *P. elongatus* and *P. stormi* as sister species, and *P. dunni* and *P. vehiculum* as sister species was strongly supported as in previous studies, but no further support arose regarding the relationship of these lineages to others or each other. A close relationship was weakly supported between *P. vandykei* and *P. larselli* but no further relationships were supported between these lineages. *Plethodon elongatus* and *P. stormi* were strongly supported as sister species (Mahoney 2004), with an average of 8.1% sequence divergence between the two using sequence data of the mtDNA genes, cytochrome b, NADH 4, and ATPase 6. The third species of this group, *P. asupak,* was differentiated from both *P. elongatus* and *P. stormi* by 10-14% sequence divergence, higher than the degree of differentiation between *P. elongatus* and *P. stormi*, (Mead et al. 2005). Also using mtDNA sequence data, Carstens et al. (2004) explored the levels of divergence and time of speciation among the *P. vandykei* and *P. idahoensis* sister species and found deep divergence within this group (8.5-10.6%) dating the split to 5-2 million years before present. These studies offer evidence of the overall relationship and timing of speciation but, the relationships between species groups are still vague and the processes underlying the high degree of sympatric speciation in salamanders remains to be understood.

Cytochrome b (cytb) has shown considerable variation within species of other salamanders, often leading to the description of new taxa (Mahoney 2004, Mead et al. 2005, Wagner et al. 2005), and therefore has strong potential for reconstructing phylogenetic hypotheses regarding the PNW *Plethodon* salamanders.

Aims and Significance

Losses of biodiversity are at an historical high as a consequence of human activities (Hedrick and Miller 1992). The most immediate threat to populations with dwindling numbers is clearly chance extinction due to demographic processes (i.e. sex ratio, age structure), but the loss of genetic variability is of equal concern as it determines the ability for a population to persist over time. Studies of species in fragmented habitats are needed to better understand how loss of genetic diversity contributes to species endangerment, as well as describe speciation events stemming from isolation, and the most appropriate organism for such studies are those that are rather sedentary and which occupy well-defined patches of habitat (Brussard 1991).

Animals, such as salamanders, that have such specific habitat requirements will often occur in small, patchy distributions, and although adequate habitat area appears sufficient, habitat corridors may not exist, keeping these small patches of populations isolated (Stacey and Taper 1992). As a result, in a topographically diverse region like the Pacific Northwest, salamanders may exhibit considerable genetic variation. Genetic studies of amphibians often reveal cryptic genetic diversity due to vicariant events, even on small geographic scales (Mahoney 2004, Mead et al. 2005,

Wagner et al. 2005). Because of terrestrial salamanders' lack of mobility, they are easily divided into genetically isolated populations (Larson et al. 1984). By describing the population structure of *P. vehiculum* we can infer levels of divergence and investigate variation among regions of the PNW. The identification of populations as evolutionary significant units (ESUs) and management units (MUs) has been commonly made with the use of mtDNA sequence data (Moritz 1994, Paetkau 1999). An ESU is the recognition of a population or group of populations to be protected due to the evolutionary potential that group holds (genetically, geographically, phenotypically, or a combination of the three), thus the evolutionary trajectories of these groups must be preserved. MUs signify a population or group of populations displaying high levels of differentiation and should therefore be monitored and managed accordingly.

Almost three quarters of forested ecosystems in North America are considered endangered because of threats to their integrity (Davic and Welsh 2004). Habitat loss and fragmentation are threats to biodiversity around the world. The world's human population numbers 6.5 billion people and estimates by the United Nations are that it will reach 9.1 billion by the middle of the century (http://unstats.un.org 2009). Altering the forest ecosystem home to terrestrial salamanders may destabilize regional environmental cycles, affecting the viability of many organisms, decreasing local biodiversity (Brussard 1991, Davic and Welsh 2004). As amphibian numbers decline worldwide it becomes ever more important to understand their role in our ecosystems, including their population dynamics. In some areas (such as western North America)

amphibians are at a greater risk for extinction than mammals or birds (Wind 1999). Habitat modifications are the most cited reason for salamander declines (Davic and Welsh 2004). Terrestrial amphibians have low powers of dispersal and are sensitive to extreme shifts in the environment because they are often narrowly adapted to life in particular microhabitats (Dumas 1956, Wind 1999). Because terrestrial salamanders have limited ability to respond to environmental fluctuations, they are increasingly being recommended for use as indicators of ecosystem health and integrity of naturally forested areas. Studies have shown that population trends for salamanders can be detected more quickly than other vertebrate species (Davic and Welsh 2004). Understanding the population dynamics of terrestrial salamanders can prove useful in the future as long-term monitoring programs are implemented. For example, the issue of whether populations form a metapopulation is extremely relevant to their conservation and management (Smith and Green 2005).

Although *P. vehiculum* is not in any known danger at this time, it is important to develop baseline data and an understanding of the population structure of this salamander species. Any disturbances that reduce an ecologically dominant salamander species, such as *P. vehiculum*, could result in profound alteration of ecosystem functions (Davic and Welsh 2004). Understanding the intraspecific phylogeography of organisms that are not currently at risk for extinction can also serve as model systems for species that have the ability to persist over time. With measurements of regional population differentiation, we can investigate the genetic variation that may be important for the success of species, local populations, and

natural lineages. By implementing phylogenetic techniques interspecifically as well as intraspecifically with ecological and spatial data, the timing and cause of speciation events (Moritz 1995, Barraclough and Nee 2001) of *Plethodon* salamanders in the PNW can be inferred. Due to the evolutionary characteristics of mtDNA, it successfully serves the dual role as a tool for exploring questions in both phylogenetics and population genetics (Avise et al. 1987).

My research aims to investigate levels of divergence and genetic variation within and among populations of *P. vehiculum* and relate these patterns to contemporary and/or historical barriers to dispersal. Using a species such as *P. vehiculum* that encompasses a large geographical area to explore among-population mtDNA haplotype variation and distribution for the purpose of identifying past and present geographical or climatic barriers to dispersal will allow us to better understand the dispersal capabilities and demographic history of salamanders in general in the PNW. Samples from the parks and greenspaces within Portland, OR will be analyzed separately to assess microgeographic patterns of variation that may be associated with topographic or hydrologic barriers to dispersal (e.g. the Willamette River). This information will shed light on the pattern of isolation among the populations of this salamander species, and may inform assumptions about regional habitat connectivity. Essential baseline data will be generated which can assist in the development of a management plan that maintains recent and historical patterns of gene flow in *Plethodon* salamanders. It is hoped that the results of this phylogeographic study will allow for better protection of native populations of amphibians by assisting in future

management policies and long-term monitoring programs to allow more efficient conservation and management plans of parks, green spaces, and forested areas in the PNW.

The relationship between *Plethodon* salamanders of the PNW will also be explored. This will be the first study to evaluate the relationship among all known *Plethodon* species from the PNW using mitochondrial sequence data, and with multiple samples from each species. Increasing the size of data sets and using different mtDNA regions can provide a higher resolution of *Plethodon* phylogenetic relationships. This will allow for more accurate estimates of divergence times of these species and the formulation of hypotheses regarding the cause of speciation in this exceptional group of animals. In addition, levels of variation between these species will be evaluated in order to compare differences in dispersal capabilities and/or demographic history.

Study Sites and Sample Collection

The PNW experiences a relatively mild climate with an average precipitation of 90-254 cm per year but can vary among regions, with summers being dry (NOAA 2009). Terrestrial salamanders typically occur in areas with considerable canopy and ground cover (Dumas 1956, Burton and Likens 1975a, personal observation). Most forested areas where terrestrial salamanders occur are dominated by western hemlock (*Tsuga heterophylla*), Douglas-fir (*Pseudotsuga menziesii*), western red cedar (*Thuja plicata*), Sitka spruce (*Picea sitchensis*), red alder (*Alnus rubra*), or bigleaf maple

(*Acer macrophyllum*). Dominant understory shrub species include salal (*Gaultheria shallon*), dwarf Oregongrape (*Mahonia nervosa*), vine maple (*Acer circinatum*), Pacific rhododendron (*Rhododendron macrophyllum*), salmonberry (*Rubus spectabilis*), trailing blackberry (*R. ursinus*), red elderberry (*Sambucus racemosa*), fools huckleberry (*Menziesia ferruginea*), beargrass (*Xerophyllum tenax*), oval-leaf huckleberry (*Vaccinium ovalifolium*), evergreen huckleberry (*V. ovatum*), and red huckleberry (*V. parvifolium*).

To encompass the entire range of *P. vehiculum*, ten regions were chosen for sampling, each separated by at least 64 kilometers or a large barrier to dispersal (i.e. the Columbia River, the Strait of Juan de Fuca, the Strait of Georgia, or high elevation changes) (Fig. 3, Appendix A). All collections were done under permits issued by the Oregon Department of Fish and Wildlife, Washington Department of Fish and Wildlife, or the British Columbia Ministry of Environment, and approved by IACUC. Within each region, *P. vehiculum* sample collections were most often less than 1km apart but no more than 3km apart. In western British Columbia, where the only Plethodontid salamander is *P. vehiculum,* samples were collected from two regions: 1) Vancouver Island, and 2) the southern mainland around Cultus Lake. Along the foothills of the Coast Range four regions were sampled: 1) northwest Washington in the Olympic National Forest, where *P. vehiculum* may be found in sympatry with *P. vandykei*, 2) southwest Washington, just north of the Columbia River in Skamokawa Vista Park, where it can be found in sympatry with *P. dunni* and *P. vandykei,* 3) northwest Oregon in the Tillamook Forest, in sympatry with *P dunni,* and

Figure 3. *Plethodon vehiculum* collection localities. BC= Mainland British Columbia; V= Vancouver Isalnd; SL= North WA Cascades at Silver Lake, NW= North WA coast/Olympic Peninsula; WC= South WA Cascades; S= South WA coast at Skamakawa; A= North OR Cascades at Angel's Rest, Mt. Hood National Forest; T= North OR coast, Tillamook Forest; LR= Portland Parks and Greenspaces; SO= Southern OR.

4) southern OR in the Siskiyou National Forest, where it is known to be parapatric with *P. elongatus*. Along the foothills of the Cascade Range three regions were sampled: 1) north Washington at Silver Lake, 2) south Washington in Multon Falls County Park, in sympatry with *P. dunni* and *P. larselli*, and 3) north Oregon on private property in the Mount Hood National Forest in sympatry with *P. dunni*. In addition three *P. dunni* samples were collected in the Mt. Hood National Forest.

During a previous study, salamander toe clips were collected from five parks in the Portland Metro area (Roberts 2005) where it is in sympatry with *P. dunni*. Four of the five parks are located west of the Willamette River – Marshall Park, George Himes Park, Tryon Creek, and West Portland Park, all within Multnomah County. One location was sampled east of the Willamette River – SE Sunnybrook Blvd. in Clackamas County (Fig. 4, Appendix A).

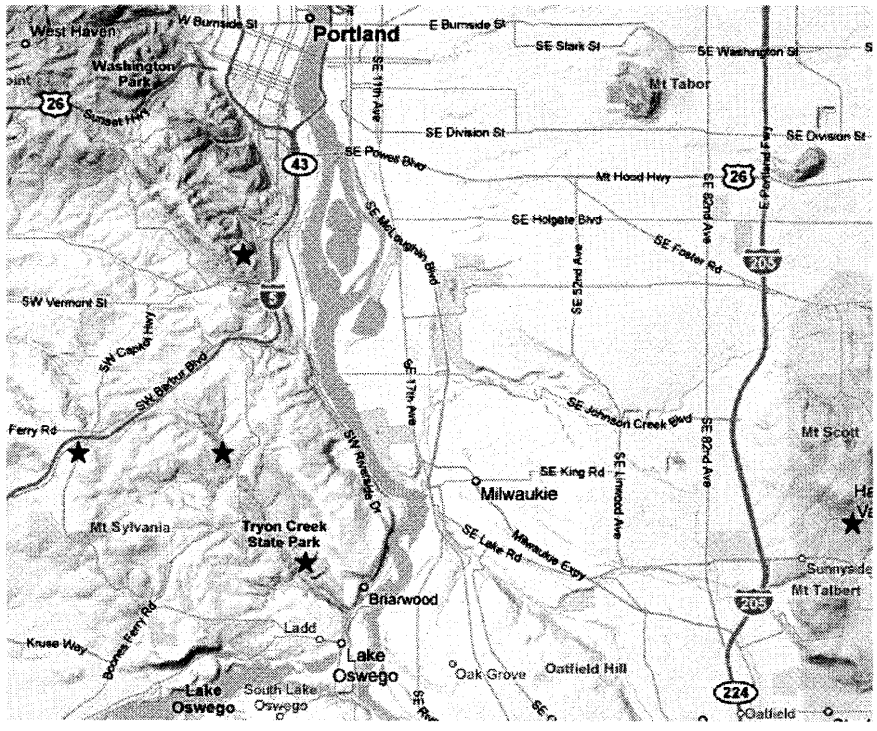


Figure 4. Collection localities of *P. vehiculum* in the Portland Metro region (Roberts 2005).

Chapter Two explores the phylogeography of *P. vehiculum* in the PNW using sequence data from the mtDNA D-loop. Results will be reviewed in light of other *Plethodon* salamander phylogeographic studies in the region to increase our understanding of historical geography and the processes shaping the current population structure of these animals. Genetic variation, differentiation, and haplotype distribution will be assessed at the regional scale as well as on a smaller geographic scale within the Portland Metro area. In Chapter Three, the D-loop will be further explored in *P. vehiculum* using phylogenetic techniques to define exclusive lineages. In addition, relationships of Plethodontid salamanders in the PNW deserve more attention to fully grasp the timing and mode of speciation in these unique animals. The cytb gene will be used to reevaluate the relationship of all *Plethodon* salamanders in the PNW using parsimony and likelihood methods.

Chapter 2: Population structure and genetic variation of *Plethodon vehiculum* in the Pacific Northwest using the highly variable mtDNA control region

Introduction

The Pacific Northwest (PNW) has a substantial history of geographic disturbance due to volcanism, glaciation, and flooding which has greatly influenced the contemporary patterns of species abundance and distribution in many taxa (Pielou 1991). Therefore, in such a topographically diverse region like the PNW, amphibian populations may exhibit considerable genetic variation given the harsh landscape that often surrounds suitable habitat (i.e. high elevation changes, rivers) and as a consequence of historical vicariant events. Due to the narrow physiological requirements of amphibians, genetic studies often reveal cryptic genetic diversity, indicating restricted gene flow and discrete evolutionary trajectories (Mahoney 2004, Mead et al. 2005, Wagner et al. 2005).

It is important to understand the levels of variation within and among populations to determine how habitat fragmentation and historical processes have shaped the population structure of a species (Larson et al. 1984, Avise et al. 1987). Field studies alone do not provide a complete enough picture in describing the population dynamics of amphibians due to the complexity of measuring all the variables that may contribute to these dynamics (i.e. population density, sensitivity to environmental variation, dispersal ability, and the amount of suitable habitat available).

Phylogeographic studies of terrestrial salamanders have contributed greatly in recognizing their population structuring in the PNW. For example, three highly divergent haplotype groups were defined in both *Plethodon elongatus* and *P. stormi* in a north-south fashion (Mahoney 2004) throughout their range in southern Oregon and northern California. The three sister species in this region, *P. elongatus*, *P. stormi*, and *P. asupak*, all displayed relatively high levels of genetic variation within and among populations using mitochondrial DNA (mtDNA) sequence data (Mahoney 2004, Mead et al. 2005). Monophyletic groups were found north and south of the Columbia River in *P.larselli* (Wagner et al. 2005), in a very narrow range, indicating long-term separation between populations. On the other hand, Carstens et al. (2004) explored the levels of genetic variation within *P. idahoensis* where most of the genetic variation was observed within river drainages, yet a large portion of the variation was also observed between the northern and southern river drainages throughout its entire range of northern Idaho and southern British Columbia.

Given the wide distribution of *P. vehiculum* in the PNW (see Fig. 1), significant genetic structuring is expected due to the assorted geography and intricate geologic history; however, no genetic studies have been reported to date. Similar patterns to those of other *Plethodon* species in the PNW are predicted. For example, phylogeographic breaks are anticipated north and south of the Columbia River, and between the mainland and Vancouver Island. Regions more distant from one another are projected to be more distant genetically as well, particularly in a north-south direction as this has been observed in other salamander species in western North

America (Mahoney 2004, Wagner 2005, Miller et al. 2006). Multiple genetically distinct populations (multiple reciprocally monophyletic groups) would be evidence of multiple refugia during the Pleistocene glaciations (Brunsfeld et al. 2001, Hewitt 2004), where populations have not had substantial gene flow since, and remain genetically differentiated. On the other hand, admixture of genetic material may be indicative of long-range dispersal allowing populations to remain genetically parallel, or of a recent range expansion from only one or a few glacial refugia. Little genetic variation may be representative of a single small refugium.

This study will be the first to determine the phylogeographic structure of *P. vehiculum*. Diversity statistics and levels of differentiation will be assessed as a means to compare population level processes on a large and small scale. This will be used to make inferences about the geographic impacts on population subdivision.

MtDNA sequence data will be developed to assess the variation present within this species throughout its range in the PNW and explore haplotype distribution and genetic diversity within the Portland Metro area. The high mutation rate, lack of recombination, and maternal mode of inheritance make this a useful tool for inferring species spatial patterns (Hedrick and Miller 1992), often showing high levels of geospatial correlation (Moritz et al. 1987). Historical biogeographic factors will be explored that may influence the structure of *P. vehiculum*, as well the possibility of recent range expansion or retraction.

Materials and Methods

Sample Collection

Plethodon vehiculum collections (see Fig. 3) were made by hand in the spring and fall 2007, and spring 2008 seasons after the rains started and salamander surface activity began, by random search of the leaf litter and under rocks and logs. Sample sizes ranged from one to eight individuals per site (Appendix A), with an average n=3. This gave an average n=6 per region, with a total of 20 individuals collected from Portland's Parks and Greenspaces (see Fig. 4). Tail clips (3-5 mm) were taken, preserved in 95% ethanol and stored at -20° C until DNA was extracted. Tails clips were collected because the tail has the ability to regenerate, and this sampling poses minimal stress to the animal. It has been proven a viable and humane field technique in salamanders (Kinkhead et al. 2006). All tails were taken with a new sterile razor blade and bactine was applied to prevent infection (Heyer et al. 1994). Snout-vent length (SVL), total length (TL), body mass and stripe color were recorded for all individuals. Sex, head length, width and height, and femur and humerous length of all adult salamanders were also recorded. Due to low sample numbers and high number of juvenile captures, no statistical analyses were carried out using morphological measurements. Animals were handled as briefly as possible, put in containers with moist substrate for sampling, and released where found. Juveniles smaller than 20mm were not sampled due to the possible negative effects of handling and tail clipping. Tail clipping prevented resampling of previously caught individuals.

Samples from the Portland city parks and greenspaces were collected during a previous study by Roberts (2002) using similar methods, although toe clips were made instead of tail clips. The latter samples were preserved in 95% ethanol. Snout-vent length, body mass, and sex were recorded.

DNA processing

Tail samples were retrieved from their tubes with sterile forceps and washed free of ethanol with STE (sodium chloride-tris-EDTA) buffer. DNA was extracted using a Qiagen DNeasy extraction kit (Valencia, CA) following their animal tissue spin-column protocol. Extractions were stored in the refrigerator.

Primers were developed for the mtDNA control region D-loop (Fig. 5). These primers were designed using the conservative flanking tRNA regions of the mtDNA

Amplification of the extracted DNA consisted of a total of 25μL reaction volume: 1μL of DloopL2 (25μM), 1μL of DloopR2 (25μM), 22 μL dH2O and 1μL of DNA was added to PuRe Taq™ Ready-To-Go Bead tube (GE Healthcare, Piscataway, NJ). The following program was run on a PTC-100 Peltier thermocycler (MJ Research, Ramsey, MN): initial denaturation for 5 minutes at 94°C; 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C (primers were tested at a range from 47°C to 58°C) for 1 minute, and extension at 72°C for 2 minutes; an additional final extension was run at 72°C for 10 minutes and held at 4°C indefinitely.

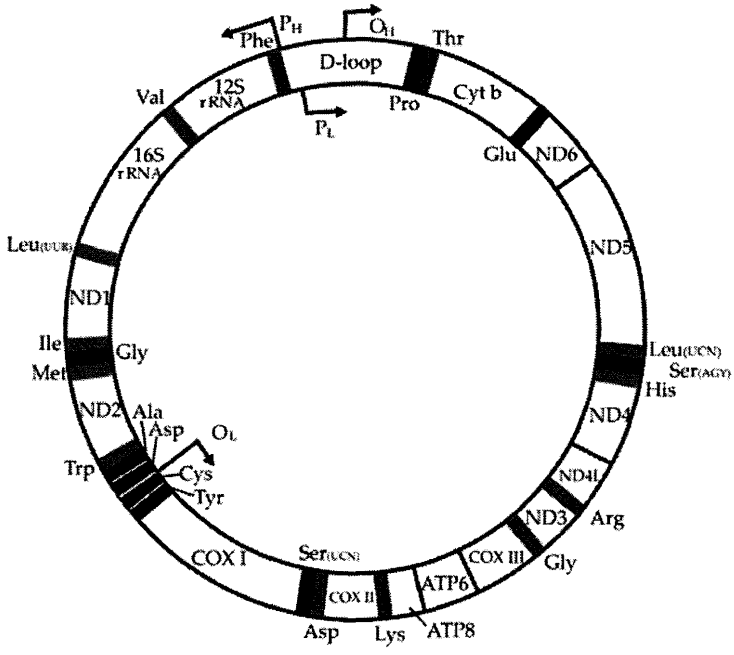


Figure 5. Diagram of vertebrate mitochondrial DNA. White regions represent coding genes or the control region, and gray or black regions represent tRNA's.

PCR product was confirmed on a 2% agarose E-gel (Invitrogen, Carlsbad, CA) and cleaned using the QIAquick PCR purification spin protocol (Qiagen, Valencia, CA). Cleaned PCR product was sequenced in both the 5' and 3' directions. The 10μL sequencing reaction consisted of 2.5μL of DloopL2 (2.5μM), 2.5μL of DloopR2 (2.5μM), 2μL of dH2O, 1μL of 5X buffer, 2μL of Big Dye Terminator 3.1, and 2.5μL of amplified DNA. Initial denaturation was 96°C for 5 minutes; then 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes, then held at 4°C. Sequencing reactions were sent to Oregon State University, Center for Genome

Research and Biocomputing Core Laboratory Facility (Corvallis, OR) and processed on an ABI 3730 capillary sequencer.

Sequence Analysis

An 823-bp portion of the D-loop was successfully sequenced. Sequences were cleaned using the SeqMan program, saved as EditSeq files for import into MegAlign (DNASTAR© Lasergene v6 software package, Branford, CT). Due to variable sequence readability near one of the primers, only 688-bp were used for analysis. MegAlign was used to align sequences using the ClustalV method (Higgins et al. 1991) and imported into MacClade 4.08 (Maddison and Maddison 2005) where the alignment was adjusted by visual inspection.

Arlequin 2.0 (Schneider et al. 2000) was used to determine diversity statistics (θ): average number of pairwise nucleotide differences (θ_π), and the number of segregating sites (θ_S). The diversity statistic θ_π is sensitive to haplotype frequency, while θ_S is not. The difference between these two measures are used to determine Tajima's D-statistic. A negative Tajima's D-statistic ($\theta_\pi < \theta_S$) is indicative of a recent population expansion because rare alleles are more abundant; a positive value ($\theta_S > \theta_\pi$) is indicative of a recent population bottleneck because rare alleles are not present. To test for significance of the D-statistic, 1000 replicates under the null hypothesis of population stability were simulated. The more conservative Fu's Fs was also used to test for range expansion. This is a test against the null model of demographic stability, the probability of having a number of alleles greater or equal to the observed number

in a sample drawn from a stationary population. To test for significance 1000 replicates were carried out. Diversity statistics were calculated for each region (10 total) while Tajima's D-statistic and Fu's Fs was calculated for the entire range of *P. vehiculum,* and within each region.

To investigate population structure, analysis of molecular variance (AMOVA) was conducted and F_{st} values among populations were determined also using Arlequin 2.0. When using haplotype data, F_{st} represents the difference in variation within a population compared to that among populations. Significance was tested for using the default setting of 3024 permutations. In addition, isolation by distance was then examined using IBD 3.16 (Jensen et al. 2005) to determine whether populations were more closely related as a factor of distance. A Mantel Test was done on an uncorrected pairwise genetic distance matrix and a pairwise geographic distance matrix. Percent sequence divergence was measured using the Tamura-Nei distance method in Arlequin 2.0 and was chosen based on the Model Test results in PAUP 4.0b10 (see Chap. 3).

A haplotype network was constructed in TCS 1.21 (Clement et al. 2000), based on statistical parsimony, to infer the relationship of haplotypes observed in this dataset. The probability of parsimony is calculated for DNA pairwise differences until the probability exceeds 0.95 and the haplotypes are connected into a network. Gaps were treated as a fifth state, and prior to analysis sequences with large amounts of missing or ambiguous data were removed (Appendix C).

Results

MtDNA control region sequences were obtained from a total of 60 individuals from the ten PNW regions sampled: Vancouver Island (V; n=4), mainland British Columbia (BC; n=4), northwest Washington (NW; n=7), southwest Washington (S; n=3), northwest Oregon (T; n=7), southern OR (SO; n=4), north Washington Cascades (SL; n=3), south Washington Cascades (WC; n=8), north Oregon Cascades (A; n=1), and from the Portland Parks and Greenspaces (PPGS/LR; n=20). These sequences were used to compare diversity statistics and to do phylogeographic analysis. The section of the D-loop analyzed had 178 polymorphic sites, but most of the variability was observed between the 500^{th} and 700^{th} bps sequenced. Base frequency was: A= 0.3037, C= 0.2465, G= 0.1289, and T= 0.3209, showing higher frequencies of A+T as is common in mtDNA. The transition/transversion ratio was 0.84, with most sequence differences being C to T (28 out of 178) and T to A (26 out of 128). Twenty-one indels were observed. Two individuals, LR107 and S2, remained in some of the analyses with only 663-bp and 642-bp respectively.

Diversity statistics were calculated for all ten PNW regions sampled (Table 1). Over the entire region of *P. vehiculum*, θ_S was 17.93 (n=58) and θ_π was 8.25 (n=60). Diversity statistics were also calculated for the entire range excluding the southern OR populations (see phylogeographic results) and were 14.26 for θ_S and 2.18 for θ_π. Within regional values ranged between 0.66 and 9.30 for θ_S and 0.66 and 8.17 for θ_π. The northern Cascade population of WA (θ_S = 0.66 and θ_π = 0.66) and mainland BC (θ_S = 1.67 and θ_π = 2.0) displayed the lowest diversity levels. The highest levels were

observed in the Portland Parks at $\theta_S = 9.30$ and $\theta_\pi = 5.47$ and on Vancouver Island at $\theta_S = 7.09$ and $\theta_\pi = 8.17$. The average for within region diversity measures was $\theta_S = 4.66$ and $\theta_\pi = 4.54$.

Diversity statistics were also calculated for the two parks within the Portland Metro area that had greater than 2 individuals (Table 2). Diversity indices were very different between these two parks despite the similar sample size. Marshall Park diversity statistics were $\theta_S = 9.93$ and $\theta_\pi = 9.19$ (n=9) and for George Himes Park, $\theta_S = 1.22$ and $\theta_\pi = 1.09$ (n=7).

Differentiation within and between populations was calculated using F_{st} for halpotypic data; overall, F_{st} values ranged from -0.37 to 0.92 and many pairwise comparisons were found to be significantly different from zero (Table 3). The highest levels of differentiation occurred between the southern OR population versus all the others (F_{st} range 0.61 to 0.89; all significant). Many of the northern group pairwise population comparisons had low F_{st} values, however, only the lowest value between the lower WA Cascade range and northern coast range was significant ($F_{st} = 0.08$). In general, the Vancouver Island population and the northwest WA population from the Olympic Peninsula were more highly differentiated from the other populations. Isolation by distance measurements revealed a correlation between genetic and geographical distance, however this relationship was not significant.

Percent sequence divergence showed variable levels of divergence between regions (Table 4). The average uncorrected pairwise difference between the southern Oregon group and the other populations was very high and ranged from 19.2 to 24.8.

Table 1. Diversity statistics across the entire range of *Plethodon vehiculum*.

	Sample Size	θ_S	θ_π
A	n=1	-	-
BC	n=4	1.67 ± 1.18	2.0 ± 1.67
LR	n=20	9.30 ± 3.47	5.47 ± 3.06
NW	n=7	2.04 ± 1.22	2.28 ± 1.62
S	n=3	9.0 ± 6.0	8.0 ± 8.0
SL	n=3	0.66 ± 0.66	0.66 +- 0.66
SO	n=4	4.36 ± 2.68	5.0 ± 3.66
T	n=7	5.71 ± 2.89	4.86 ± 3.08
V	n=4	7.09 ± 4.15	8.17 ± 5.73
WC	n=7	2.24 ± 1.4	2.19 ± 1.5
All pops	n=60	17.93 ± 5.12	8.25 ± 4.3

A= Oregon; Mt Hood National Forest; Angels Rest
BC= British Columbia (mainland); Cultus Lake
LR= Portland Parks and Greenspaces
NW= Washington; Olympic National Forest Peninsula
S= Washington; Wahkiakum Co; Skamokawa Vista Park
SL= Washington; Mt Baker National Forest; Silver Lake
SO= Oregon; Siskiyou National Forest; Coos Co
T= Oregon; Tillamook State Forest/Clatstop State Forest
V= British Columbia; Vancouver Island
WC= Washington; Gifford Pinchot National Forest; Multon Falls

Table 2. Diversity statistics for Portland Parks and Greenspaces.

	Sample Size	θ_S	θ_π
GHP	n=7	1.22 ± 0.83	1.09 ± 0.92
MP	n=9	9.93 ± 4.46	9.19 ± 5.30
WPP	n=2	-	-
TC	n=1	-	-
SESB	n=1	-	-
All Parks	n=20	9.30 ± 3.47	5.47 ± 3.06

GHP= George Himes Park; MP= Marshall Park;
WPP= West Portland Park; TC= Tryon Creek;
SESB= Southeast Sunnybrook Blvd.

Table 3. Pairwise Fst values between sampled populations of *P. vehiculum* in the PNW.

	BC	LR	NW	S	SL	SO	T	V	WC
BC	-								
LR	-0.16	-							
NW	0.35*	0.18*	-						
S	0.07	0.32*	0.24*	-					
SL	-0.05	-0.08	0.38	0.00	-				
SO	0.88*	0.82*	0.89*	0.83*	0.89*	-			
T	-0.07	0.03	0.25*	0.17*	-0.13	0.84*	-		
V	0.25*	0.22*	0.20*	0.08	0.21	0.81*	0.18*	-	
WC	-0.08	0.07	0.08*	0.02	-0.17	0.61*	0.00	0.03	-

Fst on lower diagonal and significance values (*=p<0.05)

The Northern OR Cascade population was discarded from this analysis due to only one sample from the area.

BC= British Columbia (mainland); Cultus Lake

LR= Portland Parks and Greenspaces

NW= Washington; Olympic National Forest Peninsula

S= Washington; Wahkiakum Co; Skamokawa Vista Park

SL= Washington; Mt Baker National Forest; Silver Lake

SO= Oregon; Siskiyou National Forest; Coos Co

T= Oregon; Tillamook State Forest/Clatstop State Forest

V= British Columbia; Vancouver Island

WC= Washington; Gifford Pinchot National Forest; Multon Falls

Distances among the rest of the PNW regions, excluding southern Oregon, ranged from 1.1 to 9.5. Pairwise distance measures within regions were the highest in the southern WA coast range at 7.3 and the lowest in the northern WA Cascades at 0.68. Sequence divergence patterns were comparable to those observed using the diversity statistics and F_{st} estimates within and among populations.

In examination of the distribution and relationship among haplotypes, a haplotype network was conducted using TCS. This program is sensitive to missing data and ambiguous basepairs and therefore only 58 individual sequences were analyzed (see Appendix C). Thirty-three unique haplotypes were recovered (Appendix D). One haplotype (H1) was found in 18 out of 58 individuals (frequency = 0.31) and was observed in six regions (Fig. 6). This haplotype was shared among individuals from the British Columbia mainland, multiple Portland Metro area parks, northwest OR, southwest WA, and the northern and southern populations of the Cascade range. Additional haplotypes were shared among the northern and southern Cascade range populations. The northern coast range of WA had three unique haplotypes only found in this region, Vancouver Island contained three haplotypes unique to the island, and southern Oregon had three haplotypes all unique to its region. No one park had unique haplotypes to its location. One haplotype (H16), was shared between Marshall Park and George Himes Park, while another haplotype (H1), was shared by Tryon Creek, Marshall Park, SE Sunnybrook, and West Portland Park.

Table 4. Tamura & Nei distance measure within and between all regions of *P. vehiculum*.

	A	BC	LR	NW	S	SL	SO	T	V	WC
A	0	6.02	7.35	7.36*	9.56	5.58	24.82	7.04	8.79	6.28*
BC		1.53	2.87	2.74*	4.41	1.1	19.55*	2.81	4.67*	1.78
LR			4.12	4.02	5.44	2.45	20.49*	4.14	5.73	3.08
NW				1.75	5.71*	2.38*	18.83*	4.09*	3.89*	3.02*
S					7.26	3.97	22.24*	5.7	7.21	4.66
SL						0.68	19.2*	2.38	4.23	1.16
SO							4.12	20.7*	20.85*	19.91*
T								4.09	5.6	3.06
V									5.58	4.88*
WC										1.94

Above diagonal: Average number of pairwise differences between populations (PiXY)
Diagonal elements: Average number of pairwise differences within population (PiX)
* Indicates p values < 0.05

A= Oregon; Mt Hood National Forest; Angels Rest
BC= British Columbia (mainland); Cultus Lake
LR= Portland Parks and Greenspaces
NW= Washington; Olympic National Forest Peninsula
S= Washington; Wahkiakum Co; Skamokawa Vista Park
SL= Washington; Mt Baker National Forest; Silver Lake
SO= Oregon; Siskiyou National Forest; Coos Co
T= Oregon; Tillamook State Forest/Clatstop State Forest
V= British Columbia; Vancouver Island

Figure 6. Distribution of mtDNA D-loop haplotypes of *P. vehiculum* in the PNW.

The haplotype network recovered two haplotype groups, a southern Oregon group, and a northern group containing all other populations. These two groups could not be joined into a single network with 95% confidence (Fig. 7), and are identical to the major clades defined by phylogenetic analyses based on parsimony and maximum likelihood (see Chap. 3). The larger northern haplotype group was connected with three loops. Three haplotypes from this group, H1, H16, and H18 are apparent ancestors to other haplotypes in that area. The maximum number of connection steps at 95% confidence was 11.

According to the AMOVA, 83.24% ($p<0.001$) of the genetic variation occurred between the northern and southern groups, and only 2.59% ($p<0.001$) was observed among populations within these two groups. When analyzing the Vancouver Island and Olympic Peninsula populations as separate groups, still most of the variation is observed among groups (64%; $p<0.001$), and very little variation was observed among populations within those groups (3%; $p<0.001$); true also when Vancouver Island and the Olympic Peninsula were combined together as one group.

With respect to estimates of recent range expansion, Tajima's D-statistic were calculated separately on both the north and south clades because the southern Oregon population formed a distinct clade. In the northern group Tajima's D was -2.55 ($p<0.001$; $n=60$) and Fu's Fs was -0.82 ($p=0.124$) (Table 5). Tajima's D-statistic for the southern OR population was -0.82 ($p=0.12$), but Fu's Fs was 1.16 ($p=0.65$) indicating no range expansion. Other populations showing significant evidence of

range expansion include the Portland region with Tajima's D at -2.34 (p=0.001; n=20) and Fu's Fs was

-1.85 (p=0.18), the N. OR coast with Tajima's D at -1.65 (p=0.012; n=7), and the S. WA Cascades with Fu's Fs at -3.14 (p=0.004; n=7). Therefore, Tajima's D and Fu's Fs both support recent range expansion over the entire PNW range, especially in the northern group, and within the southern Cascade/coastal populations; while in the southern OR region neither Tajima's D or Fu's Fs strongly supports a recent range expansion.

Table 5. Tajima's D-statistics and Fu's Fs

	Tajima's D	**Fu's Fs**
Northern group	-2.55 (p<0.001)	-7.28 (p=0.023)
PPGS	-2.34 (p=0.001)	-1.85 (p=0.18)
N. OR coast	-1.65 (p=0.012)	-0.13 (p=0.36)
S. WA Cascades	-1.13 (p=0.17)	-3.41 (p=0.004)
S. OR group	-0.82 (p=0.12)	1.16 (p=0.65)

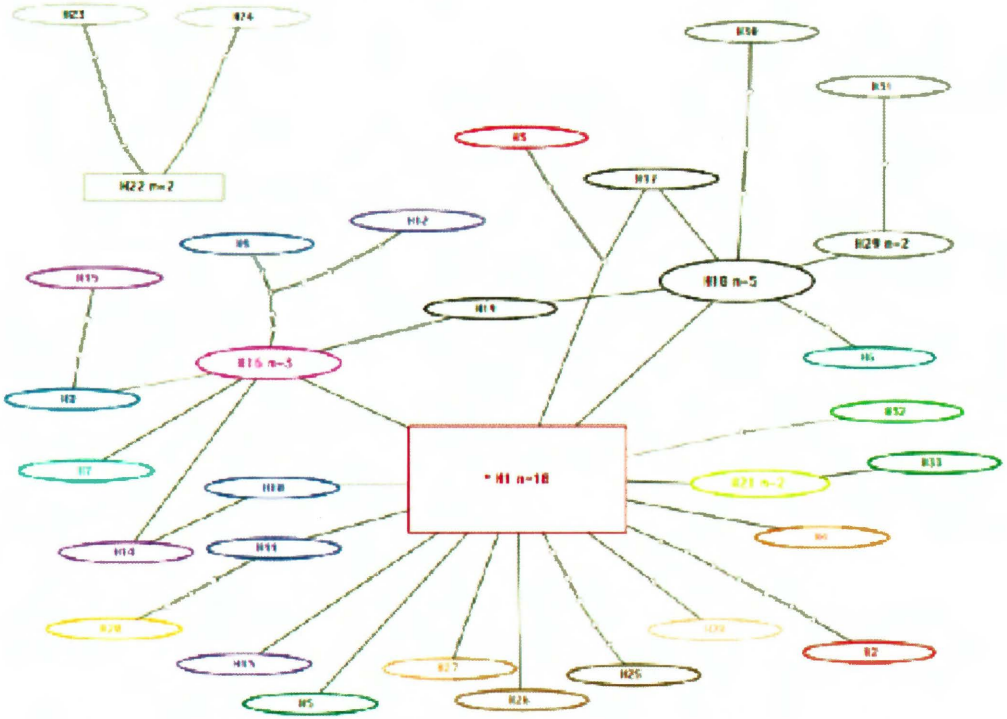


Figure 7. Haplotype network of mtDNA D-loop haplotypes of *P. vehiculum* in the PNW.

Discussion

Climate changes associated with glacial cycles and anthropogenic factors almost certainly influence the distribution of salamanders, which are sensitive to temperature and moisture fluctuations. Deep genetic divergence and varying levels of genetic diversity across narrow and broad geographic ranges have been observed in many *Plethodon* salamanders in the PNW (Carstens et al. 2004, Mahoney 2004, Mead et al. 2005, Wagner et al. 2005). Furthermore, human population growth fuels habitat loss, and with this destruction, once continuously distributed populations become smaller and isolated, and more prone to demographic and stochastic events that may lead to extinction. Dealing with the long-term ecological and genetic consequences of geographically structured populations is important. Understanding these long-term consequences will give us insight as how to better manage contemporary patterns of population structure and preserve remaining biodiversity and evolutionary processes (Templeton et al. 1990). Many analyses of population genetic structure of salamanders have established that substantial subpopulation structure exists (Templeton et al. 1990, Phillips 1994, Donovan et al. 2000, Schaffer et al. 2004, Miller et al. 2005, Miller et al. 2006, Kuchta et al. 2009). Studies at a small geographic scale have described the population genetic structure of most western *Plethodon* species and phylogeographic studies using mtDNA at the intraspecific level have increasingly gained popularity. In general, salamanders tend to display high levels of genetic differentiation and do not consist of populations with high levels of gene flow, while maintaining minimal morphological differences (Larson et al. 1984,

Highton 1995). Intraspecific genetic differentiation and variation due to geographic isolation may be more prevalent than it appears even among species, like *P. vehiculum*, that are abundant and widely distributed (Gibbs 1998). This study develops the phylogeographic information necessary to investigate this possibility.

Diversity and differentiation among the PNW

Diversity statistics differed highly between regions in this species even when sample sizes were similar. The Cascade range populations from WA to BC displayed the lowest levels of diversity while populations along the coast, including the Portland Metro area, had higher overall genetic diversity levels. The values and variation of θ_S and θ_π of the mtDNA D-loop among populations seen in this study are similar to other *Plethodon* species in the PNW (Carstens et al. 2004, Mahoney 2004). Interestingly the cytb gene, as used in these studies, did not show levels of variation in *P. vehiculum* that is even close to the other *Plethodon* species (see Chap. 3). Levels of variation within the D-loop in the highly differentiated southern OR clade was the same as the average of all the other areas, however, the diversity statistics for *P. vehiculum* when excluding the southern OR individuals is lowered slightly meaning that the southern OR population was contributing to the overall diversity levels seen in this species, but not driving it. Overall, diversity measures in this species appear to be high when using this neutral marker. In general, large population size and species with relatively wide distributions will show higher levels of genetic variation (Frankham 1996). These patterns were also seen in the within region sequence divergence estimates. F_{st} values

(0.0 – 0.89) showed relationships between populations ranging from very high to very low; an F_{st} value below 0.05 generally represents little to no differentiation between populations, values between 0.05 and 0.25 represent intermediate differentiation, and values above 0.25 represent high levels of genetic differentiation (Conner and Hartl 2004).

Highest levels of genetic differentiation were observed between the southern OR population and all the other populations indicating that there is no current gene flow between this population and populations from the northern group. The divergence estimates in *P. vehiculum* showed southern OR to be very highly divergent from all the northern populations (average = 20.7%). This finding is consistent to that observed in the cytb gene using distance measures, where sequence divergence between the north-south division in *P. vehiculum* is very high using both the mtDNA d-loop and the cytb gene (see Chap. 3). The southern group of *P. vehiculum* may deserve reclassification to separate species or subspecies status.

The northwest coastal WA population of the Olympic Peninsula showed significant levels of F_{st} ranging from low to high in pairwise comparisons to the other PNW populations and this is probably the result of the unique haplotypes found in this area. This level of differentiation may be due to the disjunction of this population during glacial cycles while the relatively high elevation, glaciated Olympic Mountains (up to 2100 m) present here continue to limit current exchange of haplotypes. Vancouver Island also had relatively high significant values of F_{st} compared to the

other PNW populations, possibly attributed to the fact that it is an island with historical separation and unique haploypes.

Excluding the southern OR population, all pairwise sequence divergence estimates throughout the northern range of *P. vehiculum* were relatively low (average = 4.4%). Percent sequence divergence among populations of terrestrial salamanders in general tend to vary. For example, percent sequence divergence within major haplotype groups of *P. elonagatus* ranged between 1.56 and 5.38 and was overall 8.83 within the species, and in this same study, the percent sequence divergence within *P. stormi* was 4.16 (Mahoney 2004). Sequence divergence within *P. larselli* falls between 0.2 and 5.2 (Wagner et al. 2005). *Plethodon asupak* displays an average of 0.58 sequence divergence between populations within a very small geographic distribution (Mead et al. 2005).

Isolation by distance measurement, although not significantly showed a slight correlation between geographic and genetic distance. However, this method does not take into account large geographic barriers, such as the Pacific Ocean or mountain ranges and may not be an accurate means to measure genetic distance against geographic distance on such a large scale. AMOVA results indicate that most of the genetic variation is observed between the northern and southern groups. Very little variation is observed among populations within the northern group indicating very little population differentiation. Similarly, in the large distribution of *P. idahoensis* little genetic variation was observed among river drainages and only slightly more between the northern and southern river drainages (Carstens et al. 2004). Moreover,

when the Vancouver Island and Olympic Peninsula populations are removed from the northern group and analyzed as separate groups, most of the genetic variation is still observed among groups and very little among the populations within these groups. This may be indicative of the differentiation levels observed between Vancouver Island and the remaining populations and between the Olympic Peninsula and the remaining populations, albeit theses are still lower than divergence estimates between the southern OR group and any other population. In summary, the southern population deserves in the very least recognition as an ESU and probably subspecies or species status, while the populations sampled from the Olympic Peninsula and Vancouver Island warrant further investigation and possibly separate management protocols, particularly in the Olympic Peninsula where diversity statistics are low compared to those of other coastal populations.

Haplotype distribution

In *P. vehiculum*, haplotypes are shared among the BC mainland all the way south into the northern Oregon populations, and as a result, cross the Columbia River. This is highly unexpected due to the significant structuring discovered within other *Plethodon* salamanders in the PNW and the lack of haplotype sharing between populations on even smaller geographic scales using even more highly conservative regions of the mtDNA genome (cytb, NADH, ATPase 6, Mahoney et al. 2004; cytb, Miller et al. 2005; cytb, Mead et al. 2005; cytb, Wagner et al. 2005). For instance, three distinct haplotype groups were defined in *P. elongatus* among distant

populations in northern California and Southern Oregon (Mahoney 2004). Three distinct haplotype groups were also defined in *P. stormi* in a relatively small geographic range in northern CA and southern OR (Mead et al. 2005). In its extremely restricted range, significant genetic structuring has been found in *P. larselli* (Wagner et al. 2005) and distinct clades were detected north and south of the Columbia River. It was suggested that limited contemporary gene flow within these salamander species contributed to the high levels of divergence observed. In this study, low divergence and high haplotype sharing in the northern populations may be indicative of high habitat connectivity for these animals or a more recent population structure, not allowing enough time for sufficient lineage sorting. In spite of this, haplotypes unique to a region were not only observed in southern OR, but were also found in the, the northwest coastal region of WA on the Olympic Peninsula, and on Vancouver Island. Lack of phylogenetic breaks, but definite spatial discontinuity can be explained by limited long-term gene flow (i.e. no long-term *firm* barrier - the Pacific Ocean or high elevation mountain ranges) (Avise et al. 1987) or historical separation due to past climatic and/or geographical barriers to dispersal.

Rivers as barriers to dispersal have been highly disputed (Highton 1972) and they do not seem to act as a barrier to dispersal in *P. vehiculum*, even large rivers, such as the Columbia River, and over long periods of time. Conversely, morphological differences, supported by genetic differences, have been observed in *P. larselli* on either side of the Columbia River (Wagner et al. 2005) indicating selection or drift acting on these populations and isolation by a large barrier. This higher level of

differentiation could be attributed both to low population size, as this salamander species is currently listed as 'near threatened', or may be due to different selection pressures on either side of the river. *Plethodon vehiculum* and *P. dunni* are the only other *Plethodon* species' whose distribution crosses the Columbia River and no comparative morphological or genetic studies have been done in either species.

The haplotype network supports the finding that the southern OR population is highly divergent from the rest of the species range as the networks formed by the northern and southern groups could not be joined. This phylogeographic break is also observed in this species using phylogenetic techniques (see Chap. 3), and is consistent with similar phylogeographic breaks in the PNW shown for a variety of taxa from mammal, to birds, and plants (Swenson and Howard 2005). The Klamath-Siskiyou region (Swenson and Howard 2005, Mahoney 2004) is the proposed refugia in this species for the southern population. During glacial cycles of the Pleistocene, ice sheets extended and retracted into the current range of *P. vehiculum*, as well as contributed to glaciation of high mountains south of the ice sheet. This dramatically altered the climate by lowering global temperatures and reducing water availability, in turn altering the suitable habitat for many North American taxa and presumably *P. vehiculum*, forcing them into these small pockets of available space and consequently fragmenting continuous populations (Hewitt 2004).

Three hypotheses have been proposed by Brunsfeld et al. (2001) to explain the historical genetic patterns observed in taxa inhabiting the coastal and Cascade region of the PNW. The 'single coastal refugium' hypothesis can be rejected in this study as

there is evidence of at least two glacial refugia in this species, one in the northern portion of its range, and one in the southern Klamath-Siskiyou region, therefore supporting the 'multiple refugia hyothesis'. A subhypothesis of the 'multiple refugia hypothesis' states that there may have may been more than one refugia in the north along the coast and the lowlands of the Cascades. The site of northern refugia have yet to be established, but results from this study suggest a southern glacial refugium and multiple northern refugia during the last glacial cycles of the Pleistocene for *P. vehiculum*. Multiple refugia are suggested by several haplotypes (H1, H16, and H18) exhibiting a starlike pattern which is often attributed to demographic expansion. In addition, the distribution of *P. vandykei* and *P. larselli* suggests that in this area of *P. vehiculum's* range, many pockets of suitable habitat existed for terrestrial salamanders during even the most recent glacial cycles (see Fig. 1). The unique haplotypes of *P. vehiculum* with higher divergence estimates observed in the Olympic Peninsula and Vancouver Island indicate that these populations have been separated from the remaining northern population relatively recently. These areas may also have served as glacial refugia during recent glacial cycles.

Negative values of Tajima's D and Fu's Fs represent evidence of recent range expansion based on an excess of rare alleles relative to the number of segregating sites. There is evidence of recent, large range expansion within the entire northern region of *P. vehiculum*, and also along areas of the coast and in the Cascade range in northern OR and southern WA. The higher diversity statistics, and significant Tajima's D and Fu's Fs in these regions, offer additional support to the idea that more

then one refugia may have existed here. Large range expansions since the Pleistocene, through juvenile movement and/or the rapid establishment of suitable habitat due to the melting of glaciers, are possible in these animals despite apparently limited dispersal capabilities and may explain the large distribution in this species. For example, evidence of population expansion in *P. idahoensis* populations of over 600 km since the end of the Pleistocene was observed by Carstens et al. (2004). Southern OR, on the other hand, displays only weak evidence of recent range expansion. This could be due to low sample size of the southern OR population or the presence of a stable population in this region, as Fs will only detect range expansion if it is large as in the case of the northern group.

Genetic diversity in wild populations can best be preserved by maintaining healthy local populations in a well-distributed manner (Gibbs 2001). Focusing on habitat preservation and population management is needed to conserve genetic variation in wild populations. The southern OR population deserves particular attention due to its smaller range compared to the rest *P. vehiculum* distribution and the need to preserve the genetic exclusivity observed in this study. Further, with more rigorous genetic and ecological studies of this salamander species, there is potential for using this species as an indicator of habitat connectivity and ecosystem heath of forested areas in the PNW (Davic and Welsh 2004). Because of its large distribution and more panmictic population in the north, this species can be very useful to use across a large area encompassing multiple national forests.

Diversity and differentiation within PPGS

There was a large difference in θ_S and θ_π between the two parks with adequate sampling in the Portland Metro area (i.e. George Himes and Marshall Parks). Both these parks are similar in shape, elevation, and the amount of potential solar radiation, but differ in size (Roberts 2005). Marshall Park is larger by approximately 15,000 square meters and therefore probably provides more adequate habitat for terrestrial salamanders or even the possibility of more fragmentation within the park. Overall, nucleotide diversity of the PPGS was higher than average compared to other PNW populations for θ_S and close to the average for θ_π. The larger than average value of θ_S could be due to the higher sampling number in this area, but since θ_S is not sensitive to haplotype frequency, this increase could indeed be the result of direct high nucleotide diversity in Marshall Park.

Miller et al. (2006) found the Willamette River acted as a barrier to dispersal in Torrent salamanders, but in this study the one haplotype (H1) that was the most widespread haplotype found in this study was found in three of the Portland Parks (including those crossing the Willamette River). Future studies with increased sampling and the addition of other molecular markers are needed to evaluate how anthropogenic disturbance on a smaller scale may contribute to differences in genetic variation in *P. vehiculum.*

It is not clear at this point how anthropogenic habitat fragmentation might have effected the genetic structuring of these populations due to its relatively short time frame. However, computer modeling has demonstrated significant population genetic

substructure over short time periods in highly disturbed habitats (Gibbs 2001) and I would expect this for animals such as salamanders that have limited dispersal capabilities. The use of more highly variable markers, such as microsatellites, may be the only way to investigate population substructure on such a local scale. It is important to note that *P. vehiculum* may have higher dispersal capabilities than other terrestrial salamander species. This may be central to further explaining differences in genetic and population structuring among terrestrial salamanders in the PNW.

Chapter 3: Phylogenetics of *Plethodon* species in the Pacific Northwest using the mtDNA control region and the mtDNA gene cytochrome b

Introduction

Both recurrent and historical events influence how genes are spread through time and space. Phylogenetics seeks to answer questions about speciation in evolutionary biology; the use of genetic techniques allows us to expand our knowledge of the processes leading to speciation events and local adaptation. When utilizing phylogenetic trees in conjunction with ecological, and geographic data, we can make relevant hypotheses regarding these processes – mutation, migration, drift, selection, and vicariance. In addition, due to the linear nature of mutation in many genes, we can make inferences as to the timing of speciation, compare these data with known geologic data and confidently determine events in the past promoting speciation, and hence, the roots of biodiversity (Avise and Ellis 1986, Highton and Larson 1979).

Divergence in *Plethodon* species in western North America is hypothesized to have taken place during the Oligocene and Miocene (Highton and Larson 1979). Currently, *P. vehiculum* and *P. dunni* are known sister species (allozymes, Highton and Larson 1979), *P. stormi*, *P. elongatus* and *P. asupak* are known sister species (allozymes, Highton and Larson 1979; mtDNA, Mead et al 2005), and *P. idahoensis* and *P. vandykei* are known sister species (allozymes, Highton and Larson 1979; mtDNA, Carstens et al. 2004). *Plethodon larselli* is suspected to be sister to the *P.*

vandykei group, and the *P. vehiculum* group is suspected to be sister to the *P. elongatus* group (allozymes, Highton and Larson 1979). Although most of these splits are suspected to predate the Pleistocene, these relationships have yet to display high support and no attempt has been made at defining these speciation events. The species *P. dunni* and *P. vehiculum* have displayed levels of divergence higher than that of most congeneric vertebrates using allozyme data (Feder 1978). Interestingly, no further studies have examined the relationship between *P. vehiculum* and *P. dunni* considering the extreme range overlap between these species. It continues to be important to use new and/or extended data sets to test new and old hypotheses. This study reevaluates the western *Plethodon* lineage using mtDNA sequence data, and seeks to measure the level of divergence between the species groups that are not strongly supported.

Molecular markers are commonly used to explore the relationships among groups (populations or different taxa), geographically and historically (Hedrick 2001). Mitochondrial DNA (mtDNA) especially, is commonly used to elucidate phylogenetic relationships; the effective population size of mtDNA is generally ¼ that of nuclear DNA, resolving phylogenies less distant in time.

Due to the high mutation rate of the mtDNA control region (see Chap. 2), the D-loop will be used to examine the phylogenetic structure of *P. vehiculum* in the Pacific Northwest (PNW) in order to define distinct lineages in this species that because of past geologic and/or climatic events have followed unique evolutionary

trajectories. These findings will be used to further query results from the genetic divergence estimates and haplotype network outlined in Chapter Two.

To fully understand the speciation events of a clade, all extant taxa need to be sampled (Barraclough and Nee 2001). This is the first study to employ all *Plethodon* species in the PNW, including many individuals from each species, using the mtDNA cytb gene. Cytb is commonly used in phylogeographic reconstruction and conservation of terrestrial salamanders (Carstens et al. 2004, Mahoney 2004, Mead et al. 2005, Wagner et al. 2005). Therefore, sequence variation of this gene will be used to explore the timing and cause of speciation events in the PNW *Plethodon* salamanders and variation will be compared across taxa.

Materials and Methods

Sample Collection and DNA processing

Tissue collection and DNA extractions are as in Chapter Two for *P. vehiculum* and *P. dunni* (see Appendix A). A combination of sequence and geographic data from previous studies and the newly generated data were used. Other *Plethodon* species sequence data was retrieved from GenBank with accession numbers and citations found in Appendix E. All unique haplotypes from each species in the PNW available on GenBank were used for phylogenetic analyses. *Ensatina eschscholztii* was chosen for the outgroup as it belongs in the family Plethodontini but does not form a monophyletic group with species from the Genus *Plethodon* (Wake 1993).

A 656-bp region of the mtDNA cytb gene (see Fig. 4) was amplified using the primers MVZ-15 (5'- GAA CTA ATG GCC CAC ACW WTA CGN AA -3') and MVZ-16 (5'- AAA TAG GAA ATA TCA TTC TGG TTT AAT -3') (Moritz et al. 1992). This region was chosen based on other studies of *Plethodon* salamanders in the PNW and is the only gene for which data exists on all the species GenBank. For a total of 25μL reaction volume, 1μL of MVZ 15 (25μM), 1μL of MVZ 16 (25μM), 22 μL dH2O and 1μL of DNA were added to PuRe Taq™ Ready-To-Go Bead tubes. The following program was run on a thermocylcer: initial denaturation ran for 5 minutes at 95°C; 36 cycles of denaturation at 95°C for 1 minute, annealing at 45°C for 1 minute, and extension at 72°C for 90 seconds; an additional final extension was run at 72°C for 10 minutes and held at 4°C. PCR purification and sequencing are as described in Chapter Two.

Sequence Analysis

Phylogenetic analyses were done with unique *P. vehiculum* D-loop haplotypes to visualize distinct lineages in this species. A parsimony tree was constructed in PAUP 4.0b10 (Swafford 2000) using the heuristic search default settings: stepwise addition, swap on the best trees only, simple addition sequence, and tree branch swapping. All characters were weighted equally and unordered. A bootstrap analysis was done with 100 pseudoreplicates. Model Test 3.8 (Posada 1998) was then used for testing 56 models of evolution, to determine the best-fit model and model parameters for maximum likelihood (ML) analysis using a Jukes-Cantor69 Neighbor-joining (NJ)

tree as a starting tree. The Hierarchical Likelihood Ratio Test determined the best-fit model to be TIM+I+G (Transition Model plus Invariant sites plus Gamma) while the Akaike Information Criterion determined the best-fit model to be GTR+G (General Time Reversible plus Gamma). The GTR+G model of evolution was used for ML analysis and includes six parameters so that each possible substitution has its own probability and allows for unequal base frequencies and estimates the shape parameter of the gamma distribution. The estimates for this dataset are as follows: Base=(0.3036 0.2415 0.1338), Nst=6, Rmat=(1.0000 2.9371 1.7877 1.7877 2.0269), Rates=gamma, Shape=0.9049, Pinvar=0.3892. Bootstrap analysis was not carried out under the ML method.

Cytb sequences were cleaned with SeqMan of the DNASTAR© Lasergene v6 software, saved as EditSeq files for import into MegAlign. MegAlign was used to align sequences using the ClustalV method (Higgins et al. 1991) and imported in MacClade 4.08 (Maddison and Maddison 2005). The alignment was then translated to amino acid sequence and adjusted by visual inspection. After alignment, 696-bp of cytb were used for phylogenetic analysis.

A parsimony tree was constructed in PAUP 4.0b10 (Swafford 2000) as the D-loop of *P. vehiclulm* with 100 bootstrap psuedoreplicates. Model Test 3.8 (Posada 1998) determined the best-fit model to be TrN+G (Tamura-Nei plus Gamma; Hierarchical Likelihood Ratio Test) and GTR+G (General Time Reversible plus Gamma; Akaike Information Criterion). The GTR+G model of evolution was used for ML analysis, also as in Chapter Two. The estimates for this dataset are as follows:

Base=(0.2932 0.2392 0.1390), Nst=6, Rmat=(2.6495 6.9439 1.5818 1.0704 10.5983), Rates=gamma, Shape=0.5167, Pinvar=0. Starting branch length was obtained using the Rogers-Swofford approximation method and one-dimensional Newton-Raphson with a pass limit of 20 was used for branch-length optimization. Starting trees were obtained by step-wise addition and branch swapping was done using the tree-bisection-reconnection (TBR) algorithm. Bootstrap analysis was not carried out under the ML method.

A pairwise distance matrix was calculated in PAUP using the GTR model of evolution between species. Using a standard molecular clock of 2% sequence divergence per million years for mtDNA suggested by Avise et al. (1998), divergence times were estimated for all lineages. The use of a molecular clock has been debated and many ectotherms exhibit divergence rates much slower than the standard rate. Less is known regarding the evolutionary rates of mtDNA in ectotherms (Tan and Wake 1995, Avise et al. 1998, Gerber et al. 2001), therefore the 2% sequence divergence rate is used only as a rough estimate for this study.

Diversity statistics and sequence divergence in all species using cytb found in the literature are compiled and summarized with a focus on the data collected in this study for *P. vehiculum*. A 361-bp region for which there was complete overlap of all individual sequences was used to compare sequence characteristics in MacClade 4.08 and input into Arelquin 2.0 (Schneider et al. 2000) to compare values of θ_S, the diversity measure which is not sensitive to haplotype frequency. Sequence divergence and the proportion of unique haplotypes within each species was also compared.

Results

Control region parsimony analysis

Of the 721 characters sequenced for the mtDNA control region of *P. vehiculum*, 553 (77%) were constant, 88 (12%) were variable but parsimony uninformative, and 80 (11%) were parsimony informative. After 73,672,842 rearrangements were tried, 1000 most parsimonious trees with a score of 234 were saved. One strict consensus tree was used for interpretation (Fig. 8). A strict consensus shows only those relationships that are unambiguous and the relationships where the trees disagree are displayed as polytomies. The southern Oregon population formed a monophyletic clade separate from all other PNW populations with high support (bootstrap = 82). Strong relationships were observed between certain individuals in the northwest WA population (bootstrap = 88), and between individuals from the southern coast and Cascades of WA (bootstrap = 100). Otherwise, no other strong phylogenetic signal was detected between populations and the northern clade is represented by one large polytomy.

Cytb sequence characteristics

When analyzing 696 characters of the cytb gene in all *Plethodon* species, 366 (53%) were constant, 56 (8%) were variable but parsimony uninformative, and 274 (39%) were parsimony informative. The third codon position displayed the highest rate of change, which would be expected, followed by the first and then second codon

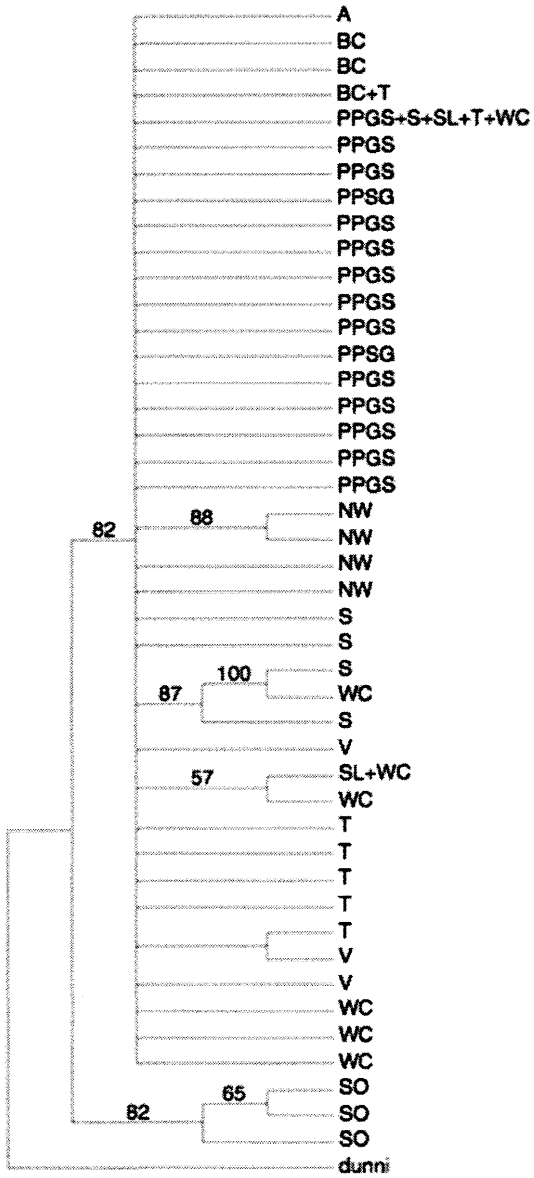


Figure 8. Maximum parsimony tree of *P. vehiculum* based on the mtDNA D-loop. Parsimony analysis score = 234. Parsimony bootstrap values based on 100 pseudoreplicates are represented by numbers above branches, only values greater than 50 are represented. A strict consensus of 1000 MP trees is shown. Outgroup is *P. dunni*.

positions. The region of cytb gene that showed the highest rates of variability is around 300-bp of the 696-bp analyzed. Base frequency was as follows: A= 0.29320, C= 0.23920, G= 0.13900, and T= 0.32860, showing higher frequencies of A+T, common in mtDNA. The transition/transversion ratio was 2.06, with most mutations C to T (99) and T to C (94).

Cytb parsimony analysis

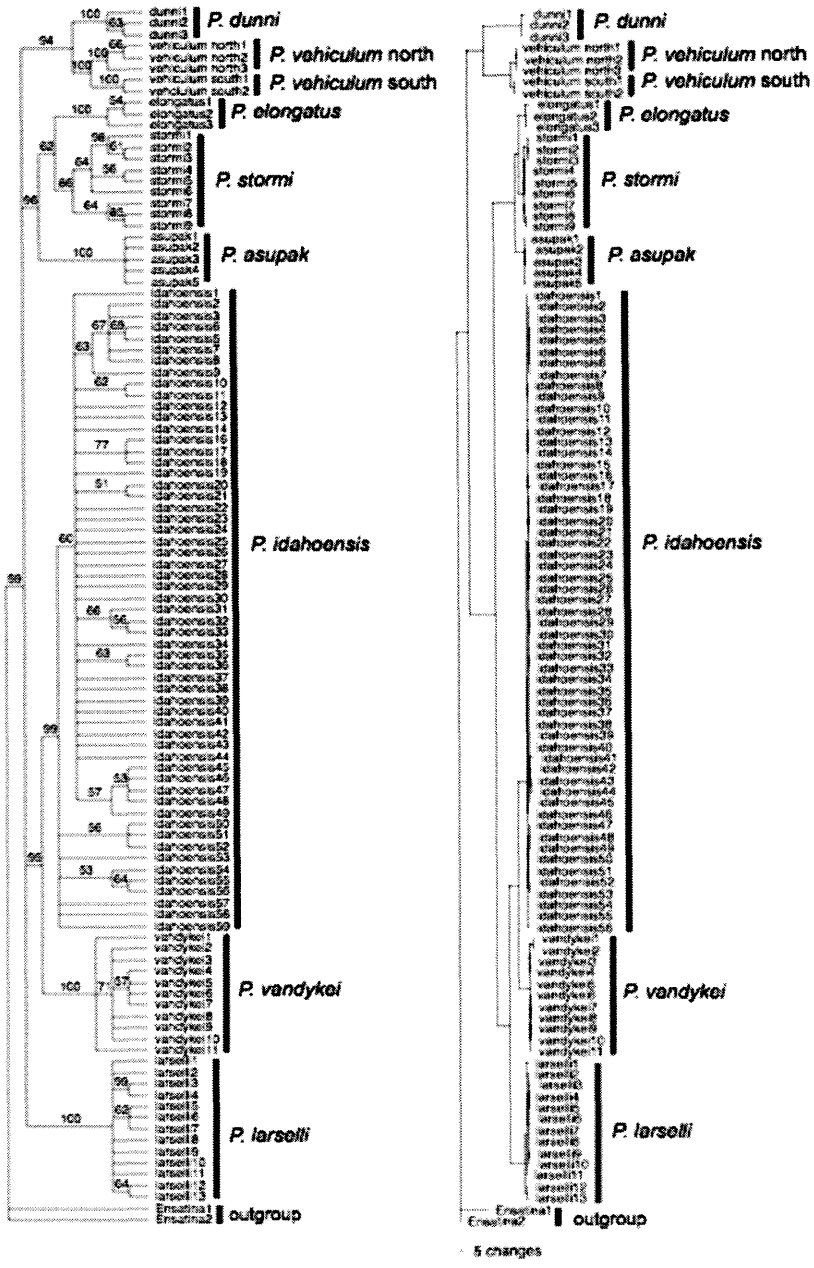
After 262,733,355 rearrangements were tried, 1000 most parsimonious trees were saved with a score of 721. The strict consensus tree of all 1000 trees was used (Fig. 9A), as it was not important to determine the relationship within species but only between them. *Plethodon dunni* and *P. vehiculum* were sister species with high bootstrap support of 94. The southern Oregon clade of *P. vehiculum* was formed a separate monophyletic clade to the rest of the *P. vehiculum* individuals (bootstrap = 100), also observed using the D-loop. Within the *P. elongatus* group, *P. elongatus* and *P. stormi* were the most closely related (bootstrap = 62), and *P. asupak* was sister to these two (bootstrap = 96). The sister relationship between *P. idahonensis* and *P. vandykei* was highly supported (bootstrap = 95). However, a polytomy was observed at the node for a common ancestor between the *P. vehiculum/dunni*, *P. elongatus/stormi/asupak*, *P. idahoensis/vandykei* and *P. larselli* groups.

Cytb maximum likelihood analysis

Using the GTR+G model of evolution, two ML trees were saved with a score of 4616.39646, after 441,906 rearrangements. Only one of these trees is shown, whereas the only differences between trees were within the *P. idahoensis* group (Fig. 9B). Relationships between *P. vehiculum* and *P. dunni* were the same as in the parsimony analysis, as well as the north-south split in *P. vehiculum*. *Plethodon larselli* is sister to the *P. idahoensis/vandykei* group. Moreover, the *P. elongatus/stormi/asupak* and *P. idahoensis/vandykei/larselli* are closely related, while the *P. vehiculum/dunni* group was sister to these two. Therefore, the ML analysis was better able to resolve relationships among the major *Plethodon* groups in this study.

Cytb divergence estimates and diversity statistics

Based on GTR distance measure, individual sequence divergence ranged from 0.002, in *P. dunni* to 0.024 in *P. stormi*, within species, and from 0.090 (*P. elongatus* and *P. stormi*) to 0.285 (*P. larselli* and *P. vehiculum*) between species (Table 6). The lowest divergence estimates within the major groups was within the *P. elongatus/stormi/asupak* group at 0.09 (*P. elongatus* and *P. stormi*), while the others were larger at 0.156 (*P. stormi* and *P. asupak*), and 0.188 (*P. elongatus and P. asupak*). Using these divergence estimates, divergence times are estimated at 4.5 mya, 7.8 mya, and 9.4 mya, respectively. The split between *P. idahoensis* and *P. vandykei* was relatively recent (0.113; 5.7 mya) while the split within the *P. vehiculum* and *P. dunni* group is relatively deep (0.155; 7.8 mya).



A. B.

Figure 9. Maximum parsimony and maximum likelihood trees of western *Plethodon* based on the mtDNA cytb gene. A. Parsimony analysis (score = 721). Bootstrap values are based on 100 pseudoreplicates and are represented by numbers above branches, only values greater than 50 are represented. A strict consensus of 1000 MP trees is shown. B. Maximum likelihood analysis (score=) based on the GTR+G model of evolution. Outgroup is *Ensatina eschcholztii*.

Sequence divergence between the major groups ranged from 0.226 for *P. vandykei/idahonesis* and *P. elongatus/stormi/asupak* to 0.278 for *P. vehiculum/dunni* and *P. larselli.* Estimated divergence times ranged from 11.3 mya for *P. vandykei/idahonesis* and *P. elongatus/stormi/asupak* to 13.9 mya for *P. vehiculum/dunni* and *P. larselli* (Table 7). The *P. vehiculum/dunni* group as a monophyletic group to the others has a high estimate of divergence at 0.243 and divergence time of 12.2 mya. As potential sister groups, *P. elongatus/stormi/asupak* and *P. vandykei/idahoensis/larselli* are 0.241 divergent and an estimated divergence time of 12.1 mya.

The proportion of unique cytb haploytpes observed in all *Plethodon* species is given in Table 9. The proportion of unique haplotypes ranged from 0.15 to 0.62. *Plethodon vehiculum* had approximately half that of all other species. For diversity estimates, *P. dunni* has the lowest estimate of θ_S, however sampling among this species was very low (Table 8). The θ_S value of *P. vehiculum* was very low at 3.25 especially compared to the range found in the other species (9.33 to 62.84). The highest was found in *P. vandykei.*

When the southern OR individuals are removed from the *P. vehiculum* analysis, the within species divergence estimates and diversity statistics fell to zero. Divergence within the southern OR clade was 0.005 and $\theta_S = 2.0 \pm 1.7$. Divergence estimates between the northern and southern clade of this species is 0.038 (1.9 mya).

Table 6. Divergence estimates based on the GTR model of evolution within and between western *Plethodon* salamanders.

	P. vehiculum	*P. dunni*	*P. larselli*	*P. vandyke*	*P. idahoensis*	*P. elongatus*	*P. stormi*	*P. asupak*
P. vehiculum	0.023	7.8	14.3	11.5	11.1	12.0	11.3	10.9
P. dunni	0.155	0.002	13.6	12.6	12.8	12.0	11.8	12.8
P. larselli	0.285	0.271	0.022	11.8	12.0	12.9	12.4	15.7
P. vandyke	0.229	0.252	0.235	0.008	5.7	11.3	11.2	13.1
P. idahoensis	0.222	0.256	0.239	0.113	0.010	11.0	9.6	11.7
P. elongatus	0.239	0.228	0.258	0.225	0.219	0.015	4.5	9.4
P. stormi	0.225	0.235	0.247	0.224	0.191	0.090	0.024	7.8
P. asupak	0.218	0.255	0.313	0.261	0.234	0.188	0.156	0.011

Above diagonal: Divergence times estimated in millions of years ago (mya).

Diagonal elements: Within population divergence estimates.

Below diagonal: Among population divergence estimates.

Table 7. Divergence estimates between major groups of western *Plethodon* salamanders based on the GTR model of evolution.

Species Group Relationship	Sequence Divergence	Divergence Time (mya)
Plethodon vehiculum/dunni and *larselli*	0.278	13.9
Plethodon vehiculum/dunni and *vandykei/idahonesis*	0.240	12.0
Plethodon vehiculum/dunni and *elongatus/stormi/asupak*	0.233	11.7
Plethodon larselli and *vandykei/idahonesis*	0.237	11.9
Plethodon larselli and *elongatus/stormi/asupak*	0.273	13.7
Plethodon vandykei/idahonesis and *elongatus/stormi/asupak*	0.226	11.3
Plethodon elongatus/stormi/asupak and *vandykei/idahoensis/larselli*	0.241	12.1
Plethodon vehiculum/dunni and *elongatus/stormi/asupak/vandykei/idahensis/larselli*	0.243	12.2

Table 8. Proportion of unique haplotypes discovered in all *Plethodon* species of the PNW and diversity estimates.

	# Unique Haplotypes	Percentage	Citation	θ_S
P. larselli	13/44	30	Wagner et al. 2005	9.667
P. vandykei	10/28	36	Carstens et al. 2004, 2005	62.838
P. idahoensis	62/243	26	Carstens et al. 2004, 2005	9.325
P. elongatus/stormi	76/122	62	Mahoney 2004	18.947/5.419
P. asupak	6/18	33	Mead et al. 2005	16.204
P. vehiculum	5/32	15		3.254
*P. dunni**				0.545

*No studies currently available. One sequence was obtained from GenBank (Mahoney 2004). Three others were obtained from the Mt Hood National Forest during the duration of this study.

Discussion

Plethodon vehiculum phylogenetics

Both parsimony and maximum likelihood analyses of the mtDNA D-loop support the findings from Chapter Two that the southern OR population of *P. vehiculum* forms a unique phylogenetic clade with high support. A north-south split in *P. vehiculum* was also observed in the more highly conservative cytb gene (see below). Thus, there are two major groups in *P. vehiculum* species in the PNW. Distinct clades represent survival over several ice ages and can provide signs of range changes during interglacials (Hewitt 2004). This is a common pattern described in many western *Plethodon* species. For example, *P. larselli*, *P. elongatus*, and *P. idahoensis* formed distinct clades in a north-south fashion with high levels of genetic variation within and between populations using the cytb gene (Carstens et al. 2004, Mahoney 2004, Wagner et al. 2005). However, in contrast to these studies, there is a striking lack of diversity in the cytb gene of *P. vehiculum* throughout its northern range. Life history and dispersal capabilities can strongly influence the signature of mtDNA genetic structuring (Avise and Ellis 1986). Age at maturity, dispersal behavior, clutch size, frequency and survivorship, might all contribute to low levels of genetic variation. Yet, ongoing dispersal does not tend to swamp out the historical structure of populations (Avise and Ellis 1986) and mtDNA is therefore useful in constructing phylogenies and inferring information about how population history has contributed to the observed patterns of local adaptation and speciation. Rarely are animals, even in a small geographic range, genetically homogenous throughout their

range. Results of this study serve as evidence of a recent population bottleneck and/or very recent range expansion in the northern clade given the phylogenetic star shaped pattern of the D-loop and lack of diversity in the cytb gene, while the southern population has a long history of separation as it forms a distinct clade in multiple analyses, with a divergence time estimated at 1.9 mya, at the start of the Pleistocene.

Plethodon phylogenetics

In this study higher support is offered to the major groups described in the literature: 1) *P. vehiculum* and *P. dunni*, 2) *P elongatus*, *P. stormi*, and *P. asupak*, and 3) *P. vandykei* and *P. idahoensis* (Highton and Larson 1979, Mahoney 2001) are all well supported sister species. All groups formed one large polytomy based on parsimony analysis, however, the ML tree indicates that the *P. vehiculum/dunni* group is the sister group to all the other western *Plethodon* species and also suggests a closer relationship between *P. larselli* and the *P. vandykei/idahoensis* group. This is in contrast with Weins et al. (2006) and Chippendale et al. (2004), but consistent with Mahoney (2001; although weakly supported) and needs to be further studied. The former studies only used a subset of western *Plethodon sp.* and one individual, while the latter had more extensive sampling. This study incorporated the use of all extant western *Plethodon* taxa using the cytb gene, but by using more molecular markers and other analytical methods, we may better resolve the relationships in this group. In any case, it is important to understand species relationships within this group as they are

unique having undergone these speciation events, yet are morphologically constrained and demonstrate considerable differences in their range distributions.

Divergence estimates and diversity statistics

Sequence divergence measurements were used to estimate divergence times among and within groups of *Plethodon* salamander species and all seem to have diverged from each other at a similar time (range among: 11.7-13.9 mya), range within: 4.5-7.8 mya) before the start of the Pleistocene (1.8 mya). These divergence times are consistent with divergence estimated in other studies for terrestrial salamanders in the PNW (i.e. divergence between *P. vandykei* and *P. idahonesis* is estimated at 5 mya, Carstens et al. 2005). However, if the mutation rate of ectotherm mtDNA is indeed slower than other mammals, the speciation events of western *Plethodons* will be even deeper (Gerber et al. 2001). In any case, this study indicates that *P. vehiculum* as well as other extant *Plethodon* salamander species were present in the PNW before and survived through the recorded Pleistocene glaciations. Still, questions remain as to how these species evolved from each other: Were these allopatric events resulting in range expansion allowing for subsequent extensive range overlap in this group? Were these speciation events driven by selection and niche specialization elevated by high levels of philopatry? Does niche specialization and/or competition play a part in shaping the differences in abundance and distribution of these salamanders? According to results of this study, speciation events of these salamanders took place during the late Miocene-early Pliocene where an increase in

temperature and aridity (Cerling et al. 1997) would have caused contraction and fragmentation of suitable habitat. A sound hypothesis is that any clade with long-term maintainence of an ecological niche, in this case, terrestrial salamanders and the forest floor since the Eocene, will have higher rates of vicariant lineage splitting (Kozak et al. 2006). This is because they are so narrowly adapted, that when they are exposed to temporal fluctuations in climate, periods of range expansion and contraction will be the result as they follow suitable habitat.

Divergence estimates based on cytb, support the relatively deep separation of the north-south division of *P. vehiculum*. The divergence levels of these two groups are not necessarily as high as the divergence estimates between the other closely related *Plethodon* species, but given the conservative nature of cytb in *P. vehiculum* this might be high enough to support the claim that it should be given subspecies or species status. Although deep divergence has been observed within many *Plethodon* salamanders (Moritz et al. 1992, Jocusch and Wake 2002), similar levels of genetic divergence are observed between species (Mahoney 2004). Collection of type specimens, and additional genetic sampling in combination with extensive morphological measurements of the southern clade are recommended.

Divergence estimates and diversity statistics within species were the smallest in *P. dunni* and *P. vehiculum*. The small sample size of *P. dunni* may have contributed to the low level of genetic sequence divergence within this species and should therefore be more widely sampled to: 1) gather a more accurate measure of genetic diversity within this species, and 2) to be sure sampling has occurred from

throughout its range to better realize its placement in the *Plethodon* phylogeny. However, even given more complete sampling, *P. vehiculum* showed levels of sequence divergence and diversity estimates lower than that of any other *Plethodon* species. Excluding the southern OR clade, divergence estimates and diversity statistics were zero across the entire PNW range. Low diversity levels of coding regions of the mtDA have been found in other salamander species like the European *Salamandra lanzai*, however, this species is an aquatic salamander (Riberon et al. 2002). Temperate species tend to have less genetic variation as they expanded after ice ages (Hewitt 2004), but the degree of variation may depend on niche specialization and dispersal capabilities of the species and on geography. In addition, life history traits may influence the structuring in diverse taxa, for example, a long generation time will mean a slower rate of evolution. Life history traits are rather unknown in terrestrial salamanders as they spend the majority of their time underground, but nonetheless may vary across taxa. At any rate, neutral theory predicts that genetic variation will increase with population size. If it is lower than expected, it may be because the population size has undergone a recent bottleneck, or that insufficient time has elapsed since their establishment and the population has not reached an evolutionary equilibrium (Mitton 1994). Highly structured populations are not consistent with dramatic range expansion and it is clear that *P. vehiculum* has undergone recent range expansion after the Pleistocene.

Phylogenetics provides us with a means to document and study speciation events, and in conjunction with ecological and geologic data, is a powerful way to

better understand the patterns and processes shaping the biodiversity observed among extant taxa. Including multiple individuals spanning a species range allows for greater sampling of current haplotype diversity and is a more accurate way to measure relationships among species. To date, no study has incorporated all western *Plethodon* species over their geographic range. This study provides additional support to the sister groups previously defined, although the relationships among these groups are yet to be strongly supported. Differences in the levels of sequence variation among taxa in the mtDNA cytb gene that is commonly used in phylogeographic and phylogenetic studies was also found to differ across taxa, emphasizing the importance of understanding mtDNA evolution in constructing phylogeographic hypotheses.

Chapter 4: General Conclusions

Phylogeographic and phylogenetic studies further increase our understanding of evolution in natural populations. The information gained using mitochondrial DNA (mtDNA) sequence data not only describes contemporary species structuring, but also gives us insight into the historical patterns exhibited by a species or group of closely related species. Both these patterns, historical and contemporary, help shape the diversity and evolution of organisms. Plethodontid salamanders display some of the highest diversity of animals in North America; understanding the patterns and processes of local adaptation and speciation is an extraordinary way of explaining this diversity (Gibbs 2001).

Producing more tissue, and of a higher protein content annually than mammals or birds of similar size, salamanders are an important food for predators (Burton and Likens 1975a,b). In moist terrestrial environments, salamanders are the primary consumers of invertebrates, and therefore act as a delivery system of invertebrate food sources up the food chain; many species of reptiles (mainly snakes), small mammals, and birds prey upon these salamanders that would otherwise be unlikely to access this food source directly. Their low energy demands, long life span and relative abundance suggests they are important nutrient pools in forests (Davic and Welsh 2004). As consumers of detritivores, salamanders also greatly affect rates of nutrient cycling and energy flow through communities and ecosystems (Stebbins and Cohen 1995). Spending much of their time underground they greatly affect soil dynamics through the translocation of nutrients, fungi, and other microorganisms (Davic and

Welsh 2004). Understanding the population dynamics of salamanders and further acknowledging each species dispersal capabilities will not only help design effective management plans, but will also shed light onto the ecology and evolutionary pathways of these organisms.

The phylogoegraphic and phylogenetic patterns observed in this study suggests the possibility that long-distance dispersal events, previously considered extremely rare among salamander populations, have directed the population structure of *P. vehiculum* in the northern clade. Perceived physiological constraints, small stature, and site fidelity of amphibians (Blaustein et al. 1994) make these data extremely surprising, especially considering large historical barriers to dispersal (i.e. the Columbia River and dramatic elevation changes). Nevertheless, even in the absence of genetic structure, source-sink dynamics need to be taken into account; dispersal may be possible but if populations that are considered source populations are harmed, the sink populations will not be recolonized and neither will be able to recover, resulting in regional extinctions. Immigrations could be the sole reason for one deme to survive, especially if it is a small population (Stacey and Taper 1992) as very small numbers of migration are required to keep a population stable.

Strong spatial structure of mtDNA implies high site fidelity of females and demogragrahic autonomy over time. Because mtDNA is maternally inherited, significant genetic structuring is expected in animals with male-biased dispersal. *Plethodon vehiculum* may not be a salamander that exhibits this life history characteristic although it is common among many salamander species. Molecular

markers with differing modes of inheritance as well as different rates of mutation combined with morphological and ecological data can give insight to sex-biased dispersal and explore further the reasons for low genetic differentiation of *P. vehiculum* in the PNW, particularly in the northern clade.

Most genetic species differentiation that can be easily explained geographically involves some type of long-term barrier to gene flow (i.e. the Columbia River) or the extinction of lineages within species that are widespread with limited gene flow/dispersal (Avise et al 1987). The north-south phylogeographic break of *P. vehiculum* established in this study is best explained by historic events. The divergence estimates between the southern and northern clades is extremely high using the mtDNA D-loop and similar to that between north-south clades found in *P. elongatus* using the cytb gene. The genetic structuring *in P. elongatus* was proposed to be the result of recent glacial cycles during the Pleistocene, as the Siskiyou mountaintops in this region were glaciated at that time (Mahoney 2004).

The genetic structuring in the northern range of *P. vehiculum* is not consistent with other terrestrial salamander studies of the Pacific Northwest (PNW). Differences may be explained by different phenotypic characteristics, which have yet to be explored, different demographic histories, as brought to light in this study, and different rates of mtDNA evolution. Assuming neutral mutation, low mtDNA diversity may mean a recent population bottleneck or strong female dispersal (Moritz et al 1987), and high recent gene flow; all of these seem to be playing a factor in shaping the distribution and structure of *P. vehiculum*.

Other types of molecular markers may be necessary to determine answers to the phylogeographic questions posed here because mtDNA is a single type of molecular marker with its own history, and this history may differ from the history of other markers and that of the true history of a species (Ballard and Whitlock 2004). On a small timescale, mtDNA may not accurately reconstruct gene genealogies because the number of mutational differences may be too small in a population (Ballard and Whitlock 2004). In the future, using both molecular markers that are neutral and under selection (Hedrick 2001) will help to fully understand the nature of population divergence. Although there might be significant gene flow, strong selection pressure might still be acting to shape populations. The comparison of the D-loop with the cytb gene emphasizes the importance of using more than one marker with slightly different evolutionary characteristics.

In summary, the western *Plethodons*, although appearing morphologically similar may differ highly in their physiology and life history traits, features than can greatly influence speciation. Microhabitat selection and niche occupation may also play roles in shaping the distribution and abundance of these salamander species.

Future Directions

The more we know about a particular system, the more doors we open for future exploration. This study provides an excellent framework for developing more intricate hypotheses and these data represent a necessary first step to directing more complex ecological, morphological, and genetic sampling. With additional data,

advances in theoretical modeling will allow us to accurately incorporate more parameters, take into account stochastic events, and evaluate the usefulness of molecular markers pertaining to specific questions to better support phylogenetic hypotheses and more accurately estimate divergence times and rates (Carstens et al. 2004, 2005, Townsend 2007). In addition, niche modeling is a means of determining whether niche spaces have been filled and if these salamanders are displaced ecologically. For example, *P. vandykei* has been known only to be found in wet logs in areas where they are sympatric with *P. vehiculum* (Carstens 2009, personal communication) as opposed to a broader selection of microhabitat types when found alone.

Studies are lacking that measure physiological differences and competitive abilities in terrestrial salamanders especially *P. vehiculum*; these types of traits will greatly influence their dispersal capabilities as well as their ability to respond to environmental fluctuations. Furthermore, although salamanders are generally known for having constrained variation in morphological characters, Carr (1996) found significant morphological variation in the *Plethodon glutinosis* group in eastern North America and Gibbs (1998) found definite enhanced morphotype diversity in fragmented populations of *P. cinereus*. This signifies that morphological variation among species/populations may exist but not enough studies have been done with terrestrial salamanders in the PNW to describe its extent. With further research we may identify additional characters of importance that can answer questions for this

Genus pertaining to range shifts, modes of speciation, species boundaries, and the presence of phenotypic clusters and elucidate what is shaping them.

MtDNA coding regions may show high rates of selective sweeps (Gerber 2001). With more markers we may better be able to differentiate between selective sweeps and other processes (i.e. range expansion). For example, without the use of the mtDNA D-loop in Chapter Two, the ability to differentiate between these processes of *P. vehiculum* would be impossible and there is obvious evidence of range expansion. For that reason, more extensive analyses of mtDNA in ecotherms are necessary. Studies have found that the stability of mtDNA in salamanders is not as consistent as previously thought (Mueller and Boore 2005). There are major differences in the characteristics of cytb in this salamander Genus, and although the gene rearrangements found in this study do not affect the variability of the cytb gene, this study still highlights the importance of understanding the natural history of mtDNA and having a broader representation of the mtDNA genome.

Given the diversity found in the D-loop of *P. vehiculum* (see Chap. 2), and its widespread distribution, the lack of variation found in the cytb gene is probably not a cause for concern and the listing of this species as 'least concern' is appropriate. But this does raise questions as to what has caused the different population structural patterns and distribution of this species compared to that of other terrestrial salamanders in the PNW. Although terrestrial salamanders tend to be morphologically, ecologically, and behaviorally constrained, data concerning differences in their physiology (i.e. temperature and moisture, requirements and limits)

and life history traits are lacking. A particular focus on *P. dunni*, as it is the sister species to *P. vehiculum* and because it shares a widespread distribution in the PNW, would be most interesting allowing for direct comparison of habitat specialization, dispersal capabilities, and demographic history.

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Appendix A. *Plethodon vehiculum* collection localities with longitude and latitude. Three *P. dunni* species were collected in the Mt. Hood National Forest. One PPGS sample was misidentified as *P. vehiculum* but upon sequence data of cytb was confirmed as *E. ensatina*.

Sample ID	Locality	Latitude (North)	Longitude (West)
A1 *(P.dunni)*	Oregon; Mt Hood National Forest; Angels Rest	45° 34' 13.56"	122° 06' 50.6"
A2 *(P.dunni)*	Oregon; Mt Hood National Forest; Angels Rest	45° 34' 13.56"	122° 06' 50.6"
A3 *(P.dunni)*	Oregon; Mt Hood National Forest; Damascus	45° 34' 13.56"	122° 06' 50.6"
A4	Oregon; Mt Hood National Forest; Damascus	45° 34' 13.56"	122° 06' 50.6"
BC1	British Columbia (mainland); Cultus Lake	49° 01' 21.22"	122° 06' 59.29"
BC2	British Columbia (mainland); Cultus Lake	49° 01' 21.22"	122° 06' 59.29"
BC3	British Columbia (mainland); Cultus Lake	49° 01' 21.22"	122° 06' 59.29"
BC4	British Columbia (mainland); Cultus Lake	49° 01' 21.22"	122° 06' 59.29"
LR106	Oregon, Portland; George Himes Park; Multnomah Co	45° 28' 42.19"	122° 41' 0.672"
LR107	Oregon, Portland; George Himes Park; Multnomah Co	45° 28' 42.19"	122° 41' 0.672"
LR133	Oregon, Portland; George Himes Park; Multnomah Co	45° 28' 42.19"	122° 41' 0.672"
LR18	Oregon; Portland; Marshall Park; Multnomah Co	45° 27' 10.02"	122° 41' 30.47"
LR19	Oregon; Portland; Marshall Park; Multnomah Co	45° 27' 10.02"	122° 41' 30.47"

LR23	Oregon; Portland; Marshall Park; Multnomah Co	45° 27' 10.02"	122° 41' 30.47"
LR24	Oregon; Portland; Marshall Park; Multnomah Co	45° 27' 10.02"	122° 41' 30.47"
LR25 (*E.ensatina*)	Oregon; Portland; Marshall Park; Multnomah Co	45° 27' 10.02"	122° 41' 30.47"
LR29	Oregon, Portland; George Himes Park; Multnomah Co	45° 28' 42.19"	122° 41' 0.672"
LR31	Oregon, Portland; George Himes Park; Multnomah Co	45° 28' 42.19"	122° 41' 0.672"
LR32	Oregon, Portland; George Himes Park; Multnomah Co	45° 28' 42.19"	122° 41' 0.672"
LR33	Oregon, Portland; George Himes Park; Multnomah Co	45° 28' 42.19"	122° 41' 0.672"
LR6	Oregon; Portland; Tryon Creek; Multnomah Co	45° 26' 23.22"	122° 40' 50.86"
LR61	Oregon; Portland; SE Sunnybrook Blvd; Clackamas Co	45° 25' 50.54"	122° 33' 13.63"
LR74	Oregon; Portland; West Portland Park; Multnomah Co	45° 26' 36.48"	122° 43' 12.01"
LR76	Oregon; Portland; West Portland Park; Multnomah Co	45° 26' 36.48"	122° 43' 12.01"
LR82	Oregon; Portland; Marshall Park; Multnomah Co	45° 27' 10.02"	122° 41' 30.47"
LR83	Oregon; Portland; Marshall Park; Multnomah Co	45° 27' 10.02"	122° 41' 30.47"
LR84	Oregon; Portland; Marshall Park; Multnomah Co	45° 27' 10.02"	122° 41' 30.47"

LR86	Oregon; Portland; Marshall Park; Multnomah Co	45° 27' 10.02"	122° 41' 30.47"
LR87	Oregon; Portland; Marshall Park; Multnomah Co	45° 27' 10.02"	122° 41' 30.47"
NW1	Washington; Olympic National Forest; Klahowya Campground	48° 04' 53.38"	124° 06' 54.73"
NW2	Washington; Olympic National Forest; Klahowya Campground	48° 04' 53.38"	124° 06' 54.73"
NW3	Washington; Olympic National Forest; Klahowya Campground	48° 04' 53.38"	124° 06' 54.73"
NW4	Washington; Olympic National Forest; Klahowya Campground	48° 04' 53.38"	124° 06' 54.73"
NW5	Washington; Olympic National Forest; Lake Quinalt	47° 57' 06.66"	124° 23' 07.48"
NW6	Washington; Olympic National Forest; Lake Quinalt	47° 57' 06.66"	124° 23' 07.48"
NW7	Washington; Olympic National Forest; Lake Quinalt	47° 57' 06.66"	124° 23' 07.48"
S1	Washington; Wahkiakum Co; Skamokawa Vista Park	46° 17' 58.49"	123° 26' 16.97"
S2	Washington; Wahkiakum Co; Skamokawa Vista Park	46° 17' 58.49"	123° 26' 16.97"
S3	Washington; Wahkiakum Co; Skamokawa Vista Park	46° 17' 58.49"	123° 26' 16.97"
S4	Washington; Wahkiakum Co; Skamokawa Vista Park	46° 17' 58.49"	123° 26' 16.97"
S5	Washington; Wahkiakum Co; Skamokawa Vista Park	46° 17' 58.49"	123° 26' 16.97"

SL1	Washington; Mt Baker National Forest; Silver Lake	48° 52' 40.23"	121° 28' 52.76"
SL2	Washington; Mt Baker National Forest; Silver Lake	48° 52' 40.23"	121° 28' 52.76"
SL3	Washington; Mt Baker National Forest; Silver Lake	48° 52' 40.23"	121° 28' 52.76"
SO1	Oregon; Siskiyou National Forest; Coos Co	42° 53' 11.51"	124° 04' 25.61"
SO2	Oregon; Siskiyou National Forest; Coos Co	42° 53' 11.51"	124° 04' 25.61"
SO3	Oregon; Siskiyou National Forest; Coos Co	42° 53' 11.51"	124° 04' 25.61"
SO4	Oregon; Siskiyou National Forest; Coos Co	42° 53' 11.51"	124° 04' 25.61"
T2	Oregon; Tillamook State Forest; Tillamook Co	44° 11' 06.27"	123° 57' 57.22"
T3	Oregon; Tillamook State Forest; Tillamook Co	45° 36' 49.98"	123° 10' 9.42"
T4	Oregon; Tillamook State Forest; Tillamook Co	45° 36' 49.98"	123° 10' 9.42"
T5	Oregon; Tillamook State Forest; Tillamook Co	45° 36' 49.98"	123° 10' 9.42"
T6	Oregon; Tillamook State Forest; Tillamook Co	45° 36' 49.98"	123° 10' 9.42"
T7	Oregon; Clatstop State Forest; Saddle Mountain	45° 48' 14.09"	123° 27' 41.79"
T8	Oregon; Clatstop State Forest; Saddle Mountain	45° 48' 14.09"	123° 27' 41.79"

T9	Oregon; Clatstop State Forest; Saddle Mountain	45° 48' 14.09"	123° 27' 41.79"
V1	British Columbia; Vancouver Island; Port Renfrew	48° 33' 29.72"	124° 23' 58.87"
V2	British Columbia; Vancouver Island; Port Renfrew	48° 33' 29.72"	124° 23' 58.87"
V3	British Columbia; Vancouver Island; Port Renfrew	48° 33' 29.72"	124° 23' 58.87"
V4	British Columbia; Vancouver Island; Cedar Creek Campground	48° 33' 29.72"	124° 23' 58.87"
WC1	Washington; Gifford Pinchot National Forest; Multon Falls	46° 28' 14.25"	122° 20' 17.05"
WC2	Washington; Gifford Pinchot National Forest; Multon Falls	46° 28' 14.25"	122° 20' 17.05"
WC3	Washington; Gifford Pinchot National Forest; Multon Falls	46° 28' 14.25"	122° 20' 17.05"
WC4	Washington; Gifford Pinchot National Forest; Multon Falls	46° 28' 14.25"	122° 20' 17.05"
WC5	Washington; Gifford Pinchot National Forest; Multon Falls	46° 28' 14.25"	122° 20' 17.05"
WC6	Washington; Gifford Pinchot National Forest; Multon Falls	46° 28' 14.25"	122° 20' 17.05"
WC7	Washington; Gifford Pinchot National Forest; Multon Falls	46° 28' 14.25"	122° 20' 17.05"
WC8	Washington; Gifford Pinchot National Forest; Multon Falls	46° 28' 14.25"	122° 20' 17.05"

Appendix B. Sequences obtained from GenBank to construct D-loop primers based on the conserved flanking tRNA regions.

Accession #	Species	Citation
AY728222	*Plethodon petraeus*	Mueller et al. 2004
NC_006343	*Plethodon cinereus*	Mueller et al. 2004
AY728232	*Plethodon cinereus*	Mueller et al. 2004
AY728223	*Plethodon elongatus*	Mueller et al. 2004
NC_006335	*Plethodon elongatus*	Mueller et al. 2004
NC_006328	*Ensatina eschscholtzii*	Mueller et al. 2004

Appendix C. Sequences removed for analysis due to large amounts of missing or ambiguous data.

Sample ID	Locality
LR107	Oregon, Portland; George Himes Park; Multnomah Co
S2	Washington; Wahkiakum Co; Skamokawa Vista Park
S3	Washington; Wahkiakum Co; Skamokawa Vista Park
S5	Washington; Wahkiakum Co; Skamokawa Vista Park
WC5	Washington; Gifford Pinchot National Forest; Multon Falls

Appendix D. List of haplotypes observed in TCS analysis in the mitochondrial D-loop of *P. vehiculum* in the PNW.

Haplotype	Sample ID
H1	BC3, BC4, LR6, LR18, LR61, LR74, LR76, LR76, S1, SL2, SL3, T3, T6, T7, WC1, WC6, WC7, WC8
H2	A4
H3	BC1
H4	BC2
H5	LR23
H6	LR24
H7	LR29
H8	LR31
H9	LR82
H10	LR83
H11	LR84
H12	LR86
H13	LR87
H14	LR106
H15	LR133
H16	LR19, LR32, LR33
H17	NW1
H18	NW2, NW3, NW5, NW6, NW7
H19	NW4
H20	S4
H21	SL1, WC4
H22	SO1, SO3
H23	SO2
H24	SO4
H25	T2
H26	T5
H27	T8
H28	T9
H29	V1, V4
H30	V2
H31	V3
H32	WC2
H33	WC3

Appendix E. Sequences obtained from GenBank for phylogenetic analysis and diversity statistics.

Acession #	Species	Citation
AY183763	*dunni*	Mahoney 2004
AY691759	*vandykei*	Wiens et al. 2004
AY962291	*vandykei*	Carstens et al. 2005
AY962290	*vandykei*	Carstens et al. 2005
AY982289	*vandykei*	Carstens et al. 2005
AY572045	*vandykei*	Carstens et al. 2004
AY572044	*vandykei*	Carstens et al. 2004
AY572043	*vandykei*	Carstens et al. 2004
AY572042	*vandykei*	Carstens et al. 2004
Ay572041	*vandykei*	Carstens et al. 2004
AY572040	*vandykei*	Carstens et al. 2004
AY572039	*vandykei*	Carstens et al. 2004
AY183762	*vandykei*	Mahoney 2004
AY691746	*elongatus*	Wiens et al. 2006
NC_006335	*elongatus* ^	Mueller et al. 2004
AY728223	*elongatus* ^	Mueller et al. 2004
AY183885	*elongatus*	Mahoney 2004
AY183884	*elongatus*	Mahoney 2004
AY183883	*elongatus*	Mahoney 2004
AY183876	*elongatus*	Mahoney 2004
AY183875	*elongatus*	Mahoney 2004
AY183874	*elongatus*	Mahoney 2004
AY183873	*elongatus*	Mahoney 2004
AY183872	*elongatus*	Mahoney 2004
AY183871	*elongatus*	Mahoney 2004
AY183870	*elongatus*	Mahoney 2004
AY183869	*elongatus*	Mahoney 2004
AY183868	*elongatus*	Mahoney 2004
AY183867	*elongatus*	Mahoney 2004
AY183866	*elongatus*	Mahoney 2004
AY183865	*elongatus*	Mahoney 2004
AY183864	*elongatus*	Mahoney 2004
AY183863	*elongatus*	Mahoney 2004
AY183862	*elongatus*	Mahoney 2004
AY183861	*elongatus*	Mahoney 2004
AY183860	*elongatus*	Mahoney 2004
AY183859	*elongatus*	Mahoney 2004
AY183858	*elongatus*	Mahoney 2004
AY183857	*elongatus*	Mahoney 2004
AY183856	*elongatus*	Mahoney 2004
AY183855	*elongatus*	Mahoney 2004
AY183854	*elongatus*	Mahoney 2004

AY183853	*elongatus*	Mahoney 2004
AY183852	*elongatus*	Mahoney 2004
AY183851	*elongatus*	Mahoney 2004
AY183850	*elongatus*	Mahoney 2004
AY183849	*elongatus*	Mahoney 2004
AY183848	*elongatus*	Mahoney 2004
AY183847	*elongatus*	Mahoney 2004
AY183846	*elongatus*	Mahoney 2004
AY183844	*elongatus* ^	Mahoney 2004
AY183842	*elongatus*	Mahoney 2004
AY183841	*elongatus*	Mahoney 2004
AY183840	*elongatus*	Mahoney 2004
AY183839	*elongatus*	Mahoney 2004
AY183838	*elongatus*	Mahoney 2004
AY183837	*elongatus*	Mahoney 2004
AY183836	*elongatus*	Mahoney 2004
AY183834	*elongatus*	Mahoney 2004
AY183833	*elongatus*	Mahoney 2004
AY183832	*elongatus*	Mahoney 2004
AY183830	*elongatus*	Mahoney 2004
AY183829	*elongatus*	Mahoney 2004
AY183928	*elongatus*	Mahoney 2004
AY183812	*elongatus*	Mahoney 2004
AY183811	*elongatus*	Mahoney 2004
AY183810	*elongatus*	Mahoney 2004
AY183808	*elongatus*	Mahoney 2004
AY183807	*elongatus*	Mahoney 2004
AY183806	*elongatus*	Mahoney 2004
AY183805	*elongatus*	Mahoney 2004
AY183804	*elongatus*	Mahoney 2004
AY183802	*elongatus*	Mahoney 2004
AY183801	*elongatus*	Mahoney 2004
AY183799	*elongatus* ^	Mahoney 2004
AY183798	*elongatus*	Mahoney 2004
AY183797	*elongatus*	Mahoney 2004
AY183796	*elongatus*	Mahoney 2004
AY183795	*elongatus*	Mahoney 2004
AY183794	*elongatus*	Mahoney 2004
AY183793	*elongatus*	Mahoney 2004
AY183791	*elongatus*	Mahoney 2004
AY183790	*elongatus*	Mahoney 2004
AY183789	*elongatus*	Mahoney 2004
AY183788	*elongatus*	Mahoney 2004
AY183779	*elongatus*	Mahoney 2004

AY183778	*elongatus*	Mahoney 2004
AY183777	*elongatus*	Mahoney 2004
AY183776	*elongatus*	Mahoney 2004
AY183775	*elongatus*	Mahoney 2004
AY183774	*elongatus*	Mahoney 2004
AY183773	*elongatus*	Mahoney 2004
AY183772	*elongatus* ^	Mahoney 2004
AY183771	*elongatus* ^	Mahoney 2004
AY183770	*elongatus*	Mahoney 2004
AY183769	*elongatus*	Mahoney 2004
AY183768	*elongatus*	Mahoney 2004
AY183767	*elongatus*	Mahoney 2004
AY183766	*elongatus*	Mahoney 2004
AY183765	*elongatus*	Mahoney 2004
AY183764	*elongatus* ^	Mahoney 2004
PLEMTCYTB	*elongatus* ^	Moritz et al. 1992
PEU89628	*elongatus* ^	Jackman et al. 1997
AY572107	*idahonesis*	Carstens et al. 2004
AY572106	*idahonesis*	Carstens et al. 2004
AY572105	*idahonesis*	Carstens et al. 2004
AY572104	*idahonesis*	Carstens et al. 2004
AY572103	*idahonesis*	Carstens et al. 2004
AY572102	*idahonesis*	Carstens et al. 2004
AY572101	*idahonesis*	Carstens et al. 2004
AY572100	*idahonesis*	Carstens et al. 2004
AY572099	*idahonesis*	Carstens et al. 2004
AY572098	*idahonesis*	Carstens et al. 2004
AY572097	*idahonesis*	Carstens et al. 2004
AY572096	*idahonesis*	Carstens et al. 2004
AY572095	*idahonesis*	Carstens et al. 2004
AY572094	*idahonesis*	Carstens et al. 2004
AY572093	*idahonesis*	Carstens et al. 2004
AY572092	*idahonesis*	Carstens et al. 2004
AY572091	*idahonesis*	Carstens et al. 2004
AY572090	*idahonesis*	Carstens et al. 2004
AY572089	*idahonesis*	Carstens et al. 2004
AY572088	*idahonesis*	Carstens et al. 2004
AY572087	*idahonesis*	Carstens et al. 2004
AY572086	*idahonesis*	Carstens et al. 2004
AY572085	*idahonesis*	Carstens et al. 2004
AY572084	*idahonesis*	Carstens et al. 2004
AY572083	*idahonesis*	Carstens et al. 2004
AY572082	*idahonesis*	Carstens et al. 2004
AY572081	*idahonesis*	Carstens et al. 2004

AY572080	*idahonesis*	Carstens et al. 2004
AY572079	*idahonesis*	Carstens et al. 2004
AY572078	*idahonesis*	Carstens et al. 2004
AY572077	*idahonesis*	Carstens et al. 2004
AY572076	*idahonesis*	Carstens et al. 2004
AY572075	*idahonesis*	Carstens et al. 2004
AY572074	*idahonesis*	Carstens et al. 2004
AY572073	*idahonesis*	Carstens et al. 2004
AY572072	*idahonesis*	Carstens et al. 2004
AY572071	*idahonesis*	Carstens et al. 2004
AY572070	*idahonesis*	Carstens et al. 2004
AY572069	*idahonesis*	Carstens et al. 2004
AY572068	*idahonesis*	Carstens et al. 2004
AY572067	*idahonesis*	Carstens et al. 2004
AY572066	*idahonesis*	Carstens et al. 2004
AY572065	*idahonesis*	Carstens et al. 2004
AY572064	*idahonesis*	Carstens et al. 2004
AY572063	*idahonesis*	Carstens et al. 2004
AY572062	*idahonesis*	Carstens et al. 2004
AY572061	*idahonesis*	Carstens et al. 2004
AY572060	*idahonesis*	Carstens et al. 2004
AY572059	*idahonesis*	Carstens et al. 2004
AY572058	*idahonesis*	Carstens et al. 2004
AY572057	*idahonesis*	Carstens et al. 2004
AY572056	*idahonesis*	Carstens et al. 2004
AY572055	*idahonesis*	Carstens et al. 2004
AY572054	*idahonesis*	Carstens et al. 2004
AY572053	*idahonesis*	Carstens et al. 2004
AY572052	*idahonesis*	Carstens et al. 2004
AY572051	*idahonesis*	Carstens et al. 2004
AY572050	*idahonesis*	Carstens et al. 2004
AY572049	*idahonesis*	Carstens et al. 2004
AY572048	*idahonesis*	Carstens et al. 2004
AY572047	*idahonesis*	Carstens et al. 2004
AY572046	*idahonesis*	Carstens et al. 2004
AY183827	*stormi*	Mahoney 2004
AY183826	*stormi*	Mahoney 2004
AY183825	*stormi*	Mahoney 2004
AY183824	*stormi*	Mahoney 2004
AY183823	*stormi*	Mahoney 2004
AY183822	*stormi*	Mahoney 2004
AY183821	*stormi*	Mahoney 2004
AY183820	*stormi*	Mahoney 2004
AY183819	*stormi*	Mahoney 2004

AY183818	*stormi*	Mahoney 2004
AY183817	*stormi*	Mahoney 2004
AY183816	*stormi*	Mahoney 2004
AY183815	*stormi*	Mahoney 2004
AY183814	*stormi*	Mahoney 2004
AY183813	*stormi*	Mahoney 2004
AY183787	*stormi*	Mahoney 2004
AY183786	*stormi*	Mahoney 2004
AY183785	*stormi*	Mahoney 2004
AY183784	*stormi*	Mahoney 2004
AY183783	*stormi*	Mahoney 2004
AY183782	*stormi*	Mahoney 2004
AY183781	*stormi*	Mahoney 2004
AY183780	*stormi*	Mahoney 2004
AY688300	*asupak*	Mead et al. 2005
AY688299	*asupak*	Mead et al. 2005
AY688296	*asupak*	Mead et al. 2005
AY688292	*asupak*	Mead et al. 2005
AY688290	*asupak*	Mead et al. 2005
AY688287	*asupak*	Mead et al. 2005
AY691744	*Ensatina eschschcoltzii*	Wiens et al. 2006
*	*larselli*	Wagner et al. 2005

*All *Plethodon larselli* sequences received by Mark Miller (Wagner et al. 2005)

^ Indicates *P. elongatus* samples only used for phylogenetic analysis.