

**Genetic Evidence for a Population Bottleneck in the Olympic
Mudminnow (*Novumbra hubbsi*)**

by

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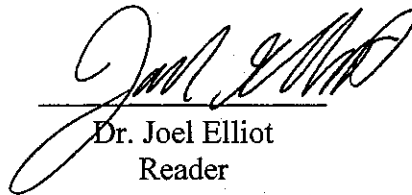
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Abstract. The Olympic Mudminnow, *Novumbra hubbsi*, is the only species of the genus *Novumbra* and is endemic to the lowlands of the Olympic Peninsula. The distribution and natural history of the species has been extensively studied, but no population genetics studies have been performed. I investigated population structure using comparisons of partial DNA sequences of the mitochondrial gene coding for the transmembrane protein cytochrome B. Sequences from five individuals from each of seven populations throughout the range of the species were examined and yielded only one nucleotide substitution in one individual. The extreme homogeneity of *N. hubbsi* suggests the occurrence of population bottlenecks during the last ice age with subsequent limited range expansions. These bottlenecks were likely caused by massive glaciations of the Olympic Peninsula and Puget Sound during the advance of the Vashon Ice sheet about 14,000 years ago. Future studies should examine genetic variation at nuclear DNA loci to determine the extent of homogeneity and whether the lack of variation has fitness consequences.

INTRODUCTION

TAXONOMY

The Olympic Mudminnow, *Novumbra hubbsi*, is the only surviving species of the genus *Novumbra* and is endemic to Washington State. The genus *Novumbra* contains only one other fossil species (Cavender 1969). Traditionally, *Novumbra* is assigned to the family Umbridae, which is comprised of *Novumbra*, *Dallia*, and *Umbra*. This family is thought to be a monophyletic sister to the family Esocidae (pikes) (Lopez et al. 2000). Traditionally, the family Umbridae was comprised of four species living in North America and one that is found in Europe (Mongillo and Hallock 1999). However, a recent phylogenetic study using mtDNA variation among members of the family Umbridae and Esocidae suggest that *Novumbra hubbsi* is the last remnant of an ancient lineage that should be reassigned to the family Esocidae (Fig. 1, Lopez et al. 2000).

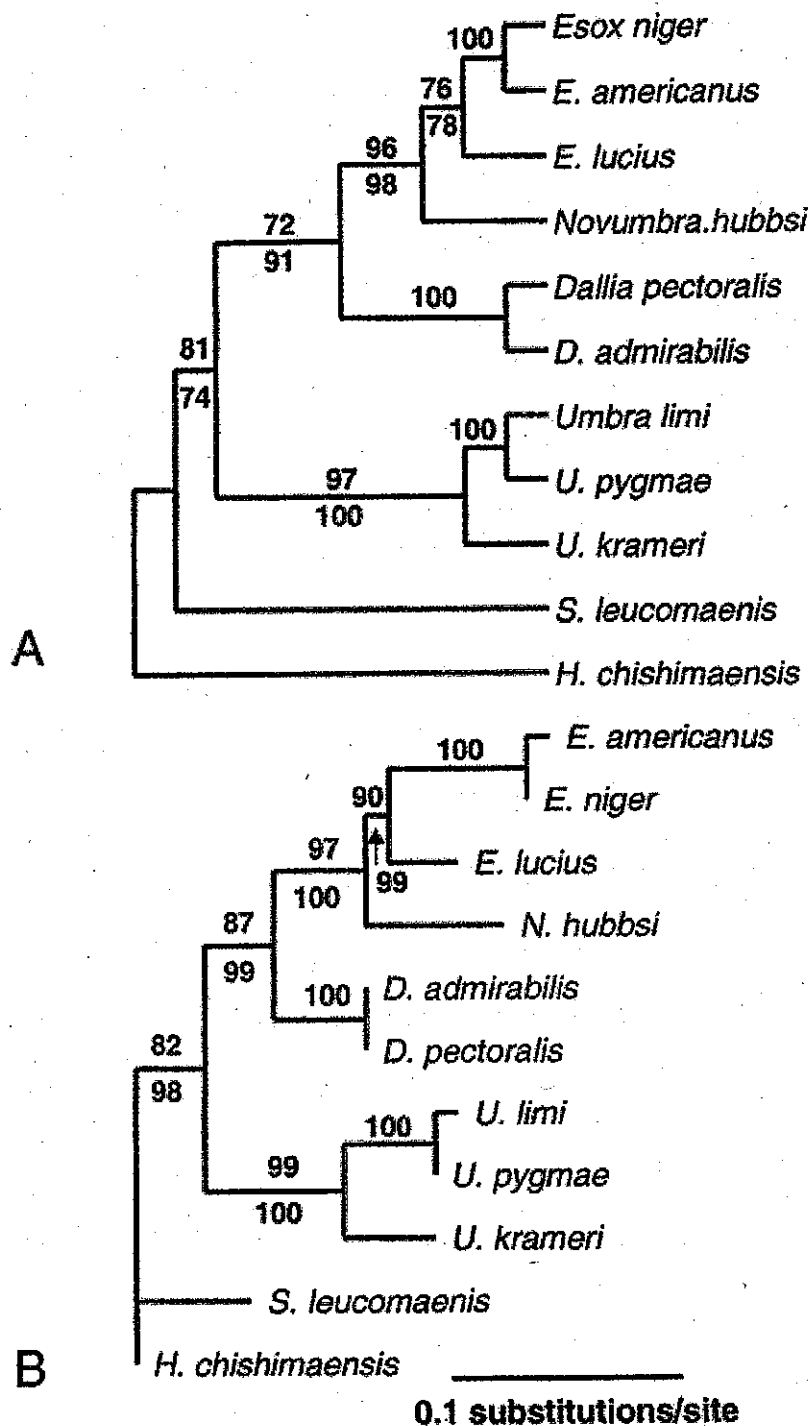


FIGURE 1. Phylogenetic relationships among species of the family Umbridae and Esocidae using mtDNA sequence comparison. (A) maximum parsimony tree and (B) maximum likelihood tree. The values on trees indicate percent occurrences of the clade. *Novumbra hubbsi* is remnant of a more ancient lineage closely related to *Esox* in the family Esocidae. *S. leucomaenis* and *H. chishimaensis* are outgroups for rooting (Lopez et al. 2000).

PHYSICAL DESCRIPTION

N. hubbsi is a small carnivorous fish that closely resembles a pike, except for its substantially smaller size. A study conducted by WDFW found the average length of *N. hubbsi* to be 52 mm (2.05 inches) with a maximum length of 88 mm (3.46 inches) and a minimum length of 22 mm (0.86 inches) (Mongillo and Hallock 1999). Under normal conditions, both male and female *N. hubbsi* are drab olive-green with about 15 faint vertical bars along their sides and the dorsal and anal fins are soft-rayed and set far back on the body (Wydosky and Whitney 1979). However, during the spawning season they display sexual dimorphism (Fig. 2). Males become dark chocolate brown to black and the faint vertical bars become brightly colored to an iridescent green-blue or white and the females remain a drab cryptic color (Mongillo and Hallock 1999). The edges of male's dorsal and anal fins brighten to a conspicuous sky-blue, which is most brilliant once eggs are deposited within their territory (Wydosky and Whitney 1979). At the same time, subordinate males lighten to a brown color and closely resemble females (Wydosky and Whitney 1979).



FIGURE 2. Sexual dimorphism observed in the *N. hubbsi*. The left image is of a male mudminnow in spawning colors and the right image is a female in typical cryptic colors.

HABITAT REQUIREMENTS

The mudminnow is found in quiet waters with a mud substrate and dense vegetation (Wydosky and Whitney 1979). Typical habitats include bogs and swamps with water currents of less than 0.03 m/s and temperatures of 0° to 21° C. However, *N. hubbsi* is often found in cold waters and areas with underground springs. Compounding its

intolerance to currents, *N. hubbsi* is also intolerant of salinity, which significantly limits its dispersal abilities (Meldrim 1968).

NATURAL HISTORY

N. hubbsi is strictly a carnivore and feeds non-selectively, although only seven items make up the majority of its diet. It consumes mostly annelids (Oligochaeta), crustaceans (Ostracoda, Mysidacea, and Isopoda), insects (Megaloptera and Diptera), and mollusks (Wydosky and Whitney 1979). The fish are ambush predators similar to pike. The fish stalks its prey with an almost motionless movement until it nears the prey and then, with a rapid strike, engulfs the prey (Wydosky and Whitney 1979). Also, *N. hubbsi* consumes only moving prey. A laboratory study found that if prey did not move, then *N. hubbsi* would not strike and after several minutes it would move to another prey item (Meldrim 1968).

Spawning begins in early March, peaks in late March and April, and ends in mid-June. Sexual dimorphism has been observed during the breeding season. During this time, males undergo dramatic physiological changes and become aggressive and territorial. Males compete with other males for territorial plots that average 32 to 64 cm² (Wydosky and Whitney 1979). To defend one's ground; a male expands and vibrates its brilliantly colored fins as a warning to other males. If the warning display is ignored then the defending male will rush toward the intruder and strike him with his head or body. The impact is often strong enough to overturn the intruder and the process is repeated until the intruder leaves the territory.

When courting, males perform the "wigwag" dance, which is characterized by moving his body side to side with his fins fully extended. When a female becomes receptive, she will enter the male's territory and spawn. Spawning occurs through physical

contact between the male and female, when a few adhesive eggs are deposited near the bottom per contact. After 7 to 9 days, fry emerge from the eggs and remain attached to the bottom or to vegetation until maturation (Mongillo and Hallock 1999).

GEOGRAPHICAL DISTRIBUTION

The distribution of *N. hubbsi* is considered to be dictated by the last glaciations of Western Washington during the Pleistocene era (McPhail 1967, Meldrim 1968). Pleistocene glaciations had an enormous impact on the phylogeographic structure and evolution of many Pacific Northwest biotas (Nielson et al. 2000). Expanding glaciers causing swift habitat and range alterations forced many species into refugia during colder conditions and allowed recolonization and dispersal during warmer interglacials (Hewitt 1996). As ice swept south through Puget Sound and covered much of the Olympic Peninsula, *N. hubbsi* was likely restricted to small ice-free refuges, such as Lake Ozette and the Chehalis River (Mongillo and Hallock 1999). When glaciers receded north about 14,000 years ago, the Olympic mudminnow distributed to suitable habitats reflecting the distribution observed today (McPhail 1967). The present restricted distribution of the species is likely the result of limited dispersal abilities due to intolerance of salinity and fast-moving currents and habitat preference (Meldrim 1968).

Compounding the distinctiveness of *N. hubbsi* is the fact that it is the only species of Umbridae found in Washington State and its range is limited to the wetlands, ponds, and slow-moving streams of the Olympic and south Puget Sound lowlands (Mongillo and Hallock 1999, Harris 1974). *N. hubbsi* occurs primarily in coastal lowlands of the western Olympic Peninsula, from the Queets River south to Grays Harbor and up the north side of the Chehalis River valley to the Skookumchuck River (Wydosky and Whitney 1979, Fig. 3). Other populations also have been found in the Deschutes River in southern Puget

Sound, the Issaquah and Cherry creek drainages of Snohomish and King Counties, and Lake Ozette on the northwest point of the Olympic Peninsula (Trotter et. al 1998). WDFW suspects the Issaquah and Cherry Creek populations to have been illegally introduced for three reasons. First, the creeks are far outside the typical range of the species and, since mudminnows are intolerant of current, it is very unlikely that they would have swam up the southerly flowing waters of receding glaciers from the Puget Trough 10,000 years ago (Meldrim 1968, Mongillo and Hallock 1999). Second, WDFW has 100 elevation measurements of *N. hubbsi* collection sites and only one is higher than 100m, whereas Cherry Cr. is 240m and Issaquah Cr. is 135m above sea level. Third, mudminnows have recently been killed in the rotenone treatments of Margaret Lake in the Cherry Creek drainage, but none were killed in the treatment of 1962 (Mongillo and Hallock 1999). The Deschutes River populations are thought to have been the result of high water years when the Chehalis River flooded southern Puget Sound Streams (Wydosky and Whitney 1979).

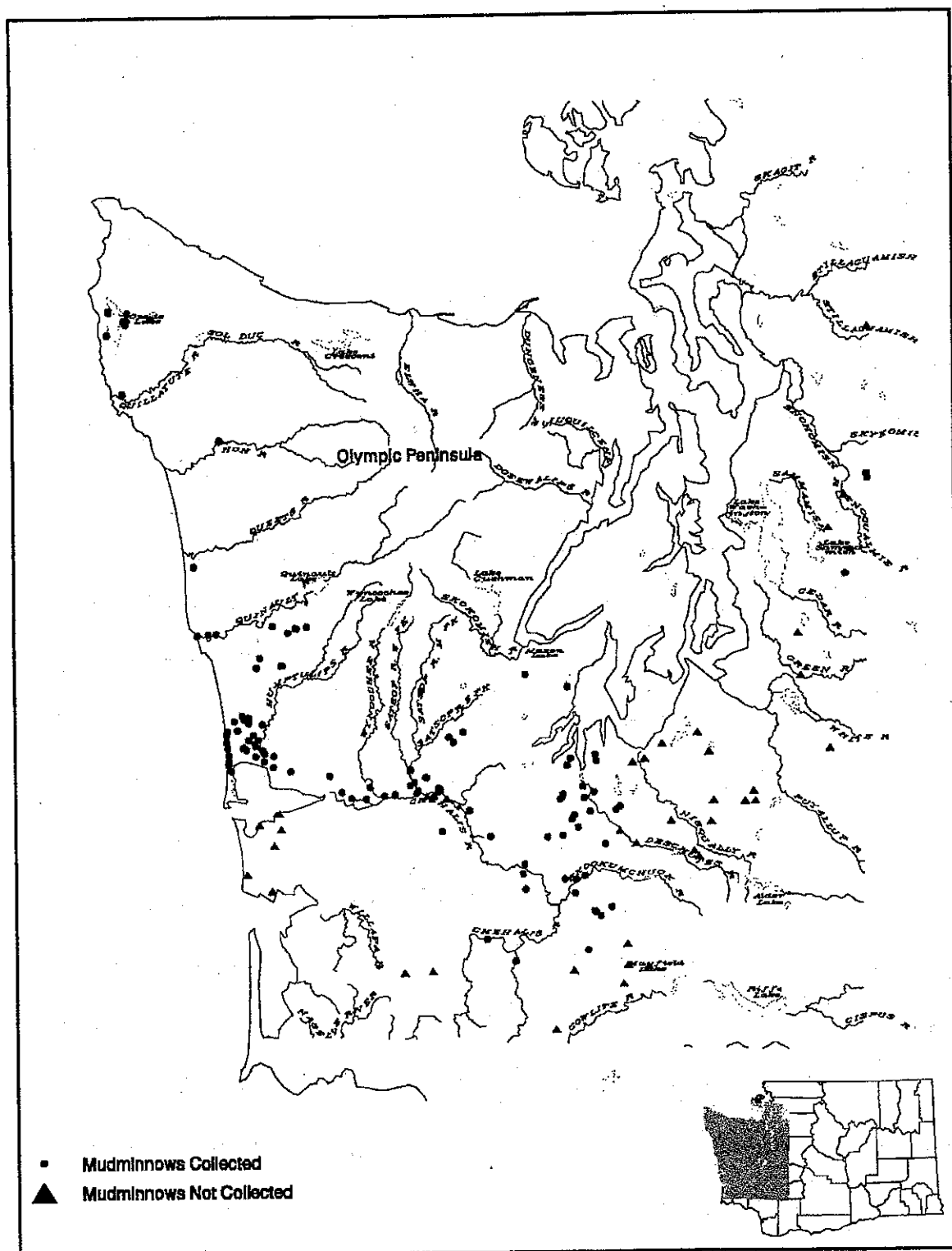


FIGURE 3. Distribution of WDFW collection sites and efforts for *N. hubbsi* in Western Washington (Mongillo and Hallock 1999).

Geologic History of N. hubbsi Range

The Chehalis River Basin is the second largest in the state of Washington and drains 2,660 square miles (Michaud 2000). It is bounded on the west by the Pacific Ocean, the east by the Deschutes River basin, the north by the Olympic Mountains, and the south by the Willapa hills and Columbia River basin. The river flows through three ecoregions: Mountains (Cascade and Olympic), Puget lowland, and Coast Range before emptying into Grays Harbor near Aberdeen. The Puget lowland is characterized by slow moving streams and wetland habitats and is where the majority of known *N. hubbsi* populations are found (Mongillo and Hallock 1999).

The Chehalis Basin has several distinct geologic regions that have unique geological histories. For example, the headwaters in the Willapa Hills are comprised of marine volcanic rocks while much of the remaining basin was glacially influenced (Michaud 2000). During the climax of the Wisconsin Ice Age (15,000 to 20,000 yrs. ago), a massive ice sheet, forming the Vashon Glacier, pushed south from British Columbia through the Puget Lowlands and extended to a few miles south of present day Olympia (Danner 1995). The Vashon Glacier filled the lowlands between the Cascade and the Olympic Mountains to a considerable height, thereby inundating Puget Sound. When temperatures warmed the ice rapidly melted and with mountains flanking on both the east and west, and glaciers blocking the north, the overflow pushed through the Chehalis Valley toward Grays Harbor, scouring the basin (Danner 1995). The advancement and retreat of the massive glaciers occurred frequently throughout the Pleistocene era (Danner 1995).

While the Vashon Glacier pushed south, another ice sheet pushed west across the Strait of Juan de Fuca and reached nearly 3,000 feet in depth along the northern Olympic Mountains. As the ice pushed westward it deepened former valleys, creating large lakes

such as Lake Crescent and Lake Sutherland (Danner 1995). It is not certain how far west the ice advanced but evidence suggests there were small glacial refugia near Lake Ozette (Danner 1995, Mongillo and Hallock 1999). Also, the Hoh, Blue, and White glaciers of the Olympic Mountains advanced west down the Hoh river valley about 30 miles to the Pacific Ocean and inundated wetland habitats (Danner 1995). The extensive glaciations and melt offs on the Olympic Peninsula could have significantly influenced the distribution of the regions species, including *N. hubbsi*.

HABITAT AND CURRENT STATUS

The destruction of an estimated 33 to 50 percent of the wetland habitat within the range of *N. hubbsi* and its limited dispersal abilities has elicited concern from state agencies about the status of the species. In response to this concern and the continued destruction of an estimated 700 to 2000 acres of wetland a year, the WDFW produced a status report for *N. hubbsi* (Washington Department of Ecology 1992). The report concluded that 90% of the current known *N. hubbsi* populations are stable; however, their continued existence depends upon the immediate protection of wetland habitat within the species range (Mongillo and Hallock 1999). Although the fish is abundant where found, it is vulnerable to extinction because it occurs only in a few areas (Meldrim 1968). Given their limited distribution, and phylogenetic distinctiveness, *N. hubbsi* has been given special conservation consideration (Lopez et al. 2000).

WDFW placed *N. hubbsi* on the Sensitive Species List, which includes "any wildlife species native to the state of Washington that is vulnerable or declining and is likely to become endangered or threatened in a significant portion of its range within the state without cooperative management or removal of threats (WDFW 1997)". Once a species is listed, WDFW acts to develop a management plan to ensure preservation of the

species. Development of an effective management plan requires understanding of the natural history, habitat requirements, geographic distribution, and population structure of the species (A vise and Hamrick 1996). The natural history, habitat needs, and geographic distribution of *N. hubbsi* have been well studied (Mongillo and Hallock 1999, Meldrim 1968, Harris 1974). However, there have been no population genetic studies performed to determine the population structure of *N. hubbsi*. Such studies could provide insight into the evolutionary history of the species as well as gene flow, degree of isolation, and fitness (A vise and Hamrick 1996, Nielson et. al 2000). In general, the ability of a species to survive is dependent upon the fitness and environmental adaptability of the species, which is derived from genetic variation. Species with greater genetic variation often exhibit lower probability of extinction (A vise 1994, A vise and Hamrick 1996). Therefore, understanding and preserving the greatest possible genetic diversity within a species is crucial for survival.

Knowledge of population structure can also demonstrate the existence of Evolutionary Significant Units (ESU), which are defined as "distinct population segments" under the Endangered Species Act. Such "distinct population segments" can harbor unique haplotypes that are crucial to the evolutionary legacy of the species (Ruckleshaus et al. 2002). If ESU's do exist then conservation efforts should be made to preserve populations representing the different ESU's (Haig 1998). On the other hand, if a population is not recognized as an ESU then conservation resources could be directed to more critical needs (Haig, 1998).

STUDY OBJECTIVES

Among the tools available to evolutionary biology, molecular phylogeography has distinguished itself as a means of understanding population structure and evolutionary processes within a species (Avice 1994). By taking advantage of information contained in the geographical distribution and topological relationships of genetic lineages (which reflect the long-term structure and demographic history of populations) phylogeography provides a useful strategy for understanding the historical factors leading to existing patterns of diversity (Wooding and Ward 1997). I investigated the population structure and phylogeography of *N. hubbsi* using mitochondrial DNA (mtDNA) sequence variation. mtDNA is used because it is maternally inherited, evolves quickly, and does not recombine. The faster rate of evolution of mtDNA relative to nuclear DNA makes mtDNA useful for high-resolution analyses of recent evolutionary events (Brown et al. 1979). Also, mtDNA does not recombine and provides a simpler data set than nuclear DNA and is an extremely popular genetic tool with which to study gene flow, hybrid zones, and population structure (Beaumont 1994).

Specifically, I conducted an analysis of mtDNA polymorphisms of the cytochrome-B gene among populations within river systems and among different river systems. My analysis focused on two aspects of genetic diversity. First, I assessed the degree of genetic variation within and among populations and generated a phylogenetic tree to illustrate population structure. Second, I estimated divergence times between individuals and inferred major evolutionary processes that may have influenced population structure. The analysis provided insight into the origin of the Cherry Creek population and whether there are populations of mudminnows that could qualify as Evolutionary Significant Units.

MATERIALS AND METHODS

SAMPLE COLLECTION

A total of 64 fin clips of *N. hubbsi* were collected from the Copalis River, Wynoochee River, Wishkah River, Deschutes River, Cherry Creek, Cook Creek, and Campell Slough (Table 1). Andres Lopez previously collected the Cherry Creek samples and the remaining samples were collected in conjunction with the WDFW. Samples were preserved in 95% EtOH at -20°C. Collection occurred by means of electro-shocking, dip netting, and trapping.

TABLE 1. Sampling information and location of population.

Sample Location	Latitude NN°NN'NN"	Longitude NNN°NN'NN"	# Sequenced
Campell Slough, WA	47 29 01N	123 40 50W	5
Cherry Creek, WA	47 46 07N	121 57 33W	5
Cook Creek, WA	47 22 55N	123 51 07W	5
Copalis River, WA	47 17 20N	123 57 42W	5
Salmon Creek, WA	46 55 59N	123 00 09W	5
Wishkah River, WA	47 10 01N	123 44 57W	5
Wynoochee River, WA	47 07 31N	123 38 59W	5

DNA EXTRACTION

DNA was extracted from five fin clips of each population using protocols provided with a Qiagen DNeasy DNA Extraction Kit. Approximately 1mg of tissue was digested with 20µL of Proteinase K and incubated at 55°C overnight. DNA quality was assessed by electrophoresis on 0.8% TBE-buffered agarose gel with a 1kb Promega DNA ladder and visualized with ethidium bromide (5 µmol/ml).

DNA AMPLIFICATION

A 437 bp fragment of mtDNA from the tRNA-Glu and cytochrome B genes was amplified using the primers: (B-GLUDG-L M13F, Lento et al. 1994)

5'-CACGACGTTGTAAAACGACTGACCTGAARAACCA YCGTTG-3' and (CYB2-HM13R, Lento et al. 1994) 5'-GGATAACAATTTACAGGCCCTCAGAATGAT-ATTTGTCCTCA-3'. Both primers were labeled with M13 florescent tails that are shown in bold. The PCR reaction conditions and amplification profile were as follows: reaction mixture contained 2.5µl 10x buffer, 1µl dNTP [0.2 mM], 1µl [0.1 pmole] of each primer, 0.15µl (0.75 units) Taq polymerase (Qiagen), 17.35µl dH₂O, and 2µl DNA template to a total volume of 25µl; amplification profile - initial denaturing (95.0 for 5 min), 35 cycles (94.0 for 30 sec, 50.6 for 60 sec, 72.0 for 80sec), and terminal extension (72.0 for 5 min). All PCR reactions included a negative control with additional dH₂O substituting for DNA template. PCR products were separated on 1.5% agarose gel with a 1kb DNA ladder and visualized with ethidium bromide. All amplified products were purified with a Qiagen Quick Purification Kit and suspended in 35µL of buffer (10mM TrisCl, pH 8.5).

DNA SEQUENCING

Sequencing reactions were performed with additional labeled primers, M13R (IR = 800) and M13F (IR = 700), and a SequiTherm Excel DNA sequencing Kit L-C. The forward and reverse primers were both used to sequence the heavy and light strands for greater accuracy and were used separately to achieve maximum sequence quality. All reverse reactions contained 1.8µL supplied buffer, 0.25µL DNA polymerase, 2µL of the appropriate termination mix, 0.25µL [0.25 pmole] of reverse primer, 0.25µL DNA template, and dH₂O to a final volume of 6µL. Each forward reaction contained equal quantities of reactants for the reverse reactions except for 0.5µl [0.5 pmole] of forward primer. Sequencing profiles were as follows: initial denaturation (95 °C for 5 min) and 30

cycles (95 °C for 30s, 50 °C for 15s, and 70 °C for 1 min). The products were suspended in 3µL of stop/loading buffer and stored in the dark at -20 °C.

Sequencing was performed on a Li-COR Long Readir 4200 IR² automated sequencer using a 41cm gel (6% acrylamide, 0.25mm thickness, 64-well sharkstooth disposable comb). Prior to sequencing, reaction products were denatured at 70 °C for 5 minutes and then loaded onto a polyacrylamide/urea gel with 10x TBE buffer. To improve accuracy each sample was loaded and sequenced twice.

SEQUENCE ALIGNMENT

The forward and complementary sequences of each individual were determined. Complementary sequences were reverse complemented and aligned with forward sequences using the program AlignIR. Consensus sequences were then generated.

PHYLOGENETIC ANALYSIS

Phylogenetic analysis was rather simple given the lack of observed genetic variation. Phylogenetic relationships between populations were illustrated with a neighbor-joining tree using PAUP Version 4.0 (Swofford 2002, Figure 2). *N. hubbsi* sequence from GenBank (accession number # AF060438) was also included in the analysis (Lopez et al, 2000).

Results

A 437 bp segment of mtDNA was sequenced, which contained 30 bp in the tRNA-glu gene and 407 bp in the cytochrome B gene (Table 2, Fig. 4). Only one variable site was detected and only one specimen expressed the substitution, which was a silent transition from an adenine to a guanine and was in the third codon position (Fig. 5). Of the 35 specimens sampled there were only two haplotypes with a sequence divergence of 0.00229. Phylogenetic analysis was rather simple because of the limited number of haplotypes (Fig. 6). The Cherry Creek population could not be distinguished from the

other populations and overall genetic variation was insignificant and did not provide significant information for phylogeography.

TABLE 2. Complete sequence of the 437 bp portion of mtDNA (CSL58).

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CCACCGTTGTAAATTCAACTATAAGAACTCATGACCAGCCTCCGAAAAACCCA
CCCTGTCCTTAAAATTGTCAATGATGCACTAATTGACCTCCCTGCCCTGCAAA
CATTTCTGTATGATGAAATTTTCGGCTCCCTATTAGGACTATGTTTAATTACCCA
AATTCTAACAGGCTTATTCCTAGCCATACACTATACTTCCGACATTTCAACAGC
CTTCTCCTCAGTATGCCATATCTGCCGAGATGTAACTATGGCTGACTCATCCG
AAATGTCCACGCCAACGGTGCCTCTCTTTTTTTCATTTGTATTTATCTACATATT
GCCCGAGGCCTATACTACGGCTCTTACCTCTATAAAGAAACCTGGAATATTGG
AGTAATTCTATTTCTTCTAACCATAATAACTGCCTTTGTAGGATATGTACTTCC
ATGAGG
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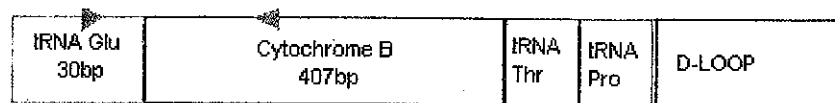


FIGURE 4. mtDNA gene map for *N. hubbsi* showing the number of base pairs sequenced from each gene. 30bp from 3' end of the tRNA-Glu and 407bp from 5' end of the Cytochrome B was sequenced. Green arrows represent sequenced portion.

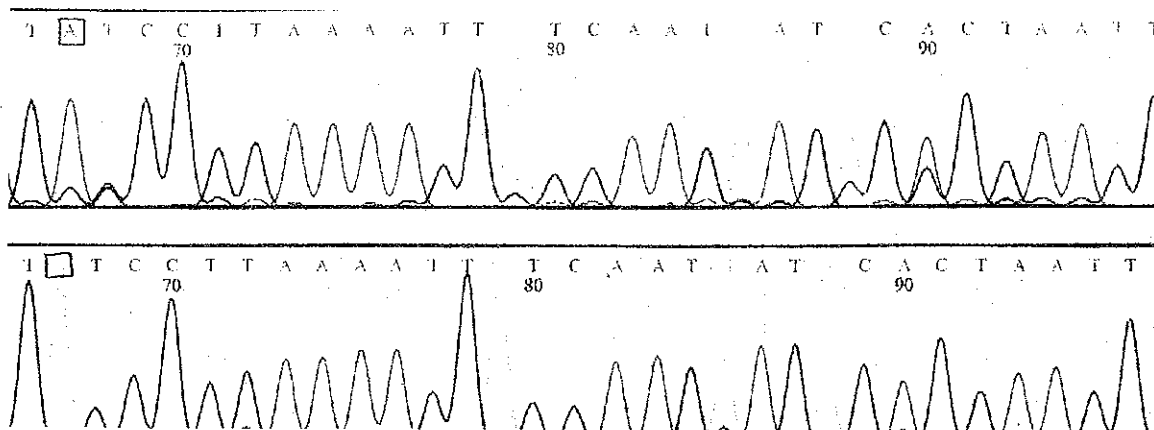


FIGURE 5. Comparison of the absorption curves for the only substitution found between the two observed haplotypes. The top image represents the haplotype shared by 34 test individuals and the obtained sequence from genbank. The lower image is the curve for the alternate haplotype of WYN43, which had a guanine substituted for an adenine at bp#67. The orange boxes indicate the substituted bp position.

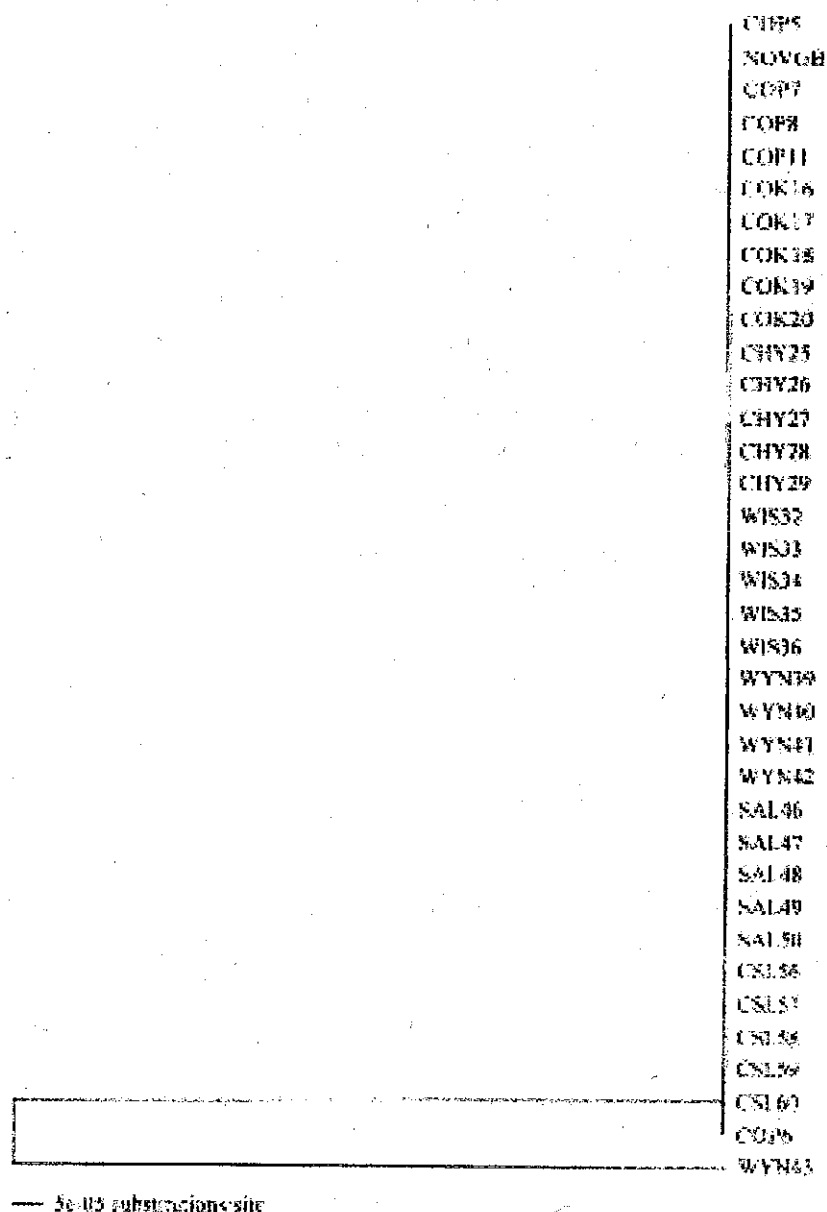


FIGURE 6. Distance Neighbor/Joining UPGMA tree for 35 sequenced samples and genbank sequence (NOVGB). There was divergence at only one base pair in a single individual (WYN43). The average divergence between the two haplotypes was 0.00229.

DISCUSSION

The observed transition from adenine to guanine in the third position of WYN43 is a silent mutation. Of the three kinds of sequence changes, substitutions are more frequent than additions or deletions, and rearrangements (Beaumont 1994). Of substitutions, transitions are much more common than transversions and especially at the third positions of codons where transitions quickly accumulate and become saturated (Beaumont 1994).

The overall results of this study revealed extreme homogeneity in mtDNA of *N. hubbsi*, which could not be used to identify evolutionary significant units. Also, the origin of the Cherry River population could not be identified. However, results do suggest that mtDNA in *N. hubbsi* mutates very slowly or that there was a recent and severe population bottleneck that extirpated other haplotypes of *N. hubbsi*. It is very unlikely that *N. hubbsi* mtDNA mutates at such a slow rate because the species is closely related to the genus *Esox* in the family Esocidae, which has shown to have mtDNA mutation rates of 2% per million years (Lopez et al. 2000). If *N. hubbsi* does have exceptionally slow evolving mtDNA then it must have acquired the trait within its single lineage, which is extremely unlikely. The reduced genetic variation found in the cytochrome B gene of *N. hubbsi* is likely the result of recent and severe population bottlenecks.

Population bottlenecks are events that severely deplete or extirpate entire populations, leaving few surviving individuals or populations. A typical consequence of bottlenecks is the loss of genetic diversity within the species due to genetic drift and extirpation of haplotypes. The extreme homogeneity observed in *N. hubbsi* suggests the occurrence of a recent and severe bottleneck or bottlenecks. Assuming a mutation rate of 2% per million years, the observed restriction of variation likely occurred between 10,000 and 100,000 years ago during the glacial expansions of the Pleistocene era.

During the climax of the Wisconsin Ice Age (15,000 to 20,000 yrs. ago), massive ice sheets forming the Vashon Glacier expanded south from British Columbia through the Puget lowlands and extended to within a few miles of present day Olympia (Danner 1995). The immense ice sheets smothered the Puget Lowlands between the Cascade and Olympic Mountains. At the same time, glacial expansion from the Cascade and Olympic Mountains

and freezing temperatures severely restricted the available habitat of the region's species, especially freshwater aquatic species (Danner 1995). The harsh environmental conditions could have severely decimated *N. hubbsi* populations, which are physically unable to migrate because of intolerance to salinity and currents (Meldrim 1968). Surviving populations would be restricted to limited glacial refugia that were capable of supporting them, such as Lake Ozette and parts of the Chehalis River Basin. The repeated advancements and withdrawals of glaciers, which occurred several times, likely restricted available habitat of *N. hubbsi* and resulted in few surviving individuals or a population bottleneck.

When the ice melted, the resulting water flowed through the Chehalis River Basin and emptied into Grays Harbor (Danner 1995). The massive floods could have quickly dispersed the species throughout the basin resulting in swift range expansion of an already restricted gene pool. Nonetheless, impoundment by glaciers and subsequent melt off may have significantly influenced the distribution and population structure of *N. hubbsi*.

CONSERVATION

Species representing remnants of ancient lineages should be given conservation priority in order to maximize preservation of character richness (Lopez et al. 2000). Lopez et al. (2000) suggest that *N. hubbsi* should be given special conservation consideration because of their limited distribution, lack of extant diversity, and phylogenetic distinctiveness. Also, the extreme homogeneity of *N. hubbsi*, as demonstrated in this study, should increase concern for the conservation of this species. Reduced genetic variation has been attributed to reduced fitness of other species, such as the African Cheetah (*Acinonyx jubatus*), which suffered severe population reduction and extreme inbreeding several thousands years ago (Awise and Hamrick 1996). Correlated with homogeneity of cheetahs

are physiological impairments that contribute to the difficulty of establishing self-sustaining captive populations (Avis and Hamrick 1996).

When populations drop to very low numbers the associated genetic depletion and subsequent inbreeding increases the risks of inbreeding depression, which is the expression of rare deleterious alleles (Beaumont 1994). When expressed, these genes can negatively affect development, survivorship, and reproduction (Beaumont 1994). Also, inbreeding homogenizes variation within polymorphic genes that mediate the immune response, such as the MHC locus, thereby reducing individual immune response (Beaumont 1994). All of these effects can act together to decrease individual and mean population fitness.

The fitness consequences of reduced genetic variability depend upon the genetic history of the species. Species with local populations that are highly adapted to homogeneity should suffer less deleterious consequences than those with high levels of gene diversity (Avis and Hamrick 1996). In fact, some heterozygous populations that have experienced a bottleneck have adapted to local inbreeding after an initial period of inbreeding depression (Templeton 1987). Deleterious recessive alleles can be purged by mild inbreeding over several generations and can be sufficient to eliminate subsequent inbreeding depression (Templeton 1987). For *N. hubbsi*, it may be possible that it is highly adapted to its environment and doesn't face fitness disadvantages as a consequence of low genetic variability. However, further studies will be necessary to estimate fitness effects.

CONCLUSION

Using sequence variation of the cytochrome B gene I found virtually zero genetic variation in *N. hubbsi* populations collected throughout its range. Because of the observed extreme homogeneity and its limited distribution to the lowlands of the Olympic Peninsula and Southern Puget Sound, *N. hubbsi* likely suffered severe population bottlenecks during

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the ice ages of the Pleistocene Era. Further sampling should incorporate specimens from Lake Ozette, which potentially served as separate glacial refugia and may contain distinct haplotypes due to separation prior to the ice ages.

mtDNA evolves differently than nuclear DNA and since it is maternally inherited it may be reflecting a bottleneck of female haplotypes. It is possible for genetic variation to be conserved within nuclear DNA genes. Future population genetics studies should investigate the amount of genetic diversity at nuclear DNA loci. Future studies should also investigate the extent of inbreeding depression in order to estimate fitness disadvantages caused by homogeneity.

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