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DISPERSAL AND PHYLOGEOGRAPHY OF *CANCER MAGISTER* USING DNA SEQUENCING

A Thesis

Submitted to

The Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Honey J. Dedhia

May 2005

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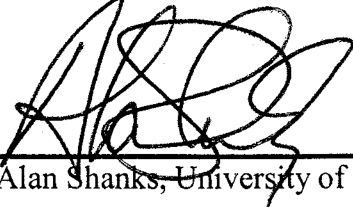
Dr. Leslee Parr

Dr. Robert Fowler



Dr. Alan Shanks, University of Oregon

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ABSTRACT

DISPERSAL AND PHYLOGEOGRAPHY OF *CANCER MAGISTER*

USING DNA SEQUENCING

By Honey Dedhia

Mitochondrial DNA sequencing of the cytochrome *c* oxidase subunit I gene was used to assess the genetic variation of Oregonian populations of the Dungeness crab, *Cancer magister*. When this data was combined with the previous data from the laboratory a total of 261 haplotypes were identified from 652 individuals. The nucleotide diversity (0.8%-5.1%) and the haplotype diversity (70% -100%) were high. There was a large occurrence of the haplotypes that were present in only one individual (singletons). Significant interpopulation differentiation was seen in many of the adult populations. Spatial and temporal genetic distinctness was found among the recruiting larvae. Seven haplotypes were found consistently over the six years of sampling. Significantly negative Fu's Fs statistics supported a recent population expansion. A series of small reserves spaced along the Pacific coast might best preserve the dynamics that have led to these genetically distinct populations.

DEDICATION

This thesis is dedicated with love to my husband and best friend, Jeinay, who has truly been an inspiration to me. My deepest sentiments of gratitude go to him for his love, patience, and for believing in me. He has listened to my moaning and complaining and has supported me in each and every step of the way. I just couldn't have done it without him. He has been always there for me and has helped me keep my life in proper balance.

This thesis is dedicated to my loving mom, who above all others deserves my deepest thanks and respect for being a constant source of encouragement, support, and care over the years. Her life sets an example of how perseverance and strong belief can make everything possible. She always has had faith in me even when I doubted myself and has believed that I could do anything I set my mind to.

This thesis is dedicated to the loving and fond memories of my dad. He is a part of every page and every thought.

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The opportunity to study at San Jose State University, California, has been one of the most wonderful experiences of my life. I will always treasure my memories of graduate studies in the United States of America.

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It is the end of my M.S. endeavor. However, it is the beginning of another journey in my life with the love of God.

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INTRODUCTION

The Dungeness crab, *Cancer magister* (order Decapoda, class Crustacea), is a large mobile crustacean that lives in bays, inlets, and around estuaries from Amchitka Islands in the Aleutians (Hoopes 1973) to the vicinity of Point Conception in California (Pauley *et al.* 1986, 1989). *Cancer* crabs are abundant on the sandy and silt-sandy bottoms and in shallow waters around eelgrass, although they may be sometimes found on gravel and mud (Harrison and Crespi 1999a).

Cancer magister provides an important commercial and recreational fishery along the west coast of North America. Habitat alterations due to the anthropomorphic activities have a strong probability of reducing crab populations unless proper precautions are taken (Fernandez *et al.* 1993; Roegner *et al.* 2003). The loss of vital estuarine habitat could significantly reduce the recruitment to the offshore fishery.

Cancer magister has a relatively long (2.5-4.0 month) pelagic phase. The length of the pelagic period is temperature dependent (Wild 1983) with faster development times in southern regions (Moloney *et al.* 1994). Any spatio-temporal variations in this planktonic dispersal phase would likely have an influence on the gene flow, speciation, and population dynamics of this species (Avise 1994). The knowledge of ocean conditions associated with the planktonic phase occurrence is necessary in order to understand the complex patterns of the crab dispersal and settling. Studies have revealed significant variation in the genetic composition of marine populations over many spatial and temporal scales (Palumbi and Benzie 1991; Van Syoc 1994; Tam *et al.* 1996; Moran

et al. 1997; Rocha Olivares and Vetter 1998; Bunch *et al.* 1998). The larval transport between populations may be restricted by either behavioral or physical mechanisms. Alternatively, the larval transport may be unrestricted but the differential mortality of the progeny may be selecting for the different genotypes between areas (Avise 1994).

With the long-term climatic trends and changing technologies, it is uncertain at what level the Dungeness fishery can be sustained. Because of the economic importance of this fishery to the west coast of North America, there is great interest in improving the Dungeness crab fishery management. The establishment of marine reserves has been proposed as a way to accomplish this. However, a better understanding of larval dispersal is required in order to develop an effective reserve design (Shanks *et al.* 2003).

Life History

The adult *Cancer magister* occur from the low line to depths as much as 230 meters, but are most abundant near shores at depths less than 90 meters (Morris *et al.* 1980). Like most marine invertebrate species, the Dungeness crab exhibits a complex life cycle with a planktonic larval phase and a benthic juvenile and adult phase.

The Dungeness crabs migrate offshore during the winter months and return to the near shore waters in the spring season for breeding. The female crabs spawn between late September and December (Wild 1983). The eggs remain attached to the abdominal pleopods of the female until they hatch between October and March depending on the variability in the hatching time. An egg mass may contain about one to two million eggs

(Wild 1983) and a female may produce up to five million eggs in three to four broods during her lifetime (MacKay 1942). Eggs mature in about two to three months. The hatching season commonly shortens from north to south along the west coast of North America.

The larvae release occurs between December and May, depending on the latitude, earlier in the south and later in the north. The crabs undergo five zoeal stages before molting to the megalopae (Reed 1969). In oceanic waters, the zoeae make typical diel migrations in the water column with high concentrations at the surface at night and low numbers during the day. Early stage zoeae are transported offshore because of the seaward movement of surface waters (Reilly 1983). Once larvae have molted into megalopae they procure a settlement site at the bottom (Jamieson *et al.* 1989; McConnaughey *et al.* 1992). In Oregon and Washington waters, the megalopae are most abundant from the months of April through June (Booth *et al.* 1985; Hobbs and Botsford 1992).

The megalopae settle and metamorphose into first crab instar exclusively in the nearshore zone and estuaries, usually in less than 20 meters depth (McConnaughey *et al.* 1992; Roegner *et al.* 2003). High settlement densities occur in the estuaries compared to the coastal sites and both the growth and the survival are enhanced in estuaries (Dumbauld *et al.* 1993).

Over a period of four to twelve months, the larvae undergo a series of six molts before manifesting the short-tailed form of a juvenile crab. After molting, the juveniles

are found in shallow coastal waters and estuaries, and large numbers live in shallow subtidal and intertidal areas hiding beneath or among plants, rocks, shell debris, and eelgrass beds that provides protection and harbors food (Fernandez *et al.* 1993; Stevens and Armstrong 1984, 1985). The crabs mature after two years. At four years of age most crabs are marketable (Cleaver 1949; Williams 1979) and the maximum lifespan of Dungeness crabs averages ten years.

Dispersal and Distribution of Dungeness Crabs

A life cycle with long pelagic phase maximizes the potential for high levels of mixing and dispersal among populations (Avise 1994; Hedgecock 1986; Shanks 1995). In the past it was thought that populations with widely dispersing larvae would be genetically similar as compared to populations with restricted dispersal. However, high dispersal potential may not always result in high genetic similarity. There are many reasons to expect detectable genetic variation in populations of this species.

Cancer magister larvae are active vertical migrators. Larval swimming behavior can affect both the direction and the timing of the transport of crabs in oceanic (Peterson *et al.* 1979; Bucklin *et al.* 1989) and estuarine systems (Cronin and Forward 1982; Bousfield 1955). Adult crabs are also capable of moving distances as much as 100 miles along the coast (Jamieson and Phillips 1988).

It has been suggested that marine populations form where the oceanographic conditions together with the life history assure that enough larvae recruit back; thus

maintaining the population (Sinclair 1988). Natural long-term shifts in oceanographic conditions have been recently recognized (Francis *et al.* 1998; McGowan *et al.* 1998). Physical oceanographic conditions may have strong influence on the dynamics of these populations. The prevalent current systems tend to restrict water movement and perhaps larval dispersal. Major currents flow at speeds up to 12.5 miles per day. Therefore, movement of larvae from one population to another is a distinct possibility (Johnson *et al.* 1986).

The physical processes can transport offshore developing larvae to nearshore settlement sites. Larvae aggregate into discrete patches in the ocean (Shenker 1988) and megalopae recruit to coastal sites episodically (Roegner *et al.* 1999) in discrete pulses (recruitment cohort). If the larvae are segregated at sea and if there is non-uniform delivery among the coastal sites, then variation may result. Abundant young crabs are located where currents are likely to concentrate megalopae. The degree of genetic distinctness in the populations will allow for measure of larval dispersal and biogeographic patterns of Dungeness crabs.

The geographic range of *Cancer magister* encompasses three main oceanographic systems: The Gulf of Alaska (GOA), Puget Sound (PS), and the California Current System (CCS) (Hickey 1979; Thomson *et al.* 1981; Thomson 1994). Off the west coast of North America transition from winter to summer wind regimes occur as the Aleutian low-pressure system weakens and moves northwestward. The North Pacific high-pressure system strengthens and moves to the north, resulting in southward winds along

the west coast of North America (Huyer 1983). There may be a major biogeographic boundary between the Gulf of Alaska and the California current, which likely reflects reduced larval exchange between these current regimes (Bunch *et al.* 1998; Rocha-Olivares and Vetter 1998).

Thomson *et al.* (1981, 1989) reviewed oceanographic patterns in the Gulf of Alaska and Strait of Georgia. West of Vancouver Island, the eastward flowing subarctic current splits in two, forming the southward flowing California Current System and the Alaskan current which flows northward into the Gulf of Alaska. This current divergence may correspond to the division of Aleutian and Oregonian biogeographical provinces. Rocha-Olivares and Vetter (1998) found genetically variable rosethorn rockfish populations on either side of this boundary despite the fact that the larval pelagic period is probably on the order of months. They concluded that the divergence in flow prevented the movement of larvae between the CCS and GOA.

Circulation of the CCS has been reviewed by Hickey *et al.* (1989), Landry *et al.* (1989), Largier *et al.* (1993) and Strub *et al.* (1987a,b). The California current system off Washington, Oregon and northern California comprises the southward flowing California current and the northward flowing California undercurrent which surfaces as the Davidson current in fall and winter (Hickey 1979; Hickey *et al.* 1989). Better understanding of the interaction between the crab behavior and physical processes is needed to explain variation in recruitment dynamics in the west coast of North America.

The dispersal distance or directionality is thus unpredictable (Botsford *et al.* 1994). By measuring the genetic variability of the Dungeness populations it is possible to assess the degree of gene flow and distinguish the effects of larval dispersal from post larval mortality.

Genetic Analysis of Dispersal

The use of sensitive molecular techniques has opened up many possibilities for examining genetic population structure in natural populations. Mitochondria contain circular DNA molecules that are maternally inherited in most species. The sequencing of mitochondrial DNA (mtDNA) has advantages over other techniques. It often shows greater resolution of genetic differences than nuclear genes (Moritz 1994) and is more prone to genetic drift than are nuclear loci (Birky *et al.* 1989). It is usually not subject to recombination and has a rapid rate of evolution. In addition, it is transmitted as a haploid mode from generation to generation.

Genetic markers that evolve rapidly are used with the expectation that they will provide greater sensitivity for the detection of genetic variation within a species. Cytochrome *c* oxidase is a mitochondrial respiratory protein. The first subunit of this protein comprises the active pocket. It is possible that some sequence variations may confer conformational changes in the protein, which may confer some selective advantage. Significant amount of genetic variation in the mitochondrial cytochrome *c* oxidase subunit I (COI) gene has been observed in several taxa related to Dungeness crab including Tanner crabs, Mole crabs, Penaeid shrimps, and Barnacles (Palumbi and

Benzie 1991; Van Syoc 1994; Tam *et al.* 1996; Bunch *et al.* 1998; Hebert *et al.* 2003). Primers used for the COI gene amplification have also been designed for many invertebrate species (Folmer *et al.* 1994). For these reasons, a base pair segment of the COI gene was used in this study.

Long-term Project Objectives

There are three long-term objectives of this project.

- The first objective is to ascertain the degree of genetic variability along the biogeographic range of *Cancer magister*.
- The second objective is to determine the origin, route, and transport mechanisms of *Cancer magister* arriving along the west coast of North America. An enhanced study of the dispersal trajectories and the magnitude of larval transport are crucial in understanding the fate of larvae and also the sources and rates of mortality.
- The third objective is to make recommendations for size and spacing of marine reserves along the west coast of North America. Reserves that retain the protected populations and enhance the populations that are not protected have been suggested. Reserves should be large enough to accommodate short distance dispersing organisms and should be far enough apart so that long distance dispersing individuals from one reserve may settle in adjacent reserves (Shanks *et al.* 2003).

Current Project Objectives

There are three objectives of the current study.

- The first objective is to provide a preliminary examination of the genetic variation among adult *Cancer magister* along the west coast of North America. Studying the adult population structure is important since it allows inferences to be drawn about the net effects of gene flow and local selection (Roughgarden *et al.* 1988; Grosberg and Levitan 1993).
- The second objective of this study is to detect genetic variation across the life stages of *Cancer magister* at two sites, Coos Bay and Columbia River. This information will help to determine whether the recruiting larvae suffer post-settlement mortality or selection pressure. This will determine how the genetic composition of larvae in the settlement pulses affects the genetic composition of the adult populations.
- The third objective of this study is to detect within and between sites genetic variation of megalopae at Coos Bay and Columbia River. Studies of larvae will characterize the sources of genetic variation in adult populations. The genetic characteristics of adults are reflected in the progeny. The genetic composition within and between larval settlement cohorts reveals the crab dispersal trajectories and oceanic mixing processes. The genetic composition of recruiting megalopae at a given location may predict the future genetic composition of that adult population.

Project Hypotheses

- Populations of adult *Cancer magister* across the biogeographic range are genetically distinct. This distinctness could be due to restricted larval transport due to the physical barriers, behavior that enhances regional retention, recruitment of larvae from different source populations or pre- and post- settlement natural selection.
- There is a variation in the genetic composition of settling larvae and adults. This could be due to the likelihood of post-settlement mortality or selection pressure or variances in reproductive success.
- There is spatial and temporal genetic distinctness among the recruiting larvae of *Cancer magister.* This could result due to differences in spawning season or developmental times or due to the effects of the coastal current patterns.

Relation to the State of Knowledge in this Field

Genetic analyses have revealed astonishing relationships between marine populations.

Kordos and Burton (1993) sampled both megalopae and adults of blue crabs along the coast of Texas. They found that the allelic frequencies in the recruiting megalopae were different from those in the adjacent adult populations and also that the frequencies changed with season. They concluded that the source of larvae was not local and varied with season. This difference in allelic frequency at different life stages may be due to selection, but because they were unable to sample megalopae continuously it may also be

due to missing critical settlement pulses of megalopae (Kordos and Burton 1993). Allelic frequencies in different adult populations were significantly different indicating that interpopulation gene flow was not sufficient to overcome population differentiation.

Hedgecock (1994a,b) proposed that genetic variation among populations might be due to large variance in reproductive success among adults, so that a resulting year class is the result of spawning by only a small portion of adult population. This "instantaneous drift effect" (David *et al.* 1997) would then lead to reduction in genetic variation among recruits, to differences between the genetic composition of recruits and the adult population as a whole and to the extent that different portions of the adult populations contribute successful progeny in different years.

Where oceanographic barriers do not exist, population genetic analyses often suggest widespread gene flow. Examples include populations of the bivalves *Mya arenaria* in New England (Caporale *et al.* 1997) and *Donex* deltoids in SE Australia (Murray-Jones and Ayre 1997).

Rawson *et al.* (1999) sampled two *Mytilus spp.* and found that a few alleles were being exchanged outside a hybridization zone centered on San Francisco Bay. The researchers concluded that restricted larval transport due to oceanographic barriers led to this genetic variation.

Rocha-Olivares and Vetter (1998) compared mtDNA sequences of rockfish populations from the coasts of California, Oregon, British Columbia and the Bearing Sea.

It was found that significant population structure existed between these populations of rockfish and that a biogeographic division between the Oregonian and Aleutian Provinces might be restricting transport of larvae.

Moberg and Burton (2000) looked at the genetic composition of populations of adult and settling red urchins, *Strongylocentrotus franciscanus*. They found that adult populations were distinct but there was no geographic pattern to the differences. Settling larvae were different from the adjacent adult population and varied between years. This indicated that these differences might be due to pre- and post- settlement natural selection and large variances in reproductive success (sweepstakes recruitment).

Li and Hedgecock (1998) found evidence for sweepstakes recruitment due to pre- and post- settlement natural selection and large variances in reproductive success amongst the larvae of the oyster *Crassostrea gigas*. They point out the role of sweepstakes recruitment and the possible role of post-settlement selection in establishing the genetic composition of populations.

Gomez-Uchida *et al.* (2003) studied genetic diversity within and between adult populations of Hairy edible crab, *Cancer setosus,* using allozyme and AFLP markers along the Chilean coast. Genetic diversity estimates within populations were low for allozyme loci but much higher for AFLP loci. They also found that the differentiation among samples was statistically significant for allozymes but not for AFLPs. They concluded the crab stock off Chile to be genetically similar even though allozyme data suggest small but significant population differentiation.

Star *et al.* (2003) noticed abrupt genetic structuring among the greenshell mussel *Perna canaliculus* populations between the North and South Islands of New Zealand.

Laminaria digitalis populations in the English Channel were found to be similar when in isolated stands due to habitat discontinuities, but continuous forests exhibited genetic variability that was not associated with the current regime (Billot *et al.* 2003).

MATERIALS AND METHODS

Sample Collection

Whole body samples of megalopae and juveniles were collected during the natural settlement seasons. Adults were collected opportunistically during the respective recruitment seasons. Non-lethal sampling of adult crabs was employed; the fifth walking leg of individuals was removed and the crabs were then released. All samples were kept frozen at –20°C.

Samples for this project were collected from the following marine stations and government agencies:

- University of Alaska, Juneau Center, School of Fisheries and Ocean Sciences, Fairbanks, AK
- National Marine Fisheries, Hammond Marine Station, OR:
  Puget Sound, Columbia River, and Newport
- University of Oregon, Oregon Institute of Marine Biology, Coos Bay, OR
- California Department of Fish and Game:
  Fort Bragg, Bodega Bay, and San Francisco Bay

Table 1: The collection years, the sample sizes and the respective life stages from Bodega Bay, Coos Bay, Juneau, Newport, Puget Sound, San Francisco, Fort Bragg, and Columbia River.

Year of collection	Life stage	Sample size	Location
1997	Adults	2	Bodega Bay
1997	Adults	12	Coos Bay
1997	Adults	5	Juneau
1998	Adults	7	New Port
2002	Adults	2	Puget Sound
2003	Adults Megalopae	16 47	Coos Bay
2004	Adults	4	San Francisco
2004	Adults	12	Fort Bragg
2004	Megalopae	29	Coos Bay
2004	Adults Juveniles Megalopae	24 75 29	Columbia River

The use of megalopae will eliminate any possible post-settlement mortality effects on measures of variability. The degree of genetic variation found between populations and groups of larvae will allow for an unparalleled measure of the consequences of larval dispersal in this system. Within estuaries, light traps were used to collect time series of larval crabs (Roegner *et al.* 2002; Miller and Shanks 2004). Light traps function as behavioral samplers and depend upon the positive phototropism of organisms towards artificial illumination. The traps that floated at the surface were made from semi-opaque

5L plastic jugs, and plankton gained entry through 1 times 3 cm openings cut into apex of translucent plastic funnels. Source of light was a 6-watt AC fluorescent bulb sealed in an acrylic tube. Captured specimens were concentrated in a cod end, 250 μm mesh, attached to the base of the trap and sorted in the laboratory. Larvae tended to occur in the light traps in distinct pulses of abundance.

Adult Dungeness crabs were caught in circular pots baited with either herring or clams. Pots are usually about 40 inches in diameter and 14 inches high. They were made of 3/4 inch round steel frames wrapped in rubber tubing and then covered with stainless steel wire mesh (Williams 1979). Two 4-3/8 inches diameter escape rings are usually built in each pot to keep the pot from filling with undersize crabs.

DNA Extraction

DNA was extracted using the Wizard Genomic Purification Kit (Promega). About 0.5-1.0 cm adult tissue was taken in 1.5 ml tube. A whole body sample was used in case of megalopae and juveniles. 0.5 M EDTA, nuclei lysis solution and Proteinase K (20 mg/ml) were then added and incubated at 55°C with gentle shaking for 24 hours (Sambrook *et al.* 1989). RNAse was then added and tubes were incubated at 37°C for 30 minutes. Protein precipitation solution was then added to the samples. After centrifugation the supernatant containing the DNA was transferred to a 1.5 ml tube. Precipitation of DNA included washings with 95% isopropanol and 70% ethanol. DNA pellets were dried at 55°C. DNA rehydration solution was then added to the dried pellet and incubated at 65°C for one hour. The DNA was stored at –20°C.

PCR Amplification

Amplification using 25µl reactions was conducted in the Personal thermal mastercycler (Eppendorf). The standard PCR reaction consisted of 2.0 µl DNA, 0.5 µM each of forward and reverse primers, 1X PCR buffer (10X with 1.5 mM Mg was supplied with MasterTaq Kit, Eppendorf), 3.0 µl Mgcl2, 300.0 ng/µl BSA, 0.2 mM each dNTPs, and 1.0 U Taq DNA polymerase (Mastertaq Kit, Eppendorf). BSA was added since the reaction mixture was suspected to contain substances that inhibited amplification. Marine water samples are found to have substances that inhibit enzyme activity. Humic substances that are a mixture of complex polyphenolics produced during the decomposition of organic matter are commonly found in water and may contaminate any material exposed to the environment (Kreader 1996). Since all the samples used in the present study were lysed with Proteinase K, added protease may also be a possible source of inhibition. BSA may provide an alternate substrate and thereby protect the enzyme, Taq DNA polymerase (Kreader 1996). PCR reactions were performed for 30 cycles: hot start for 5 minutes, denaturing at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 60 seconds and final extension at 72°C for 10 minutes. PCR product was stored at –20°C until needed. Negative control was set up along with the DNA samples. In this reaction mixture 2.0 µl DNA grade water was used instead of template DNA.

Primers were designed based on the sequence published by Harrison and Crespi (1999b) and were used to amplify a ~ 415 bp subunit of COI gene which is a non-protein coding gene of mitochondrial DNA. These primers are specific to Dungeness crabs.

Mitochondrial Forward primer: 5´-GGAGGATTTGGAAATTGATT-3´

Mitochondrial Reverse primer: 5´-GTACAGGAAGGGATAGTAGT-3´

Agarose Gel Electrophoresis

A 100 bp ladder (Biolabs) was run on 2% agarose gels to help estimate the mass of DNA amplified in nanograms and the size of the PCR product in base pairs. A negative control was also run to check for the purity of PCR reagents. Generally, 400 bp PCR product was obtained. Running of the gel also showed if there was successful PCR amplification. Gels were stained with ethidium bromide for 30 minutes and then the electrophoretic patterns of the gels were recorded using the Gel Doc 1000 Image Analyzer (BioRad).

Sequencing

10 μl of the PCR product was then sent to Gene Gateway, LLC, Hayward, CA, a private biotechnology company. Sequences were obtained through the use of the ABI Prism Model 377 (version 3.2) automated sequencer (Smith *et al.* 1986) using Big Dye.

Data Analyses

Sequences were aligned using ClustalW (Pearson and Lipman 1988) in the computer program BioEdit. A particular sequence that varied by at least one nucleotide substitution from any other sequence was called a haplotype. Haplotypes were determined by using the program DnaSp version 4.0.

Haplotype diversity (h: eq. 8.4 and 8.12 in Nei 1987) and nucleotide diversity (Pi: eq. 10.5 in Nei 1987) were calculated using the program DnaSp version 4.0 (Rozas and Rozas 1999). Haplotype diversity is a measure of the frequency of each haplotype occurring within a population. It is a measure of heterozygosity at sequence level. Nucleotide diversity is a measure of the mean number of nucleotide differences per site between two sequences (Nei and Li 1979; Nei and Tajima 1981). It is a measure of heterozygosity at nucleotide level.

The Fu's Fs test (equation 1 in Fu 1997) was performed to determine whether samples from different sites were at equilibrium with respect to mtDNA. A significant amount of deviation from mtDNA genetic equilibrium is expected due to recent population expansion or bottleneck or selection. Fu and Li's D and F tests were further performed since these tests could indicate the likely mechanism responsible for the observed deviation (Fu 1997). Fu and Li's D test is based on the differences between number of singletons and number of mutations whereas Fu and Li's F test is based on the differences between number of singletons and average number of nucleotide differences between pairs of sequences (Rozas and Rozas 1999).

Linkage disequilibrium was run to calculate nonrandom association between nucleotide variants at different polymorphic sites (Rozas *et al.* 2001). It may be a result of earlier ancestral recombination events. A population is said to be in linkage disequilibrium when the observed frequencies of haplotypes in a population deviate from the frequencies of haplotypes calculated by multiplying the frequencies of individual genetic markers in each haplotype together. Chi-square test was performed to determine whether the associations between polymorphic sites were or were not significant (Sokal and Rohlf 1981). The Bonferroni correction for multiple tests (Weir 1996) was used to avoid spurious rejections of the null hypothesis in multiple tests, assuming that all tests are independent.

Population genetic analysis of the sequence data was conducted by the use of modified hierarchical F-statistics (Felsenstein 1993). Diversity indices were calculated using DnaSp version 4.0 (Rozas and Rozas 1999). Differences among populations were quantified using Nei's coefficient of gene variation, Gst (equation 9 in Nei *et al.* 1973) and Wright's inbreeding coefficient, Fst (equation 3 in Hudson *et al.* 1992).

Gst and Fst are usually used in a similar fashion as indices of genetic difference among populations. Both these indices are functions of how heterozygosity can be partitioned within and between populations (Crow 1986). In groups with low Gst values the majority of variation is found within the population. High Gst values indicate that individuals within a population are relatively similar but populations are significantly different. Fst, a measure of population divergence, ranges from zero to one. Zero

indicates no population differentiation and one indicates population divergence. If no migration exists in the populations then alleles remain fixed and Fst value reaches 1.

The evolutionary relationships among a group of organisms are usually illustrated by means of a phylogenetic tree. The phylogenetic relationships between haplotypes were assessed using the program MEGA version 3.0 (molecular evolutionary genetic analysis, Kumar *et al.* 2001, 2004) based on the model of Tamura-Nei (Tamura and Nei 1993).

The COI data set was analyzed using neighbor-joining method (NJ) (Saitou and Nei 1987) and unweighted pair-group method with arithmetic mean (UPGMA) (Li *et al.* 1997), which are two methods of clustering (Felsenstein 1985). The robustness of trees inferred from these analyses was evaluated using bootstrap analyses with heuristic searching (500 replicates; Felsenstein 1985). Felsenstein (1985) introduced Bootstrapping which is a computational technique frequently used as a means to estimate the confidence level as it re-samples the data to empirically infer the variability of the estimate obtained by the tree-making methods.

The NJ method produces an unrooted tree because it does not require the assumption of a constant rate of evolution. It constructs trees by successive clustering of the lineages, setting branch lengths as these lineages are joined (Felsenstein 1985). The principle of NJ is to find neighbors (OTUs connected through a single internal node) in a sequential manner that may minimize the total length of the tree. UPGMA method produces a rooted tree since it assumes molecular clock. It converts sequence data into a

set of discrete pairwise distance values, arranged into matrix. Successive clustering using an average linkage method of clustering is involved. Phylogenetic trees give an estimate of the degree of genetic dissimilarity among various populations. Longer the branches, the more dissimilar are individuals from those years.

Genetic distances were measured by number of nucleotide substitutions per site between a pair of sequences. It provides a means for visualizing the degree of genetic divergence since they would have shared a common ancestor.

RESULTS

One hundred and twenty nine haplotypes were obtained from a total of 264 individuals. All the haplotypes had one unique sequence associated with them. There was a large occurrence of rare haplotypes, haplotypes that are found in only one individual (Boom *et al.* 1994). Li and Hedgecock (1998) coined the term "singletons" for such haplotypes.

Table 2: The representation of the number and frequency of the haplotypes.

Haplotype name	Number of haplotypes	Haplotype frequency (%)
H1	5	1.89%
H2	43	16.29%
H3	1	0.38%
H4	1	0.38%
H5	1	0.38%
H6	1	0.38%
H7	57	21.59%
H8	1	0.38%
H9	1	0.38%
H10	7	2.65%
H11	1	0.38%
H12	1	0.38%
H13	1	0.38%
H14	1	0.38%
H15	12	4.55%
H16	1	0.38%
H17	1	0.38%
H18	1	0.38%
H19	1	0.38%
H20	1	0.38%
H21	1	0.38%
H22	1	0.38%
H23	1	0.38%

H24	1	0.38%
H25	1	0.38%
H26	1	0.38%
H27	1	0.38%
H28	1	0.38%
H29	1	0.38%
H30	1	0.38%
H31	2	0.76%
H32	1	0.38%
H33	1	0.38%
H34	1	0.38%
H35	2	0.76%
H36	1	0.38%
H37	1	0.38%
H38	1	0.38%
H39	1	0.38%
H40	1	0.38%
H41	1	0.38%
H42	1	0.38%
H43	1	0.38%
H44	1	0.38%
H45	1	0.38%
H46	1	0.38%
H47	1	0.38%
H48	1	0.38%
H49	1	0.38%
H50	1	0.38%
H51	1	0.38%
H52	3	1.14%
H53	1	0.38%
H54	1	0.38%
H55	1	0.38%
H56	1	0.38%
H57	1	0.38%
H58	1	0.38%
H59	1	0.38%
H60	3	1.14%

H61	1	0.38%
H62	1	0.38%
H63	1	0.38%
H64	1	0.38%
H65	1	0.38%
H66	2	0.76%
H67	1	0.38%
H68	1	0.38%
H69	1	0.38%
H70	1	0.38%
H71	1	0.38%
H72	1	0.38%
H73	1	0.38%
H74	1	0.38%
H75	1	0.38%
H76	2	0.76%
H77	1	0.38%
H78	1	0.38%
H79	2	0.76%
H80	2	0.76%
H81	1	0.38%
H82	1	0.38%
H83	1	0.38%
H84	1	0.38%
H85	1	0.38%
H86	1	0.38%
H87	1	0.38%
H88	1	0.38%
H89	3	1.14%
H90	2	0.76%
H91	1	0.38%
H92	1	0.38%
H93	1	0.38%
H94	1	0.38%
H95	1	0.38%
H96	1	0.38%
H97	2	0.76%

H98	1	0.38%
H99	1	0.38%
H100	1	0.38%
H101	1	0.38%
H102	1	0.38%
H103	2	0.76%
H104	1	0.38%
H105	1	0.38%
H106	1	0.38%
H107	1	0.38%
H108	1	0.38%
H109	1	0.38%
H110	1	0.38%
H111	1	0.38%
H112	1	0.38%
H113	1	0.38%
H114	1	0.38%
H115	1	0.38%
H116	1	0.38%
H117	1	0.38%
H118	1	0.38%
H119	1	0.38%
H120	1	0.38%
H121	1	0.38%
H122	2	0.76%
H123	1	0.38%
H124	1	0.38%
H125	1	0.38%
H126	1	0.38%
H127	1	0.38%
H128	1	0.38%
H129	1	0.38%

Sequence Diversity

The results of two measures of genetic diversity, nucleotide diversity and haplotype diversity are shown in the following table. The highest haplotype diversity values were obtained in many of the adult populations. The nucleotide diversity ranged from 0.8% to 5.1% whereas the haplotype diversity ranged from 70.0% to 100.0%. Thus the diversity values of 1.00000 may not be statistically significant. The adults collected from San Francisco Bay in the year 2004 had the highest nucleotide diversity. The adults collected in the year 1997 from Juneau had the lowest haplotype diversity whereas the lowest nucleotide diversity was found in the megalopae collected in the year 2004 from Coos Bay.

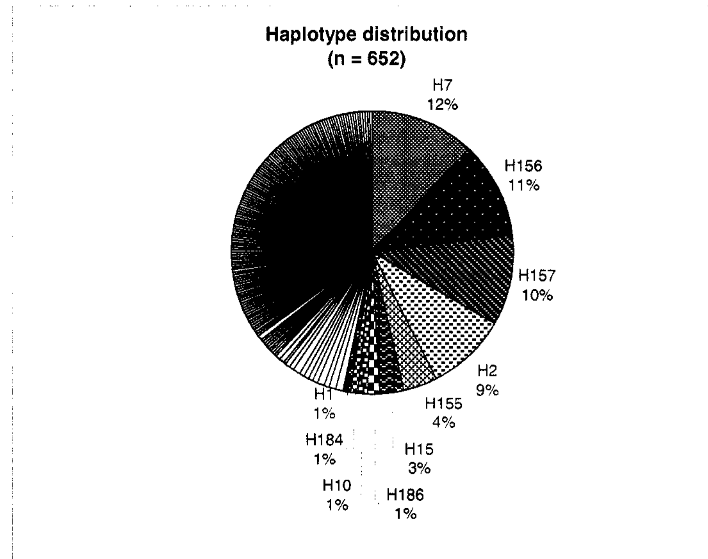
Table 3: The representation of the haplotype diversity and the nucleotide diversity of the populations by the collection years and the locations.

Grouping	Sample size	Haplotype diversity	Nucleotide diversity
All Samples	264	0.96126	0.01697
All Adults	84	0.98336	0.02035
1997 Bodega Bay (BB) Adults	2	1.00000	0.01923
1997 Coos Bay (CB) Adults	12	0.96970	0.01583
1997 Juneau (JN) Adults	5	0.70000	0.01731
1998 Newport (NP) Adults	7	0.95238	0.01282
2002 Puget Sound (PS) Adults	2	1.00000	0.02564
2003 Coos Bay (CB) Adults	16	0.97500	0.01976
2004 San Francisco (SF) Adults	4	1.00000	0.05128
2004 Fort Bragg (FB) Adults	12	1.00000	0.01884
2004 Columbia River (CR) Adults	24	0.98913	0.01430
2003 Coos Bay (CB) Megalopae and Adults	63	0.99437	0.02232
2003 Coos Bay (CB) Megalopae	47	0.99352	0.02092
2004 Columbia River (CR) Megalopae, Juveniles & Adults	128	0.93135	0.01186
2004 Coos Bay (CB) Megalopae	29	0.88177	0.00829
2004 Columbia River (CR) Megalopae	29	0.92118	0.01599
2004 Columbia River (CR) Juveniles	75	0.91171	0.00840
All Megalopae	105	0.97179	0.01842

This data was combined with the previous *Cancer magister* data from the laboratory for a total of 652 Dungeness samples. Different haplotypes were identified

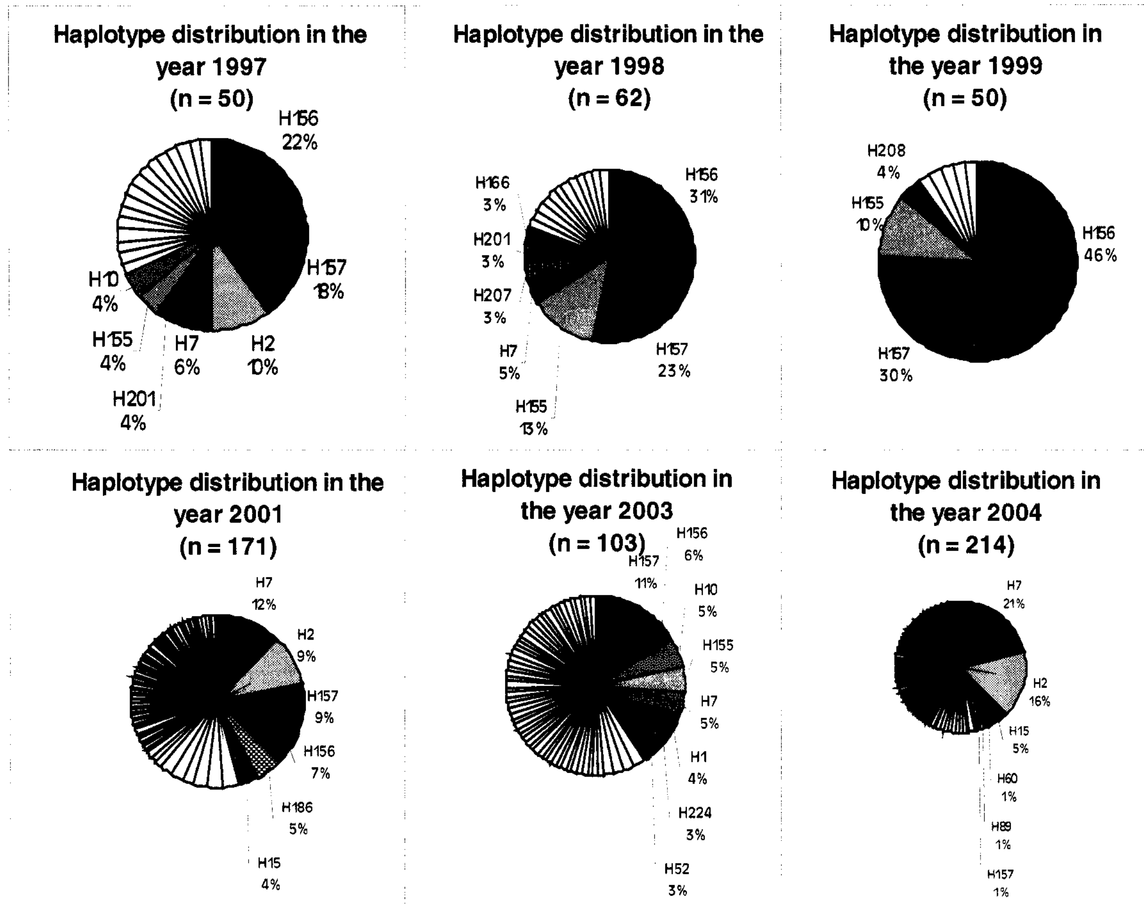
based on a minimum of 1-5 base pair difference between them. 261 haplotypes were identified.

Figure 1: Pie chart illustrating the distribution of the haplotypes in 652 individuals.



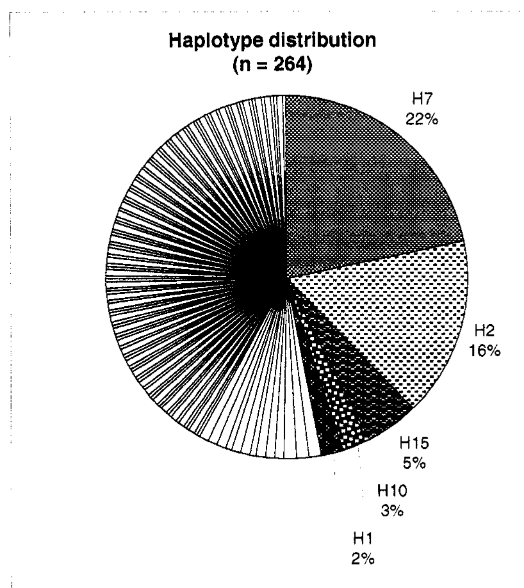
This pie chart indicated that H7 was the most common haplotype (12%) followed by H156 (11%), H157 (10%), H2 (9%), H155 (4%), and H15 (3%). H186, H10, H184, and H1 occurred in 1% of the total population. All other haplotypes were present in either one or two individuals.

Figure 2: Pie charts illustrating the distribution of the adults and the megalopae combined haplotypes collected across the years 1997, 1998, 1999, 2001, 2003, and 2004.



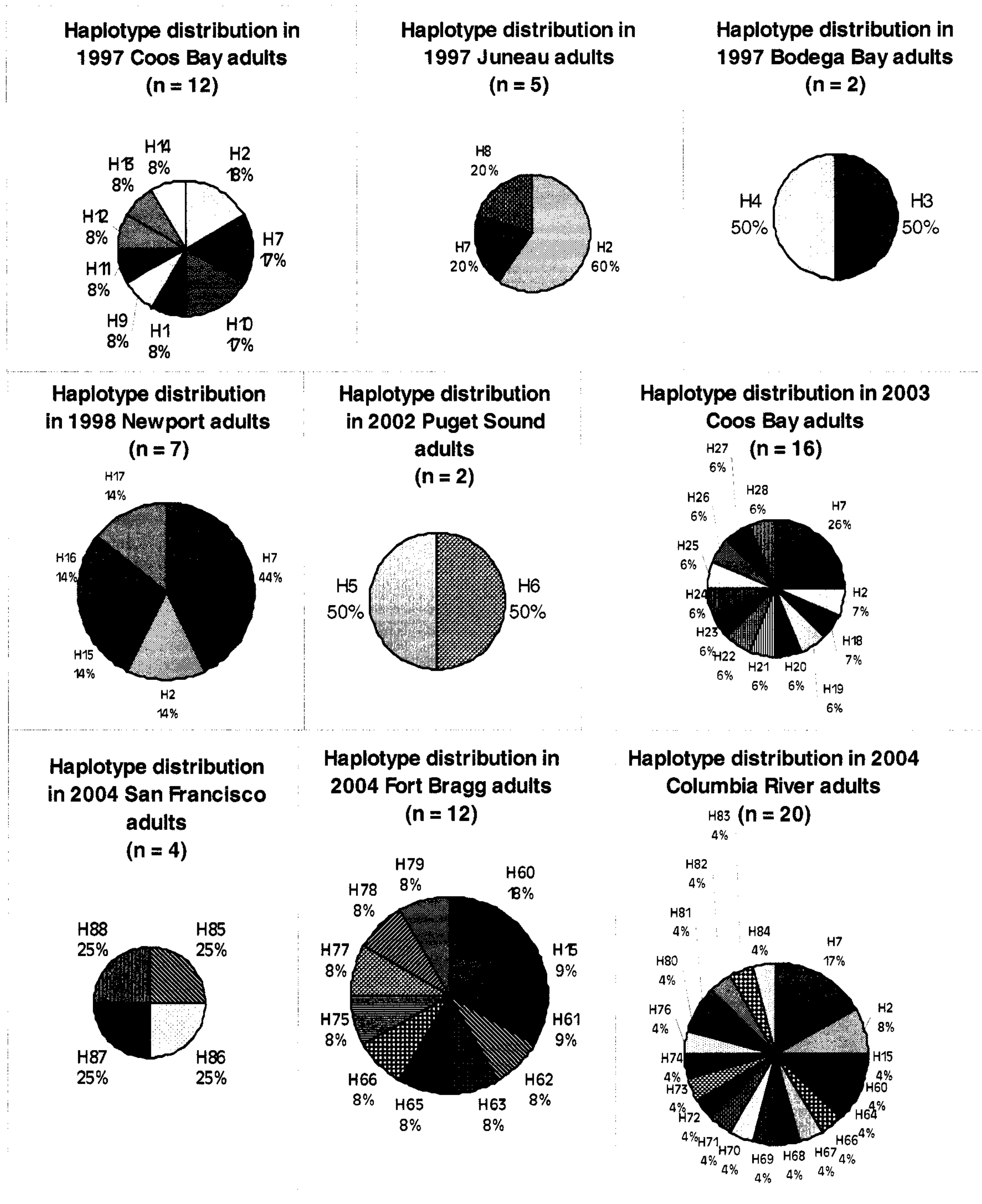
The haplotypes H2, H7, H10, H15, H155, H156, and H157 were consistently found in all populations and hence these haplotypes were called "Oregon local haplotypes." As the sample size began to increase, the presence of large number of singletons was observed.

Figure 3: Pie chart illustrating the distribution of the haplotypes in 264 individuals.



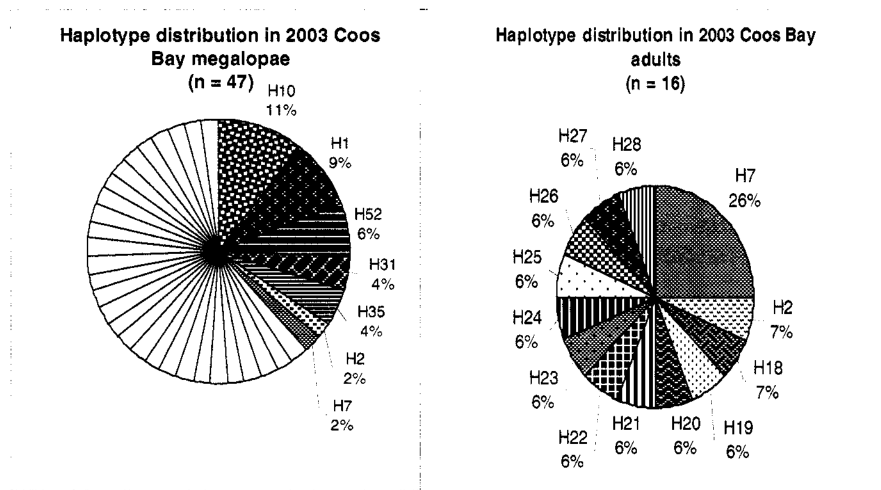
H7 (21.59%) was found to be the most common haplotype followed by H2 (16.29%) and then H15 (4.55%). H10 was found in 2.65% of the population whereas H1 was found in 1.89% of the population. H52, H60, and H89 were present in 1.14% of the total individuals. All other haplotypes were present in either one or two individuals.

Figure 4: Pie charts illustrating the spatial distribution of the adult haplotypes by year from Coos Bay, Juneau, Bodega Bay, Newport, Puget Sound, San Francisco, Fort Bragg, and Columbia River.



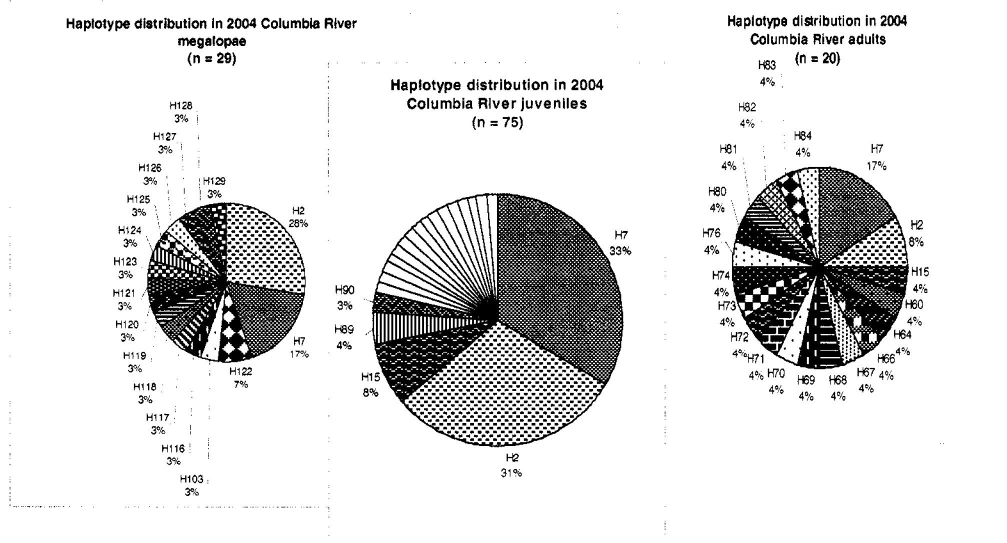
The two common "Oregon local haplotypes," H2 and H7, were also observed in Juneau, Alaska. One of the "Oregon local haplotype," H15, was sampled from Fort Bragg, California.

Figure 5: Pie charts illustrating the distribution of the haplotypes comparing the megalopae to the adults at Coos Bay in the year 2003.



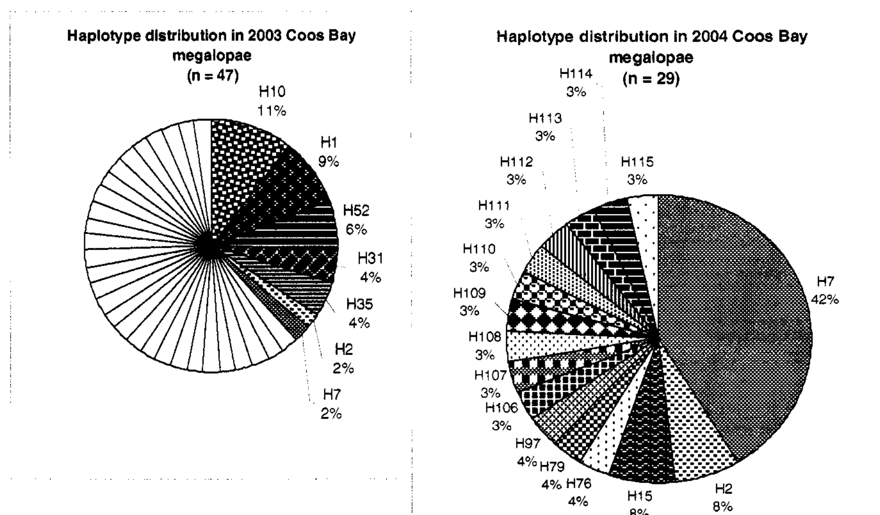
H10 and H1 were seen in megalopae but not in adults. H2 and H7 were present in small numbers in megalopae but in large proportions in adults. The haplotypes H19, H20, H21, H22, H23, H24, H25, H26, H27, and H28 were seen in 2003 Coos Bay adults only.

Figure 6: Pie charts illustrating the distribution of the haplotypes comparing the megalopae, the juveniles, and the adults at Columbia River in the year 2004.



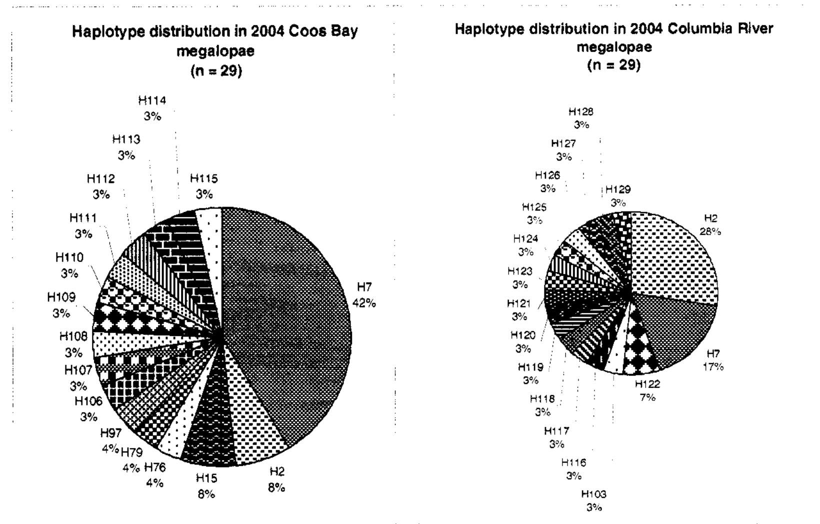
The juveniles exhibited least amount of genetic variation as compared to the adults and the megalopae.

Figure 7: Pie charts illustrating the temporal distribution of the megalopal haplotypes in the years 2003 and 2004 from Coos Bay.



H10 and H1 occurred in the year 2003 but not in 2004. Haplotypes H2 and H7 were present in small numbers in the year 2003 but were found in large proportions in the year 2004.

Figure 8: Pie charts illustrating the spatial distribution of the megalopal haplotypes in the year 2004 from Coos Bay and Columbia River.



H15 was seen in Coos Bay but not in Columbia River.

H2 and H7 were found to occur in large numbers in almost all of the populations. The haplotype H10 was seen in populations from Coos Bay only (1997 adults and 2003 megalopae). H15 was seen in almost all the 2004 populations. The 1998 Newport adults and the 2003 Coos Bay megalopae also had occurrence of H15. None of the 2004 populations had the presence of haplotype H1. H1 was seen in the 1997 Coos Bay adults, the 1997 Juneau adults and in the 2003 Coos Bay megalopae.

Table 4: The estimates of the Fu's Fs and the Fu and Li's D and F statistical tests.

Grouping	Total number of sites (excluding sites with gaps/missing data)	Number of polymorphic (segregating) sites	Fu's Fs statistical values	Fu and Li's D* test	Fu and Li's F* test
All adults	312	58	-83.173	-3.15589	-3.14984
2003 Coos Bay megalopae and adults	313	40	-66.675	-1.89652	-1.88458
2004 Columbia River megalopae, juveniles, and adults	312	53	-89.935	-3.38298	-3.39134
All megalopae	311	54	-93.490	-1.90333	-2.16150

The test showed a high number of polymorphic sites indicating a significant amount of genetic variation. Significant Fu's Fs values were seen in all the populations. Significant Fu and Li's statistical values were seen in the adults and in the samples from Columbia River collected in the year 2004.

Table 5: Linkage disequilibrium: The letter B indicates significant disequilibrium by the Bonferroni procedure. ***P<0.001

Site 1	Site 2	Chi-square values
2	125	264.000***B
5	39	23.087***B
5	43	23.087***B
5	44	23.087***B
8	277	131.498***B
14	29	37.021***B
19	65	32.921***B
19	83	87.331***B
19	118	87.331***B
19	131	42.830***B
19	150	28.000***B
19	197	87.331***B

19	222	87.331***B
19	251	87.331***B
19	277	42.830***B
29	65	77.076***B
39	45	131.498***B
39	68	21.080***B
39	127	87.331***B
39	137	131.498***B
39	160	43.164***B
43	44	264.000***B
45	127	42.830***B
45	137	64.996***B
45	160	20.668***B
52	204	31.748***B
52	234	31.748***B
65	150	32.921***B
65	197	25.497***B
65	209	25.497***B
65	242	25.497***B
68	83	21.080***B
68	98	21.080***B
68	118	21.080***B
68	184	55.617***B
68	222	21.080***B
68	251	21.080***B
76	111	87.331***B
76	241	28.000***B
83	105	28.441***B
83	118	264.000***B
83	130	23.087***B
83	222	264.000***B
83	251	264.000***B
83	277	131.498***B
95	105	28.441***B
95	241	87.331***B
100	108	28.441***B
100	127	87.331***B
100	287	43.164***B
105	118	28.441***B
105	122	28.441***B
105	221	21.925***B
105	222	28.441***B

105	251	28.441***B
106	107	131.498***B
108	127	36.875***B
108	160	40.473***B
108	287	74.608***B
111	241	87.331***B
118	130	23.087***B
118	222	264.000***B
118	251	264.000***B
118	277	131.498***B
127	137	42.830***B
127	160	56.652***B
127	184	20.527***B
127	241	28.000***B
130	222	23.087***B
130	251	23.087***B
131	150	42.830***B
131	197	131.498***B
150	197	87.331***B
150	209	87.331***B
150	265	42.830***B
153	265	131.498***B
160	184	41.532***B
184	287	27.162***B
204	234	64.996***B
209	265	131.498***B
222	251	264.000***B
222	277	131.498***B
251	277	131.498***B
253	261	87.331***B
287	301	43.164***B
287	304	43.164***B
287	318	43.164***B
301	304	264.000***B
301	318	264.000***B
304	318	264.000***B

A total of 63 polymorphic sites were analyzed while testing for linkage disequilibrium. Sites segregating for three or four nucleotides were not considered. Chi-square values were shown for significant polymorphic sites only. The number of significant comparisons using the Bonferroni procedure was 91.

Table 6: The estimates of Gst and Fst values between the adults from 1997 Bodega Bay, 1997 Coos Bay, 1997 Juneau, 1998 Newport, 2002 Puget Sound, 2003 Coos Bay, 2004 San Francisco, 2004 Fort Bragg, 2004 Columbia River and the megalopae from 2003 Coos Bay, 2004 Columbia River and 2004 Coos Bay.

		1997 BB Adults	1997 CB Adults	1997 JN Adults	1998 NP Adults	2002 PS Adults	2003 CB Adults	2004 SF Adults	2004 FB Adults	2004 CR Adults	2003 CB Meg	2004 CB Meg	2004 CR Meg
1997 BB Adults		–											
1997 CB Adults	Gst	0.07254	–										
	Fst	0.03476											
1997 JN Adults	Gst	0.09915	0.04067	–									
	Fst	0.05000	-0.01036										
1998 NP Adults	Gst	0.04595	0.01111	0.06483	–								
	Fst	0.01408	-0.01201	-0.04777									
2002 PS Adults	Gst	0.00000	0.07254	0.09915	0.05495	–							
	Fst	0.00000	0.03557	0.09459	0.06667								
2003 CB Adults	Gst	0.08054	-0.00107	0.07222	0.00420	0.08054	–						
	Fst	0.07302	0.017606	0.14002	0.11457	0.10053							
2004 SF Adults	Gst	0.02041	0.02596	0.08280	0.01833	0.02041	0.03120	–					
	Fst	0.13725	0.19075	0.20741	0.19540	0.11111	0.10776						
2004 FB Adults	Gst	0.06925	0.00763	0.07435	0.01497	0.06925	0.00691	0.02041	–				
	Fst	0.17604	0.16605	0.05748	0.09998	0.19930	0.15409	0.20920					
2004 CR Adults	Gst	0.08903	0.00022	0.06153	0.01556	0.08903	-0.00165	0.03712	0.00587	–			
	Fst	0.02318	0.05874	-0.00443	-0.01522	0.10999	0.11018	0.18903	0.05352				
2003 CB Meg	Gst	0.09923	0.01500	0.05919	0.02592	0.09923	0.01048	0.04642	0.01015	0.00553	–		
	Fst	0.14866	0.17853	0.16307	0.16116	0.18133	0.17436	0.19272	0.22253	0.20625			
2004 CB Meg	Gst	0.09851	0.00658	0.06678	0.01770	0.09519	0.00221	0.05078	0.02731	0.00622	0.03007	–	
	Fst	0.08203	0.10446	0.01882	0.00056	0.16327	0.21765	0.24251	0.13182	0.03127	0.32040		
2004 CR Meg	Gst	0.09625	-0.00131	0.04012	0.02717	0.09625	0.01143	0.04747	0.02186	0.00701	0.02082	0.01228	–
	Fst	0.17432	0.10619	0.00753	0.04749	0.19160	0.19802	0.22996	0.10072	0.08418	0.24774	0.09530	

Table 7: The estimates of Gst and Fst values between the megalopae and the adults collected in the year 2003 from Coos Bay.

		2003 CB Megalopae	2003 CB Adults
2003 CB Megalopae	Gst Fst	–	
2003 CB Adults	Gst Fst	0.01048 0.17896	–

Table 8: The estimates of Gst and Fst values between the megalopae, the juveniles, and the adults collected in the year 2004 from Columbia River.

		2004 CR Megalopae	2004 CR Juveniles	2004 CR Adults
2004 CR Megalopae	Gst Fst	–		
2004 CR Juveniles	Gst Fst	0.00147 0.11806	–	
2004 CR Adults	Gst Fst	0.00701 0.08418	0.00781 0.02999	–

The genetic differentiation was calculated among different groups of populations. Gst and Fst values of 0 were observed between the adults collected in 1997 from Bodega Bay and the adults collected in 2002 from Puget Sound. In general, in the groups with low Gst values, variation was found to occur within the population. High Gst values indicated that individuals within the population were genetically similar. Low Fst values

indicated lesser genetic differentiation and high Fst values indicated greater genetic differentiation between populations.

Phylogenetic Analyses

NJ and UPGMA methods were performed using bootstrap of 500 replicates (Kumar *et al.* 2001, 2004). Both the NJ and UPGMA trees were built using the COI haplotype sequences. The trees obtained were similar irrespective of whether the neighbor-joining dendogram was built using Jukes-Cantor, Kimura-2-parameter, Tamura-Nei, Tajima-Nei, or Tamura-3-parameter model. The phylogenetic trees built using Tamura-Nei were shown. Longer branches in the NJ trees suggested that those individuals were more genetically distinct.

Figure 9: The MEGA NJ tree relating all the haplotypes obtained in the collection year 1997. Tree constructed based on the model of Tamura-Nei (1993). Numbers at specific nodes represent bootstrap values after 500 replicates. The scale bar represents divergence using Tamura-Nei correction.

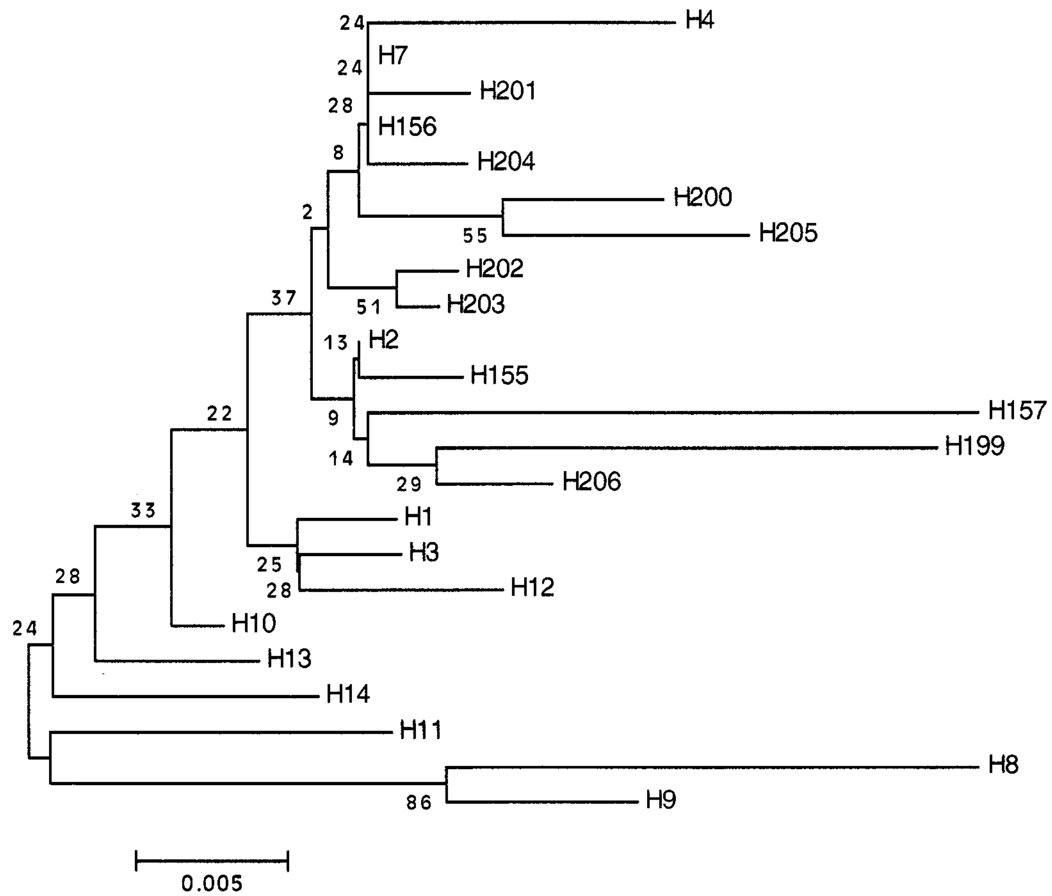


Figure 10: The MEGA UPGMA tree relating all the haplotypes obtained in the collection year 1997. Tree constructed based on the model of Tamura-Nei (1993). Numbers at specific nodes represent bootstrap values after 500 replicates. The scale bar represents divergence using Tamura-Nei correction.

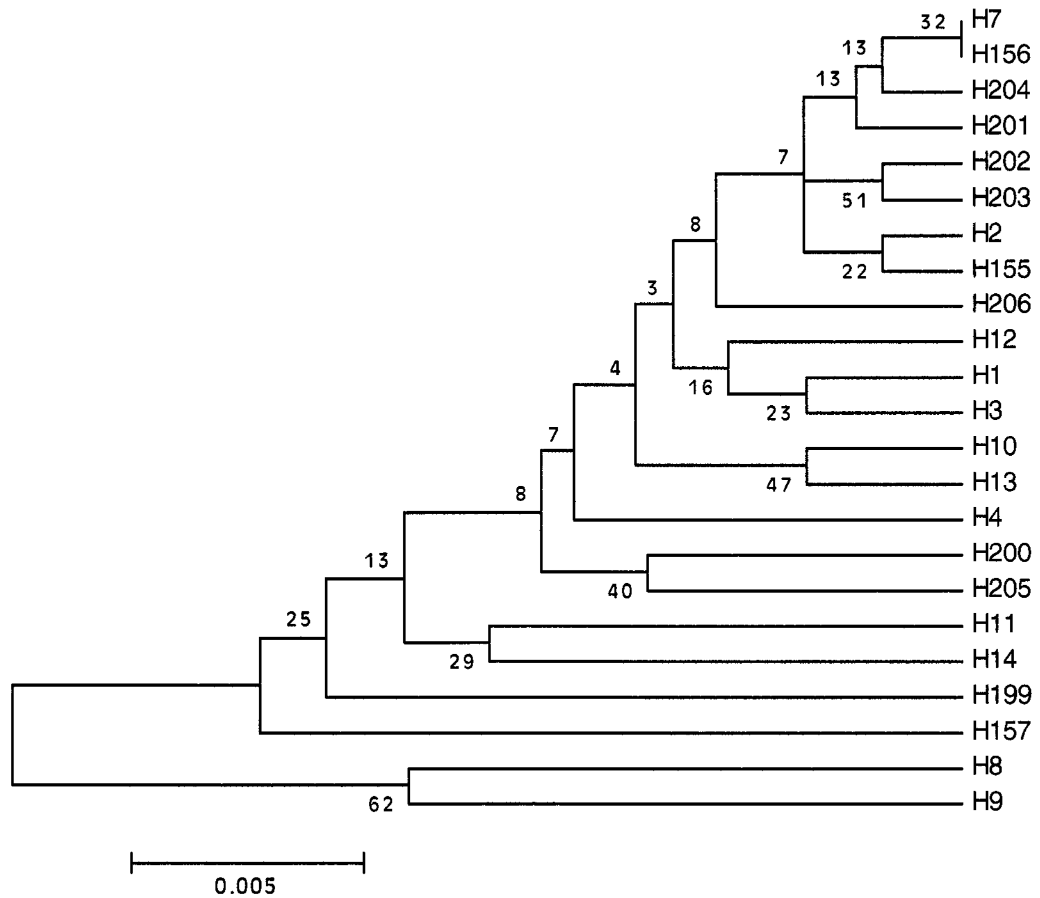


Figure 11: The MEGA NJ tree relating all the haplotypes obtained in the collection year 1998. Tree constructed based on the model of Tamura-Nei (1993). Numbers at specific nodes represent bootstrap values after 500 replicates. The scale bar represents divergence using Tamura-Nei correction.

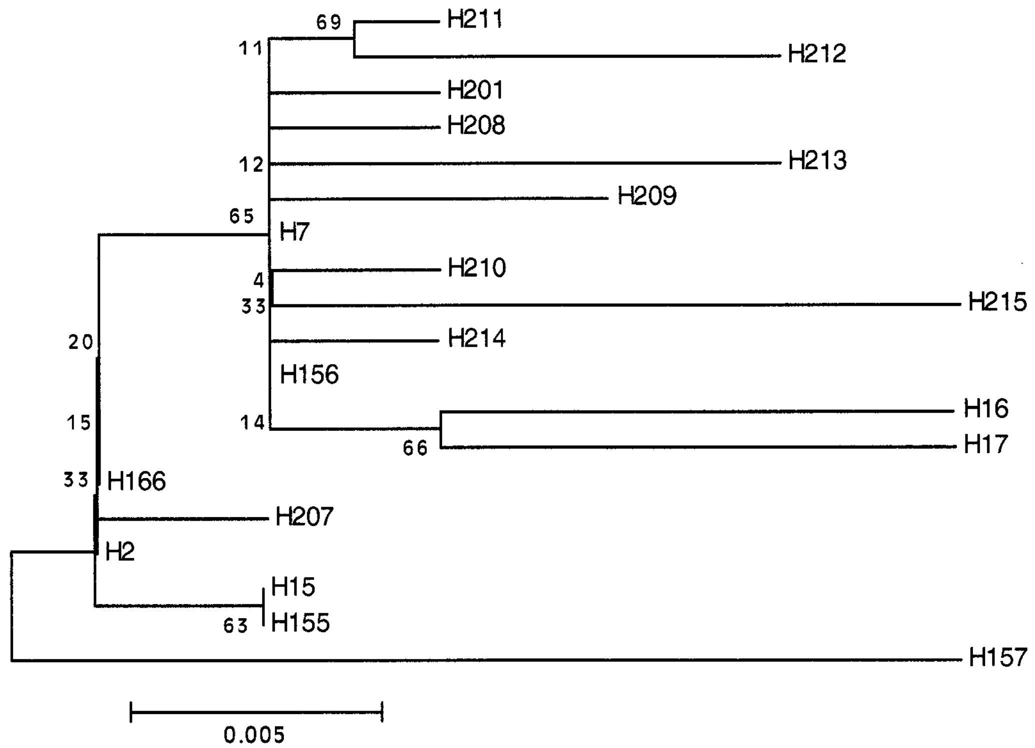


Figure 12: The MEGA UPGMA tree relating all the haplotypes obtained in the collection year 1998. Tree constructed based on the model of Tamura-Nei (1993). Numbers at specific nodes represent bootstrap values after 500 replicates. The scale bar represents divergence using Tamura-Nei correction.

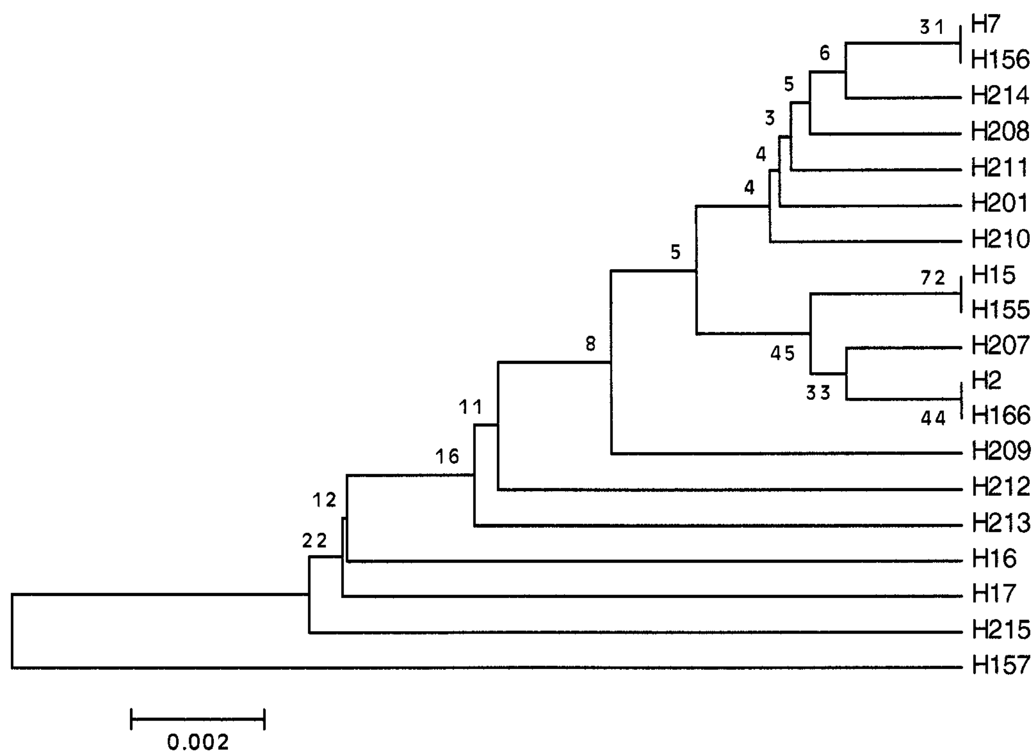


Figure 13: The MEGA NJ tree relating all the haplotypes obtained in the collection year 1999. Tree constructed based on the model of Tamura-Nei (1993). Numbers at specific nodes represent bootstrap values after 500 replicates. The scale bar represents divergence using Tamura-Nei correction.

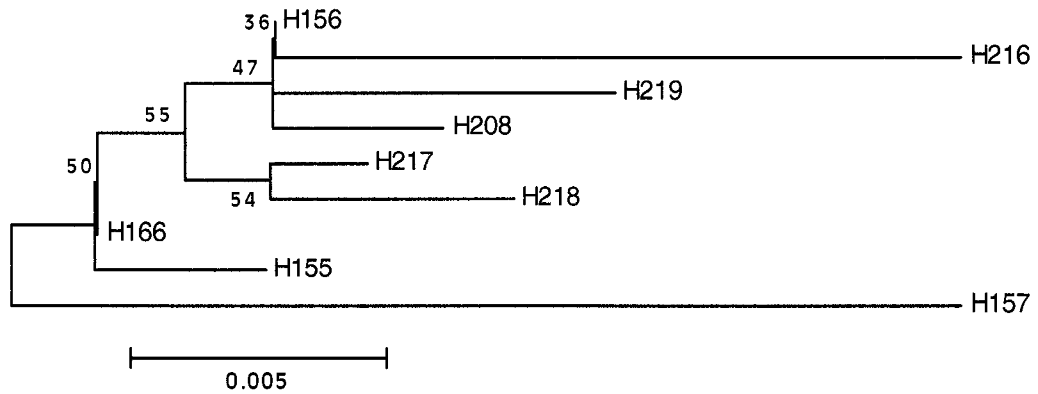
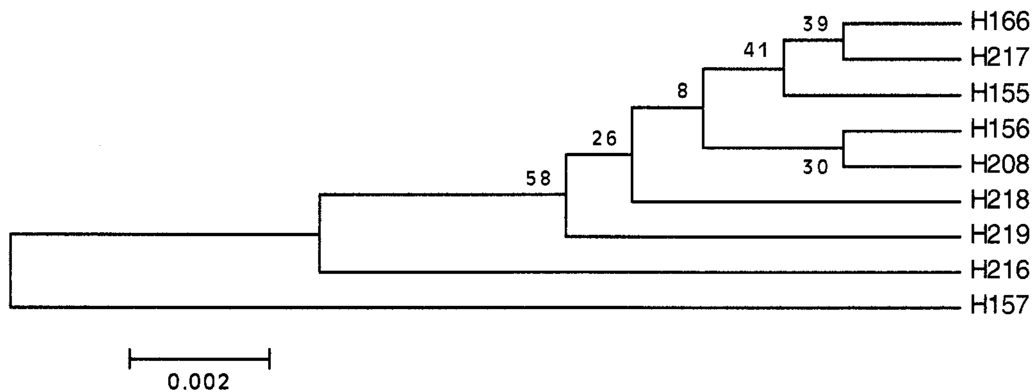


Figure 14: The MEGA UPGMA tree relating all the haplotypes obtained in the collection year 1999. Tree constructed based on the model of Tamura-Nei (1993). Numbers at specific nodes represent bootstrap values after 500 replicates. The scale bar represents divergence using Tamura-Nei correction.



The year 1999 showed the haplotypes H155, H156, and H157 to be on three different maternal lineages.

Figure 15: The MEGA NJ tree relating all the haplotypes obtained in the collection year 2001. Tree constructed based on the model of Tamura-Nei (1993). Numbers at specific nodes represent bootstrap values after 500 replicates. The scale bar represents divergence using Tamura-Nei correction.

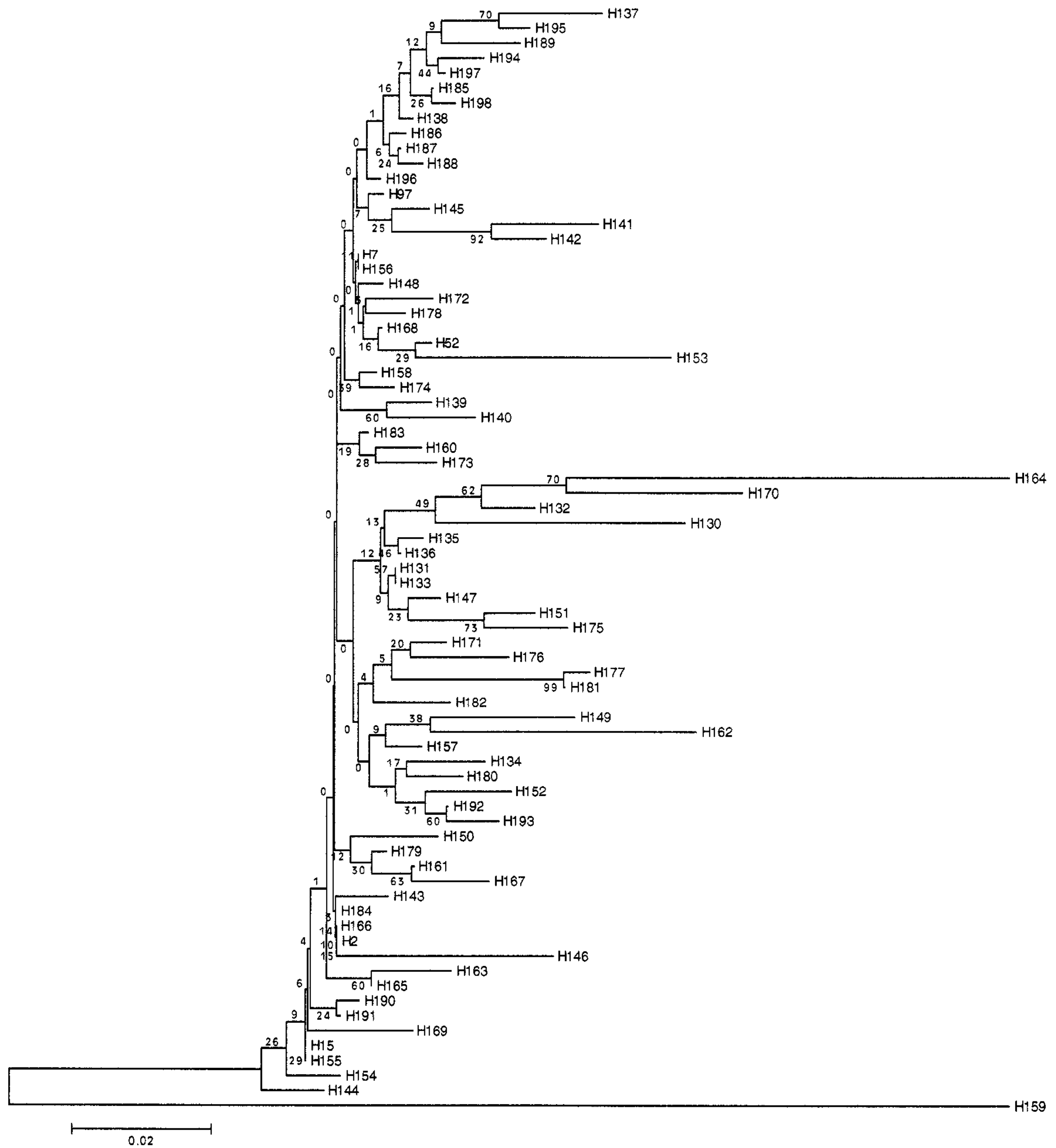


Figure 16: The MEGA UPGMA tree relating all the haplotypes obtained in the collection year 2001. Tree constructed based on the model of Tamura-Nei (1993). Numbers at specific nodes represent bootstrap values after 500 replicates. The scale bar represents divergence using Tamura-Nei correction.

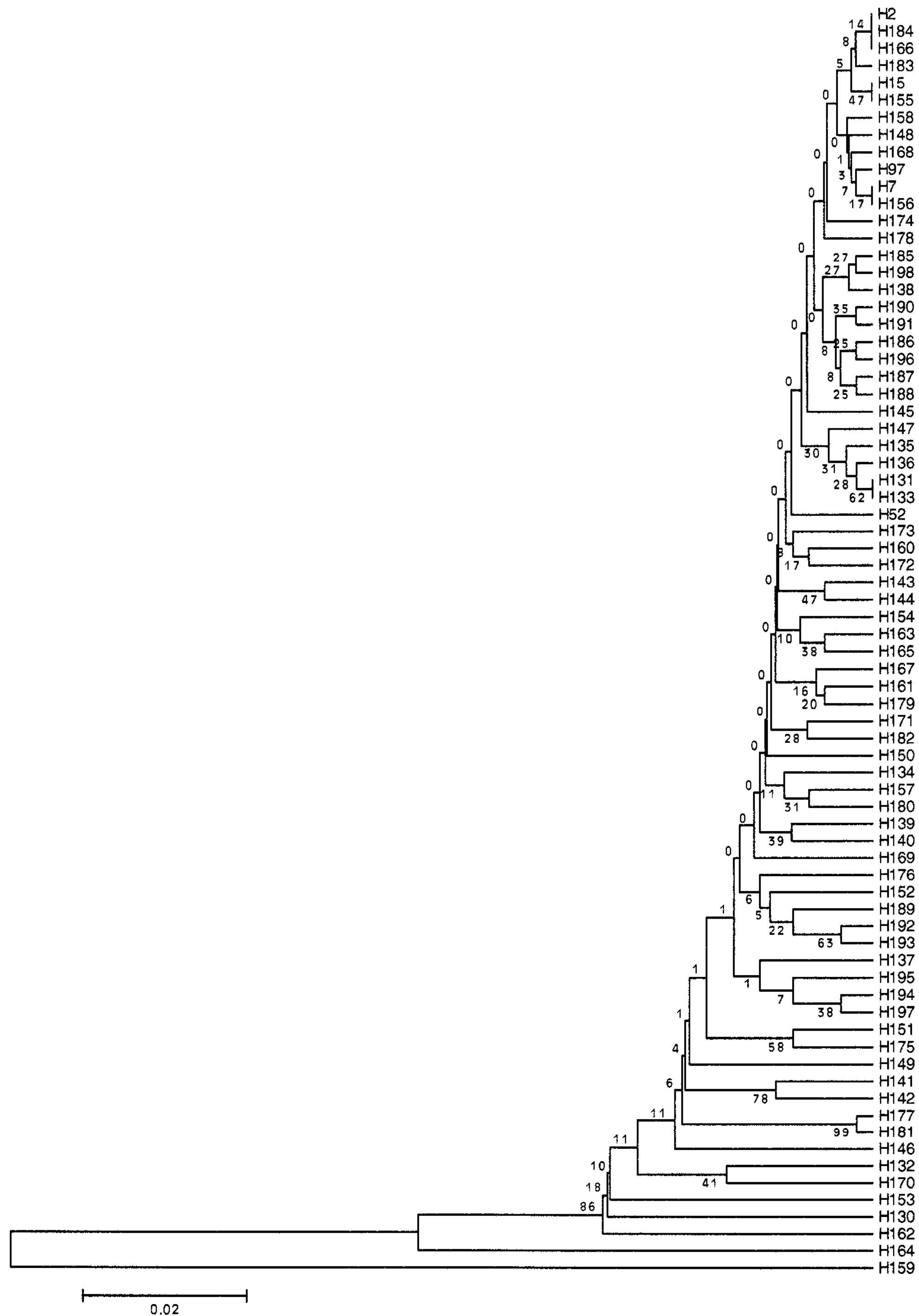
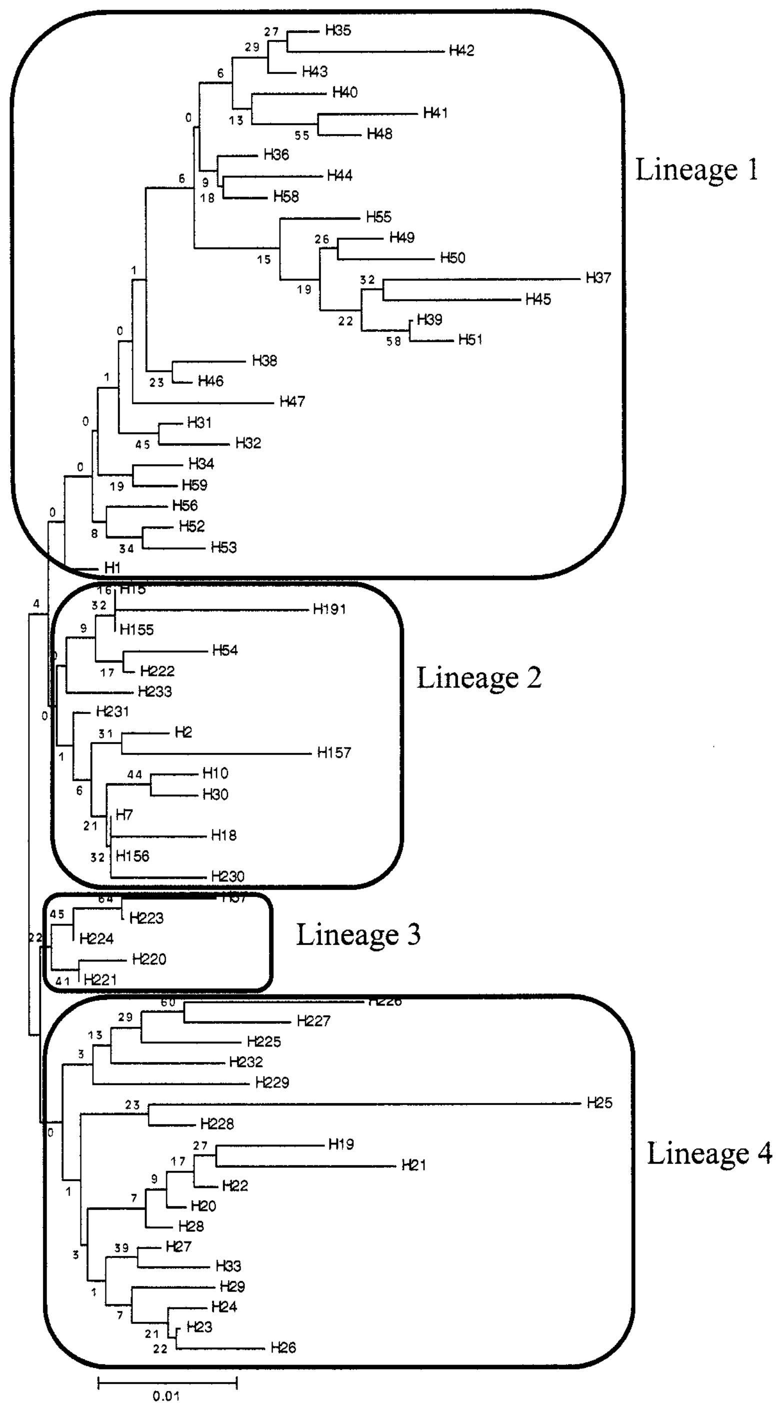


Figure 17: The MEGA NJ tree relating all the haplotypes obtained in the collection year 2003. Tree constructed based on the model of Tamura-Nei (1993). Numbers at specific nodes represent bootstrap values after 500 replicates. The scale bar represents divergence using Tamura-Nei correction.



Four mitochondrial lineages were designated.

Figure 18: The MEGA UPGMA tree relating all the haplotypes obtained in the collection year 2003. Tree constructed based on the model of Tamura-Nei (1993). Numbers at specific nodes represent bootstrap values after 500 replicates. The scale bar represents divergence using Tamura-Nei correction.

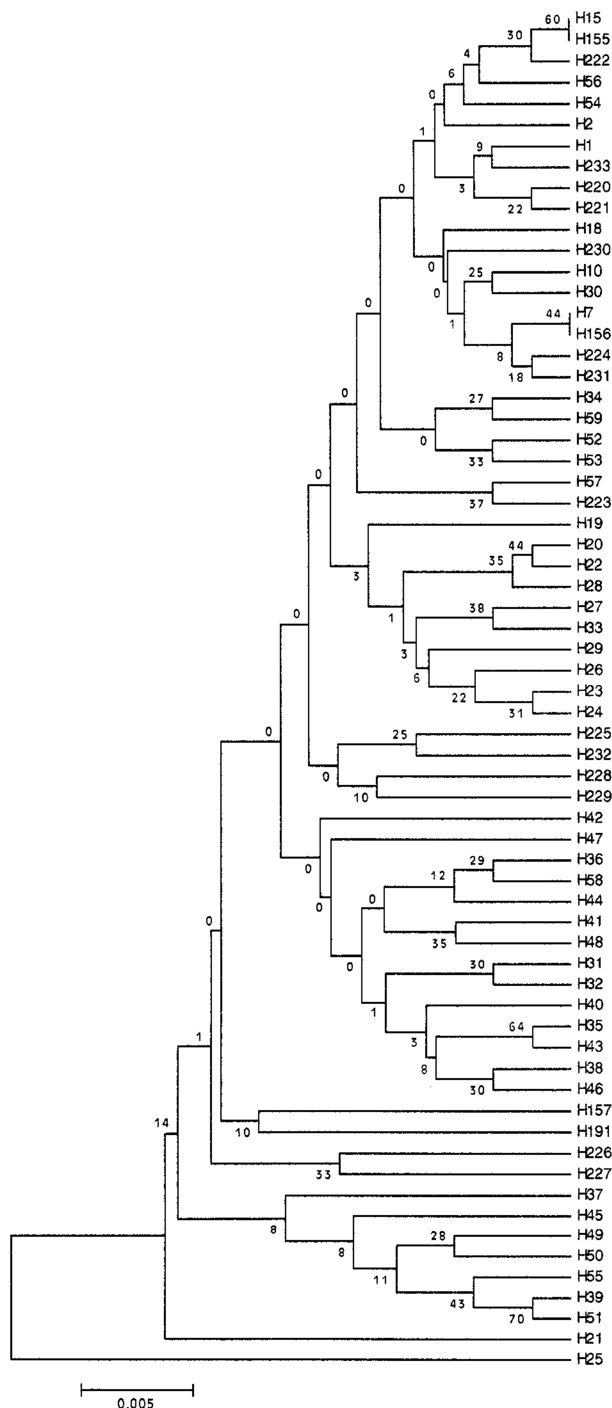
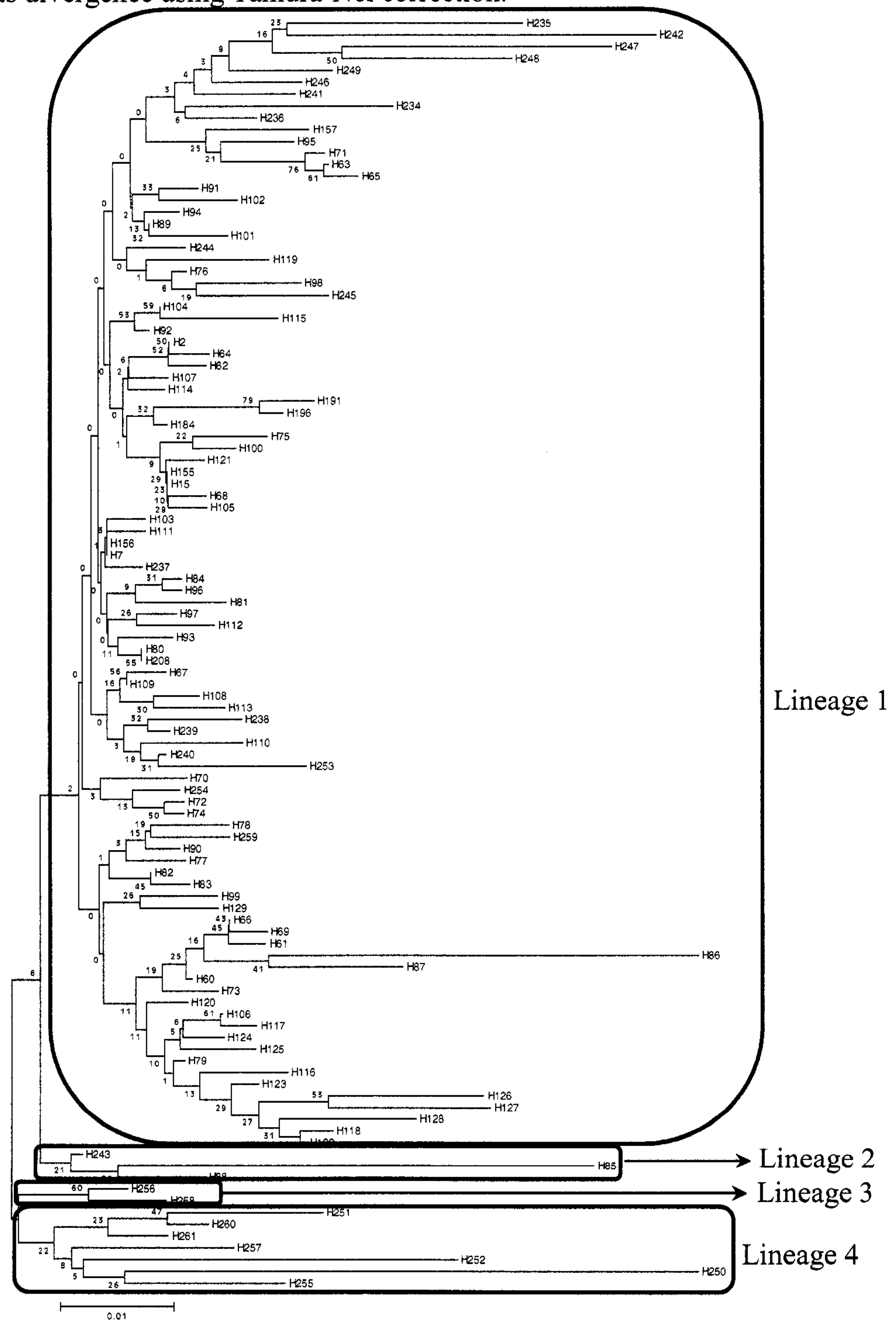
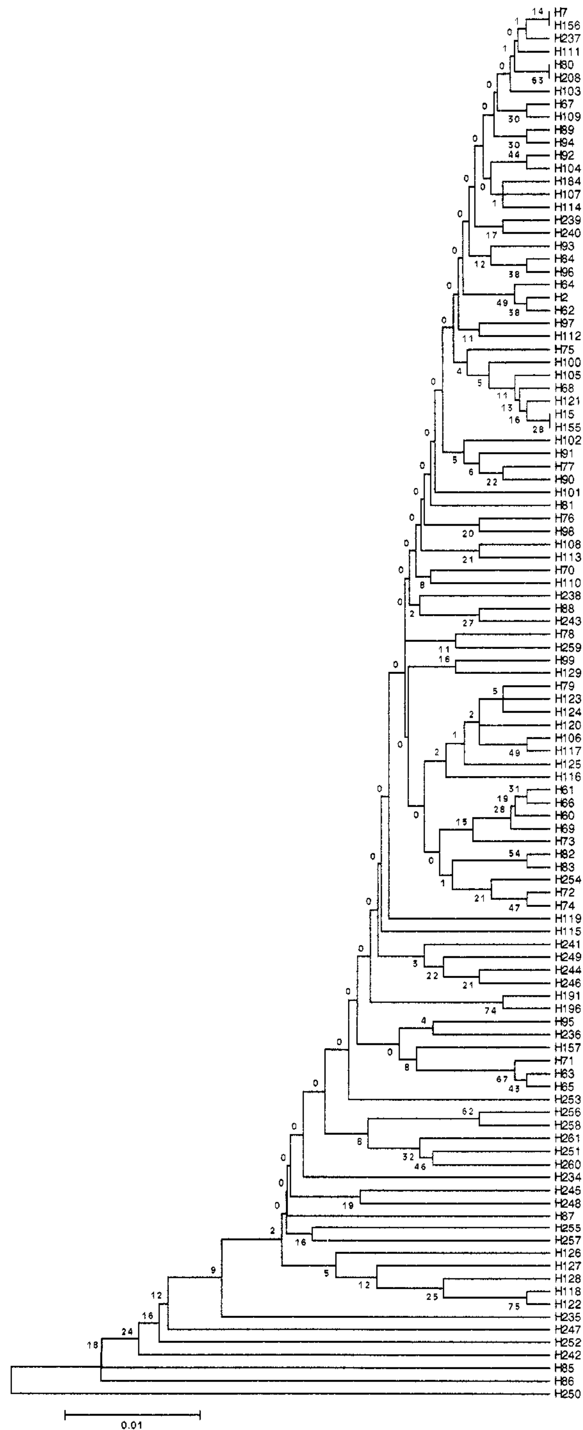


Figure 19: The MEGA NJ tree relating all the haplotypes obtained in the collection year 2004. Tree constructed based on the model of Tamura-Nei (1993). Numbers at specific nodes represent bootstrap values after 500 replicates. The scale bar represents divergence using Tamura-Nei correction.



Four mitochondrial lineages were designated.

Figure 20: The MEGA UPGMA tree relating all the haplotypes obtained in the collection year 2004. Tree constructed based on the model of Tamura-Nei (1993). Numbers at specific nodes represent bootstrap values after 500 replicates. The scale bar represents divergence using Tamura-Nei correction.



The haplotypes H2 and H7 were seen closer to the base of the tree. Moreover, H2 and H7 were on the same lineage as seen in the NJ tree from the year 2004. The lineage 1 in the NJ tree from the year 2004 was relatively larger with the accumulation of most of the singletons on it. H8, H9, H16, H17, H25, H37, H86, H157, H159, H164, H199, H215, H216, H242, and H250 were found to be the most derived haplotypes.

Table 9: The genetic distances within the 1997 Coos Bay adults, 1997 Bodega Bay adults, 1997 Juneau adults, 1998 Newport adults, 2002 Puget Sound adults, 2003 Coos Bay adults, 2003 Coos Bay megalopae, 2004 Fort Bragg adults, 2004 San Francisco adults, 2004 Columbia River adults, 2004 Columbia River juveniles, 2004 Columbia River megalopae, and 2004 Coos Bay megalopae.

Grouping	Genetic distance within the population
1997 Coos Bay Adults	0.0162
1997 Bodega Bay Adults	0.0197
1997 Juneau Adults	0.0264
1998 Newport Adults	0.0180
2002 Puget Sound Adults	0.0131
2003 Coos Bay Adults	0.0195
2003 Coos Bay Megalopae	0.0194
2004 Fort Bragg Adults	0.0146
2004 San Francisco Adults	0.0193
2004 Columbia River Adults	0.0537
2004 Columbia River Juveniles	0.0085
2004 Columbia River Megalopae	0.0084
2004 Coos Bay Megalopae	0.0164

Genetic distance was greatest within adults collected in 2004 from Columbia River and lowest genetic distance was observed within megalopae collected in 2004 from Columbia River.

Table 10: The genetic distances between the 1997 Coos Bay adults, 1997 Bodega Bay adults, 1997 Juneau adults, 1998 Newport adults, 2002 Puget Sound adults, 2003 Coos Bay adults, 2003 Coos Bay megalopae, 2004 Fort Bragg adults, 2004 San Francisco adults, 2004 Columbia River adults, 2004 Columbia River juveniles, 2004 Columbia River megalopae, and 2004 Coos Bay megalopae.

Year Site Life stage	97 CB A	97 BB A	97 JN A	98 NP A	02 PS A	03 CB A	03 CB M	04 FB A	04 SF A	04 CR A	04 CR J	04 CR M	04 CB M
97 CB A	-												
97 BB A	0.0186	-											
97 JN A	0.0221	0.0231	-										
98 NP A	0.0169	0.0198	0.0245	-									
02 PS A	0.0145	0.0166	0.0212	0.0148	-								
03 CB A	0.0218	0.0212	0.0256	0.0219	0.0185	-							
03 CB M	0.0217	0.0230	0.0281	0.0223	0.0194	0.0236	-						
04 FB A	0.0164	0.0175	0.0231	0.0162	0.0136	0.0192	0.0215	-					
04 SF A	0.0213	0.0237	0.0287	0.0198	0.0180	0.0231	0.0250	0.0179	-				
04 CR A	0.0433	0.0427	0.0453	0.0453	0.0415	0.0411	0.0454	0.0422	0.0464	-			
04 CR J	0.0140	0.0150	0.0216	0.0133	0.0111	0.0185	0.0203	0.0119	0.0159	0.0417	-		
04 CR M	0.0143	0.0158	0.0226	0.0137	0.0110	0.0186	0.0205	0.0121	0.0162	0.0420	0.0088	-	
04 CB M	0.0183	0.0219	0.0266	0.0174	0.0155	0.0225	0.0239	0.0170	0.0199	0.0457	0.0141	0.0137	-

The greatest genetic distance was found between populations of 2004 Columbia River adults and 2004 San Francisco adults whereas the lowest genetic distance was found between populations of 2004 Columbia River megalopae and 2002 Puget Sound adults.

DISCUSSION

The overall sequence diversity of the analyzed samples of Dungeness crabs was high. Nucleotide diversity ranged between 0.8% and 5.1% and haplotype diversity ranged from 70.0% to 100.0%. The Chi-square values denoted that there were many significant polymorphic sites present thus showing the presence of significant genetic variation. Bunch *et al.* (1998) measured high haplotype diversity (29.4% to 93.3%) in Tanner crab populations within the Gulf of Alaska using the COI gene and related the pattern of genetic distinctness to the larval transport. Rocha-Olivares and Vetter (1998) compared the mtDNA sequences of rosethorn rockfish populations from the coasts of California, Oregon, British Columbia and the Bearing Sea. They observed high levels of nucleotide diversity (0.3% to 1.17%) and high haplotype diversity (80% to 96%). Exceedingly high levels of diversity were also found for the marine snail *Nucella lapillus* from the coastline of South Devon (Kirby *et al.* 1997). Avise (1994) reported that in rapidly evolving genomes such as mtDNA COI, haplotype diversity could sometimes even reach 100%.

Large numbers of singletons were initially unexpected in a small population (Boom *et al.* 1994). However, in the current study as the sample sizes began to increase, the presence of a large number of singletons became evident. This large number of singletons indicated rapid mutation of the COI gene (Beckenbach 1994).

Other studies have also reported the presence of large numbers of singletons in marine organisms. While studying genetic heterogeneity among larvae of Pacific oysters

in Dabob Bay, Li and Hedgecock (1998) observed that over 70% of the haplotypes were singletons. McMillen-Jackson *et al.* (2004) reported that 79% of the haplotypes identified in a population of Blue crabs along the east coast of United States were singletons. Genetic diversity they obtained was notably high; nucleotide diversities were in the range of 1.0% to 2.0%. Douglas *et al.* (2003) studied Flannelmouth Sucker, *Catostomus latipinnis*. They found only 49 unique haplotypes, 53% of which represented single individuals.

Such high numbers of singletons could be due to rapid mutation of the COI gene. Perhaps these mutations were not passing from the generation of origin to the next generation. Though this phenomenon might be simply due to the relatively small sample size compared to large population sizes due to a sampling error. There is a possibility that each haplotype would have resulted from a new mutation transmitted locally to a small number of individuals. Depending on the transport rate from a spawning area, the haplotypes might not have had enough time to establish themselves in these populations.

Significantly negative Fu's Fs values indicated recent population expansion following genetic bottleneck. Such large Fs values could also indicate recombination (Fu 1997), but the mitochondrial genome does not undergo recombination events, so one can expect the presence of linkage disequilibrium with recent origins.

It is likely that the population expansion coincided with the last Ice Age, which started about 70,000 years ago and ended about 10,000 years ago (Wilson 1964). During the last glaciation event the sea level dropped to 100-160 meters worldwide (Fairbridge

1966). During this time the Oregon coast had no continental shelf or shallow estuaries. This would have caused a dramatic decline in the Oregon crab populations (a genetic bottleneck). The population expansion likely occurred once the sea levels had risen to the current levels, re-established the shelf, current bays and estuaries. The Alaskan coastlines are shallower and the continental shelves are wider which would have maintained the bays and the estuaries even with the lower sea levels (Jacobs and Schagian 1993).

Paleontological research (Nations 1975, 1979) suggested that the genus *Cancer* originated in the North Pacific about 20-25 million years ago. Harrison and Crespi (1999b) used a cytochrome *c* oxidase subunit I-based time-scale to show that the majority of the diversification within the genus occurred about five million years ago, the Pacific species was the basal taxa. *Cancer* crabs invaded the Atlantic from the north Pacific, six to twelve million years ago. From the north Pacific, *Cancer* species diversified quite rapidly, expanding their range southwards in the evolutionary time. The species diversified quite rapidly, dispersing south along the north and south American coasts, west towards Japan, and north across the Arctic into the Atlantic Ocean.

Fu's Fs and Fu and Li's D and F tests suggested that the populations were at mutation-migration-drift genetic disequilibrium with respect to mtDNA alleles suggesting the occurrence of selection. This might be due to some of the sequences of the cytochrome *c* oxidase protein conferring a selective advantage (Fu 1997). Cytochrome *c* oxidase is a protein found in the inner membrane of the mitochondria. It is the terminal

enzyme used in the oxidative phosphorylation of many respiratory chains. It is a respiratory enzyme catalyzing the reduction of molecular oxygen to water. The system catalyzes the final electron transfer steps from cytochrome *c* to molecular oxygen (Voet and Voet 2004).

Estuaries and tide pools have lower oxygen levels as the water warms in the summer. Dr. Roegner and Dr. Shanks (personal communication) observed that many juvenile crabs died in the warm tidal pools when the tide was low in the summer of the year 2004. Low oxygen levels could act to select for or against some COI gene variants (haplotypes). Reduction of the genetic variation in the juveniles as compared to the adult populations from the year 2004 collected from Columbia River was observed. This suggested that surviving young-of-the-year were products of only a small fraction of the adult population.

If there were no selective advantage, the juvenile and the adult populations would actually represent a mixture of the megalopae recruited continuously during the year 2004 in Columbia River. But it was found that the megalopal frequencies did not accurately predict the juvenile or the adult frequencies. One possible reason could be that the sporadic major recruitment events would have occurred along with low continuous recruitments, with major pulses having great effects on the frequencies (Kordos and Burton 1993). Pre- and post- settlement natural selection due to the selective advantage for some of the COI sequences or large variances in the reproductive success (sweepstakes recruitment) could also be the possible causes of this discrepancy in the

frequencies. The variances in the time and the place of spawning might have caused random variation in the portion of the adult population that produced the surviving offspring, and hence variation in the genetic composition of recruits seen in a chaotic genetic patchiness (Li and Hedgecock 1998; Hedgecock 1994a,b). When this happens, populations a few miles apart may be genetically different by as much as those hundreds of miles apart.

Julian (1996) found reduced genetic variability in the late pelagic juveniles of shortbelly rockfish relative to both the adults and the larvae off Central California, thus supporting that the recruits usually show a smaller degree of genetic variation than the adult population as a result of the "instantaneous drift" experienced between spawning and the establishment of the year class.

Seven common haplotypes, H2 (9%), H7 (12%), H10 (1%), H15 (3%), H155 (4%), H156 (11%), and H157 (10%) were found consistently over the six years of sample collection. They were present in large proportions in the Oregon populations as compared to the Californian and Alaskan populations. They were called the "Oregon local haplotypes." These commonly occurring haplotypes were the offspring of locally successful reproducing adults. In the future, these "signature" haplotypes might serve to identify the crabs from the Oregonian regions. Two Oregon local haplotypes (H2 and H7) were found in Juneau, Alaska. H2 (9%) and H7 (12%) were found in almost all the populations and in relatively large numbers. In general, these two haplotypes, H2 and H7 may be abundant in Dungeness populations. One of the common Oregon haplotypes

(H15) was found in the individuals sampled from Fort Bragg, California. However, more samples will be needed from Alaska and California to confirm the numbers of such shared haplotypes. If the low number of the shared haplotype is maintained with larger sample sizes, local haplotype frequencies might serve, as a biogeographical indicator thus would help to determine the source of the megalopal haplotypes in the future.

This indicated that the late arriving (August through September) megalopae in Coos Bay might be arriving from the northern waters where the larval developmental time is slower. The months of August and September are the normal recruiting times in Alaska (Moloney *et al.* 1994). Studies have shown interannual variation in the timing of the recruitment pulses where megalopae have been sampled in Coos Bay as late as November (Roegner *et al.* 1999). It was thought that this might have some relation with the tidal period or differences in temperatures between estuary and nearshore waters suggesting a synoptic regional forcing or sometimes even due to reversals in the mean southward surface currents (Roegner *et al.* 1999).

These two haplotypes, H2 and H7, were found to be very close to the base of the trees and were also seen to be on a single maternal lineage. The species are related by descent from a common ancestor. The root of the trees represented the common ancestor. The rooted UPGMA trees identified where the lineage originated and thus established the order in which the divergence events would have occurred. Again, this was evidence that H2 and H7 seemed to be the ancestral haplotypes.

Shifts in the haplotype patterns across various years of collections and locations were observed. Temporal variation in the megalopae arriving at Coos Bay throughout the study sampling years was observed. This might be a result of the population differences in the spawning season or developmental times or even perhaps from the changes in the coastal current patterns (Kordos and Burton 1993). The El Niño-Southern Oscillation is an interannual phenomenon dominated by the weak upwelling and warmer surface temperatures in the equatorial Pacific Ocean, which occurs every four to seven years (Trenberth and Caron 2000). El Niño is followed by La Niña, which is dominated by the strong upwelling and low temperatures. During El Niño, the warm water moves north and the offshore water moves towards the coast. Studies have shown that the megalopae collected during the year 1997 (neutral year) were likely from the local sources. The megalopae collected during the year 1998 (El Niño year) were from southern populations and the megalopae collected during the year 1999 (La Niña year) were the ones that had a reduced transport to the collection site (Chockalingam 2004).

The presence of the unique haplotypes (H19, H20, H21, H22, H23, H24, H25, H26, H27, and H28) in the adults collected in the year 2003 from Coos Bay suggested that the megalopae of these adults might be dispersing to some other location or these adults might not be successfully reproducing.

Spatial variation was seen in the year 2004 between Coos Bay and Columbia River. The presence of H15 in Coos Bay but not in Columbia River indicated an occurrence of clinal variation (the gradual and continuous geographical variation

observed within a species). Previously it was thought unusual to find a cline in invertebrate species with a high dispersal potential. However, Sotka *et al.* (2004) found a genetic cline between the populations of acorn barnacle *Balanus glandula* across the regions of central California. They concluded that the cline would have been produced when the populations separated in the evolutionary time and diverged by drift or local selection. This could have happened at the height of the last Ice Age when sections of the North American coastlines that are currently connected were isolated. Similarly in the present study, when the glaciers would have receded, the Columbia River population would have expanded along the coast to Coos Bay to produce clinal genetic variation. Strong selection along with the high dispersal potential could together maintain the cline (Sotka *et al.* 2004). A similar study was reported for the American oyster *Cassostrea virginica* across the phylogeographical break at Cape Canaveral, Florida (Hare and Avise 1996).

High Fst values (greater than 0.2) were seen between the 2004 Fort Bragg adults and the 2004 San Francisco adults and between the 2004 San Francisco adults and the 1997 Juneau adults. The samples from Fort Bragg, California, shared one haplotype (H15) with the Oregonian samples. Therefore, at least one marine reserve was recommended to be designed in California so that the long distance dispersing organisms from Oregon could settle in this Californian reserve. However, more samples will be needed from California and Alaska to suggest effective and sound reserve designs across the entire Pacific coast.

Fst values of 0 between the 2002 Puget Sound adults and the 1997 Bodega Bay adults indicated that a single reserve would be sufficient to accommodate these two populations. Fst values between 0-0.1 indicated a lot of migration, thus no population divergence, probably because of lots of gene flow. Fst values between 0.1-0.2 indicated a reduction in gene flow that would have allowed the accumulation of many allele frequency differences but few if any fixed allelic differences. Fst values greater than 0.2 between the populations such as the 2003 Coos Bay megalopae and the 2004 Fort Bragg adults, the 2004 Columbia River megalopae and the 2004 San Francisco adults, the 2004 Coos Bay megalopae and the 2004 San Francisco adults indicated population isolation between these groups thus allowing the accumulation of fixed allelic differences. Following such population isolations, the random genetic drift might enhance genetic differentiation especially in the populations with small sizes (Sarre 1995).

The current study suggested the establishment of distinct marine reserves in Coos Bay and Columbia River. High Fst values (greater than 0.2) were observed between the 2003 Coos Bay megalopae and the 2004 Coos Bay megalopae, between the 2003 Coos Bay megalopae and the 2004 Columbia River megalopae, between the 2003 Coos Bay megalopae and the 2004 Columbia River adults and between the 2004 Coos Bay megalopae and the 2003 Coos Bay adults. This indicated that there was very little gene flow between these populations. Small marine reserves at Coos Bay and Columbia River that can accommodate both these area's populations were suggested.

Significant interpopulation differentiation was seen among the adult populations despite the high potential for the dispersal of larvae and thus adults. This suggested that the interpopulation gene flow was not sufficient to overcome the population differentiation. A reserve is usually designed with an intention to preserve the habitat and environment that allows evolutionary processes to continue in the future, as they existed in the past. Since there was very little gene flow between the populations, a series of small reserves spaced along the Pacific coast of North America might best preserve the dynamics that have led to these distinct populations. The sample sizes were relatively small in some adult populations. Large sample sizes will be needed in future studies to reach a conclusive explanation.

The lineage 1 from the year 2004 was large and most of the singletons were seen to be accumulating in this lineage. The presence of a large number of singletons in this lineage 1 from the year 2004 added further support to a population expansion (Rand 1996). This lineage contained the closely related haplotypes that were thought to be mutating rapidly as compared to the haplotypes from the distant lineages. The lineage 1 from the year 2004 appeared to have evolved due to the rapid mutation of the COI gene. The presence of only H2 and H7 in Juneau, Alaska indicated that they might be the ancestral haplotypes that have now established themselves in Oregon. The northern populations would have expanded along the coast and would have dispersed southwards in the evolutionary time. Thus the presence of the haplotypes H2 and H7 could either be due to the range expansion if these were the ancestral haplotypes or this could be due to these haplotypes recruiting late into the estuaries from the northern waters in the

contemporary time. The UPGMA phylogenetic trees showed that the mtDNA haplotypes lacked the deep phylogenetic divergences observed in other animals (Avise 1994). This indicated that the variation was recently derived by mutation from a common ancestor.

CONCLUSIONS AND FUTURE RESEARCH GOALS

Cancer magister is a chief crab species taken in the west coast of North America. Adverse environmental effects on *Cancer* crab populations due to the anthropomorphic activities have become a serious concern for fishery management (Dumbauld *et al.* 1993). Commercial fisheries landing of Dungeness crabs have fluctuated in a cyclic manner since the 1940s (Wild 1983). A 9- to 10- year cycle has been observed (Johnson *et al.* 1986). Because of the economic importance of the Dungeness crab fishery, there is a great deal of interest in improving the crab fishery management.

Seven common haplotypes, H2 (9%), H7 (12%), H10 (1%), H15 (3%), H155 (4%), H156 (11%), and H157 (10%) were found consistently over the six years of sample collection. They were present in large proportions in the Oregon populations as compared to the Californian and Alaskan populations. These "Oregon local haplotypes" were the offspring of locally successful reproducing adults. The presence of H2 and H7 in Alaska and evidences from phylogenetic trees revealed that H2 and H7 were the most ancestral haplotypes. H2 and H7 could be the late arriving megalopae in Coos Bay or they might be the ancestral haplotypes that would have dispersed south in the evolutionary time. From the North Pacific, the *Cancer* species dispersed quite rapidly and because of this, the southern populations were seen to be expanding. The presence of a large number of singletons added further support to the population expansion on a broad geographic scale. The populations would have started to expand about 10,000 years ago at the end of the last Ice Age.

The high levels of genetic diversity were due to a recent population expansion or due to the rapid mutation of the COI gene. Excess of recent mutations were present. The natural selection of certain haplotypes, most likely due to the subunit I pocket of the cytochrome *c* oxidase protein conferring the selective advantage was seen.

Based on all the evidences from the current study, the three project hypotheses were accepted.

- It was found that populations of adult *Cancer magister* across the biogeographic range were genetically distinct. Restricted larval transport due to the effects of current patterns, genetic differentiation among the retention areas or pre- and post-settlement natural selection due to the selective advantage of some of the COI sequences might be the likely causes for such an event.
- There was variation in the genetic composition of the settling larvae and the adults. This implied the occurrence of the post-settlement selection or a large variance in the reproductive success of the adults.
- Spatial and temporal genetic distinctness was found among the recruiting larvae of *Cancer magister*. This could implicate factors acting prior to the settlement including the existence of the physical barriers to larval dispersal or the differences in spawning or developmental times.

The present study showed that mtDNA analysis can be important not only for studying the genetic composition of a population, but also for suggesting effective management strategies for that particular species. The current study suggested distinct

marine reserves to be designed in Coos Bay and Columbia River. A reserve was also recommended in California to accommodate the long distance dispersing organisms from Coos Bay.

Tracking individual crabs in the open ocean waters is impractical because of the massive sampling required and the difficulty to quantitatively identify crab larvae on a huge scale (Bunch *et al.* 1998). In marine environments, the distances over which the larvae disperse are usually indirectly inferred. One of the future goals is to determine the origin, route, and transport mechanisms of *Cancer magister* arriving at the west coast of North America. A detailed understanding of dispersal trajectories and the magnitude of larval transport are crucial in understanding the fate of larvae and also the sources and rates of mortality. A variety of methods (Ennevor and Beames 1993; Levin *et al.* 1993; Kennedy *et al.* 1997; Anastasia *et al.* 1998) have been proposed for tagging and thus tracking marine larvae such as the use of trace elements and radiotracers (Levin 1990).

A second goal is to continue to determine the genetic differentiation within and between populations of *Cancer magister* across the geographic range. More samples will be needed from California and Alaska to reach a conclusive explanation and to be able to suggest effective management strategies. Moreover, besides expanding megalopal and adult studies, collections of zoeal stages (Shirley *et al.* 1987) can also be subjected to genetic analysis.

Although the life cycle of Dungeness crabs requires that large scale studies be used to track dispersal and recruitment patterns, genetic analysis continues to hold promise in revealing the structure of these populations.

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