UNIVERSITY OF CALIFORNIA, SAN DIEGO

Conservation genetics of California abalone species

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Marine Biology

by

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The dissertation of Kristen Marie Gruenthal is approved, and is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

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DEDICATION

For my Mom and Dad

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My education in population and conservation genetics at Scripps Institution of Oceanography (SIO) is largely due to the tutelage of my doctoral dissertation advisor, Professor Ronald S. Burton. Before arriving at SIO, I only had knowledge of human disease genetics and no education in my future chosen field of study. It has been only through Ron's patience and support and expertise in population genetic theory that I have been able to accomplish my educational goals. Ron also generously provided me with research funding and California Sea Grant traineeships throughout my graduate career.

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Chapter III was done in collaboration with Lauren K. Acheson, a former undergraduate student at the University of California, San Diego, and current Masters student in the College of Landscape Architecture at the University of Washington. The dissertation author was the primary investigator and author of this paper. Chapter III in full is a reprint of the material as it appears in Gruenthal KM, Acheson LK, Burton RS (2007) Genetic structure of natural populations of California red abalone (*Haliotis rufescens*) using multiple genetic markers. *Marine Biology*, DOI 10.1007/s00227-007-0771-4. *Marine Biology* is © by Springer/Kluwer Academic Publishers. Reprinting was granted with kind permission of Springer Science and Business Media.

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PUBLICATIONS

Gruenthal KM, Acheson LK, Burton RS (2007) Genetic structure of natural populations of California red abalone (*Haliotis rufescens*) using multiple genetic markers. *Marine Biology*, DOI 10.1007/s00227-007-0771-4

Demissie S, Cupples LA, Shearman AM, **Gruenthal KM**, Peter I, Schmid CH, Karas RH, Housman DE, Mendelsohn ME, Ordovas JM (2006) Estrogen receptor-alpha variants are associated with lipoprotein size distribution and particle levels in women: The Framingham Heart Study. *Atherosclerosis*, 185, 210-218

Gruenthal KM, Burton RS (2005) Genetic diversity and species identification in the endangered white abalone (*Haliotis sorenseni*). *Conservation Genetics*, 6, 929-939

Shearman AM, Karasik D, **Gruenthal KM**, Demissie S, Cupples LA, Housman DE, Kiel DP (2004) Estrogen receptor beta polymorphisms are associated with bone mass in women and men: the Framingham Study. *Journal of Bone and Mineral Research*, 19, 773-781

Karasik D, Shearman AM, Cupples LA, Demissie S, **Gruenthal K**, Housman D, Kiel DP (2003) Association of aromatase gene polymorphisms with bone mineral density in men and women: the Framingham Offspring Study. *Journal of Bone and Mineral Research*, 18, S325

Shearman A, Karasik D, Cupples LA, Demissie S, Coven S, **Gruenthal K**, Housman DE, Kiel DP (2003) Smoking influences the association between estrogen receptor alpha gene polymorphisms and bone mineral density: a gene-environment interaction in the Framingham Offspring Study. *Journal of Bone and Mineral Research*, 18, S52

Shearman A, Karasik D, Cupples LA, Coven S, **Gruenthal K**, Housman DE, Kiel DP (2003) Smoking status and estrogen receptor genotype show significant interaction with bone mineral density in the Framingham Offspring Study. *American Journal of Human Genetics*, 73, 1988

Karasik D, Shearman AM, Cupples LA, **Gruenthal K**, Housman D, Kiel DP (2002). Intronic polymorphisms of the estrogen receptor beta gene are associated with bone mineral density: the Framingham Study. *Journal of Bone and Mineral Research*, 17, M122

PUBLICATIONS continued

Samowitz W, Holden J, Curtin K, Edwards S, Walker A, Lin J, Robertson M, Nichols M, **Gruenthal K**, Lynch B, Leppert M, Slattery M (2001) Inverse relationship between microsatellite instability and K-ras and p53 gene alteration in colon cancer. *American Journal of Pathology*, 158, 1517-1524

Samowitz W, Curtin K, Lin H, Robertson M, Schaffer D, Nichols M, **Gruenthal K**, Leppert M, Slattery M (2001) The colon cancer burden of genetically defined hereditary nonpolyposis colon cancer. *Gastroenterology*, 121, 830-838

Peiffer A, Thompson J, Charlier C, Otterud B, Varvil T, Pappas C, Barnitz C, **Gruenthal K**, Kuhn R, Leppert M (1999) A locus for febrile seizures (FEB3) maps to chromosome 2q23-24. *Annals of Neurology*, 46, 671-678

ABSTRACT OF THE DISSERTATION

Conservation genetics of California abalone species

by

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Doctor of Philosophy in Marine Biology

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Over the past three decades, five species of abalone (genus *Haliotis*) in California waters have gone from harvestable resources to rare or even endangered species status. Both anthropogenic (overexploitation, habitat destruction) and natural (disease, predation) factors have contributed to the declines. This dissertation explores several aspects of abalone recovery and conservation along the coast of California through population genetic theory and techniques. Chapters I and II deal with the captive breeding of two California abalone species. Chapter I focuses on ensuring the specific purity of broodstock and maintaining genetic diversity in hatchery-bred progeny of the endangered white abalone (*H. soreseni*). Using DNA from these animals, genetic markers were developed, including five nuclear microsatellite loci and partial sequences of one nuclear (VERL) and two mitochondrial (COI and CytB) genes, to assess genetic variability in the species, aid in species identification (ergo prevent broodstock contamination), and potentially track the success of future outplanting of captive-reared animals in restocking operations. Chapter II quantifies the loss of genetic diversity

resulting from the captive breeding of green abalone (*H. fulgens*). No change in overall heterozygosity was evident, but significant losses in allelic richness were found in the captive-bred green abalone versus that in wild populations. Chapters III, IV, and V center on inferring realized connectivity (gene flow) among natural populations of red (*H. rufescens*), black (*H. cracherodii*), and pink (*H. corrugata*) abalone. For red abalone (Chapter III), COI sequencing and microsatellite genotyping did not show significant genetic divergence among populations. In contrast, data from AFLPs became the first to suggest there is significant genetic differentiation among California red abalone populations. In black abalone (Chapter IV), data from AFLPs and one microsatellite locus showed significant divergence among multiple populations and exhibited a signal of isolation by distance consistent with a stepping-stone model of connectivity. Pink abalone (Chapter V) also showed evidence of restricted gene flow among populations. Finally, Chapters VI and VII focus on the fatal disease withering syndrome (WS). Chapter VI involves characterizing the post-esophageal microbiomes of red and black abalone with and without WS. Significant differences between the clone libraries isolated from healthy and diseased abalone suggested that WS has significant impact on the bacterial composition of the abalone post-esophageal microbiome, and specific membership of the microbiomes suggests that infection by Candidatus *Xenohaliotis californiensis*, the etiological agent of WS, may not be the sole cause of morbidity and mortality due to WS in abalone. Chapter VII is a preliminary study of genetic variation within C. *X. californiensis* samples from wild and captive-bred abalone species collected from multiple locations along the coast of California. Thus far, no variation has been found in partial sequences of either the 16S rDNA or recombinase A (recA) genes.

INTRODUCTION

The application of genetics to the decline of California abalone species

Abalone are marine gastropods in the genus *Haliotis* found in temperate and tropical rocky macroalgal habitats worldwide (Geiger 2000). All haliotids share particular life-history characteristics that maximize reproductive capacity, survival, and dispersal without parental care in the marine environment. Reproductively mature abalone broadcast spawn, releasing millions of gametes *en masse* into the water column during a seasonally synchronized event. Fertilization and early development is followed by free-swimming trochophore and veliger larval phases lasting four to 15 days (Leighton 2000). Although the free-swimming larval period is short and the larvae are lecithotrophic, high connectivity between widely geographically separated populations is not impossible. Ocean currents and the lack of major oceanographic barriers have been implicated in maintaining dispersal among populations of marine organisms over long geographic distances (Waples 1998).

Abalone species found off California, USA, are representative of a paradox, however, as these life-history traits do not equate to high realized survival rates. Intuitively, severely depleted species do not spawn seasonally once reproductive maturity is reached, theoretically produce millions of viable offspring per spawning event, or have long-distance dispersal potential. However, over the past three decades, red (*H. rufescens*), black (*H. cracherodii*), pink (*H. corrugata*), green (*H. fulgens*), and white (*H. sorenseni*) abalone in southern California waters have gone from harvestable resources to rare or even endangered species status (CDFG 2005).

There are circumstances external to life-history confounding California abalone survival and successful reproduction, however. Both anthropogenic (overexploitation, habitat destruction) and natural (disease, predation) factors have contributed to the

declines (CDFG 2005). Overexploitation during the latter half of last century led to severe depletion of abalone abundance (Hobday and Tegner 2000; CDFG 2005), and serial fishery crashes in all five formerly commercially-fished abalone species resulted in a moratorium on the fishery enacted by the California Department of Fish and Game in 1997 (CDFG 2005). White abalone abundances were so reduced by overfishing that the species was added to the US Fish and Wildlife Service's Endangered Species list in 2001, the list's first marine invertebrate (Hobday and Tegner 2000). Currently, only a heavily-regulated red abalone sport fishery north of San Francisco Bay remains.

The black abalone, in particular, also saw extremely high mortalities starting in the mid-1980's due to a fatal wasting disease called withering syndrome (Haaker et al. 1992; see Friedman et al. 2000 for a clinical description of the disease and etiological agent). Affected populations were found throughout much of the species' range, which extends from central Baja California, Mexico, to near San Francisco Bay, California, USA (Geiger 2000). Areas in the Channel Islands off the southern California mainland saw mortality rates as high as 97% (Haaker et al. 1992; Friedman et al. 1997; CDFG 2005). In response, the Federal government deemed the black abalone a Species of Concern in 1999 and a candidate for listing as an Endangered Species in June 2006 (see http://www.nmfs.noaa.gov/pr/pdfs/species/black_abalone.pdf).

Critical to the natural persistence and recovery of abalone species is whether decimated populations and formerly occupied habitats receive recruits from remnant populations. Exacerbating straightforward depletion, however, populations may become functionally extinct if within-population densities fall below the minimum number of animals required for successful reproduction (i.e. the Allee effect; Stephens et al. 1999).

Research on a marine invertebrate in California with characteristics similar to abalone (e.g. optimal kelp forest habitat, broadcast-spawning), the red urchin (*Strongylocentrotus franciscanus*), showed that fertilization success is positively correlated with greater numbers and densities of animals within aggregations (Levitan 1991; Levitan et al. 1992) and fertilization rate drops precipitously with increasing current speed, even when the animals are as little as one meter apart (Levitan et al. 1992). Severely impacted species such as white and black abalone or species with high census size but low aggregation densities may not be reproducing effectively enough to maintain or recover the species in current and/or former habitat.

Recent evidence suggests that recruitment failure is indeed occurring in abalone within the state of California. Surveys of juvenile abalone recruiting to abalone recruitment modules (ARMs) and baby abalone recruitment traps (BARTs) in the California Channel Islands have shown that very few juvenile abalone are present (Rogers-Bennett et al. 2004). The recruitment failure is not a function of the inefficacy of the ARMs or BARTs either as these tools have been used successfully for the pinto abalone (*H. kamtschatkana*) in the Queen Charlotte Islands off British Columbia, Canada, for several years (DeFreitas 2003).

If the depletion of California abalone species continues even under the moratorium-mediated cessation of fishery pressure and an unlikely eradication of disease, recovery of locally extirpated populations will only occur if stock enhancement operations are initiated. Although stock enhancement efforts have met with little success both in California (Burton and Tegner 2000; Lapota et al. 2000) and elsewhere (Kristiansen et al. 2000; Gutierrez-Gonzalez and Perez-Enriquez 2005; see also Hilborn

1998; Travis et al. 1998; Lorenzen 2005), such operations would likely need to involve outplanting of hatchery reared animals. This type of active involvement by the Federal and/or California state government in the conservation and recovery of these commercially and ecologically important marine invertebrate species may be the only recourse.

Integral to today's wildlife management and stock enhancement strategies are the tools and techniques of population genetics (Avise 2004). The design of marine reserves, assessing recruitment patterns, and quantifying the success of stock enhancement programs are all potential arenas for the application of population genetic information (Travis et al. 1998; CDFG 2005; Palumbi 2003). In this dissertation, I explored several aspects of abalone recovery and conservation along the coast of California through population genetic theory and techniques. The first chapter focuses on ensuring the specific purity of broodstock and maintaining genetic diversity in hatchery-bred progeny of the white abalone. Chapter II discusses the reduction of genetic diversity in captive-bred green abalone and the potential consequences of outplanting these progeny. The third, fourth, and fifth chapters center on inferring realized connectivity (gene flow) among natural populations of red, black, and pink abalone. Finally, the sixth and seventh chapters focus on the fatal wasting disease withering syndrome (WS); Chapter VI characterizes the post-esophageal microbiomes of red and black abalone with and without WS and Chapter VII explores the genetic diversity of the bacterium that causes WS.

References

Avise JC (2004) Molecular markers, natural history, and evolution (second edition). Sinauer Associates, Sunderland, Massachusetts

Burton RS, Tegner MJ (2000) Enhancement of red abalone *Haliotis rufescens* stocks at San Miquel Island: reassessing a success story. *Marine Ecology Progress Series*, 202, 303-308

California Department of Fish and Game, Marine Region (2005) Final abalone recovery and management plan. The Resources Agency, Sacramento, California

DeFreitas B (2003) Estimating juvenile northern abalone (*Haliotis kamtschatkana*) abundance using artificial habitats. *Journal of Shellfish Research*, 22, 819-823

Friedman CS, Thomson M, Chun C, Haaker PL, Hedrick RP (1997) Withering syndrome of the black abalone, *Haliotis cracherodii* (Leach): water temperature, food availability, and parasites as possible causes. *Journal of Shellfish Research*, 16, 203-411

Friedman CS, Andree KB, Beauchamp KA, Moore JD, Robbins TT, Shields JD, Hedrick RP (2000) 'Candidatus *Xenohaliotis californiensis*', a newly described pathogen of abalone, *Haliotis* spp., along the west coast of North America. *Journal of Systematic and Evolutionary Microbiology*, 50, 847-855

Geiger DL (2000) Distribution and biogeography of the recent Haliotidae (Gastropoda: Vetigastropoda) worldwide. *Bolletino Malocologico*, 35, 57-120

Gutierrez-Gonzalez JL, Perez-Enriquez R (2005) A genetic evaluation of stock enhancement of blue abalone *Haliotis fulgens* in Baja California, Mexico. *Aquaculture*, 247, 233-242

Haaker PL, Parker DO, Chun CSY (1992) Growth of black abalone, *Haliotis cracherodii* Leach, at San Miguel Island and Point Arguello, California. *Journal of Shellfish Research*, 14, 519-525

Hilborn R (1998) The economic performance of marine stock enhancement projects. *Bulletin of Marine Science*, 62, 661-674

Hobday AJ, Tegner MJ (2000) Status review of white abalone (*Haliotis sorenseni*) throughout its range in California and Mexico. NOAA Tehnical Memorandum NOAA-TM-NMFS-SWR-035. U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Southwest Region

Kristiansen TS, Otterå, Svåsand T (2000) Size-dependent mortality of juvenile Atlantic cod, estimated from recaptures of released reared cod and tagged wild cod. *Journal of Fish Biology*, 56, 687-712

Lapota D, Rosen G, Chock J, Liu CH (2000) Red and green abalone seed growout for reseeding activities off Point Loma, California. *Journal of Shellfish Research*, 19, 431-438

Leighton DL (2000) The biology and culture of the California abalones. Dorrance Publishing, Co., Inc., Pittsburg, Pennsylvania

Levitan DR (1991) Influence of body size and population density on fertilization success and reproductive output in a free-spawning invertebrate. *Biological Bulletin*, 181, 261-268

Levitan DR, Sewell MA, Fu-Shiang C (1992) How distribution and abundance influence fertilization success in the sea urchin *Strongylocentrotus franciscanus*. *Ecology*, 73, 248-254

Lorenzen K (2005) Population dynamics and potential of fisheries stock enhancement: practical theory for assessment and policy analysis. *Philosophical Transactions of the Royal Society B*, 360, 171-189

Palumbi SR (2003) Population genetics, demographic connectivity, and the design of marine reserves. *Ecological Applications***,** 13, S146-S158

Rogers-Bennett L, Allen BL, Davis GE (2004) Measuring abalone (*Haliotis* Spp.) recruitment in California to examine recruitment overfishing and recovery criteria. *Journal of Shellfish Research*, 23, 1201-1207

Stephens PA, Sutherland WJ, Freckleton RP (1999) What is the Allee Effect? *Oikos*, 87, 185-190

Travis J, Coleman FC, Grimes CB, Conover D, Bert TM, Tringali M (1998) Critically assessing stock enhancement: an introduction to the Mote Symposium. *Bulletin of Marine Science*, 62, 305-311

Waples RS (1998) Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. *Journal of Heredity*, 89, 438-450

CHAPTER I

Genetic diversity and species identification in the endangered white abalone (*Haliotis sorenseni*)

Abstract

In 2001, the white abalone *Haliotis sorenseni* became the first marine invertebrate in United States waters to receive federal protection as an endangered species. Prior to the endangered species listing, twenty abalone were collected as potential broodstock for a captive rearing program. Using DNA from these animals, we have developed genetic markers, including five nuclear microsatellite loci and partial sequences of one nuclear (VERL) and two mitochondrial (COI and CytB) genes, to assess genetic variability in the species, aid in species identification, and potentially track the success of future outplanting of captive-reared animals. All five microsatellite loci were polymorphic and followed expectations of simple Mendelian inheritance in laboratory crosses. Each of the wild-caught adult abalone exhibited a unique composite microsatellite genotype, suggesting that significant genetic variation remains in natural populations. A combination of nuclear and mitochondrial gene sequencing demonstrated that one of the original wild-caught animals was, in fact, not a white abalone, but *H. kamtschatkana* (possibly subspecies *assimilis*). Similarly, another animal of uncertain identity accidentally collected by dredging was also shown to be *H. kamtschatkana.* Inclusion of these two animals as broodstock could have resulted in unintentional hybridizations detrimental to the white abalone recovery program. Molecular genetic identifications will be useful both in preventing broodstock contamination and as markers for future restocking operations.

Introduction

The white abalone *Haliotis sorenseni* (a marine gastropod mollusk in the family Haliotidae) and four other commercially-fished abalone species off the coast of California, USA, experienced severe population declines largely due to overfishing in the late 1970's and 1980's (Hobday and Tegner 2000; CDFG 2002). Currently, white abalone abundance may be only one percent of that found 25 years ago (CDFG 2002). In response, a moratorium was placed on abalone fishing by the California Department of Fish & Game (CDFG) in 1997; all abalone harvest was prohibited except for heavily-regulated sport diving for the red abalone *H. rufescens* north of San Francisco Bay. By 2001, the dramatic decline in adult numbers combined with evidence of long-term recruitment failures resulted in the listing of the white abalone by the United States Fish & Wildlife Service (USFWS) as an endangered species under the Federal Endangered Species Act (ESA) of 1973. It is the first marine invertebrate species to receive Federal protection under the ESA. Recovery efforts for the white abalone are in the planning stage and may include a captive breeding program with subsequent outplanting to enhance and/or reestablish populations across the species' documented range in central and southern California and Baja California in Mexico (current range extends from Pt. Conception to Baja California; CDFG 2002).

Population genetics is becoming increasingly important in marine conservation biology and can potentially play several roles in white abalone conservation and recovery plans. First, identification of stock structure would have important implications for the collection of broodstock and breeding programs as well as for the design of marine reserves for natural populations (Waples 1998; Travis et al. 1998; Palumbi 2003).

Second, genetic tools can be applied to assess stock enhancement programs (Milbury et al. 2004). Finally, molecular forensics can be used in law enforcement cases, such as prosecution of suspected cases of illegal harvesting (CDFG 2002).

We have developed a panel of genetic markers useful for both species identification and analyses of within-species variation. The panel includes five nuclear microsatellite loci and partial sequences of two mitochondrial and one nuclear gene. These markers may have several applications in the future management of white abalone as well, including parentage testing of hatchery-bred progeny, maintaining genetic diversity in captive breeding programs, and engineering offspring with unique genetic signatures to measure outplant success. For example, microsatellite loci, tandemly repeated short (2-10 basepair) sequences of DNA, are often highly variable between individuals, populations, and species; hence, they are often used for parentage and pedigree assessment as well as for determining the genetic structure of natural populations (Avise 2004). Here we make use of existing microsatellite primer sets designed for another abalone species (*H. kamtschatkana*, Miller et al. 2001) to assess nuclear genetic variation in the white abalone.

Potentially high homoplasy and high within-species variation make microsatellites imperfect markers for species-level identification (Estoup *et al*. 2002; Avise 2004). Mitochondrial DNA (mtDNA) sequences often make effective markers for species identification (Hebert et al. 2003a, b, 2004). We have employed both cytochrome oxidase subunit I (COI) and cytochrome b (CytB) sequences for this purpose. Although mitochondrial genes are often used for species identification, the fact that they are typically maternally inherited makes mtDNA insufficient for identification of

interspecific hybrids. A biparentally inherited or male-specific nuclear gene marker is required to complement mtDNA data. The gene encoding VERL, the egg vitelline envelope receptor for sperm lysin, plays a role in species-specific sperm/egg interactions (Lee and Vacquier 1992; Galindo et al. 2002, 2003) and seems a good candidate gene for species identification.

Materials and Methods

Sample collection and DNA extraction

General information concerning the abalone included in this study can be found in Table 1.1. Adult samples consisted of tentacle, epipodial, or foot tissue obtained from 20 unrelated wild-caught abalone originally held in aquaculture facilities in Oxnard and Santa Barbara, CA. These animals were collected primarily at Farnsworth Bank (33º 26' N, 118º 30' W) prior to the June 28, 2001, listing of the white abalone as an endangered species and were intended for use as potential broodstock in propagation studies and an enhancement/recovery program (USDOC NOAA permit no. 1346). Foot or epipodial tissue was cut from the animal after death or epipodial tentacles were clipped from live animals (non-lethal sampling). The single animal (SWFSC, accidentally collected by dredging) held at the NOAA Southwest Fisheries Science Center in La Jolla, CA, was sampled by rubbing the live animal's mantle and epipodium with a cotton swab to obtain epithelial cells.

Samples from two successive generations of white abalone reared in captivity (obtained from TB McCormick, Channel Islands Marine Resource Institute, Port Hueneme, CA) were used to study genetic marker inheritance. The P_0 parental generation consisted of two females (895 and 1193) spawned separately with a single male (4239). The F_1 generation sample was composed of 24 randomly chosen animals from each of the initial P_0 crosses (895 X 4239, 1193 X 4239). Samples were obtained from foot tissue of whole F_1 juvenile abalone frozen at -80ºC. The F_2 generation sample consisted of veliger larvae resulting from the spawning of half-sibs; an F_1 female from

the 1193 X 4239 P_0 was crossed with an F_1 male from the 895 X 4239 P_0 cross. Individual larvae (N = 12) were chosen at random and frozen at -80ºC.

Adult and juvenile DNA was extracted from approximately 25 milligrams of tissue or from a cotton swab using a DNeasy Tissue Kit (Qiagen Inc., Valencia, CA) and eluted in 200 µl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). Larval DNA was extracted by incubating a single whole larva at 65ºC for one hour in 10 µL of a buffer solution (10mM Tris pH 8.3, 50 mM KCl, 0.5% Tween 20) containing 200 µg/mL Proteinase K followed by 20 minutes at 80ºC to inactivate the enzyme. A 5 µL aliquot of the stock DNA was diluted with 45 µL of DNA-grade water, placed in 96-well trays, and stored at 4ºC until use in the polymerase chain reaction (PCR).

PCR methods

Reactions were carried out in 10 µL volumes, including 1 µL of template DNA, 0.2 U REDTaq DNA polymerase (Sigma-Aldrich Corporation, St. Louis, MO), 1 µL 10X PCR buffer, 2.5 mM $MgCl_2$ final concentration, 2 µM each dNTP final concentration, and 2 pmoles each primer. Thermal cycling was performed in Thermo-Hybaid PCRExpress and Px2 thermal cyclers (Thermo Electron Corporation, Boston, MA).

One compound tri-/tetranucleotide (*Hka3*) and four dinucleotide (*Hka*28, *Hka*40, *Hka*56, and *Hka*80) microsatellite loci were amplified in all 21 adults, 48 juveniles, and 12 larvae by PCR (Table 1.2). Primers used were originally developed for a closely-related species, the pinto abalone *H. kamtschatkana* (Miller et al. 2001). Forward primers were 5' end-labeled with one of three dyes (HEX, TET, or 6-FAM) for

fluorescent visualization. PCR cycling conditions from Miller et al. (2001) were optimized for use with white abalone DNA.

We amplifed 580 basepairs (bp) of the COI gene and 388 bp of the CytB gene in all 21 adults, two F_1 juveniles per cross, five F_2 larvae, and a panel of seven other Eastern Pacific abalone species. PCR primer sequence for COI were from Metz et al. (1998): AB-COIF (forward: 5'-TGATCCGGCTTAGTCGGACTGC) and AB-COIR (reverse: 5'-GATGTCTTGAAATTACGGTCGGT). PCR cycling conditions included an initial two minute 95ºC denaturation followed by 35 cycles of 95ºC for 30 s, 50ºC for 30 s, and 72ºC for 30 s.

The CytB primer set was developed in our lab based on conserved regions found in alignments of invertebrate (mollusk and insect) CytB sequences in the NCBI GenBank database: CYTBFA (forward: 5'-CCCTGGGGACAAATGTCTTT) and CYTBR (reverse: 5'-GCAAAGAGAAAATACCACTCAGG). Touchdown PCR cycling conditions included an initial two minute 95ºC denaturation followed by 10 cycles of 95ºC for 30 s, 64ºC for 30 s (-1ºC per cycle), and 72ºC for 30 s. The remaining 25 cycles were conducted at 95ºC for 30 s, 54ºC for 30 s, and 72ºC for 30 s.

Approximately 1040 bp of the 5' portion of repeat 1 of the VERL gene were amplified in the same sample group using the published primers from Galindo et al. (2003): VERL-F2 (forward: 5'-GATACCCCAGACCCCAGAGTG) and VERL-R2 (reverse: 5'-TTGGCTGGAATGCTCTC). Since VERL is nearly monomorphic within haliotid species, the purpose of amplifying VERL was for species identification only. Therefore, no larval DNA was sequenced for VERL. Four juveniles were sequenced as further confirmation of monomorphism. Touchdown PCR cycling conditions included an

initial two minute 95ºC denaturation; 10 cycles of 95ºC for 30 s, 60ºC for 30 s (-1ºC per cycle), and 72ºC for 30 s; and 25 cycles of 95ºC for 30 s, 50ºC for 30 s, and 72ºC for 30 s.

Microsatellite genotyping

Following amplification, samples were loaded onto vertical large format (33 x 39 cm, 0.4 mm thick) 6% polyacrylamide denaturing gels containing 33% formamide and 7M urea. ROX-labeled Genemark 350 Fluorescent DNA Ladder (Northern Biotechnology, Schofield, WI) was run concurrently as a size standard. Gels were electrophoresed at 60 watts for two to three hours and scanned on a Typhoon 9410 Variable Mode Imager (Molecular Dynamics Inc., Sunnyvale, CA).

Hka3 allele sizes were scored using FragmeNT Analysis v1.2 (Molecular Dynamics Inc., Sunnyvale, CA) software. *Hka*28, *Hka*40, *Hka*56, and *Hka*80 allele sizes were viewed with ImageQuaNT (Molecular Dynamics Inc., Sunnyvale, CA) and scored by hand. The data were analyzed with GENEPOP v3.4 (Raymond and Rousset 1995, 2003). Samples that did not amplify or whose genotypes could not be scored after three rounds of PCR were not included in the analysis.

DNA sequencing

PCR products were sequenced on a MegaBACE™ 500 DNA sequencer (Amersham Biosciences Corp., Piscataway, NJ) using the forward primers and the DYEnamic™ ET dye terminator sequencing kit (Amersham Biosciences Corp., Piscataway, NJ). Sequences were aligned and edited using Sequencher 3.1 (Gene Codes

Corporation, Ann Arbor, MI) and submitted to the NCBI GenBank database (accessions AY817689-AY817735; see Table 1.4 for a partial list). Previous NCBI GenBank accessions used for COI and VERL, respectively, include pinto (AF060845, AF490761), white (AF060844, AF490760), flat (AF060846, AF490762), red (AF060842, AF453553), black (AF060848, AF490765), pink (AF060849, AF490764), and green (AF060850, AF490766). No prior COI sequences were available for threaded abalone, and no prior CytB sequences were available in the database for any abalone species. The final edited sequences per individual were composed of 774 bp of the nuclear VERL gene and 663 bp (376 bp COI and 287 bp CytB) of the combined mitochondrial genes for phylogenetic analysis. The data were analyzed with PAUP* 4.0b10 (Swofford 1991, 1998).

Results

Microsatellite loci

All five nuclear microsatellite loci were polymorphic, in Hardy-Weinberg equilibrium, and exhibited heterozygosities > 0.50 (Table 1.2) in our sample of 19 wild-caught white abalone (sample named "Abigail" excluded, see below). Further, each of the adults had unique composite genotypes when information from all five microsatellites was combined. Pairwise comparisons for linkage disequilibrium between microsatellite loci were not significant ($P > 0.05$). The loci appear to be independently segregating (unlinked) and informative. Inheritance of the microsatellite alleles through both the F_1 and F_2 generations in the pedigree was straightforward; no null alleles or extra alleles were evident. Genotypic ratios for each locus in the F_1 conformed to expectations of simple Mendelian inheritance. Chi-square tests of observed versus expected genotypic ratios were not significantly different at any locus for either cross ($P > 0.05$; Table 1.3).

All 48 F_1 juveniles could be assigned female parentage (895 or 1193) unambiguously using the combined microsatellite data. The male (4239) was the same in both crosses and, therefore, irrelevant to parentage assessment in this case. The key microsatellite in determining female parentage was *Hka*80, as both females were heterozygous at this locus and did not have any alleles in common.

DNA sequencing

To employ genetics to determine species identity, we used a set of samples and published data comprising eight Eastern Pacific abalone species (white *H. sorenseni*, threaded *H. k. assimilis*, pinto *H. k. kamtschatkana*, flat *H. walallensis*, red *H. rufescens*,

pink *H. corrugata*, green *H. fulgens*, and black *H. cracherodii*). Combined, the three sequenced gene fragments totaled 1437 bp and contain 225 variable sites in our set of sequences (excluding extensive insertion/deletions found in pink and green VERL). Genetic diversity within the sample of white abalone was assessed by sequencing portions COI and CytB. CytB/COI contained 130 variable sites in 663 bp of sequence from the eight Eastern Pacific abalone species. However, there was only one high frequency polymorphism within all 28 aligned white abalone sequences, including 19 adults, four juveniles, and five larvae, indicating that mitochondrial diversity is not high in the white abalone at these genes (Table 1.4). Unfortunately, sequencing of COI and CytB was not particularly informative with respect to parentage assessment in the pedigree. It was confirmed, however, that mitochondrial DNA is inherited through the female parent in the white abalone. Both female parents (895 and 1193) had the same mtDNA haplotype, which was different from the male parent (4239), and no F_1 or F_2 offspring carried the male parental haplotype (Table 1.4).

VERL was used only for interspecific comparisons. It contained 95 variable sites (excluding the pink and green insertion/deletions) in 774 bp of sequence from eight Eastern Pacific abalone species.

Species identification

Initially, nuclear microsatellite data revealed that two DNA samples (Abigail and SWFSC) contained alleles at all five loci that were unique to these particular animals. Our original efforts to confirm whether these animals were indeed white abalone focused on mtDNA sequencing. In 663 bp of CytB/COI sequence, only four nucleotide sites are

phylogenetically informative in distinguishing among the white, threaded, and pinto abalone in our sample (Table 1.4). All four of these sites differed between three wild caught white abalone and three threaded abalone samples (CytB at position 54 and COI at positions 129, 222, and 435). Polymorphism within the pinto abalone samples left only two diagnostic sites (CytB at position 54 and COI at position 435) to distinguish pinto from white abalone. The potential for additional polymorphism within species seems high, since we have only a few representative sequences per species. However, Abigail matched with threaded abalone sequence at all four sites, making this appear a likely species identification. The SWFSC sample best matches with pinto, but its identification based on mtDNA sequence alone would be considered tentative at best (see analysis below).

Neighbor Joining trees were initially generated in PAUP* for the mitochondrial and nuclear DNA sequence alignments. The two trees were then optimized using Maximum Likelihood under the general time-reversible model of nucleotide substitution with a gamma distribution of rates (ML, heuristic search, GTR + G; Figure 1.1). ML analysis of the CytB/COI sequence revealed that Abigail belonged closely to the clade containing the threaded and pinto abalone sequences (Figure 1.1a). Although placed with threaded and pinto abalone, ML analysis failed to definitively resolve the relationship between the SWFSC CytB/COI sequence and white, threaded, and pinto sequence. The signal from VERL, however, was unambiguous. Heterozygotes are a potentially confounding issue, though, since VERL is a diploid nuclear gene, and two of 21 sites variable between white, threaded, and pinto abalone were excluded from analysis. While the VERL primers did not successfully amplify DNA from Abigail, the SWFSC abalone

and the eight white abalone included in the sequence alignment differed at 17 phylogenetically informative sites in this gene (Table 1.4). All 17 matched with the threaded/pinto abalone, and ML analysis of VERL placed the SWFSC DNA within this clade (Figure 1.1b)

In order to test the strength of the relationships presented in the original ML trees (Figure 1.1), constraints were placed on the tree topologies by forcing the Abigail and SWFSC sequences into monophyletic relationships with each of the other species sequence groups using PAUP*. The "best fit" tree produces the highest -*ln*L value. A Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa 1999) as implemented in PAUP* was performed on the differences in -*ln*L between each of the trees, including the unconstrained tree, using 1000 RELL bootstrap replicates.

In the cases of both Abigail and the SWFSC sequences, the best fit to the CytB/COI sequence data was the original unconstrained tree (–*ln*L = 1684.39; Table 1.5). For Abigail, constraining each sequence into monophyly with the threaded and pinto sequences was equivalent to the relationships found in the unconstrained tree. While constraining Abigail's sequence with white abalone did not produce a significant difference in –*ln*L ($P = 0.185$), the –*ln*L scores were markedly higher for this constraint than for threaded and pinto abalone, further suggesting that Abigail is not a white abalone. For SWFSC, the difference in –*ln*L from the unconstrained tree for CytB/COI was not significant for the pinto, threaded, white, or flat abalone constraints, emphasizing that the signal from the mtDNA sequence was not strong enough to resolve this relationship. The SH test on VERL, however, produced highly significant differences in –*ln*L values ($P \leq 0.002$) for all constraints other than those involving threaded and pinto

sequences, implying that SWFSC also is not a white abalone (Table 1.5).

Discussion

Genetic analysis can play diverse roles in conservation and recovery plans for threatened and endangered species. Patterns of within and between population genetic variation can inform both captive breeding programs and design of networks of marine protected areas. In the case of white abalone, we do not have samples from multiple natural populations, although the current distribution is assumed to extend from Monterey, CA, USA, to Punta Eugenia in Baja California Sur, Mexico (Geiger 2000). Additional white abalone samples are difficult to obtain due to current endangered species status, low abundances, and habitat depth (25 m to 60 m). Collection and survey work with SCUBA is inefficient, necessitating surveys with ROVs before divers can be sent to sample animals (White Abalone Recovery Team personal communication). We are largely restricted to studies of within-population diversity on the animals (i.e. Farnsworth Bank, Table 1.1). Access to three laboratory crosses has permitted verification of inheritance patterns of the markers we employ. This is important since previous studies of inheritance using microsatellite markers in abalone have proven problematic. Several markers have failed to conform to simple Mendelian patterns of inheritance (Li et al. 2003a, b). This was not the case here; all markers followed expected patterns of biparental inheritance (nuclear microsatellite loci) or maternal inheritance (mtDNA).

The combination of sequencing multiple genes and genotyping multiple microsatellites revealed a unique composite genotype or genetic fingerprint for all adult abalone analyzed. Unique genetic fingerprints indicate that it may be possible to use genetic analysis to monitor success of future outplanting programs. If animals are bred to

carry particular unique multi-locus microsatellite genotypes, offspring could be tracked after outplanting, as could their wild-bred progeny (Burton and Tegner 2000; Milbury et al. 2004). Ideally, more microsatellite loci would be added in the future to provide greater detection power.

The most important result of the present study was the discovery that one animal (Abigail) originally included in a set of 20 wild-caught white abalone had been misidentified. Microsatellite analyses revealed that Abigail had unique alleles at each locus, causing us to further investigate its specific status. Sequences from the mtDNA loci CytB/COI indicate that Abigail was most likely a threaded or pinto abalone. Abigail's identity, based upon morphological characteristics, was apparently not in question until this study. In a second case requiring species identification, the SWFSC abalone was confiscated by the National Oceanic and Atmospheric Administration (NOAA) after being tentatively identified morphologically as a white abalone. Color and morphology, however, can be plastic traits in abalone depending upon location, habitat, and diet, and hybrids often have intermediate morphologies (Leighton 2000). Again, unique microsatellite alleles made the identification suspect, and sequence of the nuclear VERL gene confirmed that the animal was not a white abalone.

Based on this work, it is clear that morphology alone is insufficient for species identification of some abalone species, including the endangered white abalone. Although neither of the two animals ultimately found not to be white abalone were used in the captive rearing program (Abigail died before spawning and SWFSC was of questionable identity), their inclusion as broodstock could have had serious consequences. At a minimum, erroneously using the wrong animals in laboratory culture

would lead to substantial waste of effort for the captive rearing program as interspecific hybrids (even if viable) are often infertile. More importantly, inadvertent hybridization could potentially contaminate the white abalone gene pool if fertile hybrids are obtained, with the captive breeding program doing more harm than good with regard to species recovery.

As noted above, mitochondrial DNA sequences are widely used for species identification, and the concept of developing a "barcode" system of species identification based on mtDNA has received much attention (Mallet and Willmott 2003; Tautz et al. 2003; Hebert et al. 2003a, b, 2004; Janzen 2004; Moritz and Cicero 2004). Although barcodes based on partial COI sequences will often distinguish species, cases where mtDNA divergence is low have been widely acknowledged (Russo et al. 1996; Zardoya and Meyer 1996). We could not make the critical distinction between the endangered white abalone and the closely related *H. kamtschatkana* (including both putative subspecies, threaded *H. k. assimilis* and pinto *H. k. kamtschatkana*) based on partial COI sequence. Sequence data from an additional mtDNA locus, CytB, also failed to resolve species identity.

As discussed previously, even if mtDNA could distinguish all taxa satisfactorily, nuclear markers are required to assess if individuals are of interspecific hybrid origin. For abalone, we have found that the VERL locus is especially useful in this regard. Although direct DNA sequencing of the VERL PCR product could be confounded by heterozygosity in diploids, the high level of divergence observed at this locus suggests heterozygotes would be readily distinguished by sequence ambiguity at a large number of sites in interspecific hybrids. We did not see any evidence of such ambiguity and

conclude that the suspect individual (SWFSC) was not a hybrid. Since VERL could not be amplified from DNA extracted from Abigail, hybrid status cannot be fully ruled out. In future work, restriction digest assays will be developed for genotyping individual animals (e.g. the 5' end of repeat 1 of VERL displays multiple potential diagnostic restriction enzyme sites, per Bikandi et al. 2004). Such assays could more quickly assess species (or hybrid) identity.

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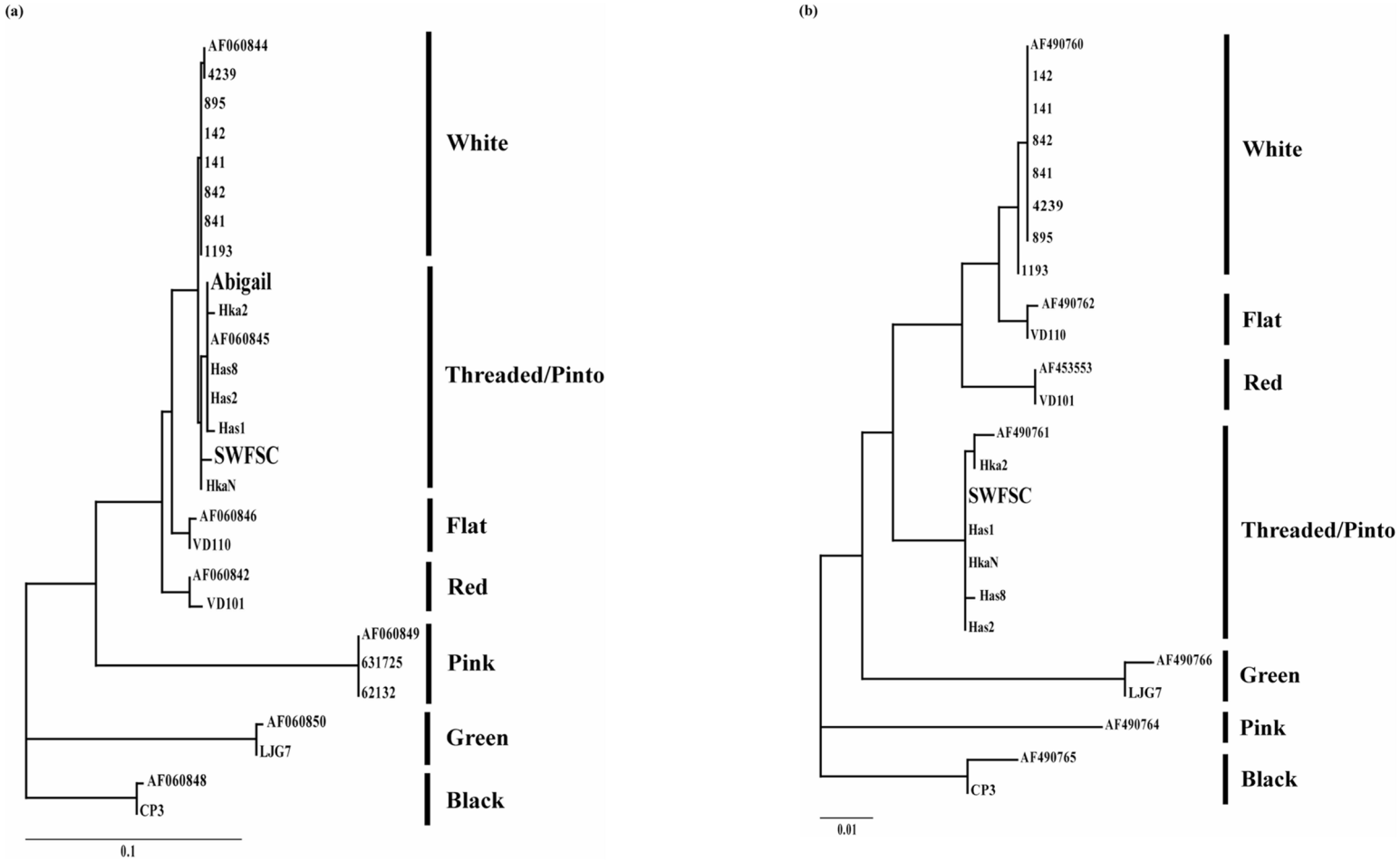

Figure 1.1: Maximum Likelihood phylograms. (a) Combined of CytB and COI (663 bp). (b) The 5' end of protein repeat 4 of VERL (774 bp). Species are indicated by the vertical bars. Branch lengths are to scale. Sample names correspond to one another in each tree. Sequence from the black abalone *H. cracherodii* is used as the outgroup.

Table 1.1: General information regarding the abalone included in this study (TB McCormick personal communication).

Population	ID#	N	Pedigree	DOB	Location	Sex
Adults	SWFSC	1			Horseshoe Kelp near San Pedro	F
	Abigail	1			Farnsworth Bank	F
	Bird Rock	1			Farnsworth Bank	F
	890	1			Santa Catalina Island	M
	891	1			Farnsworth Bank	M
	892	1			Farnsworth Bank	F
	893	1			Farnsworth Bank	F
	894	1			Farnsworth Bank	M
	895	1	P_0: female		Farnsworth Bank	F
	1118	1			Farnsworth Bank	F
	1193	1	P_0: female		Farnsworth Bank	F
	1405	1			Farnsworth Bank	M
	1494	1			Farnsworth Bank	
	1638	1			Farnsworth Bank	F
	3184	1			Farnsworth Bank	M
	3326	1			Farnsworth Bank	F
	3432	1			Farnsworth Bank	M
	3563	1			Farnsworth Bank	M
	3588	1			Farnsworth Bank	F
	4017	1			Farnsworth Bank	F
	4239	1	P_0: male		Farnsworth Bank	M
Juveniles	J841 - J8424	24	F_1: 895 X 4239	4/23/2001	Proteus SeaFarms	mixed
	J141 - J1424	24	F_1: 1193 X 4239	4/23/2001	Proteus SeaFarms	mixed
Larvae	L1 - L12	12	F_1: 84 male X 14 female	6/3/2003	Proteus SeaFarms	mixed

Table 1.2: Nuclear microsatellite loci. Nucleotide repeat sequence, number of alleles genotyped per locus (N), expected (H_e) and observed heterozygosities (H_o), and Hardy-Weinberg equilibrium probability test P-values per locus in the 19 prelisted white abalone are included. All loci were originally isolated in the northern abalone *H. kamtschatkana* (Miller *et al.* 2001).

Locus	Repeat	N	He	Ho	P
***Hka* 3**	(GTA)n(GAGT)n	38	0.56	0.74	0.07
***Hka* 28**	(CA)n	38	0.56	0.53	0.58
***Hka* 40**	(CA)n	36	0.73	0.78	0.98
***Hka* 56**	(CA)n	38	0.65	0.68	0.85
***Hka* 80**	(CA)n	38	0.76	0.79	1.00
Multi-locus		188	0.65	0.70	0.75

Table 1.3: Chi-square (χ^2) test. Individual microsatellite loci were tested for conformation to expectations of simple Mendelian inheritance in the F_1 generation.

Cross	Locus	ratio	exp	obs	Chi-square	P
895 X 4239	*Hka* 3	1	6	9	2.00	0.35
		2	12	10		
		1	6	5		
	Hka 28	1	12	10	0.67	0.45
		1	12	14		
	Hka 40	1	12	12	0.00	1.00
		1	12	12		
	Hka 56	1	6	4	1.33	0.60
		2	12	12		
		1	6	8		
	Hka 80	1	12	14	0.67	0.45
		1	12	10		
1193 X 4239	*Hka* 3	1	12	11	0.17	0.75
		1	12	13		
	Hka 28	1	24	24	0.00	1.00
	Hka 40	1	12	11	0.17	0.75
		1	12	13		
	Hka 56	1	6	6	0.50	0.93
		1	6	7		
		1	6	5		
		1	6	5		
	Hka 80	1	12	12	0.00	1.00
		1	12	12		

Table 1.4: Mitochondrial CytB and COI and nuclear VERL partial gene sequences. White adult 895 sequence is the reference sequence for CytB, and GenBank accessions AF060844 and AF460760 are used as references for COI and VERL, respectively. All constant and heterozygous sites have been removed. Numbers in *italics* below gene names are site numbers for each base change in our alignment for CytB, AF060844 for COI (basepair 1 in our alignment is equivalent to basepair 129 in the full AF060844 sequence), and AF490760 for the 5' end of repeat 4 of VERL (basepair 1 in our alignment is equivalent to basepair 46 in the full AF490760 sequence). Data for more distantly related California abalone species (*H. walallensis*, *H. rufescens*, *H. corrugata*, *H. fulgens*, and *H. cracherodii*) are not included. A period implies no base change and blank space means no data was collected for this individual.

Group	ID	Accession	CytB	Accession	COI	Accession	VERL
			27 54 147 189		*129 222 235 455 456*		*120 125 215 230 232 278 329 336 363 376 380 401 415 423 443 445 458 478 495*
White				AF060844	T G T C C		
White						AF490760	C A T G C T A A G C C A T C C A T T A
White Adults	895	AY817722	C A C T	AY817710	. . C . .	AY817690	. . . .
White Adults	1193	AY817723	. . . .	AY817711	. . C . .	AY817691	. . . .
White Adults	4239	AY817724	. . . .	AY817712	. . . . .	AY817692	. . . .
White Juveniles	J841		. . . .		. . C . .	AY817693	. . . .
White Juveniles	J842		. . . .		. . C . .	AY817694	. . . .
White Juveniles	J141		. . . .		. . C . .	AY817695	. . . .
White Juveniles	J142		. . . .		. . C . .	AY817696	. . . .
White Larvae	L1		. . . .		. . C . .		
White Larvae	L2				. . C . .		
White Larvae	L3		. . . .		. . C . .		
White Larvae	L4		. . . .		. . C . .		
White Larvae	L5		. . . .		. . C . .		
Threaded	Has 1	AY817725	. G T .	AY817713	C A C T T	AY817697	A C G A T G T G A . T C A A A C . A G
Threaded	Has 2	AY817726	. G . .	AY817714	C A C T .	AY817698	A C G A T G T G A . T C A A A C . A G
Threaded	Has 8	AY817727	. G . .	AY817715	C A C T .	AY817699	A C G A T G T G A . T C A A A C . A G
Pinto				AF060845	C A C T .		
Pinto						AF490761	A C G A T G T G A T T C A A A C C A G
Pinto	Hka2	AY817728	T G . .		C A C T .	AY817704	A C G A T G T G A T T C A A A C . A G
Pinto	HkaN	AY817729	. G . .		. . C T .	AY817705	A C G A T G T G A . T C A A A C . A G
Unknown	SWFSC	AY817720	T G . C	AY817708	. . C T .	AY817689	A C G A T G T G A . T C A A A C . A G
Unknown	Abigail	AY817721	. G . .	AY817709	C A C T .		

Table 1.5: SH test on the constrained trees for the VERL and combined CytB/COI sequences. Constraints were placed on the tree topology by forcing the Abigail and SWFSC sequences (ID) into monophyletic relationships with each of the other species sequence groups (Tree). "Unconstrained" refers to the original tree, containing no topological constraints (Figure 1). Negative log-likelihood (–*ln*L) scores of the trees, difference in –*ln*L from the unconstrained tree, and SH test P-values are included. A * indicates a significant difference in –*ln*L at the P = 0.05 level.

Gene	ID	Tree	-lnL	Difference	P
CytB/COI	Abigail	Unconstrained	1684.39	(best)	
		Threaded	1684.39	0.00	0.992
		Pinto	1684.39	0.00	0.992
		White	1707.86	23.47	0.185
		Flat	1719.93	35.53	0.030*
		Red	1738.82	54.43	0.002*
		Green	1780.72	96.33	0.000*
		Black	1793.84	109.45	0.000*
		Pink	1824.39	140.00	0.000*
	SWFSC	Unconstrained	1684.39	(best)	
		Threaded	1692.19	7.80	0.727
		Pinto	1692.19	7.80	0.727
		White	1695.69	11.30	0.572
		Flat	1706.89	22.50	0.180
		Red	1726.31	41.91	0.006*
		Green	1771.30	86.91	0.000*
		Black	1783.50	99.11	0.000*
		Pink	1810.47	126.08	0.000*
VERL	SWFSC	Unconstrained	1721.73	(best)	
		Threaded	1721.73	0.00	0.965
		Pinto	1727.16	5.43	0.772
		Pink	1766.78	45.05	0.002*
		White	1800.18	78.45	0.000*
		Red	1801.85	80.12	0.000*
		Flat	1805.53	83.80	0.000*
		Black	1816.22	84.49	0.000*
		Green	1830.53	108.80	0.000*

References

Avise JC (2004) Molecular markers, natural history, and evolution (second edition). Sinauer Associates, Sunderland, Massachusetts

Bikandi, J, San Millán R, Rementeria A, Garaizar J (2004) *In silico* analysis of complete bacterial genomes: PCR, AFLP-PCR, and endonuclease restriction. *Bioinformatics*, 20, 798-799

Burton RS, Tegner MJ (2000) Enhancement of red abalone *Haliotis rufescens* stocks at San Miquel Island: reassessing a success story. *Marine Ecology Progress Series*, 202, 303-308

California Department of Fish and Game, Marine Region (2002) Draft abalone recovery and management plan

Estoup A, Jarne P, Cornuet J-M (2002) Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. *Molecular Ecology*, 11, 1591-1604

Galindo BE, Swanson WJ, Vacquier VD (2002) Both abalone sperm lysine and its egg receptor are subjected to positive selection. *Molecular Biology of the Cell*, 13,111A-111A

Galindo BE, Vacquier VD, Swanson WJ (2003) Positive selection in the egg receptor for abalone sperm lysine. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 4639-4643

Geiger DL (2000) Distribution and biogeography of the recent Haliotidae (Gastropoda: Vetigastropoda) worldwide. *Bolletino Malocologico*, 35, 57-120

Hebert PDN,Cynwinska A, Ball SL, deWaard JR (2003a) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London* Series B: Biological Sciences, 217, 313-322

Hebert PDN, Ratnasingham S, deWaard JR (2003b) Barcoding animal life: cytochrome *c* oxidase subunit I divergences among closely related species. *Proceedings of the Royal Society of London Series B: Biological Sciences* (Supplement), 270, S96-S99

Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of birds through DNA barcodes. *Public Library of Science Biology*, 2, e312

Hobday AJ, Tegner MJ (2000) Status review of white abalone (*Haliotis sorenseni*) throughout its range in California and Mexico. NOAA Technical Memorandum

NOAA-TM-NMFS-SWR-035. U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Southwest Region

Janzen DH (2004) Now is the time. *Philosophical Transactions of the Royal Society of London Series B: Biological Sciences*, 359, 731-732

Lee Y, Vacquier VD (1992) The divergence of species-specific abalone sperm lysins is promoted by positive Darwinian selection. *Biological Bulletin*, 182, 97-104

Leighton DL (2000) The biology and culture of the California abalones. Dorrance Publishing, Co., Inc., Pittsburg, Pennsylvania

Li Q, Park C, Kobayashi T, Kijima A (2003a) Inheritance of microsatellite DNA markers in the Pacific abalone *Haliotis discus hannai*. *Marine Biotechnol*ogy, 5, 331-338

Li Q, Park C, Kijima A (2003b) Allelic transmission of microsatellites and kinship analysis in newly hatched Pacific abalone larvae. *Fisheries Scienc*e, 69, 883-889

Mallet J, Willmott K (2003) Taxonomy: Renaissance or Tower of Babel. *Trends in Ecology and Evolution*, 18, 57-59

Metz EC, Robles-Sikisaka R, Vacquier VD (1998) Nonsynonymous substitution in abalone sperm fertilization exceeds substitution in introns and mitochondrial DNA. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 10676-10681

Milbury CA, Meritt DW, Newell RIE, Gaffney PM (2004) Mitochondrial DNA markers allow monitoring of oyster stock enhancement in the Chesapeake Bay . *Marine Biology*, 145, 351-359

Miller KM, Laberee K, Kaukinen KH, Li S, Withler RE (2001) Development of microsatellite loci in pinto abalone (*Haliotis kamtschatkana*). *Molecular Ecology Notes*, 1, 315-317

Moritz C, Cicero C (2004) DNA barcoding: promise and pitfalls. *Public Library of Science Biology*, 2, 1526-1528

Palumbi SR (2003) Population genetics, demographic connectivity, and the design of marine reserves. *Ecological Applications*, 13 Supplement, S146-S158

Raymond M, Rousset F (1995) GENEPOP: population genetics software for exact tests and ecumenicism. *Journal of Heredity*, 86, 248-249

Raymond M, Rousset F (2003) GENEPOP (version 3.4): population genetics software for exact tests and ecumenicism. Updated from Raymond and Rousset (1995)

Russo CAM, Takezaki N, Nei M (1996) Efficiencies of different genes and different tree-building methods in recovering a known vertebrate phylogeny. *Molecular Biology and Evolution*, 13, 525-536

Shimodaira H, Hasegawa M (1999) Multiple comparison of log-likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution*, 16, 1114-1116

Swofford DL (1991) PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1 Computer program distributed by the Illinois Natural History Sruvey, Champaign, Illinois

Swofford DL (1998) PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts

Tautz D, Arctander P, Minelli A, Thomas RH, Vogler AP (2003) A plea for DNA taxonomy. *Trends in Ecology and Evolution*, 18, 70-74

Travis J, Coleman FC, Grimes CB, Conover D, Bert TM, Tringali M (1998) Critically assessing stock enhancement: an introduction to the Mote Symposium. *Bulletin of Marine Science*, 62, 305-311

Waples RS (1998) Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. *Journal of Heredity*, 89, 438-450

Zardoya R, Meyer A (1996) Phylogenetic performance of mitochondrial protein-coding genes in resolving relationships among vertebrates. *Molecular Biology and Evolution*, 13, 933-942

CHAPTER II

Loss of genetic diversity in cultivated green abalone (*Haliotis fulgens*) from Southern California

Abstract

Hatchery propagation of a species can result in the potential loss of genetic variation due to a severe artificial bottleneck in population size, and outplanting hatchery-bred progeny can result in swamping the natural gene pool with hatchery-based alleles and/or deleterious alleles may be introduced. We examined the genetic diversity of hatchery vs. wild samples of green abalone (*Haliotis fulgens* Philippi 1845) from Southern California using two microsatellite loci and partial mitochondrial COI sequence. A hatchery sample of 50 green abalone originally purchased from an aquaculture facility in Santa Barbara, CA, showed reductions in allelic richness and gene diversity and an increase in individual pairwise relatedness relative to a small set of samples from four wild populations. However, there was no evidence of a reduction in heterozygosity. Multiple instances of outplanting progeny of these captive-bred green abalone have been performed since 2000 off Point Loma, CA, and future efforts are planned. It may be possible to track the survival of and successful reproduction by the outplanted green abalone using a panel of genetic markers that includes those used in this study.

Introduction

Single species recovery efforts through stock enhancement of natural populations are based on the outplanting (release) into the environment of captive-bred individuals from various life stages (Travis et al. 1998). When possible, broodstock are harvested from local populations to ensure that the genetic background of the future outplanted progeny matches that of the local gene pool. Even so, hatchery propagation of a species can result in the potential loss of genetic variation resulting from the elimination of primarily low frequency alleles and/or shifts in allele frequencies and allelic diversity due to the interplay of random genetic drift in a population with a small effective population size (N_e; Nei et al. 1975; Allendorf 1986).

The loss of genetic diversity and reduction in N_e in hatchery-bred animals is of concern to government and environmental agencies overseeing enhancement efforts of natural populations (Allendorf 1986; Tringali and Bert 1998; Utter 1998). The native gene pool may become swamped with hatchery-based alleles and/or deleterious alleles may be introduced into the gene pool. Various genetic effects of hatchery rearing have been documented in multiple hatchery-propagated species (Allendorf and Phelps 1980; Ryman and Stahl 1980; Cross and King 1983), including abalone (Smith and Conroy 1992; Evans et al. 2004; Sekino et al. 2005).

Multiple instances of outplanting green abalone have been performed since 2000 off the southern California mainland coast and future efforts are planned (Lapota et al. 2000; D Lapota pers. comm.). We study the effects captive breeding in the green abalone (*Haliotis fulgens* Philippi 1845) has had on various measures of genetic diversity and how it may influence stock enhancement efforts for this species off Point Loma in San

Diego, CA. We relate our results to the possibility of tracking outplanted individuals and their potential progeny in local native populations of the species.

Materials and Methods

Sample collection and DNA extraction

Collection sites are summarized in Figure 2.1. A sample of cultivated green abalone (SPAWAR, N = 50) originally purchased from The Cultured Abalone, an aquaculture facility in Santa Barbara, CA, was obtained from D Lapota of the US Navy Space and Naval Warfare Systems Command in 2001. Wild abalone were sampled from four southern California populations. Collection from Santa Catalina Island (SCAT, N = 15) and San Clemente Island (SCLE, N = 4) was done by the California Department of Fish and Game in 2003. Mainland samples collected by E Kisfaludy of Scripps Institution of Oceanography in 2002 included La Jolla (LJ, N = 4) and Point Loma (PL, N = 3). Epipodial tentacle or foot clippings were preserved in ethanol or water and stored at -80ºC.

DNA was extracted from approximately 25 milligrams of tissue using a DNeasy Tissue Kit (Qiagen Inc., Valencia, CA) and eluted in 200 µl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). A 5 µL aliquot of the concentrated stock DNA was diluted with 45 µL of DNA-grade water, placed in 96-well trays, and stored at 4ºC for use in the polymerase chain reaction (PCR).

Microsatellites

Microsatellite loci, including the dinucleotide repeats *Hka*28 and *Hka*56, were amplified in all 76 abalone (Table 2.1). The primers used were originally developed for a related eastern Pacific species, the pinto abalone *H. kamtschatkana* (Miller et al. 2001). Forward primers were 5' end-labeled with TET for fluorescent visualization. Reactions

were carried out in 25 μL volumes, including 5 μL of dilute template DNA, 0.5 U REDTaq DNA polymerase (Sigma-Aldrich Corporation, St. Louis, MO), 2.5 μL 10X PCR buffer, 2.5 mM $MgCl_2$ final concentration, 2 mM each dNTP final concentration, and 5 pmoles each primer. Thermal cycling was performed in Thermo-Hybaid PCR Express and Px2 thermal cyclers (Thermo Electron Corporation, Boston, MA). Cycling conditions were modified slightly from Miller et al. (2001) for optimization.

Following amplification, 2 μL of a denaturing solution containing xylene cyanol and bromophenol blue dyes in 99% formamide were added to each 5 μL of PCR product. Samples were loaded onto vertical 0.4mm thick large format (33 x 39 cm) 6% polyacrylamide denaturing gels containing 33% formamide and 7M urea. ROX-labeled MegaBACE™ ET900-R Size Standard (Amersham Pharmacia Inc., Piscataway, NJ) was run concurrently as a size standard. Gels were electrophoresed at 60 Watts for two to three hours and scanned on a Typhoon 9410 Variable Mode Imager (Molecular Dynamics Inc., Sunnyvale, CA). The data were analyzed using GENEPOP v3.4 (Raymond and Rousset 1995, 2003), ML-Relate (Kalinowski and Taper 2006), and STRUCTURE v2.0 (Pritchard et al. 2000).

COI

A 580 basepair (bp) fragment of the mitochondrial cytochrome oxidase subunit one (COI) gene was amplified from 19 SPAWAR green abalone and 25 wild green abalone. PCR primers and cycling conditions were taken from Metz et al. (1998). Reactions were carried out in 15 μL volumes, including 5 μL of dilute template DNA, 0.5

U REDTaq DNA polymerase (Sigma-Aldrich Corporation, St. Louis, MO), 1.5 μL 10X PCR buffer, 2.5 mM $MgCl_2$ final concentration, 2 mM each dNTP final concentration, and 3 pmoles each primer. Thermal cycling was performed in Thermo-Hybaid PCR Express and Px2 thermal cyclers (Thermo Electron Corporation, Boston, MA).

The amplified fragments were sequenced on a MegaBACE™ 500 DNA sequencer (Amersham Biosciences Corp., Piscataway, NJ) using the forward primer and a DYEnamic™ ET dye terminator sequencing kit (Amersham Pharmacia Inc., Piscataway, NJ). Sequences were aligned and edited to 490 bp using Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI). Analyses were conducted with Arlequin v.2.000 (Scheider et al. 2000) and DnaSP v4.0.0 (Rozas et al. 2003).

Results

Microsatellites

There were no departures from Hardy-Weinberg Equilibrium (HWE) at either locus in the wild sample or at *Hka*56 in the SPAWAR sample. *Hka*28 did depart from HWE ($p = 0.002$) in SPAWAR, perhaps due to heterozygote deficiency, but tests of both heterozygote deficiency and excess were not significant. There was no evidence for linkage disequilibrium between the loci in any of the samples.

*Hka*28 contained 10 alleles and *Hka*56 contained 12 alleles in the full sample set, including both wild and hatchery samples (Table 2.2a, b). The SPAWAR sample showed reduced allelic richness (number of alleles per locus) vs. the wild samples, exhibiting the same number of alleles per locus as each of wild samples containing less than or equal to four individuals (SCLE, LJ, and PL). Gene diversity (mean expected heterozygosity (D) as per Nei 1973) was reduced in the SPAWAR sample as well ($D_{SPAWAR} = 0.870$, $D_{SCLE} = 0.964$, $D_{SCAT} = 0.977$, $D_{LJ} = 0.964$, and $D_{PL} = 1.000$). Private alleles were present only in SCAT, but SCAT had largest sample size of any wild population. There was no significant shift in allele frequencies among the samples (i.e. common alleles were the same regardless of sample).

Coefficients of relatedness (percentage of genes that two individuals theoretically share by common descent (*r*)) for each possible individual pairwise comparison were estimated under a maximum likelihood framework using the program ML-Relate (Table 2.3). Estimates for *r* ranged from 0.00 to 1.00 within each sample. In *t*-tests, the mean pairwise *r* was significantly higher for SPAWAR vs. SCLE or SCAT ($p < 0.05$) but not vs. LJ or PL.

The Bayesian model-based clustering program STRUCTURE was used to determine whether the SPAWAR aquaculture animals had a unique genetic signature as compared to the wild animals. Multiple runs were performed under the various combinations of ancestry and allele frequency models with no marked change in clustering. The default program settings (admixture model, inferred initial $\alpha = 1.0$ with a uniform prior across populations, correlation of allele frequencies within populations) were ultimately used for the analyses. An initial burnin of 5×10^5 Markov Chain Monte Carlo (MCMC) iterations followed by an 5×10^6 MCMC iterations was chosen to ensure stabilization of the summary statistics (α, F_{ST}, and likelihood). Multiple runs were performed for each number of clusters inferred (K) from two to five. There were two distinct clusters in the data, with SPAWAR individuals belonging primarily to Cluster 1 and wild individuals belonging primarily to Cluster 2 (Figure 2.2).

COI

The distribution of COI haplotype counts is displayed in Table 2.2c. Only one haplotype was present in SPAWAR. The SPAWAR haplotype was the common haplotype in the wild samples, and the only haplotype present in the LJ and PL samples. SCLE and SCAT, however, exhibited four additional low frequency haplotypes.

Arlequin was used to perform an analysis of molecular variance (AMOVA) on the interhaploytpe distances. The variance was partitioned among groups (aquaculture vs. wild combined), among populations within the groups, and within each of the populations. The bulk of the variance (~92%) was found within populations, and the

remainder was divided equally among the groups (~4%) and among populations within the groups (~4%).

Discussion

The hatchery sample (SPAWAR) in our study showed reductions in allelic richness and gene diversity and an increase in individual pairwise relatedness, but there was no evidence of a reduction in heterozygosity vs. the wild green abalone populations. This pattern is not unusual and has been seen in two other captive-bred abalone species as well, *H. rubra* and *H. midae* (Evans et al. 2004; see also Allendorf 1986). There was no also observable shift in allele frequencies with respect to the most common microsatellite alleles, and the single COI haplotype in SPAWAR was the most common haplotype in the wild samples. However, three of the wild samples contained four or fewer individuals, so allele frequency distributions were not well-sampled and cannot be considered representative of the natural populations (Ruzzante 1998).

Unfortunately, we do not know the exact pedigree of the animals bought by D Lapota from the aquaculture facility in Santa Barbara, CA, although they are likely to be from an F_1 generation (D Lapota pers. comm). Culture facilities often harvest adult broodstock from the wild, spawn the animals in house, and raise F_1 progeny for sale. Progeny are seldom mated to produce successive generations in a commercial hatchery as it takes several years to raise abalone to reproductive maturity and grow the animals to a size that is competitive in fecundity with wild-caught broodstock. The wild broodstock can also be repeatedly spawned without harm (Vic Vacquier pers. comm.).

Since the common purpose of F_1 abalone is for consumption, maintaining genetic variability was not an issue for the culture facility, and it would not be inconceivable that all or most of the F_1 generation was from a single spawning event. The hatchery abalone used in this study were originally purchased from The Cultured Abalone, an aquaculture

facility in Santa Barbara, CA, and were used to function as broodstock for the production of larvae for toxicological research (Chapman et al. 1995). Many larvae that survived the toxicity studies were settled and raised for further permitted research on the success of outplanting hatchery-reared green abalone near Point Loma, CA (Lapota et al. 2000).

Because progeny from the F_1 animals have been repeatedly outplanted into the Point Loma, CA, area since 1999, potential shifts in effective population size and swamping out the local gene pool with hatchery-based alleles may be irrelevant. Recent outplant survival rates exceed 50% after one year (D Lapota pers. comm.), and changes in genetic variation of the natural populations may have already been realized if the surviving outplanted individuals are actively reproducing in the wild. As such, the genetic markers we employed may be more useful for monitoring outplant success.

Thus far, outplanted animals have been monitored through periodic diving inspections (D Lapota pers. comm.). However, we have no way of knowing if the outplants are contributing to the local gene pool through reproduction without using genetic techniques. While all of the microsatellite alleles in the SPAWAR sample are also present in the wild samples (i.e. SPAWAR had no private alleles), SPAWAR had only a fraction of the total number of microsatellite alleles and only one mitochondrial COI haplotype. A distinct genetic signature to the hatchery-bred animals, evidenced by the cluster analysis using the program STRUCTURE, was illustrated by a split in the genetic signal between SPAWAR and wild green abalone samples. A genetic signature of captive breeding may offer us the opportunity to study the success of these stock enhancement programs over time. Unique combinations of alleles at multiple genetic loci and/or common alleles in outplants that are rare in the native population can be

exploited in order to track survivorship in and, potentially, successful reproduction by outplanted individuals, although using more microsatellite loci than we have here would be preferable in order to determine a purely hatchery-based signature.

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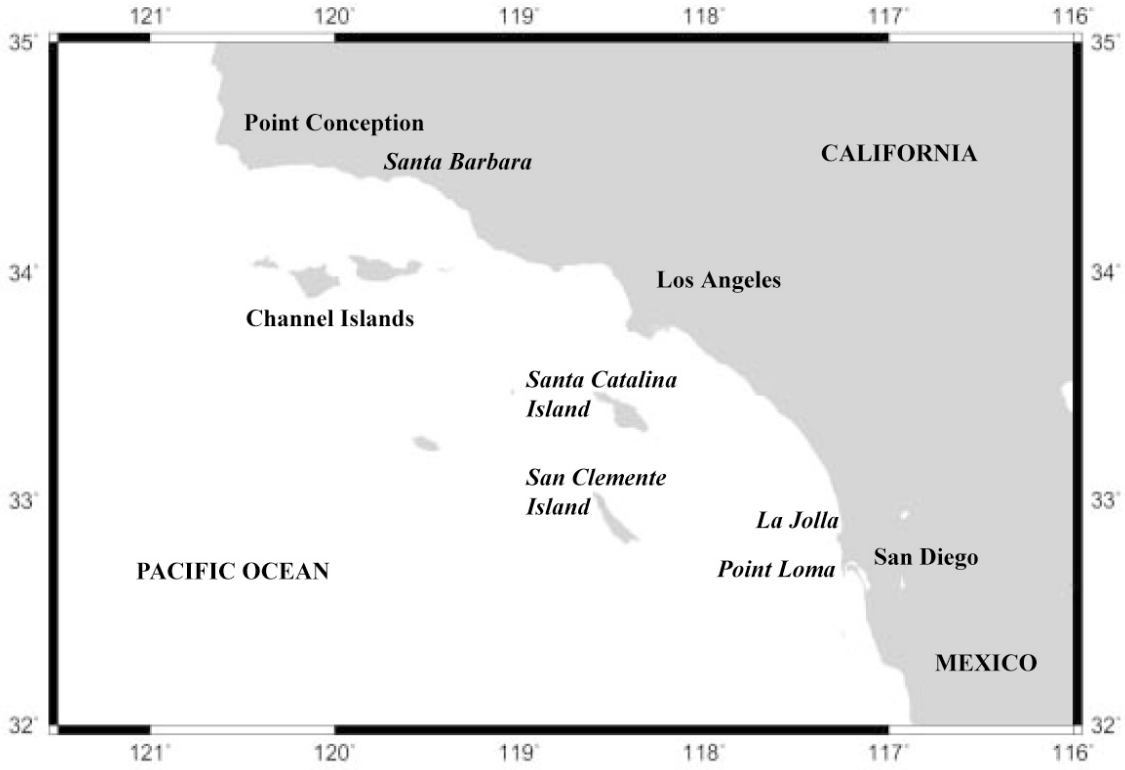

Figure 2.1: Map of southern California collection sites for *H. fulgens*. Site names are in *italics*. The aquaculture facility is located in Santa Barbara, and the four wild abalone sites include Santa Catalina Island (SCAT), San Clemente Island (SCLE), La Jolla (LJ), and Point Loma (PL).

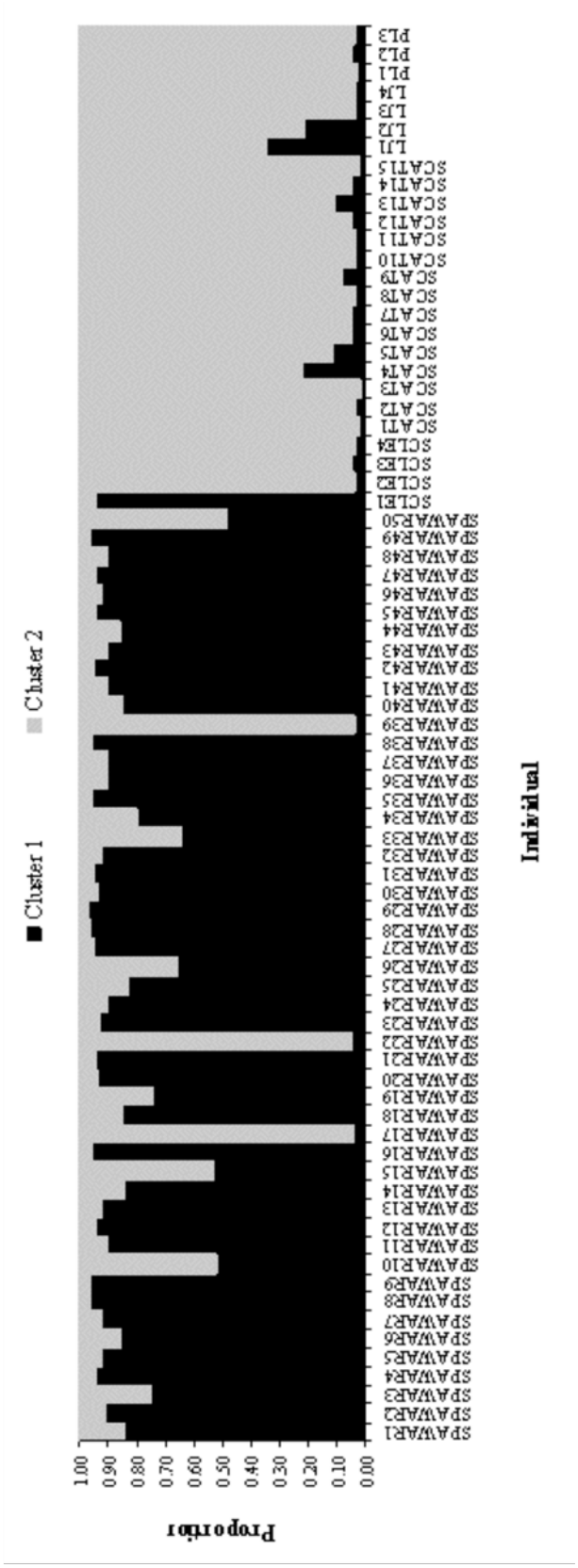

Figure 2.2: Cluster analysis on *H. fulgens* samples. Shading indicates proportion of an individual multilocus microsatellite genotype belonging to either Cluster 1 (black) or Cluster 2 (grey).

Table 2.1: Basic statistical information regarding the *Hka*28 and *Hka*56 microsatellite loci in *H. fulgens*.

Locus	Repeat	N	Alleles	H_e	H_o	p	Null
***Hka* 28**	$(CA)_n$	73	10	0.68	0.75	0.06	0.00
***Hka* 56**	$(CA)_n$	76	12	0.75	0.82	0.95	0.02

Table 2.2: Microsatellite allele frequencies for *H. fulgens* samples shown for (a) *Hka*28 and (b) *Hka*56, and COI haplotype occurrences shown in (c).

(a)

Sample	Allele									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
SPAWAR	0.04	–	–	–	0.54	–	0.08	0.34	–	–
SCLE	0.38	0.13	–	–	0.25	–	–	0.13	–	0.13
SCAT	0.10	0.13	0.03	0.10	0.33	0.17	0.07	0.03	0.03	–
LJ	–	–	0.13	–	0.13	0.38	0.25	–	0.13	–
PL	0.17	–	–	–	–	0.17	0.33	–	0.33	–

(b)

Sample	Allele											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
SPAWAR	–	0.26	–	–	–	0.36	0.23	0.13	0.02	–	–	–
SCLE	–	0.50	–	0.17	–	–	0.17	0.17	–	–	–	–
SCAT	0.10	0.30	0.07	0.13	0.17	0.10	–	–	0.03	0.03	0.03	0.03
LJ	–	0.17	–	–	–	0.33	0.33	–	–	0.17	–	–
PL	–	0.50	–	–	0.25	–	–	0.25	–	–	–	–

(c)

Haplotype	Sample					Total
	SPAWAR	**SCLE**	**SCAT**	**LJ**	**PL**	
1	19	3	10	4	3	39
2	-	-	1	-	-	1
3	-	-	1	-	-	1
4	-	-	2	-	-	2
5	-	1	-	-	-	1
Total	19	4	14	4	3	44

Table 2.3: Pairwise relatedness (*r*) among *H. fulgens* individuals within samples. Mean and the median pairwise *r* and the variance for each sample are presented. Last row includes significance results for a *t*-test on the mean of each sample distribution vs. the mean of the SPAWAR sample distribution.

Statistic	**Sample**					
	SPAWAR	**All wild**	**SCLE**	**SCAT**	**LJ**	**PL**
Mean	0.37	0.13	0.08	0.14	0.25	0.33
Median	0.44	0.00	0.00	0.00	0.25	0.50
Variance	0.08	0.05	0.04	0.05	0.07	0.08
p	-	0.00	0.02	0.00	0.31	0.83

References

Allendorf FW, Phelps SR (1980) Loss of genetic variation in a hatchery stock of cutthroat trout. *Transactions of the American Fisheries Society*, 109, 537-543

Allendorf, F.W., 1986. Genetic drift and the loss of alleles versus heterozygosity. *Zoo Biology*, 5, 181-190

Bilodeau AL, Felder DL, Neigel JE (2005) Population structure at two geographic scales in the burrowing crustacean *Callichirus islagrande* (Decapoda, Thalassinidea): historical and contemporary barriers to planktonic dispersal. *Evolution*, 59, 2125-2128

Bravington MV, Ward RD (2004) Microsatellite DNA markers: evaluating their potential for estimating the proportion of hatchery-reared offspring in a stock enhancement programme. *Molecular Ecology*, 13, 1287-1297

Chapman G, Denton D, Lazorchak J (1995) Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms. U.S. Environmental Protection Agency, Washington, D.C., EPA/600/R-95/136 (NTIS PB96261665)

Colson I, Hughes RN (2004) Rapid recovery of genetic diversity of dogwhelk (*Nucella lapillus* L.) populations after local extinction and recolonization contradicts predictions from life-history characteristics. *Molecular Ecology*, 13, 2223-2233

Cross TF, King J (1983) Genetic-effects of hatchery rearing in Atlantic salmon. *Aquaculture*, 33, 33-40

Evans B, Barlett J, Sweijd N, Cook P, Elliot NG (2004) Loss of genetic variation at microsatellite loci in hatchery produced abalone in Australia (*Haliotis rubra*) and South Africa (*Haliotis midae*). *Aquaculture*, 233, 109-127

Gruenthal KM, Burton RS (2005) Genetic diversity and species identification in the endangered white abalone (*Haliotis sorenseni*). *Conservation Genetics*, 6, 929-939

Kalinowski ST, Taper ML (2006) Maximum likelihood estimation of the frequency of null alleles at microsatellite loci. *Conservation Genetics*, 7, 991-995

Lapota D, Rosen G, Chock J, Liu CH (2000) Red and green abalone seed growout for reseeding activities off Point Loma, California. *Journal of Shellfish Research*, 19, 431-438

Li Q, Park C, Kijima A (2003) Allelic transmission of microsatellites and application to kinship analysis in newly hatched Pacific abalone larvae. *Fisheries Science*, 69, 883-889

Nei M (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences of the USA*, 70, 3321-3323

Nei M, Maruyama T, Chakraborty R (1975) The bottleneck effect and genetic variability in populations. *Evolution*, 29, 1-10

Nei M (1987) Molecular Evolutionary Genetics. Columbia University Press, New York, New York

Peakall R, Smouse PE (2006) GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6, 288-295

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, 155, 945-959

Raymond M, Rousset F (1995) GENEPOP: population genetics software for exact tests and ecumenicism. *Journal of Heredity*, 86, 248-249

Raymond M, Rousset F (2003) GENEPOP (version 3.4): population genetics software for exact tests and ecumenicism. Updated from Raymond and Rousset (1995)

Rozas J, Sanches-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, 19, 2496-2497

Ruzzante DE (1998) A comparison of several measure of genetic distance and population structure with microsatellite data: bias and sampling variance. *Canadian Journal of Fisheries and Aquatic Sciences*, 55, 1-14

Ryman N, Stahl G (1980) Genetic changes in hatchery stocks of brown trout (*Salmo trutta*). *Canadian Journal of Fisheries and Aquatic Sciences*, 37, 82-87

Schneider S, Roessli D, Excoffier L (2000) Arlequin ver.2.000: A software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland

Schug MD, Hutter CM, Noor MAF, Aquadro CF (1998) Mutation and evolution of microsatellites in *Drosophila melanogaster*. *Genetica*, 102-103, 359-367

Sekino M, Takahiro S, Fujita T, Kobayashi T, Takami H (2005) Microsatellite DNA markers of Ezo abalone (*Haliotis discus hannai*): a preliminary assessment of natural populations sampled from heavily stocked areas. *Aquaculture*, 243, 33-47

Smith PJ, Conroy AM (1992) Loss of genetic variation in hatchery-produced abalone, *Haliotis iris*. *New Zealand Journal of Marine and Freshwater Research*, 26, 81-85

Travis J, Coleman FC, Grimes CB, Conover D, Bert TM, Tringali M (1998) Critically assessing stock enhancement: an introduction to the Mote Symposium. *Bulletin of Marine Science*, 62, 305-311

Tringali M, Bert TM (1998) Risk to genetic effective population size should be an important consideration in fish stock-enhancement programs. *Bulletin of Marine Science*, 62, 641-659

Utter F (1998) Genetic problems of hatchery-reared progeny released into the wild, and how to deal with them. *Bulletin of Marine Science*, 62, 623-640

CHAPTER III

Genetic structure of natural populations of California red abalone (*Haliotis rufescens*) using multiple genetic markers

Abstract

Mitochondrial cytochrome oxidase subunit one (COI) sequence, nuclear microsatellites, and amplified fragment length polymorphisms (AFLPs) were used to evaluate connectivity among nine red abalone (*Haliotis rufescens*) populations sampled between August 1998 and November 2003 along approximately 1300 kilometers of California coastline from Crescent City (41°46'N, 124°12'W) to San Miguel Island (34°02'N, 120°22'W). COI sequences and microsatellite genotypes did not show significant genetic divergence among nine sampled populations. A subset of five populations spanning the geographic range of the study was scored for 163 polymorphic AFLP markers. Of these, 41 loci showed significant divergence ($p < 0.001$) among populations. Still, no AFLP markers were diagnostic for any of the study populations, and assignment tests did not consistently assign individuals to the correct population. Although the AFLP data are the first to suggest there is significant genetic differentiation among California red abalone populations, the discordance between the different genetic markers needs further study before unambiguous conclusions can be drawn with respect to connectivity among the populations.

Introduction

Natural history considerations fail to make clear predictions regarding population connectivity in the red abalone (*Haliotis rufescens*), an ecologically important, broadcast spawning marine gastropod. Ocean currents suggest that dispersal among populations of this and other marine species may occur over long geographic distances (Waples 1998). Red abalone spawn from October through February in northern California and year-round in southern California (CDFG 2005), leaving their pelagic larvae subject to diverse coastal current systems. Even with a relatively short pelagic phase at 4 – 15 days, larvae entrained in the north-flowing Davidson Current of the California Current system could be transported at 0.2 – 0.3 m s^{-1} in January (Glickman 1999), theoretically traveling as far as 350 km, nearly one quarter of the California coastline. In addition, although red abalone larvae are competent to settle in four days at 18°C, an 8 – 18°C temperature range is tolerated (Leighton 2000), and cooler water temperatures (hence, longer dispersal times) are typical of winter months when the bulk of the species' spawning takes place.

Despite the potential for extensive dispersal, habitat characteristics and other early life-history traits favor local retention of abalone larvae. Adults are predominantly found in or near rocky macroalgal communities where water flow is attenuated by kelp (Gaylord et al. 2004), and abalone eggs are negatively buoyant (Moffet 1978; Leighton 2000). Furthermore, the potential for predation and starvation (abalone larvae are lecithotrophic) increases and the probability of encountering suitable habitat decreases the longer the larvae remain in the plankton. Although little is known about the capabilities of larvae to select specific habitats, larval settlement occurs in response to

chemical inducers such as the phycobilins produced by crustose coralline red algae (Morse et al. 1979; Morse and Morse 1984; Leighton 2000). Crustose corallines are found commonly in rocky low intertidal and subtidal zones that are often coincident with the macroalgal habitat where adult abalone are found, again suggesting a mechanism favoring local larval retention.

Past genetic research on the red abalone has indicated little or no differences in allelic frequencies among populations, consistent with high gene flow and exchange of migrants. Kirby et al. (1998) describe results for a single microsatellite locus genotyped in 74 individuals randomly selected from northern and southern California. Although the authors note that several rare alleles were unique to either the northern or southern California samples, no statistically significant genetic differences were observed. Burton and Tegner (2000) genotyped three allozyme loci and partially sequenced the mitochondrial cytochrome oxidase subunit one (COI) gene in samples from three California locales (two from northern California and one from San Miguel Island) to assess whether individuals from a 1979 outplanting of red abalone on San Miguel Island persisted through the following two decades as suggested by Gaffney et al. (1996). No genetic signal from the 1979 outplanting was evident, and allele frequencies and genic diversity did not differ among the samples.

We expand on the limited scope of previous work by analyzing the genetic connectivity among red abalone populations using a combination of nuclear and mitochondrial loci. We use mtDNA sequences, microsatellite polymorphisms, and amplified fragment length polymorphisms (AFLPs) to increase resolution of gene flow among red abalone populations. Geographically, our sample range starts near the

Oregon, USA, border at Crescent City (41°46'N, 124°12'W), California, and spans approximately 1300 kilometers of California coastline, ending at San Miguel Island (34°02'N, 120°22'W) in the Southern California Bight. This encompasses over half of the species' range, which extends from northern California, USA, to central Baja California, Mexico (Geiger 2000).

Materials and Methods

Samples and DNA Extraction

Samples of red abalone tissue collected and archived between the years 1998 and 2003 from nine locations ($N = 474$) were obtained from the California Department of Fish and Game (CDFG) and L Rogers-Bennett at the University of California, Davis. Although abalone densities are > 8000 per hectare in some northern California locales (Rogers-Bennett et al. 2004), samples south of San Francisco Bay (SFB) are limited by low natural abundances. Collection sites, including sample sizes and site abbreviations, are shown in Figure 3.1 (see also Table 3.1 for sample collection information). From north to south along the California coast, sites included Crescent City, Trinidad Head, Shelter Cove, 7 House Cove near Caspar, Van Damme State Park, Salt Point State Park, Horseshoe Cove, Monterey, and San Miguel Island. A second independent sample of smaller abalone was obtained in 2001 from Van Damme State Park to examine sampling effects on genetic composition.

Epipodial tentacle or foot clippings were preserved in ethanol or water and stored at -80ºC. DNA was extracted from approximately 25-mg of tissue using a DNeasy Tissue Kit (Qiagen Inc., Valencia, California) and eluted in 200-µl of TE buffer (10-mM Tris pH 8.0, 1-mM EDTA). A 5-µl aliquot of the stock DNA was diluted with 45-µl of DNA-grade water, placed in 96-well trays, and stored at 4ºC for use in the polymerase chain reaction (PCR).

Cytochrome Oxidase Subunit One (COI)

A 580 basepair (bp) fragment of the mitochondrial COI gene was amplified from approximately 30 abalone per population ($N = 309$). Polymerase chain reaction (PCR) primers and cycling conditions were taken from Metz et al. (1998). Reactions were carried out in 25-µl volumes, including 5-µl of dilute template DNA, 0.5-U REDTaq DNA polymerase (Sigma-Aldrich Corporation, St. Louis, Missouri), 2.5-µl 10X PCR buffer, 2.5-mM $MgCl_2$ final concentration, 2-mM each dNTP final concentration, and 5-pmoles each primer. The amplified fragments were sequenced on a MegaBACE™ 500 DNA sequencer (Amersham Biosciences Corp., Piscataway, New Jersey) using the forward primer AB-COIF and a DYEnamic™ ET dye terminator sequencing kit (Amersham Pharmacia Inc., Piscataway, New Jersey). Sequences were aligned and edited to 483-bp using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, Michigan).

A UPGMA dendrogram was generated in PAUP* 4.0b10 (Swofford 1998) and visualized with TREEVIEW (Page 1996) to define the COI haplotypes. To evaluate levels of genetic structure, exact tests of differentiation (Markov chain parameters: 1000 dememorization steps, 10000 permutations) and an hierarchical analysis of molecular variance (AMOVA: 1000 permutations) were performed in Arlequin v2.000 (Schneider et al. 2000) on the haploid COI sequence data. Significance of pairwise Φ_{ST} estimates was calculated based on χ^2 tests of the parameter value being significantly different from zero (i.e. no genetic divergence; $\chi^2 = 2N\Phi_{ST}$, df = 1). Results were sequential Bonferroni corrected at the $\alpha = 0.05$ level (Rice 1989). A COI haplotype network (Figure 3.2) that takes into account both distance between haplotypes and haplotype frequency was generated using parsimony in the program TCS v1.18 (Clement et al. 2000). The hierarchical clades of the network were described topologically according to Templeton

et al. (1995). Nested clade analysis (NCA) was performed on each cladistic level in order to find geographic structure among the haplotypes using the program GeoDis 2.2 (Posada et al. 2000).

Microsatellites

Genotyping was attempted at five microsatellite loci in all 474 red abalone. Basic information regarding the microsatellite loci is presented in Table 3.2. Loci included one compound tri-/tetranucleotide repeat (*Hka3*) and four dinucleotide repeats (*Hka*28, *Hka*40, *Hka*56, and *Hka*80). PCR primers used were originally developed for a related eastern Pacific species, the pinto abalone *H. kamtschatkana* (Miller et al. 2001). Reactions were carried out in 25-µl volumes, including 5-µl of dilute template DNA, 0.5-U REDTaq DNA polymerase, 2.5-µl 10X PCR buffer, 2.5-mM $MgCl_2$ final concentration, 2-mM each dNTP final concentration, and 5-pmoles each primer. Forward primers were 5' end-labeled with one of three dyes HEX, TET, or 6-FAM for fluorescent visualization. Cycling conditions were modified slightly from Miller et al. (2001) for optimization.

Following amplification, 2-µl of a denaturing solution containing xylene cyanol and bromophenol blue dyes in 99% formamide were added to each 5-µl of PCR product. Samples were loaded onto vertical 0.4-mm thick large format (33 x 39 cm) 6% polyacrylamide denaturing gels containing 33% formamide and 7-M urea. ROX-labeled MegaBACE™ ET900-R Size Standard (Amersham Pharmacia Inc., Piscataway, New Jersey) or Genemark 350 Fluorescent DNA Ladder (Northern Biotechnology, Schofield, Wisconsin) was run concurrently as a size standard. Gels were electrophoresed at 60-

Watts for two to three hours and scanned on a Typhoon 9410 Variable Mode Imager (Molecular Dynamics Inc., Sunnyvale, Califonia). *Hka*3 allele sizes were scored using FragmeNT Analysis v1.2 software (Molecular Dynamics Inc., Sunnyvale, California). *Hka*28, *Hka*40, *Hka*56, and *Hka*80 allele sizes and AFLP banding patterns were viewed with ImageQuaNT software (Molecular Dynamics Inc., Sunnyvale, California) and scored by hand.

A maximum-likelihood estimate of the inferred frequency of the null allele was calculated for each locus using ML-NullFreq (Kalinowski and Taper 2006). Global and pairwise exact tests of genotypic differentiation (Markov Chain parameters: 10000 dememorization steps, 1000 batches, 10000 permutations per batch) were performed in GENEPOP v3.4 (Raymond and Rousset 1995, 2003). GENEPOP was also used to calculate estimates of F_{ST} and the F_{ST} analogue ρ_{ST} (which takes into account microsatellite allele size assuming the stepwise mutation model (Weir and Cockerham 1984; Slatkin 1995; Rousset 1996; Michalakis and Excoffier 1996)). Estimates of Φ_{ST} were calculated via AMOVA in Arlequin. Levels of significance were again calculated based on χ^2 tests of the parameter value being significantly different from zero ($\chi^2 = 2N\rho_{ST}$, df = 1), and results were sequential Bonferroni corrected at the $\alpha = 0.05$ level.

Amplified Fragment Length Polymorphisms (AFLPs)

AFLP analyses were limited to a subset of 209 red abalone chosen to represent the full breadth of our geographic sampling effort; the full sample sets were included for CC, 7HC, HSC, MY, and SMI (minus 11 SMI DNAs that failed repeatedly in PCR; see also Figure 3.1 for sample locations and abbreviations). Time constraints associated with

scoring the allele banding patterns by hand prevented scoring AFLPs in all samples genotyped by mtDNA sequencing and microsatellites. Protocols were performed according to Vos et al. (1995). Multiple pairs of restriction enzymes were tested on two individuals per population; *Eco*RI and *Mse*I were found to give bands in the appropriate size range (≤ 2000 bp). After ligation of adapters complementary to the restriction sites, all sixteen possible pre-amplification primer combinations were tested. Pre-amplification products were diluted 10-fold with 10-mM Tris-EDTA pH 8.0 for use in the final amplification step. *Eco*RI amplification primers were 5' end-labeled with the fluorescent dye HEX for visualization.

Both pre-amplification and amplification reactions were carried out in 20 μL volumes, including 5-μl of template (concentrated stock DNA or pre-amplification product), 1-U MasterTaq DNA polymerase (Eppendorf AG, Hamburg, Germany), 1X Taq Buffer, 1X TaqMaster PCR Enhancer, 2-mM each dNTP final concentration, 6-pmoles of *Mse*I primer, and 6-pmoles *Eco*RI pre-amplification primer or 1-pmole of *Eco*RI amplification primer. Touchdown PCR cycling conditions for the pre-amplification included an initial two minute 95°C denaturation followed by 10 cycles of 95°C for 30-s, 50°C for 30-s (-1ºC per cycle), and 72°C for 30-s. The annealing temperature for the remaining 25 cycles was 40ºC. A touchdown protocol was also used for the final amplification, but initial annealing temperatures were raised to 56°C or 58°C depending on the primer combination. Final amplification primer combinations chosen are presented in Table 3.3.

Following amplification, 1-μl of a denaturing solution containing xylene cyanol and bromophenol blue dyes in 99% formamide were added to each 5-μl of PCR product.

Samples were loaded onto vertical 0.4-mm thick large format (33 x 39 cm) 7.5% polyacrylamide denaturing gels containing 33% formamide and 7-M urea, electrophoressed (3-4 hours, 60-Watts), and scanned as with the microsatellite gels.

Initial calculations of divergence assumed HWE and were based on allele frequency differences; corrected allele frequencies were calculated in TFPGA v1.3 (Miller 1997) using the square root of the frequency of the null (recessive) genotype. TFPGA was also used to estimate per-locus global F_{ST} values (with jackknifing over the loci) and perform exact tests of global and pairwise differentiation (Markov Chain parameters: 2000 dememorization steps, 20 batches, 5000 permutations per batch). An AMOVA was performed in the Microsoft ® Excel add-in program GenAlEx 6 (Peakall and Smouse 2006) to calculate estimates of the F_{ST} analogue Φ_{PT} ($\equiv \Phi_{ST}$). All results were sequential Bonferroni corrected at the $\alpha = 0.05$ level. A UPGMA cladogram was generated in TFPGA with 5000 bootstrap replicates to determine the topological relationships among the five populations.

An outlier test of selection was conducted on our AFLP loci to establish whether the pattern of divergence seen in the AFLPs may be due primarily to neutral variation in allele frequencies or directional selection on the loci or linked regions of the genome. The program fdist2 (Beaumont and Nichols 1996) was used as in Murray and Hare (2006). Simulations of neutral F_{ST} under mutation-drift equilibrium were run using the infinite alleles model for 100 demes (islands), 5 populations, sample sizes of 50, 50000 realizations (loci), and an expected F_{ST} equivalent to the global F_{ST} value estimated above ($F_{ST} = 0.035$). The medians and upper and lower 99^{th} quantiles (percentiles) were

calculated for the simulated F_{ST} data, and the simulated and actual per locus F_{ST} data were plotted against the inferred heterozygosity (Figure 3.4).

Assignment tests were performed in AFLPOP 1.1 (Duchesne and Bernatchez 2002) using all 163 AFLP loci. In this analysis, no correction was made for dominance; each individual's genotype consisted of the presence/absence of the 163 amplified fragments. Each individual was removed in turn from the data set and allele frequencies were recalculated across the loci. Log-likelihoods (lnL) were then estimated for each individual as a member of each candidate population, based on the likelihood that the genotype matched the allele frequency profile of a candidate population better than other candidate populations (with a specified difference in minimum acceptable lnL (lnL_{min})).

An evaluation of the spatial distribution of individuals within populations was performed using the multivariate technique principle coordinates analysis (PCA) option in GenAlEx. A pairwise genetic distance matrix was generated from the binary data file, where the total pairwise distance between individuals is the sum of the pairwise distances for each locus (either one or zero). The PCA was performed directly on the matrix after distances were standardized by dividing each by $(n - 1)^{-2}$, where n is the number of pairwise comparisons.

AFLP Reproducibility Test

A set of the first 12 individuals from the northern California and southern California populations ($N = 24$) were subjected to a complete replication of the AFLP protocol for 11 markers from the first four primer combinations listed in Table 3.3. The presence or absence of product bands was independently rescored by hand, and the

genotypes were compared between the original scoring and the rescoring. Of 264 genotypes, five (< 2%) were scored differently between runs. Gels were visually identical in side-by-side comparisons, so variability was due to scoring error and not irreproducibility in the AFLP assay.

Validation of Genetic Markers

No breeding experiments were carried out to assess the genetic bases of the markers employed in this study. However, we have previously confirmed the maternal inheritance of mtDNA and the Mendelian inheritance of microsatellite polymorphisms in the closely related white abalone (*H. sorenseni*), using is same genetic markers in laboratory crosses (Gruenthal and Burton 2005). Although we have no family data on AFLPs, Liu et al. (2006) found that 95% of 387 AFLP markers exhibited normal Mendelian segregation in the Pacific abalone (*H. discus hannai*).

Results

COI

In 483-bp of partial COI sequence obtained for 309 red abalone, 43 haplotypes resulting from 39 individual mutations were observed (Figure 3.2; see also Table 3.4 for haplotype counts). Representative haplotype sequences were deposited in GenBank under the accessions DQ297507 – DQ297549. There was one common haplotype at a frequency of 0.38, and two additional haplotypes at mid-frequency (0.16 and 0.15). The frequencies of the remaining 40 haplotypes were ≤ 0.08. The variation resulted in five amino acid changes within the species (translation based on the invertebrate mitochondrial amino acid code; first codon started at basepair 3 of our 483-bp of sequence). Nonpolar to polar singleton changes occurred twice (Ala1Thr, Phe48Cys), but the remaining three low-frequency base changes did not alter polarity or charge (Ile30Met, Ala91Gly, Ala146Gly).

The mitochondrial data provided little evidence for population genetic structure in global or pairwise comparisons among populations (Table 3.5). Regardless of grouping, over 99% of variation was within populations rather than among populations or groups (global $\Phi_{ST} = 0.007$). There was no signal of variation between the two Van Damme samples, VD and VDJ. No significant association was found between haplotype and geographic location at any level; the clades appeared to be randomly distributed with respect to population samples.

Microsatellites

See Table 3.2 for basic statistical information. The compound repeat *Hka*3 locus contained 75 alleles in our sample. The remaining four dinucleotide repeat loci contained 26 – 36 alleles. There was no evidence for linkage disequilibrium (LD) between any loci in any of the populations after sequential Bonferroni correction at the table-wide $\alpha = 0.05$ level. Significant departures from Hardy-Weinberg equilibrium (HWE) due to heterozygote deficiency were found at *Hka*56 and *Hka*80 (Table 3.2). Null alleles, nonrandom mating, inbreeding, or temporal genetic variation of recruits are possible explanations (Miller et al. 2001). Missing genotypes at microsatellite loci were only scored as null homozygotes if the same DNA sample yielded scoreable results at other loci. Only *Hka*56 exhibited a high inferred null allele frequency at 0.31, which did not ultimately affect significance levels in our analyses. Analysis of all microsatellite loci combined revealed global differentiation ($p = 0.002$) but no pairwise divergence (Table 3.5) and no pattern of isolation by distance. Minor pairwise population divergence between VDJ and HSC was not significant after sequential Bonferroni correction. There was no significant variation in allele frequencies between the two Van Damme samples, VD and VDJ.

AFLPs

A total of 163 polymorphic loci from the eight final amplification primer combinations was scored in a subset of 209 abalone from five populations (CC, 7HC, HSC, MY, and SMI). A reproducibility test on a subset of abalone showed that there was < 2% error in independent scoring between runs. There were no fully diagnostic markers (private alleles) for any of the populations. There was no evidence for redundancy

among the loci. Uncorrected dominant band allele frequencies for the markers ranged from 0.041 – 0.994 in the full sample set. Inferred (corrected) allele frequencies at some loci varied by as much as 80% between populations.

Out of 163 markers, 41 (~25%) exhibited significant F_{ST} estimates based on inferred allele frequencies (all $p < 0.001$; data not shown). Only two loci (< 2%) that fell outside of the upper 99th quantile in the outlier test of selection were considered outliers potentially under selective pressure. Approximately 4% of variation was between populations in the AMOVA, and estimates of Φ_{ST} indicated a significant level of global divergence among the five populations with patchy pairwise divergence (Table 3.5). Global differentiation over all five samples was significant as well ($\chi^2 = 1032$, df = 326, $p < 0.001$), and nine of ten pairwise comparisons were highly significant (all $p < 0.001$). The tenth comparison (7HC vs. HSC) was borderline significant ($p = 0.055$). In the cladogram, distances were small and no geographic structure was apparent, but the branches were well-supported, suggesting that the populations are genetically distinct (Figure 3.3).

In assignment tests, only MY was successfully reassigned at a rate of 0.792 (Figure 3.5). Even when the $\ln L_{min}$ was dropped to $\ln L_{min} = 0.001$, the remaining populations only reassigned at rates of between 0.257 (SMI) and 0.580 (CC). The success of these global assignment tests was generally low, but specific pairwise assignment tests with the MY population fared better. For example, comparing the two southern populations, SMI vs. MY, yielded correct assignment of 0.742 and 0.833 of the individuals, respectively. In principle coordinates analysis, despite great overlap among

the populations, MY was closely clustered in space, consistent with the results of the assignment tests (Figure 3.6).

Although available resources limited the number of abalone populations subjected to AFLP analysis, the higher level of population differentiation observed for AFLP markers relative to COI and microsatellites was not a result of the difference in population sampling. The five populations sampled for AFLPs spanned the full geographic range of the study, and when COI and microsatellite data sets were reanalyzed in the truncated set of five populations, global divergence at COI was not significant ($\Phi_{ST} = 0.010$). Global divergence at the microsatellite loci was also not significant ($\rho_{ST} = -0.003$).

Discussion

In studies of species with high potential gene flow such as broadcast spawning marine invertebrates (Strathmann 1985; Hedgecock 1994; Waples 1998), the power of genetic analyses for detecting population structure can be increased by using both various types and greater numbers of genetic markers. Prior, small-scale projects typing a nuclear microsatellite locus (Kirby et al. 1998), mtDNA sequence, and allozyme loci (Burton and Tegner 2000) in red abalone found no significant genetic differences among populations, consistent with high interpopulation gene flow. Results presented here, including more extensive analyses of mtDNA sequence (expanded sample size compared to previous studies) and microsatellite loci (increased number of loci and increased sample size), are consistent with the earlier work: the hypothesis of population homogeneity on a broad geographic scale could not be rejected based on these genetic markers (although global microsatellite differentiation hinted at the possibility).

In addition to mtDNA sequence and microsatellite data, we present the results of the first AFLP study on red abalone. Despite their widespread use on other organisms (e.g. plants and bacteria), application of AFLP methods to animal populations in general and marine animal populations in particular has been limited (Bensch and Åkesson 2005). However, several recent investigations suggest that AFLP methods provide an efficient approach to increasing the power of genetic analyses of population structure (Baus et al 2005; Brazeau et al 2005; Shank and Halanych 2007; Sønstebø et al. 2007; Weetman et al. 2007). We found that AFLP protocols offered a reliable and efficient method for scoring genetic variation at 163 loci, presumably spread genome-wide (Vos et al. 1995; Mueller and Wolfenbarger 1999; Campbell et al. 2003; Avise 2004). Using

AFLPs, we expanded the number of markers in the red abalone by nearly 70-fold with respect to earlier studies and gained power for detecting significant population genetic divergence. In fact, 25% of the AFLP markers revealed significant differentiation among populations.

The nature of different genetic markers dictates that different evolutionary forces may differentially impact each marker's respective levels of diversity within and between populations. For example, because mtDNA is maternally inherited, it experiences reduced effective population size (potentially increasing random genetic drift) and sex-specific gene flow (only female dispersal impacts mtDNA connectivity measures). Hence, it is not surprising that several studies have found that different genetic markers give contradictory results with regard to population structure (e.g. Karl and Avise 1992; Shank and Halanych 2007). Our finding of population differentiation at AFLP loci but not with other molecular markers is consistent with some recent studies of marine organisms such as the hydrothermal vent tubeworm *Riftia pachyptila* (Shank and Halanych 2007) and the shrimp *Crangon crangon* (Weetman et al 2007). It is important to note that AFLPs do not always find more pronounced population differentiation than other markers. Gomez-Uchida et al. (2003) found significant differentiation among *Cancer setosus* crab populations with allozymes, which was not observed in parallel AFLP analyses.

As individual markers, one significant weakness of AFLPs is that they are dominant markers (dominant homozygotes cannot be distinguished from heterozygotes). Although dominant markers convey less information than co-dominant markers, Campbell et al. (2003) have shown that the ability to score large numbers of markers can

compensate for the information lost at each dominant locus. A consequence of the dominant nature of AFLPs is that the data can be analyzed in different ways. Using an assumption of Hardy-Weinberg equilibrium (HWE), the frequency of the recessive "allele" (no band) can be calculated as the square root of the homozygous recessive frequency. These inferred frequencies can then be used in standard population genetic analyses. Similar approaches are common in analyses of microsatellite null alleles and were used in our own microsatellite analyses.

Is the assumption of HWE defensible? Although many marine populations show deviations from HWE at one or more gene loci, all three allozyme loci scored by Burton and Tegner (2000) in red abalone were found to be in HWE and three of five microsatellite loci scored in this study were in equilibrium (deviations at the other two are best explained by the presence of null alleles). Based on these observations, we do not believe that the assumption of HWE used in calculating AFLP allelic frequencies invalidates our general conclusions. Alternative methods of AFLP analysis do not make the assumption of HWE, and, instead, treat each AFLP band as a simple dominant (present/absent) character. This approach underlies the assignment tests and PCA analyses, which also showed evidence of population differentiation, especially with regard to the MY sample.

A second issue is that inconsistencies in banding intensity in this PCR-based method could potentially lead to inaccuracies in the data matrix. Where variation in banding intensity proves to be a significant issue, any suspect loci can simply be eliminated from the data set. For example, in the shrimp (*Crangon crangon*), relatively few AFLPs could be reliably scored (Weetman et al. 2007). In our own reproducibility

studies, scoring error was < 2%, suggesting that such problems were unlikely to impact subsequent analyses and interpretation. Furthermore, recent work on abalone using AFLPs found that the preponderance of loci scored showed normal segregation in families (Liu et al. 2006), again attesting to the validity of AFLP studies in abalone.

These AFLP data provide the first evidence for genetic differentiation among populations of red abalone along the California coast. F_{ST} estimates and exact tests of population differentiation were significant even after adjustment for multiple tests, clearly rejecting the hypothesis that red abalone populations are genetically homogeneous across the sampled locales. However, no easily discernable geographic patterns (e.g. isolation by distance) were apparent. The five population samples analyzed lie within the Oregonian biogeographic province and the Transition Zone between the Oregonian and Californian provinces. Cape Mendocino is a reputed phylogeographic break for marine species (Edmands 2001; Cope 2004), and our samples from north of the Cape showed significant divergence from the two northern California populations sampled south of the Cape (7HC and HSC, the only pair of populations that could not be distinguished by the AFLP markers).

Genetically, MY was the most distinctive sample. The observed genetic differentiation of the MY sample is consistent with observations by Marko (1998), Dawson and Jacobs (2001), and Dawson (2001), suggesting that the Monterey Peninsula is another phylogeographic break reinforced by hydrographic characteristics different from the open coast (see http://newark.cms.udel.edu/~brucel/realtimemaps/) that could result in larval retention (Sponaugle et al. 2002). In addition, while genetic homogeneity is maintained in the larger populations, a small population such as that found at MY (see

below) would be more responsive to differentiation due to random genetic drift. Although size-age relationships in abalone are not straightforward (Leighton 2000), the divergence at MY may also be due to narrower cohort structure (i.e. fewer year classes). The MY sample consisted of individuals of < 150-mm shell length (see Table 3.1), whereas several other samples encompassed larger size ranges. Unfortunately, our lack of cohort sampling prevented assessment of stochastic events such as sweepstakes recruitment (Hedgecock 1994). There was no difference between our two cohorts from Van Damme (VD, VDJ), but the result cannot be extrapolated sample range-wide.

Despite its geographic distance, our SMI sample was genetically more similar to the northern California populations than to MY. San Miguel Island is south of Point Conception, a biogeographic and disputed phylogeographic break (see Burton 1998; Dawson 2001). However, faunal/floral survey evidence suggests that SMI, on the outskirts of the Southern California Bight (SCB) and in the path of the south-flowing California Current, is ecologically more similar to northern California cold water habitats than warmer waters in the SCB (Clark et al. 2005; see also http://www.nps.gov/archive/chis/rm/HTMLPages/MarineResources.htm). Given the data in hand, we can only speculate that the SMI population has experienced relatively recent or on-going recruitment from northern populations. Further, surveys have indicated high natural abundances of red abalone throughout northern California and on SMI (populations perhaps less responsive to random genetic drift), while many central and southern California populations were historically less abundant and have been severely depleted or extirpated in recent decades due to overfishing and, at some locations, sea otter predation (CDFG 2005).

Patterns of connectivity can be obscured if the genetic markers employed are subject to selection (e.g. Karl and Avise 1992). We considered the possibility that the 41 markers exhibiting significant divergence among the red abalone populations were subject to higher levels of selection than the bulk of markers showing no genetic structure (see Lewontin and Krakauer 1973). To address a similar question, Murray and Hare (2006) used a mutation-drift equilibrium model to test for selection on AFLP loci across a secondary contact zone in two *Crassostrea virginica* oyster populations. Their results indicate that AFLPs can efficiently characterize the genomic distribution of F_{ST} and that equilibrium models can be used to evaluate outliers experiencing divergent selection. Our results using this same model suggested that < 2% (2 of 163) of our AFLP loci may be under directional selection (i.e. the majority of the allelic frequency variation fits the neutral model), similar to that found in other studies (Wilding et al. 2001; Campbell and Bernatchez 2004; Murray and Hare 2006). Even those two markers, however, could represent extremes in a broad distribution of neutral variation that is not well sampled (Murray and Hare 2006), a possible consequence of insufficient sampling of loci.

Assignment test results also suggested that we may have genotyped too few AFLP loci to successfully identify source populations for individual abalone. Unfortunately, despite considerable effort, we were unable to obtain population samples from additional southern California sites to further investigate the possibility that region-wide assignment tests might be more successful in central and southern California where geographic distances among extant populations might be greater than in northern California. Campbell et al. (2003) pointed out that larger numbers of loci are required when larger numbers of weakly differentiated populations are used in the tests. Further work is

needed to explore the possibility that additional AFLP markers will enhance assignment success. Although we have substantially increased our total marker set for this species, an additional 100+ markers may be required to successfully assign a high proportion of individuals to the proper population (Campbell et al. 2003).

Understanding the genetic structure of a long-lived species with population admixture can be facilitated by sampling specific recruitment events or size/age cohorts (Flowers et al. 2002; Hellberg et al. 2002). Unfortunately, we were not able to obtain such samples for red abalone; all of our samples were composed of multiple year classes and they were collected in different years. Few abalone larvae are ever seen in the field (Prince et al. 1987) and juvenile abalone are cryptic, hiding under other biota (e.g. urchins) or rocks and in crevices (Leighton 2000; CDFG 2005). Abalone recruitment modules (ARMs) and baby abalone recruitment traps (BARTs) consisting of cinder block "habitat" in a wire cage have been set up along the coast of California at several locations to monitor settlers (Davis et al. 1997), but they have met with limited success due to presumed recruitment failure and not failure of the devices (Rogers-Bennett et al. 2004; see also DeFreitas 2003). Even collecting adult samples was difficult in some locales; our total sample size south of SFB is small as most extant populations south of SFB have extremely low abundances (CDFG 2005). In fact, accidental resampling of individuals is a concern (L Rogers-Bennett pers. comm.); several red abalone from the Monterey area where samples have been collected over multiple years exhibited identical multilocus microsatellite genotypes (data not included in these analyses).

Recent evidence from other marine species indicates that homogeneity among populations of species with planktonic larvae may not be as common as previously

thought, and long-distance larval dispersal may be sporadic at best (Warner and Cowen 2002; Gilg and Hilbish 2003; Veliz et al. 2006; see also Sponaugle et al. 2002). World-wide, several abalone species have shown evidence of genetic subdivision. The blacklip abalone (*H. rubra*) from southern Australia exhibited divergence between populations from Victoria and Tasmania (Ward and Elliot 2001), and the perlemoen (*H. midae*) from South Africa shows regional divergence east and west of Cape Agulhas (Evans et al. 2004). Most recently, the tropical abalone (*H. asinina*) was shown to be divided into populations from the Gulf of Thailand and the Andaman Sea (Tang et al. 2005). Previous studies of abalone species along the Pacific coast of North America have revealed considerably less population divergence. Using allozyme electrophoresis, Hamm and Burton (2000) and Chambers et al (2006) have found some evidence for population structure in California black abalone (*H. cracherodii*). Withler et al. (2003) found little evidence for genetic structure in microsatellite allele frequencies in northern abalone (*H. kamtschatkana*), and, as discussed above, Burton and Tegner (2000) found no evidence for population divergence in the red abalone. As such, this study presents the first evidence of significant population differentiation in red abalone, but continued research is needed to further examine the spatial scale and temporal stability of genetic subdivision in this species.

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Chapter III was done in collaboration with Lauren K. Acheson, a former undergraduate researcher from the University of California, San Diego. Lauren performed DNA extractions and some microsatellite genotyping. Chapter III in full is a reprint of the material as it appears in Gruenthal KM, Acheson LK, Burton RS (2007) Genetic structure of natural populations of California red abalone (*Haliotis rufescens*) using multiple genetic markers. *Marine Biology*, DOI 10.1007/s00227-007-0771-4.

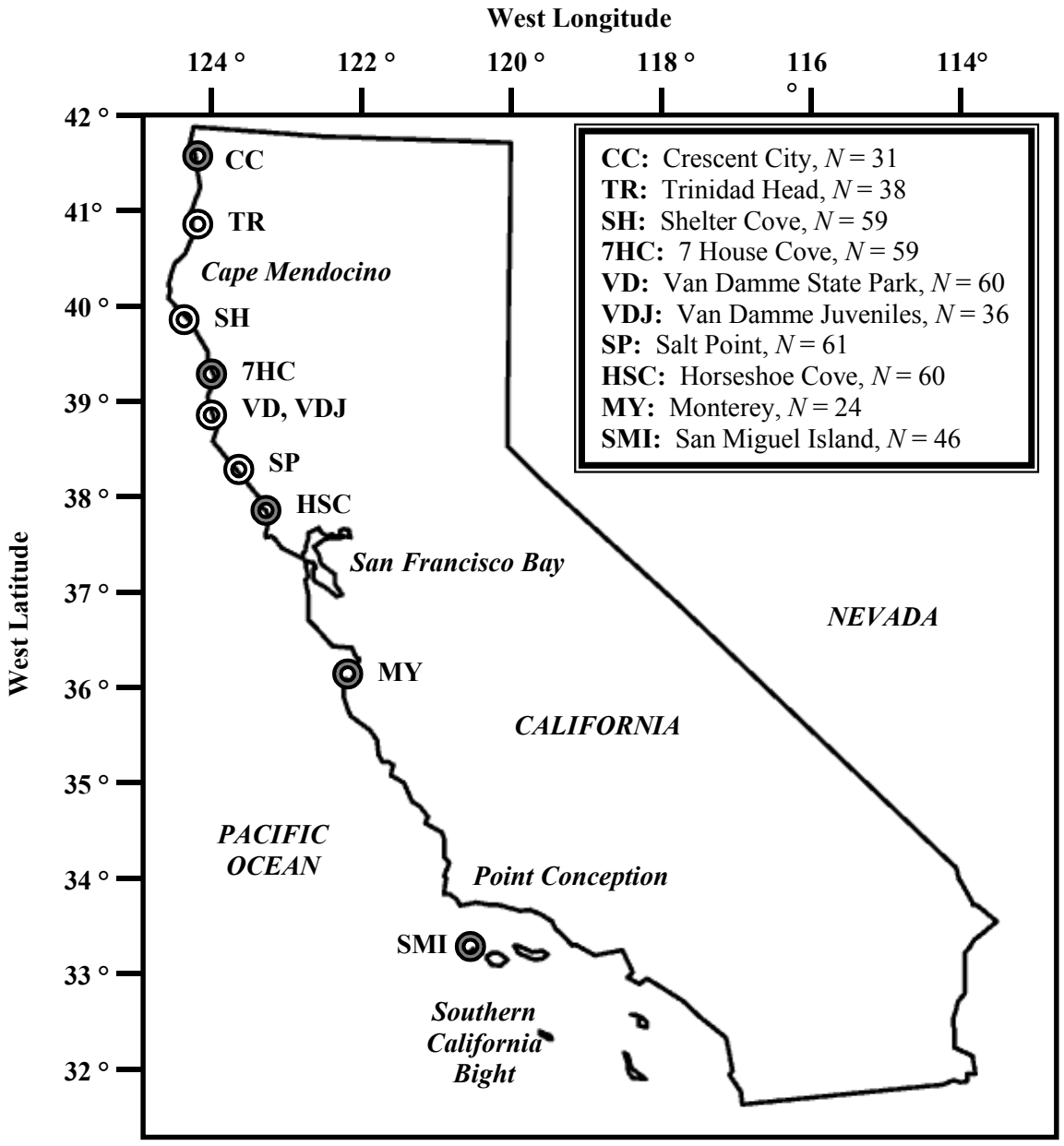

Figure 3.1: Map of California collection sites for *H. rufescens*. Key includes sample size and abbreviation. Filled circles indicate population used in AFLP analyses.

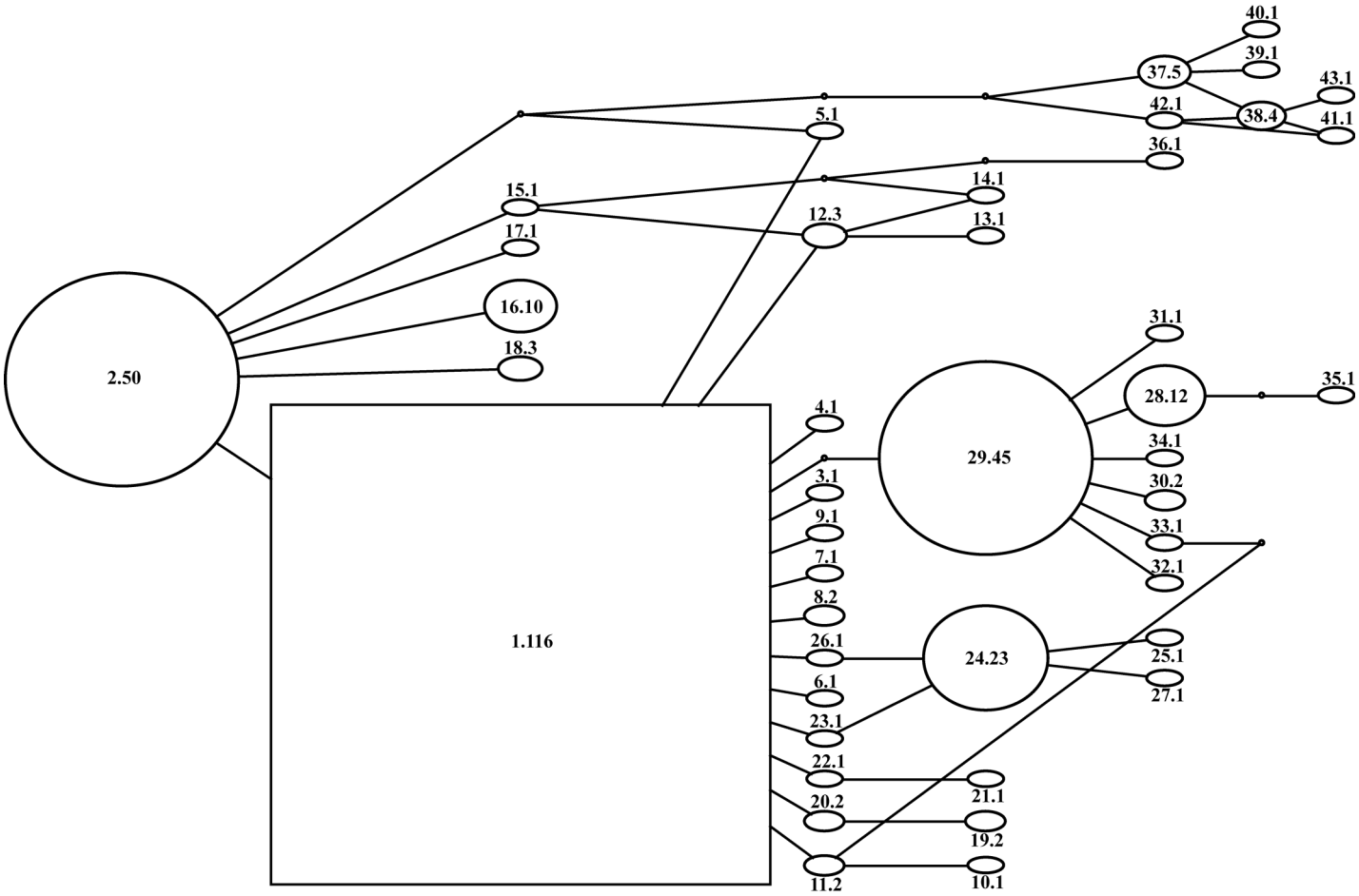

Figure 3.2: COI haplotype network for *H. rufescens* from nine California sites. Highest frequency haplotype represented with a square; size of the ovals represents relative frequency of remaining haplotypes. Ovals labeled with haplotype names, including haplotype number, followed by a period and number of times that haplotype occurred. Lines represent single nucleotide substitutions. Black dots indicate missing intermediate haplotypes.

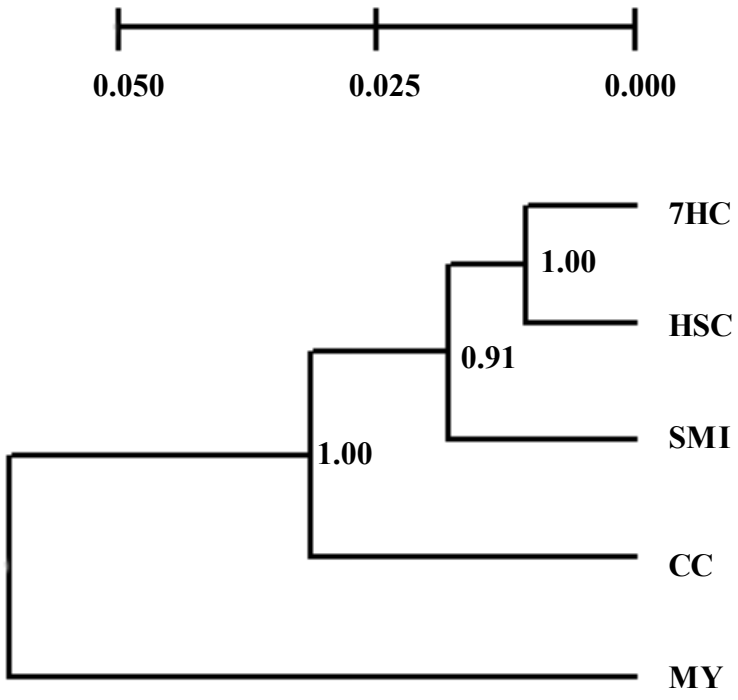

Figure 3.3: UPGMA dendrogram of AFLP genotyped *H. rufescens* samples. Branch lengths according to Nei's distance (1972). Branch support values based on 5000 bootstrap replicates.

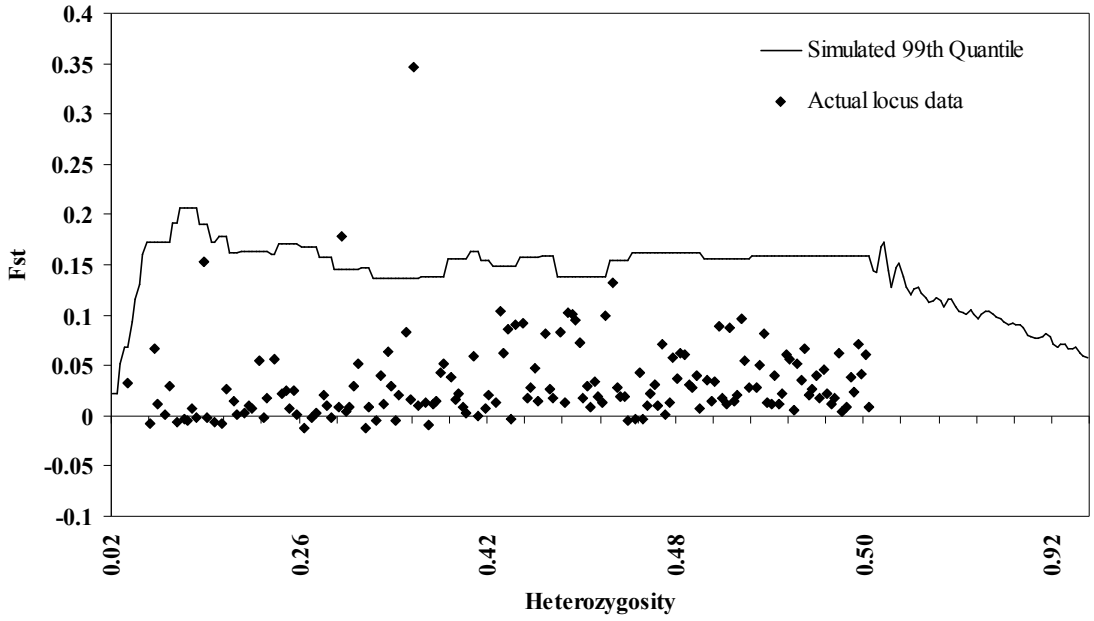

Figure 3.4: Graph of AFLP locus-specific F_{ST} estimates versus corrected heterozygosity used as an outlier test of selection on AFLP loci in *H. rufescens*. Actual data points (black diamonds) are presented for the loci. The upper 99th quantile for the loci (black line) is based on simulated data for 50000 loci. Points above the 99th quantiles are considered outliers.

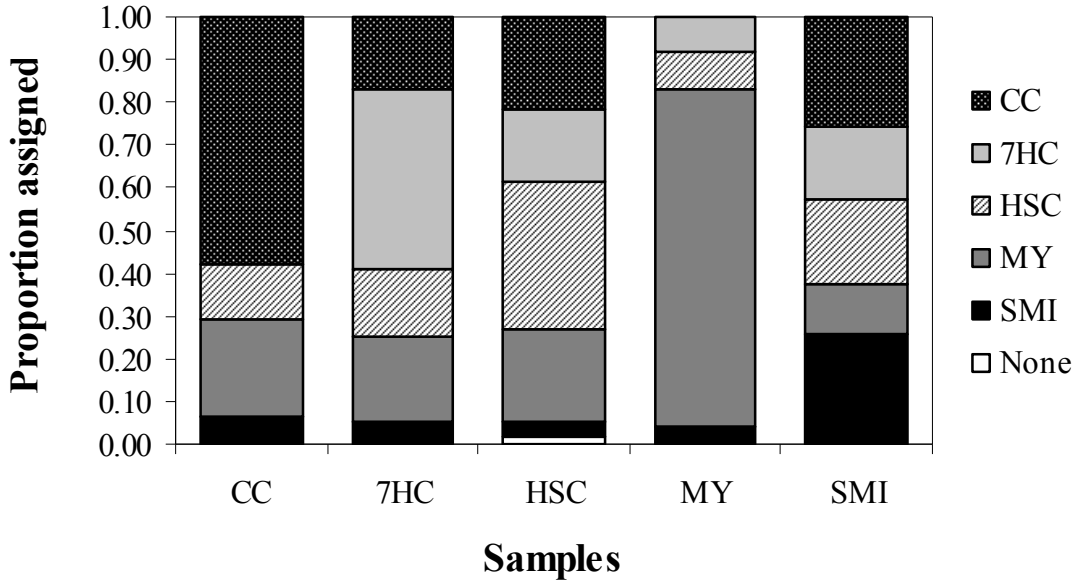

Figure 3.5: Assignment test allocation proportions for AFLP genotyped *H. rufescens* samples. Allocation rates based on a minimum log-likelihood difference of 0.001. Shading in vertical bars represents proportion of individuals in a population or group assigned to a population or group.

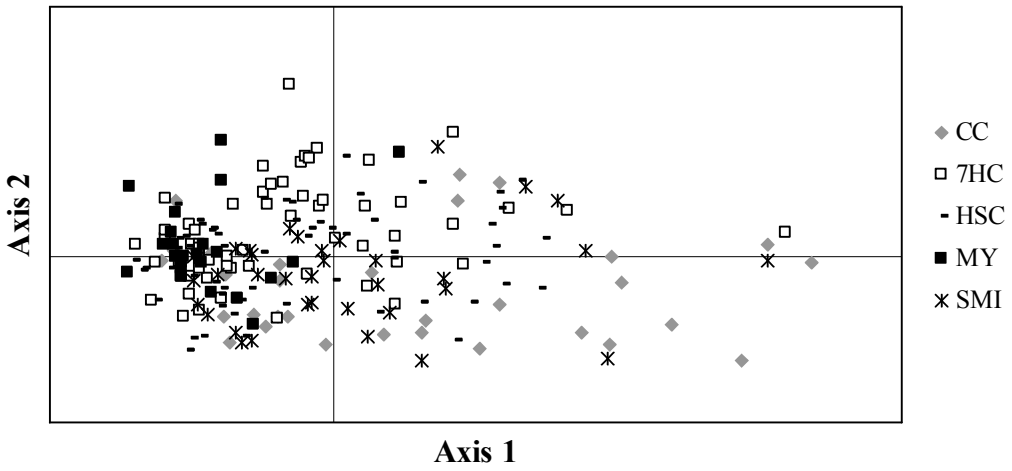

Figure 3.6: Principal coordinates analysis on pairwise genetic distances between *H.rufescens* individuals. All 163 AFLP markers were used in analysis. Plot offers maximum separation among populations. Coordinate exes 1 and 2 are shown.

Table 3.1: Sample collection information for *H. rufescens* from nine California sites ranging from Crescent City (CC) to San Miguel Island (SMI). For other site abbreviations see Figure 3.1. Samples were taken at Van Damme (VD) on two separate dates, and the juvenile samples from 2001 are labeled as VDJ.

Population	N	Date Collected	Shell Length (mm)
CC	31	18-Aug-99	144.0-241.0
TR	38	22-Oct-99	144.0-244.0
SH	59	18-Dec-98	130.8-205.0
7HC	58	27-Oct-99	21.4-190.0
VD	60	30-Sep-99	25.7-314.0
VDJ	36	2001	< 50
SP	61	25-Mar-00	115.2-211.8
HSC	60	18-Mar-99	150.0-226.0
MY	24	10-May-03	< 150
SMI	46	Jun-99	N/A

Table 3.2: Descriptive statistics for nuclear microsatellite loci in *H. rufescens*. Nucleotide repeat sequence, number of alleles, number of individuals genotyped out of 474 total (excludes null homozygotes), number of expected and observed heterozygotes, expected (H_e) and observed heterozygosities (H_o), Hardy-Weinberg equilibrium probability test p-values (p) with standard errors (S.E.) in parentheses, and null allele frequencies (Null) per locus are included. All microsatellite loci originally isolated in the northern abalone *H. kamtschatkana* (Miller et al. 2001).

Locus	**Repeat**	**Alleles**	**Individuals**	**H_e**	**H_o**	**p(S.E.)**	**Null**
Hka 3	$(GTA)_n(GAGT)_n$	75	445	0.96	0.90	0.06(0.02)	0.03
Hka 28	$(CA)_n$	29	429	0.88	0.86	0.67(0.04)	0.01
Hka 40	$(CA)_n$	28	448	0.91	0.91	0.60(0.04)	0.00
Hka 56	$(CA)_n$	26	401	0.80	0.42	0.00(0.00)	0.31
Hka 80	$(CA)_n$	36	396	0.83	0.73	0.00(0.00)	0.06

Table 3.3: AFLP final amplification primer combinations used on five *H. rufescens* samples. *Eco*RI core sequences consist of 5'-GACTGCGTACCAATTC-3' and *Mse*I core is 5'-GATGAGTCCTGAGTAA-3'. All markers chosen were polymorphic.

Primer combination	***Mse* I primer**	***Eco* RI primer**	**Approx. size range (basepairs)**	**Number of markers**
1	*Mse* I core + CCC	*Eco* RI core + CCC	110 - 225	10
2	*Mse* I core + CCC	*Eco* RI core + CGG	150	1
3	*Mse* I core + CCG	*Eco* RI core + CCC	90 - 310	5
4	*Mse* I core + CCG	*Eco* RI core + CGG	120 - 375	12
5	*Mse* I core + CTG	*Eco* RI core + ACC	195 - 425	41
6	*Mse* I core + CTG	*Eco* RI core + ACG	125 - 225	20
7	*Mse* I core + CTG	*Eco* RI core + AGC	150 - 350	35
8	*Mse* I core + CTG	*Eco* RI core + AGG	115 - 300	38

Table 3.4: COI haplotype counts per population sample for *H. rufescens* from nine California sites ranging from Crescent City (CC) to San Miguel Island (SMI). For other site abbreviations see Figure 3.1. Samples were taken at Van Damme (VD) on two separate dates, with the juvenile samples from 2001 labeled as VDJ. The number of times a specific COI haplotype occurred in a population and the total number of sequences obtained per population ("Total" row) and per haplotype ("Total" column) are presented. Columns in grey are haplotype counts for samples included in AFLP analysis.

Haplotype	Population										Total
	CC	TR	SH	7HC	VD	VDJ	SP	HSC	MY	SMI	
1	12	7	10	12	18	11	10	9	14	13	116
2	5	7	8	4	3	1	5	8	2	7	50
3	-	1	-	-	-	-	-	-	-	-	1
4	-	1	-	-	-	-	-	-	-	-	1
5	-	-	1	-	-	-	-	-	-	-	1
6	-	-	-	1	-	-	-	-	-	-	1
7	-	-	-	1	-	-	-	-	-	-	1
8	-	-	-	-	1	-	-	-	1	-	2
9	-	-	-	-	-	-	-	-	1	-	1
10	-	1	-	-	-	-	-	-	-	-	1
11	-	2	-	-	-	-	-	-	-	-	2
12	1	-	-	-	-	-	2	-	-	-	3
13	-	1	-	-	-	-	-	-	-	-	1
14	-	-	-	-	-	-	1	-	-	-	1
15	-	-	-	-	-	-	1	-	-	-	1
16	3	1	1	-	-	1	1	2	-	1	10
17	-	-	-	1	-	-	-	-	-	-	1
18	-	-	-	-	-	2	-	1	-	-	3
19	1	1	-	-	-	-	-	-	-	-	2
20	-	2	-	-	-	-	-	-	-	-	2
21	-	-	-	-	1	-	-	-	-	-	1
22	-	-	-	-	-	-	1	-	-	-	1
23	1	-	-	-	-	-	-	-	-	-	1
24	-	-	2	6	3	3	1	3	1	4	23
25	-	-	-	-	-	1	-	-	-	-	1
26	-	-	-	-	-	-	-	1	-	-	1
27	-	-	-	-	-	-	-	1	-	-	1
28	4	-	2	1	-	1	-	3	-	1	12
29	3	5	7	1	5	9	2	6	3	4	45
30	-	1	1	-	-	-	-	-	-	-	2
31	-	-	1	-	-	-	-	-	-	-	1
32	-	-	-	-	1	-	-	-	-	-	1
33	-	-	-	-	-	-	1	-	-	-	1
34	-	-	-	-	-	-	-	1	-	-	1
35	-	-	-	-	-	-	1	-	-	-	1
36	-	-	-	-	-	-	1	-	-	-	1
37	-	1	-	1	-	1	1	-	1	-	5
38	-	-	1	-	1	-	-	1	1	-	4
39	-	-	-	-	-	1	-	-	-	-	1
40	-	-	-	-	-	-	1	-	-	-	1
41	-	-	-	1	-	-	-	-	-	-	1
42	-	-	-	-	-	-	-	-	-	1	1
43	-	-	-	1	-	-	-	-	-	-	1
Total	30	31	34	30	33	31	29	36	24	31	309

Table 3.5: Global and pairwise sample comparisons for ten samples of *H. rufescens* from nine California sites. Samples at Van Damme taken in 1999 (VD) and 2001 (juveniles < 50 mm shell length, VDJ). Table includes p-value results from exact tests of differentiation and estimates of parameter(s) Φ_{ST} for mitochondrial COI; F_{ST}, ρ_{ST}, and Φ_{ST} for nuclear microsatellites (μsats); and Φ_{PT} for AFLPs. Significant p-values and parameter estimates after sequential Bonferroni correction at the $\alpha = 0.05$ level in *italics*.

Comparison			Exact tests			F-statistics				
			COI p	μsats p	AFLPs p	COI Φ_{ST}	F_{ST}	μsats ρ_{ST}	Φ_{ST}	AFLPs Φ_{PT}
Global			0.066	*0.002*	*< 0.001*	0.007	0.002	0.001	-0.001	*0.035*
CC	&	TR	0.154	0.033		-0.008	0.007	-0.006	0.015	
CC	&	SH	0.355	0.505		-0.010	0.001	-0.002	-0.004	
CC	&	7HC	0.040	0.418	*< 0.001*	0.052	0.001	0.003	-0.002	*0.058*
CC	&	VD	0.018	0.013		0.012	0.007	-0.014	0.005	
CC	&	VDJ	0.024	0.010		0.007	0.005	-0.001	0.015	
CC	&	SP	0.392	0.075		0.007	0.002	-0.005	-0.005	
CC	&	HSC	0.462	0.484	*< 0.001*	-0.009	0.001	-0.003	-0.003	*0.049*
CC	&	MY	0.116	0.112	*< 0.001*	0.017	0.001	-0.020	0.003	*0.112*
CC	&	SMI	0.211	0.896	*< 0.001*	-0.001	-0.001	-0.010	-0.004	0.022
TR	&	SH	0.473	0.556		0.001	0.002	0.004	0.008	
TR	&	7HC	0.008	0.228		0.036	0.001	-0.008	0.003	
TR	&	VD	0.009	0.029		0.008	0.003	0.000	-0.001	
TR	&	VDJ	0.027	0.025		0.015	0.000	-0.002	-0.010	
TR	&	SP	0.421	0.104		-0.007	0.003	0.003	0.004	
TR	&	HSC	0.234	0.051		0.006	0.001	0.009	0.002	
TR	&	MY	0.080	0.282		-0.002	0.003	-0.004	0.003	
TR	&	SMI	0.111	0.057		0.000	0.004	0.010	0.003	
SH	&	7HC	0.084	0.690		0.050	0.001	0.007	-0.003	
SH	&	VD	0.202	0.254		0.012	0.002	0.000	0.001	
SH	&	VDJ	0.154	0.092		-0.017	0.000	0.020	0.002	
SH	&	SP	0.313	0.497		0.018	0.002	-0.007	0.000	
SH	&	HSC	1.000	0.081		-0.020	0.002	-0.007	0.000	
SH	&	MY	0.263	0.408		0.020	0.001	-0.011	0.005	
SH	&	SMI	0.871	0.589		0.002	-0.001	-0.005	0.000	
7HC	&	VD	0.218	0.064		0.016	0.002	0.007	-0.004	
7HC	&	VDJ	0.043	0.152		0.032	0.002	0.001	-0.005	
7HC	&	SP	0.284	0.118		0.011	0.004	0.007	-0.005	
7HC	&	HSC	0.123	0.781	0.055	0.025	-0.002	0.010	-0.004	0.011
7HC	&	MY	0.315	0.479	*< 0.001*	-0.002	0.006	0.002	-0.001	0.034
7HC	&	SMI	0.597	0.406	*< 0.001*	-0.007	0.001	0.017	-0.002	0.021
VD	&	VDJ	0.243	0.387		-0.001	-0.001	0.004	-0.006	
VD	&	SP	0.165	0.077		0.018	0.007	-0.002	0.000	
VD	&	HSC	0.117	0.003		0.010	0.004	-0.001	-0.002	
VD	&	MY	0.979	0.116		-0.020	0.007	-0.015	-0.002	
VD	&	SMI	0.492	0.114		-0.009	0.004	-0.007	0.000	
VDJ	&	SP	0.066	0.076		0.024	0.002	0.010	-0.002	
VDJ	&	HSC	0.254	0.251		-0.015	0.001	0.026	-0.004	
VDJ	&	MY	0.337	0.212		0.012	0.002	0.005	-0.006	
VDJ	&	SMI	0.156	0.012		0.002	0.003	0.016	0.002	
SP	&	HSC	0.235	0.223		0.011	0.002	-0.002	-0.005	
SP	&	MY	0.637	0.590		-0.002	0.001	-0.013	0.002	
SP	&	SMI	0.504	0.410		-0.003	0.002	-0.009	-0.001	
HSC	&	MY	0.125	0.664	*< 0.001*	0.018	0.001	-0.012	-0.003	0.055
HSC	&	SMI	0.842	0.070	*< 0.001*	-0.014	0.002	-0.004	-0.001	0.021
MY	&	SMI	0.299	0.066	*< 0.001*	-0.008	0.000	-0.015	0.000	*0.072*

References

Avise JC (2004) Molecular markers, natural history, and evolution (second edition). Sinauer Associates, Sunderland, Massachusetts

Baus E, Darrock DJ, Bruford MW (2005) Gene-flow patterns in Atlantic and Mediterranean populations of the Lusitanian sea star *Asterina gibbosa*. *Molecular Ecology*, 14, 3373-3382

Beaumont MA, Nichols RA (1996) Evaluating loci for use in the genetic analysis of population structure. *Proceedings of the National Academy of Sciences of the USA* 263, 1619-1626

Bensch S, Åkesson M (2005) Ten years of AFLP in ecology and evolution: why so few animals? *Molecular Ecology*, 14, 2899-2914

Brazeau DA, Sammarco PW, Gleason DF (2005) A multi-locus genetic assignment technique to assess sources of *Agaricia agarictes* larvae on coral reefs. *Marine Biology*, 147, 1141-1148

Burton RS (1998) Intraspecific phylogeography across the Point Conception biogeographic boundary. *Evolution*, 52, 734-745

Burton RS, Tegner MJ (2000) Enhancement of red abalone *Haliotis rufescens* stocks at San Miquel Island: reassessing a success story. *Marine Ecology Progress Series*, 202, 303-308

California Department of Fish and Game, Marine Region (2005) Final abalone recovery and management plan. The Resources Agency, Sacramento, California

Campbell D, Duchesne P, Bernatchez L (2003) AFLP utility for population assignment studies: analytical investigation and empirical comparison with microsatellites. *Molecular Ecology*, 12, 1979-1991

Campbell D, Bernatchez L (2004) Generic scan using AFLP markers as a means to assess the role of directional selection in the divergence of sympatric whitefish ecotypes. *Molecular Biology and Evolution*, 21, 945-956

Chambers MD, VanBlaricon GR, hauser L, Utter F, Friedman CS (2006) Genetic structure of black abalone (Haliotis cracherodii) populations in the California island and central California coast: impacts of larval dispersal and decimation from withering syndrome. *Journal of Experimental Marine Biology and Ecology*, 331, 173-185

Clark R, Christensen J, Caldow C, Allen J, Murray M, MacWilliams S (2005) A biogeographic assessment of the Channel Island National Marine Sanctuary: a review of boundary expansion concepts for NOAA's National Marine Sanctuary Program. NOAA Technical Memorandum NOS NCCOS 21

Clement M, Posada D, Crandall K (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, 9, 1657-1660

Cope JM (2004) Population genetics and phylogeography of the blue rockfish (*Sebastes mystinus*) from Washington to California. *Canadian Journal of Fisheries and Aquatic Sciences*, 61, 332-342

Davis GE, Kushner DJ, Mondragon JM, Mondragon JE, Lerma D, Richards DV (1997) Kelp Forest Monitoring Handbook, Volume 1, Sampling Protocol. Channel Islands National Park, Ventura, California

Dawson MN (2001) Phylogeography in coastal marine animals: a solution from California. *Journal of Biogeography*, 28, 723-736

Dawson MN, Jacobs DK (2001) Molecular evidence for cryptic species of *Aurelia aurita* (Cnidaria, Scyphozoa). *Biological Bulletin*, 200, 92-96

DeFreitas B (2003) Estimating juvenile northern abalone (*Haliotis kamtschatkana*) abundance using artificial habitats. *Journal of Shellfish Research*, 22, 819-823

Duchesne P, Bernatchez L (2002) AFLPOP: A computer program for simulated and real population allocation based on AFLP data. *Molecular Ecolology Notes*, 3, 380-383

Edmands S (2001) Phylogeography of the intertidal copepod *Tigriopus californicus* reveals substantially reduced population differentiation at northern latitudes. *Molecular Ecology*, 10, 1743-1750

Evans BS, Sweijd NA, Bowie RCK, Cook PA, Elliot NG (2004) Population genetic structure of the perlemoen Haliotis midae in South Africa: evidence of range expansion and founder events. *Marine Ecology Progress Series*, 270, 163-172

Flowers JM, Schroeter SC, Burton RS (2002) The recruitment sweepstakes has many winners: genetic evidence from the sea urchin *Strongylocentrotus purpuratus*. *Evolution*, 56, 1445-1453

Gaffney PM, Rubin VP, Hedgecock D, Powers DA, Morris G, Hereford L (1996) Genetic effects of artificial propagation: signals from wild and hatchery populations of red abalone in California. *Aquaculture*, 143, 257–266

Gaylord B, Reed DC, Washburn L, Raimondi PT (2004) Physical-biological coupling in spore dispersal of kelp forest macroalgae. *Journal of Marine Systems*, 49, 19-39

Geiger DL (2000) Distribution and biogeography of the recent Haliotidae (Gastropoda: Vetigastropoda) worldwide. *Bolletino Malocologico*, 35, 57-120

Gilg MR, Hilbish TJ (2003) Dispersal patterns of mussel larvae throughout a hybrid zone in Southwest England. *Evolution*, 57, 1061-1077

Glickman TS (1999) Glossary of Meteorology, 2nd Edition. American Meteorological Society, Cambridge, Massachusetts

Gomez-Uchida D, Weetman D, Hauser L, Galleguillos R, Retamal M (2003) Allozyme and AFLP analyses of genetic population structure in the hairy edible crab *Cancersetosus* from the Chilean coast. *Journal of Crustacean Biology*, 23, 486-494

Hamm DE, Burton RS (2000) Population genetics of black abalone, *Haliotis cracherodii*, along the central California coast. *Journal of Experimental Marine Biology and Ecology*, 254, 235-247

Hedgecock D (1994) Temporal and spatial genetic structure of marine animal populations in the California Current. *CalCOFI Investigative Reports*, 35, 73-81

Hellberg ME, Burton RS, Neigel JE, Palumbi SR (2002) Genetic assessment of connectivity among marine populations. *Bulletin of Marine Science*, 70, 273-290

Kalinowski ST, Taper ML (2006) Maximum likelihood estimation of the frequency of null alleles at microsatellite loci. *Conservation Genetics*, 7, 991-995

Karl SA, Avise JC (1992) Balancing selection at allozyme loci in oysters: implications from nuclear RFLPs. *Science*, 256, 100-102

Kirby VL, Villa R, Powers DA (1998) Identification of microsatellites in the California red abalone, *Haliotis rufescens*. *Journal of Shellfish Research*, 17, 801-804

Leighton DL (2000) The biology and culture of the California abalones. Dorrance Publishing, Co., Inc., Pittsburg, Pennsylvania

Lewontin RC, Krakauer J (1973) Distribution of gene frequency as a test of the theory of the selective neutrality of polymorphisms. *Genetics*, 74, 175-195

Liu X, Liu Xi, Guo X, Gao Q, Zhao H, Zhang G (2006) A preliminary genetic linkage map of the pacific abalone *Haliotis discus hannai* Ino. *Marine Biotechnology*, 8, 386-397

Marko PB (1998) Historical allopatry and the biogeography of speciation in the prosobranch snail genus *Nucella*. *Evolution*, 52, 757-774

Metz EC, Robles-Sikisaka R, Vacquier VD (1998) Nonsynonymous substitution in abalone sperm fertilization exceeds substitution in introns and mitochondrial DNA. *Proceedings of the National Academy of Sciences of the USA*, 95, 10676-10681

Michalakis Y, Excoffier L (1996) A generic estimation of population subdivision using distances between alleles with special reference for microsatellite loci. *Genetics*, 142, 1061-1064

Miller KM, Laberee K, Kaukinen KH, Li S, Withler RE (2001) Development of microsatellite loci in pinto abalone (*Haliotis kamtschatkana*). *Molecular Ecology Notes*, 1, 315-317

Miller M (1997) Tools for Population Genetic Analyses (TFPGA) version 1.3: a Windows® program for the analysis of allozyme and molecular population genetic data. Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona

Moffet MG (1978) A review of the fishery biology of abalones. Washington Report. Fish Tech Report 37

Morse DE, Hooker N, Duncan H, Jensen L (1979) Gamma-aminobutyric acid, a neurotransmitter, induces planktonic abalone larvae to settle and begin metmorphosis. *Science*, 204, 407-410

Morse ANC, Morse DE (1984) Recruitment and metamorphosis of *Haliotis* larvae induced by molecules uniquely available at the surfaces of crustose red algae. *Journal of Experimental Marine Biology and Ecology*, 75, 191-215

Mueller UG, Wolfenbarger LL (1999) AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution*, 14, 389-394

Murray MC, Hare MP (2006) A genomic scan for divergent selection in a secondary contact zone between Atlantic and Gulf of Mexico oysters, *Crassostrea virginica*. *Molecular Ecology*, 15, 4229-4242

Nei M (1972) Genetic distance between populations. *American Naturalist*, 106, 283-292

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences*, 12, 357-358

Peakall R, Smouse PE (2006) GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6, 288-295

Posada D, Crandall KA, Templeton AR (2000) GeoDis: A program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, 9, 487-488

Prince JD, Sellers TL, Ford WB, Talbot SR (1987) Experimental evidence for limited dispersal of haliotid larvae (genus *Haliotis*; Mollusca: Gastropoda). *Journal of Experimental Marine Biology and Ecology*, 106, 243-263

Raymond M, Rousset F (1995) GENEPOP: population genetics software for exact tests and ecumenicism. *Journal of Heredity*, 86, 248-249

Raymond M, Rousset F (2003) GENEPOP (version 3.4): population genetics software for exact tests and ecumenicism. Updated from Raymond and Rousset (1995)

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, 43, 223-225

Rogers-Bennett L, Allen BL, Davis GE (2004) Measuring abalone (*Haliotis* Spp.) recruitment in California to examine recruitment overfishing and recovery criteria. *Journal of Shellfish Research*, 23, 1201-1207

Rousset F (1996) Equilibrium values of measures of population subdivision for stepwise mutation processes. *Genetics*, 142, 1357-1362

Schneider S, Roessli D, Excoffier L (2000) Arlequin ver.2.000: A software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland

Shank TM, Halanych KM (2007) Toward a mechanistic understanding of larval dispersal: insights from genomic fingerprinting of the deep-sea hydrothermal vent tubeworm *Riftia pachyptila*. *Marine Ecology*, 28, 25-35

Slatkin M (1987) Gene flow and the geographic structure of natural populations. *Science*, 236, 787-792

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, 139, 457-462

Sønstebø JH, Borgstrøm R, Heun M (2007) A comparison of AFLPs and microsatellites to identify the population structure of brown trout (*Salmo trutta* L.) populations from Hardangervidda, Norway. *Molecular Ecology*, 16, 1427-1439

Sponaugle S, Cowen RK, Shanks A, Morgan SG, Leis JM, Pineda J, et al. (2002) Predicting self-recruitment in marine populations: biophysical correlates and mechanisms. *Bulletin of Marine Science*, 70, S341-S375

Strathmann SR (1985) Feeding and nonfeeding larval development and life-history evolution in marine invertebrates. *Annual Review of Ecology and Systematics*, 16, 339-361

Swofford DL (1998) PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts

Tang S, Popongviwat A, Klinbunga S, Tassanakajon A, Jarayabhand P, Menasveta P (2005) Genetic heterogeneity of the tropical abalone (*Haliotis asinina*) revealed by RAPD and microsatellite analysis. *Journal of Biochemistry and Molecular Biology*, 38, 182-190

Templeton AR, Routman E, Phillips CA (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics*, 140, 767-782

Veliz, D, Duchesne P, Bourget E, Bernatchez L (2006) Stable genetic polymorphism in heterogeneous environments: balance between asymmetrical dispersal and selection in the acorn barnacle. *Journal of Evolutionary Biology*, 19, 589-599

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, 23, 4407-4414

Waples RS (1998) Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. *Journal of Heredity*, 89, 438-450

Ward RD, Elliot NG (2001) Genetic population structure of species in the South East Fishery of Australia. *Marine and Freshwater Research*, 52, 563-573

Warner RR, Cowen RK (2002) Local retention of production in marine populations: evidence, mechanisms, and consequences. *Bulletin of Marine Science*, 70, S245-S249

Weetman DW, Ruggerio A , Mariani S, Shaw PW, Lawler AR, Hauser L (2007) Hierarchical population structure in the commercially exploited shrimp *Crangon crangon* identified by AFLP analysis. *Marine Biology*, 151, 565-575

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, 38, 1358-1370

Wilding CS, Butlin RK, Grahame J (2001) Differential gene exchange between parapatric morphs of *Littorina saxatilis* detected using AFLP markers. *Journal of Evolutionary Biology*, 14, 611–619

Withler RE, Campbell A, Li SR, Brouwer D, Supernault KJ, Miller KM (2003) Implications of high levels of genetic diversity and weak population structure for the rebuilding of northern abalone in British Columbia, Canada. Journal of Shellfish Research, 22, 839-847

Wright JS (1978) Variability within and among natural populations. Evolution and the genetics of natural populations: Vol. 4. University of Chicago Press, Chicago, Illinois

CHAPTER IV

Detecting restricted gene flow among natural populations of the California black abalone (*Haliotis cracherodii*), a candidate for listing as an endangered species

Abstract

The black abalone (*Haliotis cracherodii*) has been severely depleted across much of its historic range by a combination of overexploitation and disease. Natural recovery of extirpated populations along the southern California coast will depend on the extent to which remnant populations can serve as larval sources to geographic locations formerly supporting abalone populations. Population genetic analyses of mitochondrial cytochrome oxidase subunit one (COI) DNA sequences, nuclear microsatellites, and amplified fragment length polymorphisms (AFLPs) were used to evaluate connectivity among populations of *H. cracherodii* sampled from the central California coast and four islands in the Southern California Bight. Global divergence among populations was significant at COI and 142 AFLP loci. The *Hka*28 microsatellite locus and AFLP data showed significant divergence in multiple pairwise population comparisons and exhibited a signal of isolation by distance consistent with a stepping-stone model of connectivity. Patterns of differentiation were correlated with local ocean currents during the *H. cracherodii* spawning season. The low level of interpopulation connectivity suggests that larval dispersal is restricted and that natural recovery of decimated black abalone populations along the coast of California may not occur in the near future.

Introduction

A combination of overexploitation (Hobday and Tegner 2000; CDFG 2005) and disease (Haaker et al. 1992; Friedman et al. 1997; CDFG 2005) contributed to severe declines in abundance of black abalone (*Haliotis cracherodii*) along the central and southern California coasts and islands over the course of the past three to five decades. The black abalone has been considered a Species of Concern under the Endangered Species Act of 1973 (ESA) since 1999 and became a candidate for listing as an Endangered Species under the ESA in June 2006 (see http://www.nmfs.noaa.gov/pr/pdfs/species/black_abalone.pdf). *H. cracherodii* is the second species of abalone found along the coast of California, USA, to warrant potential Federal protection; the white abalone (*H. sorenseni*) was declared as Endangered in 2001, the first marine invertebrate ever to be listed in the USA (Hobday and Tegner 2000). A moratorium placed on the entire California abalone fishery in 1997 (except a red abalone (*H. rufescens*) sport fishery north of San Francisco Bay) relieved fishing pressure on the black abalone, but it is unknown whether extant populations of the species will be able to initiate recovery of extirpated populations in southern California.

Abalone are broadcast spawning marine gastropods from the monogeneric genus *Haliotis* found in temperate and tropical macroalgal communities worldwide (Geiger 2000). Early development includes a free-swimming lecithotrophic veliger larval phase lasting approximately 4 – 15 days before the larva settles out of the water column and undergoes metamorphosis (Morse et al. 1979; Leighton 2000). Based on this larval duration and a range of ocean current velocities, planktonic larval dispersal could result in a panmictic population across broad geographic ranges. However, many studies have

suggested that broad dispersal is not as common as might be predicted (e.g., Warner and Cowen 2002; Gilg and Hilbish 2003; Veliz et al. 2006; see also Levin 2006). If black abalone larvae are not as highly dispersive as their pelagic duration suggests, recovery of natural populations along the coast of California may be extremely slow even if disease and fishing mortality are reduced to zero.

Population genetic analyses can provide indirect measures of connectivity among populations (Bossart and Prowell 1998; Waples 1998; Hellberg et al. 2002; Thorrold et al. 2002). Prior research on the California black abalone indicated a restriction in gene flow and an inferred lack of interpopulation dispersal. Hamm and Burton (2000) studied the genetic variation in black abalone populations along the central coast of California and found significant genetic differentiation at two of three allozyme loci, but no differentiation in a modest sample of cytochrome oxidase subunit one (COI) DNA sequences. Chambers et al. (2006) used five allozyme loci to demonstrate that several island populations in the Southern California Bight were significantly differentiated from one another and from two mainland populations just south of SFB. However, allozymes are genetic markers that have garnered much attention in neutrality-selection debates (Avise 2004); geographic variation in selective pressures could result in population differentiation that is largely unrelated to dispersal and recruitment processes (e.g. Hilbish et al. 1982; Karl and Avise 1992).

We gathered data from three additional types of genetic markers – mtDNA sequence, microsatellites, and amplified fragment length polymorphisms (AFLPs) – to more fully assess natural genetic variation within and among samples taken from the species. We also expand on previous research by combining the sampling distributions of

Hamm and Burton (2000) and Chambers et al. (2006) to include 588 individual black abalone from 11 populations. Our geographic sampling range covers nearly 750 km and includes seven mainland sites along the central coast of California from Scotts Creek south of San Francisco Bay to Vandenberg Marine Ecological Reserve near Point Conception and four Southern California Bight (SCB) island sites, including three of the northern Channel Islands and San Nicolas Island. The samples span interpopulation distances from 10 km between Asilomar and Monterey (and Monterey and Carmel Point) to approximately 750 km between Scotts Creek and San Nicolas Island. The sites also encompass two different coastal oceanographic flow regimes experienced by 1) the linear central California coastline and 2) the nonlinear distribution of the islands in the SCB.

Our goal is to address three research questions related to the black abalone: 1) do all three genetic marker types used yield identical genetic structure, 2) what evolutionary and ecological aspects of interpopulation connectivity can be inferred from each of these markers, and 3) does the magnitude of gene flow correlate with geography and/or local hydrographic conditions? We compare and add our results to the allozyme data from earlier research and discuss our genetic marker choices in light of the interpopulation divergence and phylogeographic patterns we detect in *H. cracherodii*. Finally, the implications of our results to the potential recovery of this ecologically and commercially valuable marine invertebrate species are briefly discussed.

Materials and Methods

Population sampling and DNA extraction

Collection sites are summarized in Figure 4.1a and Table 4.1. California mainland tissue samples were collected in 1997 and 1998 at five locations by D Hamm and two locations by the California Department of Fish & Game. Collection sites from north to south included Scotts Creek (SC, N = 63), Asilomar (ASL, N = 56), Monterey (MY, N = 54), Carmel Point (CP, N = 23), Big Creek (BC, N = 14), Cambria (CA, N = 58), and Vandenberg Air Force Base (VB, N = 48). Island tissue samples were collected in the Southern California Bight (SCB) in 2002 by GR VanBlaricom and M Chambers from the University of Washington. Sites included San Miguel Island (SMI, N = 101), Santa Rosa Island (SRI, N = 55), Santa Cruz Island (SCI, N = 29), and San Nicolas Island (SNI, N = 87).

Epipodial tentacle or foot clippings were preserved in ethanol or water and stored at -80ºC. DNA was extracted from approximately 25 mg of tissue using a DNeasy Tissue Kit (Qiagen Inc., Valencia, CA), eluted in 200 µl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA), and stored at -80ºC. A 5 µL aliquot of the stock DNA was diluted with 45 µL of DNA-grade water, placed in 96-well trays, and stored at 4ºC for use in the polymerase chain reaction (PCR). All PCR and sequencing reactions were performed in Thermo-Hybaid PCR Express and Px2 thermal cyclers (Thermo Electron Corporation, Boston, MA).

Gene sequencing

A 580 basepair (bp) fragment of the mitochondrial cytochrome oxidase subunit one (COI) gene was amplified in a subset of 238 individuals (Table 4.1). PCR primers for abalone COI were from Metz et al. (1998): AB-COIF (forward: 5'-TGATCCGGCTTAGTCGGACTGC) and AB-COIR (reverse: 5'-GATGTCTTGAAATTACGGTCGGT). Reactions were carried out in 25 μL volumes, including 5 μl of the dilute template DNA, 0.5 U REDTaq DNA polymerase (Sigma-Aldrich Corporation, St. Louis, MO), 2.5 μl 10X PCR buffer, 2.5 mM $MgCl_2$ final concentration, 2 μM each dNTP final concentration, and 5 pmoles each primer.

PCR cycling conditions included an initial two minute 95ºC denaturation followed by 35 cycles of 95ºC for 30 s, 50ºC for 30 s, and 72ºC for 30 s. The amplified fragments were sequenced on a MegaBACE™ 500 DNA sequencer (Amersham Biosciences Corp., Piscataway, NJ) DNA sequencer using the forward primer AB-COIF and a DYEnamic™ ET dye terminator sequencing kit (Amersham Pharmacia Inc., Piscataway, NJ). Sequences were aligned and edited to 403 bp using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI). Coding sequence was determined by aligning our COI sequences to the NCBI GenBank database accession AY679073, the partial coding sequence for the *H. cracherodii* Hcr01 COI haplotype. The amino acid translation table used corresponded to the invertebrate mtDNA code of *Drosophila melanogaster*.

Basic summary statistics such as the number of segregating sites and the number of haplotypes were calculated in DnaSP v4.0.0 (Rozas et al. 2003). A UPGMA tree (not shown) was generated in PAUP* 4.0b10 (Swofford 1998) and visualized in TREEVIEW (Page 1996) to define the COI haplotypes, and representative sequences for the

haplotypes were deposited in GenBank under the accessions DQ297475 – DQ297506. A haplotype network was generated using parsimony in the program TCS v1.18 (Clement et al. 2000). To evaluate potential geographic structure among the COI haplotypes, hierarchical clades were described on the haplotype network according to Templeton et al. (1995) and nested clade analysis (NCA) was performed on the hierarchical clades using the program GeoDis 2.2 (Posada et al. 2000).

An hierarchical analysis of molecular variance (AMOVA) was performed with 1000 permutations of the data using Arlequin v2.000 (Schneider et al. 2000) to generate global and pairwise estimates of the parameter Φ_{ST}, an F_{ST} analogue that takes into account allelic identity. Levels of significance were calculated based upon χ^2 tests ($\chi^2 = 2N\Phi_{ST}$, df = 1) of the parameter value being significantly different from zero (i.e. no genetic divergence). Results were sequential Bonferroni corrected at the table-wide $\alpha = 0.05$ level to account for multiple comparisons (Rice 1989). A Mantel test (1000 permutations) was performed in Arlequin to assess the level of correlation between population pairwise genetic divergence and geographic distance matrices (e.g. isolation by distance, IBD). Mainland interpopulation geographic distances were calculated in km as the shortest along-shoreline distance and inter-island distances were calculated as the shortest over-water distance using a Rand McNally road atlas. The parameter Φ_{ST} was standardized to $\Phi_{ST} / (1-\Phi_{ST})$ and plotted against the natural log of the pairwise distance between populations.

Microsatellites

We tested ten microsatellite loci originally isolated from four abalone species closely related to the black abalone (Kirby et al. 1998; Miller et al. 2001; Sekino et al. 2004; Dennis Petersen pers. comm.) in a subset of 12 black abalone. We proceeded to PCR amplify in the full sample set four dinucleotide repeat microsatellite loci that worked reliably in the tests (Table 4.2). Primers for the *Hka*28, *Hka*40, *Hka*56, and *Hka*80 loci were originally developed for a related Eastern Pacific abalone species, the pinto *H. kamtschatkana* (Miller et al. 2001). All 588 individuals were genotyped (Table 4.1); a sample size of ≥ 50 is recommended to effectively assess microsatellite variation within a population (Ruzzante 1998). Reactions were carried out in 25 µl volumes, including 5 µl of the dilute template DNA, 0.5 U REDTaq DNA polymerase, 2.5 µl 10X PCR buffer, 2.5 mM $MgCl_2$ final concentration, 2 µM each dNTP final concentration, and 5 pmoles each primer. The forward primers were 5' end-labeled with one of three dyes HEX, TET, or 6-FAM for fluorescent visualization. PCR cycling conditions were per Miller et a. (2001).

Following amplification, 15 µl of a denaturing solution containing xylene cyanol and bromophenol blue dyes in 99% formamide and 1% NaOH were added to each 25 µl PCR product. Samples were then loaded onto vertical 0.4-mm thick large format (33 x 39 cm) 5% polyacrylamide denaturing gels containing 33% formamide and 7 M urea. ROX-labeled MegaBACE™ ET900-R Size Standard (Amersham Pharmacia Inc., Piscataway, NJ) or Genemark 350 Fluorescent DNA Ladder (Northern Biotechnology, Schofield, WI) was run concurrently as a size standard. Gels were electrophoresed at 60 Watts for two to three hours and scanned on a Typhoon 9410 Variable Mode Imager

(Molecular Dynamics Inc., Sunnyvale, CA). Allele sizes were visualized with ImageQuaNT (Molecular Dynamics Inc., Sunnyvale, CA) and scored by hand.

The frequency of the null allele was calculated using the maximum likelihood program ML-Nullfreq (Kalinowski and Taper 2006). Because null alleles may elevate perceived levels of genetic divergence due to overrepresentation of homozygotes, the genotypes for three loci exhibiting high null frequencies were corrected for the presence of nulls using 1000 bootstrap replicates in the program FreeNA (Chapuis and Estoup 2007). All subsequent analyses were performed on both the full corrected microsatellite data set of four loci and on the *Hka*28 locus (the only microsatellite in HWE) alone.

Global and pairwise estimates of ρ_{ST}, an F_{ST} analogue which takes into account allele size under a stepwise mutation model (Weir and Cockerham 1984; Slatkin 1995; Michalakis and Excoffier 1996), were calculated using GENEPOP v3.4 (Raymond and Rousset 1995, 2003). Levels of significance were calculated based on χ^2 tests ($\chi^2 = 2N\rho_{ST}$, df = 1) of the ρ_{ST} estimate being significantly different from zero. Results were sequential Bonferroni corrected at the table-wide $\alpha = 0.05$ level. Mantel tests (1000 permutations) were run in GENEPOP to assess whether there was a signal of IBD. The parameter ρ_{ST} was standardized to $\rho_{ST} / (1-\rho_{ST})$ and plotted against the natural log of the pairwise distance between populations.

Amplified fragment length polymorphisms (AFLPs)

A subset of 156 individuals from six populations {SC (N = 30), CP (N = 23), BC (N = 14), CA (N = 30), SCI (N = 29), and SNI (N = 30)} was chosen for AFLP genotyping (Table 4.1); although financial constraints prevented genotyping of all

samples, the populations used in the analyses span the full sampled range of ~750 km. The decision to use sample sizes of ~30 was based on two points: 1) some types of analyses (e.g. assignment tests) can be skewed by unequal sample sizes (Davies et al. 1999) and 2) AFLP allele frequency variation within populations may be effectively assessed from ~ 30 individuals per sample (Medina et al. 2006). Protocols were performed according to Vos et al. (1995). After ligation of adapters complementary to the *Eco*RI and *Mse*I restriction sites, pre-amplification was performed and the products were diluted 10-fold with 10 mM Tris-EDTA pH 8.0 for use in the final amplification step.

Both pre-amplification and amplification reactions were carried out in 20 μL volumes, including 5 μl of template (concentrated stock DNA or pre-amplification product), 1 U MasterTaq DNA polymerase (Eppendorf AG, Hamburg, Germany), 1X Taq Buffer, 1X TaqMaster PCR Enhancer, 2 mM each dNTP final concentration, 6 pmoles of *Mse*I primer, and 6 pmoles *Eco*RI pre-amplification primer or 1 pmole of *Eco*RI amplification primer. *Eco*RI amplification primers were 5' end-labeled with the fluorescent dye HEX for visualization. PCR cycling conditions for the pre-amplification included an initial two minute 95°C denaturation followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s. A similar protocol was also used for the final amplification, but initial annealing temperatures were raised to 56°C or 58°C depending on the primer combination.

Following amplification, 1 μl of a denaturing solution containing xylene cyanol and bromophenol blue dyes in 99% formamide and 1% NaOH was added to each 5 μl of PCR product. Samples were loaded onto vertical 0.4 mm thick large format (33 x 39 cm)

5% polyacrylamide denaturing gels containing 33% formamide and 7 M urea. ROX-labeled Genemark 350 Fluorescent DNA Ladder was run concurrently as a size standard. Gels were electrophoresed at 60 Watts for three to four hours and scanned on a Typhoon 9410 Variable Mode Imager.

Tests were performed to ensure that the AFLP banding patterns were consistent. A set of 12 individuals from SNI were subjected to two complete replications of the AFLP protocol for four final primer combinations. The presence or absence of product bands was scored by hand, and the genotypes were compared between the three independent scoring events. One of the four primer combinations did not produce clean amplification products and could not be reproducibly scored; it was dropped from all analyses. For the remaining three final amplification primer combinations (Table 4.3), independent hand scoring was different in 1.6% of the tests; gels were visually identical in side-by-side comparisons. Variability was due to human error in recording scores and not irreproducibility in the AFLP assay.

In estimating genetic divergence among populations, allele frequencies at the AFLP loci were calculated assuming HWE; the frequency of the recessive alleles was calculated in TFPGA v1.3 (Miller 1997) based on the square root of the frequency of the null homozygote genotype. The corrected frequencies were used to generate per-locus F_{ST} estimates in TFPGA (5000 bootstrap replicates). An outlier test of selection was conducted to determine whether significant locus-specific divergences were due to neutral variation or directional selection. The program fdist2 (Beaumont and Nichols 1996) was used as in Murray and Hare (2006). Simulations of neutral F_{ST} under mutation-drift equilibrium were run using the infinite alleles model for 100 demes

(islands), 6 populations, sample sizes of 30, 50000 realizations, and an expected F_{ST} equivalent to the global Φ_{PT} value estimated in an AMOVA described below (F_{ST} = 0.044). The medians and upper and lower 99th quantiles were calculated for the simulated F_{ST} data, and the simulated and actual per locus F_{ST} data were plotted against the inferred heterozygosity.

An AMOVA was performed using the Microsoft Excel add-in program GenAlEx 6 (Peakall and Smouse 2006) to generate global and pairwise estimates of the parameter Φ_{PT}, an analogue of F_{ST} equivalent to Φ_{ST} calculated in the COI analyses using Arlequin. Results were sequential Bonferroni corrected at the $\alpha = 0.05$ level. A Mantel test (999 permutations) was run in GenAlEx to assess whether interpopulation geographic distance played a role in structuring the AFLP data. Geographic distances were calculated from latitudinal and longitudinal coordinates as straight line distances between populations. Interindividual genetic distance was plotted against interpopulation geographic distance in km for all loci and for the 16 loci with significant global F_{ST} estimates.

Spatial clustering of the multilocus genotypes was assessed to obtain a visual representation of the genetic relationships among the populations. Principal coordinates analysis (PCA) in GenAlEx was used to partition individual multilocus AFLP genotypes composed of the 16 significant AFLP loci along vectors in eigenspace (including all 142 markers provided less spatial resolution among samples). An individual pairwise genetic distance matrix was generated from the binary data file, where the total pairwise distance between individuals is the sum of the pairwise distances for each locus (either one or zero). The PCA was then performed on the matrix after distances were standardized by dividing each by $(n - 1)^{-2}$, where n is the number of pairwise comparisons.

To test the magnitude of genetic separation between the samples mentioned above, assignment tests based on all 142 loci were performed in AFLPOP 1.1 (Duschesne and Bernatchez 2002) using maximum likelihood. Each multilocus AFLP genotype was removed from the data set in turn and dominant band allele frequencies were recalculated across loci. Log-likelihoods (lnL) were estimated for each genotype as a member of each candidate population. Genotypes were allocated to a specific population based on the likelihood an individual genotype matched the uncorrected allele frequency profile of a candidate population and according to a user-defined minimum difference in membership lnL between candidate populations (lnL_{min}).

Validation of Mendelian inheritance

No breeding experiments were carried out to assess the genetic bases of the markers employed in this study. However, we have previously confirmed the maternal inheritance of mtDNA and the Mendelian inheritance of microsatellite alleles in another eastern Pacific abalone species, the white abalone (*H. sorenseni*), using the same genetic markers in laboratory crosses (Gruenthal and Burton 2005). Although we have no pedigree data on AFLPs, Liu et al. (2006) found that 95% of 387 AFLP markers exhibited normal Mendelian segregation in the Pacific abalone (*H. discus hannai*).

Results

COI

Summary statistics included the number of segregating/polymorphic sites (S = 31), total number of mutations ($\eta = 32$), number of haplotypes (h = 32), haplotype diversity (Hd = 0.630), nucleotide diversity ($\pi = 0.002$), and the average number of nucleotide differences (k = 0.948). Only one singleton mutation resulted in an amino acid change within our sample; G168A changed glycine (non-polar) to serine (polar) at amino acid position 56 in partial sequence from one MY individual.

The topology of the haplotype network was star-like, with a single common haplotype surrounded by an array of low-frequency haplotypes one or more mutational steps removed (Figure 4.2). The common haplotype had a frequency of 0.59, with two additional haplotypes at moderate frequencies (0.15 and 0.04). The remaining 29 haplotypes occurred at frequencies of less than 0.02. Additional analysis using DnaSP indicated that our mitochondrial sample was not in an equilibrium state; Tajima's D (Tajima 1989) and Fu and Li's D* and F* Fu and Li 1993) were all significantly negative at the $p < 0.02$ level, and a plot of the pairwise sequence mismatch distribution reveals a peak in the number of mismatches ($\tau = 0.986$; not shown). NCA indicated no significant association with geography at any cladistic level.

From the AMOVA, the highest among-group variation (~6%) resulted when BC and SCI were treated as individual groups and the remaining populations were combined into a third group; over 99% of the variation was within populations for all other partitions of the data. Global divergence was significant ($\Phi_{ST} = 0.014$, $p = 0.010$), but there were no significant population pairwise comparisons after sequential Bonferroni

correction. Pairwise results are presented in Table 4.4a. The Mantel test indicated a non-significant negative trend to the data ($R^2 = 0.003$) and no evidence of isolation by distance (IBD; Figure 4.4a).

Microsatellites

The four dinucleotide repeat loci were highly polymorphic and contained between 33 and 51 alleles each. There was no evidence for linkage disquilibrium between any loci in any of the populations after sequential Bonferroni correction. Heterozygote deficiencies ($p < 0.05$, Table 4.2) were found at three of four loci (*Hka*40, *Hka*56, *Hka*80); only the *Hka*28 locus was in Hardy-Weinberg equilibrium (HWE). We assumed the departures from HWE were a result of null alleles, since null homozygotes were observed at single loci in individuals where other loci amplified. Excluding *Hka*28, the three loci all exhibited null allele frequencies in excess of 37% (Table 4.2).

Using corrected allele frequencies, global divergence over all loci was not significant ($\rho_{ST} = 0.002$), but analysis of *Hka*28 alone was significant ($\rho_{ST} = 0.026$, $p < 0.001$). The full microsatellite data set exhibited no significant pairwise divergences (Table 4.4b). In contrast, analysis of *Hka*28 alone indicated a generalized break centered near BC or CA (both divergent in pairwise tests with only one or few populations); pairing a population north of BC/CA with a population south of BC/CA often resulted in significant ρ_{ST} estimate (ASL to the north and SCI to the south were exceptions). The full microsatellite data set indicated a slight positive but non-significant trend between genetic divergence and geographic distance ($R^2 = 0.003$), while *Hka*28 alone showed a

significant positive correlation ($R^2 = 0.311$, $p = 0.001$) and evidence for IBD (Figure 4.4b).

AFLPs

A total of 143 polymorphic loci from three final amplification primer combinations were scored in 156 animals (22,308 presence/absence cells in a binary data matrix). Scored as dominant traits, the frequency of the marker bands within the full data set ranged from 0.071 – 0.974. There were no fully diagnostic markers (private markers) for any population out of 143 loci. There was one redundant locus within the populations; locus 140 repeated the presence/absence pattern of locus 30 and was deleted, resulting in a final data set of 142 polymorphic AFLP markers. Out of 142 markers, 16 (~11%) exhibited significant F_{ST} estimates (standardized variance in allele frequencies) after sequential Bonferroni correction (all $p \leq 0.0002$; data not shown). Only three loci (~2%) fell outside of the upper 99th quantile and were considered outliers potentially under selective pressure (Figure 4.3).

The AMOVA indicated that ~4% of the variation was among populations, and the remaining 96% was within populations. Global population divergence was significant ($\Phi_{PT} = 0.044$, $p = 0.001$), and 14 out of 15 pairwise population comparisons were significant after sequential Bonferroni correction at the table-wide $\alpha = 0.05$ level (Table 4.4c); only CP and BC were not significantly divergent at the marker loci. There was a slight positive trend and significant correlation between genetic and geographic distance for all loci combined ($R^2 = 0.011$, $p = 0.001$; Figure 4.4c) and for the subset of 16

significant AFLP loci ($R^2 = 0.025$, $p = 0.001$; graph not shown), indicating a subtle signal of IBD in the data.

From the PCA, vectors one and two account for the bulk of the variation and are presented in Figure 4.5. Points within each population visually clustered in space along the coordinate axes; some population point distributions were nearly fully separated (e.g. the point distribution for SC is separate from that of BC and barely overlaps that of CA; see Figure 4.5 inset). A portion of the distribution for SNI, in particular, is distinct, whereas the other populations form a continuum in the presented 2D space, with BC at the negative extreme of axis one and SC at the positive extreme of axis two.

As would be predicted by the PCA analysis, assignment tests showed variable success. Reassignment rates were not high (≤ 0.64) when all samples were included in a single analysis, but several tested sample pairs reassigned at high rates for $\ln L_{min} = 0.001$ (≥ 0.80), indicating that particular population pairs are indeed genetically distinct. Test results are presented in Table 4.5. Multilocus AFLP genotypes for SC and BC were reassigned successfully at rates of 0.87 and 0.79, respectively, and for SC and CA were reassigned at rates of 0.80 and 0.83, respectively. SNI reassigned at only ~0.60 vs. the most of the rest of the samples. However, a portion of the SNI individuals may be indistinguishable from the remaining samples; approximately half of the SNI point distribution overlapped that of other samples in the Figure 4.5 PCA. SNI did reassign at a rate of 0.80 when tested, for example, against BC alone, the point distribution of which does not overlap with SNI (BC reassigned at a rate of 0.79).

Discussion

Samples of black abalone collected along the coast of central California and from four islands in the Southern California Bight (SCB) showed significant global but patchy pairwise population genetic structure. The specific genetic pattern obtained differed somewhat among marker types, but the overall pattern is consistent with restricted gene flow among populations of black abalone along the California coast. No easily discernable regional geographic structure was apparent (e.g., mainland populations as a group were not significantly different from the islands as a group). Positive and significant correlations were seen, however, between interpopulation geographic distance and pairwise population genetic divergences with the *Hka*28 microsatellite locus and the AFLP loci, indicating a pattern of isolation by distance (IBD); historical gene flow may have followed a stepping-stone type of model, with higher interpopulation connectivity occurring among populations that are geographically closer together.

Detecting population structure

Different Genetic markers can provide different ecological and demographic information about a species (Avise 2004). We measured population differentiation using three types of genetic markers – mitochondrial gene (COI) sequence, microsatellites, and AFLPs – as a proxy for realized connectivity among black abalone populations along the coast of California. COI sequences did not give clear evidence for restricted gene flow among natural populations; despite significant global divergence and borderline significance of several pairwise population comparisons using mitochondrial COI, we could not definitively identify any phylogeographic structure. Our 238 sequence data set

showed the same generalized haplotype topology as the small set of 51 sequences obtained by Hamm and Burton (2000); a single common haplotype was surrounded by multiple low-frequency haplotypes, the majority resulting from single base differences from the common haplotype. Although the low-frequency haplotypes were often restricted to single geographic locales, most were singletons and the ubiquitous presence of the common haplotype drowned out any significant signal of frequency variation and interpopulation divergence. COI, the standard interspecific barcode tool (e.g. COI is utilized by the Consortium for the Barcode of Life and Census of Marine Life molecular barcoding efforts; see also Hebert et al. 2003a; Hebert et al. 2003b) and highly useful in many population-level phylogeographic studies, did not prove to be an informative marker for our intraspecific study of the black abalone. In fact, COI has not provided strong resolution in population studies of other California abalone species where even species-level distinctions can be difficult based on mtDNA sequences alone (Gruenthal and Burton 2005; see also Gruenthal et al. 2007). Perhaps a more rapidly-evolving region of the mitochondrial genome (such as the D-loop) may have been more useful, but attempts in our laboratory to PCR amplify the abalone D-loop have proved unsuccessful thus far.

In addition, Bazin et al. (2006) contend that the mtDNA is not a neutrally-evolving (Kimura 1991) genome and that mitochondrial markers are only appropriate for studying species over the time period since the last mitochondrial selective sweep. As such, the mtDNA may not reflect the true evolutionary history of a species. In fact, the star-like topology of our COI haplotype network indicated that black abalone mtDNA may have undergone a selective sweep (or range expansion) relatively recently and

throughout a large portion of the species' range. The cursory analysis of Tajima's D and Fu and Li's D* and F* (all three significantly negative) and the peak the pairwise sequence mismatch distribution further support the hypothesis of a recent sweep (Rogers and Harpending 1992). Hence, a true restriction in gene flow in the black abalone (potentially seen with allozymes, microsatellites, and AFLPs) may have been masked by selection if only mtDNA markers were included in the study.

Presumed neutral nuclear markers, such as microsatellites, may be more suited to assessing population structure in the black abalone. Microsatellites are also highly polymorphic and, as such, are often used in population genetic and fingerprinting studies (Avise 2004). We detected significant pairwise population divergence scattered throughout our sampled range at one of four microsatellites (and with AFLPs), further supporting the results found in previous allozyme-based studies (Hamm and Burton 2000; Chambers et al. 2006). A primary concern regarding the microsatellite loci used here was the high occurrence of null alleles found at three of four loci. Null alleles may alter perceived levels of population differentiation by increasing the frequency of homozygotes and distorting alleleic frequencies (Chapuis and Estoup 2007). In fact, prior analysis not presented here on the uncorrected microsatellite data set indicated significant global and sporadic pairwise divergence throughout the sampled range. These apparent population differences disappeared when the data was corrected for the presence of nulls, attesting to the power of null alleles in driving divergence estimates. A potential reason for the abundance of null alleles may be that the markers were isolated from a congener (*H. kamtschatkana*; Miller et al. 2001) and not the black abalone itself. Cross-species amplification can be problematic (e.g. oysters per Hedgecock et al. 2004) and

microsatellite markers are often developed *de novo* in each new species (Meuller and Wolfenbarger 1999). However, the nulls may also be a function of the loci themselves as all four microsatellites used here exhibited observed heterozygosities less than expected in *H. kamtschatkana*, as well (Miller et al. 2001).

The results from the *Hka*28 microsatellite locus are intriguing. This marker indicated that northern mainland populations appear to be significantly divergent from populations in and near the SCB. Chambers et al. (2006) saw suggestive evidence of this pattern with allozymes where two mainland populations just south of SFB were significantly differentiated from those sampled in the SCB. In addition, a strong signal of IBD is apparent in the *Hka*28 data, supporting a stepping-stone model for gene flow among black abalone populations. Selection cannot be ruled out in structuring the *Hka*28 data (or data from any other locus), however, as it is unknown where the locus is located within the black abalone genome (e.g., it may be linked to or within a selected region). Evidence from allozymes in prior research and the AFLPs included here (discussed below), though, suggests that the signal of restricted gene flow we see in the microsatellite data is also evident in data from other genetic markers.

AFLPs have proven useful in a variety of evolutionary and ecological genetic studies (Campbell et al. 2003; Campbell and Bernatchez 2004; Baus et al. 2005; Brazeau et al. 2005; Murray and Hare 2006). Several studies have shown population structure using AFLP analysis in marine invertebrate species where other markers had indicated high gene flow (Lopez et al. 1999; Shank and Halanych 2007; Weetman et al. 2007; Gruenthal et al. 2007). A particularly attractive attribute of AFLP methods is that specific knowledge of the target genome is not necessary, so the taxon-specific start-up

costs (time and money) associated with development of microsatellite primer sets is circumvented. AFLP banding patterns are highly repeatable (< 2% scoring error in our own analyses), and genome-wide variation is assessed (Vos et al. 1995; Meuller and Wolfenbarger 1999; Avise 2004). AFLPs were employed in this study primarily to further assess the genetic divergence evident in microsatellites and allozymes and to address the uncertainty associated with the microsatellite null alleles. We obtained 142 polymorphic AFLP markers in our subset of six black abalone samples, of which 16 markers showed evidence of significant interpopulation divergence. These data provided further support for IBD among these samples and a stepping-stone model of gene flow for the black abalone (although subtle, the correlation was significant). Of course, selection cannot be ruled out in influencing our AFLP data either, but recent evidence indicates that typically < 5% of AFLP loci are under selective pressure (Wilding et al. 2001; Campbell and Bernatchez 2004; Murray and Hare 2006), including evidence from a related eastern Pacific abalone species, *H. rufescens*, (Gruenthal et al. 2007) and the black abalone here.

Despite the dominant nature of the markers, information lost due to dominance can be minimized by 1) assuming HWE and calculating the frequency of the null allele and/or 2) genotyping a larger number of loci (Campbell et al. 2003). HWE was assumed for all estimations of divergence (locus-specific, global, and pairwise). Regarding the number of loci, we obtained reassignment rates for several pairwise tests in excess of 80%, but a minimum of 250 loci may be required to assign individuals at rates of ≥ 90% for the number of population samples we included (Campbell et al. 2003). Alternatively, it is possible that incorrectly assigned or unassigned individuals are migrants (Paetkau et

al. 1995; Waser and Strobeck 1998; Davies et al. 1999) and evidence of extant gene flow. Waser and Strobeck (1998) report that assignment rates correlate well with F-statistics in empirical studies, and estimates of divergence in the range of those among our black abalone populations do indicate gene flow exists among populations.

Allozyme variation was not assessed here because it was well-characterized in these populations in Hamm and Burton (2000) and Chambers et al. (2006). Debate has occurred over whether allozymes make a true account of neutral variation (Avise 2004), but, as discussed, the same may be said for the other marker types as well. Although we did not find exactly the same genetic patterns found in earlier allozyme-based research (e.g. divergence of the BC population in Hamm and Burton (2000) was not apparent in our data), results among all three studies correlate well in general: *H. cracherodii* experiences some measure of restricted gene flow across much of its sampled range along the coast of California.

Mapping genetic structure onto local hydrography

Hamm and Burton (2000) contrasted population genetic structure in black abalone and red abalone and tied it to seasonal changes in hydrography and corresponding interspecific differences in spawning season. The genetic trends in our black abalone sample are consistent with ocean circulation off of the coast of California as well, despite the expansion in sampling range. Sporadic divergence was found throughout the sampled range and all three marker types indicated significant global divergence among populations. See Figure 4.1b for basic California coastal circulation patterns. Upwelling develops along the California coast during the summer months, creating eddying and

offshore jets that coincide with the black abalone spawning season in late spring through summer (Glickman 1999; CDFG 2005). Abalone larvae are likely the main vector for dispersal, and larvae that are not locally retained have a greater chance of being transported offshore away from suitable habitat during this timeframe, potentially resulting in the patchy genetic divergence we see along the central California coast in the microsatellite and AFLP data.

We found little evidence for divergence among the islands using microsatellites even though waters in the Southern California Bight (SCB) experience increased small-scale eddying (under 50-km in diameter) in summer as the California Current strengthens and becomes more jet-like on the outskirts of the SCB (see http://seis.natsci.csulb.edu/bperry/scbweb/circulation.htm for a description of circulation in the SCB). Offshore jets in the SCB are nearly non-existent, but eddying may provide the opportunity for transport between islands and/or between islands and the southern California mainland. Larvae experiencing transport away from natal grounds in the SCB may have a greater chance of encountering suitable habitat because habitat is not linearly distributed as along the mainland coast. For example, the semi-permanent Catalina Eddy in the Santa Barbara Channel flows counterclockwise past SMI, SRI, SCI, along the mainland just south of Point Conception, and back towards SMI. The eddy may help to maintain gene flow among these islands and, potentially, the mainland near Point Conception (see also Chambers et al. 2006). SNI and SCI were divergent, however, in pairwise analyses using AFLPs. SNI exhibited a more unique PCA signature, but both samples could be reassigned at a rate > 0.80 in specific pairwise analyses. Despite relatively more gene flow than the central California coast, populations may still be

primarily self-recruiting. Indeed, a drift-tube study by Tegner and Butler (1985) designed to mimic larval dispersal in the Channel Islands revealed that the bulk of drift-tubes were recovered within a few kilometers of the deployment site, while only 0.4% reached mainland California within an appropriate timespan for larval duration. A drift-card study during the black abalone spawning season by Chambers et al. reached similar conclusions (MD Chambers pers. comm.).

The black abalone is not alone in exhibiting population genetic patterns consistent with local hydrographic features. Blacklip abalone (*H. rubra*) from southern Australia show differentiation between populations from Victoria and Tasmania due to a potential disruption in gene flow by the sluggish, unidirectional currents of the Bass Straight (Ward and Elliot 2001), and the perlemoen (*H. midae*) from South Africa shows regional divergence east and west of Cape Agulhas consistent with the complex oceanography off of the Cape (Evans et al. 2004).

Implications for fishery management

Miner et al. (2007) conclude that the prospect of black abalone recovery is poor in populations decimated by withering syndrome due to a combination of documented recruitment failure and shifts in community composition away from habitat suitable for abalone. Our data corroborate this conclusion; low interpopulation connectivity and a potential stepping-stone model for dispersal suggest that recovery of extirpated black abalone populations along the coast of California is unlikely to occur in the near future. The 1997 fishery moratorium may be a necessary but not a sufficient measure for ensuring recovery and persistence. If existing natural populations are unable to provide

sufficient numbers of recruits, locally extirpated populations may only recover if stock enhancement operations are initiated in conjunction with ecosystem-based management methods as suggested by Miner et al. (2007). Such operations would likely need to involve outplanting of hatchery reared animals, a procedure that has only met with limited success in previous efforts both in California (Burton and Tegner 2000; Lapota et al. 2000) and elsewhere (Kristiansen et al. 2000; Gutierrez-Gonzalez and Perez-Enriquez 2005; see also Hilborn 1998; Travis et al. 1998; Lorenzen 2005). Furthermore, efforts to induce black abalone to spawn in culture have yet to be successful, although research is ongoing (TB McCormick pers. comm.).

Maximizing the number, types, and polymorphism levels of the markers genotyped increased our chances of encountering meaningful genetic structure among black abalone populations and, ultimately, added confidence to conclusions from past and present research. However, while often generally consistent (global divergence, IBD), specific results among the three marker types varied. More genetic research is needed before a definitive spatial model can be developed for larval dispersal patterns and interpopulation connectivity in the black abalone along the California coast. Application of alternative methods for studying connectivity, such as trace elemental fingerprinting and hydrographic modeling (see Levin 2006), may also prove useful in analyses of abalone populations as demonstrated in other molluscan species (e.g. *Mytilus* mussel spp. (Becker et al. 2005) and the marine gastropods *Concholepas concholepas* (Zacherl et al. 2003) and *Kelletia kelletii* (Zacherl 2005)).

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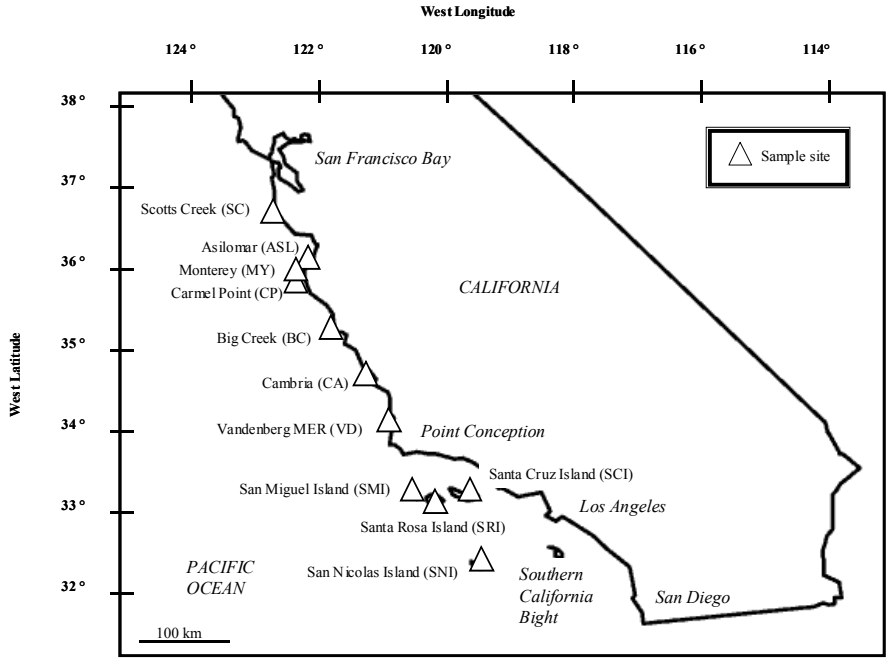
(a)



(b)

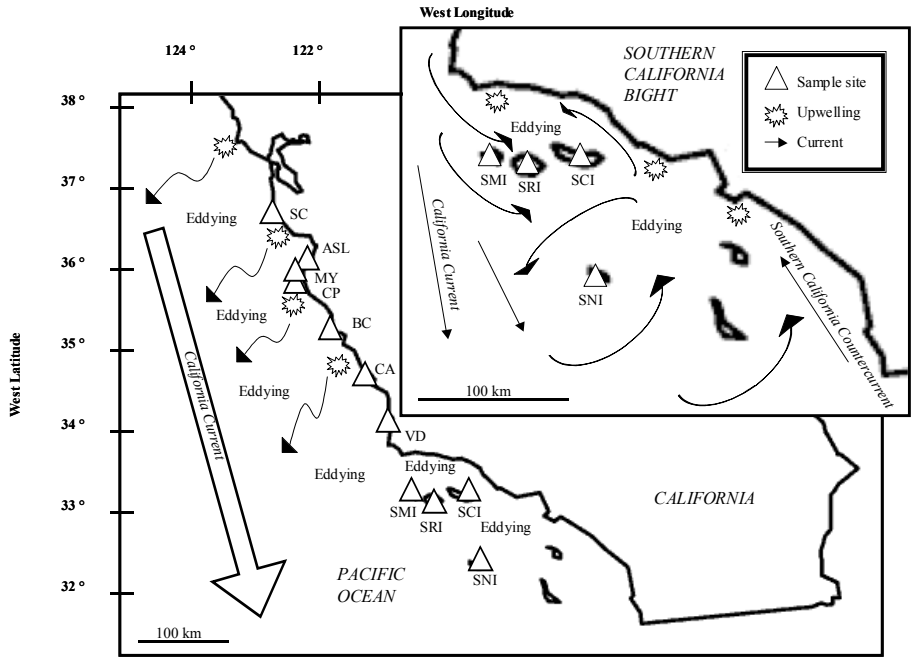


Figure 4.1: Map of California collection sites for *H. cracherodii*. In (a), collection site names are followed by population abbreviations in parentheses. Current systems and upwelling during late spring and summer are represented in (b). Names of places and bodies of water used for reference are in *italics*. Latitude and longitude are included.

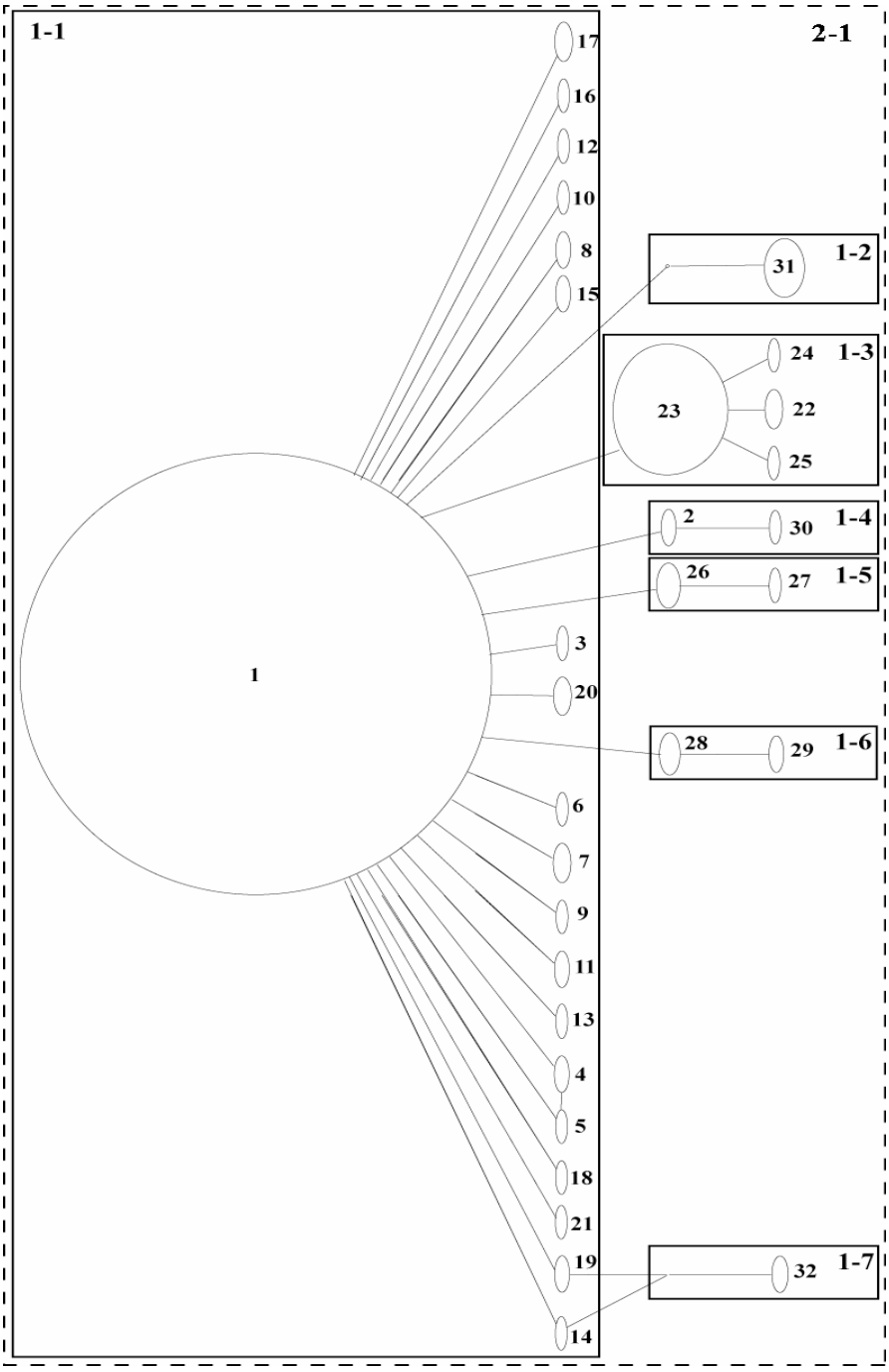


Figure 4.2: COI haplotype network for *H. cracherodii* samples. The network is based on 403 bp of sequence (32 haplotypes). Single lines represent single nucleotide substitutions. Black dots indicate missing intermediate haplotypes. Size of the ovals represents the relative frequency of the haplotype. Nested clades are indicated by the solid (first level) and dashed (second level) lines.

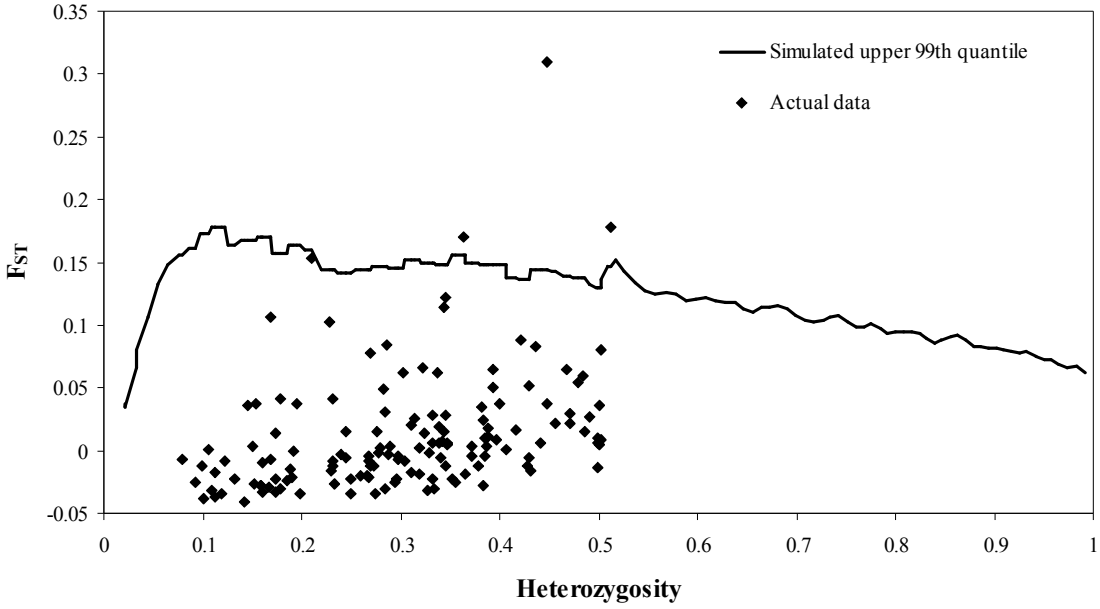


Figure 4.3: Graph of AFLP locus-specific F_{ST} estimates versus inferred heterozygosity used as an outlier test of selection on AFLP loci in *H. cracherodii*. Actual data points (black diamonds) presented for loci. Upper 99th quantile for loci (black line) based on data simulated for 50000 loci. Points above 99th quantile are outliers.

(a)

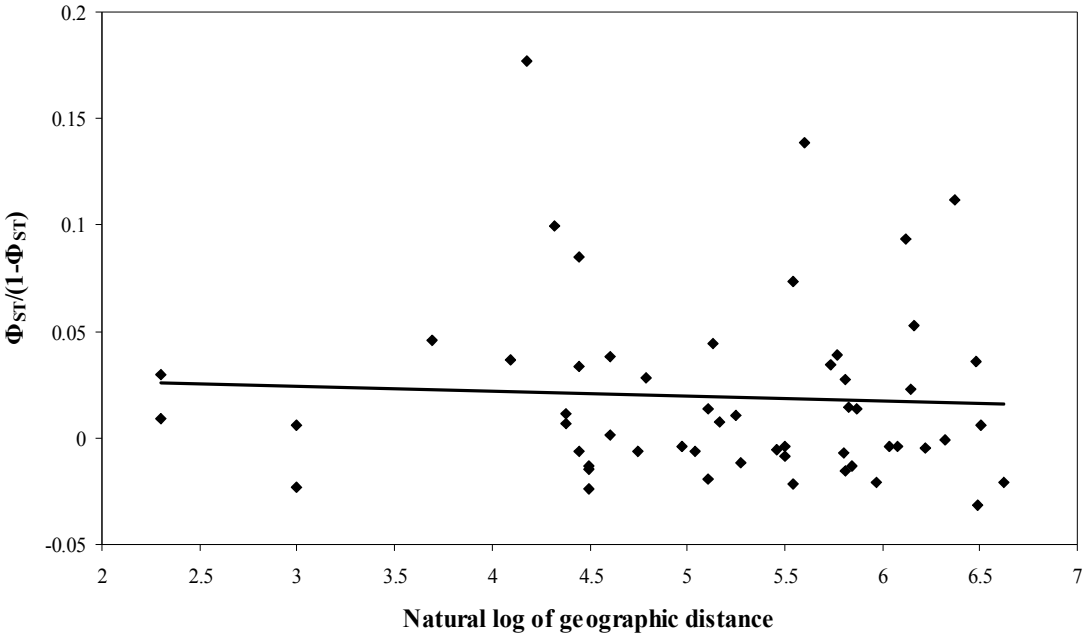
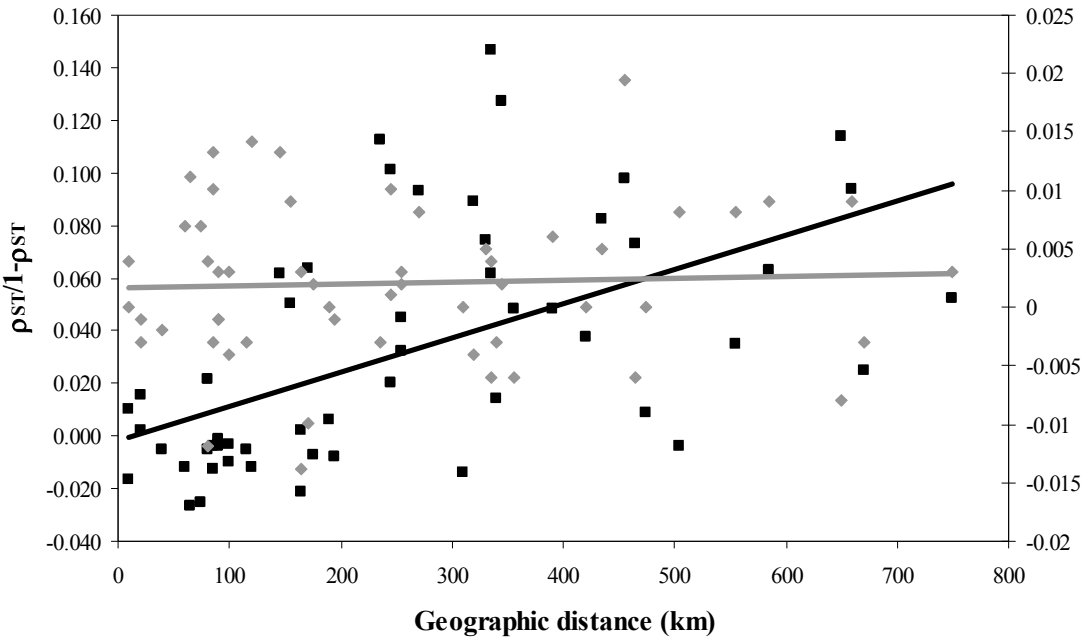


Figure 4.4: Isolation by distance in *H. cracherodii* samples. The pairwise divergence estimates for each marker type are plotted versus geographic distance. Regression equations are (a) y = -0.002x + 0.032 for COI, (b) y = 0.000002 + 0.0016 for all microsatellites (grey) and y = 0.0001x - 0.0017 for *Hka*28 alone (black), and (c) y = 0.005x + 55.552 for all 142 AFLP loci.

(b)



(c)

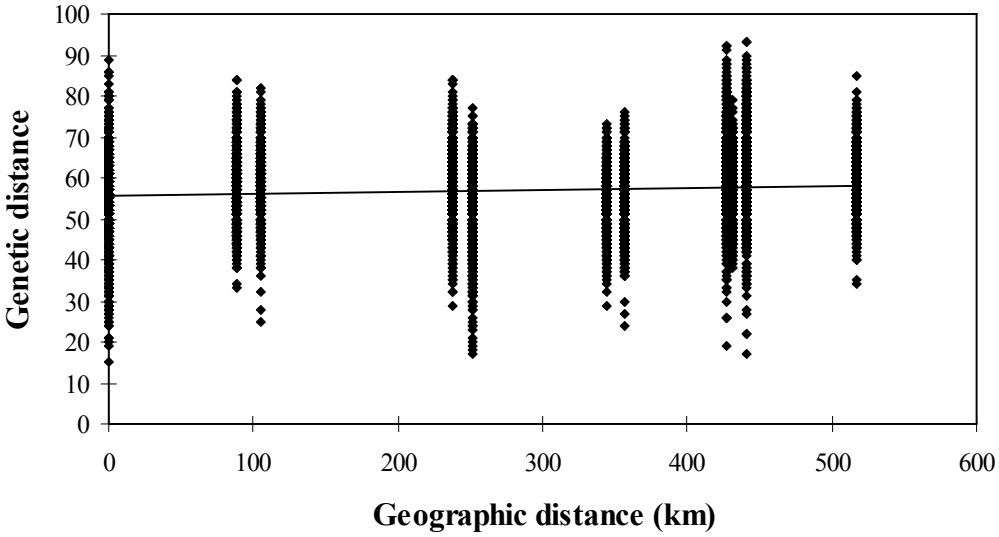


Figure 4.4 continued.

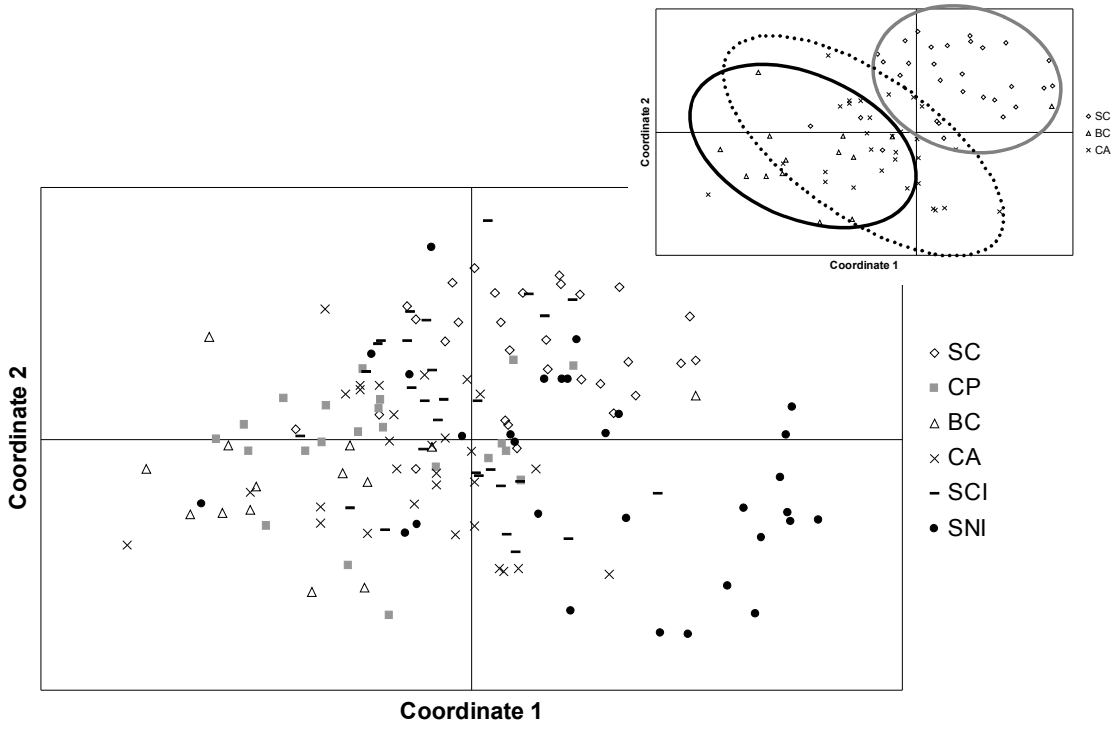


Figure 4.5: Principle Coordinates Analysis on AFLP genotyped *H. cracherodii* samples. Multilocus AFLP genotypes for each individual consisted of 16 AFLP markers with significant F_{ST} estimates. Sample memberships coded by color and shape (see key). Point distributions plotted on axes one and two.

Table 4.1: Summary of collection site names and number of samples run for each marker type for *H. cracherodii*.

Sample	**Abbreviation**	$\mathbf{N_{Total}}$	$\mathbf{N_{COI}}$	$\mathbf{N_{Microsatellites}}$	$\mathbf{N_{AFLP}}$
Scotts Creek	SC	63	24	63	30
Asilomar	ASL	56	24	56	
Monterey	MY	54	20	54	
Carmel Point	CP	23	22	23	23
Big Creek	BC	14	11	14	14
Cambria	CA	58	24	58	30
Vandenberg MER	VD	48	24	48	
San Miguel Island	SMI	101	27	101	
Santa Rosa Island	SRI	55	21	55	
Santa Cruz Island	SCI	29	20	29	29
San Nicolas Island	SNI	87	21	87	30

Table 4.2: Nuclear microsatellite loci genotyped in *H. cracherodii*. Nucleotide repeat sequence, number of alleles, number of chromosomes genotyped, number of expected and observed heterozygotes, expected (H_e) and observed heterozygosities (H_o), Hardy-Weinberg equilibrium (HWE) probability test p-values, and null allele frequencies per locus are included.

Locus	**Repeat**	**Alleles**	**Individuals**	**H_e**	**H_o**	**p**	**Null**
Hka 28	$(CA)_n$	33	579	0.95	0.88	0.08	0.04
Hka 40	$(CA)_n$	51	437	0.94	0.21	0.00	0.54
Hka 56	$(CA)_n$	48	522	0.94	0.34	0.00	0.39
Hka 80	$(CA)_n$	51	520	0.96	0.40	0.00	0.37

Table 4.3: AFLP final amplification primer combinations used on *H. cracherodii* samples. Number of markers genotyped per primer combination and approximate size range of markers included.

Primer combination	*Mse* I primer	*Eco* RI primer	Approx. size range (basepairs)	Number of markers
1	*Mse* I core + CTG	*Eco* RI core + ACG	90 - 325	33
2	*Mse* I core + CTG	*Eco* RI core + AGC	90 - 375	56
3	*Mse* I core + CTG	*Eco* RI core + AGG	110 - 500+	54

Table 4.4: Pairwise population divergences for all three marker types in *H. cracherodii*. Estimates of parameters ρ_{ST} and are provided. Tables include pairwise estimates of (a) Φ_{ST} for h-1fg above diagonal and COI below diagonal, (b) ρ_{ST} for *Hka*28 above diagonal and all microsatellite loci combined below diagonal, and (c) Φ_{PT} for all 142 AFLP markers. Significant parameter estimates after sequential Bonferroni correction at the $\alpha = 0.05$ level are indicated by a *.

(a)

	SC	**ASL**	**MY**	**CP**	**BC**	**CA**	**VD**	**SMI**	**SRI**	**SCI**	**SNI**
SC	-										
ASL	0.011	-									
MY	-0.015	0.009	-								
CP	0.037	-0.024	0.029	-							
BC	0.014	0.078	0.091	0.150	-						
CA	-0.004	-0.020	-0.006	-0.004	0.007	-					
VD	-0.015	-0.022	-0.009	-0.006	0.043	-0.025	-				
SMI	-0.004	0.014	-0.007	0.038	0.068	0.007	-0.007	-			
SRI	-0.004	0.013	-0.014	0.027	0.122	0.010	0.002	0.006	-		
SCI	-0.001	0.050	0.022	0.086	-0.021	0.033	0.027	0.035	0.044	-	
SNI	-0.022	0.006	-0.033	0.035	0.101	-0.005	-0.012	-0.006	-0.014	0.033	-

Table 4.4 continued.

(b)

	SC	ASL	MY	CP	BC	CA	VD	SMI	SRI	SCI	SNI
SC	-	-0.005	-0.003	-0.003	-0.022	0.020	0.058 *	0.036 *	0.076 *	0.034	0.050 *
ASL	0.004	-	0.010	0.015	-0.013	0.002	0.031	0.014	0.046	0.009	0.024
MY	0.003	0.000	-	-0.017	-0.026	0.048 *	0.092 *	0.069 *	0.113 *	0.068 *	0.086 *
CP	-0.004	-0.001	0.004	-	-0.027	0.058	0.101 *	0.082 *	0.128 *	0.089	0.102 *
BC	-0.014	0.010	0.007	0.011	-	0.021	0.060	0.043	0.085 *	0.046	0.059 *
CA	0.001	0.003	0.009	0.013	-0.012	-	-0.001	-0.007	0.006	-0.014	-0.004
VD	-0.006	0.003	0.010	-0.003	-0.010	-0.001	-	-0.004	-0.010	-0.012	-0.008
SMI	0.000	-0.003	0.005	-0.004	0.002	0.002	-0.003	-	0.002	-0.012	-0.005
SRI	0.005	-0.006	0.002	0.004	0.008	0.000	0.003	-0.003	-	-0.005	-0.004
SCI	0.008	0.000	-0.006	0.019	0.006	0.000	0.014	0.007	-0.002	-	-0.013
SNI	0.003	-0.003	0.009	-0.008	0.009	0.008	-0.001	-0.003	-0.001	0.013	-

(c)

	SC	CP	BC	CA	SCI	SNI
SC	-					
CP	0.057 *	-				
BC	0.109 *	0.014	-			
CA	0.032 *	0.019 *	0.053 *	-		
SCI	0.026 *	0.016 *	0.062 *	0.020 *	-	
SNI	0.029 *	0.073 *	0.128 *	0.047 *	0.039 *	-

Table 4.5: Assignment test results for *H. cracherodii*. Tests are presented in rows. Only samples included per test have assignment values. Column labeled 'pooled' includes all samples pooled except sample being tested against.

Test	SC	CP	BC	CA	SCI	SNI	Pooled
1	0.60	0.30	0.64	0.47	0.48	0.50	
2	0.77	0.57					
3	0.87		0.79				
4	0.80			0.83			
5	0.67				0.76		
6	0.90					0.63	
7	0.57						0.80
8		0.70	0.71				
9		0.61		0.73			
10		0.61			0.79		
11		0.70				0.67	
12		0.52					0.78
13			0.79	0.77			
14			0.71		0.93		
15			0.79			0.80	
16			0.71				0.87
17				0.70	0.76		
18				0.87		0.63	
19				0.57			0.86
20					0.83	0.57	
21					0.55		0.83
22						0.57	0.81

References

Avise JC (2004) Molecular markers, natural history, and evolution (second edition). Sinauer Associates, Sunderland, Massachusetts

Baus E, Darrock DJ, Bruford MW (2005) Gene-flow patterns in Atlantic and Mediterranean populations of the Lusitanian sea star *Asterina gibbosa*. *Molecular Ecology*, 14, 3373-3382

Beaumont MA, Nichols RA (1996) Evaluating loci for use in the genetic analysis of population structure. *Proceedings of the National Academy of Sciences of the USA* 263, 1619-1626

Becker BJ, Fodrie FJ, McMillan PA, Levin LA (2005) Spatial and temporal variation in trace elemental fingerprints of mytilid mussel shells: a precursor to invertebrate larval tracking. *Limnology and Oceanography*, 50, 48-60

Bazin E, Glémin S, Galtier N (2006) Population size does not influence mitochondrial genetic diversity in animals. *Science*, 312, 570-572

Bensch S, Åkesson M (2005) Ten years of AFLP in ecology and evolution: why so few animals? *Molecular Ecology*, 14, 2899-2914

Bossart JL, Prowell DP (1998) Genetic estimates of population structure and gene flow: limitations, lessons and new directions. *Trends in Ecology and Evolution*, 13, 202-206

Brazeau DA, Sammarco PW, Gleason DF (2005) A multi-locus genetic assignment technique to assess sources of *Agaricia agarictes* larvae on coral reefs. *Marine Biology*, 147, 1141-1148

Burton RS, Tegner MJ (2000) Enhancement of red abalone *Haliotis rufescens* stocks at San Miquel Island: reassessing a success story. *Marine Ecology Progress Series*, 202, 303-308

California Department of Fish and Game, Marine Region (2005) Final abalone recovery and management plan. The Resources Agency, Sacramento, California

Campbell D, Duchesne P, Bernatchez L (2003) AFLP utility for population assignment studies: analytical investigation and empirical comparison with microsatellites. *Molecular Ecology*, 12, 1979-1991

Campbell D, Bernatchez L (2004) Generic scan using AFLP markers as a means to assess the role of directional selection in the divergence of sympatric whitefish ecotypes. *Molecular Biology and Evolution*, 21, 945-956

Chambers MD, VanBlaricon GR, hauser L, Utter F, Friedman CS (2006) Genetic structure of black abalone (Haliotis cracherodii) populations in the California island and central California coast: impacts of larval dispersal and decimation from withering syndrome. *Journal of Experimental Marine Biology and Ecology*, 331, 173-185

Chapuis M-P, Estoup A (2007) Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution*, 24, 621-631

Clement M, Posada D, Crandall K (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, 9, 1657-1660

Davies N, Villablanca FX, Roderick GK (1999) Determining the source of individuals: multilocus genotyping in nonequilibrium population genetics. *Trends in Ecology and Evolution*, 14, 17-21

Duchesne P, Bernatchez L (2002) AFLPOP: A computer program for simulated and real population allocation based on AFLP data. *Molecular Ecolology Notes*, 3, 380-383

Evans BS, Sweijd NA, Bowie RCK, Cook PA, Elliot NG (2004) Population genetic structure of the perlemoen Haliotis midae in South Africa: evidence of range expansion and founder events. *Marine Ecology Progress Series*, 270, 163-172

Friedman CS, Thomson M, Chun C, Haaker PL, Hedrick RP (1997) Withering syndrome of the black abalone, *Haliotis cracherodii* (Leach): water temperature, food availability, and parasites as possible causes. *Journal of Shellfish Research*, 16, 403-411

Fu Y-X, Li W-H (1993) Statistical tests of neutrality of mutations. *Genetics*, 133, 693-709

Geiger DL (2000) Distribution and biogeography of the recent Haliotidae (Gastropoda: Vetigastropoda) worldwide. *Bolletino Malocologico*, 35, 57-120

Gilg MR, Hilbish TJ (2003) Dispersal patterns of mussel larvae throughout a hybrid zone in Southwest England. *Evolution*, 57, 1061-1077

Glickman TS (1999) Glossary of Meteorology, 2nd Edition. American Meteorological Society, Cambridge, Massachusetts

Gruenthal and Burton (2005) Genetic diversity and species identification in the endangered white abalone (*Haliotis sorenseni*). *Conservation Genetics*, 6, 929-939

Gruenthal KM, Acheson LK, Burton RS (2007) Genetic structure of natural populations of California red abalone (*Haliotis rufescens*) using multiple genetic markers. *Marine Biology*, DOI 10.1007/s00227-0771-007-4

Gutierrez-Gonzalez JL, Perez-Enriquez R (2005) A genetic evaluation of stock enhancement of blue abalone *Haliotis fulgens* in Baja California, Mexico. *Aquaculture*, 247, 233-242

Haaker PL, Parker DO, Chun CSY (1992) Growth of black abalone, *Haliotis cracherodii* Leach, at San Miguel Island and Point Arguello, California. *Journal of Shellfish Research*, 14, 519-525

Hamm DE, Burton RS (2000) Population genetics of black abalone, *Haliotis cracherodii*, along the central California coast. *Journal of Experimental Marine Biology and Ecology*, 254, 235-247

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003a) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B Biology Sciences*, 270, 313-321

Hebert PDN, Ratnasingham S, deWaard JR (2003b) Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related taxa. *Proceedings of the Royal Society of London B Biology Sciences*, 270, S96-S99

Hedgecock D, Li G, Hubert S, Bucklin K, Ribes V (2004) Widespread null alleles and poor cross-species amplification of microsatellite DNA loci cloned from the Pacific oyster, *Crassostrea gigas*. *Journal of Shellfish Research*, 23, 379-385

Hellberg ME, Burton RS, Neigel JE, Palumbi SR (2002) Genetic assessment of connectivity among marine populations. *Bulletin of Marine Science*, 70 Supplement, 273-290

Hilbish TJ, Deaton LE, Koehn RK (1982) Effect of an allozyme polymorphism on regulation of cell volume. *Nature*, 298, 688-689

Hilborn R (1998) The economic performance of marine stock enhancement projects. *Bulletin of Marine Science*, 62, 661-674

Hobday AJ, Tegner MJ (2000) Status review of white abalone (*Haliotis soreseni*) throughout its range in California and Mexico. NOAA Tehnical Memorandum NOAA-TM-NMFS-SWR-035. U.S. Department of Commerce, National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Southwest Region

Kalinowski ST, Taper ML (2006) Maximum likelihood estimation of the frequency of null alleles at microsatellite loci. *Conservation Genetics*, 7, 991-995

Karl SA, Avise JC (1992) Balancing selection at allozyme loci in oysters: implications from nuclear RFLPs. *Science*, 256, 100-102

Kimura M (1991) Recent development of the neutral theory viewed from the Wrightian tradition of theoretical population genetics. *Proceedings of the National Academy of Science of the USA*, 88, 5969-5973

Kirby VL, Villa R, Powers DA (1998) Identification of microsatellites in the California red abalone, *Haliotis rufescens*. *Journal of Shellfish Research*, 17, 801-804

Kristiansen TS, Otterå, Svåsand T (2000) Size-dependent mortality of juvenile Atlantic cod, estimated from recaptures of released reared cod and tagged wild cod. *Journal of Fish Biology*, 56, 687-712

Lapota D, Rosen G, Chock J, Liu CH (2000) Red and green abalone seed growout for reseeding activities off Point Loma, California. *Journal of Shellfish Research*, **19**(1), 431-438.

Leighton DL (2000) The biology and culture of the California abalones. Dorrance Publishing, Co., Inc., Pittsburg, Pennsylvania

Levin L (2006) Recent progress in understanding larval dispersal: new directions and digressions. *Integrative and Comparative Biology*, 46, 282-297

Liu X, Liu Xi, Guo X, Gao Q, Zhao H, Zhang G (2006) A preliminary genetic linkage map of the pacific abalone *Haliotis discus hannai* Ino. *Marine Biotechnology*, 8, 386-397

Lopez JV, Kersanach R, Rehner SA, Knowlton N (1999) Molecular determination of species boundaries in corals: genetic analysis of the *Montastraea annularis* complex using amplified fragment length polymorphisms and a microsatellite marker. *Biological Bulletin*, 196, 80-93

Lorenzen K (2005) Population dynamics and potential of fisheries stock enhancement: practical theory for assessment and policy analysis. *Philosophical Transactions of the Royal Society B*, 360, 171-189

Medina RF, Barbosa P, Christman M, Battisti A (2006) Number of individuals and molecular markers to use in genetic differentiation studies. *Molecular Ecology Notes*, 6, 1010-1013

Metz EC, Robles-Sikisaka R, Vacquier VD (1998) Nonsynonymous substitution in abalone sperm fertilization exceeds substitution in introns and mitochondrial DNA. *Proceedings of the National Academy of Sciences of the USA*, 95, 10676-10681

Meuller UG, Wolfenbarger LL (1999) AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution*, 14, 389-394

Michalakis Y, Excoffier L (1996) A generic estimation of population subdivision using distances between alleles with special reference for microsatellite loci. *Genetics*, 142, 1061-1064

Miller KM, Laberee K, Kaukinen KH, Li S, Withler RE (2001) Development of microsatellite loci in pinto abalone (*Haliotis kamtschatkana*). *Molecular Ecology Notes*, 1, 315-317

Miller M (1997) Tools for Population Genetic Analyses (TFPGA) version 1.3: a Windows® program for the analysis of allozyme and molecular population genetic data. Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona

Miner CM, Alstatt JM, Raimondi PT, Minchinton TE (2007) Recruitment failure and shifts in community structure following mass mortality limit recovery prospects of black abalone. *Marine Ecology Progress Series*, 327, 107-117

Morse DE, Hooker N, Duncan H, Jensen L (1979) Gamma-aminobutyric acid, a neurotransmitter, induces planktonic abalone larvae to settle and begin metmorphosis. *Science*, 204, 407-410

Murray MC, Hare MP (2006) A genomic scan for divergent selection in a secondary contact zone between Atlantic and Gulf of Mexico oysters, *Crassostrea virginica*. *Molecular Ecology*, 15, 4229-4242

Paetkau D, Calvert W, Stirling I, Strobeck C (1995) Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology*, 4, 347-354

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences*, 12, 357-358

Peakall R, Smouse PE (2006) GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6, 288-295

Posada D, Crandall KA, Templeton AR (2000) GeoDis: A program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, 9, 487-488

Raymond M, Rousset F (1995) GENEPOP: population genetics software for exact tests and ecumenicism. *Journal of Heredity*, 86, 248-249

Raymond M, Rousset F (2003) GENEPOP (version 3.4): population genetics software for exact tests and ecumenicism. Updated from Raymond and Rousset (1995)

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, 43, 223-225

Rogers AR, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution*, 9, 552-569

Rozas J, Sanches-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, 19, 2496-2497

Ruzzante DE (1998) A comparison of several measure of genetic distance and population structure with microsatellite data: bias and sampling variance. *Canadian Journal of Fisheries and Aquatic Sciences*, 55, 1-14

Schneider S, Roessli D, Excoffier L (2000) Arlequin ver.2.000: A software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland

Sekino M, Saido T, Fujita T, Kobayashi T, Takami H (2004) Microsatellite DNA markers of Ezo abalone (*Haliotis discus hannai*): a preliminary assessment of natural populations sampled from heavily stocked areas. *Aquaculture*, 243, 33-47

Shank TM, Halanych KM (2007) Toward a mechanistic understanding of larval dispersal: insights from genomic fingerprinting of the deep-sea hydrothermal vent tubeworm *Riftia pachyptila*. *Marine Ecology*, 28, 25-35

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, 139, 457-462

Streit K, Geiger DL, Lieb B (2005) Molecular phylogeny and the geographic origin of Haliotidae traced by haemocyanin sequences. *Journal of Molluscan Studies*, 72, 105-110

Swofford DL (1998) PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 125, 585-595

Tegner MJ, Butler RA (1985) Drift-tube study of the dispersal potential of green abalone (*Haliotis fulgens*) larvae in the southern California Bight: implications for recovery of depleted populations. *Marine Ecology Progress Series*, 26, 73-84

Templeton AR, Routman E, Phillips CA (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics*, 140, 767-782

Thorrold SR, Jones GP, Hellberg ME, Burton RS, Swearer SE, Neigel JE, Morgan SG, Warner RR (2002) Quantifying larval retention and connectivity in marine populations with artificial and natural markers. *Bulletin of Marine Science*, 70 Supplement, 291-308

Travis J, Coleman FC, Grimes CB, Conover D, Bert TM, Tringali M (1998) Critically assessing stock enhancement: an introduction to the Mote Symposium. *Bulletin of Marine Science*, 62, 305-311

Veliz, D, Duchesne P, Bourget E, Bernatchez L (2006) Stable genetic polymorphism in heterogeneous environments: balance between asymmetrical dispersal and selection in the acorn barnacle. *Journal of Evolutionary Biology*, 19, 589-599

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, 23, 4407-4414

Waples RS (1998) Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. *Journal of Heredity*, 89, 438-450

Ward RD, Elliot NG (2001) Genetic population structure of species in the South East Fishery of Australia. *Marine and Freshwater Research*, 52, 563-573

Warner RR, Cowen RK (2002) Local retention of production in marine populations: evidence, mechanisms, and consequences. *Bulletin of Marine Science*, 70, S245-S249

Waser PM, Strobeck C (1998) Genetic signatures of interpopulation dispersal. *Trends in Ecology and Evolution*, 13, 43-44

Weetman DW, Ruggerio A , Mariani S, Shaw PW, Lawler AR, Hauser L (2007) Hierarchical population structure in the commercially exploited shrimp *Crangon crangon* identified by AFLP analysis. *Marine Biology*, 151, 565-575

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, 38, 1358-1370

Wilding CS, Butlin RK, Grahame J (2001) Differential gene exchange between parapatric morphs of *Littorina saxatilis* detected using AFLP markers. *Journal of Evolutionary Biology*, 14, 611–619

Zacherl DC, Manriquez PH, Paradis G, Day RW, Castilla JC, Warner RR, Lea DW, Gaines SD (2003) Trace elemental fingerprinting of gastropod statoliths to study larval dispersal trajectories. *Marine Ecology Progress Series*, 248, 297-303

Zacherl DC (2005) Spatial and temporal variation in statolith and protoconch trace elements as natural tags to track larval dispersal. *Marine Ecology Progress Series*, 290, 145-163

CHAPTER V

Population structure of pink abalone (*Haliotis corrugata*) in southern California and Mexico

Abstract

Samples were collected from three natural populations of the pink abalone (*Haliotis corrugata* Wood 1828) separated by approximately 600 km, including San Clemente Island in the Southern California Bight, Point Loma near San Diego off the California mainland coast, and Punta Eugenia midway down the Baja California peninsula in Mexico to assess levels of interpopulation genetic divergence in the species as a proxy for realized connectivity. Samples from San Clemente Island were taken from multiple locations around the island to examine potential microgeographic genetic structure. While no interpopulation differences were seen using COI, evidence for restricted gene flow was seen in pairwise tests using microsatellites. San Clemente Island and Point Loma were different at the *Hka*40 locus, but the Punta Eugenica sample was not different from either southern California sample. On San Clemente Island, the subsample of Seal Cove was different from both the west end of the airstrip runway and Pyramid Head at *Hka*3, and the east end of the runway was different from west end of the runway at *Hka*40. Our microsatellite data supports the hypothesis that the pink abalone may experience some restriction in gene flow along the California, USA, and Baja California, Mexico, coasts, but more research is needed before any definitive conclusions can be reached.

Introduction

Abalone populations in the state of California once supported a valuable commercial fishery. Serial fishery crashes, however, in all five formerly commercially fished abalone species during the latter half of last century prompted the California Department of Fish and Game to declare a moratorium on the abalone fishery in 1997. A sport fishery for the red abalone (*Haliotis rufescens*) north of San Francisco Bay is all that remains. The crashed were so severe, that the white abalone (*H. sorenseni*) was declared an Endangered Species in 2001 under the Endangered Species Act (ESA) of 1973 and the black abalone (*H. cracherodii*) has been considered a candidate for listing since 2006.

The pink abalone (*Haliotis corrugata*), once common in southern California, is now considered a Species of Concern by the US Federal government. The species once supported a viable fishery in California that peaked in 1952, but abundances have dropped since then, with severe declines starting in about 1985 (CDFG 2005). Pink abalone is still one of the main commercial species of abalone (along with the green, *H. fulgens*) caught in Mexico, however (Del Río-Portilla and González-Avilés 2001). The species' range extends from around Point Conception in Calfornia, USA, to the tip of the Baja California peninsula in Mexico (Geiger 2000), and as the range crosses USA/Mexico political boundary, both countries have vested interest in the pink abalone's persistence and recovery. Persistence depends upon successful recruitment of abalone larvae to suitable habitat. Abalone larvae are likely the main vector for dispersal, and the pelagic larval period for California abalone lasts for approximately four to 15 days (Leighton 2000). It is often unknown, however, whether the larval period is a good

predictor of realized dispersal distance (i.e. whether populations of the pink abalone primarily self-recruiting despite time spent in the plankton).

A method for inferring the actual interpopulation dispersal is to use population genetic techniques to indirectly measure gene flow. Previous research by Del Río-Portilla and González-Avilés (2001) using allozyme loci indicated that there may be restricted dispersal among populations of pink abalone on Cedros and San Benito Islands off Punta Eugenia on Baja California, Mexico. In this study, we use two other types of genetic markers: mitochondrial cytochrome oxidase subunit one (COI) DNA sequencing and nuclear microsatellite genotyping. We include samples from three natural populations of the pink abalone separated by approximately 600 km, including San Clemente Island in the Southern California Bight, Point Loma near San Diego off the California mainland coast, and Punta Eugenia midway down the Baja California peninsula in Mexico. Samples from San Clemente Island were taken from multiple locations around the island to assess potential microgeographic genetic structure around the island.

Materials and Methods

Sample collection and DNA extraction

Collection sites are summarized in Figure 5.1. Samples were collected from San Clemente Island (SCLE, N = 77) by the California Department of Fish and Game in 2003, Point Loma (PL, N = 36) by E Kisfaludy of Scripps Institution of Oceanography in 2002, and Punta Eugenia (MEX, N = 14), Mexico, by D Richards of the US National Park Service in 1998. The SCLE sample was divided into seven subpopulations: Castle Rock (CR, N = 8), west of Bird Rock (WBR, N = 4), east end of the airport runway (EER, N = 9), Pyramid Head (PH, N = 10), Seal Cove (SC, N = 25), Mail Point (MP, N = 5), and the west end of the airport runway (WER, N = 11) to test microgeographic genetic structure around the island. Epipodial tentacle or foot clippings were preserved in ethanol or water and stored at -80ºC. DNA was extracted from approximately 25 milligrams of tissue using a DNeasy Tissue Kit (Qiagen Inc., Valencia, CA) and eluted in 200 µl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). A 5 µL aliquot of the concentrated stock DNA was diluted with 45 µL of DNA-grade water, placed in 96-well trays, and stored at 4ºC for use in the polymerase chain reaction (PCR).

COI

A 580 basepair (bp) fragment of the mitochondrial cytochrome oxidase subunit one (COI) gene was amplified in all individuals. PCR primers and cycling conditions were taken from Metz et al. (1998). Reactions were carried out in 15 µL volumes, including 5 µL of dilute template DNA, 0.5 U REDTaq DNA polymerase (Sigma-Aldrich Corporation, St. Louis, MO), 1.5 µL 10X PCR buffer, 2.5 mM $MgCl_2$ final

concentration, 2 mM each dNTP final concentration, and 3 pmoles each primer. Thermal cycling was performed in Thermo-Hybaid PCR Express and Px2 thermal cyclers (Thermo Electron Corporation, Boston, MA). The amplified fragments were sequenced on a MegaBACE™ 500 DNA sequencer (Amersham Biosciences Corp., Piscataway, NJ) using the forward primer AB-COIF and a DYEnamic™ ET dye terminator sequencing kit (Amersham Pharmacia Inc., Piscataway, NJ). Sequences were aligned and edited to 319 bp using Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI). Analyses were conducted with Arlequin v.2.000 (Scheider et al. 2000) and DnaSP v4.0.0 (Rozas et al. 2003).

Microsatellites

Microsatellite loci, including one compound repeat *Hka*3 and two dinucleotide repeats *Hka*40 and *Hka*56, were amplified in all individuals (Table 5.1). The primers used were originally developed for a related eastern Pacific species, the pinto abalone *H. kamtschatkana* (Miller et al., 2001). Forward primers were 5' end-labeled with HEX or TET for fluorescent visualization. Reactions were carried out in 25 μL volumes, including 5 μL of dilute template DNA, 0.5 U REDTaq DNA polymerase (Sigma-Aldrich Corporation, St. Louis, MO), 2.5 μL 10X PCR buffer, 2.5 mM $MgCl_2$ final concentration, 2 mM each dNTP final concentration, and 5 pmoles each primer. Thermal cycling was performed in Thermo-Hybaid PCR Express and Px2 thermal cyclers (Thermo Electron Corporation, Boston, MA). Cycling conditions were modified slightly from Miller et al. (2001) for optimization.

Following amplification, 2 μL of a denaturing solution containing xylene cyanol and bromophenol blue dyes in 99% formamide were added to each 5 μL of PCR product. Samples were loaded onto vertical 0.4mm thick large format (33 x 39 cm) 6% polyacrylamide denaturing gels containing 33% formamide and 7M urea. ROX-labeled MegaBACE™ ET900-R Size Standard (Amersham Pharmacia Inc., Piscataway, NJ) was run concurrently as a size standard. Gels were electrophoresed at 60 Watts for two to three hours and scanned on a Typhoon 9410 Variable Mode Imager (Molecular Dynamics Inc., Sunnyvale, CA). The data were analyzed using GENEPOP v3.4 (Raymond and Rousset 1995, 2003) and ML-Relate (Kalinowski et al. 2006).

Results

COI

The COI data set was composed of 100 sequences (62 from SCLP, 27 from PL, and 11 from MEX) of 319 bp in length. There were four total haplotypes and three base change mutations (59G→A, 209A→G, and 269G→A). Protein sequence was determined according to the invertebrate mitochondrial code for *Drosophila melanogaster*. None of the base changes resulted in amino acid changes within the sample set.

To evaluate levels of genetic structure, an hierarchical analysis of molecular variance (AMOVA) was performed in Arlequin to estimate the F_{ST} analogue Φ_{ST} (Weir and Cockerham 1984), which takes into account the genetic distance between haplotypes. Significance of the Φ_{ST} values was calculated based upon χ^2 tests of the parameter value being significantly different from zero (i.e. no genetic divergence; $\chi^2 = 2N\Phi_{ST}$, df = (k – 1)(s – 1), where k is the number of alleles at a locus and s is the number of populations). The mitochondrial data provided little evidence for population genetic structure. Regardless of grouping, over 99% of variation was within populations rather than among populations or groups (global $\Phi_{ST} = 0.002$), and there was no evidence for microgeographic structure around San Clemente Island (global $\Phi_{ST} = -0.018$). Pairwise estimates of Φ_{ST} between populations or SCLE subpopulations were also not significant (data not shown).

Microsatellites

Basic statistical information regarding the microsatellite data used here is presented in Table 5.1. The final data set consisted of 127 total multilocus microsatellite genotypes, including 77 from SCLE, 36 from PL, and 14 from MEX. Significant departures from Hardy-Weinberg equilibrium (HWE) were found at two of three loci due to a deficiency of heterozygotes in all three populations for the *Hka*56 locus and only at SCLE for the *Hka*40 locus. The departures from HWE were assumed to be the result of null alleles due to the presence of null homozygotes at a locus for individuals where other loci amplified. Since null alleles can artificially elevate the signal of genetic structure among populations, the frequency of the null was calculated using the maximum likelihood program ML-Relate (Table 5.1). Only one of the loci, *Hka*56, showed a high frequency of null alleles in excess of 26%. *Hka*40 and *Hka*56 also exhibited significant linkage disequilibrium (LD) in SCLE; all other locus pairs exhibited no evidence of LD.

Estimates of a second F_{ST} analogue ρ_{ST}, which takes into account microsatellite allele size assuming the stepwise mutation model, were calculated in GENEPOP (Weir and Cockerham 1984; Slatkin 1995; Michalakis and Excoffier 1996). Levels of significance were again calculated based on χ^2 tests of the parameter value being significantly different from zero. There was no evidence for significant global divergence among populations ($\rho_{ST} = 0.0129$) or among subpopulations on SCLE ($\rho_{ST} = 0.0120$). Pairwise estimates of ρ_{ST}, however, showed sporadic significant divergence between populations and SCLE subpopulations after sequential Bonferroni correction at the table-wide $\alpha = 0.05$ level (Rice 1989). Significance depended on the microsatellite marker (Table 5.2). SCLE and PL were different at the *Hka*40 locus, but the MEX sample (despite having the smallest sample size) was not different from either SCLE or

PL. On SCLE, the subpopulation of SC was different from both WBR and PH at *Hka*3, and EER was different from WER at *Hka*40.

Discussion

Del Río-Portilla and González-Avilés (2001) found significant divergence among populations pink abalone on Cedros and San Benito Islands off Punta Eugenia on Baja California, Mexico, using eight allozyme loci. We saw no evidence of divergence using mitochondrial COI, but COI has met with limited success in other California abalone species that have shown significant interpopulation divergences using other genetic marker types (Hamm and Burton 2000; Gruenthal et al. in review; Gruenthal and Burton in review). Nuclear microsatellites, however, did exhibit sporadic pairwise divergence among three pink abalone populations, including San Clemente Island (SCLE), Point Loma (PL), and Punta Eugenia (MEX).

Unexpectedly, interpopulation geographic distance was not a factor in the levels of pairwise divergence; SCLE was different from PL, but, at a distance of ~600 km, the MEX sample was not diverged from either SCLE or PL. There are a few potential explanations: 1) the small size of the MEX sample (N = 14) could result in a Type II error, or erroneously accepting the null hypothesis of no genetic divergence among populations; 2) samples do not represent the true allele frequency profile of the natural population; and 3) the Punta Eugenia area exchanges migrants with populations near southern California, but genetic homogeneity must be interpreted with caution, since as few as one to ten migrants per generation can homogenize allele frequencies between populations that are primarily self-recruiting (Wright 1978; Slatkin 1987; Mills and Allendorf 1996).

We also saw evidence for microgeographic genetic structure around San Clemente Island, but the sample sizes were extremely small for microsatellite analyses

(Ruzzante 1998). Divergence levels here could be the result of a Type 1 error, or erroneously rejecting the null hypothesis that there is no genetic divergence among populations, due to an incomplete sampling of genetic variation within a population. Del Río-Portilla and González-Avilés (2001) address the issue of small samples sizes for their data and conclude that small sample size did not affect their results in the context of their study. We cannot make that same assumption for our microgeographic data.

Null alleles may also artificially elevate estimates of divergence (as mentioned previously). In our case, however, the locus that was primarily affected (*Hka*56 exhibited null alleles in excess of 26% frequency) showed no evidence of genetic divergence (significant divergence was restricted to the other two loci with null frequencies of ≤5%), indicating that null alleles are not a driving force in our results. The convergence of the Del Río-Portilla and González-Avilés (2001) allozyme results and our results suggests that a restriction in gene flow among pink abalone populations may indeed exist at least at mid-range spatial scales, although more research is needed before any definitive conclusions about interpopulation connectivity in the pink abalone can be reached.

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Figure 5.1: Map of California and Baja California collection sites for *H. corrugata*. Inset shows subpopulation sample collection sites for San Clemente Island (SCLE). San Clemente Island map adapted from Jorgensen and Ferguson (1984).

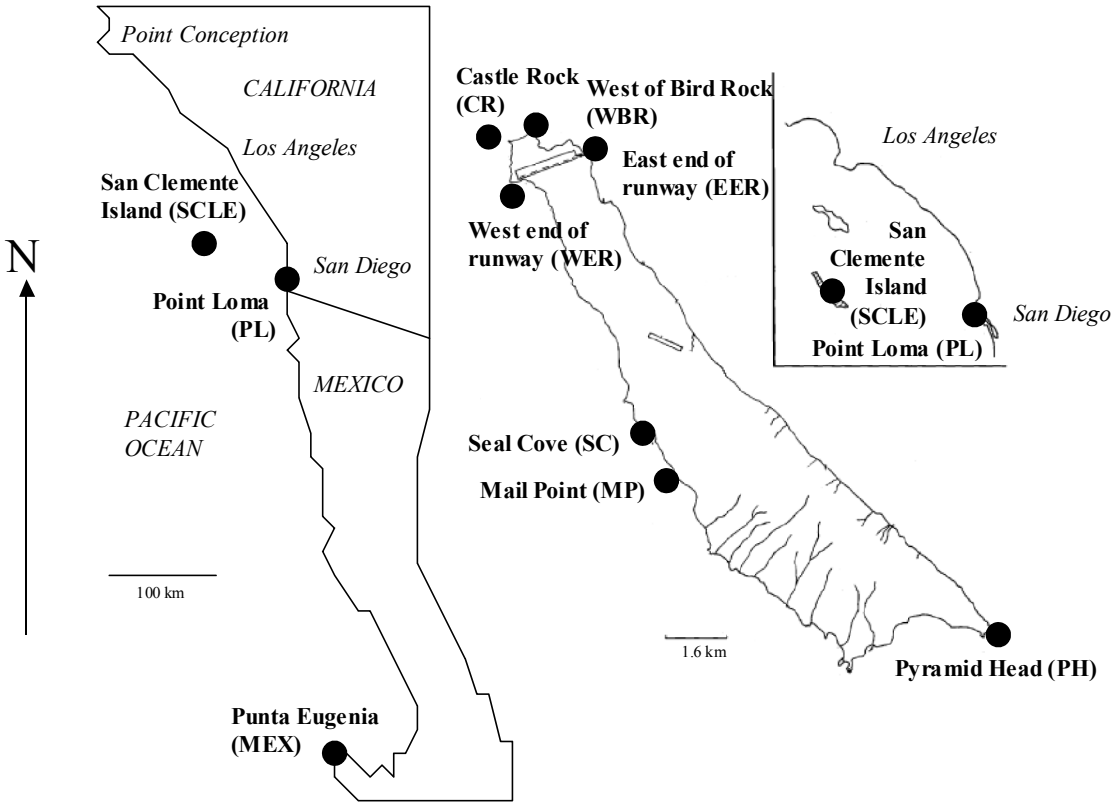

Table 5.1: Descriptive statistics for nuclear microsatellite loci in *H. corrugata*. Nucleotide repeat sequence, number of alleles ($N_{alleles}$), number of individuals genotyped out of 127 total ($N_{individuals}$), number of expected and observed heterozygotes, expected (H_e) and observed heterozygosities (H_o), Hardy-Weinberg equilibrium probability test p-values (p), and null allele frequencies (Null) per locus are included. All microsatellite loci originally isolated in the northern abalone *H. kamtschatkana* (Miller et al. 2001).

Locus	**Repeat**	**$N_{alleles}$**	**$N_{individuals}$**	**H_e**	**H_o**	**p**	**Null**
Hka 3	$(GTA)_n(GAGT)_n$	87	106	0.987	0.953	0.179	0.014
Hka 40	$(CA)_n$	60	104	0.978	0.975	0.013	0.054
Hka 56	$(CA)_n$	33	97	0.948	0.557	<0.001	0.261

Table 5.2: Pairwise population divergences for the microsatellites in *H. corrugata*. Tables include pairwise estimates of ρ_{ST} for *Hka*3, *Hka*40, *Hka*56, and all microsatellites combined. Significant parameter estimates after sequential Bonferroni correction at the $\alpha = 0.05$ level are indicated by a *.

Pop. 1	**Pop. 2**	**Microsatellite**			
		***Hka* 3**	***Hka* 40**	***Hka* 56**	**All**
SCLE	PL	-0.002	0.052 *	-0.018	0.029 *
SCLE	MEX	-0.010	-0.020	-0.050	-0.019
PL	MEX	-0.035	0.041	-0.069	0.019
CR	WBR	0.110	0.038	0.004	0.073
CR	EER	-0.056	0.178	-0.236	0.063
CR	PH	0.067	-0.143	-0.156	-0.055
CR	SC	-0.061	-0.039	-0.129	-0.052
CR	MP	-0.102	-0.124	-0.233	-0.123
CR	WER	0.001	-0.079	-0.119	-0.035
WBR	EER	0.182	-0.091	-0.115	0.046
WBR	PH	-0.103	-0.030	-0.071	-0.061
WBR	SC	0.443 *	-0.002	-0.024	0.166 *
WBR	MP	-0.058	0.065	-0.211	-0.015
WBR	WER	-0.228	0.191	0.027	-0.054
EER	PH	-0.006	0.117	-0.118	0.054
EER	SC	0.038	0.079	-0.081	0.054
EER	MP	-0.098	0.168	-0.178	0.040
EER	WER	-0.040	0.314 *	-0.086	0.111
PH	SC	0.185 *	-0.043	-0.058	0.028
PH	MP	-0.052	-0.137	-0.128	-0.104
PH	WER	-0.054	-0.086	-0.053	-0.066
SC	MP	0.013	-0.042	-0.060	-0.029
SC	WER	0.098	0.014	-0.065	0.041
MP	WER	-0.080	-0.082	-0.048	-0.078

References

California Department of Fish and Game, Marine Region (2005) Final abalone recovery and management plan. The Resources Agency, Sacramento, California

Geiger DL (2000) Distribution and biogeography of the recent Haliotidae (Gastropoda: Vetigastropoda) worldwide. *Bolletino Malocologico*, 35, 57-120

Gruenthal KM, Burton RS (2007) The influence of genetic marker choice on detecting gene flow among populations of the California black abalone (*Haliotis cracherodii*), *Molecular Ecology*, in review

Gruenthal KM, Acheson LK, Burton RS (2007) Genetic structure of natural populations of California red abalone (*Haliotis rufescens*) using multiple genetic markers. *Marine Biology*, DOI 10.1007/s00227-007-0771-4

Jorgensen PD, Ferguson HL (1984) The birds of San Clemente Island. *Western Birds*, 15, 111-130

Kalinowski ST, Wagner AP, Taper ML (2006) ML-Relate: a computer program for maximum likelihood estimation of relatedness and relationship. *Molecular Ecology Notes*, 6, 576-579

Leighton DL (2000) The biology and culture of the California abalones. Dorrance Publishing Company, Incorporated, Pittsburg, Pennsylvania

Michalakis Y, Excoffier L (1996) A generic estimation of population subdivision using distances between alleles with special reference for microsatellite loci. *Genetics*, 142, 1061-1064

Miller KM, Laberee K, Kaukinen KH, Li S, Withler RE (2001) Development of microsatellite loci in pinto abalone (*Haliotis kamtschatkana*). *Molecular Ecology Notes*, 1, 315-317

Mills LS, Allendorf FW (1996) The one-migrant-per-generation rule in conservation and management. *Conservation Biology*, 10, 1509-1518

Raymond M, Rousset F (1995) GENEPOP: population genetics software for exact tests and ecumenicism. *Journal of Heredity*, 86, 248-249

Raymond M, Rousset F (2003) GENEPOP (version 3.4): population genetics software for exact tests and ecumenicism. Updated from Raymond and Rousset (1995)

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, 43, 223-225

Rozas J, Sanches-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, 19, 2496-2497

Ruzzante DE (1998) A comparison of several measure of genetic distance and population structure with microsatellite data: bias and sampling variance. *Canadian Journal of Fisheries and Aquatic Sciences*, 55, 1-14

Schneider S, Roessli D, Excoffier L (2000) Arlequin ver.2.000: A software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland

Slatkin M (1987) Gene flow and the geographic structure of natural populations. *Science*, 236, 787-792

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, 139, 457-462

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, 38, 1358-1370

Wright JS (1978) Variability within and among natural populations. Evolution and the genetics of natural populations: Vol. 4. University of Chicago Press, Chicago, Illinois

CHAPTER VI

The impact of withering syndrome on gut microbiomes of California abalone

Abstract

Resident microbiomes (i.e. the sum total bacterial population found in or on an organism) can significantly influence the health of their hosts. The bacterial compositions of the gut microbiomes of healthy black abalone and red abalone and those diagnosed with the fatal disease withering syndrome (WS) were examined using universal 16S rDNA clone libraries. Healthy abalone tended toward higher bacterial species richness than abalone diagnosed with WS (although differences were not significant), and the microbiomes of healthy and diseased abalone have significantly different bacterial species composition comprising their microbiomes. The microbiomes of diseased red and diseased black abalone were more similar to one another than either microbiome was to its respective healthy abalone species partner. Abundances of several environmentally common bacterial genera, including *Escherichia*, *Delftia*, and *Geobacillus*, were substantially reduced in the libraries from diseased abalone. Differences between the clone libraries isolated from healthy and diseased abalone suggested that WS has significant impact on the bacterial composition of the post-esophageal microbiome. Specific membership further suggests that infection by Candidatus *Xenohaliotis californiensis*, the etiological agent of WS, may not be the sole cause of WS morbidity and mortality in abalone. Finally, nested PCR trials indicate that a commonly-used diagnostic test for detection of C. *X. californiensis* DNA using a standard PCR may not be stringent enough to detect low levels of infection.

Introduction

The black abalone (*Haliotis cracherodii*) experienced severe declines in abundance in the Channel Islands of California, USA, starting in the mid-1980's (Haaker et al. 1995; Gardner et al. 1995; Friedman et al. 1997). Unlike other commercially fished abalone species in California, however, which experienced population crashes primarily due to overfishing during the latter half of last century, the declines in black abalone were caused in large part by a fatal wasting disease called withering syndrome (WS). In some areas of the Channel Islands, mortalities due to WS were as high as 97%. The combined effects of WS and overfishing depleted populations so severely that the black abalone was designated a Species of Concern by the Federal government in 2001 and became a candidate for listing as an Endangered Species in 2006 under the Endangered Species Act of 1973. The 1997 moratorium on the California abalone fishery relieved existing fishery pressure on the black abalone (and the other commercially fished abalone species), but mortality due to WS persists. The geographic range of the disease has since expanded as well; WS has extirpated black abalone populations on the southern California mainland coast south of Point Conception and has spread as far north as Monterey and San Simeon on the central coast (Moore et al. 2001; CS Friedman and PL Haaker pers. comm.).

Unfortunately, while mortality due to WS is highest in the black abalone, it is not the only species of California abalone affected. Visibly withered red (*H. rufescens*), pink (*H. corrugata*), and green (*H. fulgens*) abalone have been found in the wild, and all species of abalone held in aquaculture in California are known to be susceptible to the disease. The endangered white abalone (*H. sorenseni*), for example, is suspected to contract WS only in aquaculture at this time (T McCormick pers. comm.), and the

etiological agent of the disease is found in almost all abalone aquaculture facilities in the state of California (Moore et al. 2001).

WS was experimentally shown to be due to infection by a Rickettsiales-like prokaryote (RLP) in the α-subclass of Proteobacteria, which includes other disease-causing bacterial genera such as *Rickettsia*, *Ehrlichia*, *Anaplasma*, and *Wolbachia*. The bacterium, described as Candidatus *Xenohaliotis californiensis* (Friedman et al. 2000), is a gram-negative, obligate intracellular, pleomorphic coccobacillus. It is non-motile and forms spherical to oblong, intracytoplasmic membrane-bound inclusions within posterior esophagus and digestive gland transport ducts of abalone (Gardner et al. 1995; Moore et al. 2000; Friedman et al. 2000). The effects that uncontrolled infection by the RLP has on abalone are well-documented. Disease progression results in degeneration of the digestive gland and depletion of glycogen reserves in the abalone. This leads to starvation, muscular autophagy (hence, wasting or withering of the foot), pedal atrophy, and ultimately death (Friedman et al. 2000).

It is unknown, however, how RLP infection may impact the natural gastrointestinal bacteria found in the abalone digestive tract. A host and its associated "microbiome", or the sum total microbial population found in or on an organism, are so closely intertwined – often collaborative – that they can be considered a "superorganism" with an essentially chimeric genome (Lederberg 2000; Goodacre 2007). Perturbation by some factor (diet, disease, stress, heredity), however, can cause shifts in the bacterial composition (i.e. species membership) of a microbiome, a phenomenon well-studied in humans (Mai and Morris, Jr. 2004; Tumbaugh et al. 2006; see also Goodacre 2007) but

occurring in other species, as well, from rats (Dumas et al. 2007) to western Pacific abalone (Tanaka et al. 2003; Tanaka et al. 2004).

Is there is a significant difference in the bacterial composition of the post-esophageal microbiome between healthy abalone and abalone clinically diagnosed with WS? To address this question, we use universal eubacterial 16S rDNA clone libraries to assess differences in bacterial species and proportionality found within and between two species known to be affected by WS in the wild, the red and the black abalone. We hypothesize that 1) healthy red (*H. rufescens*) and black (*H. cracherodii*) abalone have species-specific post-esophageal microbiomes and 2) the post-esophageal microbiomes of healthy abalone are different from those diagnosed with WS. We assess our results in the context of the disease's etiology, discuss what role the loss of beneficial microbes may have on the abalone, and question the potential for secondary infection as an agent of morbidity and mortality in withered abalone.

Materials and Methods

Sample collection and DNA extraction

A list of samples used can be found in Table 6.1. Samples of digestive gland and/or post-esophageal tissue from 17 healthy red abalone (N = 22 total tissue samples) and ~50 wild red and black abalone diagnosed with withering syndrome were provided by J Moore from Bodega Marine Laboratories at Bodega Bay, CA. C Friedman from the University of Washington School of Aquatic and Fisheries Sciences provided post-esophageal tissue samples from four healthy black abalone. Finally, three healthy red abalone held at Scripps Institution of Oceanography were sacrificed and digestive gland and post-esophageal tissue samples were taken from each (N = 6). Tissues were preserved in ethanol or water and stored at -80ºC. Whole DNA was extracted from approximately 25 mg of tissue using a DNeasy Tissue Kit (Qiagen Inc., Valencia, CA), eluted in 200 µl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA), and stored at -80ºC.

Universal 16S rDNA gene cloning and sequencing

DNA samples were diluted 1:20 with DNA-grade water from stock extractions for use in PCR. All thermal cycling was performed in Thermo-Hybaid PCR Express and Px2 thermal cyclers (Thermo Electron Corporation, Boston, MA). PCR products were visualized under ultraviolet light on 2% agarose gels stained with ethidium bromide (EtBr).

All samples were tested twice for the presence of the RLP by PCR with the species-specific primers RA5-1 (forward) and RA3-6 (reverse) developed by Andree et al. (2000) that amplify a diagnostic 160 basepair (bp) region of the RLP 16S rDNA gene.

Reactions were carried out in 15 μL volumes, including 1 μl of dilute template DNA, 1X final concentration GoTaq ® Green Master Mix (Promega Corporation, Madison, WI), 1.5 μg bovine serum albumin (BSA), and 3 pmoles each primer. PCR cycling conditions included an initial two minute 95ºC denaturation; 40 cycles of 95ºC for 30 s, 62ºC for 1 min, and 72ºC for 2 min; and a final extension for 10 min at 72ºC. Samples of post-esophageal tissue from four each of healthy black and red abalone (tested RLP negative) and four each of diseased black and red abalone (tested RLP positive) were included subsequent cloning assays (Table 6.1; see also Table 6.2 for clone library abbreviations).

A 1507 bp portion of the 16S rDNA gene was PCR amplified at least twice in all 12 samples using the universal eubacterial primers EubA (forward) and EubB (reverse) designed by Lane (1991). Reactions were carried out in 15 μL volumes, including 1 μl of dilute template DNA, 1X final concentration GoTaq ® Green Master Mix, 1.5 μg BSA, and 3 pmoles each primer. PCR cycling conditions included an initial two minute 95ºC denaturation; 40 cycles of 95ºC for 30 s, 62ºC for 1 min, and 72ºC for 2 min; and a final extension for 10 min at 72ºC.

Fresh 16S PCR products were pooled prior to each of the four cloning assays (blkRLP-, blkRLP+, redRLP-, and redRLP+; see Table 6.2). Transformation and cloning was performed with a TOPO TA Cloning ® Kit for Sequencing with pCR®4- TOPO® vector (Invitrogen, Carlsbad, CA). All steps were performed according to the kit protocol for transforming One Shot® TOP10 Chemically Competent E. coli. Two volumes (20 μl and 40 μl) of each transformation mixture were spread on Luria broth (LB) agar plates with 50 mg/ml kanamycin to select for inserts. Plates were incubated overnight at 37°C.

Colonies were picked the following morning using sterile pipet tips. Pipet tips were touched to a single well containing 10 µl of water in a 96-well plate (for PCR) and transferred to a single well containing 170 µl of LB with 50mg/ml Kanamycin in a 96-well tissue culture plate (for glycerol stocks). The 96-well LB plates were incubated overnight at 37°C and 30 µl of glycerol was added per well the following morning. Glycerol stock plates were stored at -80°C.

The water plate was incubated at 99°C for 10 min to lyse the bacterial cells, and 5 µl per well was immediately PCR amplified using T3 and T7 universal primers to screen for cloned inserts of the appropriate size (~1600 bp). Reactions were carried out in 15 µL volumes, including the 5 µl of template, 1X final concentration GoTaq ® Green Master Mix, and 3 pmoles each primer. PCR cycling conditions included an initial two minute 95ºC denaturation and 35 cycles of 95ºC for 30 s, 50ºC for 1 min, and 72ºC for 2 min. PCR products from positive clones were sequenced on a MegaBACE™ 500 DNA sequencer (Amersham Biosciences Corp., Piscataway, NJ) DNA sequencer using the universal eubacterial 16S reverse primer (EubB) and a DYEnamic™ ET dye terminator sequencing kit (Amersham Pharmacia Inc., Piscataway, NJ). Sequences were edited using Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI).

Phylogenetic and statistical analyses

Best hit matches for each clone sequence were gathered from the National Center for Biotechnology Information (NCBI) nucleotide collection (nr/nt) database using the BLASTN suite of programs (http://www.ncbi.nlm.nih.gov/BLAST/). Based on the

BLAST hit information, full-length 16S rDNA gene sequences from 58 taxa were downloaded from the Ribosomal Database Project II (RDP) website (http://rdp.cme.msu.edu/); these sequences then served to classify the sequences obtained from the clone libraries. ClustalX v1.83 (Chenna et al. 2003) was used to generate a neighbor-joining (NJ) trees over all clone libraries and for each library individually. Full-length 16S rDNA sequence from the archaea *Sulfolobus acidocaldarius* DSM 639 (from GenBank accession NC_007181) used as the outgroup. The trees were visualized using HyperTree (Bingham and Sudarsanam 2000), and taxa that were not representative of any sample sequences were pruned. The program EstimateS v8.0 (Colwell 2005) was used to calculate true species richness (S) in each clone library separately by the Chao1 (Chao 1984) and ACE (Chao and Lee 1992) richness estimators. Operational taxonomic units (OTUs) per library were defined according to the original BLAST results, which generally conformed to the clades in each library-specific NJ tree.

Comparisons were made between clone libraries isolated from the microbiomes of 1) diseased and healthy black abalone (blkRLP+-), 2) diseased and healthy red abalone (redRLP+-), 3) healthy black and red abalone (blkredRLP-), and 4) diseased black and red abalone (blkredRLP+). See Table 6.2 for reference. Alignment files for each comparison were created in ClustalX and input into the DNADIST executable from the Phylip suite of programs (Felsenstein 1989, 2004) to create a distance matrix using the Jukes-Cantor method. The distance matrix was then analyzed using the online program webLIBSHUFF v1.22 (Singleton et al. 2001; Henriksen 2004) to determine whether the libraries were significantly different from one another as defined by the extent of reciprocal sequence coverage.

Further inter-library comparisons were made using the online UniFrac suite of tools (Luzupone and Knight 2005; Luzupone et al. 2006; Luzupone et al. 2007). A NJ tree was first created in ClustalX with the sequences from all four libraries, with full-length 16S rDNA sequence from the archaeal *Sulfolobus acidocaldarius* DSM 639 again used as the outgroup. The tree was then converted to NEXUS format using TreeView v1.6.6 (Page 1996) and input into UniFrac along with an environmental file detailing from which library each clone originated (blkRLP-, blkRLP+, redRLP-, or redRLP+). UniFrac was then used to 1) measure the overall phylogenetic distance between each library via the unique fraction metric (i.e. the fraction of a branch length of a tree that leads to descendants from one library or another, but not both), 2) determine how the libraries cluster together via the unweighted pair group method with arithmetic mean (UPGMA, performed with jackknifing, 58 sequences kept, 100 permutations), and 3) perform multivariate principle coordinates analysis to see how the libraries separate out in multi-dimensional space. Abundance weights were not used in any analyses because each sequence was considered unique (i.e. there were no duplicate sequences within and between libraries).

Nested PCR diagnostic test

In response to finding RLP clones from healthy red abalone tissue, a nested PCR test was performed that used two diagnostic RLP-specific primer sets from Andree et al. (2000). The RA5-1 (forward) and RA3-1 (reverse) set amplifies a 930 bp portion of the RLP 16S rDNA gene and was run on the full set of healthy red and black abalone (N = 32 tissue samples). Reactions were carried out in 15 µL volumes, including 1 µl of dilute

template DNA, 1X final concentration GoTaq ® Green Master Mix, 1.5 μg BSA, and 3 pmoles each primer. PCR cycling conditions included an initial two minute 95ºC denaturation; 40 cycles of 95ºC for 30 s, 62ºC for 1 min, and 72ºC for 2 min; and a final extension for 10 min at 72ºC. PCR products were subjected to a second round of PCR using the RA5-1 and RA3-6 primer set as described in the cloning section above. The RA5-1 and RA3-6 primer set was also used in a solo PCR on the original template DNA. Pictures taken of the EtBr-stained gels were compared among runs.

Results

Universal 16S rDNA gene cloning

Similar numbers of clones were sequenced from each library (blkRLP- = 70, blkRLP+ = 58, redRLP- = 66, and redRLP+ = 58; see Tables 4.2 and 4.3). A list of the best match bacterial species identified by the BLAST algorithm on the NCBI database is presented in Table 6.3, and the data are presented graphically in Figures 4.1 and 4.2. Several general observations follow from the data. First, there was lower species richness (number of species within a region) in black abalone ($S_{blkRLP-} = 16$, $S_{blkRLP+} = 8$) vs. red abalone ($S_{redRLP-} = 21$, $S_{redRLP+} = 16$), but greater species richness in healthy abalone ($S_{blkRLP-} = 16$, $S_{redRLP-} = 21$) vs. diseased abalone ($S_{blkRLP+} = 8$, $S_{redRLP+} = 16$). None of the differences were significant, however. Second, an absence of the *Escherichia* genus and abundance of C. *X. californiensis*, *Mycoplasma*, and Bacteroidetes was seen in diseased abalone. Finally, there were differences in low abundance taxa among the clone libraries.

The ACE and Chao1 richness estimators indicated that we may have mildly but significantly undersampled our blkRLP- library despite the fact that we sequenced the greatest number of clones from that library (Table 6.4). The LIBSHUFF program, however, with which we calculate whether the libraries are statistically different (see below) is designed to work with potentially undersampled libraries (Singleton 2001). All other observed values of S fell within the Chao1 upper and lower 95% confidence intervals.

Statistically, all four clone libraries were significantly different from one another in paiwise comparisons, although both black abalone libraries were statistically subsets of

the red abalone libraries (Table 6.5). The differences between the libraries appeared to be less dependent on the abalone species the library was isolated from than whether the abalone had been diagnosed with WS. Estimates of interlibrary genetic distance (Table 6.6) and a tree on the four clone libraries (Figure 6.3) indicated that the diseased libraries (blkRLP+, redRLP+) were indeed more similar to one another than either library was to its respective species-specific healthy abalone counterpart (blkRLP- or redRLP-).

Principle coordinates analysis was used to tease out the respective contributions of disease and abalone species to the differences between libraries. Approximately 79% of the variation among libraries was accounted for by the first two principle components (Figure 6.4a). Nearly 50% of the variation was associated with the presence of disease (component one), while another ~30% separated the healthy clone libraries on the basis of abalone species (component two). The third principle component, which accounted for ~21% of the variation, separated the diseased libraries on the basis of abalone species (Figure 6.4b).

Diagnostic test using nested PCR

Low levels of the C. *X. californiensis* bacterium were found in red abalone not diagnosed with WS (healthy) and whose feces originally tested negative for the presence of the RLP prior to sacrifice for this study. Approximately 31% more of the samples from apparently healthy abalone tested positive for RLP DNA in the nested PCR assay (46% RLP+) vs. the original diagnostic PCR assay (15% RLP+).

Discussion

The bacterial composition of a gut microbiome can affect and be affected by the health of the host, and shifts in the microbiome can be the result of host genetics, changes in diet, stress, and/or disease that can, in turn, cause metabolic changes and induce or prevent disease (e.g. Lederberg 2000; Oxley et al. 2002; Tanaka et al. 2003; Blaser and Atherton 2004; Dillon and Dillon 2004; Mai and Morris, Jr. 2004; Tanaka et al. 2004; Tumbaugh et al. 2006; Dumas et al. 2007; Goodacre 2007). Uncontrolled infection by Candidatus *Xenohaliotis californiensis*, the bacterium that causes fatal disease withering syndrome in California abalone species, appears to be associated with a significant shift in the bacterial composition of an abalone's post-esophageal microbiome. Healthy red and black abalone tended toward higher bacterial species richness than abalone of the same species diagnosed with WS, and healthy and diseased abalone have significantly different bacterial members comprising their microbiomes.

Although the microbiomes are determined in part by the abalone species itself, that factor appears to play a greater role in healthy abalone than in diseased abalone. The microbiome of healthy black abalone had lower species richness and was significantly different from that of healthy red abalone, while the microbiomes of diseased red and black abalone were more similar to one another than either microbiome was to its respective healthy abalone species partner. Differences between the healthy species representatives may be due to diet and/or artifacts of their ambient environments. Black abalone are prefer the algae *Macrocystis* and *Egregia* as food sources whereas red abalone have a more generalized algal diet that includes *Nereocystis*, *Macrocystis*, *Laminaria*, *Egregia*, *Pterygophora*, and *Ulva* (CDFG 2005). In addition, our healthy

black abalone samples were from Carmel Point on the central coast while the reds were from two northern California populations.

A deficit of beneficial microbes and the potential role of secondary infection

Abundances of several environmentally common bacterial genera, including *Escherichia*, *Delftia*, and *Geobacillus*, were substantially reduced in the libraries from diseased abalone. The shift was greatest for *Escherichia*. While making up >30% of the clones sequenced from healthy red and black abalone, this genus was nearly absent in clinically diseased abalone (0 – 3% of clones). Does the loss of these or other microbes from the microbiome of red and black abalone negatively affect the abalone superorganism? As mentioned previously, the gut microbiome has been found to be critical to the health of species ranging from humans (Goodacre 2007) to insects (Dillon and Dillon 2004). Unfortunately, we do not know what disorders may result from a lack of these bacteria in the gastrointestinal tract of an abalone, but deficits in metabolism and the development of disease can result from the absence of specific microbes. *Escherichia coli*, for example, a gram-negative, anaerobic bacterium commonly found in the lower gastrointestinal tract of many animals, assists with food digestion and vitamin K production, and vitamin K deficiency has been linked to bleeding and connective tissue disorders in humans (Masterjohn 2007).

Nested PCR test results combined with the RLP positive clones from healthy red abalone pose interesting questions regarding the environmental role that the bacterium that causes WS may play. First, is C. *X. californiensis* ubiquitous in the marine environment? It is unlikely that the bacterium can live for long outside of a host since it

is a member of a clade of bacteria that tends to have reduced genomes (~0.8 – 1.4Mb) and whose species are obligately intracellular (McGraw-Hill 2002). However, its presence in healthy abalone may indicate that the RLP is a low-abundance gut bacterium that is benign or quickly shed under normal circumstances. Following that assumption is a second question: does C. *X. californiensis* pathogenicity and the clinical onset of WS in abalone require an outside perturbation of some physical or biological factor? For example, clinical onset of WS and mortality can be induced by water temperature; survival is 100% at 15°C but approximately 33% die at 18°C (Moore et al. 2000). Tissue and fecal samples from healthy red, pink, and green abalone in our own facility tested positive for the presence of the RLP (hence, none of the red abalone samples were used in cloning), but none of our animals exhibited signs of the disease, likely due to the fact that the seawater is chilled to 12.5°C. If water temperature plays a role, perhaps the severe El Niño over the winter of 1982-83 could have aided the onset of the WS epidemic in black abalone populations in the Channel Islands in the mid-1980's (Haaker et al. 1995). However, there is no published evidence that California coastal waters are experiencing increased temperatures other than normal seasonal fluctuation, and neither the 1982-83 El Niño nor successive El Niño Southern Oscillation (ENSO) events correspond to the spread of the bacterium along the southern and central California mainland coast (although mortalities were higher during El Niño years per Raimondi et al. (2002)). Hence, higher temperature, while potentially accelerating declines, is probably not the ultimate perturbation leading to mortality in natural abalone populations infected with the RLP.

Disease can also result from opportunistic infections (OI) in already immunocompromised individuals. For example, human morbidity and mortality due to auto-immune deficiency syndrome (AIDS) is not due to the human immunodeficiency virus (HIV) itself, but is often the result of a secondary infection (e.g. infection by the ubiquitous unicellular eukaryote *Pneumocystic carinii* causes AIDS-related pneumonia; e.g. Thomas 2007). Uncontrolled growth of C. *X. californiensis* may be an OI resulting from immunosuppression by some other agent. In addition, although degeneration of digestive tract tissue due to inclusion formation by the RLP is likely the primary cause of starvation and wasting in abalone with WS, we cannot rule out the possibility that one or more of the bacteria associated with the shift in the microbiome of diseased abalone may result in a secondary or tertiary OI. Several microbial clades isolated from abalone with WS in our study can cause disease in other species (e.g. the family Bacteroidetes and genus *Mycoplasma*). The genus *Bacteroides* in the Bacteroidetes, in particular, is known to be an opportunistic pathogen in immunosuppressed humans (Finegold and George 1989; Rocha et al. 2003). Unfortunately, our universal 16S clone libraries represent a unique time point in the full course of the disease. Future work should include a series of universal 16S clone libraries to see how the gut microbiome of an abalone shifts over the course of infection by C. *X. californiensis*.

Alternatively, some microbial species may represent opportunists in abalone that take advantage of a secondary aspect of WS: starvation. Tanaka et al. (2004) focused on the differences in gut bacteria seen in the Japanese Ezo abalone (*H. discus hannai*) fed natural algae, processed pellets, and starved abalone. Interestingly, several of the bacteria

in our diseased red and black abalone had the greatest BLAST hit to bacteria in the guts of starved Ezo abalone (e.g. the *Mycoplasma* and uncultured α-proteobacteria groups).

Assessing bias in our clone libraries

We must be somewhat cautious in using universal 16S clone libraries to characterize the bacterial membership of environmental samples. Cloning may reduce the selective error inherent in culture-dependent techniques where only bacterial strains that grow on artificial media are represented, but cloning cannot be considered a quantitative technique either. The initial PCR step potentially overrepresents or underrepresents particular bacterial taxa (i.e. PCR bias) because of variation in the ability of different templates to be PCR amplied. Bias can occur when there are more abundant bacterial species and/or species with a higher number of 16S repeats (although PCR may counterintuitively result in 1:1 mixtures of template-specific PCR products regardless of initial template ratio, which may underrepresent higher copy numbers; Suzuki and Giovannoni 1996); differences in G+C content between species-specific templates; differences in the binding efficiencies of universal, as opposed to species-specific, PCR primers to specific templates; or the formation of heteroduplexed PCR products (Ishii and Fukui 2001; Kanagawa 2003; Acinas et al. 2005). In addition, the cloning efficiency of different amplicons can introduce bias at a later stage (Acinas et al. 2005).

To lower bias in PCR and subsequent clone libraries, a review by Kanagawa (2003) recommended that primer degeneracy be avoided (difficult for universal eubacterial PCR), the number of PCR cycles be reduced (attempted here with ≥20 cycles, but we could not obtain visible product with less than 35-40 cycles), replicate

amplifications be performed (we pooled product from at least two replicate reactions per sample), and/or real-time quantitative PCR be used (not attempted here). Currently, techniques such as environmental shotgun sequencing that propose to further reduce species or strain bias are also being explored, but they can be expensive and technologically intensive (von Mering et al. 2007).

Despite possible bias in our assay techniques, prior research on the gut microbiomes of other gastropod species, including abalone, supports our results. Several bacterial taxa represented in this study were also seen in enrichment culture and culture-independent studies of the gut microflora of western Pacific abalone like the Ezo (*H. discus hannai*) of Japan (Tanaka et al. 2003, 2004) and greenlip (*H. laevigata*) of Australia (Harris et al. 1998). Some genera were even found in the guts of the distantly-related terrestrial brown garden snail, *Helix aspersa* (Watkins and Simkiss 1990).

A note on diagnostic PCR testing for the presence of the RLP

The RLP-specific PCR primers developed by Andree et al. (2000) have been used in diagnostic testing for the presence of the RLP in abalone. The PCR test is typically performed on DNA extractions from tissue or feces and is based on the presence/absence of the 160 bp amplicon generated by the RA5-1 and RA3-6 primer set. The fact that the test can be performed on feces reduces the need for sacrificing abalone, and greatly increases the utility and ease of the test for diagnostic purposes. Unfortunately, our results indicate that standard PCR amplification may not be sufficient for detecting very low levels of RLP DNA. A nested PCR may be necessary for detection when using UV visualization of ethidium bromide-stained agarose gels.

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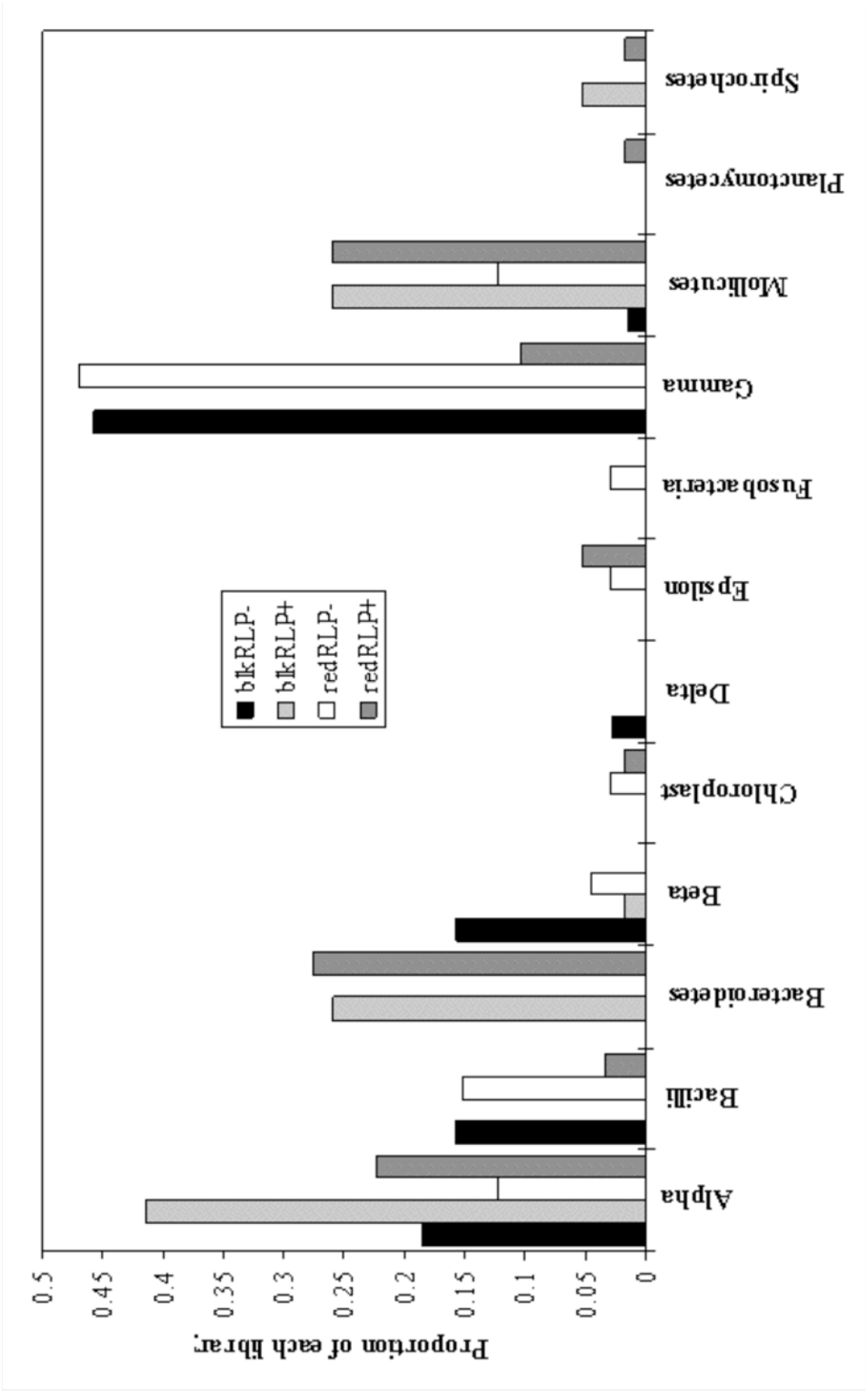

Figure 6.1: Bar graph showing proportional membership of each class per universal 16S clone library. Color of bars coded according to clone library.

(a)

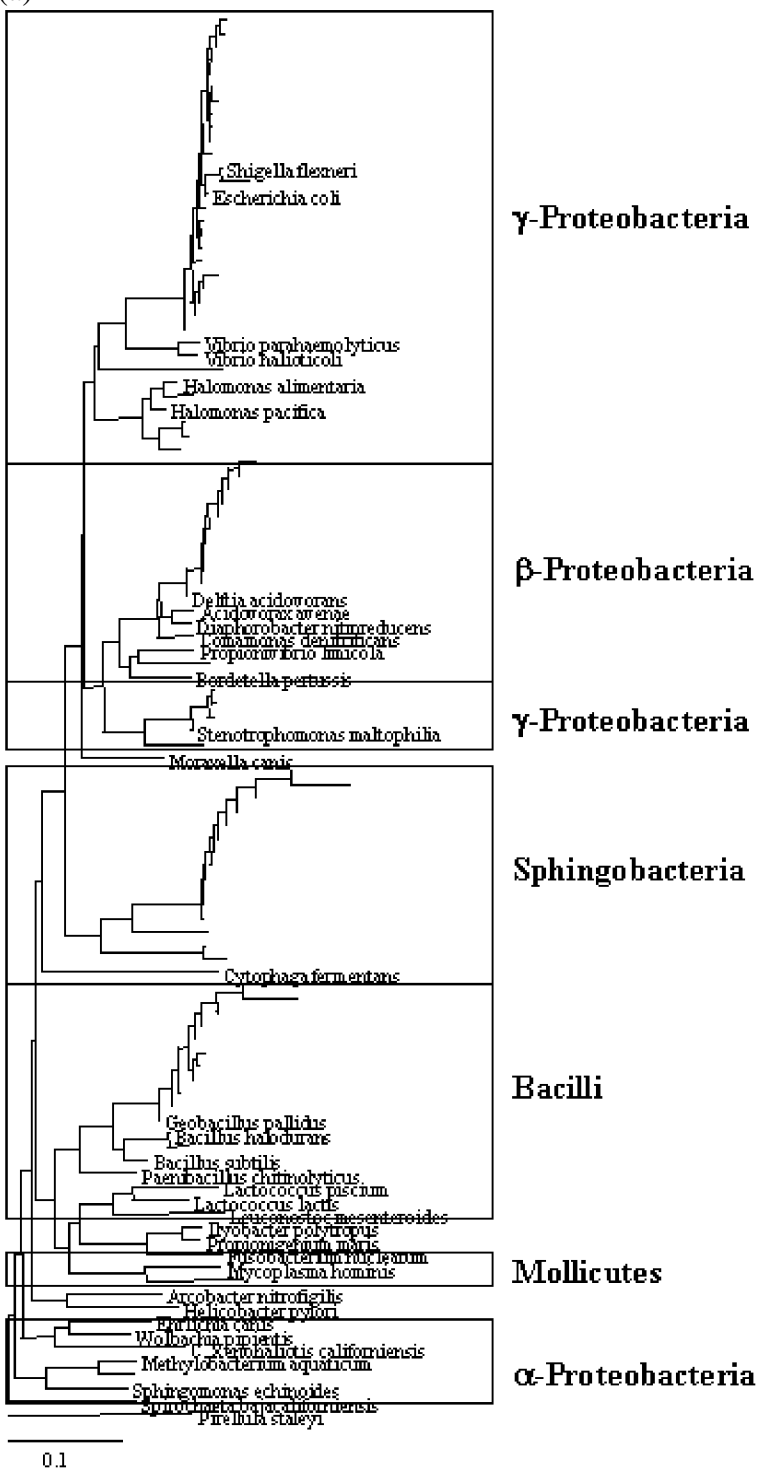

Figure 6.2: Neighbor joining (NJ) trees for each universal 16S clone library: (a) blkRLP-, (b) blkRLP+, (c) redRLP-, and (d) redRLP+. Branches with no label are clone library sequences. Representative RDP taxa are labelled; all trees include the same RDP taxa. Boxes indicate bacterial classes represented in the clone library.

(b)

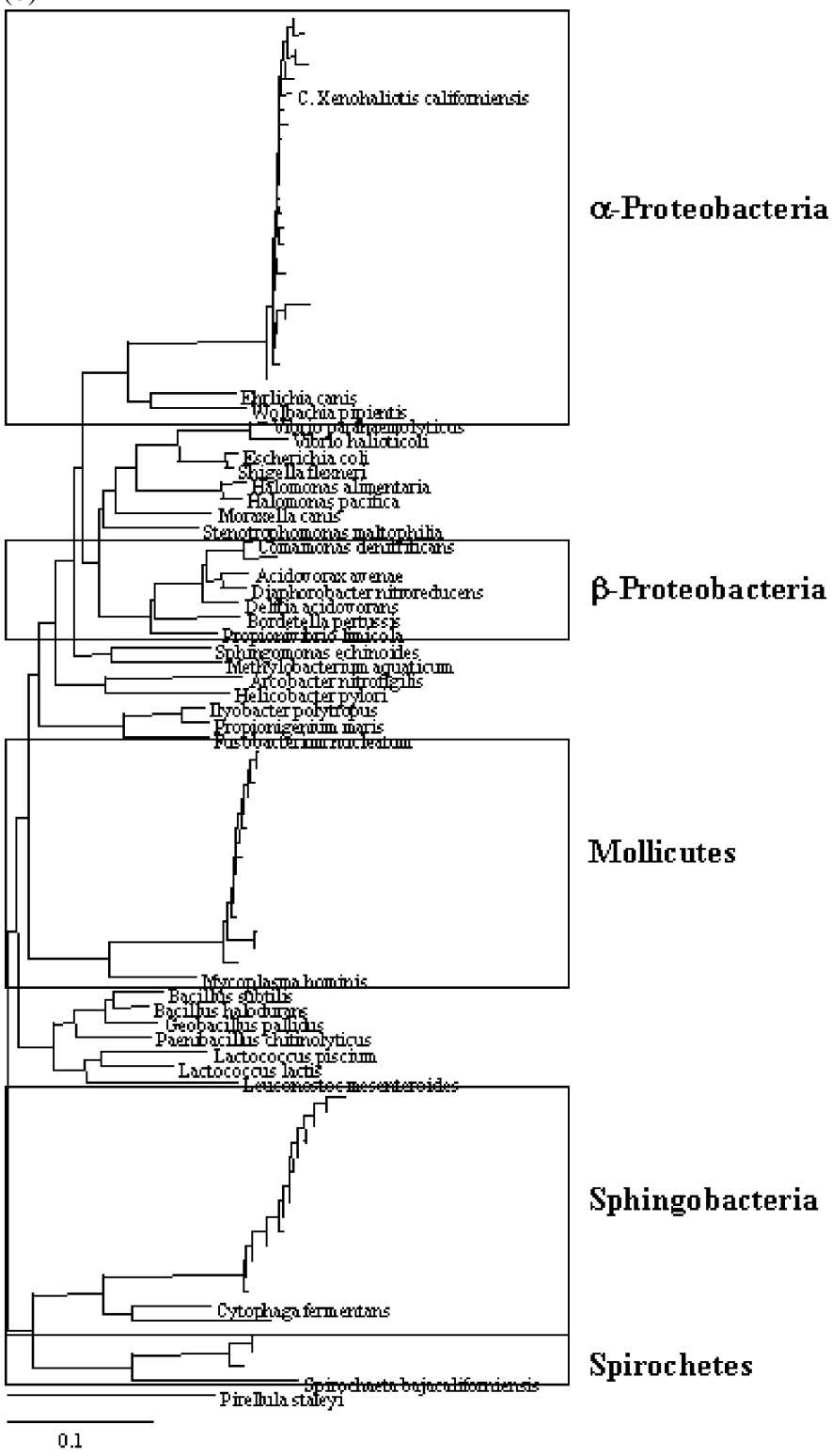

Figure 6.2 continued.

(c)

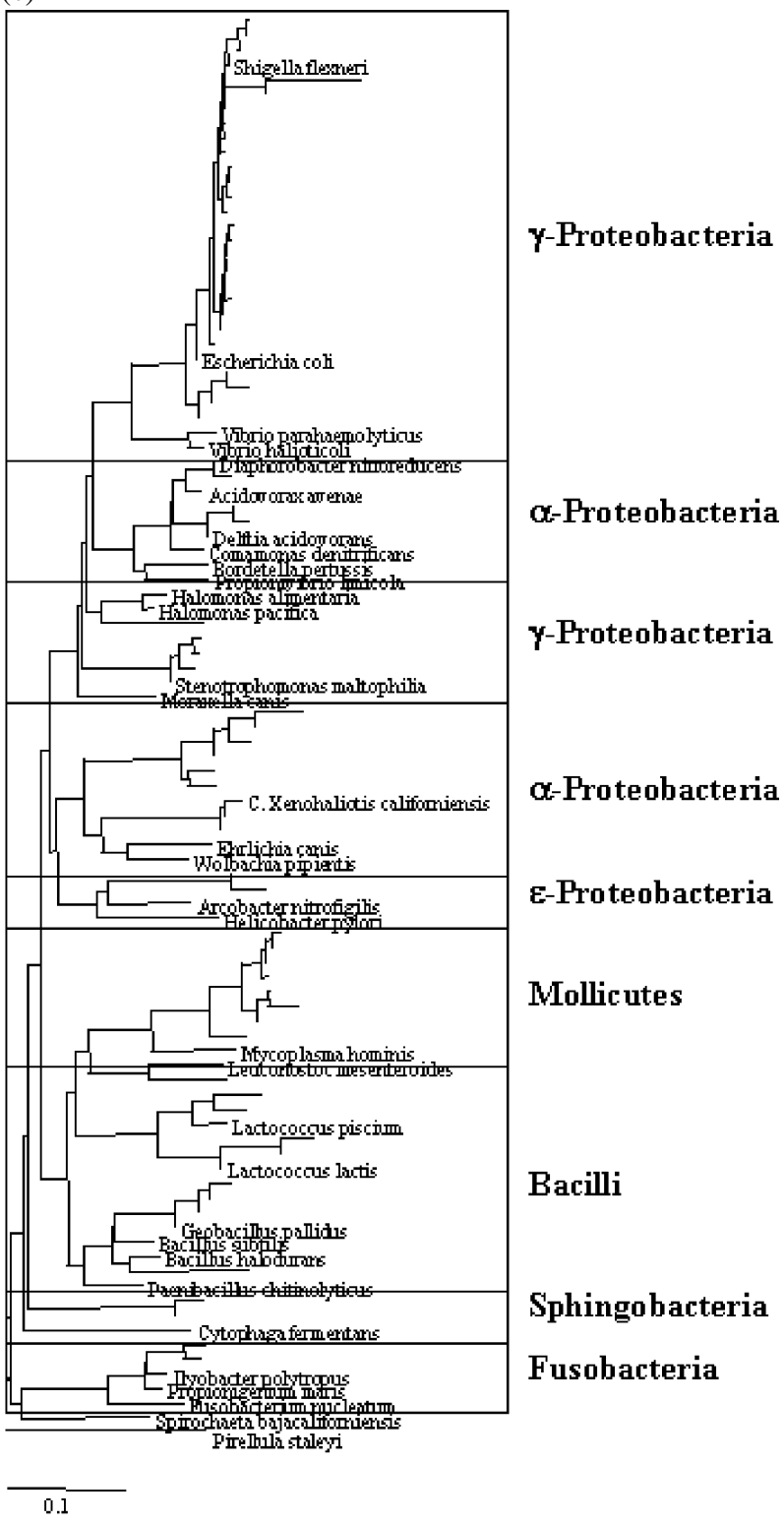

Figure 6.2 continued.

(d)

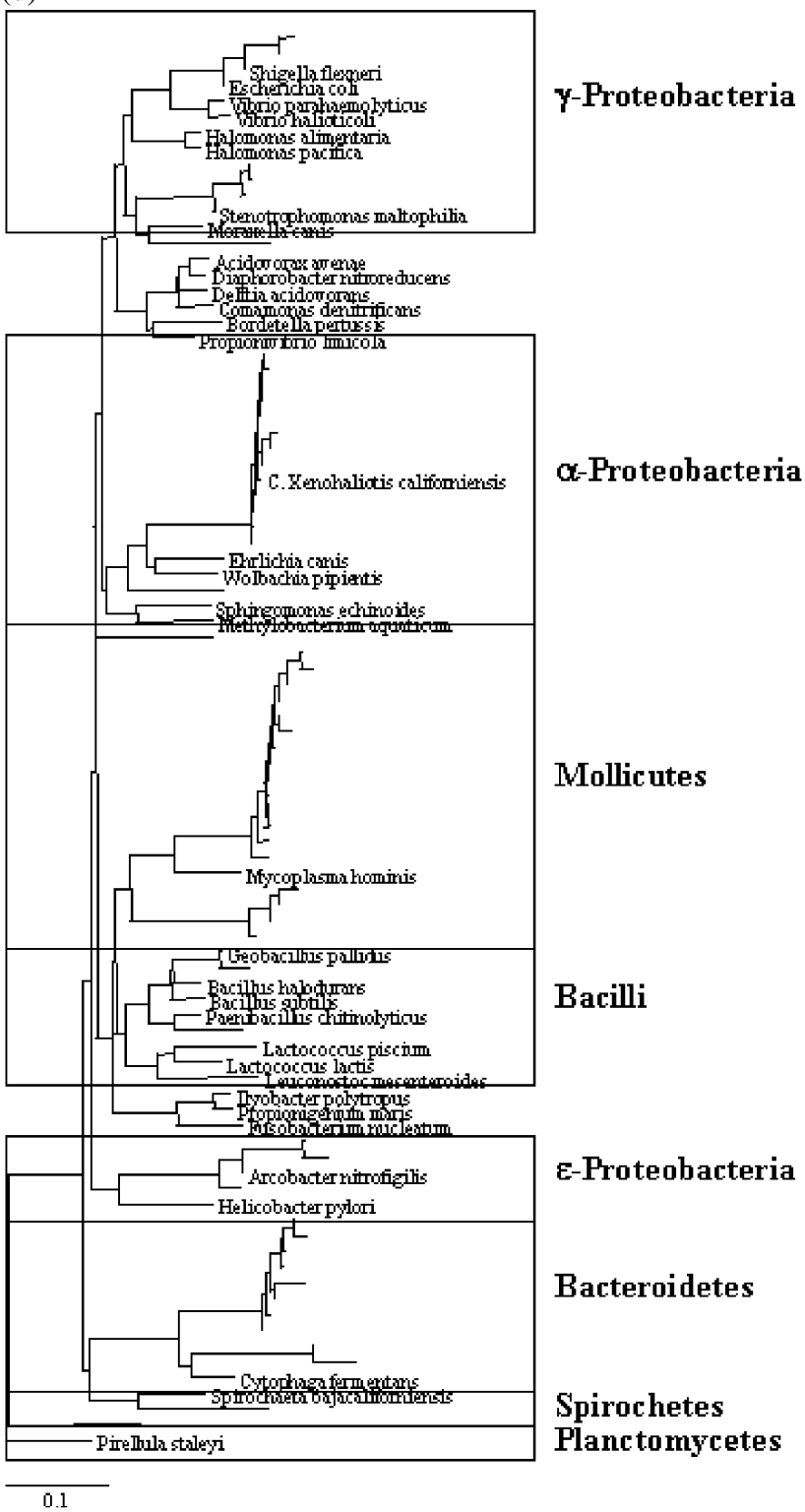

Figure 6.2 continued.

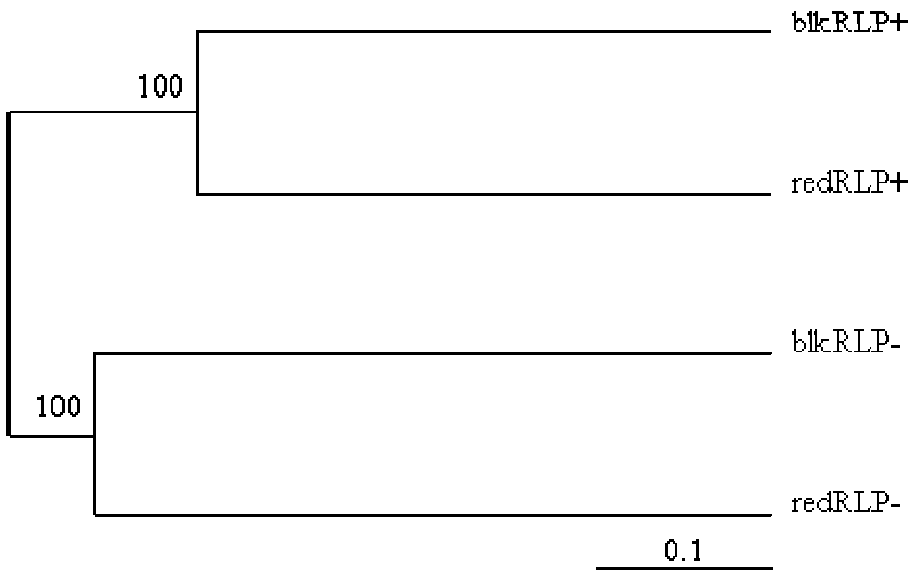

Figure 6.3: UPGMA dendrogram created using UniFrac showing cluster relationships among universal 16S rDNA clone libraries.

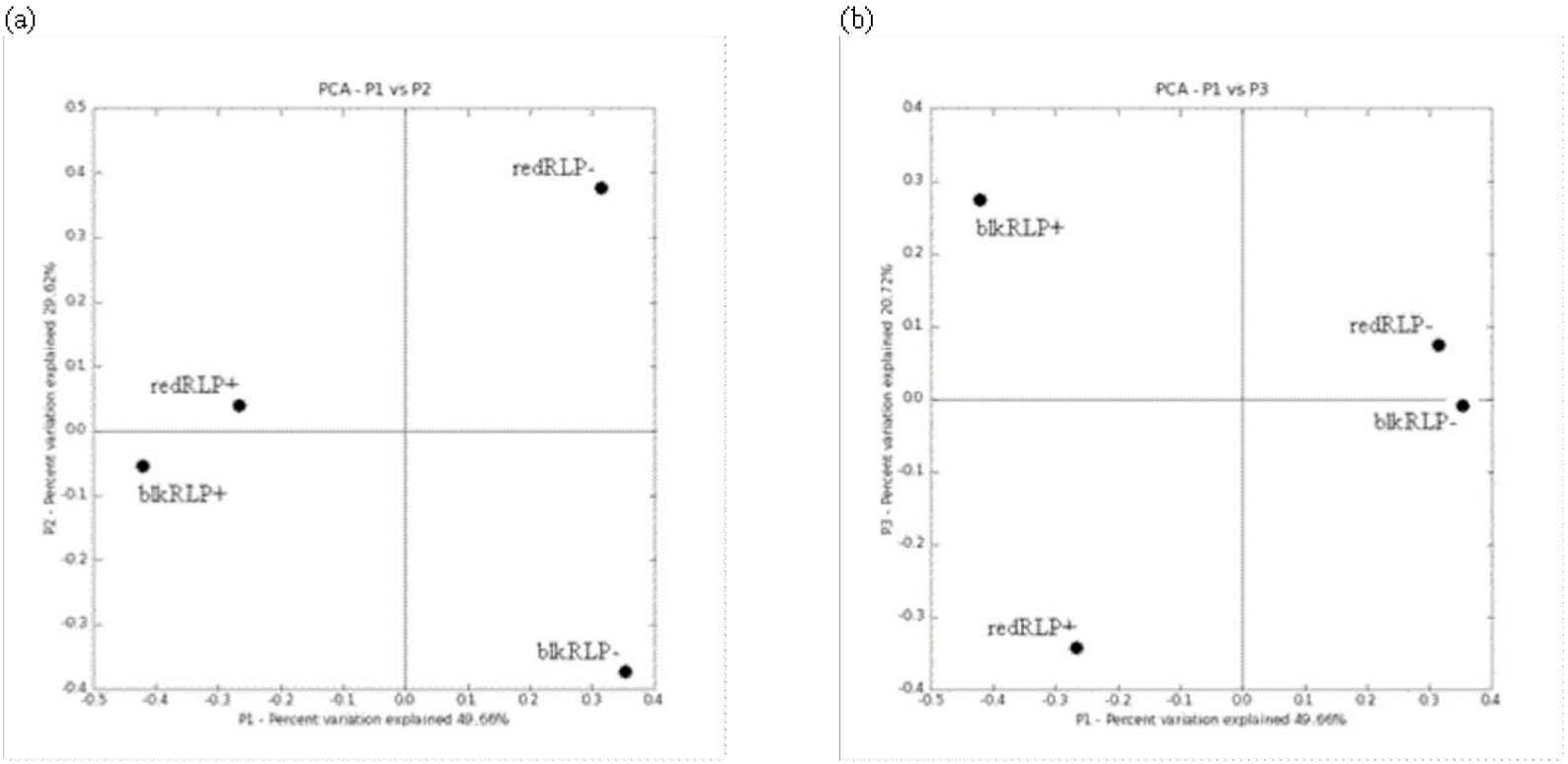

Figure 6.4: Principle coordinates analysis (PCA) on the universal 16S rDNA clone libraries. Coordinate axes one and two presented for (a), axes one and three for (b).

Table 6.1: List of samples used in universal 16S clone libraries. All samples from wild abalone collected at several locations along the California coast.

ID	Abalone species	Location	Diseased/healthy
UW06:6 25	Black	Carmel Point	Healthy
UW06:6 26	Black	Carmel Point	Healthy
UW06:6 27	Black	Carmel Point	Healthy
UW06:6 28	Black	Carmel Point	Healthy
SF98-35 #26	Black	Scotts Creek	Diseased
SF98-35 #44	Black	Scotts Creek	Diseased
VDBASac	Black	Vandenberg	Diseased
SF98-35 #20	Black	Scotts Creek	Diseased
SF99-38 #51	Red	Caspar	Healthy
SF99-38 #52	Red	Caspar	Healthy
SF00-13 #51	Red	Salt Point	Healthy
SF00-13 #52	Red	Salt Point	Healthy
SF98-35-7 #3	Red	Scotts Creek	Diseased
JF4	Red	N/A	Diseased
JF8	Red	N/A	Diseased
JF6	Red	N/A	Diseased

Table 6.2: List of abbreviations for each universal 16S clone library and interlibrary comparison.

Abbreviation	# of libraries involved	Description
blkRLP-	1	Healthy black abalone
blkRLP+	1	Diseased black abalone
redRLP-	1	Healthy red abalone
redRLP+	1	Diseased red abalone
blkRLP+-	2	Comparison of healthy and diseased black abalone
redRLP+-	2	Comparison of healthy and diseased red abalone
blkredRLP-	2	Comparison of healthy red and black abalone
blkredRLP+	2	Comparison of diseased red and black abalone

Table 6.3: List of best hit BLAST taxon matches for healthy and diseased red and black abalone universal 16S clone libraries. Taxa alphabetically ordered. Numbers under each clone library indicate number of clones matching that taxon.

Best BLAST hit	% Identity (BLAST)	Clone library				General information
		blkRLP-	blkRLP+	redRLP-	redRLP+	
Acidovorax/Diaphorobacter	99			1		denitrifying
Arcobacter	98				3	normal intestinal bacterium
Bacillus	97			1		ubiquitous
Bacillus halodurans	99	1				ubiquitous in salt water
Bacillus/Geobacillus	99	10		3	1	ubiquitous
Bacteroides	96		1		2	normal intestinal bacterium
Bacteroidetes	<95		14		10	normal intestinal bacterium
C. *X. californiensis*	**99**		**24**	**2**	**12**	**etiological agent of withering syndrome**
Comamonas	100		1			ubiquitous
Cytophaga	98				4	digests cellulose
Delftia acidovorans	99	10		2		ubiquitous
Escherichia	99	22		26	2	normal intestinal bacterium
Fucus vesiculosus chloroplast	97			2		alga
Fusobacteria/Ilyobacter	98			2		normal intestinal bacterium
Halomonas haplotype 1	99	1				ubiquitous in salt water
Halomonas haplotype 2	98	3				ubiquitous in salt water
Halomonas haplotype 3	<95	1				ubiquitous in salt water
Lactococcus haplotype 1	99			1		fermentation
Lactococcus haplotype 2	<95			1		fermentation
Lactococcus lactis	98			3		fermentation
Leuconostoc	99			1		fermentation
Methylobacteria	99	1				ubiquitous in water
Moraxella	<95				1	respiratory bacterium
Mycoplasma haplotype 1	97		2	4		ubiquitous (best hit to *Mycoplasma* from *H. d. hannai*)
Mycoplasma haplotype 2	95	1	13	4	15	ubiquitous (best hit to *Mycoplasma* from *H. d. hannai*)
Paenibacillus	98				1	normal plant probiotic bacterium
Palmaria palmata chloroplast	98				1	alga
Pirellula	<95				1	ubiquitous in salt water
Spirochaeta	97		3		1	digests plant polysaccharides
Stenotrophomonas	99	3		3	3	ubiquitous
uncultured alpha haplotype 1	<95			2	1	unknown (best hit to uncultured alpha from *H. d. hannai*)
uncultured alpha haplotype 2	98	12		4		unknown (best hit to uncultured alpha from *H. d. hannai*)
uncultured beta	99	1				unknown
uncultured delta	<95	2				unknown
uncultured epsilon	97			2		unknown
uncultured gamma haplotype 1	97	1		1		unknown
uncultured gamma haplotype 2	<95	1				unknown
Vibrio halioticoli	99			1		alginolytic bacterium originally described from *H. d. hannai*
TOTAL		70	58	66	58	

Table 6.4: ACE and Chao1 estimates of species richness for each universal 16S clone library. OTU_{obs} indicates number of operational taxonomic units (OTUs) observed in our data. An OTU_{obs} outside of the Chao1 upper and lower 95% confidence interval (CI) is considered significantly over- or undersampled.

Clone library	**OTU_{obs}**	**ACE**	**Chao1 (95% CI)**
blkRLP-	16	24.5	38.5 (17.58 - 181.21)
blkRLP+	8	6.0	6.5 (6.02 - 14.44)
redRLP-	21	21.1	22.0 (19.53 - 35.81)
redRLP+	16	19.0	23.0 (15.52 - 67.27)

Table 6.5: Statistical comparisons between pairs of universal 16S clone libraries. See Table 6.2 for a description of each comparison. Columns under XY indicate coverage of library X by library Y and columns under YX indicate coverage of library Y by library X. When ΔC_{XY} is not significant but ΔC_{YX} is, library X is considered to be a subset of library Y. P-values in *italics* are significant after Bonferroni correction at the $\alpha = 0.05$ level.

Comparison	**XY**		**YX**		**Significant?**
	ΔC	**p**	**ΔC**	**p**	
blkRLP+-	14.294	*0.001*	16.061	*0.001*	Yes
redRLP+-	2.514	*0.001*	1.167	*0.001*	Yes
blkredRLP+	0.025	0.723	1.076	*0.002*	Yes
blkredRLP-	0.105	0.219	1.330	*0.001*	Yes

Table 6.6: Pairwise phylogenetic distance matrix for the universal 16S clone libraries. Calculations based on the unique fraction (UniFrac) metric.

	blkRLP+	**blkRLP-**	**redRLP+**	**redRLP-**
blkRLP+	-			
blkRLP-	0.880	-		
redRLP+	0.641	0.823	-	
redRLP-	0.876	0.752	0.796	-

References

Acinas SG, Sarma-Rupavtarm R, Klepac-Ceraj V, Polz MF (2005) PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. *Applied and Environmental Microbiology*, 71, 8966-8969

Andree KB, Friedman CS, Moore JD, Hedrick RF (2000) A polymerase chain reaction assay for the detection of genomic DNA of a *Rickettsiales*-like prokaryote associated with Withering Syndrome in California abalone. *Journal of Shellfish Research*, Supplement, 213-218

Bigham J, Sudarsanam S (2000) Visualizing large hierarchical clusters in hyperbolic space. *Bioinformatics*, 16, 660-661

Blaser MJ, Atherton JC (2004) Helicobacter pylori persistence: biology and disease. *Journal of Clinical Investigation*, 113, 321-333

Chao A (1984) Nonparametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics*, 11, 265-270

Chao A, Lee S-M (1992) Estimating the number of classes via sample coverage. *Journal of the American Statistical Association*, 87, 210-217

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research*, 31, 3497-3500

Colwell RK (20057) EstimateS: statistical estimation of species richness and shared species from samples. Version 7.5. User's guide and application published at: http://purl.oclc.org/estimates

Dillon RJ, Dillon VM (2004) The gut bacteria of insects: nonpathogenic interactions. *Annual Review of Entomology*, 49, 71-92

Dumas ME, Wilder SP, Bihoreau MT, Barton RH, Fearnside JF, Argoud K, D'Amato L, Wallis RH, Blancher C, Keun HC, Baunsgaard D, Scott J, Sidelmann UG, Nicholson JK, Gaugier D (2007) Direct quantitative trait locus mapping of mammalian metabolic phenotypes in diabetic and normoglycemic rat models. *Nature Genetics*, 39, 666-672

Felsenstein J (1989) PHYLIP – Phylogeny Inference Package (Version 3.2). *Cladistics*, 5, 164-166

Felsenstein J (2004) PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle

Finegold SM, George WL (1989) Anaerobic infections in humans. Academic Press, San Diego

Gardner GR, Harshbarger JC, Lake JL, Sawyer TK, Price KL, Stephenson MD, Haaker PL, Togstad HA (1995) Association of prokaryotes with symptomatic appearance Withering Syndrome in black abalone *Haliotis cracherodii*. *Journal of Invertebrate Pathology*, 66, 111-120

Friedman CS, Thomson M, Chun C, Haaker PL, Hedrick RP (1997) Withering syndrome of the black abalone, *Haliotis cracherodii* (Leach): water temperature, food availability, and parasites as possible causes. *Journal of Shellfish Research*, 16, 203-411

Friedman CS, Andree KB, Beauchamp KA, Moore JD, Robbins TT, Shields JD, Hedrick RP (2000) 'Candidatus *Xenohaliotis californiensis*', a newly described pathogen of abalone, *Haliotis* spp., along the west coast of North America. *Journal of Systematic and Evolutionary Microbiology*, 50, 847-855

Goodacre R (2007) Metabolomics of a superorganism. *Journal of Nutrition*, 137, 259S-266S

Haaker PL, Parker DO, Chun CSY (1995) Growth of black abalone, *Haliotis cracherodii* Leach, at San Miguel Island and Point Arguello, California. *Journal of Shellfish Research*, 14, 519-525

Harris JO, Burke CM, Maguire GB (1998) Characterization of the digestive tract of greenlip abalone, *Haliotis laevigata* Donovan. II. Microenvironment and bacterial flora. *Journal of Shellfish Research*, 17, 989-994

Henriksen JR (2004) webLIBSHUFF (http://libshuff.mib.uga.edu/)

Ishii K, Fukui M (2001) Optimization of annealing temperature to reduce bias caused by a primer mismatch in multitemplate PCR. *Applied and Environmental Microbiology*, 67, 3753-3755

Kanagawa T (2003) Bias and artifacts in multitemplate polymerase chain reactions (PCR). *Journal of Bioscience and Bioengineering*, 96, 317-323

Lane DJ (1991) 16S/23S rRNA sequencing. Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Ltd., New York, 115-175

Lederberg J (2000) Infectious history. *Science*, 288, 287-293

Lozupone C, Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*, 71, 8228-8235

Lozupone C, Hamady M, Knight R (2006) UniFrac – an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics*, 7, 371

Lozupone CA, Hamady M, Kelley ST, Knight R (2007) Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Applied and Environmental Microbiology*, 73, 1576-1585

Mai V, Morris, Jr. JG (2004) Colonic bacterial flora: changing understandings in the molecular age. *Journal of Nutrition*, 134, 459-464

Masterjohn C (2007) Vitamin D toxicity redefined: vitamin K and the molecular mechanism. *Medical Hypotheses*, 68, 1026-1034

McGraw-Hill, Parker SP (2002) Dictionary of Scientific and Technical Terms 6th Edition. The McGraw-Hill Companies, Columbus

Moore JD, Robbins TT, Friedman CS (2000) Withering Syndrome in farmed red abalone *Haliotis rufescens*: thermal induction and association with a gastrointestinal Rickettsiales-like prokaryote. *Journal of Aquatic Animal Health*, 12, 26-34

Oxley APA, Shipton W, Owens L, McKay D (2002) Bacterial flora from the gut of the wild and cultured banana prawn, *Penaeus merguiensis*. *Journal of Applied Microbiology*, 93, 214-223

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences*, 12, 357-358

Raimondi PT, Wilson CM, Ambrose RF, Engle JM, Minchinton TE (2002) Continued declines of black abalone along the coast of California: are mass mortalities related to El Niño events? *Marine Ecology Progress Series*, 242, 143-152

Rocha ER, Herren CD, Smalley DJ, Smith CJ (2003) The complex oxidative stress response of *Bacteroides fragilis*: the role of OxyR in control of gene expression. *Physiology and Microbial Chemistry*, 9, 165-173

Singleton DR, Furlong MA, Rathbun SL, Whitman WB (2001) Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. *Applied and Environmental Microbiology*, 67, 4374-4376

Suzuki MT, Giovannoni SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Applied Environmental Microbiology*, 62, 625-630

Tanaka R, Sugimura I, Sawabe T, Yoshimizu M, Ezura Y (2003) Gut microflora of abalone *Haliotis discus hannai* in culture changes coincident with a change in diet. *Fisheries Science*, 69, 961-968

Tanaka R, Ootsubo M, Sawabe T, Ezura Y, Tajima K (2004) Biodiversity and in situ abundance of gut microflora of abalone (*Haliotis discus hannai*) determined by culture-independent techniques. *Aquaculture*, 241, 453-463

Thomas CF (2007) Current insights into the biology and pathogenesis of *Pneumocystis* pneumonia. *Nature Reviews Microbiology*, 5, 298-308

Tumbaugh PJ, Ley RE, Mahovald MA, Magrini V, Mardis ER, Gordon JI (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, 444, 21-28

von Mering C, Hugenholz P, Raes J, Tringe SG, Doerks T, Jensen LJ, Ward N, Bork P (2007) Quantitative phylogenetic assessment of microbial communities in diverse environments. *Science*, 315, 1126-1130

Watkins B, Simkiss K (1990) Interactions between soil bacteria and molluscan alimentary tract. *Journal of Molluscan Studies*, 56, 267-274

CHAPTER VII

Population genetic structure of Candidatus *Xenohaliotis californiensis*, the bacterium that causes withering syndrome in California abalone species: a preliminary study

Abstract

Withering syndrome (WS) is a fatal wasting disease affecting several California abalone species in the wild and/or in aquaculture. The causative agent is a Rickettsiales-like prokaryote described as Candidatus *Xenohaliotis californiensis*. In a preliminary study of the genetic diversity of C. *X. californiensis*, there were no polymorphic sites in 913 bp of 16S rDNA sequence and 470 bp of Rickettsiales-specific recA sequence from bacteria isolated from three abalone species, both in the wild and in aquaculture and from multiple locations around the state of California. While it is possible that all affected natural populations and aquaculture facilities within the state of California are infected with the same strain of the bacterium, more research is needed before we can answer the question as to whether the C. *X. californiensis* bacterium is truly invariant and whether the withering syndrome epidemic in abalone in the state of California is caused by one or more strains of this bacterium.

Introduction

Although preventing over-exploitation is frequently the focus of marine conservation efforts, diseases present yet another threat to marine ecosystems. Withering syndrome (WS) is a fatal wasting disease affecting several California abalone species in the wild and/or in aquaculture. First discovered in populations of black abalone (*Haliotis cracherodii*) in the California Channel Islands in the mid-1980's (Haaker et al. 1995; Gardner et al. 1995; Friedman et al. 1997), the range of WS has spread to mainland California, reaching as far north as Monterey on the central California coast (Moore et al. 2001; CS Friedman and PL Haaker pers. comm.). The impact of WS on natural populations of other abalone species currently appears to be low, but this may change in the future. Besides extensive mortality in the black abalone, red (*H. rufescens*), pink (*H. corrugata*), and green (*H. fulgens*) abalone apparently suffering from WS have been seen in the wild in California and Mexico and in aquaculture (I Taniguchi, PL Haaker, and E Kisfaludy pers. comm.); white abalone (*H. sorenseni*) are known to contract the disease in aquaculture only, at this time (TB McCormick pers. comm.).

WS has been experimentally shown to be due to infection by a Rickettsiales-like prokaryote described as Candidatus *Xenohaliotis californiensis* (Friedman et al. 2000). A critical disease-related issue is the transmission of C. *X. californiensis* among culture facilities and between culture facilities and the environment. At least two northern California red abalone populations are known to be infected with C. *X. californiensis,* quite possibly due to anthropogenic introduction after hatchery-bred animals were stocked in the wild (Moore et al. 2000; Friedman and Finley 2003). C. *X. californiensis* is present in most abalone aquaculture facilities in California, regardless of the species

being cultivated (Moore et al. 2001). Most aquaculture facilities in California grow abalone for human consumption, and mortality due to WS in commercial aquaculture is costly (withered animals cannot be sold and are a direct loss to the company). Consequently, there is heightened commercial interest in disease research. C. *X. californiensis* endemic to a particular area may be introduced to culture facilities via infected (wild captured) brood stock or from the local seawater supply, generally pumped into the facility from an offshore intake. There is also often exchange of stock abalone between facilities from multiple regions around the state, increasing the potential spread of C. *X. californiensis*.

Affects on the white abalone (*H. sorenseni*), which was listed as endangered in 2001 by the US Fish & Wildlife Service, is another concern. *H. sorenseni* is the first marine invertebrate to be declared an endangered species under the Endangered Species Act of 1973 (CDFG 2005). A permitted captive breeding program is currently underway with the ultimate goal of outplanting progeny in suitable habitat off the California coast in an attempt to replenish the species. Although white abalone are not known to contract WS in the wild, this species has been found to be susceptible to the disease in culture (T McCormick pers. comm.). Under an outplanting scenario, unintentionally releasing hatchery bred animals with a harmful bacterium into the environment would be extremely detrimental to the recovery of the species, particularly if the bacterial strain is not locally endemic.

Little is known about the genetic variability within and between natural populations of this bacterium (Robles-Sikisaka 2003), so the potential risk of introducing non-native strains of the C. *X. californiensis* bacterium into the local environment

through aquaculture and other human activities is difficult to assess. Population genetic research may help to identify possible source areas and populations for the bacterium. Here, we used 16S rDNA and recA gene sequencing to study diversity in C. *X. californiensis* in natural and hatchery samples of abalone from California. Hopefully, knowledge gained from studies such as this can be applied toward the development of effective management strategies for California abalone and other anthropogenically and naturally compromised coastal species.

Materials and Methods

Sample collection and DNA extraction

A list of samples used can be found in Table 7.1. Samples of abalone digestive gland and/or post-esophageal tissue diagnosed with withering syndrome (WS) from 105 individuals were provided by J Moore from Bodega Marine Laboratories at Bodega Bay, CA. C Friedman from the University of Washington School of Aquatic and Fisheries Sciences provided four more post-esophageal tissue samples. Tissues were preserved in ethanol or water and stored at -80ºC. Whole DNA was extracted from approximately 25 mg of tissue using a DNeasy Tissue Kit (Qiagen Inc., Valencia, CA), eluted in 200 µl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA), and stored at -80ºC. A set of 71 pre-extracted DNA samples was also provided by J Moore and stored at -80ºC.

16S rDNA gene sequencing

A 930 bp fragment of the 16S gene was PCR amplified in all 176 DNA samples. C. *X. californiensis*-specific PCR primers for the 16S gene were from Andree et al. (2000): RA5-1 (forward: 5'- GTTGAACGTGCCTTCAGTTTAC) and RA3-1 (reverse: 5'- CTGAGGCCATCTGTTAAAATGG). Reactions were carried out in 15 µL volumes, including 1 µl of concentrated template DNA, 0.3 U REDTaq DNA polymerase (Sigma-Aldrich Corporation, St. Louis, MO), 1.5 µl 10X PCR buffer, 2.5 mM $MgCl_2$ final concentration, 2 µM each dNTP final concentration, and 3 pmoles each primer.

Thermal cycling was performed in Thermo-Hybaid PCR Express and Px2 thermal cyclers (Thermo Electron Corporation, Boston, MA). PCR cycling conditions included an initial two minute 95ºC denaturation; 40 cycles of 95ºC for 30 s, 62ºC for 1 min, and

72ºC for 2 min; and a final extension for 10 min at 72ºC. PCR products were visualized under ultraviolet light on 2% agarose gels stained with ethidium bromide. PCR products from samples positive for C. *X. californiensis* DNA (N = 99) were included in subsequent DNA sequencing assays. The amplified fragments were sequenced in both the forward and reverse directions on a MegaBACE™ 500 DNA sequencer (Amersham Biosciences Corp., Piscataway, NJ) DNA sequencer using a DYEnamic™ ET dye terminator sequencing kit (Amersham Pharmacia Inc., Piscataway, NJ). Sequences were aligned and edited to 913 bp using Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI).

RecA cloning and sequencing

Due to invariance in the 16S gene, several other candidate loci were chosen for testing, including pCS20 (Peter et al. 1995; Alsopp et al. 2003; Steyn et al. 2003); hsp60/cpn60 (Goh et al. 1996; Hill et al. 2002); ITS-2 (i.e. the 5S-23S intergenic spacer region, Vitorino et al. 2003), 23S (Van Camp et al. 1993); and fusA, recA, and rpoB (Santos and Ochman 2004). These eight loci include both gene and intergenic regions that are 1) universally conserved protein coding regions common to nearly all bacteria (e.g. housekeeping genes) and/or 2) more typically used for bacterial population genetics due to higher mutation rates. Rickettsiales-specific and universal bacterial PCR primer sets for each locus were PCR amplified on a set of eight DNAs (four red abalone and four black abalone). Products were visualized under ultraviolet light on 2% agarose gels stained with ethidium bromide. Only the universal prokaryote recA primer set from Santos and Ochman (2004) reliably produced products in the appropriate size range from our sample set.

Fresh recA PCR products from the eight DNAs used in the above testing were pooled for cloning using a TOPO TA Cloning ® Kit for Sequencing with pCR®4-TOPO® vector (Invitrogen, Carlsbad, CA). All steps were performed according to the kit protocol for transforming One Shot® TOP10 Chemically Competent E. coli. Two volumes (20 µl and 40 µl) of each transformation mixture were spread on Luria broth (LB) agar plates with 50 mg/ml Kanamycin to select for inserts. Plates were incubated overnight at 37°C. Colonies were picked the following morning using a pipet tip, touched to a single well containing 10 µl of water in a 96-well plate (for PCR), and then transferred to a single well containing 170 µl of LB with 50mg/ml Kanamycin in a 96-well tissue culture plate (for glycerol stocks). The 96-well LB plates were incubated overnight at 37°C, 30 µl of glycerol was added per well the following morning, and the plates were stored at -80°C. The water plate was incubated at 99°C for 10 min to lyse the bacterial cells, and 5 µl per well was immediately PCR amplified using T3 and T7 universal primers to screen for inserts of the appropriate size (~450 bp). Positive clones were sequenced using the universal recA forward primer (recABDUP1).

A set of clone sequences that grouped phylogenetically with Rickettsiales recA sequences from the NCBI GenBank database were used to create two specific forward primers (recACXcalF1: 5'-TAGATGCAGAACATGCACTAGACTC and recACXcalF2: 5'-CATTTATAGATGCAGAACATGCAC) that, in conjunction with either the recABGDN2 or recABHDN1 universal recA reverse primer from Santos and Ochman (2004), amplified ~380 bp or ~500 bp of the recA gene, respectively. A set of five DNAs was sequenced in both forward and reverse directions from PCR products generated from all four possible primer combinations.

Results

Partial sequence from the C. *X. californiensis* 16S gene was successfully obtained for 56 out of 176 total individuals (99 of which tested positive for the bacterium in a single diagnostic PCR reaction). The data set was comprised of three species of abalone (red, black, and white) and included both wild and captive individuals (Table 7.1). The small set of five samples successfully sequenced for recA included wild red and black abalone from two sites (Scotts Creek and Vandenberg). Despite the range of samples, there were no polymorphic sites in 913 bp of *C. X. californiensis* 16S rDNA sequence or 470 bp of Rickettsiales-specific recA sequence (although the recA data set is extremely small). Due to the invariance of both genes, no population genetic analyses could be performed.

Discussion

All samples of Candidatus *Xenohaliotis californiensis* from which we were able obtain data were genetically invariant at over 900 bp of the 16S rDNA gene, regardless of the host abalone species or whether the abalone was wild or held in an aquaculture facility. Our data supports an earlier small-scale study by Robles-Sikisaka (2003) who studied ~400 bp of the same portion of the 16S rDNA gene in a set of 16 individuals. Robles-Sikisaka (2003) also found no variation within the C. *X. californiensis* species except a single nucleotide change in one individual from Vandenberg. This, of course, does not imply that there is no genetic variation within the C. *X. californiensis* species. While it is possible that all affected natural populations and aquaculture facilities within the state of California are infected with the same strain of the bacterium, this cannot be assumed from our data set. The 16S gene is typically and effectively used for a variety of interspecific purposes, such as bacterial community structure, species-level phylogenetics, and genome characterization (Lane 1991; Weisberg et al. 1991; Kirchman et al. 2001; Massung et al. 2002), but 16S is not often used for intraspecific studies likely due to its invariability (Devers et al. 2005), although there are exceptions (Allsopp and Allsopp 2001; Allsopp et al. 2003; Whitaker et al. 2003). Indeed, a large portion of the 16S gene (913 bp, >56% of the full length of the gene) was not variable in our C. *X. californiensis* samples and did not provide us with any information regarding strain variation within the species.

As a result, we attempted to find genetic variation in a second gene, the recombinase A (recA) gene, which has been used for bacterial population genetics in past research (Solca et al. 2001; Santos and Ochman 2004). RecA produces a protein that

catalyzes the pairing of single-stranded DNA with homologous double-stranded DNA in order to facilitate recombination as a method of maintaining genetic diversity. The recA gene, however, was invariant in a sample of five individuals, including one wild red and two wild black abalone from Scotts Creek near San Francisco and two wild black abalone from Vandenberg near Point Conception.

Although recA did not look promising, before further sequencing was performed, it was necessary to establish a link between the C. *X. californiensis* 16S and the targeted recA gene (i.e. prove that the two genes were both from C. *X. californiensis*). While sequences from the two genes grouped phylogenetically within the same set of α-proteobacteria (i.e. genuses within the Order Rickettsiales, such as *Anaplasma*, *Wolbachia*, and *Ehrlichia*), stronger evidence had not been gathered. Unfortunately, techniques such as fluorescence in situ hybridization (FISH) to show co-localization of genes or proteins are not commonly used with single-copy genes like recA, and a preliminary attempt at separating C. *X. californiensis* DNA from whole DNA extract by using biotinylated oligonucleotide gene-specific capture probes and streptavidin-coated magnetic beads (e.g. Millar et al. 1995; MacGregor 2006) failed. Because we have not established this link as yet and the five DNAs sequenced were invariant, no further recA sequencing was performed.

More research is needed before we can answer the question as to whether the C. *X. californiensis* bacterium is truly invariant and whether the withering syndrome epidemic in abalone in the state of California is caused by one or more strains of this bacterium. As to the possibility of introducing non-native strains of the bacterium to new culture facilities or into the natural environment, this research cannot answer that

question, either (although it is always appropriate to be cautious when transferring any species to a new environment). Recent evidence suggests that the bacterium can be eliminated from gastrointestinal tissue using the antibiotic oxytetracycline (Friedman et al. 2006), however, which may ultimately lessen the risk of introducing non-native strains into the environment or in transferring abalone among aquaculture facilities.

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Table 7.1: Collection information for C. *X. californiensis* samples. Final two columns indicate samples sequenced for 16S and recA.

ID	Tissue (T) or DNA	Abalone Species	Wild or Captive (W or C)	Location	Facility	16S	recA
WSTF6	T	black	W	Big Sur		x	
WSTF15	T	black	W	Santa Rosa Island		x	
WSTF19	T	black	W	Año Nuevo Island		x	
WSTF21	T	black	W	Vandenberg MER		x	
WSTF28	T	red	C	Cayucos	The Abalone Farm	x	
WSTF30	T	red	C	Cayucos	The Abalone Farm	x	
WSTF32	T	red	C	Cayucos	The Abalone Farm	x	
WSTF35	T	black	W	Vandenberg MER		x	
WSTF36	T	black	W	Vandenberg MER		x	
WSTF38	T	black	W	Vandenberg MER		x	
WSTF40	T	black	W	Vandenberg MER		x	
WSTF44	T	black	W	Vandenberg MER		x	x
WSTF45	T	red	W	Scotts Creek		x	
WSTF46	T	red	W	Scotts Creek		x	x
WSTF47	T	black	W	Vandenberg MER		x	
WSTF49	T	black	W	Scotts Creek		x	x
WSTF50	T	black	W	Scotts Creek		x	x
WSTF53	T	red				x	
WSTF58	T	black	W	Carmel Point		x	
WSTF59	T	red	C	Estero Americano	Bodega Farms	x	
WSTF60	T	black	W	Vandenberg MER		x	x
WSTF61	T	black	W	Carmel Point		x	
WSTF65	T	black	W	Point Lobos		x	
WSTF66	T	black	W	Point Lobos		x	
WSTF67	T	red	C	Estero Americano	Bodega Farms	x	
WSTF68	T	red	C	Estero Americano	Bodega Farms	x	
WSTF69	T					x	
WSTF71	T	black	W	Carmel Point		x	
WSTF72	T	red	C	Estero Americano	Bodega Farms	x	
WSTF74	T	red	C	Estero Americano	Bodega Farms	x	
WSTF75	T	red	C	Estero Americano	Bodega Farms	x	
WSTF76	T	red	C	Estero Americano	Bodega Farms	x	
WSTF77	T	black	W	Carmel Point		x	
WSTF78	T	black	W	Vandenberg MER		x	
WSTF84	T	black	W	Scotts Creek		x	
WSTF85	T	red	C			x	
WSTF88	T	black	W	Vandenberg MER		x	
WSTF92	T	red	C			x	
WSTF93	T	red	C			x	
WSTF94	T	white	C	Oxnard	Proteus SeaFarms	x	
WSTF95	T	white	C	Oxnard	Proteus SeaFarms	x	
WSTF98	T	white	C	Oxnard	Proteus SeaFarms	x	
WSTF102	T	red				x	
JMDNA6	DNA	red	C	Cayucos	The Abalone Farm	x	
JMDNA7	DNA	red	C	Cayucos	The Abalone Farm	x	
JMDNA8	DNA	red	C	Cayucos	The Abalone Farm	x	
JMDNA10	DNA	red	C	Cayucos	The Abalone Farm	x	
JMDNA11	DNA	red	C	Cayucos	The Abalone Farm	x	
JMDNA12	DNA	red	C	Cayucos	The Abalone Farm	x	
JMDNA13	DNA	red	C	Cayucos	The Abalone Farm	x	
JMDNA14	DNA	red	C	Cayucos	The Abalone Farm	x	
JMDNA50	DNA	red	C	Arcata	Humboldt State	x	
CF14	T	black				x	
CF17	T	black				x	
CF21	T	white	C	Oxnard	Proteus SeaFarms	x	
CF25	T	white	C	Oxnard	Proteus SeaFarms	x	

References

Allsopp MTEP, Allsopp BA (2001) Novel *Ehrlichia* genotype detected in dogs in South Africa. *Journal of Clinical Microbiology*, 39, 4202-4207

Allsopp MTEP, Van Heerden H, Steyn HC, Allsopp BA (2003) Phylogeneti relationships among *Ehrlichia ruminantium* isolates. *Annals of the New York Academy of Science*, 990, 685-691

Andree KB, Friedman CS, Moore JD, Hedrick RF (2000) A polymerase chain reaction assay for the detection of genomic DNA of a *Rickettsiales*-like prokaryote associated with Withering Syndrome in California abalone. *Journal of Shellfish Research*, Supplement, 213-218

California Department of Fish and Game, Marine Region (2005) Abalone recovery and management plan

Devers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, Stackebrandt E, Van de Peer Y, Vandamme P, Thompson FL, Swings J (2005) Re-evaluating prokaryotic species. *Nature Reviews Microbiology*, 3, 733-739

Friedman CS, Thomson M, Chun C, Haaker PL, Hedrick RP (1997) Withering syndrome of the black abalone, *Haliotis cracherodii* (Leach): water temperature, food availability, and parasites as possible causes. *Journal of Shellfish Research*, 16, 203-411

Friedman CS, Andree KB, Beauchamp KA, Moore JD, Robbins TT, Shields JD, Hedrick RP (2000) 'Candidatus *Xenohaliotis californiensis*', a newly described pathogen of abalone, *Haliotis* spp., along the west coast of North America. *Journal of Systematic and Evolutionary Microbiology*, 50, 847-855

Friedman CS, Finley CA (2003) Anthropogenic introduction of the etiological agent of withering syndrome into northern California abalone populations via conservation efforts. *Canadian Journal of Fisheries and Aquatic Sciences*, 60, 1424-1431

Friedman C, Moore J, Tjeerderna R, Viant M (2006) Pharmacokinetics and efficacy of oxytetracycline in RLP-infected abalone. California Sea Grant College Program Research Profiles (University of California, San Diego), R/A-122: 3.1.2004–2.28.2007

Gardner GR, Harshbarger JC, Lake JL, Sawyer TK, Price KL, Stephenson MD, Haaker PL, Togstad HA (1995) Association of prokaryotes with symptomatic appearance Withering Syndrome in black abalone *Haliotis cracherodii*. *Journal of Invertebrate Pathology*, 66, 111-120

Goh SH, Potter S, Wood JO, Hemmingsen SM, ReynoldsRP, Chow AW (1996) HSP60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative Staphylococci. *Journal of Clinical Microbiology*, 34, 818-823

Haaker PL, Parker DO, Chun CSY (1995) Growth of black abalone, *Haliotis cracherodii* Leach, at San Miguel Island and Point Arguello, California. *Journal of Shellfish Research*, 14, 519-525

Hill JE, Seipp RP, Betts M, Hawkins L, Van Kessel AG, Crosby WL, Hemmingsen SM (2002) Estensive profiling of a complex microbial community by high-throughput sequencing. *Applied and Environmental Microbiology*, 68, 3055-3056

Kirchman DL, Liying Y, Fuchs BM, Amann R (2001) Structure of bacterial communities in aquatic systems as revealed by filter PCR. *Aquatic Microbial Ecology*, 26, 13-22

Lane DJ (1991) 16S/23S rRNA sequencing. Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Ltd., New York, 115-175

MacGregor BJ, Amann R (2006) Single-stranded conformational polymorphism for separation of mixed rRNAS (rRNA-SSCP), a new method for profiling microbial communities. *Systematic and Applied Microbiology*, 29, 661-670

Massung RF, Lee K, Mauel M, Gusa A (2002) Characterization of the rRNA genes of *Ehrlichia chaffeensis* and *Anaplasma phagocytophila*. *DNA and Cell Biology*, 21, 587-596

Millar DS, Withey SJ, Tizard MLV, Ford JG, Hermon-Taylor J (1995) Solid-phase hybridization capture of low-abundance target DNA sequences: application to the polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp. *silvaticum*. *Analytical Biochemistry*, 226, 325-330

Moore JD, Robbins TT, Friedman CS (2000) Withering Syndrome in farmed red abalone *Haliotis rufescens*: thermal induction and association with a gastrointestinal Rickettsiales-like prokaryote. *Journal of Aquatic Animal Health*, 12, 26-34

Moore JD, Robbins TT, Hedrick RP, Friedman CS (2001) Transmission of the Rickettsiales-like prokaryote "Candidatus Xenohaliotis californiensis" and its role in Withering Syndrome of California abalone, *Haliotis* spp. *Journal of Shellfish Research*, 20, 867-874

Peter TF, Deem SL, Barbet AF, Norval AI, Simbi BH, Kelly PJ, Mahan SM (1995) Development and evaluation of PCR assay for detection of low levels of *Cowdria*

ruminantium infection in *Amblyomma* ticks not detected by DNA probe. *Journal of Clinical Microbiology*, 33, 166-172

Robles-Sikisaka R (2003) Genetic diversity of Candidatus *Xenohaliotis californiensis*, causative agaent of withering syndrome in abalone (*Haliotis* spp.). BML Student Reports, ESP 124 Marine and Coastal Field Ecology, Environmental Studies, University of California, Davis

Santos SR, Ochman H (2004) Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. *Environmental Microbiology*, 6, 754-759

Solca MN, Bernasconi MV, Valsangiacomo C, Van Doorn LJ, Piffaretti JC (2001) Population genetics of *Helicobacter pylori* in the southern part of Switzerland analysed by sequencing of four housekeeping genes (atpD, glnA, scoB and recA), and by vacA, cagA, iceA and IS605 genotyping. *Microbiology*, 147, 1693-1707

Steyn HC, Van Heerden H, Allsopp MTEP, Allsopp BA (2003) Variability of pCS20 gene sequences among different *Ehrlichi ruminantium* isolates. *Annals of the New York Academy of Science*, 990, 723-725

Van Camp G, Chapelle S, De Wachter R (1993) Amplification and sequencing of variable regions in bacterial 23S ribosomal RNA genes with conserved primer sequences. *Current Microbiology*, 27, 147-151

Vitorino L, Zé-Zé L, Sousa A, Bacellar F, Tenreiro R (2003) rRNA intergenic specer regions for phylogenetic analysis of *Rickettsia* species. *Annals of the New York Academy of Science*, 990, 726-733

Weisberg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, 173, 697-703

Whitaker RJ, Grogan DW, Taylor JW (2003) Geographic barriers isolate endemic populations of hyperthermophilic archaea. *Science*, 301, 976-978

CONCLUSION

Management and recovery of California abalone species

Over the past three decades, five species of abalone (genus *Haliotis*) in California waters have gone from harvestable resources to rare or even endangered species status. A moratorium brought nearly all abalone fishing off the coast of California to a halt in 1997 (CDFG 2005) due to stock collapses in red (*H. rufescens*), black (*H. cracherodii*), white (*H. sorenseni*), green (*H. fulgens*), and pink (*H. corrugata*) abalone. Since 1997, the fishery has been restricted to heavily-regulated sport diving north of San Francisco Bay (SFB) for the red abalone only. Both anthropogenic (overexploitation, habitat destruction) and natural (disease, predation) factors contributed to the declines (CDFG 2005).

In this dissertation, I wanted to assess the role of genetics might play in the conservation and recovery of these depleted California abalone species. Each abalone species that I studied presented a unique research focus, with topics ranging from measuring genetic diversity of hatchery-bred progeny and ensuring the specific purity of broodstock in a captive-breeding program for the endangered white abalone to inferring dispersal among natural populations of abalone along the California coast to studying the microbial shifts that occur in the gut microbiomes of abalone diagnosed with withering syndrome. Here, I explore in greater detail how these topics are relevant to the management of these ecologically and commercially important marine invertebrate species.

The influence of dispersal on stock enhancement initiatives and forensics

Population genetics is often used today in conservation biology to identify breeding stocks and infer dispersal potential (Waples 1998), and there are at least two

areas associated with the management of California red and black abalone to which population genetics can be applied in this manner. First, it is currently unclear whether the fishery moratorium combined with reproduction by healthy stocks will halt declines and replenish abalone populations along the California coast. For example, northern red abalone populations appear to be self-sustaining under limited fishery pressure, although there is evidence of poor recruitment, depletion of intertidal stocks, and a decline in deep-water stocks over the past two decades (CDFG 2005). Genetic analyses of population structure could clarify whether those remnant northern stocks may also be seeding severely depleted populations to the south with recruits. Similarly, abundances of black abalone in some areas of the Southern California Bight (SCB) are at less than three percent of historical abundances seen prior to the start of the fishery and withering syndrome epidemic (Haaker et al. 1992). Hence, we assessed gene flow among existing natural populations of red and black abalone from California in order to infer the realized dispersal capability of each species.

For both species, our data suggested that red and black abalone populations are primarily self-recruiting and that recruitment from outside source populations to those nearly extirpated may be dependent on sporadic chance events. Recovery of depleted populations, if it occurs at all, will be extremely slow, potentially taking several hundred generations. Recovery of the black abalone may be slowed even further as the fatal disease withering syndrome remains a threat to natural black abalone populations. The disease is still causing mortality in populations in the SCB and its range has currently spread as far northward along the mainland coast as Point San Pedro near San Francisco (Friedman et al. 2002; Moore et al. 2002; CDFG 2005). Coupled with the recovery and

recent range expansion of sea otters, a top abalone predator, south along the central coast of California (CDFG 2005), continued decline and not recovery of natural populations of the red and the black abalone may occur.

These predictions based on genetic data will ultimately be tested via ongoing efforts to monitor abalone recruitment in regions of low local abalone abundance (e.g. the Channel Islands Kelp Forest Monitoring (KFM) Program; Davis et al. 1997). Unfortunately, a recent study by Rogers-Bennett et al. (2004) implied that recruitment is failing in depleted regions. Juvenile recruitment for all abalone species combined was found to be 30 times more abundant between 2001 and 2003 in northern California where populations are robust than in the Channel Islands in the SCB where populations are severely depleted. Although this does support the hypothesis that abalone populations along the California coast are primarily self-recruiting, it is not good news for abalone recovery.

If recruitment failure is indeed occurring for California abalone species despite the fishery moratorium, Federal and state entities might consider switching the focus of management from the conservation and natural recovery of abalone populations to active restoration. A captive-breeding program similar to the one instituted for the white abalone with the ultimate intent to augment natural populations of each species through outplanting of hatchery-bred individuals is an option. If undertaken, stock enhancement plans should seek to maintain genetic diversity in the captive-bred pool, as well as match the genetic background of the natural populations where outplanting will occur, if possible. Unlike the endangered white abalone which is so depleted and difficult to collect (due to the scarcity of individuals and a resident depth of 30m – 60m (CDFG

2005)) that matching the genetic background of extant populations is not feasible, existing populations of the red and the black abalone are likely robust enough to support more focused stock enhancement efforts in which each population or region "provides" its own broodstock.

The second area population genetics can be applied in the management of natural abalone populations primarily targets the red abalone. Since legal take is currently geographically limited to north of SFB and does not encompass the entire red abalone species range in California, genetics could have a forensic application if regions north and south of SFB differ in genetic composition. Our AFLP study on the red abalone was originally designed with only six populations to test the hypothesis that northern California populations are genetically distinct from central and southern California populations. The data, however, showed evidence of a genetic divisions among individual populations and not regionally north and south of SFB.

A recent complicating caveat to any forensic application to the disjunct red abalone fishery is that increasing pressure by interest groups (e.g. the California Abalone Association) has resulted in the potential reopening of selected commercial fisheries south of SFB. San Miguel Island (SMI), for example, is a remnant healthy population in the northern Channel Islands that has shown signs of recent recruitment. In December 2005, the California Department of Fish and Game (CDFG) proposed a timeline at the request of the California Fish and Game Commission for the development and reopening of the SMI red abalone commercial fishery by 2008 (I Taniguchi pers. comm.; see also www.dfg.ca.gov/news/news05/05125.html). If SMI populations are deemed robust enough after initial surveys to sustain the commercial fishery, total allowable catch

statistics will be calculated and a permitting program will be instituted in time for reopening by April of 2008. Regulation of the fishery and the prosecution of poachers will be more difficult under this scenario, but our AFLP data may be more applicable. The data are currently sufficient to show that red abalone populations are divergent, but if more AFLP loci are added to the current marker set, more power may be gained for assignment test purposes (Campbell et al. 2003); source populations for individual abalone could be directly identified.

A brief overview of other formerly fished California abalone species

The pink and the green abalone are two more heavily-depleted California abalone species that are primarily found south of Point Conception, which marks the northern extent of the SCB. Neither species was included in the main body of this dissertation (see Chapters II and V), but a significant portion of the catch during the former commercial abalone fishery was provided by these two species. Each is now considered Species of Concern by the Federal government (see http://www.nmfs.noaa.gov/pr/species/concern/). The situation for pink abalone is similar to that of red and black abalone; according to microsatellite analyses, populations may be primarily self-recruiting, but more research is needed before any definitive conclusions can be made.

The green abalone, however, is the only abalone species in California in which captively-bred progeny have been and will continue to be outplanted (Lapota et al. 2000; D Lapota pers. comm.). The outplant sites are off the Point Loma peninsula within San Diego, California, proper. Outplanted individuals are physically tagged and surveys are

conducted annually. One concern is that the captive-raised green abalone have lower genetic diversity than those found in the wild, and outplanting of these animals may have adverse effects on the local wild populations (e.g. swamping of the gene pool). However, since the outplanting operation is legally permitted and already in progress, the hope is that this experiment will provide the state of California with valuable information over time regarding the success of an outplanting effort for a depleted abalone species. Initial outplantings appeared to have failed (Lapota et al. 2000), but recent reports indicate that survival rates may be as high as 50% in some areas (D Lapota pers. comm.).

Withering syndrome, stock enhancement, and future directions

The problem of withering syndrome (WS) in natural populations of California abalone cannot be solved by human intervention. However, long-term monitoring of some populations, such as annual surveys of black abalone on San Nicolas Island (SNI) in the SCB conducted by G VanBlaricom from the University of Washington, provide valuable time series data on abalone density that might be used to infer the extent and impact of continuing WS infection. For example, the recent stabilization (or even increase) of black abalone abundances at specific sites on SNI (G VanBlaricom pers. comm.) may imply that morbidity and mortality due to WS is at least slowing in some areas.

A main concern now is the impact WS has on captively-raised abalone. As mentioned in Chapters VI and VII, nearly all abalone aquaculture facilities (commercial and private) in the state of California have been adversely affected by WS (Moore et al. 2001). Without the eradication of the disease in aquaculture facilities breeding abalone

for stock enhancement initiatives, no future outplanting can take place under Federal law, and captive-breeding for outplanting under this scenario would be a waste of money and resources. However, recent research indicates that the antibiotic oxytetracycline (OTC) eradicates Candidatus *Xenohaliotis californiensis*, the bacterium that causes WS, from the abalone gastrointestinal tract (Friedman et al. 2006). Therefore, is the outplanting of disease-free (via OTC or other means), captive-bred California abalone into an environment that still contains the WS pathogen a sound management decision? Unfortunately, the answer is unknown without more research. The C. *X. californiensis* bacterium seems to be present even in healthy abalone at low levels (see Chapter VI). In addition, all five formerly-fished California abalone species can contract WS in aquaculture (Moore et al. 2001), and visibly withered individuals have been found in the wild from at least four of the five abalone species (I Taniguchi, PL Haaker, and E Kisfaludy pers. comm.). Future research should seek to establish how common the bacterium is in healthy abalone gut tissue and in the marine environment.

References

California Department of Fish and Game, Marine Region (2005) Abalone recovery and management plan

Campbell D, Duchesne P, Bernatchez L (2003) AFLP utility for population assignment studies: analytical investigation and empirical comparison with microsatellites. *Molecular Ecology*, 12, 1979-1991

Davis GE, Kushner DJ, Mondragon JM, Mondragon JE, Lerma D, Richards DV (1997) Kelp Forest Monitoring Handbook, Volume 1, Sampling Protocol. Channel Islands National Park, Ventura, California

Friedman CS, Biggs W, Shields JD, Hedrick RP (2002) Transmission of withering syndrome in black abalone, *Haliotis cracherodii* Leach. *Journal of Shellfish Research*, 21, 817-824

Friedman C, Moore J, Tjeerderna R, Viant M (2006) Pharmacokinetics and efficacy of oxytetracycline in RLP-infected abalone. California Sea Grant College Program Research Profiles (University of California, San Diego), R/A-122: 3.1.2004–2.28.2007

Moore JD, Robbins TT, Hedrick RP, Friedman CS (2001) Transmission of the Rickettsiales-like prokaryote "Candidatus Xenohaliotis californiensis" and its role in Withering Syndrome of California abalone, *Haliotis* spp. *Journal of Shellfish Research*, 20, 867-874

Moore JD, Finley CA, Robbins TT, Friedman CS (2002) Withering syndrome and restoration of southern California abalone populations. *CalCOFI Report*, 43, 112-117

Rogers-Bennett L, Allen BL, Davis GE (2004) Measuring abalone (*Haliotis* spp.) recruitment in California to examine recruitment overfishing and recovery criteria. *Journal of Shellfish Research*, 23, 1201-1207

Waples RS (1998) Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. *Journal of Heredity*, 89, 438-450